Table 4
Haplotype frequencies of LIF gene of control subjects and patients with hebephrenic schizophrenia.

Haplotype rs929271–rs737812	Controls Frequency	Patients Frequency	Permutation *P*
T–C	0.4344	0.5254	0.0040
G–C	0.4267	0.3503	0.011
T–A	0.1388	0.1244	–

Haplotype analysis was performed by the permutation method. The global permutation *P* was 0.013.

were also found in healthy first-degree relatives of schizophrenic patients (Rybakowski and Borkowska, 2002). Suhr and Spitznagel (2001) reported that high negative symptoms were associated with poor performance on the WCST. Taking these findings together with ours, it can be speculated that T-carrier genotypes of rs929271 of the LIF gene may alter the pro-inflammatory cytokine response to infections during fetal or early life, which may result in neurocognitive maldevelopment of working memory in later life, which are physiological bases for thought disturbance. Finally, a LIF variant may produce susceptibility to hebephrenic schizophrenia, a prominent feature of which is thought disturbance.

Some points should be considered in the present study. We found a statistically significant association of the LIF gene with schizophrenia, but the association was marginal, and we did not apply a multiple test correction. The possibility of a type I error should be considered. As we examined only three SNPs of the LIF gene, although they cover almost the entire LIF gene, the present study does not exclude the possibility of associations between other variations in the LIF gene and schizophrenia. Further replication studies by examining of additional SNPs in large sample populations are necessary to confirm our findings.

We examined healthy subjects to examine the role of the LIF gene in neurocognition, but not in patients with schizophrenia. In spite of recommendations as to the optimal prescription of antipsychotics, antipsychotic polypharmacy and excessive dosing are still highly prevalent worldwide, especially in Japan (Faries et al., 2005; Procyshyn et al., 2001; Sim et al., 2004a,b). It was suggested that the cognitive deficits of patients with schizophrenia may result from non-standard use, polypharmacy, and overdose of antipsychotics (Hori et al., 2006). On the other hand, some studies reported that the WCST score of schizophrenic patients was improved after administration with atypical antipsychotics (Rybakowski et al., 2007; Gallhofer et al., 2007). We did not examine the association between the LIF gene and cognitive decline in patients because almost all of the patients in the present study were taking antipsychotic medications, and their effects on neurocognitive testing cannot be denied. Further studies including unmedicated patients and a more precise, advanced statistical approach are necessary to understand the effects of the LIF gene on susceptibility to schizophrenia and cognitive function. In addition, examination of association between LIF and other endophenotypes, including prepulse inhibition may be necessary for further understanding the physiological roles of the LIF gene.

In conclusion, the present study indicated that the LIF gene variant may produce susceptibility to hebephrenic schizophrenia and deterioration of working memory function.

Table 5
Results of WCST with genotype of rs929271.

	T carrier	Non-T carrier	*P*
Verbal IQ	109.5 ± 12.3	107.5 ± 12.8	0.36
Performance IQ	110.9 ± 11.3	109.8 ± 12.6	0.52
Verbal memory	111.5 ± 14.0	111.0 ± 14.9	0.48
Visual memory	109.8 ± 9.5	107.5 ± 12.5	0.23
Attention	105.1 ± 13.6	105.6 ± 13.6	0.50
Delayed recall	112.2 ± 12.1	112.8 ± 13.2	0.43
WCST-CA	3.55 ± 2.06	3.82 ± 1.89	0.16
WCST-TE	18.29 ± 8.50	16.62 ± 6.42	0.04

The scores of T-allele carriers (T/T or T/G) of rs929271 and non-carriers (G/G) were compared. WCST-CA, WCST: category achieved; WCST-TE, WCST: total errors.
P values were examined by MANOVA to adjust for age, sex and education years.

Acknowledgement

We thank the Zikei Institute of Psychiatry (Okayama, Japan).

References

Akiyama K. Serum levels of soluble IL-2 receptor alpha, IL-6 and IL-1 receptor antagonist in schizophrenia before and during neuroleptic administration. Schizophr Res 1999;37:97-106.
Badner JA, Gershon ES. Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. Mol Psychiatry 2002;7:405-11.
Bains JS, Oliet SH. Glia: they make your memories stick! Trends Neurosci 2007;30:417-24.
Barnabe-Heider F, Wasylnka JA, Fernandes KJ, Porsche C, Sendtner M, Kaplan DR, et al. Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron 2005;48:253-65.
Benes FM, Davidson J, Bird ED. Quantitative cytoarchitectural studies of the cerebral cortex of schizophrenics. Arch Gen Psychiatry 1986;43:31-5.
Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, et al. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 1997;278:477-83.
Borrell J, Vela JM, Arevalo-Martin A, Molina-Holgado E, Guaza C. Prenatal immune challenge disrupts sensorimotor gating in adult rats. Implications for the etiopathogenesis of schizophrenia. Neuropsychopharmacology 2002;26:204-15.
Brown AS. The risk for schizophrenia from childhood and adult infections. Am J Psychiatry 2008;165:7-10.
Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, et al. Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry 2004;61:774-80.
Crow TJ. Molecular pathology of schizophrenia: more than one disease process? Br Med J 1980;280:66-8.
Everett J, Lavoie K, Gagnon JF, Gosselin N. Performance of patients with schizophrenia on the Wisconsin Card Sorting Test (WCST). J Psychiatry Neurosci 2001;26:123-30.
Faries D, Ascher-Svanum H, Zhu B, Correll C, Kane J. Antipsychotic monotherapy and polypharmacy in the naturalistic treatment of schizophrenia with atypical antipsychotics. BMC Psychiatry 2005;5:26.
Fatemi SH, Reutiman TJ, Folsom TD, Huang H, Oishi K, Mori S, et al. Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders. Schizophr Res 2008;99:56-70.
Franke P, Maier W, Hain C, Klingler T. Wisconsin Card Sorting Test: an indicator of vulnerability to schizophrenia? Schizophr Res 1992;6:243-9.
Gallhofer B, Jaanson P, Mirttoux A, Tanghoj P, Lis S, Krieger S. Course of recovery of cognitive impairment in patients with schizophrenia: a randomized double-blind study comparing sertindole and haloperidole. Pharmacopsychiatry 2007;40:275-86.
Gill M, Vallada H, Collier D, Sham P, Holmans P, Murray R, et al. A combined analysis of D22S278 marker alleles in affected sib-pairs: support for a susceptibility locus for schizophrenia at chromosome 22q12. Schizophrenia Collaborative Linkage Group (Chromosome 22). Am J Med Genet 1996;67:40-5.
He F, Ge W, Martinowich K, Becker-Catania S, Coskun V, Zhu W, et al. A positive autoregulatory loop of Jak-STAT signaling controls the onset of astrogliogenesis. Nat Neurosci 2005;8:616-25.
Holmberg KH, Patterson PH. Leukemia inhibitory factor is a key regulator of astrocytic, microglial and neuronal responses in a low-dose pilocarpine injury model. Brain Res 2006;1075:26-35.
Hori H, Noguchi H, Hashimoto R, Nakabayashi T, Omori M, Takahashi S, et al. Antipsychotic medication and cognitive function in schizophrenia. Schizophr Res 2006;86:138-46.
Johnston-Wilson NL, Sims CD, Hofmann JP, Anderson L, Shore AD, Torrey EF, et al. Disease-specific alternations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. Mol Psychiatry 2000;5:142-9.
Kashima H, Handa T, Kato M, Sakura K, Yokoyama N, Murakami M, et al. Neuropsychological investigation on chronic schizophrenia—aspects of its frontal functions. In: Takahashi R, Flor-Henry P, Gruzelier J and Niwa S, (Eds.), Cerebral Dynamics, Laterality and Psychopathology, Elsevier, Amsterdam 1987, p. 337-45
Kerr BJ, Patterson PH. Potent pro-inflammatory actions of leukemia inhibitory factor in the spinal cord of the adult mouse. Exp Neurol 2004;188:391-407.
Kerr BJ, Patterson PH. Leukemia inhibitory factor promotes oligodendrocyte survival after spinal cord injury. Glia 2005;51:73-9.
Kobayashi S. Wisconsin Card Sorting Test Program Keio-F-S-version (Web site, in Japanese) 1999. Available at:http://cvddb.shimane-med.ac.jp/user/wisconsin.htm
Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, et al. Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: schizophrenia. Am J Hum Genet 2003;73:34-48.
Maes M, Bocchio Chiavetto L, Bignotti S, Battisa Tura GJ, Pioli R, Boin F, et al. Increased serum interleukin-8 and interleukin-10 in schizophrenic patients resistant to treatment with neuroleptics and the stimulatory effects of clozapine on serum leukemia inhibitory factor receptor. Schizophr Res 2002;54:281-91.
Martino DJ, Bucay D, Butnam JT, Allegri RF. Neuropsychological frontal impairments and negative symptoms in schizophrenia. Psychiatry Res 2007;59:127-8.

Meyer U, Feldon J, Yee BK. A review of the fetal brain cytokine imbalance hypothesis of schizophrenia. Schizophr Bull 2008;35:959–72.
Nawa H, Takahashi M, Patterson PH. Cytokine and growth factor involvement in schizophrenia—support for the developmental model. Mol Psychiatry 2000;5:594–603.
Otsubo T, Tanaka K, Koda R, Shinoda J, Sano N, Tanaka S, et al. Reliability and validity of Japanese version of the Mini-International Neuropsychiatric Interview. Psychiatry Clin Neurosci 2005;59:517–26.
Procyshyn RM, Kennedy NB, Tse G, Thompson B. Antipsychotic polypharmacy: a survey of discharge prescriptions from a tertiary care psychiatric institution. Can J Psychiatry 2001;46:334–9.
Pulver AE, Karayiorgou M, Wolyniec PS, Lasseter VK, Kasch L, Nestadt G, et al. Sequential strategy to identify a susceptibility gene for schizophrenia: report of potential linkage on chromosome 22q12–q13.1: part 1. Am J Med Genet 1994;54:36–43.
Romero E, Guaza C, Castellano B, Borrell J. Ontogeny of sensorimotor gating and immune impairment induced by prenatal immune challenge in rats: implications for the etiopathology of schizophrenia. Mol Psychiatry 2008; in press. doi:10.1038/m.p.2008.44.
Rybakowski JK, Borkowska A. Eye movement and neuropsychological studies in first-degree relatives of schizophrenic patients. Schizophr Res 2002;54:105–10.
Rybakowski JK, Drozdz W, Borkowska A. Long-term administration of the low-dose risperidone in schizotaxia subjects. Hum Psychopharmacol 2007;22:407–12.
Sanfilipo M, Lafargue T, Rusinek H, Arena L, Loneragan C, Lautin A, et al. Volumetric measures of the frontal and temporal lobe regions in schizophrenia: relationship to negative symptoms. Arch Gen Psychiatry 2000;57:471–80.
Segurado R, Detera-Wadleigh SD, Levinson DF, Lewis CM, Gill M, Nurnberger Jr JI, et al. Genome scan meta-analysis of schizophrenia and bipolar disorder, part III: bipolar disorder. Am J Hum Genet 2003;73:49–62.
Sim K, Su A, Fujii S, Yang SY, Chong MY, Ungvari GS, et al. Antipsychotic polypharmacy in patients with schizophrenia: a multicentre comparative study in East Asia. Br J Clin Pharmacol 2004a;58:178–83.
Sim K, Su A, Leong JY, Yip K, Chong MY, Fujii S, et al. High dose antipsychotic use in schizophrenia: findings of the REAP (research on east Asia psychotropic prescriptions) study. Pharmacopsychiatry 2004b;37:175–9.
Stark AK, Uylings HB, Sanz-Arigita E, Pakkenberg B. Glial cell loss in the anterior cingulate cortex, a subregion of the prefrontal cortex, in subjects with schizophrenia. Am J Psychiatry 2004;161:882–8.
Steffek AE, McCullumsmith RE, Haroutunian V, Meador-Woodruff JH. Cortical expression of glial fibrillary acidic protein and glutamine synthetase is decreased in schizophrenia. Schizophr Res 2008;103:71–82.
Sugiura S, Lahav R, Han J, Kou SY, Banner LR, de Pablo F, et al. Leukaemia inhibitory factor is required for normal inflammatory responses to injury in the peripheral and central nervous systems in vivo and is chemotactic for macrophages in vitro. Eur J Neurosci 2000;12:457–66.
Suhr JA, Spitznagel MB. Factor versus cluster models of schizotypal traits. II: relation to neuropsychological impairment. Schizophr Res 2001;52:241–50.
Sutherland GR, Baker E, Hyland VJ, Callen DF, Stahl J, Gough NM. The gene for human leukemia inhibitory factor (LIF) maps to 22q12. Leukemia 1989;3:9-13.
Taga T, Kishimoto T. Gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol 1997;15:797–819.
Tohmi M, Tsuda N, Watanabe Y, Kakita A, Nawa H. Perinatal inflammatory cytokine challenge results in distinct neurobehavioral alterations in rats: implication in psychiatric disorders of developmental origin. Neuro Res 2004;50:67–75.
Toyooka K, Watanabe Y, Iritani S, Shimizu E, Iyo M, Nakamura R, et al. A decrease in interleukin-1 receptor antagonist expression in the prefrontal cortex of schizophrenic patients. Neurosci Res 2003;46:299–307.
Urakubo A, Jarskog LF, Lieberman JA, Gilmore JH. Prenatal exposure to maternal infection alters cytokine expression in the placenta, amniotic fluid, and fetal brain. Schizophr Res 2001;47:27–36.
Watanabe Y, Hashimoto S, Kakita A, Takahashi H, Ko J, Mizuno M, et al. Neonatal impact of leukemia inhibitory factor on neurobehavioral development in rats. Neurosci Res 2004;48:345–53.
Webster MJ, O'Grady J, Kleinman JE, Weickert CS. Glial fibrillary acidic protein mRNA levels in the cingulated cortex of individuals with depression, bipolar disorder and schizophrenia. Neuroscience 2005;133:453–61.
Weinberger DR, Berman KF, Zec RF. Physiological dysfunction of dorsolateral prefrontal cortex in schizophrenia. I. Regional cerebral blood flow evidence. Arch Gen Psychiatry 1986;43:114–24.
Wolkin A, Choi SJ, Szilagyi S, Sanfilipo M, Rotrosen JP, Lim KO. Inferior frontal white matter anisotropy and negative symptoms of schizophrenia: a diffusion tensor imaging study. Am J Psychiatry 2003;160:572–4.

Neuroscience Letters 476 (2010) 104–109

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Cortical neurons from intrauterine growth retardation rats exhibit lower response to neurotrophin BDNF

Midori Ninomiya[a,b], Tadahiro Numakawa[b,c,*], Naoki Adachi[b,c], Miyako Furuta[b], Shuichi Chiba[b], Misty Richards[b,d], Shigenobu Shibata[a], Hiroshi Kunugi[b,c]

[a] Laboratory of Physiology and Pharmacology, School of Advanced Science and Engineering, Waseda University, Shinjuku-ku, Tokyo, Japan
[b] Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan
[c] Core Research for Evolutional Science and Technology Program (CREST), Japan Science and Technology Agency (JST), Saitama 332-0012, Japan
[d] Albany Medical College, Albany, NY 12208, USA

ARTICLE INFO

Article history:
Received 21 December 2009
Received in revised form 31 March 2010
Accepted 31 March 2010

Keywords:
BDNF
TrkB
Cell death
Intrauterine growth retardation

ABSTRACT

Intrauterine growth retardation (IUGR) is putatively involved in the pathophysiology of schizophrenia. The animal model of IUGR induced by synthetic thromboxane A2 (TXA2) is useful to clarify the effect of IUGR on pups' brains, however, analysis at the cellular level is still needed. Brain-derived neurotrophic factor (BDNF), which plays a role in neuronal survival and synaptic plasticity in the central nervous system (CNS), may also be associated with schizophrenia. However, the possible relationship between IUGR and BDNF function remains unclear. Here, we examined how IUGR by TXA2 impacts BDNF function by using dissociated cortical neurons. We found that, although BDNF levels in cultured neurons from the cerebral cortex of low birth weight pups with IUGR were unchanged, TrkB (BDNF receptor) was decreased compared with control-rats. BDNF-stimulated MAPK/ERK1/2 and PI3K/Akt pathways, which are downstream intracellular signaling pathways of TrkB, were repressed in IUGR-rat cultures. Expression of glutamate receptors such as GluA1 and GluN2A was also suppressed in IUGR-rat cultures. Furthermore, in IUGR-rat cultures, anti-apoptotic protein Bcl2 was decreased and BDNF failed to prevent neurons from cell death caused by serum-deprivation. Taken together, IUGR resulted in reductions in cell viability and in synaptic function following TrkB down-regulation, which may play a role in schizophrenia-like behaviors.

Schizophrenia is a chronic, severe and disabling brain disease, of which neuropathological basis has remained elusive [18]. Growing evidence has suggested neurodevelopmental impairments in the pathogenesis of schizophrenia [13]. Importantly, obstetric complications play a role in such impairments [8,24,35]. Among various obstetric complications, low birth weight is a strong risk factor for schizophrenia [25].

Intrauterine growth retardation (IUGR) induced by synthetic thromboxane A2 (TXA2) was associated with a delay in postnatal neurological development and learning disabilities in rats in which the neuronal density in the cortical plate was lower than that of control rats [31]. Interestingly, mRNA expression of neurotrophins such as BDNF and NT-3 (neurotrophin-3) was suppressed in the cerebral cortex of TXA2-induced IUGR-rats [14].

BDNF has critical roles in neuronal survival and synaptic plasticity [7,32] through activation of TrkB, and consequent stimulation of downstream signaling including mitogen-activated protein/extracellular signal-regulated kinase (MAPK/ERK), phosphoinositide 3-kinase/Akt (PI3K/Akt) and phospholipase Cγ (PLCγ) pathways. Recently, we reported important regulatory roles of BDNF in synaptic functions via these pathways [23,26,27]. Remarkably, altered serum levels of BDNF and its expression in the postmortem brain of schizophrenia patients have been reported [10,21,33]. Furthermore, forebrain-specific TrkB knockout mice showed schizophrenia-like behaviors, including hyperlocomotion, stereotyped behaviors and cognitive impairments [36].

Though both IUGR and dysfunction of BDNF-TrkB signaling may contribute to the pathogenesis of schizophrenia, the possible change in the BDNF-TrkB signaling in Central Nervous System (CNS) neurons of IUGR has not yet been clarified. Here, we found that cortical neurons from IUGR-rats exhibited lower levels of TrkB, Bcl2, and glutamate receptors. Interestingly, neurons from IUGR-rats showed a decreased response to BDNF when survival was examined.

Female Long-Evans rats (Institute for Animal Reproduction, Ibaraki, Japan) were purchased at 8 days of pregnancy and kept

* Corresponding author at: Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan. Tel.: +81 42 341 2711x5132; fax: +81 42 346 1744.
E-mail address: numakawa@ncnp.go.jp (T. Numakawa).

0304-3940/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2010.03.082

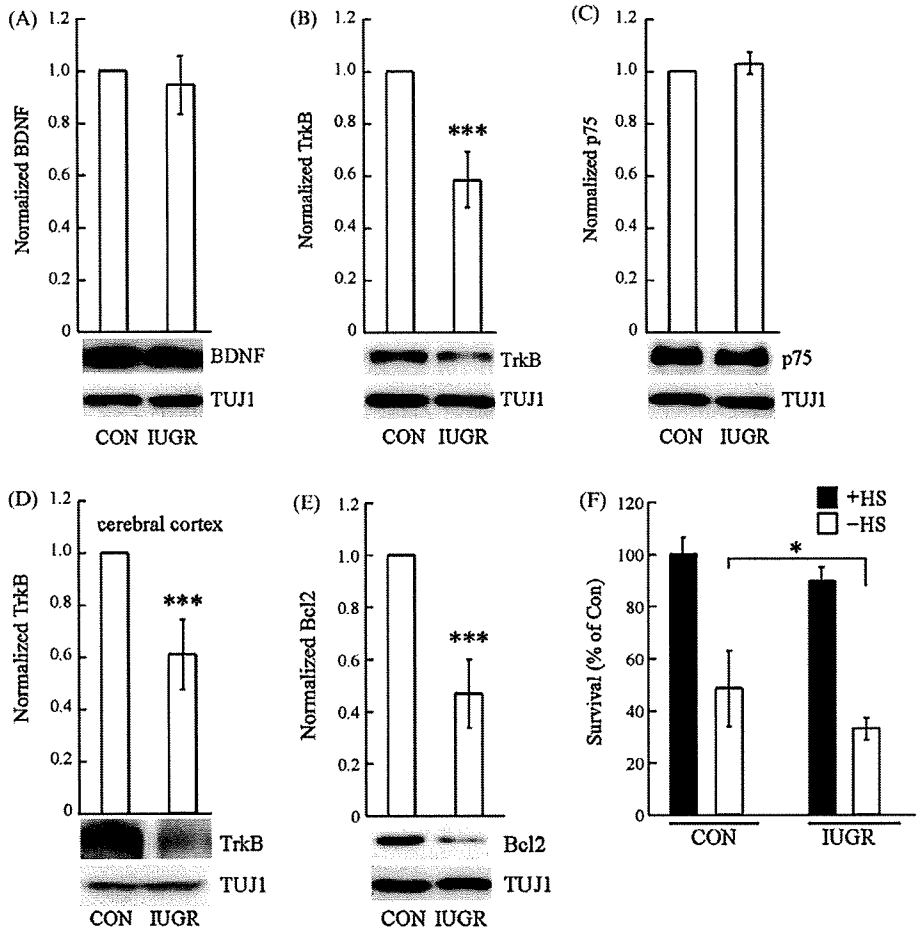

Fig. 1. Reduction in levels of TrkB, Bcl2, and cell viability in cortical cultures prepared from the cerebral cortex of low weight newborn rats with TXA2-induced IUGR. The levels of BDNF (A), TrkB (B), and p75 (C) were examined in 5DIV cortical cultures from IUGR-rats or from control-rats. TrkB was reduced in IUGR-rat neurons. Quantification was carried out after immunoblotting. Normalization to a level in control was performed. Data represent mean ± SD ($n = 6$), ***$P < 0.001$. IUGR: intrauterine growth retardation. (D) TrkB down-regulation was observed in homogenates from the cerebral cortex of IUGR-rats, ***$P < 0.001$ ($n = 4$). (E) Reduction in Bcl2 expression in cultures from IUGR-rats. Data represent mean ± SD ($n = 7$), ***$P < 0.001$. The three independent series of cultures were used for each set of immunoblotting experiments. TUJ1 levels are shown as controls in each representative blot. (F) Decrease in cell viability of cortical neurons from IUGR-rats. To induce neuronal cell death, serum-deprivation was performed. Cell survival was determined by MTT assay. Data represent mean ± SD ($n = 8$, n indicates the number of wells of a plate for each experimental condition), *$P < 0.05$. To confirm reproducibility, the three independent series of cultures were used.

in individual cages under a standard laboratory environment (12L:12D, light on at 15:30; 21–24 °C temperature; free access to food and water). IUGR was induced by TXA2 analog (9,11-dideoxy-9 a, 11a-methanoepoxy-prosta-5Z, 13E-dien-1-oic acid; Cayman Chemical, MI, USA) application on mother rats according to previous studies [20]. Briefly, an osmotic pump (2ML1, Alzet Corp., Palo Alto, CA, USA) containing 2 ml of TXA2 solution (12.5 μg/ml) or PBS for control rats was implanted into the lower portion of the peritoneal cavity under sodium pentobarbital (31.5 mg/kg b.w.) anesthesia on 13 days of pregnancy. Rats were allowed to deliver spontaneously, and pups were fed by their own mothers. Brains of pups were removed at postnatal day 1 (P1) and used for dissociated cultures. To check levels of TrkB in homogenates from the cerebral cortex, the brains were removed from the deeply anesthetized P1 IUGR- or control-rats. All the experiments were approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience, Japan.

Cultures were prepared as previously reported [28]. Dissociated cortical neurons were plated on polyethyleneimine-coated culture dishes or 48-well plates (Corning, NY, USA). The cell density was $5 \times 10^5/cm^2$, respectively. Neuronal cultures from cerebral cortex of pups of control or of IUGR were maintained with 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 5% fetal bovine serum and 5% heated-inactivated horse serum for 5 days before the survival assay or collecting samples for immunoblotting. To induce cell death, the culture media was replaced with a serum-free fresh media for 24 h. Then, to determine the cell viability, a mitochondrial-dependent conversion of the tetrazolium salt (MTT) assay was performed [30]. When glial cell contribution was checked, arabinosylcytosine (1.0 μM, SIGMA, MO, USA) was applied at 24 h after cell plating. BDNF (100 ng/ml) was applied 20 min before serum-deprivation. LY294002 (1.0 μM, Calbiochem-Novabiochem, CA, USA) was added 20 min before BDNF application.

MAP2 immunostaining was conducted [27]. Cells were fixed in 4% paraformaldehyde at room temperature for 20 min. After blocking with PBS containing 10% goat serum and 0.2% Triton X-100 for 30 min, anti-MAP2 (1:1000, SIGMA) antibody was incubated overnight at 4 °C. Alexa Fluor 594-conjugated anti-mouse IgG (1:200, Invitrogen, CA, USA) was used as a secondary antibody.

Cells 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). The protein concentration was quantified using a BCA Protein Assay Kit

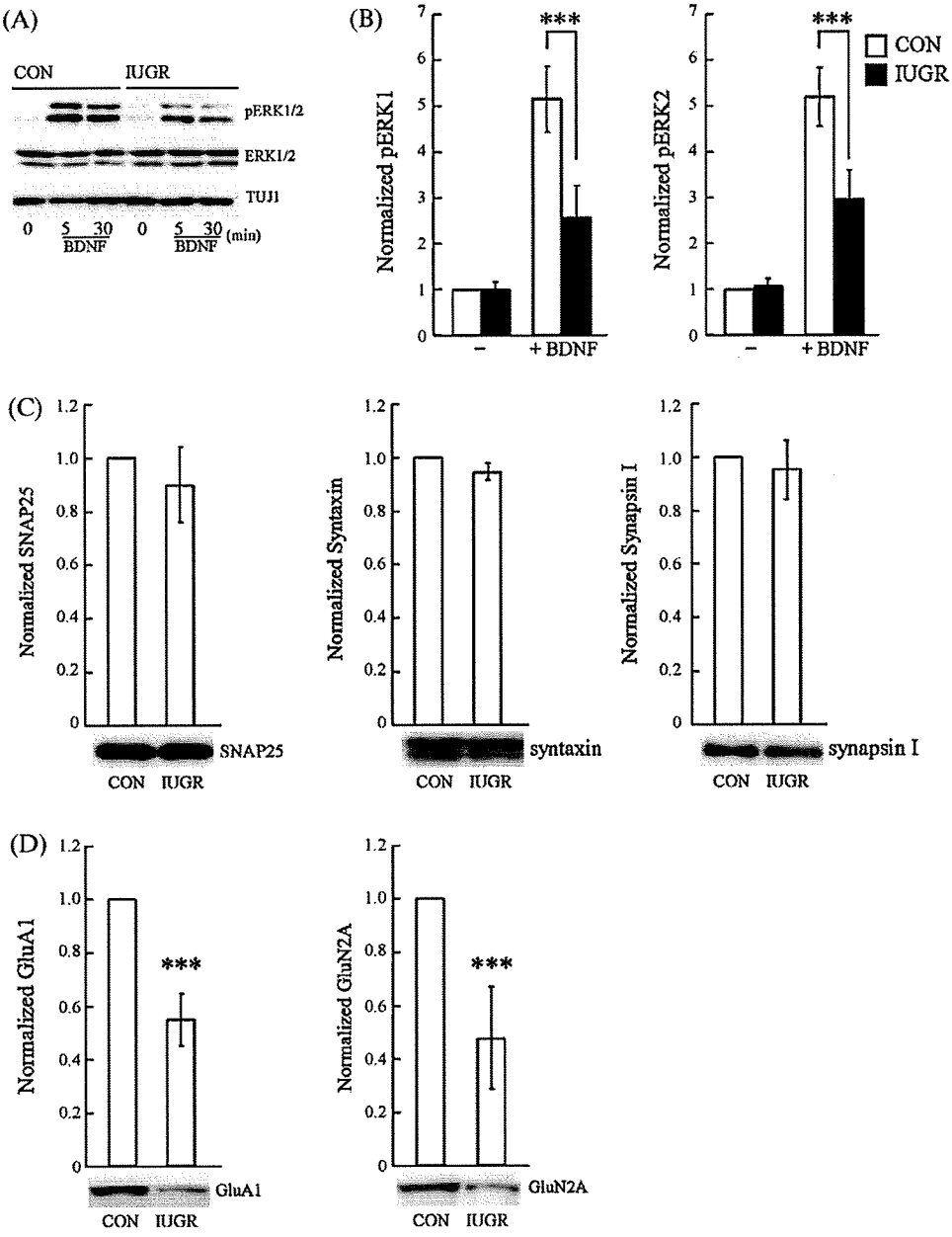

Fig. 2. Decrease in BDNF-stimulated ERK1/2 and in levels of glutamate receptors in IUGR-rat cultures. (A) and (B) Levels of activated ERK1/2 (pERK1/2) in IUGR- or control-rat cultures were examined at 5DIV. Cultured neurons with IUGR- or control-rats were stimulated by BDNF (100 ng/ml) for 0 min, 5 min, or 30 min. pERK1/2 stimulated by BDNF (5 min) was suppressed in IUGR-rat cultures. To quantify the pERK1/2 (BDNF 5 min), normalization to a level in control was performed. Data represent mean ± SD ($n = 5$), ***$P < 0.001$. Total ERK1/2 was unchanged. TUJ1 is shown as a control. (C) Presynaptic proteins including SNAP25, syntaxin, and synapsin I were unchanged in IUGR-rat cultures. Data represent mean ± SD (SNAP25, $n = 6$, syntaxin, $n = 6$, synapsin I, $n = 6$). (D) Postsynaptic glutamate receptor (GluA1 and GluN2A) levels in IUGR-rat neurons were reduced. Data represent mean ± SD (GluA1, $n = 6$, GluN2A, $n = 5$). ***$P < 0.001$. The four independent series of cultures were used for each set of experiments.

(PIERCE, IL, USA), and equivalent amounts of protein were applied for each immunoblotting. 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-GluN2A (NR2A) (1:500, SIGMA), anti-GluA1 (GluR1) (1:1000, CHEMICON, CA, USA), anti-SNAP25 (1:1000, Synaptic Systems, Gottingen, Germany), anti-syntaxin (1:10000, SIGMA), anti-synapsin I (1:2000, CHEMICON), anti-Bcl2 (1:1000, BD Biosciences, CA, USA), anti-TUJ1 (1:5000, Berkeley Antibody Company, CA, USA), anti-p75 (1:1000, Promega, WI, USA), anti-TrkB (1:1000, BD Biosciences), and anti-BDNF (1:200, Santa Cruz Biotechnology Inc., CA, USA) antibodies. The immunoreactivity was quantified by using Lane & Spot Analyzer software (ATTO Corporation, Tokyo, Japan).

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

Initially, we examined the possible change in levels of endogenous BDNF and associated receptors in 5 days in vitro (5DIV) neurons prepared from the cerebral cortex of low weight pups with TXA2-induced IUGR (IUGR-rats). Birth weight was decreased by approximately 16% due to IUGR (control rats: 7.76 ± 0.25 g;

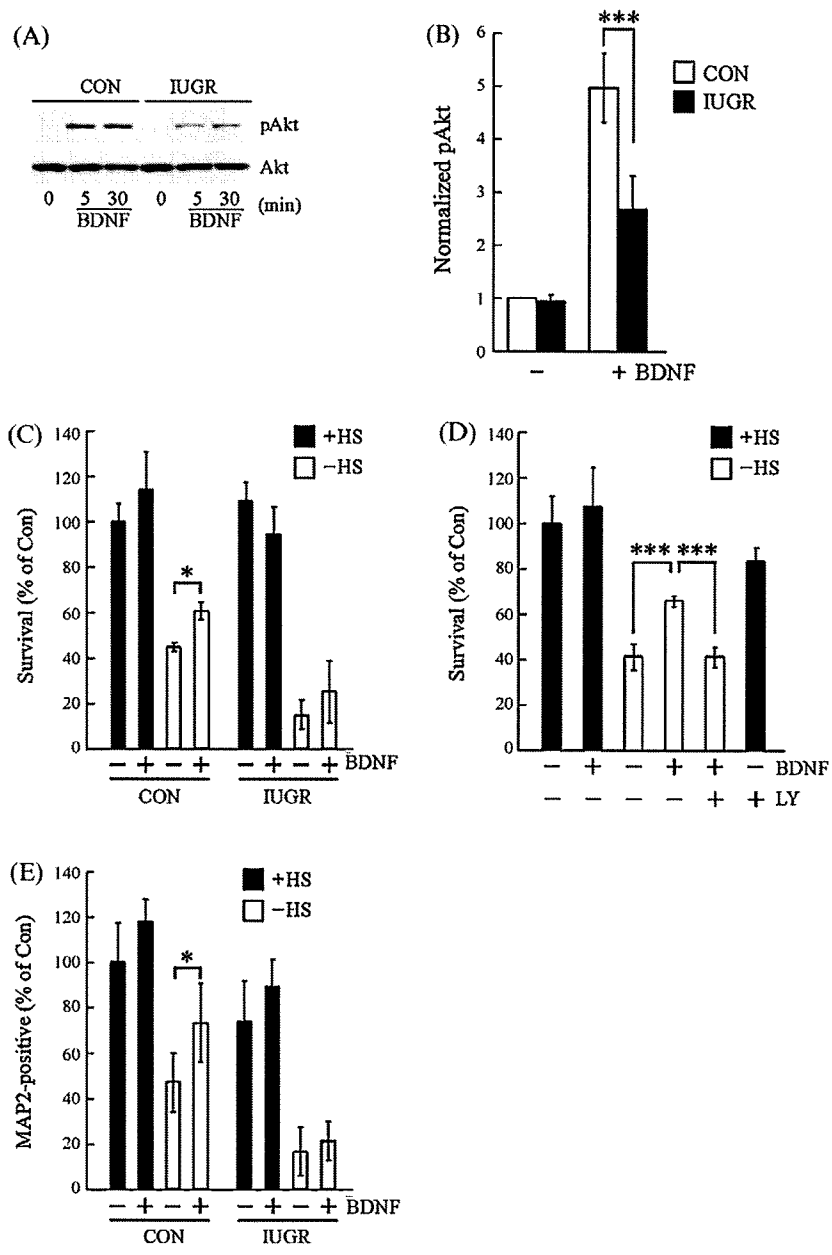

Fig. 3. Cortical neurons from IUGR-rats demonstrated a weakened response to BDNF measured through activation of the PI3K/Akt pathway and neuronal survival. (A) and (B) BDNF-stimulated Akt, a component of the PI3K pathway, was diminished in IUGR-rat cultures at 5DIV. BDNF (100 ng/ml) was applied for the indicated number of minutes. Activated Akt (pAkt, BDNF 5 min) was quantified. Normalization to a level in control was performed. Data represent mean ± SD ($n = 5$), $^{***}P < 0.001$. Three independent series of cultures were used for experiments. (C) The survival-promoting effect of BDNF was lost in IUGR-rat cultures. Cell viability was determined by MTT assay. Data represent mean ± SD ($n = 5$, n indicates the number of wells of a plate for each experimental condition), $^{*}P < 0.05$. To confirm reproducibility, the three independent series of cultures were used. (D) BDNF-dependent survival was blocked by LY294002 (1 μM), an inhibitor for PI3K. Data represent mean ± SD ($n = 6$, n indicates the number of wells of a plate for each experimental condition. MTT assay), $^{***}P < 0.001$. To confirm reproducibility, the three independent series of cultures were used. (E) Neuronal survival was determined by MAP2 immunostaining. The number of MAP2-positive cells was counted. The lower response to BDNF was confirmed in IUGR-rat cultures. Data represent mean ± SD ($n = 11$, n indicates the number of wells of a plate for each experimental condition), $^{*}P < 0.05$.

IUGR-rats: 6.54 ± 0.40 g, $n = 6$ for each). As shown in Fig. 1A, BDNF levels in neurons from IUGR-rats were unchanged compared with those from normally weighed rats (control-rats). In contrast, TrkB, a high affinity receptor for BDNF, was significantly decreased in neurons from IUGR-rats (Fig. 1B). A low affinity common receptor for neurotrophins, p75, was unchanged in cultures from IUGR-rats (Fig. 1C). Such down-regulation of TrkB was confirmed in homogenates from the cerebral cortex of IUGR-rats (Fig. 1D), suggesting that the change observed in TrkB levels in culture is not specific to culture conditions. TUJ1 (class III β-tubulin, a neuronal marker) levels are shown as a control (Fig. 1A–D). As BDNF/TrkB signaling is important for expression of Bcl2 [4], we determined the Bcl2 levels and found marked reduction in IUGR-rat cultures, though TUJ1 was unchanged (Fig. 1E), raising a possibility that IUGR makes cortical neurons vulnerable to death-inducible stimuli. Cell viability of cultures from both IUGR- and control-rats was decreased after serum-deprivation (Fig. 1F). Expectedly, the level of decrease in cell viability of IUGR-rat neurons was larger than that of control-rats. We observed a clear reduction of TrkB and Bcl2 levels in 8DIV cultures (Supplementary Fig.S1), implying that down-

regulation of TrkB and Bcl2 proteins due to IUGR is sustained during neuronal maturation. Furthermore, decreased viability of 8DIV neurons from IUGR-rats was confirmed (Supplementary Fig.S1).

Next, activation of intracellular signaling stimulated by BDNF was examined. Activation of ERK1/2 (phosphorylated ERK1/2, pERK1/2) 5 min after BDNF application was reduced in IUGR-rat cultures while total ERK1/2 and TUJ1 levels were unchanged (Fig. 2A and B). In the presence of arabinosylcytosine, which prevents glial cell proliferation, the reduced pERK1/2 in IUGR-rat cultures when BDNF was added was also observed (Supplementary Fig.S2), suggesting that this suppression of ERK1/2 activation is a neuronal, and not glial, response. We previously reported that ERK1/2 regulates synaptic protein expression [23,26]. In the current study, the expression levels of SNAP25, syntaxin, and synapsin I were unchanged in IUGR-rat neurons compared with control (Fig. 2C). Interestingly, ionotropic glutamate receptors (GluA1 and GluN2A) in IUGR-rat cultures were down-regulated (Fig. 2D). It is possible that the decreased activity of the ERK1/2 pathway via IUGR results in down-regulation of glutamate receptors.

The activation of Akt, a component of the PI3K pathway (well-known as a survival promoting pathway), was also determined. BDNF-stimulated activation of Akt (phosphorylated Akt, pAkt) was reduced in IUGR-rat neurons with or without arabinosylcytosine treatment (Fig. 3A and B, and Supplementary Fig.S2). To test whether the PI3K/Akt pathway is involved in neuronal survival [9], we examined the BDNF-dependent protection of cortical neurons from cell death caused by serum-deprivation. MTT assay revealed that BDNF inhibited the cell death in control-rat cultures, however, the protection by BDNF was not observed in IUGR-rat cultures (Fig. 3C). In control cultures, BDNF upregulated Bcl2, an anti-apoptotic protein (Supplementary Fig.S3). We confirmed that LY294002, a PI3K inhibitor, blocked BDNF-dependent survival in control cultures (Fig. 3D). Furthermore, immunostaining with anti-MAP2 (microtubule-associated protein 2, neuronal marker) antibody revealed that, though the number of MAP2-positive surviving cells was reduced after serum-deprivation in both IUGR- and control cultures, a lower response to BDNF was confirmed in IUGR-rat neurons compared with control (Fig. 3E). Taken together, it is possible that the survival-promoting effect of BDNF was weakened in IUGR-rat neurons.

We found that TrkB (not BDNF) was significantly decreased in cortical cultures from IUGR-rats. Consistent with the reduction of TrkB, BDNF-stimulated MAPK/ERK1/2 and PI3K/Akt pathways were diminished in IUGR-rat cultures compared with control. Bcl2, a survival promoting protein, was also down-regulated in IUGR-rat cultures. We also found a significant decrease in synaptic protein (GluA1 and GluN2A) levels in IUGR-rat cultures. Interestingly, cortical neurons from IUGR-rats showed vulnerability to cell death as well as a weakened response to the survival-promoting effect of BDNF.

Impairment of BDNF and TrkB functions has been implicated in the pathogenesis of schizophrenia [6,11], as well as other neuropsychiatric diseases such as depression [5]. A recent study demonstrated a reduction in BDNF in the dorsolateral prefrontal cortex of schizophrenics [34], suggesting that BDNF down-regulation may affect the functions of intrinsic cortical neurons, afferent neurons, and target neurons. Remarkably, an animal model of IUGR by TXA2 showed decreased BDNF and NT-3 mRNA in the cerebral cortex [14]. In our system, TrkB was decreased by IUGR, although BDNF levels were not altered. In addition to ligand (BDNF), a change in the expression of receptor (TrkB) may contribute to neuronal dysfunction due to IUGR.

Perhaps the down-regulation of TrkB in IUGR-rat neurons occurred as a result of post-transcriptional modifications. Ernst et al. reported that TrkB.T1 (one of the truncated types of TrkB) is down-regulated in the frontal cortex in a subset of suicide victims compared with controls and that this down-regulation is associated with methylation at specific CpG dinucleotides proximal to the coding region [12]. Indeed, several susceptibility genes for schizophrenia are subject to changes in transcriptional activity due to histone modifications and DNA methylation [15]. To date, most studies exploring DNA methylation changes in schizophrenia postmortem brain were focused on the cerebral cortex, primarily its prefrontal areas [3]. Various degrees of aberrant CpG hyper- or hypomethylation have been reported in regulatory sequences of promoters of genes involved in the cortical dysfunction of schizophrenia, including the glycoprotein *REELIN*, *COMT*, and *SOX10* [1,2,3,16,22]. Thus, TrkB reduction via IUGR in our models may be due to DNA methylation, although further studies are required to confirm this possibility.

In IUGR-rat cultures, the expression of postsynaptic proteins, GluA1 and GluN2A, was decreased. In the hippocampus of schizophrenia postmortem brains, reduced expression of subunits for ionotropic glutamate receptors (including NMDA, AMPA, and kainate type receptors) was reported [17]. Recently, we found that intracellular signaling, including the MAPK/ERK pathway, has an important role in the maintenance of synaptic proteins and is involved in schizophrenia [19,23,26,29]. The decrease of TrkB expression may lead to the reduction in postsynaptic proteins as observed in patients with schizophrenia.

TrkB down-regulation may cause reduced activation of the MAPK/ERK and PI3K/Akt pathways in response to BDNF. These pathways are critical for synaptic protein expression and neuronal survival. We confirmed that cortical neurons from IUGR-rats were vulnerable to cell death by serum-deprivation. Bcl2 expression and survival-promoting effects of BDNF were also decreased in IUGR-rat cultures. Increased vulnerability of neurons to neurotoxic damage caused by inadequate neurotrophic support is thought to be involved in the etiology of psychiatric disease [6]. In summary, our results suggest that impairment of BDNF-TrkB signaling caused by IUGR and the resultant decrease in viability of neurons and expression of glutamate receptors may be responsible, at least in part, for the cortical dysfunction observed in schizophrenia-like behaviors. We demonstrated that our in vitro system may offer a useful model for studies to investigate the cellular mechanisms of schizophrenia.

Acknowledgements

We thank Regeneron Pharmaceutical Co., Takeda Chemical Industries, Ltd., and Dainippon Sumitomo Pharma Co. Ltd. for donating the BDNF. This study was supported by the Research Grants for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, Health and Labor Sciences Research Grants (Research on Psychiatric and Neurological Diseases and Mental Health), Health and Labor Sciences Research Grants, a grant from the Japan Foundation for Neuroscience and Mental Health, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (H. K.), and a Grant-in-Aid for Young Scientists (A) (21680034) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (T. N.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2010.03.082.

References

[1] H.M. Abdolmaleky, K.H. Cheng, S.V. Faraone, M. Wilcox, S.J. Glatt, F. Gao, C.L. Smith, R. Shafa, B. Aeali, J. Carnevale, H. Pan, P. Papageorgis, J.F. Ponte, V. Sivaraman, M.T. Tsuang, S. Thiagalingam, Hypomethylation of MB-COMT promoter

is a major risk factor for schizophrenia and bipolar disorder, Hum. Mol. Genet. 15 (2006) 3132–3145.
[2] H.M. Abdolmaleky, K.H. Cheng, A. Russo, C.L. Smith, S.V. Faraone, M. Wilcox, R. Shafa, S.J. Glatt, G. Nguyen, J.F. Ponte, S. Thiagalingam, M.T. Tsuang, Hypermethylation of the reelin (RELN) promoter in the brain of schizophrenic patients: a preliminary report, Am. J. Med. Genet. B: Neuropsychiatr. Genet. 134B (2005) 60–66.
[3] S. Akbarian, The molecular pathology of schizophrenia-Focus on histone and DNA modifications, Brain Res. Bull., in press.
[4] R.D. Almeida, B.J. Manadas, C.V. Melo, J.R. Gomes, C.S. Mendes, M.M. Grãos, R.F. Carvalho, A.P. Carvalho, C.B. Duarte, Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways, Cell Death Differ. 12 (2005) 1329–1343.
[5] C.A. Altar, Neurotrophins and depression, Trends Pharmacol. Sci. 20 (1999) 59–61.
[6] F. Angelucci, S. Brenè, A.A. Mathé, BDNF in schizophrenia, depression and corresponding animal models, Mol. Psychiatry 10 (2005) 345–352.
[7] O. Arancio, M.V. Chao, Neurotrophins, synaptic plasticity and dementia, Curr. Opin. Neurobiol. 17 (2007) 325–330.
[8] P. Boksa, Animal models of obstetric complications in relation to schizophrenia, Brain Res. Brain Res. Rev. 45 (2004) 1–17.
[9] H. Dudek, S.R. Datta, T.F. Franke, M.J. Birnbaum, R. Yao, G.M. Cooper, R.A. Segal, D.R. Kaplan, M.E. Greenberg, Regulation of neuronal survival by the serine-threonine protein kinase Akt, Science 275 (1997) 661–665.
[10] N. Durany, T. Michel, R. Zochling, K.W. Boissl, F.F. Cruz-Sanchez, P. Riederer, J. Thome, Brain-derived neurotrophic factor and neurotrophin 3 in schizophrenic psychoses, Schizophr. Res. 52 (2001) 79–86.
[11] N. Durany, J. Thome, Neurotrophic factors and the pathophysiology of schizophrenic psychoses, Eur. Psychiatry 19 (2004) 326–337.
[12] C. Ernst, V. Deleva, X. Deng, A. Sequeira, A. Pomarenski, T. Klempan, N. Ernst, R. Quirion, A. Gratton, M. Szyf, G. Turecki, Alternative splicing, methylation state, and expression profile of tropomyosin-related kinase B in the frontal cortex of suicide completers, Arch. Gen. Psychiatry 66 (2009) 22–32.
[13] W.G. Frankle, J. Lerma, M. Laruelle, The synaptic hypothesis of schizophrenia, Neuron 39 (2003) 205–216.
[14] E. Fukami, A. Nakayama, J. Sasaki, S. Mimura, N. Mori, K. Watanabe, Underexpression of neural cell adhesion molecule and neurotrophic factors in rat brain following thromboxane A(2)-induced intrauterine growth retardation, Early Hum. Dev. 58 (2000) 101–110.
[15] D.P. Gavin, R.P. Sharma, Histone modifications, DNA methylation, and schizophrenia, Neurosci. Biobehav. Rev. 34 (2010) 882–888.
[16] D.R. Grayson, X. Jia, Y. Chen, R.P. Sharma, C.P. Mitchell, A. Guidotti, E. Costa, Reelin promoter hypermethylation in schizophrenia, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 9341–9346.
[17] P.J. Harrison, A.J. Law, S.L. Eastwood, Glutamate receptors and transporters in the hippocampus in schizophrenia, Ann. N. Y. Acad. Sci. 1003 (2003) 94–101.
[18] P.J. Harrison, D.R. Weinberger, Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence, Mol. Psychiatry 10 (2005) 40–68.
[19] R. Hashimoto, T. Numakawa, T. Ohnishi, E. Kumamaru, Y. Yagasaki, T. Ishimoto, T. Mori, K. Nemoto, N. Adachi, A. Izumi, S. Chiba, H. Noguchi, T. Suzuki, N. Iwata, N. Ozaki, T. Taguchi, A. Kamiya, A. Kosuga, M. Tatsumi, K. Kamijima, D.R. Weinberger, A. Sawa, H. Kunugi, Impact of the DISC1 Ser704Cys polymorphism on risk for major depression, brain morphology and ERK signaling, Hum. Mol. Genet. 15 (2006) 3024–3033.
[20] M. Hayakawa, S. Mimura, J. Sasaki, K. Watanabe, Neuropathological changes in the cerebrum of IUGR rat induced by synthetic thromboxane A2, Early Hum. Dev. 55 (1999) 125–136.
[21] S. Iritani, K. Niizato, H. Nawa, K. Ikeda, P.C. Emson, Immunohistochemical study of brain-derived neurotrophic factor and its receptor, TrkB, in the hippocampal formation of schizophrenic brains, Prog. Neuropsychopharmacol. Biol. Psychiatry 27 (2003) 801–807.
[22] K. Iwamoto, M. Bundo, K. Yamada, H. Takao, Y. Iwayama-Shigeno, T. Yoshikawa, T. Kato, DNA methylation status of SOX10 correlates with its downregulation and oligodendrocyte dysfunction in schizophrenia, J. Neurosci. 25 (2005) 5376–5381.
[23] E. Kumamaru, T. Numakawa, N. Adachi, Y. Yagasaki, A. Izumi, M. Niyaz, M. Kudo, H. Kunugi, Glucocorticoid prevents brain-derived neurotrophic factor-mediated maturation of synaptic function in developing hippocampal neurons through reduction in the activity of mitogen-activated protein kinase, Mol. Endocrinol. 22 (2008) 546–558.
[24] H. Kunugi, Obstetric and delivery complications as risk factors for schizophrenia: neurodevelopmental perspectives, in: A. Grispini (Ed.), Preventive Strategies for Schizophrenic Disorders: Basic Principles, Opportunities and Limits, Giovanni Floriti Editore, 2003, pp. 159–187.
[25] H. Kunugi, S. Nanko, R.M. Murray, Obstetric complications and schizophrenia: prenatal underdevelopment and subsequent neurodevelopmental impairment, Br. J. Psychiatry Suppl. 40 (2001) s25–29.
[26] T. Matsumoto, T. Numakawa, D. Yokomaku, N. Adachi, S. Yamagishi, Y. Numakawa, H. Kunugi, T. Taguchi, Brain-derived neurotrophic factor-induced potentiation of glutamate and GABA release: different dependency on signaling pathways and neuronal activity, Mol. Cell. Neurosci. 31 (2006) 70–84.
[27] T. Numakawa, E. Kumamaru, N. Adachi, Y. Yagasaki, A. Izumi, H. Kunugi, Glucocorticoid receptor interaction with TrkB promotes BDNF-triggered PLC-gamma signaling for glutamate release via a glutamate transporter, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 647–652.
[28] T. Numakawa, S. Yamagishi, N. Adachi, T. Matsumoto, D. Yokomaku, M. Yamada, H. Hatanaka, Brain-derived neurotrophic factor-induced potentiation of Ca(2+) oscillations in developing cortical neurons, J. Biol. Chem. 277 (2002) 6520–6529.
[29] T. Numakawa, Y. Yagasaki, T. Ishimoto, T. Okada, T. Suzuki, N. Iwata, N. Ozaki, T. Taguchi, M. Tatsumi, K. Kamijima, R.E. Straub, D.R. Weinberger, H. Kunugi, R. Hashimoto, Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia, Hum. Mol. Genet. 13 (2004) 2699–2708.
[30] Y. Numakawa, T. Numakawa, T. Matsumoto, Y. Yagasaki, E. Kumamaru, H. Kunugi, T. Taguchi, E. Niki, Vitamin E protected cultured cortical neurons from oxidative stress-induced cell death through the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase, J. Neurochem. 97 (2006) 1191–1202.
[31] A. Saito, F. Matsui, K. Hayashi, K. Watanabe, Y. Ichinohashi, Y. Sato, M. Hayakawa, S. Kojima, A. Oohira, Behavioral abnormalities of fetal growth retardation model rats with reduced amounts of brain proteoglycans, Exp. Neurol. 219 (2009) 81–92.
[32] R. Stoop, M.M. Poo, Synaptic modulation by neurotrophic factors: differential and synergistic effects of brain-derived neurotrophic factor and ciliary neurotrophic factor, J. Neurosci. 16 (1996) 3256–3264.
[33] K. Toyooka, K. Asama, Y. Watanabe, T. Muratake, M. Takahashi, T. Someya, H. Nawa, Decreased levels of brain-derived neurotrophic factor in serum of chronic schizophrenic patients, Psychiatry Res. 110 (2002) 249–257.
[34] C.S. Weickert, T.M. Hyde, B.K. Lipska, M.M. Herman, D.R. Weinberger, J.E. Kleinman, Reduced brain-derived neurotrophic factor in prefrontal cortex of patients with schizophrenia, Mol. Psychiatry 8 (2003) 592–610.
[35] G.L. Zornberg, S.L. Buka, M.T. Tsuang, Hypoxic-ischemia-related fetal/neonatal complications and risk of schizophrenia and other nonaffective psychoses: a 19-year longitudinal study, Am. J. Psychiatry 157 (2000) 196–202.
[36] B. Zorner, D.P. Wolfer, D. Brandis, O. Kretz, C. Zacher, R. Madani, I. Grunwald, H.P. Lipp, R. Klein, F.A. Henn, P. Gass, Forebrain-specific trkB-receptor knockout mice: behaviorally more hyperactive than "depressive", Biol. Psychiatry 54 (2003) 972–982.

Journal of Psychiatric Research xxx (2010) 1–7

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/psychires

Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia

Takashi Fujii [a,b,c], Hirofumi Uchiyama [a], Noriko Yamamoto [a], Hiroaki Hori [a], Masahiko Tatsumi [d], Masanori Ishikawa [e], Kunimasa Arima [e], Teruhiko Higuchi [f], Hiroshi Kunugi [a,b,*]

[a] *Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo, Japan*
[b] *Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi-shi, Saitama, Japan*
[c] *Japan Human Sciences Foundation, 13-4 Kodenma-cho Nihonbashi, Chuo-ku, Tokyo, Japan*
[d] *Yokohama Shinryo Clinic, Yamamoto Bldg. 2F, 3-28-5 Tsuruyacho, Kanagawa-ku, Yokohama, Japan*
[e] *Department of Psychiatry, National Center Hospital of Neurology and Psychiatry, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo, Japan*
[f] *National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo, Japan*

ARTICLE INFO

Article history:
Received 22 January 2010
Received in revised form
1 April 2010
Accepted 6 May 2010

Keywords:
Semaphorin
Plexin
Non-synonymous polymorphism
Schizophrenia
Haplotype

ABSTRACT

Semaphorins are ligands of plexins, and the plexin–semaphorin signaling system is widely involved in many neuronal events including axon guidance, cell migration, axon pruning, and synaptic plasticity. The plexin A2 gene (*PLXNA2*) has been reported to be associated with schizophrenia. This finding prompted us to examine the possible association between the semaphorin 3D gene (*SEMA3D*) and schizophrenia in a Japanese population. We genotyped 9 tagging single nucleotide polymorphisms (SNPs) of *SEMA3D* including a non-synonymous variation, Lys701Gln (rs7800072), in a sample of 506 patients with schizophrenia and 941 healthy control subjects. The Gln701 allele showed a significant protective effect against the development of schizophrenia ($p = 0.0069$, odds ratio = 0.76, 95% confidence interval 0.63 to 0.93). Furthermore, the haplotype-based analyses revealed a significant association. The four-marker analysis (rs2190208–rs1029564–rs17159614–rs12176601), in particular, not including the Lys701Gln, revealed a highly significant association ($p = 0.00001$, global permutation), suggesting that there may be other functional polymorphisms within *SEMA3D*. Our findings provide strong evidence that *SEMA3D* confers susceptibility to schizophrenia, which could contribute to the neurodevelopmental impairments in the disorder.

1. Introduction

The first discovered semaphorin, collapsing-1 (now Sema3A), was originally reported as a repulsive cue in axon guidance (Luo et al., 1993). To date, more than 20 semaphorins of secreted or membrane forms have been identified in various species ranging from nematodes to humans (Luo et al., 1993; Fujii et al., 2002; Yazdani and Terman, 2006). Semaphorins act as ligands for plexins, and the plexin–semaphorin signaling system has been widely investigated in nervous systems (Mann et al., 2007). Class 3 semaphorins (SEMA3A-G) have been well-studied and generally act as secreted ligands for the heterodimerized complex of the plexin A family members and neuropilins (Fujisawa, 2004). For example, Sema3A binds to neuropilin-1 and activates plexin A1 or plexin A2 to transduce a repulsive axon guidance signal (Takahashi and Strittmatter, 2001). Many studies of the plexin–semaphorin signaling system have concentrated on their roles in neuronal development and plasticity (reviewed in (Kruger et al., 2005; Halloran and Wolman, 2006; Waimey and Cheng, 2006; Mann et al., 2007)).

Recently, the relationship between schizophrenia and molecules in the plexin–semaphorin signaling system has begun to receive much attention, for several reasons (Mann et al., 2007). An increase in levels of SEMA3A was noted in the cerebellum in postmortem brains of schizophrenia patients, as measured by immunoreactivity in the inner molecular layer and by the enzyme-linked immunosorbent assay (ELISA) in cerebellar protein extract (Eastwood et al., 2003). A genome-wide association study using 25,494 single nucleotide polymorphisms (SNPs) revealed that an intronic SNP of *PLXNA2* was most consistently associated with schizophrenia in European–American populations (Mah et al., 2006). Our replication study in a Japanese sample failed to confirm such an association (Fujii et al., 2007); however, a meta-analysis combining data from previous studies of *PLXNA2* yielded a positive association with schizophrenia (Allen et al., 2008), in which it was reported that the C allele of the SNP rs752016 of *PLXNA2* showed a nominally significant protective effect (odds ratios (OR) = 0.82, 95%

* Corresponding author. Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. Tel./fax: +81 42 346 1714.

0022-3956/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jpsychires.2010.05.004

confidence interval (CI) = 0.69–0.99), and association of the SNP rs841865 approached statistical significance (OR = 0.84, 95% CI = 0.69–1.01) when samples of Mah et al. and Fujii et al. were combined (Mah et al., 2006; Fujii et al., 2007). Furthermore, in the updated online database, "SchizophreniaGene (http://www.schizophreniaforum.org/)," association of the SNP rs1327175 approached statistical significance (OR = 0.76, 95% CI = 0.57–1.00) (Mah et al., 2006; Fujii et al., 2007; Takeshita et al., 2008; Budel et al., 2008). Therefore, genes of the plexin family, the semaphorin family, and neuropilins, are intriguing candidates for schizophrenia susceptibility genes. We then focused on *SEMA3D* as a candidate gene for schizophrenia. *SEMA3D* was mapped to chromosome 7q21 (Clark et al., 2003); interestingly, a previous genome-wide scan suggested that this chromosomal region contains a susceptibility locus for schizophrenia (Ekelund et al., 2000) and recent studies have provided additional support for this possibility (Tastemir et al., 2006; Wedenoja et al., 2008, 2009; Idol et al., 2008).

The aim of the present study was to examine the possible association between *SEMA3D* and schizophrenia. *SEMA3D* has a common variant in the coding region due to an A to C base substitution (rs7800072), which results in an amino acid change (701 Lys to Gln). This SNP has previously been examined with regard to brain morphology (assessed with magnetic resonance imaging) in patients with schizophrenia (Gregorio et al., 2009). Although this study failed to find significant alterations in brain morphology, it is still unclear whether this SNP confers susceptibility to schizophrenia. We examined the possible association of schizophrenia with this non-synonymous SNP, plus 8 tagging SNPs encompassing the entire *SEMA3D* gene.

2. Subjects and methods

2.1. Subjects

Subjects were 506 patients with schizophrenia (278 males [54.9%], mean age 44.3 years [SD 14.1]) and 941 healthy controls (334 males [35.5%], mean age 44.8 years [SD 16.3]). All subjects were Japanese, biologically unrelated, and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from the same geographical area. Control individuals were interviewed and those who had a current or past history of psychiatric treatment were not enrolled in the study. The study protocol was approved by the ethics committee of the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

2.2. SNP selection

The tagging SNPs were selected using the phase III version of HapMap (http://www.hapmap.org/cgi-perl/gbrowse/). SNP genotype data for the JPT (Japanese in Tokyo, Japan) were downloaded for the genomic region of *SEMA3D* plus 2 kb 5′ and 2 kb 3′ of this region (chr7q21.11). The most centromeric and telomeric HapMap markers downloaded were rs6944966 and rs11762367, respectively. HapMap markers were analyzed using the Haploview 4.1 system (http://www.broad.mit.edu/mpg/haploview) with the following criteria of marker selection: Hardy–Weinberg (HW) p value cutoff: 0.05; minimum genotypes: 90%; maximum number of Mendelian errors: 1; minimum minor allele frequency: 0.1; minimum distance between tags: 10 kb. Tagging SNPs were selected using the Tagger function implemented in Haploview with the following criteria: pairwise tagging only and r^2 threshold 0.8. We preselected rs7800072 and rs6966472 as markers and used the Tagger function implemented in Haploview to select other markers. As a result, 9 markers were selected as suitable for analysis for *SEMA3D*. SNP rs7800072 is non-synonymous (2141A > C, Lys701Gln). The numbers of base and amino acid positions were according to NM_152754.2 and NP_689967.2, respectively.

2.3. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay; the assay ID (Applied Biosystems, Foster City, CA) of each SNP was C__15937080_10 for rs2190208, C___7585979_10 for rs1029564, C__33462384_10 for rs17159614, C__31373903_10 for rs12176601, C___2635874_10 for rs6966472, C___2635864_10 for rs17559978, C__33462432_10 for rs17159577, C__33462438_10 for rs17159556, and C__25994972_10 for rs7800072. Thermal cycling conditions for polymerase chain reaction (PCR) were 1 cycle at 95 °C for 10 min followed by 50 cycles of 92 °C for 15 s and 60 °C for 1 min. Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis.

2.4. Haplotype and statistical analysis

Deviations of genotype distributions from the HW equilibrium (HWE) were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. These tests were performed with SPSS software ver.11 (SPSS Japan, Tokyo, Japan). Haplotype-based association analyses were performed with SNPAlyze software ver.6.5 (http://www.dynacom.co.jp/e/products/package/snpalyze/about.html). The measures of linkage disequilibrium (LD), denoted as D′ and r^2, were calculated from the haplotype frequency using the expectation-maximization (EM) algorithm. Haplotypes with frequencies of less than 1% were considered to be rare and were excluded from the analyses. All p values reported are two-tailed. We performed 100,000 permutations only for some significant haplotypes (e.g., rs2190208–rs1029564–rs17159614–rs121176601) and 10,000 permutations for the other haplotypes. OR and 95% CI were also calculated. To correct the critical p value for multiple testing, we used the spectral decomposition method of SNPSpD software (http://gump.qimr.edu.au/general/daleN/SNPSpD/) (Nyholt, 2004; Li and Ji, 2005), which considers marker linkage disequilibrium information and generates an experiment-wide significance threshold required to keep the type I error rate at 5%.

3. Results

Genotype and allele distributions of the examined SNPs of *SEMA3D* in patients and controls are shown in Table 1. LD estimates of pairwise SNPs, expressed in D′ and r^2, are presented in Fig. 1. The genotype distributions did not significantly deviate from the HWE in patients and controls for any of the examined SNPs. For the non-synonymous polymorphism of *SEMA3D* (rs7800072), there were significant differences in both genotype ($\chi^2 = 8.7$, df = 2, $p = 0.013$) and allele ($\chi^2 = 7.3$, df = 1, $p = 0.0069$, OR = 0.76, 95% CI 0.63–0.93) distributions between patients and controls (Table 1). Furthermore, with respect to the other 8 SNPs (rs2190208, rs1029564,

Please cite this article in press as: Fujii T, et al., Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia, Journal of Psychiatric Research (2010), doi:10.1016/j.jpsychires.2010.05.004

Table 1
Genotype and Allelic Distribution of the SEMA3D SNPs in Japanese Patients with Schizophrenia, and Controls.

dbSNP ID	position[a]	Inter-SNP distance (bp)	Group	N	Genotype distribution (frequency)			Allele distribution (frequency)		Odds ratio (95% CI)	Chi-square test[b]		
											HWE(df = 1)[c]	GF(df = 2)[d]	AF(df = 1)[e]
rs2190208	9986227				GG	GA	AA	G	A				
	5′ promoter	____	Schizophrenia	494	186 (0.38)	231 (0.47)	77 (0.16)	603 (0.61)	385 (0.39)	0.96	$\chi2 = 0.14$, $p = 0.71$	$p = 0.48$	$p = 0.59$
			Control	930	325 (0.35)	466 (0.50)	139 (0.15)	1116 (0.60)	744 (0.40)	(0.82–1.12)	$\chi2 = 1.79$, $p = 0.18$	$\chi2 = 1.48$	$\chi2 = 0.29$
rs1029564	9974131				AA	AC	CC	A	C				
	intron 1	12096	Schizophrenia	492	334(0.68)	140 (0.28)	18 (0.04)	808 (0.82)	176 (0.18)	0.78	$\chi2 = 0.48$, $p = 0.48$	$\mathbf{p = 0.028}$	$\mathbf{p = 0.011}$
			Control	931	565 (0.61)	324 (0.35)	42 (0.05)	1454 (0.78)	408 (0.22)	(0.64–0.94)	$\chi2 = 0.27$, $p = 0.61$	$\chi2 = 7.17$	$\chi2 = 6.40$
rs17159614	9959778				GG	GA	AA	G	A				
	intron 2	14353	Schizophrenia	495	289 (0.58)	181(0.37)	25 (0.05)	759 (0.77)	231 (0.23)	1.00	$\chi2 = 0.24$, $p = 0.62$	$p = 1.00$	$p = 0.96$
			Control	931	545 (0.59)	339 (0.36)	47(0.05)	1429(0.77)	433 (0.23)	(0.83–1.19)	$\chi2 = 0.38$, $p = 0.54$	$\chi2 = 0.0034$	$\chi2 = 0.0023$
rs12176601	9948019				TT	TA	AA	T	A				
	intron 2	11759	Schizophrenia	493	166(0.34)	244 (0.49)	83 (0.17)	576 (0.58)	410 (0.42)	1.21	$\chi2 = 0.17$, $p = 0.68$	$\mathbf{p = 0.029}$	$\mathbf{p = 0.021}$
			Control	917	375 (0.41)	403 (0.44)	139 (0.15)	1153 (0.63)	681 (0.37)	(1.03–1.41)	$\chi2 = 3.16$, $p = 0.08$	$\chi2 = 7.08$	$\chi2 = 5.35$
rs6966472	9933663				AA	AG	GG	A	G				
	intron 4	14356	Schizophrenia	493	381 (0.77)	103 (0.21)	9 (0.02)	865 (0.88)	121 (0.12)	0.73	$\chi2 = 0.43$, $p = 0.51$	$\mathbf{p = 0.023}$	$\mathbf{p = 0.0075}$
			Control	931	656 (0.70)	252 (0.27)	23 (0.02)	1564 (0.84)	298 (0.16)	(0.59–0.92)	$\chi2 = 0.04$, $p = 0.84$	$\chi2 = 7.59$	$\chi2 = 7.16$
rs17559978	9912136				CC	CT	TT	C	T				
	intron 7	21527	Schizophrenia	499	339 (0.68)	138 (0.28)	22 (0.04)	816 (0.82)	182 (0.18)	0.80	$\chi2 = 2.63$, $p = 0.10$	$\mathbf{p = 0.029}$	$\mathbf{p = 0.025}$
			Control	936	571 (0.61)	322 (0.34)	43 (0.05)	1464 (0.78)	408 (0.22)	(0.66–0.97)	$\chi2 = 0.08$, $p = 0.78$	$\chi2 = 7.11$	$\chi2 = 5.05$
rs17159577	9900238				CC	CT	TT	C	T				
	intron 10	11898	Schizophrenia	494	244 (0.49)	195 (0.39)	55 (0.11)	683 (0.69)	305 (0.31)	1.05	$\chi2 = 2.79$, $p = 0.09$	$p = 0.15$	$p = 0.60$
			Control	934	453 (0.49)	403 (0.43)	78 (0.08)	1309 (0.70)	559 (0.30)	(0.88–1.24)	$\chi2 = 0.77$, $p = 0.38$	$\chi2 = 3.78$	$\chi2 = 0.27$
rs17159556	9886562				GG	GT	TT	G	T				
	intron 10	13676	Schizophrenia	496	372 (0.75)	112 (0.23)	12 (0.02)	856(0.86)	136(0.14)	0.76	$\chi2 = 1.03$, $p = 0.31$	$\mathbf{p = 0.024}$	$\mathbf{p = 0.012}$
			Control	932	635 (0.68)	271 (0.29)	26 (0.03)	1541 (0.83)	323(0.17)	(0.61–0.94)	$\chi2 = 0.21$, $p = 0.65$	$\chi2 = 7.43$	$\chi2 = 6.29$
rs7800072	9863265				AA	AC	CC	A	C				
	exon 17	23297	Schizophrenia	502	342 (0.68)	140 (0.28)	20 (0.04)	824 (0.82)	180(0.18)	0.76	$\chi2 = 1.37$, $p = 0.24$	$\mathbf{p = 0.013}$	$\mathbf{p = 0.0069}$
	Lyn701Gln		Control	934	563 (0.60)	327 (0.35)	44 (0.05)	1453(0.78)	415 (0.22)	(0.63–0.93)	$\chi2 = 0.16$, $p = 0.69$	$\chi2 = 8.67$	$\chi2 = 7.31$

[a] Chromosome position was established from the dbSNP database.
[b] Without Bonferroni's correction.
[c] HWE; Hardy–Weinberg equilibrium.
[d] GF; Genotype distribution frequency.
[e] AF; Allele distribution frequency.

Please cite this article in press as: Fujii T, et al. Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia. Journal of Psychiatric Research (2010), doi:10.1016/j.jpsychires.2010.05.004

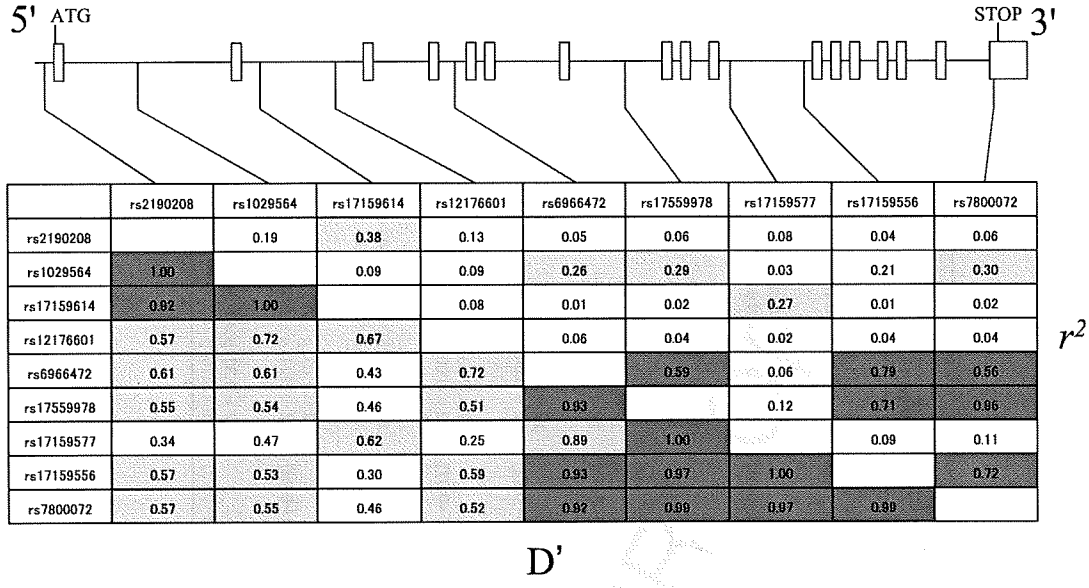

Fig. 1. The genetic structure of *SEMA3D* and location of the examined SNPs. The D' and r^2 values between paired SNPs are shown in the diagram. The exonic regions are shown as white squares. The intensity of the box color corresponds to the strength of LD or r^2.

rs17159614, rs12176601, rs6966472, rs17559978, rs17159577, and rs17159556), several significant differences in genotype and allele distributions were observed (Table 1). To correct for multiple testing, we calculated the experiment-wide significance threshold required to keep the type I error rate at 5%. As a result, the corrected p value was calculated as 0.0085. The allelic associations with the SNPs rs7800072 (Lys701Gln) and rs6966472 remained significant after the correction (Table 1). Distinguishing between the carriers and the non-carriers with respect to the Gln701 allele for patients and controls, the protective effect became clearer ($p = 0.0033$).

The results of haplotype-based analyses are shown in Table 2. There were significant haplotypic associations of the SNPs in *SEMA3D* when comparing the schizophrenic patients and control subjects. In particular, the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601) showed a statistically significant association with schizophrenia (global permutation $p = 0.00001$). Concerning this haplotype analysis, global p values of 100,000 permutations, which corrected for multiple testing, were also significant. Furthermore, the haplotype frequency of GAGA was significantly higher in schizophrenia patients than in control subjects (0.376 and 0.291, permutation $p = 0.00005$), whereas those of GAGT, AAAA, and GCGA were significantly lower in schizophrenic patients than in controls (0.050 and 0.084, permutation $p = 0.0029$; 0.007 and 0.025, permutation $p = 0.0062$; 0.007 and 0.021, permutation $p = 0.020$, respectively) (Table 3).

When we performed stratified analysis of the data for rs7800072 by sex, a significant association was observed in women ($p = 0.0089$), but not in men ($p = 0.41$) (supplementary Tables 1 and 2). In the haplotype analysis, on the other hand, the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601) showed a statistically significant association in men (global permutation $p = 0.00001$), but was at a trend level in women (global permutation $p = 0.0699$). The haplotype frequency of GAGA

Table 2
Associations with schizophrenia of the 9 SNPs and haplotypes in SEMA3D.

SNP No.	dbSNP ID	Allele model	Haplotype p[a]							
		p value	2 Locus	3 Locus	4 Locus	5 Locus	6 Locus	7 Locus	8 Locus	9 Locus
SNP1	rs2190208	0.59								
			0.019							
SNP2	rs1029564	**0.011**		0.10						
			0.029		**0.00001**					
SNP3	rs17159614	0.96		**0.00002**		**0.00005**				
			0.0004		**0.0003**		**0.00007**			
SNP4	rs12176601	**0.021**		**0.0010**		**0.0001**		**0.0003**		
			0.035		**0.0006**		**0.0016**		**0.0007**	
SNP5	rs6966472	**0.0075**		0.053		**0.0004**		**0.0001**		**0.0007**
			0.023		0.098		**0.0001**		**0.0001**	
SNP6	rs17559978	**0.025**		**0.022**		0.061		**0.0004**		
			0.030		**0.025**		0.076			
SNP7	rs17159577	0.60		0.064		**0.024**				
			0.042		0.051					
SNP8	rs17159556	**0.012**		**0.028**						
			0.020							
SNP9	rs7800072	**0.0069**								

[a] global p value.

Please cite this article in press as: Fujii T, et al., Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia, Journal of Psychiatric Research (2010), doi:10.1016/j.jpsychires.2010.05.004

Table 3
Estimated haplotype frequencies and association significance for SEMA3D.

Haplotype	rs2190208	rs1029564	rs17159614	rs12176601	% of individuals					
					Overall	Control	Schizophrenia	$\chi 2$	*p value*	Permutation *p* value
1	G	A	G	A	0.321	0.291	0.376	20.40	**0.0000063**	**0.000050**
2	A	A	A	T	0.207	0.201	0.219	1.21	0.27	0.28
3	G	C	G	T	0.190	0.199	0.172	2.98	0.085	0.089
4	A	A	G	T	0.142	0.143	0.139	0.10	0.75	0.76
5	G	A	G	T	0.072	0.084	0.050	11.23	**0.00080**	**0.0029**
6	A	A	G	A	0.034	0.036	0.031	0.37	0.54	0.59
7	A	A	A	A	0.019	0.025	0.007	10.75	**0.0010**	**0.0062**
8	G	C	G	A	0.016	0.021	0.007	7.55	**0.0060**	**0.020**

	$\chi 2$	*p value*	Permutation *p* value	Replications
Global	46.07	0.000000085	**0.00001**	100000

was significantly higher in schizophrenia patients than in control subjects in both men (0.368 and 0.272, permutation $p = 0.00053$) and women (0.384 and 0.302, permutation $p = 0.003$).

4. Discussion

Our results provide the first evidence for the possible involvement of *SEMA3D* in the pathogenesis of schizophrenia. With respect to the non-synonymous (Lys701Gln) polymorphism, we found a significant preponderance of the Lys/Lys genotype and the Lys701 allele in schizophrenia patients compared with control subjects. In the haplotype-based analyses, we also obtained evidence for an association between *SEMA3D* and schizophrenia. Interestingly, the most significant haplotype, rs2190208–rs1029564–rs17159614–rs121176601, does not include rs7800072 (Lys701Gln) (see Fig. 1). Therefore, it is likely that at least one functional polymorphism other than rs7800072, which is in linkage disequilibrium to the haplotype, could be responsible for susceptibility to schizophrenia. In stratified analysis for rs7800072 by sex, the frequency of the Gln701 allele was significantly lower in schizophrenia patients than in control subjects in women (0.17 and 0.23, $p = 0.0088$) (supplementary Table 2). Likewise, this was also lower in men, but was not statistically significant (0.18 and 0.20, $p = 0.41$) (supplementary Table 1). Regarding analysis of the four-marker haplotype (rs2190208–rs1029564–rs17159614–rs121176601), there remained a statistical significance in men (global permutation $p = 0.00001$) and a tendency in women (global permutation $p = 0.0699$). In addition, the frequency of the most major haplotype (GAGA) was significantly higher in schizophrenia patients than in control subjects in both sexes. These inconsistent results between males and females are likely to have arisen from the lack of statistical power after dividing the sexes.

The neurodevelopmental hypothesis of schizophrenia proposes that abnormalities of brain development are involved in the pathogenesis of schizophrenia (Conrad and Scheibel, 1987; Weinberger, 1987; Murray, 1994; Waddington et al., 1998). In early brain developmental stages, a number of semaphorins play important roles in axonal repulsion, axonal attraction, neuronal cell migration, and axon pruning (reviewed in Kruger et al., 2005; Waimey and Cheng, 2006; Halloran and Wolman, 2006; Mann et al., 2007). Indeed, SEMA3D has been shown to act in axon guidance and cell migration during neuronal development (Wolman et al., 2004, 2007; Liu et al., 2004; Liu and Halloran, 2005; Sakai and Halloran, 2006; Takahashi et al., 2009). With respect to neuronal cell migration, neuronal disarray and abnormal migration in the neocortical white matter were reported in postmortem studies of patients with schizophrenia (Jakob and Beckmann, 1986; Akbarian et al., 1993). Regarding pruning, Feinberg proposed that schizophrenia may arise from excessive synaptic pruning during adolescence (Feinberg, 1982; Keshavan et al., 1994). Indeed, decreased density of dendritic spines was observed in the prefrontal cortex of patients with schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000). These findings suggest that variants of *SEMA3D* may contribute to the pathogenesis of schizophrenia through affecting development of neural networks. The genotypic difference based on the Lys701Gln polymorphism of *SEMA3D* might lead to developmental differences in the brain; the Gln701 carriers would exhibit intrinsically greater protective effects against the development of schizophrenia than the Gln701 non-carriers. Although *SEMA3D* has not yet been well-studied, *SEMA3A* has been investigated in detail. In particular, an increase in the expression of SEMA3A has previously been associated with schizophrenia (Eastwood et al., 2003). Moreover, *PLXNA2*, which encodes one of the receptors for class 3 semaphorins, was identified as a candidate gene for schizophrenia in a genome-wide association study (Mah et al., 2006). Currently, this association is also supported by the meta-analysis of Allen et al. (2008). SEMA3A and SEMA3D belong to the same class and share the most similarity with each other of the class 3 semaphorin genes (Luo et al., 1995). These findings further strengthen the evidence for a possible role of *SEMA3D* in the development of schizophrenia.

It is possible that the amino acid change (Lys701Gln) may affect the function of SEMA3D protein and that this results in susceptibility to schizophrenia. Indeed, this is a substitution from a large and basic amino acid (Lys) to a medium-sized and polar one (Gln). This is likely to lead to functional differences between the two types of SEMA3D. One possibility is that this substitution might result in conformational change of SEMA3D and influence its affinity for its receptors. Another possibility is that the Lys701 and Gln701 variants of SEMA3D have different cellular localization. The basic domain of class 3 semaphorins electrostatically interacts with the proteoglycan components of the extracellular matrix (De Wit et al., 2005) and the granule matrix (de Wit et al., 2009). The substitution from the basic Lys701 to the non-basic Gln701 may affect such interactions between SEMA3D and these matrices. Alteration of the extracellular matrix may modify distribution of SEMA3D in neurons, and that of the granule matrix may affect secretion from secretory vesicles. The class 3 semaphorins not only act as axon guidance cues but also have key roles in synaptic formation and function. Therefore, these modified interactions could impact on the establishment of synaptic contacts and the formation of new synapses. Although the amino acid substitution (Lys701Gln) was predicted to be benign by Polyphen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org/) programs, its actual effects should be elucidated by cell biological or biochemical approaches.

Accumulating evidence suggests that the semaphorins are regulatory factors of tumor progression and modulators of angiogenesis (reviewed in (Neufeld and Kessler, 2008) and (Capparuccia and Tamagnone, 2009)). Recently, SEMA3D was also reported to

Please cite this article in press as: Fujii T, et al., Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia, Journal of Psychiatric Research (2010), doi:10.1016/j.jpsychires.2010.05.004

possess anti-tumorigenic and anti-angiogenic properties (Kigel et al., 2008). The hypoactivity of *SEMA3D* could be linked to increased incidence of cancer. Previous studies and reviews have partially supported the idea that the incidence of cancer in patients with schizophrenia is reduced compared with the general population (Grinshpoon et al., 2005; Dalton et al., 2005; Catts et al., 2008). It is possible that semaphorins are related to the development of schizophrenia and also contribute to the associated lower incidence of cancer, and this topic warrants further investigation.

In conclusion, we found a significant association between the Lys701Gln polymorphism of *SEMA3D* and schizophrenia. In addition, the haplotype rs2190208–rs1029564–rs17159614–rs121176601, not including the Lys701Gln variant, was shown to be associated with schizophrenia, which suggests that some other polymorphisms of *SEMA3D* play a role in the pathogenesis of schizophrenia. Taking the previous molecular and developmental findings together with the present genetic findings, *SEMA3D* appears to be a promising candidate gene related to susceptibility to schizophrenia.

Conflict of interest

All authors declare no conflict of interest that could influence their work.

Role of funding source

This study was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (T.F.), Japan Human Sciences Foundation (T.F.), Health and Labor Sciences Research Grants (Research on Psychiatric and Neurological Diseases and Mental Health; Research on Human Genome Tailor made) (H. K.), Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug innovation) (H.K.), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (H.K.), JST, CREST (H.K.), and Grant-in-Aid for Scientific Research on Priority Areas of Applied Genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H.K.). These agencies had no role in study design, acquisition and interpretation of data or writing the report.

Contributors

T.F. designed the study, performed genotyping of *SEMA3D*, made statistical analysis, managed literature search, interpreted the data, and wrote the manuscript. H.U. and N.Y. took part in genotyping. H. H., M.T., M.I., K.A., and T.H. collected samples and gave comments to the manuscript. H.K. organized recruitment and genotyping of schizophrenic patients and control subjects, and took part in analyzing the data and writing the manuscript.

Acknowledgements

We thank the patients and the healthy volunteers for their participation.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.jpsychires.2010.05.004.

References

Akbarian S, Bunney Jr WE, Potkin SG, Wigal SB, Hagman JO, Sandman CA, Jones EG. Altered distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase cells in frontal lobe of schizophrenics implies disturbances of cortical development. Archives of General Psychiatry 1993;50:169–77.

Allen NC, Bagade S, McQueen MB, Ioannidis JP, Kavvoura FK, Khoury MJ, et al. Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. Nature Genetics 2008;40:827–34.

American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 4th ed. Washington, DC: American Psychiatric Association (APA); 1994.

Budel S, Shim SO, Feng Z, Zhao H, Hisama F, Strittmatter SM. No association between schizophrenia and polymorphisms of the PlexinA2 gene in Chinese Han Trios. Schizophrenia Research 2008;99:365–6.

Capparuccia L, Tamagnone L. Semaphorin signaling in cancer cells and in cells of the tumor microenvironment–two sides of a coin. Journal of Cell Science 2009;122:1723–36.

Catts VS, Catts SV, O'Toole BI, Frost AD. Cancer incidence in patients with schizophrenia and their first-degree relatives–a meta-analysis. Acta Psychiatrica Scandinavica 2008;117:323–36.

Clark HF, Gurney AL, Abaya E, Baker K, Baldwin D, Brush J, et al. The secreted protein discovery initiative (SPDI), a large-scale effort to identify novel human secreted and transmembrane proteins: a bioinformatics assessment. Genome Research 2003;13:2265–70.

Conrad AJ, Scheibel AB. Schizophrenia and the hippocampus: the embryological hypothesis extended. Schizophrenia Bulletin 1987;13:577–87.

Dalton SO, Mellemkjaer L, Thomassen L, Mortensen PB, Johansen C. Risk for cancer in a cohort of patients hospitalized for schizophrenia in Denmark, 1969–1993. Schizophrenia Research 2005;75:315–24.

De Wit J, De Winter F, Klooster J, Verhaagen J. Semaphorin 3A displays a punctate distribution on the surface of neuronal cells and interacts with proteoglycans in the extracellular matrix. Molecular and Cellular Neurosciences 2005;29:40–55.

de Wit J, Toonen RF, Verhage M. Matrix-dependent local retention of secretory vesicle cargo in cortical neurons. The Journal of Neuroscience 2009;29:23–37.

Eastwood SL, Law AJ, Everall IP, Harrison PJ. The axonal chemorepellant semaphorin 3A is increased in the cerebellum in schizophrenia and may contribute to its synaptic pathology. Molecular Psychiatry 2003;8:148–55.

Ekelund J, Lichtermann D, Hovatta I, Ellonen P, Suvisaari J, Terwilliger JD, et al. Genome-wide scan for schizophrenia in the Finnish population: evidence for a locus on chromosome 7q22. Human Molecular Genetics 2000;9:1049–57.

Feinberg I. Schizophrenia: caused by a fault in programmed synaptic elimination during adolescence? Journal of Psychiatric Research 1982;17:319–34.

Fujii T, Iijima Y, Kondo H, Shizuno T, Hori H, Nakabayashi T, et al. Failure to confirm an association between the PLXNA2 gene and schizophrenia in a Japanese population. Progress in Neuro-Psychopharmacology & Biological Psychiatry 2007;31:873–7.

Fujii T, Nakao F, Shibata Y, Shioi G, Kodama E, Fujisawa H, Takagi S. Caenorhabditis elegans PlexinA, PLX-1, interacts with transmembrane semaphorins and regulates epidermal morphogenesis. Development 2002;129:2053–63.

Fujisawa H. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. Journal of Neurobiology 2004;59:24–33.

Garey LJ, Ong WY, Patel TS, Kanani M, Davis A, Mortimer AM, et al. Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. Journal of Neurology, Neurosurgery, and Psychiatry 1998;65:446–53.

Glantz LA, Lewis DA. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Archives of General Psychiatry 2000;57:65–73.

Grinshpoon A, Barchana M, Ponizovsky A, Lipshitz I, Nahon D, Tal O, et al. Cancer in schizophrenia: is the risk higher or lower? Schizophrenia Research 2005;73:333–41.

Halloran MC, Wolman MA. Repulsion or adhesion: receptors make the call. Current Opinion in Cell Biology 2006;18:533–40.

Idol JR, Addington AM, Long RT, Rapoport JL, Green ED. Sequencing and analyzing the t(1;7) reciprocal translocation breakpoints associated with a case of childhood-onset schizophrenia/autistic disorder. Journal of Autism and Developmental Disorders 2008;38:668–77.

Jakob H, Beckmann H. Prenatal developmental disturbances in the limbic allocortex in schizophrenics. Journal of Neural Transmission 1986;65:303–26.

Keshavan MS, Anderson S, Pettegrew JW. Is schizophrenia due to excessive synaptic pruning in the prefrontal cortex? the Feinberg hypothesis revisited. Journal of Psychiatric Research 1994;28:239–65.

Kigel B, Varshavsky A, Kessler O, Neufeld G. Successful inhibition of tumor development by specific class-3 semaphorins is associated with expression of appropriate semaphorin receptors by tumor cells. PLoS One 2008;3:e3287.

Kruger RP, Aurandt J, Guan KL. Semaphorins command cells to move. Nature Reviews. Molecular Cell Biology 2005;6:789–800.

Li J, Ji L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity 2005;95:221–7.

Liu Y, Berndt J, Su F, Tawarayama H, Shoji W, Kuwada JY, Halloran MC. Semaphorin3D guides retinal axons along the dorsoventral axis of the tectum. The Journal of Neuroscience 2004;24:310–8.

Liu Y, Halloran MC. Central and peripheral axon branches from one neuron are guided differentially by Semaphorin3D and transient axonal glycoprotein-1. The Journal of Neuroscience 2005;25:10556–63.

Luo Y, Raible D, Raper JA. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 1993;75:217–27.

Luo Y, Shepherd I, Li J, Renzi MJ, Chang S, Raper JA. A family of molecules related to collapsin in the embryonic chick nervous system. Neuron 1995;14:1131–40.

Mah S, Nelson MR, Delisi LE, Reneland RH, Markward N, James MR, et al. Identification of the semaphorin receptor PLXNA2 as a candidate for susceptibility to schizophrenia. Molecular Psychiatry 2006;11:471–8.

Please cite this article in press as: Fujii T, et al., Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia, Journal of Psychiatric Research (2010), doi:10.1016/j.jpsychires.2010.05.004

Mann F, Chauvet S, Rougon G. Semaphorins in development and adult brain: Implication for neurological diseases. Progress in Neurobiology 2007;82:57–79.

Murray RM. Neurodevelopmental schizophrenia: the rediscovery of dementia praecox. The British Journal of Psychiatry. Supplement; 1994:6–12.

Neufeld G, Kessler O. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. Nature Reviews. Cancer 2008;8:632–45.

Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. American Journal of Human Genetics 2004;74:765–9.

Sakai JA, Halloran MC. Semaphorin 3d guides laterality of retinal ganglion cell projections in zebrafish. Development 2006;133:1035–44.

Takahashi K, Ishida M, Takahashi H. Expression of Sema3D in subsets of neurons in the developing dorsal root ganglia of the rat. Neuroscience Letters 2009;455:17–21.

Takahashi T, Strittmatter SM. Plexina1 autoinhibition by the plexin sema domain. Neuron 2001;29:429–39.

Takeshita M, Yamada K, Hattori E, Iwayama Y, Toyota T, Iwata Y, et al. Genetic examination of the PLXNA2 gene in Japanese and Chinese people with schizophrenia. Schizophrenia Research 2008;99:359–64.

Tastemir D, Demirhan O, Sertdemir Y. Chromosomal fragile site expression in Turkish psychiatric patients. Psychiatry Research 2006;144:197–203.

Waddington JL, Buckley PF, Scully PJ, Lane A, O'Callaghan E, Larkin C. Course of psychopathology, cognition and neurobiological abnormality in schizophrenia: developmental origins and amelioration by antipsychotics? Journal of Psychiatric Research 1998;32:179–89.

Waimey KE, Cheng HJ. Axon pruning and synaptic development: how are they perplexin? Neuroscientist 2006;12:398–409.

Wedenoja J, Loukola A, Tuulio-Henriksson A, Paunio T, Ekelund J, Silander K, et al. Replication of linkage on chromosome 7q22 and association of the regional Reelin gene with working memory in schizophrenia families. Molecular Psychiatry 2008;13:673–84.

Wedenoja J, Tuulio-Henriksson A, Suvisaari J, Loukola A, Paunio T, Partonen T, et al. Replication of association between working memory and Reelin, a potential modifier gene in schizophrenia. Biological Psychiatry; 2009.

Weinberger DR. Implications of normal brain development for the pathogenesis of schizophrenia. Archives of General Psychiatry 1987;44:660–9.

Wolman MA, Liu Y, Tawarayama H, Shoji W, Halloran MC. Repulsion and attraction of axons by semaphorin3D are mediated by different neuropilins in vivo. The Journal of Neuroscience 2004;24:8428–35.

Wolman MA, Regnery AM, Becker T, Becker CG, Halloran MC. Semaphorin3D regulates axon axon interactions by modulating levels of L1 cell adhesion molecule. The Journal of Neuroscience 2007;27:9653–63.

Yazdani U, Terman JR. The semaphorins. Genome Biology 2006;7:211.

Please cite this article in press as: Fujii T, et al., Possible association of the semaphorin 3D gene (*SEMA3D*) with schizophrenia, Journal of Psychiatric Research (2010), doi:10.1016/j.jpsychires.2010.05.004

Neuroscience 165 (2010) 1301–1311

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

H. KAWASHIMA,[a] T. NUMAKAWA,[b]* E. KUMAMARU,[b] N. ADACHI,[b] H. MIZUNO,[a] M. NINOMIYA,[b] H. KUNUGI[b] AND K. HASHIDO[a]

[a]*Administrative Section of Radiation Protection, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan*

[b]*Department of Mental Disorder Research, NCNP, Tokyo, Japan*

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.

*Corresponding author. Tel: +81-42-341-2711 ext. (5132); fax: +81-42-346-1744.
E-mail address: numakawa@ncnp.go.jp (T. Numakawa).

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-

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2009.11.057

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

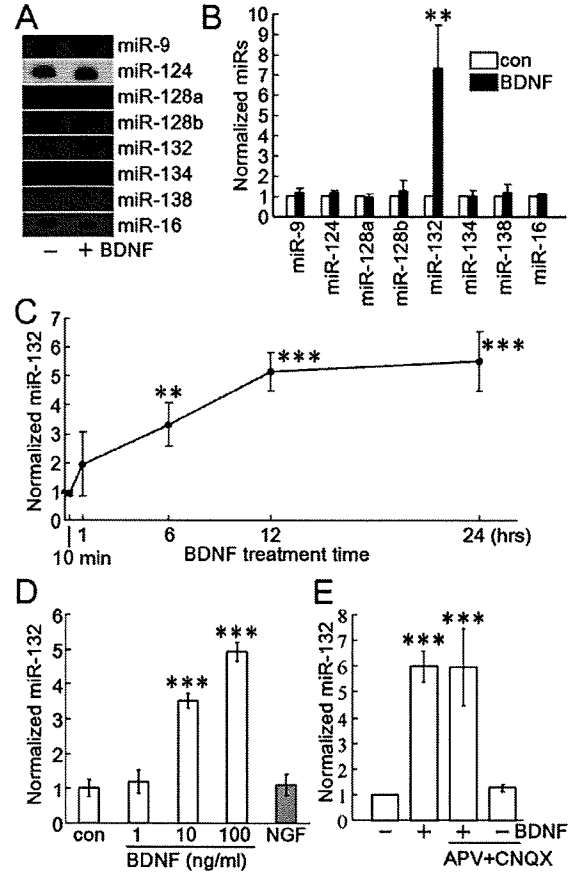

Fig. 1. Expression of miR-132 was increased by brain-derived neurotrophic factor (BDNF) in cultured cortical neurons. (A) Levels of microRNAs (miRs) including miR-9, -124, -128a, -128b, -132, -134, -138, and -16 with or without BDNF stimulation in cultured cortical neurons. The miR-132 was upregulated 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+

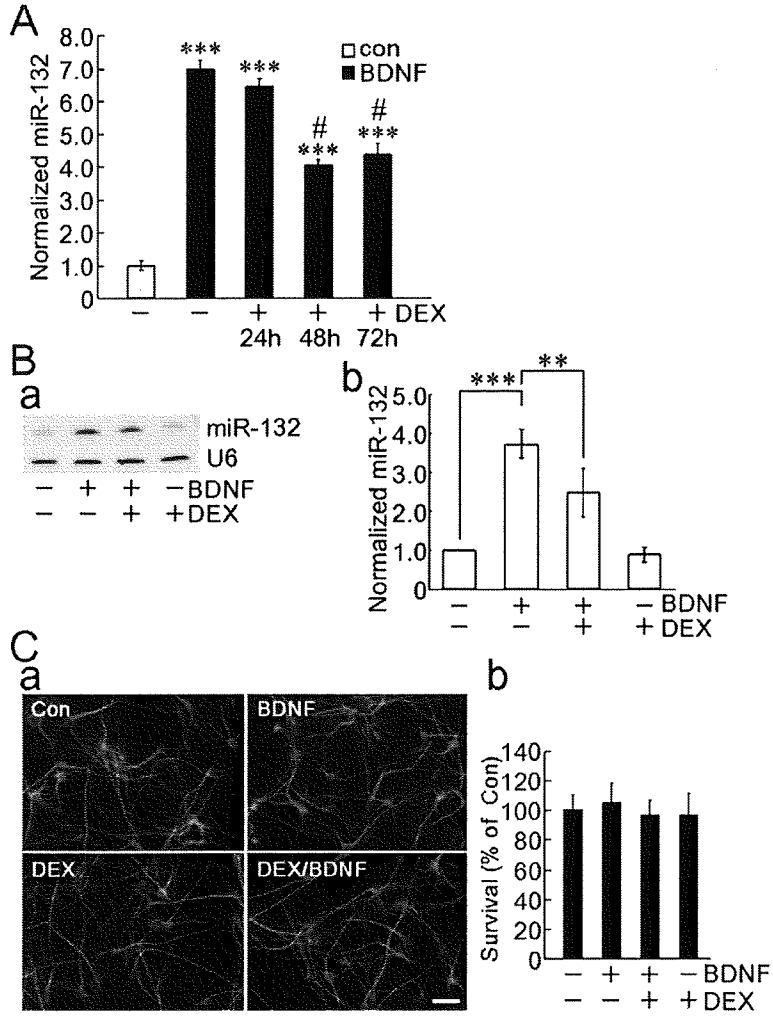

Fig. 2. Pretreatment with dexamethasone (DEX) reduced BDNF-stimulated miR-132 increase. (A) Inhibitory effect of various durations of DEX (1.0 μM) pretreatment on the BDNF-increased miR-132. DEX incubation was performed for the indicated times before BDNF addition (at DIV10, 24 h). Normalization to control (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.

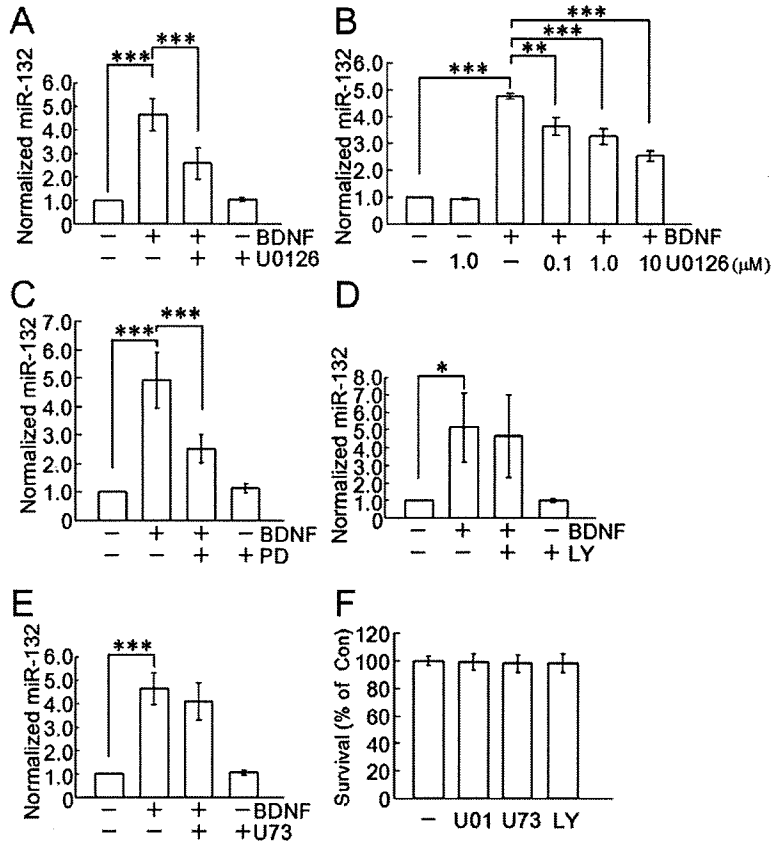

Fig. 3. Activation of the MAPK/ERK1/2 pathway was required for the BDNF-increased miR-132. (A) Effect of the MAPK/ERK1/2 pathway inhibitor, U0126, on the BDNF-dependent miR-132 increase. U0126 (final 10 μM) was applied 20 min before BDNF addition. BDNF was added at DIV10 for 24 h. Quantitative analysis 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,