interview sessions by two senior psychiatrists and a consensus diagnosis was made. Their family history of mental disorder was assessed by interviewing the proband and available relatives. Control subjects were recruited from the staff or students of participating institutes and their friends, who reported themselves to be healthy. Written informed consent was obtained from all subjects. This study was approved by the ethics committees of RIKEN and all participating institutes.

The study subjects for the initial association study consisted of 199 patients with bipolar disorder (143 bipolar I and 56 bipolar II, 76 male and 123 female, 49.8 years of age on average) and 258 healthy volunteers (129 male and 129 female, 33.0 years of age on average). An additional independent sample set in COSMO consisted of 431 patients with bipolar disorder (214 male and 217 female, 49.5 years of age on average) and 476 healthy volunteers (226 male and 250 female, 50.4 years of age on average).

Six patients with bipolar disorder with somatic symptoms suggestive of mitochondrial disorders were chosen from the first sample set for examination of the entire mtDNA. They had been recruited in our bipolar disorder study based on our inclusion criteria, having DSM-IV bipolar disorder by consensus diagnoses after two nonstructured interview sessions with senior psychiatrists, and exclusion criteria, having no clinically remarkable neurological diseases, head trauma, or comorbid Axis II diagnoses. Characteristics of these six subjects are listed below and summarized in the Table 1.

The transmitochondrial cybrids for the following functional analyses were generated from 24 subjects in the initial samples, including two patients with 3644C.

Case reports

Case 1, 38-year-old female, is a patient with bipolar I disorder without psychotic features. She had the first episode of mania with psychomotor agitation and confusion at age 17. At age 27, she was admitted to a hospital due to bilateral optic neuritis. She had no other symptoms suggestive of multiple sclerosis. Her optic neuritis was improved by steroid therapy, and final diagnosis was idiopathic optic neuropathy. Because she had no relatives with optic neuropathy and her symptoms were reversible, Leber disease was not considered by the attendant ophthalmologist.

Case 2 is a 61-year-old male diagnosed as having bipolar I disorder. At age 30, he had the onset of mania with mood-incongruent psychotic features. At age 60, after being discharged from a psychiatric hospital, he was admitted to a hospital due to stroke. Brain imaging revealed infarctions in the cerebellum and the brain stem. During this hospitalization, chest X-ray showed enlarged heart and he was diagnosed as idiopathic dilated cardiomyopathy. He also had renal failure. His attendant physician did not suspect mitochondrial disease.

Case 3 is a 56-year-old male diagnosed with bipolar I disorder. At age 49, he had the onset of depression characterized by depressive mood, fatigability, retardation, insomnia, and suicidal thought. At age 52, he suddenly became manic. During this manic episode, he caused a motor vehicle accident. He was admitted to a psychiatric ward for the treatment of mania. After the first admission, he had generalized tonic clonic seizures. Although electroencephalography (EEG) recording showed no signs of epilepsy, he was clinically diagnosed as having epilepsy. At age 54, he complained of swollen eyelid, and medical examination did not show any signs of renal failure. He also complained of muscle weakness and had an episode of falling down due to muscle weakness. A neurologist saw this patient and assessed that ptosis may be present but fluctuating and was not pathological. He also showed some tendency of disturbed movement of the eyes, but it was also fluctuating and he did not have diplopia. Muscle weakness was not objectively present. Based on these clinical examinations, the neurologist ruled out mitochondrial disease from differential diagnosis and judged that further investigation was not necessary. Electrocardiogram indicated supraventricular extrasystole, but it was not clinically remarkable.

Case 4 is a 46-year-old female diagnosed with bipolar I disorder. At age 24, she had the onset of mania. At age 40, she began rapid cycling. During her psychiatric hospitalization, EEG recording showed epileptic abnormality. However, she did not have any signs or symptoms of epilepsy and was not diagnosed as epileptic.

Case 5 is 57-year-old male diagnosed as having bipolar I disorder. He had the onset of a manic episode at age 50. Since his clinical representation resembled confusion caused by organic mental disorder, he received lumbar puncture by a neurologist during psychiatric hospitalization, which showed elevated cerebrospinal fluid protein levels. The neurologist also noted muscle weakness and slight ptosis on the left eyelid. However, these symptoms were improved without any treatment and his subsequent manic episodes were typical manic syndrome without any additional neurological features or psychotic features. He was finally diagnosed as having bipolar I disorder. After the onset of bipolar disorder, he was diagnosed as having non-insulin-dependent diabetes mellitus. His cranial magnetic resonance image showed multiple subcortical silent infarction.

Case 6 is a 38-year-old male having bipolar I disorder. His clinical record was published elsewhere [37]. He complained of ptosis during antipsychotic treatment. Both a neurologist and an ophthalmologist examined and diagnosed him as not having any mitochondrial disease, since his sign was transient and not clinically remarkable.

MtDNA sequencing

Total DNA was extracted from peripheral blood leukocytes by standard protocols. Entire mtDNA sequenc-

ing was performed as previously described [38] with some modifications. In short, each DNA sample was diluted to 10 μg/ml, and nested PCR was performed. PCR was initially performed to obtain two long PCR products, 6 and 11 kb of mtDNA; the second PCR was designed as a set of three overlapping fragments from the first 6-kb PCR product and six fragments from the first 11-kb PCR product. After the second PCR, the products were treated with a SeqDirect PCR Cleaning kit (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol. Both strands of these fragments were then sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism 3700 DNA sequencer (Applied Biosystems). Each mtDNA site was read at least three times, including at least once for each strand.

Genotyping

The two base substitutions of mtDNA, 3644T→C and 13928G→T, were genotyped using the PCR-RFLP method and sequencing. The enzymes and experimental conditions for the PCR-RFLP were as follows: 3644 was genotyped by primers 5′-GTAGAATGATGGCTAGGGTGACT-3′ and 5′-TCTAGCCACCTCTAGCCTAGACG-3′ and the restriction enzyme *Tai*I (Fermentas), 13928 by 5′-CATACTCGGATTC-TACGCTA-3′, 5′-TTTAGGTAATAGCTTTTCTA-3′, and *Nhe*I (Takara Bio, Inc., Shiga, Japan). MtDNAs 5178C→A and 10398A→G were genotyped to determine the haplogroups. Genotypes of 5178C→A and 10398A→G were examined as previously described [10].

Generation of cybrids

The 143B.TK$^-$ $\rho^0$206 cell line, lacking mtDNA and established by King and Attardi [39], was used for generating cybrids. Platelets of patients and controls were separated from peripheral blood and fused with $\rho^0$206 cells using 40% polyethylene glycol 1500 (Sigma), as previously described [40]. We used DMEM (Gibco BRL) containing 10% FBS (fetal bovine serum; Gibco BRL), penicillin/streptomycin, pyruvate (Gibco BRL), and uridine (Sigma) as the growth medium for ρ^0 cells. For the selection of transmitochondrial cybrid cell lines, we used DMEM containing 10% dialyzed FBS, penicillin/streptomycin, and pyruvate. After the harvest of individual cybrid cell lines, the integration of mtDNA was confirmed by Southern blot analysis using 18S ribosomal RNA repeating units as a reference [41]. The identity of the mtDNA of the cybrids with that of the donor was verified by sequencing the D loop and genotyping several polymorphisms. For Southern blot analysis, we used the ECL Labeling and Detection System according to the manufacturer's protocol (Amersham Biosciences Corp., NJ, USA). Cybrids were stored in liquid nitrogen for further experiments.

Measurement of MMP using JC-1

MMP was estimated using JC-1 (Molecular Probes, Eugene, OR) and flow cytometry. Cybrids stored in liquid nitrogen were thawed and incubated in an atmosphere of 5% CO_2 at 37°C in DMEM containing 10% FBS, penicillin/streptomycin, and pyruvate. Cells (1×10^6) were trypsinized and harvested in 10 ml of DMEM containing 10% FBS, washed with PBS (phosphate-buffered saline) once, and stained with DMEM containing 5 μg/ml JC-1 for 15 min at 37°C. Cells were then washed with PBS and subjected to analysis using a FACS (Epics Elite cell sorter; Beckman Coulter, Fullerton, CA, USA) as previously described [42]. The excitation wavelength was 488 nm by argon ion laser. Emissions at 590 and 527 nm were isolated by each photomultiplier detector and 10,000 cells were measured for each experiment. The experiment was performed in triplicate for each cell line. The cells with polarized mitochondria were defined by an intensity ratio of 590 nm/527 nm above 0.2.

Activities of enzymes in the electron-transport chain

For the sample preparation of the mitochondrial fraction, each line of cybrids was amplified until the cell count was 5×10^7. Cybrids were trypsinized and harvested in DMEM. After being washed once with PBS and once with isolation buffer [210 mM D-mannitol, 71 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin (fatty acid free), 5 mM Hepes, pH 7.2], the cells were suspended in 5 ml of isolation buffer. Using a chilled Dounce glass homogenizer with a loose fitting pestle, 20 passes were applied to the cell suspension on ice, which was centrifuged at 700*g* for 7 min at 4°C. The supernatant was centrifuged at 10,000*g* for 7 min at 4°C, and the mitochondrial pellet was obtained. The pellet was suspended in 250 mM sucrose, divided into aliquots, and kept at −80°C until use. Activities of complexes I, III, and IV were measured as previously described [43]. Rotenone-sensitive complex I activity was measured by the change in absorption of decylubiquinone. All samples were measured within 1 month from preparation. The activity of each complex was corrected by citrate synthase activity. All the chemical products for these assays were obtained from Sigma. We used a UVmini1240 spectrophotometer (Shimadzu, Kyoto, Japan) for this experiment.

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References

[1] P. Sklar, S.B. Gabriel, M.G. McInnis, P. Bennett, Y.M. Lim, G. Tsan, S. Schaffner, G. Kirov, I. Jones, M. Owen, et al., Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. Brain-derived neurotrophic factor, Mol. Psychiatry 7 (2002) 579–593.

[2] M. Neves-Pereira, E. Mundo, P. Muglia, N. King, F. Macciardi, J.L. Kennedy, The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: evidence from a family-based association study, Am. J. Hum. Genet. 71 (2002) 651–655.

[3] C. Kakiuchi, K. Iwamoto, M. Ishiwata, M. Bundo, T. Kasahara, I. Kusumi, T. Tsujita, Y. Okazaki, S. Nanko, H. Kunugi, et al., Impaired feedback regulation of XBP1 as a genetic risk factor for bipolar disorder, Nat. Genet. 35 (2003) 171–175.

[4] T. Kato, S. Takahashi, T. Shioiri, T. Inubushi, Alterations in brain phosphorous metabolism in bipolar disorder detected by in vivo ^{31}P and ^{7}Li magnetic resonance spectroscopy, J. Affect. Disord. 27 (1993) 53–59.

[5] C. Konradi, M. Eaton, M.L. MacDonald, J. Walsh, F.M. Benes, S. Heckers, Molecular evidence for mitochondrial dysfunction in bipolar disorder, Arch. Gen. Psychiatry 61 (2004) 300–308.

[6] G. Siciliano, A. Tessa, S. Petrini, M. Mancuso, C. Bruno, G.S. Grieco, A. Malandrini, L. DeFlorio, B. Martini, A. Federico, et al., Autosomal dominant external ophthalmoplegia and bipolar affective disorder associated with a mutation in the ANT1 gene, Neuromuscul. Disord. 13 (2003) 162–165.

[7] J.N. Spelbrink, F.Y. Li, V. Tiranti, K. Nikali, Q.P. Yuan, M. Tariq, S. Wanrooij, N. Garrido, G. Comi, L. Morandi, et al., Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria, Nat. Genet. 28 (2001) 223–231.

[8] G. Van Goethem, B. Dermaut, A. Lofgren, J.J. Martin, C. Van Broeckhoven, Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions, Nat. Genet. 28 (2001) 211–212.

[9] F.J. McMahon, O.C. Stine, D.A. Meyers, S.G. Simpson, J.R. DePaulo, Patterns of maternal transmission in bipolar affective disorder, Am. J. Hum. Genet. 56 (1995) 1277–1286.

[10] T. Kato, H. Kunugi, S. Nanko, N. Kato, Mitochondrial DNA polymorphisms in bipolar disorder, J. Affect. Disord. 62 (2001) 151–164.

[11] F.J. McMahon, Y.S. Chen, S. Patel, J. Kokoszka, M.D. Brown, A. Torroni, J.R. DePaulo, D.C. Wallace, Mitochondrial DNA sequence diversity in bipolar affective disorder, Am. J. Psychiatry 157 (2000) 1058–1064.

[12] S. Washizuka, C. Kakiuchi, K. Mori, H. Kunugi, O. Tajima, T. Akiyama, S. Nanko, T. Kato, Association of mitochondrial complex I subunit gene NDUFV2 at 18p11 with bipolar disorder, Am. J. Med. Genet. 120B (2003) 72–78.

[13] S. Anderson, A.T. Bankier, B.G. Barrell, M.H. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, et al., Sequence and organization of the human mitochondrial genome, Nature 290 (1981) 457–465.

[14] D.C. Wallace, Mitochondrial diseases in man and mouse, Science 283 (1999) 1482–1488.

[15] T.R. Prezant, J.V. Agapian, M.C. Bohlman, X. Bu, S. Oztas, W.Q. Qiu, K.S. Arnos, G.A. Cortopassi, L. Jaber, J.I. Rotter, et al., Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness, Nat. Genet. 4 (1993) 289–294.

[16] M.X. Guan, N. Fischel-Ghodsian, G. Attardi, Biochemical evidence for nuclear gene involvement in phenotype of non-syndromic deafness associated with mitochondrial 12S rRNA mutation, Hum. Mol. Genet. 5 (1996) 963–971.

[17] S.W. Chang, D. Zhang, H.D. Chung, H.P. Zassenhaus, The frequency of point mutations in mitochondrial DNA is elevated in the Alzheimer's brain, Biochem. Biophys. Res. Commun. 273 (2000) 203–208.

[18] J.M. van der Walt, K.K. Nicodemus, E.R. Martin, W.K. Scott, M.A. Nance, R.L. Watts, J.P. Hubble, J.L. Haines, W.C. Koller, K. Lyons, et al., Mitochondrial polymorphisms significantly reduce the risk of Parkinson disease, Am. J. Hum. Genet. 72 (2003) 804–811.

[19] R.M. Andrews, I. Kubacka, P.F. Chinnery, R.N. Lightowlers, D.M. Turnbull, N. Howell, Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA, Nat. Genet. 23 (1999) 147.

[20] A.M. Kogelnik, M.T. Lott, M.D. Brown, S.B. Navathe, D.C. Wallace, MITOMAP: a human mitochondrial genome database, Nucleic Acids Res. 24 (1996) 177–179.

[21] A.M. Kogelnik, M.T. Lott, M.D. Brown, S.B. Navathe, D.C. Wallace, MITOMAP: a human mitochondrial genome database—1998 update, Nucleic Acids Res. 26 (1998) 112–115.

[22] M. Tanaka, T. Takeyasu, N. Fuku, G. Li-Jun, M. Kurata, Mitochondrial genome single nucleotide polymorphisms and their phenotypes in the Japanese, Ann. N. Y. Acad. Sci. 1011 (2004) 1–20.

[23] J.K. Pritchard, M. Stephens, P. Donnelly, Inference of population structure using multilocus genotype data, Genetics 155 (2000) 945–959.

[24] Y. Aomi, C.S. Chen, K. Nakada, S. Ito, K. Isobe, H. Murakami, S.Y. Kuno, M. Tawata, R. Matsuoka, H. Mizusawa, J.-I. Hayashi, Cytoplasmic transfer of platelet mtDNA from elderly patients with Parkinson's disease to mtDNA-less HeLa cells restores complete mitochondrial respiratory function, Biochem. Biophys. Res. Commun. 280 (2001) 265–273.

[25] R.H. Swerdlow, J.K. Parks, J.N. Davis II, D.S. Cassarino, P.A. Trimmer, L.J. Currie, J. Dougherty, W.S. Bridges, J.P. Bennett Jr., G.F. Wooten, W.D. Parker, Matrilineal inheritance of complex I dysfunction in a multigenerational Parkinson's disease family, Ann. Neurol. 44 (1998) 873–881.

[26] J.P. Sheehan, R.H. Swerdlow, S.W. Miller, R.E. Davis, J.K. Parks, W.D. Parker, J.B. Tuttle, Calcium homeostasis and reactive oxygen species production in cells transformed by mitochondria from individuals with sporadic Alzheimer's disease, J. Neurosci. 17 (1997) 4612–4622.

[27] M.P. Vawter, W.J. Freed, J.E. Kleinman, Neuropathology of bipolar disorder, Biol. Psychiatry 48 (2000) 486–504.

[28] G. Rajkowska, Cell pathology in bipolar disorder, Bipolar Disord. 4 (2002) 105–116.

[29] L.V. Kessing, E.W. Olsen, P.B. Mortensen, P.K. Andersen, Dementia in affective disorder: a case-register study, Acta Psychiatr. Scand. 100 (1999) 176–185.

[30] R.C. Green, L.A. Cupples, A. Kurz, S. Auerbach, R. Go, D. Sadovnick, R. Duara, W.A. Kukull, H. Chui, T. Edeki, et al., Depression as a risk factor for Alzheimer disease: the MIRAGE Study, Arch. Neurol. 60 (2003) 753–759.

[31] F.M. Nilsson, L.V. Kessing, T.G. Bolwig, Increased risk of developing Parkinson's disease for patients with major affective disorder: a register study, Acta Psychiatr. Scand. 104 (2001) 380–386.

[32] G. Chen, W.Z. Zeng, P.X. Yuan, L.D. Huang, Y.M. Jiang, Z.H. Zhao, H.K. Manji, The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS, J. Neurochem. 72 (1999) 879–882.

[33] E.C. Thomeer, W.M. Verhoeven, C.J. van de Vlasakker, J.L. Klompenhouwer, Psychiatric symptoms in MELAS: a case report, J. Neurol. Neurosurg. Psychiatry 64 (1998) 692–693.

[34] M. Yamazaki, H. Igarashi, M. Hamamoto, T. Miyazaki, I. Nonaka, A case of mitochondrial encephalomyopathy with schizophrenic psychosis, dementia and neuroleptic malignant syndrome, Rinsho Shinkeigaku 31 (1991) 1219–1223.

[35] M.D. Brown, I.A. Trounce, A.S. Jun, J.C. Allen, D.C. Wallace, Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mitochondrial DNA mutation, J. Biol. Chem. 275 (2000) 39831–39836.

[36] P.L. Roubertoux, F. Sluyter, M. Carlier, B. Marcet, F. Maarouf-Veray, C. Cherif, C. Marican, P. Arrechi, F. Godin, M. Jamon, et al., Mitochondrial DNA modifies cognition in interaction with the nuclear genome and age in mice, Nat. Genet. 35 (2003) 65–69.
[37] T. Kato, Y. Takahashi, Deletion of leukocyte mitochondrial DNA in bipolar disorder, J. Affect. Disord. 37 (1996) 67–73.
[38] J. Akanuma, K. Muraki, H. Komaki, I. Nonaka, Y. Goto, Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene, J. Hum. Genet. 45 (2000) 337–341.
[39] M.P. King, G. Attardi, Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation, Science 246 (1989) 500–503.
[40] A. Chomyn, S.T. Lai, R. Shakeley, N. Bresolin, G. Scarlato, G. Attardi, Platelet-mediated transformation of mtDNA-less human cells: analysis of phenotypic variability among clones from normal individuals—and complementation behavior of the tRNALys mutation causing myoclonic epilepsy and ragged red fibers, Am. J. Hum. Genet. 54 (1994) 966–974.
[41] I. Nishino, O. Kobayashi, Y. Goto, M. Kurihara, K. Kumagai, T. Fujita, K. Hashimoto, S. Horai, I. Nonaka, A new congenital muscular dystrophy with mitochondrial structural abnormalities, Muscle Nerve 21 (1998) 40–47.
[42] A. Sawa, G.W. Wiegand, J. Cooper, R.L. Margolis, A.H. Sharp, J.F. Lawler Jr., J.T. Greenamyre, S.H. Snyder, C.A. Ross, Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization, Nat. Med. 5 (1999) 1194–1198.
[43] I.A. Trounce, Y.L. Kim, A.S. Jun, D.C. Wallace, Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines, Methods Enzymol. 264 (1996) 484–509.

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Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia

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Genetic variation in dysbindin (DTNBP1: dystrobrevin-binding protein 1) has recently been shown to be associated with schizophrenia. The dysbindin gene is located at chromosome 6p22.3, one of the most promising susceptibility loci in schizophrenia linkage studies. We attempted to replicate this association in a Japanese sample of 670 patients with schizophrenia and 588 controls. We found a nominally significant association with schizophrenia for four single nucleotide polymorphisms and stronger evidence for association in a multi-marker haplotype analysis ($P = 0.00028$). We then explored functions of dysbindin protein in primary cortical neuronal culture. Overexpression of dysbindin induced the expression of two pre-synaptic proteins, SNAP25 and synapsin I, and increased extracellular basal glutamate levels and release of glutamate evoked by high potassium. Conversely, knockdown of endogenous dysbindin protein by small interfering RNA (siRNA) resulted in the reduction of pre-synaptic protein expression and glutamate release, suggesting that dysbindin might influence exocytotic glutamate release via upregulation of the molecules in pre-synaptic machinery. The overexpression of dysbindin increased phosphorylation of Akt protein and protected cortical neurons against neuronal death due to serum deprivation and these effects were blocked by LY294002, a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor. SiRNA-mediated silencing of dysbindin protein diminished Akt phosphorylation and facilitated neuronal death induced by serum deprivation, suggesting that dysbindin promotes neuronal viability through PI3-kinase-Akt signaling. Genetic variants associated with impairments of these functions of dysbindin could play an important role in the pathogenesis of schizophrenia.

INTRODUCTION

Schizophrenia is a complex genetic disorder characterized by profound disturbances of cognition, emotion and social functioning. It affects ~1% of the general population worldwide. Chromosome 6p is one of the most consistently replicated susceptibility regions in linkage studies of schizophrenia (1). A recent study implicated a gene on chromosome 6p, dysbindin (DTNBP1: dystrobrevin-binding protein 1), as a susceptibility locus in the Irish pedigrees (2). Since then, four studies have reported evidence supporting the association between genetic variants in dysbindin and schizophrenia in

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German, Chinese, Swedish and Irish populations (3–6), while one study failed to replicate positive association in an Irish case–control design (7). In the present study, we attempted to perform an independent association study in a Japanese population of schizophrenic cases and controls.

The pathophysiology of schizophrenia is still unclear; however, this disease is believed to involve genetic abnormalities in developmental processes leading to abnormal synaptic plasticity, including glutamatergic transmission (8,9). Several genes, e.g. dysbindin, neuregulin 1, G72, D-aminoacid oxidase, the regulator of G-protein signaling-4, GRM3 and PPP3CC are described as susceptibility genes for schizophrenia, and those genes may have convergent effects on glutamatergic synapses (10,11). Neuregulin affects the expression and plasticity of the *N*-methyl-D-aspartate (NMDA) receptor (12,13). D-aminoacid oxidase metabolizes D-serine, an endogenous modulator of the NMDA receptor (14), and G72 is probably an activator of D-aminoacid oxidase (15). The regulator of G-protein signaling-4 is the negative regulator of G-protein-coupled receptors, including metabotropic glutamate receptors (16). GRM3 encodes the mGlu3 receptor gene. PPP3CC, the calcineurin γ-subunit, is critical for certain types of NMDA-mediated plasticity. However, no evidence of a role in glutamatergic transmission has been imputed to dysbindin, although dysbindin is believed to play a role in synaptic plasticity and signal transduction. Although dysbindin has recently been cloned as a dystrobrevin-binding protein in mouse (17), little is known about the functions in neurons. Here, we examined neuronal functions of dysbindin and found two novel actions: (1) increased glutamate release with upregulation of pre-synaptic proteins and (2) neurotrophic effect through Akt signaling pathway.

RESULTS

Genetic association analysis

We genotyped six single nucleotide polymorphisms (SNPs) in dysbindin in 670 schizophrenic patients and 588 controls in a Japanese population. The genotype distributions of the six SNPs for the schizophrenic patients and the control subjects were in Hardy–Weinberg equilibrium (data not shown). Allele frequencies of the six SNPs among the patients and controls are shown in Table 1. A significant difference in allele frequency was observed between cases and controls for four SNPs, but not for the remaining two SNPs (Table 1). The G allele of P1635 was in excess in our cases when compared with controls ($\chi^2 = 10.3$, df = 1, $P = 0.0013$, odds ratio = 2.71, 95% CI 1.46–5.79, corrected $P = 0.0078$).

To further analyze the haplotype structure in our sample, we computed the linkage disequilibrium (LD) between the SNPs using D'. D' values ranged between 0.5 and 1.0 and indicated strong to intermediate LD between the markers. Thus, adjacent combinations of up to six markers were examined for association with schizophrenia. Global and individual *P*-values corresponding to haplotypes consisting of adjacent markers and estimated haplotype frequencies in patients and controls are shown in Table 2. All haplotype combinations were significantly associated with schizophrenia, except the P1320–P1763 haplotype. Given this result, we tested the contribution of individual haplotypes to the global result. The G–G haplotype (P1635–P1325), including the G allele of P1635, which was significantly more frequent in our cases (Table 2), was enriched in patients with schizophrenia when compared with controls (estimated frequencies: patients 3.0% versus controls 0.9%, *P*-value = 0.00028, corrected $P = 0.0042$).

Functional analysis in dysbindin-overexpressing cultured neurons

To clarify the function of dysbindin in the central nervous system, we focused on the pre-synaptic machinery in neuronal transmission, as dysbindin is primarily expressed in axonal terminals of the mouse brain (17). Pre-synaptic machinery for exocytotic transmitter release is composed of membrane proteins, cytoskeletal proteins and synaptic vesicle proteins (18). SNAP25 (25 kDa synaptosomal associated protein) and syntaxin are membrane proteins implicated in the docking, priming and fusion of the vesicles. Synapsin I is a cytoskeletal protein associated with the synaptic vesicles in the reserve pool. Synaptotagmin is a synaptic vesicle protein, which has been identified as a calcium sensor protein. Thus, we examined the expression of these synaptic associated molecules after overexpression of dysbindin with virus-mediated gene transfer system. Infected neuronal cultures were doubly stained with GFP signal and immunostaining signal by anti-MAP2 (a neuronal dendritic marker) antibody (Fig. 1A). Approximately 80% of MAP2-positive cells in either control (GFP-infected) or dysbindin-overexpressing (dysbindin- and GFP-infected) cultures were GFP-positive, indicating that the majority of neurons were infected. As shown in Figure 1B, SNAP25 and synapsin I expression tended to be upregulated in dysbindin-overexpressing cultures compared with control (49 and 57%, respectively), whereas the changes of synaptotagmin and syntaxin expression were not observed (data not shown). The levels of class III β-tubulin (TUJ1, a neuronal marker) were not altered in the three conditions (Fig. 1B). We confirmed the overexpression of dysbindin (~17-fold when compared with control) in dysbindin-infected cultures and the expression of GFP in both control and dysbindin-overexpressing cultures (Fig. 1B).

Upregulation of synapsin I and SNAP25 raised the possibility that release of neurotransmitter might be increased by the overexpression of dysbindin. Therefore, we measured the release of glutamate, which is the principle neurotransmitter in these neurons. As expected, the amount of basal glutamate from dysbindin-infected cortical cultures was significantly increased when compared with the uninfected or control cultures (Fig. 1C), indicating that dysbindin overexpression resulted in an elevation of extracellular glutamate. Furthermore, high KCl (HK^+)-evoked exocytotic release of glutamate was enhanced in dysbindin-infected cultures. These results suggest that dysbindin might be one of the regulator proteins in the excitatory neurotransmission.

We then investigated the effects of dysbindin on neuronal viability. Interestingly, it was found that the phosphorylation of Akt, a molecule in the phosphatidylinositol 3-kinase (PI3-kinase) pathway, was significantly enhanced by 67% in the dysbindin-overexpressing cultures, whereas total Akt protein levels were unchanged (Fig. 2A). As the activation of Akt is

Table 1. Allele frequencies of six dysbindin SNPs between the patients with schizophrenia and controls

Marker name	dbSNP ID	Polymorphism major/minor	Location	Minor allele frequency Controls	Minor allele frequency Patients	*P*-value	Odds ratio (95% CI)
P1655	rs2619539	G/C	Int 5	0.311	0.317	0.748	1.03 (0.87–1.22)
P1635	rs3213207	A/G	Int 4	0.011	0.030	0.0013	2.71 (1.46–5.79)
P1325	rs1011313	G/A	Int 4	0.153	0.166	0.372	0.91 (0.72–1.15)
P1320	rs760761	C/T	Int 3	0.071	0.095	0.027	1.38 (1.04–1.83)
P1763	rs2619522	T/G	Int 1	0.070	0.095	0.022	1.40 (1.05–1.86)
SNPA	rs2619538	T/A	Promoter	0.024	0.040	0.025	1.69 (1.05–2.86)

Table 2. Estimated haplotype frequencies and case–control haplotype results

Markers	*P*-value Global	*P*-value Individual	Haplotype	Haplotype frequency Controls	Haplotype frequency Patients
P1655–P1635	0.0026	0.0003	G–G	0.011	0.030
P1635–P1325	0.00041	0.00028	G–G	0.009	0.030
P1325–P1320	0.0074	0.013	G–T	0.069	0.096
P1320–P1763	0.06	0.02	C–T	0.929	0.904
P1763–SNPA	0.025	0.0047	G–A	0.009	0.025
P1655–P1635–P1325	0.0055	0.001	G–G–G	0.011	0.030
P1635–P1325–P1320	0.0006	0.0009	G–G–T	0.010	0.027
P1325–P1320–P1763	0.027	0.029	G–T–G	0.068	0.095
P1320–P1763–SNPA	0.05	0.0045	T–G–A	0.009	0.025
P1655–P1635–P1325–P1320	0.011	0.0038	G–G–G–T	0.011	0.027
P1635–P1325–P1320–P1763	0.0015	0.001	G–G–T–G	0.010	0.027
P1325–P1320–P1763–SNPA	0.015	0.0019	G–T–G–A	0.007	0.025
P1655–P1635–P1325–P1320–P1763	0.025	0.0028	G–G–G–T–G	0.011	0.027
P1635–P1325–P1320–P1763–SNPA	0.003	0.0016	G–G–T–G–A	0.009	0.026
P1655–P1635–P1325–P1320–P1763–SNPA	0.024	0.0012	G–G–G–T–G–A	0.010	0.026

Case–control haplotype analysis were performed using the permutation method to obtain empirical *P*-values. Global *P*-values and individual *P*-values (lowest *P*-values among the haplotypes) are indicated. Estimated frequency for the haplotype with significant association in controls and patients were shown.

regulated by phosphorylation, overexpression of dysbindin resulted in the activation of Akt. LY294002, a PI3-kinase inhibitor, completely blocked the activation of Akt by the dysbindin overexpression, with no alteration of the expression levels of Akt and TUJ1 proteins (Fig. 2A). As the PI3-kinase pathway is involved in neuronal function and survival (19), we examined the viability of cortical neurons with our virus infection system (Fig. 2B). The overexpression of dysbindin protein itself did not alter neuronal viability when compared with control. However, dysbindin overexpression significantly blocked the reduced viability of cortical cultures by serum deprivation. Additionally, LY294002 significantly inhibited the protective effects of dysbindin, suggesting that the PI3-kinase pathway was involved in the dysbindin-dependent viability promoting effects.

Knockdown analysis of endogenous dysbindin in cultured neurons

We further examined the endogenous dysbindin function in cortical cultures using small interfering RNA (siRNA) for dysbindin. Previously, we reported siRNA-dependent down-regulation of endogenous protein expression in primary cultured neurons (20). Here, we performed transfection of siRNA for dysbindin and confirmed the robust decrease (83%) of endogenous dysbindin protein (Fig. 3A). The protein expression levels of SNAP25 and synapsin I and the phosphorylation level of Akt protein was significantly suppressed after dysbindin-siRNA transfection (43, 37 and 52% of reduction, respectively), although the expression levels of TUJ1 and Akt proteins were not altered (Fig. 3A). Thus, we investigated dysbindin function on glutamate release and neuronal viability under this condition. The amount of basal and released glutamate from dysbindin-siRNA-transfected cortical cultures significantly decreased when compared with the control (scramble) cultures (Fig. 3B), indicating that endogenous dysbindin protein plays a role in the excitatory neurotransmission. The neuronal viability was not changed by dysbindin-siRNA transfection in the presence of horse serum (Fig. 3C). However, dysbindin-siRNA transfection significantly facilitated neuronal death when horse serum was deprived (Fig. 3C), suggesting that the endogenous dysbindin protein has a promoting effect on survival.

DISCUSSION

In the present study, we report a significant association between genetic variation of dysbindin and schizophrenia in a Japanese population. In previous studies, highly significant

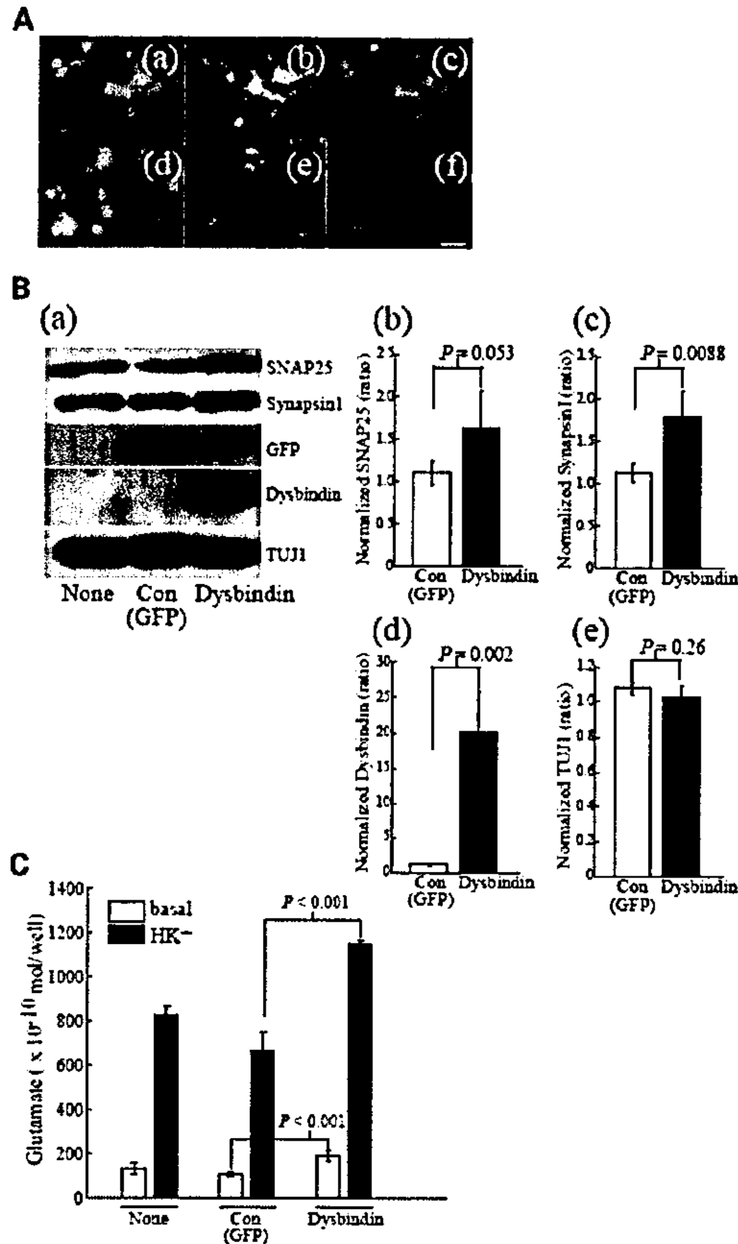

Figure 1. Dysbindin increases the expression of pre-synaptic proteins and glutamate release. (A) Double-staining of GFP and MAP2. Cortical cultures (6 days *in vitro*, DIV6) were prepared with viral infection of GFP only (a–c) or with viral infection of GFP and dysbindin (d–f) at DIV4. Images were obtained with GFP (a, d; green) and with immunostaining of anti-MAP2 antibody (b, e; red). Merged images (c, f; yellow) were also shown. (**B**) (a) Upregulation of pre-synaptic proteins. Cortical cultures (DIV6) were prepared without viral infection (None), with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dysbindin) at DIV4. The cell lysates were collected at DIV6 and SNAP25, synapsin I, GFP, dysbindin and TUJ1 were detected by western blotting. The immunoblots shown are representative of four independent experiments. (b–e) Quantification of the immunoreactivity of SNAP25, Synapsin I, dysbindin and TUJ1. Data represent mean ± SD of the immunoreactivity from four independent experiments. (C) Increase of the released glutamate in dysbindin-overexpressing cortical cultures. Cortical cultures were prepared without viral infection (None), with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dysbindin) at DIV4. Basal or HK^+ (50 mM KCl)-evoked release of glutamate was measured at DIV6 (after 48 h from infection). Data represent mean ± SD ($n = 4$).

associations were found for SNPs in introns 4–6, which is consistent with our results. The G allele of P1635, which was significantly in excess in our cases (3.0%), was also over-transmitted in Irish samples (10.2%) (2), whereas this allele was under-transmitted in German samples (17.6%) (3), suggesting that this SNP might be a marker rather than a polymorphism responsible for giving susceptibility. Notably, a high-risk haplotype in our samples was the G–G–T–G

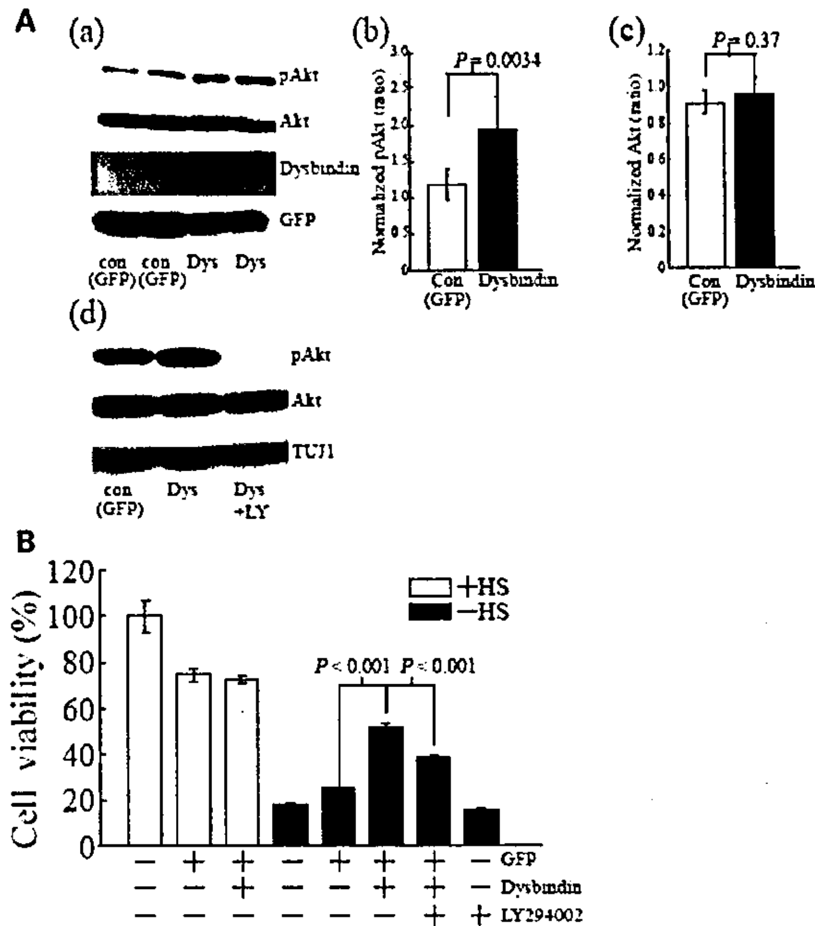

Figure 2. Dysbindin protects cortical neurons through PI3-kinase-Akt signaling. (**A**) (a) The activation of PI3-kinase pathway in dysbindin-overexpressing cultures. Cortical cultures after DIV4 were treated with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dys) for 48 h. (b, c) Quantification of the immunoreactivity of pAkt and total Akt proteins. Data represent mean ± SD of the immunoreactivity from four independent experiments. (d) The inhibitory effect of LY294002 on activation of Akt. Cortical cultures at DIV4 were treated with viral infection of GFP (Con), with viral infection of GFP and dysbindin (Dys) or with viral infection of GFP and dysbindin in the presence of LY294002 (1.0 μM) (Dys + LY) for 48 h. Cortical cultures were harvested at DIV6 for western blotting for pAkt, Akt, dysbindin, GFP or TUJ1. The immunoblots shown are representative of four independent experiments. (**B**) Neuroprotective effects of dysbindin against serum deprivation. Cortical cultures after DIV4 were treated with viral infection of GFP (Con), with viral infection of GFP and dysbindin (Dysbindin) or with LY294002 (1.0 μM) for 48 h. Deprivation of horse serum (HS) at DIV5 24 h after viral infection is indicated as −HS. Cell viability was determined using the MTT assay at DIV6 48 h after the viral infection and/or 24 h after HS deprivation. Data represent mean ± SD ($n = 8$).

haplotype (P1635–P1325–P1320–P1763), which includes the high-risk haplotype (G–G–G–G–T–G–C–C; P1635–P1325–P1765–P1757–P1320–P1763–P1578–P1792) reported in an Irish sample (6). The frequency of our high-risk haplotype (2.7% in cases versus 1.0% in controls) is lower than that in an Irish population (6%). Novel schizophrenia risk and protective haplotypes (C–A–T, C–A–A, G–G–T; P1655–P1635–SNPA) were recently identified in Cardiff and Dublin samples (21). We also analyzed these haplotypes in our sample and obtained evidence for a significant association with a different haplotype (global P-value = 0.0086, individual P-value = 0.005; G–G–A). Furthermore, the estimated frequencies of C–A–A and G–G–T haplotypes in our sample were <0.1%, although the overall frequencies in Cardiff and Dublin were 33 and 1.4%, respectively. We failed to find a significant association for the C–A–T haplotype (overall frequency, Cardiff and Dublin versus ours, C–A–T: 18 versus 32%). These differences of the haplotype frequencies might be based on the different ethnicity. A false-positive association owing to population stratification could not be excluded in our case–control study, despite the precaution of ethnic matching of this study.

It is of interest to study how genetic variation affects dysbindin function/expression. We do not know that any of the SNPs in our haplotypes are functional. Very little is known about the potential function of specific intronic sequences with regard to protein binding, stability and splicing efficacy. A recent study showed the functional possibility of intronic SNPs on gene expression. For example, an intronic SNP affects the transcriptional efficiency of SLC22A4 *in vitro*, owing to an allelic difference in affinity to Runt-related transcription factor 1, and this SNP is associated with rheumatoid arthritis, one of

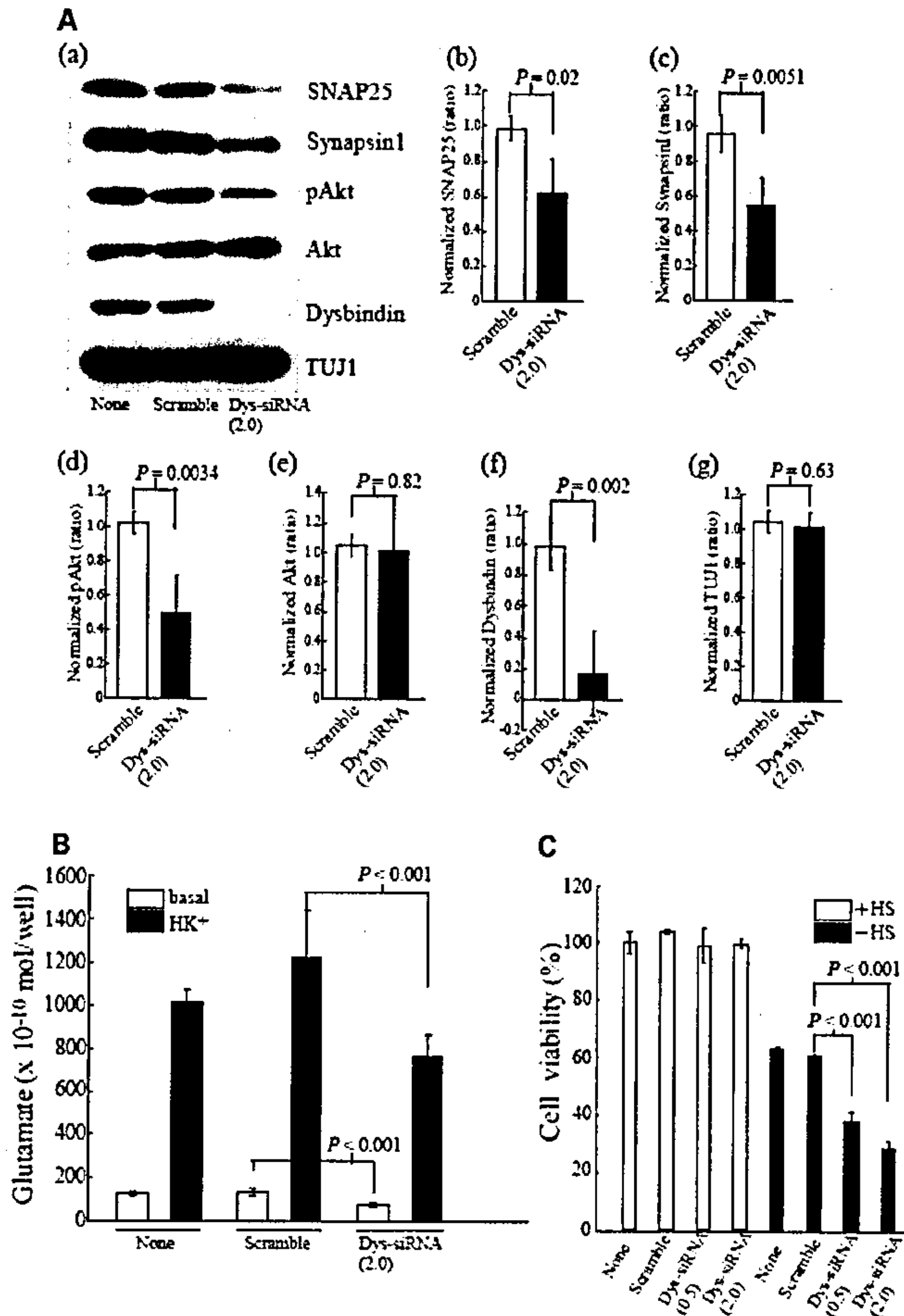

Figure 3. siRNA inhibition of endogenous dysbindin protein modulates protein expression, glutamate release and cell viability. (**A**) (a) Suppression of the pre-synaptic proteins and the phosphorylation of Akt in dysbindin-siRNA-transfected cultures. Cortical cultures after DIV4 were treated with siRNA for dysbindin (dys-siRNA; 2 mg/ml) or control (scramble; 2 mg/ml) for 72 h. Cortical cultures were harvested at DIV7 for western blotting for SNAP25, Synapsin I, pAkt, Akt, dysbindin or TUJ1. The immunoblots shown are representative of four independent experiments. (b–g) Quantification of the immunoreactivity of SNAP25, synapsin I, pAkt, total Akt, dysbindin and TUJ1. Data represent mean ± SD of the immunoreactivity from four independent experiments. (**B**) The reduced glutamate release in dysbindin-siRNA-transfected cultures. Cortical cultures were prepared without transfection (None), with transfection of control siRNA (Scramble; 2 mg/ml) or with transfection of siRNA for dysbindin (dys-siRNA; 2 mg/ml) at DIV4. Basal or HK^+ (50 mM KCl)-evoked release of glutamate was measured at DIV7 (after 72 h from transfection). Data represent the mean ± SD ($n = 6$). (**C**) Facilitation of neuronal death after serum deprivation by dysbindin-siRNA transfection. Cortical cultures after DIV4 were treated without transfection (None), with transfection of control siRNA (Scramble; 0.5 or 2 mg/ml) or with transfection of siRNA for dysbindin (dys-siRNA; 0.5 or 2 mg/ml) for 72 h. Deprivation of horse serum (HS) at DIV6 48 h after transfection is indicated as −HS. Cell viability was determined using the MTT assay at DIV7 72 h after the transfection and/or 24 h after HS deprivation. Data represent mean ± SD ($n = 8$).

the complex genetic diseases like schizophrenia (22). Alternatively, an unknown functional polymorphism, which is in LD with the SNPs and/or haplotypes, may be responsible for providing susceptibility to schizophrenia.

To date, association of dysbindin with schizophrenia has been confirmed across diverse populations. In addition, decreased expression of dysbindin mRNA and protein levels has been observed in prefrontal cortex and hippocampus of postmortem brain in schizophrenic patients (23–25). As dysbindin is distributed at least in part in axonal terminals (17), we focused on the possible role of dysbindin in neuronal transmission. We used two techniques, overexpression and knockdown, to investigate neuronal function of dysbindin. As the overexpression levels of dysbindin using sindbis virus were quite high when compared with the control level (~17-fold), the results could have non-physiological effects. However, the results from the knockdown experiments of the endogenous dysbindin protein were consistent with those from overexpression experiments. Our experiments suggest that dysbindin regulates the expression of SNAP25 and synapsin I proteins in the pre-synaptic machinery and is associated with increased glutamate release. SNAP25 is one of the fundamental molecular components of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) protein complex, which is involved in intracellular vesicle trafficking and neurotransmitter release (18). Synapsin I is localized to the synaptic vesicles that are both docked and located away from the plasma membrane (18). Reduction of SNAP25 protein has been observed in frontal cortex of schizophrenia patients (26) and synapsin I protein was found to be reduced in the hippocampus of patients with schizophrenia (27). Hypofunction of glutamatergic system has been implicated in the neuropathology in schizophrenia (8). The abuse of phencyclidine, an NMDA receptor antagonist, results in positive symptoms, negative symptoms and cognitive impairments, similar to schizophrenic patients. The postmortem brain studies suggested impaired glutamatergic systems, e.g. reduced glutamate level, decreased AMPA receptor binding and expression and reduced NMDA receptor expression in several brain areas, including frontal cortex and hippocampus.

Our experiments also suggest the survival effect of dysbindin protein on cortical neurons against serum deprivation through the PI3-kinase-Akt signaling pathway. Thus, dysbindin might play an important role in neuronal vulnerability. Impaired PI3-kinase-Akt signaling in schizophrenia has been reported recently (28). Dysbindin expression in the brain of schizophrenic patients was reduced (23–25) and our data suggested that the downregulation of dysbindin expression suppressed the phosphorylation levels of Akt. Taken together, impaired PI3-kinase-Akt signaling in the schizophrenic brain might be due, in part, to the decreased expression of dysbindin. As dysbindin may affect neuronal viability through Akt activation, dysbindin-Akt signaling might be involved in early disruptions producing long-term vulnerability that leads to the onset of schizophrenia symptoms. As PI3-kinase-Akt signaling is activated by several growth factors such as brain-derived neurotrophic factor, nerve growth factor and insulin-like growth factors through tyrosine kinase receptors (19), the regulation of this system might be associated with dysbindin.

The Hermansky–Pudlak syndrome defines a group of autosomal recessive disorders characterized by deficiencies in lysosome-related organelles complex-1 (BLOC-1). Hermansky–Pudlak type-7 is caused by a nonsense mutation of dysbindin, which is a component of the BLOC-1 (29). Biological roles of BLOC-1 are still unknown; however, it might be involved in vesicle docking and fusion. Sandy mouse, which has a deleted dysbindin gene, expresses no dysbindin (29). Thus, this mouse could be a powerful tool for investigating brain function of dysbindin *in vivo*. It is of interest to examine the pre-synaptic protein expression, glutamate release, Akt phosphorylation and neuronal vulnerability *in vivo* using this mouse.

We have demonstrated the additional support for the genetic association between dysbindin and schizophrenia in a relatively large sample and the evidence of novel functions of dysbindin in cultured neurons. Our results suggest that an abnormality of dysbindin might influence glutamatergic systems and Akt signaling. Further investigation is necessary to elucidate the mechanisms of Akt activation and upregulation of pre-synaptic molecules by dysbindin.

MATERIALS AND METHODS

Subjects

Subjects for the association study were 670 patients with schizophrenia [males: 50.6%, mean age of 44.2 years (SD 14.6)] and 588 healthy comparison subjects [males: 48.7%, mean age of 36.2 years (SD 12.4)]. All the subjects were biologically unrelated Japanese patients. Consensus diagnosis was made for each patient by at least two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria. Control subjects were healthy volunteers who had no current or past contact to psychiatric services. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees (Fujita Health University School of Medicine, Showa University School of Medicine and National Center of Neurology and Psychiatry).

SNP genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Six SNPs (P1655, P1635, P1325, P1320, P1763 and SNPA) adopted in the work of Straub *et al.* (2) and Williams *et al.* (21) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, described previously (30,31). Briefly, the probes and primers for detection of the SNP were as follows. P1655: forward primer 5′-AGTTTTTAT CACTAATCAAAATGAAACAGCCTTT-3′, reverse primer 5′-CTCATTCTGTTATAACTAGTCTGACATGGT-3′, probe 1 5′-VIC-TATTAGCTATGATAGTGTTTTAT-MGB-3′ and probe 2 5′-FAM-ATTAGCTATGATAGTCTTTTAT-MGB -3′; P1635: forward primer 5′-GGAACTTTTCTTTGAAGA CTTCCTTTCG-3′, reverse primer 5′-ACCACTAACAACC AAAAAGAAAACAAACA-3′, probe 1 5′-VIC-TAAAGCC AATAATTACC-MGB-3′ and probe 2 5′-FAM-AGCCAG

TAATTACC-MGB-3′; P1325: forward primer 5′-GATATG ACTCCTTAATTCACAGGCTACAG-3′, reverse primer 5′-GTTACTGCACACAAGCAACTGTTAA-3′, probe 1 5′-VIC -AATGGATGTTGCATTAGT-MGB-3′ and probe 2 5′-FAM -ATGGATGTTGCGTTAGT-MGB-3′; P1320: forward primer 5′-CCAATCCATTCTTTTATTGACATGGAGTTT-3′, reverse primer 5′-TGATTTTGACCAAGTCCATTGTGTCT -3′, probe 1 5′-VIC-AAAAGCACAAACAACAAG-MGB-3′ and probe 2 5′-FAM-AAAAGCACAAATAACAAG-MGB-3′; P1763: forward primer 5′-GGCAGAAGCAGTGAGTGAGA-3′, reverse primer 5′-TGGGCTCTTATGTCTACCTTTCCTAAA -3′, probe 1 5′-VIC-TCACCTGGATGTCAGC-MGB-3′ and probe 2 5′-FAM-ACCTGGCTGTCAGC-MGB-3′; SNPA: forward primer 5′-TCTGTTATGTGCCATTCACTGTTTT-3′, reverse primer 5′-TAGGGCTGGGATTGGATGA-3′, probe 1 5′-VIC-AGCAGTTTACATCTGGG-MGB-3′ and probe 2 5′-FAM-AGCAGTTTACATCAGGG-MGB-3′. PCR cycling conditions were 95°C for 10 min, 45 cycles of 92°C for 15 s and 60°C for 1 min.

Cell culture

Dissociated cortical cultures were prepared from postnatal 2- or 3-day-old rat (SLC, Shizuoka, Japan) cortex, as described previously (32,33). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml, Sigma) at 37°C. The dissociated cells were plated at a final density of 5×10^5 per cm^2 on polyethyleneimine-coated 12- or 24-well plates (4 and 2 cm^2 surface area/well, respectively; Corning, NY, USA) or cover glasses (Matsunami, Osaka, Japan) attached to flexiperm (VIVASCIENCE, Gottingen, Germany). The culture medium consisted of 5% precolostrum newborn calf serum, 5% heated-inactivated horse serum and 90% of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium containing 15 mM HEPES buffer, pH 7.4, 30 nM Na_2SeO_3 and 1.9 mg/ml of $NaHCO_3$.

Sindbis virus

A bicistronic vector plasmid (pSinEGdsp) was provided by Dr Kawamura (Niigata University, Japan). The plasmid was derived from pSinRep5 (Invitrogen, USA) and had two subgenomic promoters followed by a multiple cloning site for arbitrary gene insertion and an EGFP open reading frame, thus the virus can produce arbitrary protein and EGFP independently in the infected cell, as previously described (34). Dysbindin cDNAs amplified by RT–PCR with specific primer pairs (forward 5′-ACGCGTCAATGCTGGAGACCCTTCG-3′ and reverse 5′-GCATGCCAATTTAAGAGTCGCTGTCC-3′) were inserted at the *Mlu*I and *Sph*I sites of the plasmid. Each plasmid was cleaved with *Pac*I, and used as a template for mRNA transcription *in vitro* using mMESSAGE mMACHINE kit (Ambion, USA). Pseudovirions were produced according to the experimental procedure of Invitrogen. Baby hamster kidney (BHK) cells were transfected with each mRNA and 26S helper mRNA (Invitrogen) by electroporation (1250 V/cm, 50 μF, single pulse) using Gene Pulser2 (BioRad, USA). The cells were incubated with DMEM supplemented with 10% FCS for 24 h at 37°C, the supernatants were collected as pseudovirion-containing solutions.

Immunocytochemistry

Cultured neurons were fixed with 4% paraformaldehyde for 20 min and then rinsed three times with PBS. Subsequently, cultured cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. The primary antibodies (anti-MAP2; Sigma) with 3% skim milk in PBS were applied overnight at 4°C. After washing, cells were incubated with secondary antibodies (Alexa Fluor, Molecular Probes) for 1 h at room temperature. Fluorescent images were captured by an inverted microscope (Axiovert 200, Zeiss) with a CCD (cool SNAPfx) purchased from Zeiss. Monochrome images were turned into color and analyzed using software (Slide BookTM 3.0, Intelligent Imaging Innovations, Inc., Denver, CO, USA). The images of GFP were analyzed with the same software.

Immunoblotting

Cells were lysed in 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. Lysates were centrifuged at 15 000 rpm for 60 min at 4°C, and the supernatants were collected for analysis. Samples were heat denatured with the standard SDS sample buffer. Immunoblottings were carried out as described previously (35). Briefly, immunoblottings were carried out with anti-SNAP25 antibody (1:3000, mouse monoclonal, Synaptic System, Gottingen, Germany), anti-synapsin I antibody (1:1000, rabbit anti-serum, Chemicon), anti-synaptotagmin antibody (1:1000, mouse monoclonal, BD Transduction Laboratory), anti-syntaxin antibody (1:3000, mouse monoclonal, Sigma), anti-GFP antibody (1:1000, rabbit polyclonal, MBL, Nagoya, Japan), anti-dysbindin antibody (23) (1:100, rabbit polyclonal), anti-TUJ1 antibody (1:5000, mouse monoclonal, Berkeley antibody company, CA, USA), anti-Akt antibody (1:1000, rabbit anti-serum, Cell Signaling) and anti-phospho-Akt antibody (Ser473, 1:1000, rabbit anti-serum, Cell Signaling) in TBS containing 1% non-fat dried milk. The immunoblotting experiments were performed four times and they were quantitatively analyzed by capturing images on films using a scanner (Epson, Tokyo, Japan) in conjunction with the Lane and Spot Analyzer software (version 6.0, ATTO, Tokyo, Japan).

Anti-dysbindin antibody was produced as described previously (36). Briefly, the peptide synthesized (QSDEEEVQVDTALC: 320–333 amino acid residue of human dysbindin, with no homology in any mammalian protein) was conjugated with maleimide-activated keyhole limpet hemocyanin and immunized to two rabbits. The titer was measured by ELISA and sera of high titer against the peptide were obtained from both rabbits. The sera were affinity purified by a column conjugated with the immunized peptide.

Detection of glutamate release

The amount of glutamate released from the cultures was measured as previously reported (33,35). The glutamate released into the modified HEPES-buffered Krebs–Ringer assay buffer (KRH; 130 mM NaCl, 5 mM KCl, 1.2 mM

NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin and 25 mM HEPES, pH 7.4) were measured by HPLC (Shimadzu, Kyoto, Japan) with a fluorescence detector (excitation wavelength, 340 nm; emission wavelength, 445 nm, Shimadzu). For stimulation of cortical neurons, we used a HK^+ KRH solution consisting of 85 mM NaCl, 50 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin and 25 mM HEPES, pH 7.4. Before exposing the cultures to HK^+ solution (1 min), basal fractions were collected. The glutamate release experiments were performed three times with independent cultures to confirm reproducibility.

MTT assay

To examine the cell viability, the metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). We performed the viral infection or transfection of siRNA and then, the serum was deprived from culture medium. MTT (0.5 mg/ml in PBS) was added to each well at 24 h after serum deprivation. MTT was incubated for 1.5 h at 37°C. Then, the medium was carefully aspirated, and 200 μl of acidified isopropyl alcohol was added to solubilize the colored formazan product. Absorbance was determined at 550 nm on a scanning multi-well plate reader (Bio-Rad) after agitating the plates for 5 min on a shaker.

siRNA transfection

We used 23 nt siRNA duplexes with two 3′ overhanging nucleotides targeting position 182–204 (aagugacaagucaaga gaagcaa) of human dysbindin mRNA. Scrambled sequence (aacgaugagaacgaucaagaaga), which had no homology to any mammalian mRNA, was used as a control siRNA. Both sense and antisense strands were synthesized by Dharmacon Research Inc (Lafayette, PA, USA). SiRNA duplexes in the 2′-ACE deprotected and desalted form were dissolved in a 1× universal buffer (Dharmacon Research Inc). Transfection of both siRNAs was performed using NeuroPORTER™ (Gene Therapy Systems, Inc., San Diego, CA, USA), as reported (20).

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse (DYNACOM, Yokohama, Japan). The presence of Hardy–Weinberg equilibrium was examined by using the χ^2-test for goodness of fit. Allele distributions between patients and controls were analyzed by the χ^2-test for independence. The measure of LD, denoted as D', was calculated from the haplotype frequency using the expectation–maximization algorithm. Case–control haplotype analysis was performed by the permutation method to obtain the empirical significance (37). The global P-values represent the overall significance using the χ^2-test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by grouping all others together and applying the χ^2-test with 1 df. P-values were calculated on the basis of 10 000 replications. Statistical analysis of neurobiological assays was performed by Students' t-test. All P-values reported are two tailed. Statistical significance was defined at $P < 0.05$. To be conservative, Bonferroni corrections were applied for multiple comparisons, e.g. number of analyzed SNPs and haplotypes, although SNPs were in LD.

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REFERENCES

1. O'Donovan, M.C., Williams, N.M. and Owen, M.J. (2003) Recent advances in the genetics of schizophrenia. *Hum. Mol. Genet.*, **12**, R125–R133.
2. Straub, R.E., Jiang, Y., MacLean, C.J., Ma, Y., Webb, B.T., Myakishev, M.V., Harris-Kerr, C., Wormley, B., Sadek, H., Kadambi, B. *et al.* (2002) Genetic variation in the 6p22.3 gene *DTNBP1*, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. *Am. J. Hum. Genet.*, **71**, 337–348.
3. Schwab, S.G., Knapp, M., Mondabon, S., Hallmayer, J., Borrmann-Hassenbach, M., Albus, M., Lerer, B., Rietschel, M., Trixler, M., Maier, W. *et al.* (2003) Support for association of schizophrenia with genetic variation in the 6p22.3 gene, dysbindin, in sib-pair families with linkage and in an additional sample of triad families. *Am. J. Hum. Genet.*, **72**, 185–190.
4. Tang, J.X., Zhou, J., Fan, J.B., Li, X.W., Shi, Y.Y., Gu, N.F., Feng, G.Y., Xing, Y.L., Shi, J.G. and He, L. (2003) Family-based association study of DTNBP1 in 6p22.3 and schizophrenia. *Mol. Psychiatr.*, **8**, 717–718.
5. Van Den Bogaert, A., Schumacher, J., Schulze, T.G., Otte, A.C., Ohlraun, S., Kovalenko, S., Becker, T., Freudenberg, J., Jonsson, E.G., Mattila-Evenden, M. *et al.* (2003) The *DTNBP1* (dysbindin) gene contributes to schizophrenia, depending on family history of the disease. *Am. J. Hum. Genet.*, **73**, 1438–1443.
6. van den Oord, E.J., Sullivan, P.F., Jiang, Y., Walsh, D., O'Neill, F.A., Kendler, K.S. and Riley, B.P. (2003) Identification of a high-risk haplotype for the dystrobrevin binding protein 1 (*DTNBP1*) gene in the Irish study of high-density schizophrenia families. *Mol. Psychiatr.*, **8**, 499–510.
7. Morris, D.W., McGhee, K.A., Schwaiger, S., Scully, P., Quinn, J., Meagher, D., Waddington, J.L., Gill, M. and Corvin, A.P. (2003) No evidence for association of the dysbindin gene [*DTNBP1*] with schizophrenia in an Irish population-based study. *Schizophr. Res.*, **60**, 167–172.
8. Tsai, G. and Coyle, J.T. (2002) Glutamatergic mechanisms in schizophrenia. *Annu. Rev. Pharmacol. Toxicol.*, **42**, 165–179.
9. Weinberger, D.R., Egan, M.F., Bertolino, A., Callicott, J.H., Mattay, V.S., Lipska, B.K., Berman, K.F. and Goldberg, T.E. (2001) Prefrontal neurons and the genetics of schizophrenia. *Biol. Psychiatr.*, **50**, 825–844.
10. Moghaddam, B. (2003) Bringing order to the glutamate chaos in schizophrenia. *Neuron*, **40**, 881–884.
11. Harrison, P.J. and Owen, M.J. (2003) Genes for schizophrenia? Recent findings and their pathophysiological implications. *Lancet*, **361**, 417–419.
12. Buonanno, A. and Fischbach, G.D. (2001) Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr. Opin. Neurobiol.*, **11**, 287–296.
13. Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S.,

Ivarsson, O., Chou, T.T. *et al.* (2002) Neuregulin 1 and susceptibility to schizophrenia. *Am. J. Hum. Genet.*, **71**, 877–892.

14. Mothet, J.P., Parent, A.T., Wolosker, H., Brady, R.O., Jr, Linden, D.J., Ferris, C.D., Rogawski, M.A. and Snyder, S.H. (2000) D-serine is an endogenous ligand for the glycine site of the *N*-methyl-D-aspartate receptor. *Proc. Natl Acad. Sci. USA*, **97**, 4926–4931.
15. Chumakov, I., Blumenfeld, M., Guerassimenko, O., Cavarec, L., Palicio, M., Abderrahim, H., Bougueleret, L., Barry, C., Tanaka, H., La Rosa, P. *et al.* (2002) Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc. Natl Acad. Sci. USA*, **99**, 13675–13680.
16. De Blasi, A., Conn, P.J., Pin, J. and Nicoletti, F. (2001) Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol. Sci.*, **22**, 114–120.
17. Benson, M.A., Newey, S.E., Martin-Rendon, E., Hawkes, R. and Blake, D.J. (2001) Dysbindin, a novel coiled-coil-containing protein that interacts with the dystrobrevins in muscle and brain. *J. Biol. Chem.*, **276**, 24232–24241.
18. Turner, K.M., Burgoyne, R.D. and Morgan, A. (1999) Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends. Neurosci.*, **22**, 459–464.
19. Brunet, A., Datta, S.R. and Greenberg, M.E. (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr. Opin. Neurobiol.*, **11**, 297–305.
20. Numakawa, T., Nakayama, H., Suzuki, S., Kubo, T., Nara, F., Numakawa, Y., Yokomaku, D., Araki, T., Ishimoto, T., Ogura, A. *et al.* (2003) Nerve growth factor-induced glutamate release is via p75 receptor, ceramide, and Ca(2+) from ryanodine receptor in developing cerebellar neurons. *J. Biol. Chem.*, **278**, 41259–41269.
21. Williams, N.M., Preece, A., Morris, D.W., Spurlock, G., Bray, N.J., Stephens, M., Norton, N., Williams, H., Clement, M., Dwyer, S. *et al.* (2004) Identification in 2 independent samples of a novel schizophrenia risk haplotype of the dystrobrevin binding protein gene (*DTNBP1*). *Arch. Gen. Psychiatr.*, **61**, 336–344.
22. Tokuhiro, S., Yamada, R., Chang, X., Suzuki, A., Kochi, Y., Sawada, T., Suzuki, M., Nagasaki, M., Ohtsuki, M., Ono, M. *et al.* (2003) An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat. Genet.*, **35**, 341–348.
23. McClintock, W., Shannon Weickert, C., Halim, N.D., Lipska, B.K., Hyde, T.M., Herman, M.M., Weinberger, D.R., Kleinman, J.E. and Straub, R.E. (2003) Reduced expression of dysbindin protein in the dorsolateral prefrontal cortex of patients with schizophrenia. *Program No. 317.9. 2003 Abstract Viewer/Itinerary Planner.* Washington, DC, Society for Neuroscience (Online).
24. Talbot, K., Eidem, W.L., Tinsley, C.L., Benson, M.A., Thompson, E.W., Smith, R.J., Hahn, C.G., Siegel, S.J., Trojanowski, J.Q., Gur, R.E. *et al.* (2004) Dysbindin-1 is reduced in intrinsic, glutamatergic terminals of the hippocampal formation in schizophrenia. *J. Clin. Invest.*, **113**, 1353–1363.
25. Weickert, C.S., Straub, R.E., McClintock, B.W., Matsumoto, M., Hashimoto, R., Hyde, T.M., Herman, M.M., Weinberger, D.R. and Kleinman, J.E. (2004) Human dysbindin (*DTNBP1*) gene expression in normal brain and in schizophrenic prefrontal cortex and midbrain. *Arch. Gen. Psychiatr.*, **61**, 544–555.
26. Honer, W.G., Falkai, P., Bayer, T.A., Xie, J., Hu, L., Li, H.Y., Arango, V., Mann, J.J., Dwork, A.J. and Trimble, W.S. (2002) Abnormalities of SNARE mechanism proteins in anterior frontal cortex in severe mental illness. *Cereb. Cortex*, **12**, 349–356.
27. Vawter, M.P., Thatcher, L., Usen, N., Hyde, T.M., Kleinman, J.E. and Freed, W.J. (2002) Reduction of synapsin in the hippocampus of patients with bipolar disorder and schizophrenia. *Mol. Psychiatr.*, **7**, 571–578.
28. Emamian, E.S., Hall, D., Birnbaum, M.J., Karayiorgou, M. and Gogos, J.A. (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat. Genet.*, **36**, 131–137.
29. Li, W., Zhang, Q., Oiso, N., Novak, E.K., Gautam, R., O'Brien, E.P., Tinsley, C.L., Blake, D.J., Spritz, R.A., Copeland, N.G. *et al.* (2003) Hermansky–Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). *Nat. Genet.*, **35**, 84–89.
30. Hashimoto, R., Yoshida, M., Ozaki, N., Yamanouchi, Y., Iwata, N., Suzuki, T., Kitajima, T., Tatsumi, M., Kamijima, K. and Kunugi, H. (2004) Association analysis of the −308G>A promoter polymorphism of the tumor necrosis factor alpha (TNF-alpha) gene in Japanese patients with schizophrenia. *J. Neural Transm.*, **111**, 217–221.
31. Hashimoto, R., Straub, R.E., Weickert, C.S., Hyde, T.M., Kleinman, J.E. and Weinberger, D.R. (2004) Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia. *Mol. Psychiatr.*, **9**, 299–307.
32. Numakawa, T., Matsumoto, T., Adachi, N., Yokomaku, D., Kojima, M., Takei, N. and Hatanaka, H. (2001) Brain-derived neurotrophic factor triggers a rapid glutamate release through increase of intracellular Ca(2+) and Na(+) in cultured cerebellar neurons. *J. Neurosci. Res.*, **66**, 96–108.
33. Numakawa, T., Yamagishi, S., Adachi, N., Matsumoto, T., Yokomaku, D., Yamada, M. and Hatanaka, H. (2002) Brain-derived neurotrophic factor-induced potentiation of Ca(2+) oscillations in developing cortical neurons. *J. Biol. Chem.*, **277**, 6520–6529.
34. Kawamura, M., Namba, H., Otsu, Y., Hayashi, Y., Takei, N. and Nawa, H. (2003) Characterization of novel bicistronic sindbis virus vectors, SinEGdsp and SinIRES-EG, in cultured neurons. *Recent Res. Dev. Neurochem.*, **6**, 105–120.
35. Numakawa, T., Yokomaku, D., Kiyosue, K., Adachi, N., Matsumoto, T., Numakawa, Y., Taguchi, T., Hatanaka, H. and Yamada, M. (2002) Basic fibroblast growth factor evokes a rapid glutamate release through activation of the MAPK pathway in cultured cortical neurons. *J. Biol. Chem.*, **277**, 28861–28869.
36. Hashimoto, R., Nakamura, Y., Komai, S., Kashiwagi, Y., Tamura, K., Goto, T., Aimoto, S., Kaibuchi, K., Shiosaka, S. and Takeda, M. (2000) Site-specific phosphorylation of neurofilament-L is mediated by calcium/calmodulin-dependent protein kinase II in the apical dendrites during long-term potentiation. *J. Neurochem.*, **75**, 373–382.
37. Good, P. (2000) *Permutation Tests. A Practical Guide to Resampling Methods for Testing Hypothesis.* 2nd edn. Springer-Verlag, New York.

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Association study of the frizzled-3 (FZD3) gene with schizophrenia and mood disorders

Short Communication

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Summary. Two research groups have recently reported a significant association between schizophrenia and genetic variants of Frizzled-3 (FZD3) gene. We examined a possible association in a Japanese sample of schizophrenia, bipolar disorder, unipolar depression and controls with four single nucleotide polymorphisms (SNPs), tested in previous reports. We failed to find significant association in the four SNPs or haplotype analysis. The FZD3 gene might not play a role in conferring susceptibility to major psychosis in our sample.

Keywords: FZD3, schizophrenia, mood disorder, association study, single nucleotide polymorphism (SNP).

Introduction

Schizophrenia is a complex genetic disorder characterized by disturbances of cognition, emotion and social functioning. This disease is believed to involve genetic abnormalities in developmental/plasticity related processes during a critical period in neuronal growth (Weinberger et al., 2001). Wnt signal transduction cascades have been implicated in a variety of neurodevelopmental processes, e.g. segmentation, central nervous system patterning, and cell divisions (Wodarz and Nusse, 1998). Wnt proteins signal via cell surface transmembrane receptors, termed frizzleds, which display many properties

characteristic of members of the superfamily of G-protein-coupled receptors (Wang and Malbon, 2004). The frizzled-3 (FZD3) gene, a member of frizzles, is located on chromosome 8p21, repeatedly suggested as a positive linkage locus for schizophrenia (Lewis et al., 2003; McGuffin et al., 2003). The FDZ3 gene consists of 8 exons and 7 introns, spanning approximately 70 Kb (Kirikoshi et al., 2000). In accordance with this, two research groups have recently reported a significant association between schizophrenia and the FZD3 gene in Japanese and Chinese samples (Katsu et al., 2003; Yang et al., 2003). We tried to replicate these findings in an independent Asian sample. Furthermore, we also examined the possible association between the FZD3 gene with mood disorders, since schizophrenia and mood disorders might share the genetic vulnerability (Berrettini, 2003).

Methods and materials

Subjects

Subjects were 427 patients with schizophrenia (221 males and 206 females with mean age of 44.2 years [SD 14.5]), 91 with bipolar disorder (40 and 51; 53.6 years [SD 14.8]), and 396 with major depression (155 and 241; 53.4 years [SD 16.1]) and 473 healthy controls (228 and 245; 36.1 years [SD 12.5]). All the subjects were biologically unrelated Japanese. Consensus diagnosis was made for each patient by at least two trained psychiatrists according to the DSM-IV criteria. Controls were healthy volunteers who had no current or past contact to psychiatric services. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees.

SNP genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. We genotyped four SNPs (single nucleotide polymorphisms; dbSNP accession: rs960914 in intron3, rs2241802 in exon5: A435G, L145L, rs2323019 in intron5 and rs352203 in intron5) in the FZD3 gene, which were examined in the previous two studies (Katsu et al., 2003; Yang et al., 2003). Genotyping was performed with the TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2004a, b). Briefly, primers and probes for detection of the SNPs are: rs960914: forward primer 5′-CTTTTATAAAGAAATTTGAAACAT CAGAACATGGGA-3′, reverse primer 5′-ACTTTTTCACTGCTTGGGAGTTATTCT-3′, probe 1 5′-VIC-CTGAATGGCTGCTATC-MGB-3′, and probe 2 5′-FAM-TCTGAATGGCTACTATC-MGB-3′; rs2241802: forward primer 5′-ATGAGCCATATCCTCGACTTGTG-3′, reverse primer 5′-GGACACCAAAAACCATAGTCTCTCT-3′, probe 1 5′-VIC-TCCAGCTAAATTCAG-MGB-3′, and probe 2 5′-FAM-CAGCCAAATTCAG-MGB-3′; rs2323019: forward primer 5′-GAAT TACTTTGTTTTTCTAGATTCTTGAATTGAAAGC-3′, reverse primer 5′-CCAACCTGGTTAA TAATGGTCTTTTGG-3′, probe 1 5′-VIC-TCATTTATTGTCAATGTTTTAA-MGB-3′, and probe 2 5′-TCATTTATTGTCAATATTTTAA-MGB-3′; rs352203: forward primer 5′-CCTGAAAAAA TATTCTATATCTCTTGTTTTGCCA-3′, reverse primer 5′-CAACCAGGACATAACAGTATTA CAGTTTCTAT-3′, probe 1 5′-VIC-TCCTTCATGTCGTATTC-MGB-3′, and probe 2 5′-FAM-TTTCCTTCATATCGTATTC-MGB-3′, PCR cycling conditions were: at 95°C for 10 minutes, 45 cycles of 92°C for 15 seconds and 60°C for 1 minute.

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse software (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium was examined using the $\chi\chi^2$ test for goodness of fit. Allele distributions between patients and controls were analyzed by the

Table 1. Allele distribution for SNPs in the FZD3 gene between major psychoses and controls

dbSNP ID	SNP	Controls	Schizophrenia		Bipolar		Unipolar	
		n = 473	n = 427	*P* value	n = 91	*P* value	n = 397	*P* value
rs960914	T/C	.398	.396	.91	.352	.23	.386	.61
rs2241802	G/A	.453	.458	.85	.451	.94	.455	.96
rs2323019	A/G	.407	.420	.57	.357	.21	.409	.91
rs352203	T/C	.397	.396	.97	.352	.24	.386	.64

χ^2 test for independence. The measures of linkage disequilibrium (LD), denoted as D', was calculated from the haplotype frequency using Expectation-Maximization algorithm. Case-control haplotype analysis was performed by the permutation method to obtain empirical significance (Good, 2000). The global p-values represent the overall significance using the χ^2 test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotype was tested for association by grouping all others together and applying the χ^2 test with 1 df. P-values were calculated based on 10,000 replications. All p-values reported are two tailed. Statistical significance was defined at $p<0.05$.

Results

The obtained allele frequencies for the patients and controls are shown in Table 1. The genotype distributions for all the diagnostic groups were in Hardy-Weinberg equilibrium (data not shown). There was no significant difference in genotype distributions or allele frequencies of the four SNPs in the FZD3 gene between the controls and any patient group, although previous studies reported positive associations between schizophrenia and several SNPs (Katsu et al., 2003; Yang et al., 2003). We computed the LD between the SNPs using D', which ranged between 0.8 and 1.0, indicating strong to intermediate LD between the markers. Adjacent combinations of up to four markers were tested, however, any haplotype combination was not significantly associated with any diagnostic group (all global p-values>0.2).

Discussion

This study examined the possible association of the FZD gene with schizophrenia and mood disorders in our Japanese sample. We obtained no evidence for a significant association of the genetic variations of the FZD gene with any diagnostic group, suggesting that the examined polymorphisms play no major role in the pathogenesis of major psychoses in our sample. Our results are thus inconsistent with the results of the previous case-control study which reported a significantly higher frequency of the T allele of rs960914 in patients with schizophrenia than in controls (Katsu et al., 2003). The frequencies of the T allele were 0.62 and 0.51 in schizophrenics (n = 209) and controls (n = 200) in their Japanese sample, while the frequencies of the T allele were 0.60 and 0.60 in patients with schizophrenia (n = 427) and controls (n = 473) in ours. A highly significant association of the other three SNPs (rs2241802, rs2323019 and rs352203) and their three marker haplotypes with schizophrenia patients was reported in a family-based association study in a Chinese population

(Yang et al., 2003), while no evidence of such an association was obtained in our results (Chinese: global p-value $<$ 0.000001, GAT haplotype p value $<$ 0.000001; vs our results: global p-value $=$ 0.31, GAT haplotype p-value $=$ 0.97). Recently, the positive association between schizophrenia and the FZD3 gene has been reported in case-control study in a Chinese population (Zhang et al., 2004). This study presented that a new marker, rs880481, created the most positive results. Further analysis using this new marker should be examined in other ethnic populations.

A possible explanation for the discrepancy between the previous results and ours is a type II error in our sample. The odds ratio of the T allele of rs960914 was 1.54 in Japanese study (Katsu et al., 2003). However, power analysis revealed that our sample size could detect a significant association between the examined risk alleles (frequency of 0.4–0.6) and schizophrenia with a power of 90% when odds ratio was assumed to be 1.4 or more and the critical p-value was set at 0.05. There is only a small chance that a clinically meaningful difference would have been missed with the data. Secondly, it is possible that LD with other unknown polymorphisms, that is truly responsible for giving susceptibility to schizophrenia, may explain the discrepancy. Alternatively, the significant association observed by the previous two groups may have arisen by chance. The case-control association study is subject to the effect of population stratification, although the patients and controls were ethnically matched.

More recently, an extensive family-transmission and case-control analysis in a Japanese population with additional post-mortem mRNA expression data and family trio analysis in a British population yielded negative results for association between the FZD3 gene and schizophrenia (Ide et al., 2004; Wei and Hemmings, 2004), which is consistent with our results.

In conclusion, we obtained no evidence for an association between the FZD3 gene and schizophrenia or mood disorders, suggesting that this gene has no major role in conferring susceptibility to major psychoses in our sample.

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References

Berrettini W (2003) Evidence for shared susceptibility in bipolar disorder and schizophrenia. Am J Med Genet 123C: 59–64

Good P (2000) Permutation tests. A practical guide to resampling methods for testing hypothesis, 2nd edn. Springer, New York

Hashimoto R, Straub RE, Weickert CS, Hyde TM, Kleinman JE, Weinberger DR (2004) Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia. Mol Psychiatry 9: 299–307

Hashimoto R, Yoshida M, Ozaki N, Yamanouchi Y, Iwata N, Suzuki T, Kitajima T, Tatsumi M, Kamijima K, Kunugi H (2004) Association analysis of the −308G>A promoter polymorphism of the tumor necrosis factor alpha (TNF-alpha) gene in Japanese patients with schizophrenia. J Neural Transm 111: 217–221

Ide M, Muratake T, Yamada K, Iwayama-Shigeno Y, Iwamoto K, Takao H, Toyota T, Kaneko N, Minabe Y, Nakamura K, Kato T, Mori N, Asada T, Someya T, Yoshikawa T (2004) Genetic and expression analyses of FZD3 in schizophrenia. Biol Psychiatry 56: 462–465

Katsu T, Ujike H, Nakano T, Tanaka Y, Nomura A, Nakata K, Takaki M, Sakai A, Uchida N, Imamura T, Kuroda S (2003) The human frizzled-3 (FZD3) gene on chromosome 8p21, a receptor gene for Wnt ligands, is associated with the susceptibility to schizophrenia. Neurosci Lett 353: 53–56

Kirikoshi H, Koike J, Sagara N, Saitoh T, Tokuhara M, Tanaka K, Sekihara H, Hirai M, Katoh M (2000) Molecular cloning and genomic structure of human frizzled-3 at chromosome 8p21. Biochem Biophys Res Commun 271: 8–14

Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, Williams NM, Schwab SG, Pulver AE, Faraone SV, Brzustowicz LM, Kaufmann CA, Garver DL, Gurling HM, Lindholm E, Coon H, Moises HW, Byerley W, Shaw SH, Mesen A, Sherrington R, O'Neill FA, Walsh D, Kendler KS, Ekelund J, Paunio T, Lonnqvist J, Peltonen L, O'Donovan MC, Owen MJ, Wildenauer DB, Maier W, Nestadt G, Blouin JL, Antonarakis SE, Mowry BJ, Silverman JM, Crowe RR, Cloninger CR, Tsuang MT, Malaspina D, Harkavy-Friedman JM, Svrakic DM, Bassett AS, Holcomb J, Kalsi G, McQuillin A, Brynjolfson J, Sigmundsson T, Petursson H, Jazin E, Zoega T, Helgason T (2003) Genome scan meta-analysis of schizophrenia and bipolar disorder, part II. Schizophrenia. Am J Hum Genet 73: 34–48 (Epub 2003 Jun 2011)

McGuffin P, Tandon K, Corsico A (2003) Linkage and association studies of schizophrenia. Curr Psychiatry Rep 5: 121–127

Wang HY, Malbon CC (2004) Wnt-frizzled signaling to G-protein-coupled effectors. Cell Mol Life Sci 61: 69–75

Wei J, Hemmings GP (2004) Lack of a genetic association between the frizzled-3 gene and schizophrenia in a British population. Neurosci Lett 366: 336–338

Weinberger DR, Egan MF, Bertolino A, Callicott JH, Mattay VS, Lipska BK, Berman KF, Goldberg TE (2001) Prefrontal neurons and the genetics of schizophrenia. Biol Psychiatry 50: 825–844

Wodarz A, Nusse R (1998) Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14: 59–88

Yang J, Si T, Ling Y, Ruan Y, Han Y, Wang X, Zhang H, Kong Q, Li X, Liu C, Zhang D, Zhou M, Yu Y, Liu S, Shu L, Ma D, Wei J (2003) Association study of the human FZD3 locus with schizophrenia. Biol Psychiatry 54: 1298–1301

Zhang Y, Yu X, Yuan Y, Ling Y, Ruan Y, Si T, Lu T, Wu S, Gong X, Zhu Z, Yang J, Wang F, Zhang D (2004) Positive association of the human frizzled 3 (FZD3) gene haplotype with schizophrenia in Chinese Han population. Am J Med Genet 129B: 16–19

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Hypersomnia, asterixis and cataplexy in association with orexin A-reduced hypothalamic tumor

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Sirs: There is an absence or low level of orexin in the cerebrospinal fluid (CSF) in a majority of patients with narcolepsy-cataplexy, a chronic sleep disorder characterized by excessive daytime sleepiness (EDS) and cataplexy [6]. Orexin deficiency has been reported in several symptomatic cases of narcolepsy or hypersomnia associated with neurodegenerative, surgical, ischemic and post-infectious insults to hypothalamus [1, 5, 7].

Here we report a patient with a hypothalamic tumor and low CSF orexin A level whose symptoms improved with non-surgical therapy. We discuss the clinical presentation of orexin-reduced hypersomnia in comparison with idiopathic narcolepsy-cataplexy.

A 66-year-old woman without remarkable family history was admitted to our hospital reporting symptoms typical of EDS for 1 month including falling asleep when riding in a car and during eating. Also, her family had noted that she sustained abrupt falls without loss of consciousness. Visual hallucinations also were noted but it was not of the sleep-onset type, and there was no history suggestive of sleep paralysis. Although alert, able to follow commands, and answer questions her time orientation was disturbed and she scored 21/30 on minimental state examination (MMSE). The cranial nerves, muscle tone, motor strength, and sensory were normal. When the arms were outstretched with wrists and fingers extended, abrupt downward movements of the hands occurred bilaterally consistent with asterixis. In the ward the patient suffered from facial injury from falls at night several times and she said it had been caused by an abrupt tone reduction of legs without loss of consciousness. Routine blood chemistry analysis was normal and endocrinological studies were consistent with mild anterior hypopituitarism. CSF examination revealed a protein concentration of 57 mg/dl, mild mononuclear pleocytosis, and an orexin A concentration of 62 pg/ml measured by radioimmunoassay using methodology reported previously [6]. Magnetic resonance T2 weighted images of the brain exhibited high signal intensities in the hypothalamus, thalamus, and midbrain bilaterally, with gadolinium enhancement on T1 images (Fig. 1A, B) consistent with tumor. A daytime polysomnogram demonstrated REM sleep latency of eleven minutes with five minute sleep latency. She underwent radiation therapy (total of 46 Gy), and nimustine hydrochloride and interferon β administered. The patient improved cognitively (MMSE 28/30) and behaviorally. EDS, disturbed sleep-wake cycle, falls, arm asterixis and night confusion subsided. MR images taken 4 months after completion of her therapy demonstrated reduction of tumor size and edema (Fig. 1C, D).

Features of symptomatic narcolepsy or hypersommnia can vary widely according to histopathology, lesion size and location. Memory disturbance in our patient may have been due to dysfunction of amygdalofugal pathways to the ventromedial nuclei [2]. Her arms showed typical asterixis and although she had no walking difficulty in daytime she fell at night without disturbance of consciousness so we estimated that she had cataplexy-like drop attacks independent of emotional stimulus as well as upper asterixis. Similar atypical cataplexy has been reported in hypothalamic tumor cases with hypersomnia [5]. Patients with focal lesions of the basal ganglia or midbrain can show asterixis [3], but there has been no reported case having both upper asterixis and drop attack. Bril et al. have discussed the similarities between asterixis and drop attack or cataplexy and suggested upper asterixis is a segmental form of drop attack [3]. Our case also suggests that there might be common pathophysiology or anatomical site, within the hypothalamus or brainstem, for abrupt hypotonus, thus linking asterixis with cataplexy.

Most cases of symptomatic narcolepsy involve lesions of the hypothalamus and rostral brainstem, and only one previously reported case fulfills the narcoleptic tetrad [7]. The reduction of orexin level supports the hypothesis that hypersomnia is caused by decreased orexin. Typically, primary narcolepsy patients show extremely low, and even undetectable levels of orexin A in the CSF [4, 6]. Narcolepsy without cataplexy or idiopathic hypersomnia cases shows only relatively low orexin levels [4]. In our patient CSF orexin A (62 pg/ml) showed relative reduction compared with controls (280 ± 33pg/ml) [6].

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