今村明、岡崎祐士：精神分裂病の病態の変化～最近の知見～．医薬ジャーナル 38 巻 3 号、983-986、2002.

Matsumoto S, Sasaki T, Imamura A, Matsuo K, Kayashima T, Hashida A, Ono S, Tsujita T, Matsumoto S, Nakane Y, Tokunaga K, Okazaki Y. HLA class I distribution in Japanese patients with schizophrenia. Am J Med Genet 2002 Jan 8;114(1):42-45

Imamura A, Tsujita T, Kayashima T, Oda R, Kikuchi T, Hayashida M, Hamada A, Fujimaru K, Matsumoto S, Hashida A, Nakane Y, Okazaki Y. Lack of association between the hKCa3 gene and Japanese schizophrenia patients. Psychiatr Genet 2001 Dec;11(4):227-229

2.学会発表

Ono S, Imamura A, Hashida A, Matsumoto S, Tasaki S, Fujimaru K, Tsujita T, Hirayama R, Ozawa H, Okazaki Y: The relationship between neurotrophic factor-related genes and minor physical anomalies (MPAs) in Japanese patients with schizophrenia. Am J Med Genet 130B, 55, 2004

Imamura A, Jinno Y, Shimabukuro M, Hashida A, Matsumoto S, Ozawa H, Okazaki Y: Anticipation DNA methylation in schizophrenia. Am J Med Genet 130B, 136, 2004

Tasaki S, Imamura A, Hirayama R, Kikuchi T, Tsujita T, Okazaki Y, Ozawa H: Scanning of genomic differences in monozygotic twins discordant for schizophrenia, using CA repeat markers. Am J Med Genet 130B,147,2004

（以上３つは The XIIth World Congress of Psychiatric Genetics 2004 9th - 13th October 2004, Dublin, Ireland の抄録）

CA リピートマーカーを用いた一卵性双生児統合失調症不一致例の差異の検討－第 2 報－
田崎真也、古賀利香、橋田あおい、菊池妙子、与那城竹亮、藤丸浩輔、、今村明、辻田高宏、岡崎祐士（第 56 回九州精神神経学会、2003 年 11 月 6 日－7 日、久留米）

H.知的財産権の出願・登録状況

なし

Ⅲ　研究成果の刊行に関する一覧表　　（主要なものを選択）

書　籍

著者氏名	論文タイトル名	書籍全体の編集者	書籍名	出版社名	出版地	出版年	ページ
Ujike H, Takaki M, Kodama M, Kurida S	Gene expression related to synaptogenesis, neuritogenesis, and MAP kinase in behavioral sensitization to psychostimulants	Ali SF	Cellular and Molecular Mechanisms of Drugs of Abuse II, Cocaine, Substituted Amphetamines, GHB, and Opiates	Ann New York Acad Sci	New York	2002	55-67
Inada T, Iijima Y, Uchida N, Maeda T, Iwashita S, Ozaki N, Harano M, Komiyama T, Yamada M, Sekine Y, Iyo M, Sora I, Ujike H	No Association Found between the Type 1 Sigma Receptor Gene Polymorphisms and Methamphetamine Abuse in the Japanese Population: A Collaborative Study by the Japanese Genetics Initiative for Drug Abuse.	Ali SF	Cellular and Molecular Mechanisms of Drugs of Abuse II, Cocaine, Substituted Amphetamines, GHB, and Opiates	Ann New York Acad Sci	New York	2004	27-33
Iwata N, Inada T, Harano M, Komiyama T, Yamada M, Sekine Y, Iyo M, Sora I, Ujike H, Ozaki N	No Association Is Found between the Candidate Genes of t-PA/Plasminogen System and Japanese Methamphetamine-Related Disorder: A Collaborative Study by the Japanese Genetics Initiative for Drug Abuse.	Ali SF	Cellular and Molecular Mechanisms of Drugs of Abuse II, Cocaine, Substituted Amphetamines, GHB, and Opiates	Ann New York Acad Sci	New York	2004	34-38

Harano M, Uchimura N, Abe H, Ishibashi M, Iida N, Yanagimoto K, Tanaka T, Maeda H, Sora I, Iyo M, Komiyama T, Yamada M, Sekine Y, Inada T, Ozaki N, Ujike H	A Polymorphism of DRD2 Gene and Brain Atrophy in Methamphetamine Psychosis.	Ali SF	Cellular and Molecular Mechanisms of Drugs of Abuse II, Cocaine, Substituted Amphetamines, GHB, and Opiates	Ann New York Acad Sci	New York		307-315
Ide S, Kobayashi H, Tanaka K, Ujike H, Sekine Y, Ozaki N, Inada T, Harano M, Komiyama T, Yamada M, Iyo M, Ikeda K, Sora I	Gene polymorphisms of the mu opioid receptor in methamphetamine abusers.	Ali SF	Cellular and Molecular Mechanisms of Drugs of Abuse II, Cocaine, Substituted Amphetamines, GHB, and Opiates	Ann New York Acad Sci	New York	2004	316-324

Kobayashi H, Ide S, Hasegawa J, Ujike H, Sekine Y, Ozaki N, Inada T, Harano M, Komiyama T, Yamada M, Iyo M, Shen HW, Ikeda K	Study of Association between {alpha}-Synuclein Gene Polymorphism and Methamphetamine Psychosis/Dependence.	Ali SF	Cellular and Molecular Mechanisms of Drugs of Abuse II, Cocaine, Substituted Amphetamines, GHB, and Opiates	Ann New York Acad Sci	New York	2004	324-335

雑　誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Masui T, Hashimoto R, Kusumi I, Suzuki K, Tanaka T, Nakagawa S, Kunugi H, Koyama T:	Possible association between -116C/G polymorphism of the XBP1 gene and response to lithium in bipolar disorder.	International Journal of Neuropsychopharmacology		in press	
Hashimoto R, Okada T, Kato T, Kosuga A, Tatsumi M, Kamijima K, Kunugi H	The breakpoint cluster region (BCR) gene on chromosome 22q11 is associated with bipolar disorder	Biological Psychiatry		in press	
Hashimoto R, Suzuki T, Iwata N, Yamanouchi Y, Kitajima T, Kosuga A, Tatsumi M, Ozaki N, Kamijima K, Kunugi H	Association study of the frizzled-3 (FZD3) gene with schizophrenia and mood disorders.	Journal of Neural Transmission	112	303-307	2005
Hashimoto R, Yoshida M, Ozaki N, Yamanouchi Y, Iwata N, Suzuki T, Kitajima T, Tatsumi M, Kamijima K, Kunugi H	A missense polymorphism (H204R) of a Rho GTPase-activating protein, the chimerin 2 gene, is associated with schizophrenia in men.	Schizophrenia Research	73	383-385	2005
Munakata K, Tanaka M, Mori K, Washizuka S, Yoneda M, Tajima O, Akiyama T, Nanko S, Kunugi H, Tadokoro K, Ozaki N, Inada T, Sakamoto K, Fukunaga T, Iijima Y, Iwata N, Tatsumi M, Yamada K, Yoshikawa T, Kato T	Mitochondrial DNA 3644T-->C mutation associated with bipolar disorder.	Genomics	84	1041-1050	2004
Numakawa T, Yagasaki Y, Ishimoto T, Suzuki T, Iwata N, Ozaki N,Taguchi T, Tatsumi M, Kamijima K, Straub RE, Weinberger DR, Kunugi H, Hashimoto R	Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia.	Human Molecular Genetics	13	2699-2708	2004
Washizuka S, Iwamoto K,	Association of mitochondrial	Biological	56	483-489	2004

Kazuno AA, Kakiuchi C, Mori K, Kametani M, Yamada K, Kunugi H, Tajima O, Akiyama T, Nanko S, Yoshikawa T, Kato T	complex I subunit gene NDUFV2 at 18p11 with bipolar disorder in Japanese and the National Institute of Mental Health pedigrees	Psychiatry			
Kusumi I, Masui T, Kakiuchi C, Suzuki K, Akimoto T, Hashimoto R, Kunugi H, Kato T, Koyama T	Lack of association between XBP1 genotype and calcium signaling in the platelets of healthy subjects.	Neuroscience Letters	369	1-3	2004
Kunugi H, Iijima Y, Tatsumi M, Yoshida M, Hashimoto R, Kato T, Sakamoto K, Fukunaga T, Inada T, Suzuki T, Iwata N, Ozaki N, Yamada K, Yoshikawa T	No association between the Val66Met polymorphism of the brain-derived neurotrophic factor (BDNF) gene and bipolar disorder in Japanese: a multi-center study.	Biological Psychiatry	56	376-378	2004
Tadokoro K, Hashimoto R, Tatsumi M, Kamijima K, Kunugi H	Analysis of enhancer activity of a dinucleotide repeat polymorphism in the neurotrophin-3 gene and its association with bipolar disorder.	Neuropsycho biology	50	206-210	2004
Kakiuchi C, Nanko S, Kunugi H, Kato T	Reply to "Lack of support for a genetic association of the XBP1 promoter polymorphism with bipolar disorder in probands of European origin".	Nature Genetics	36	784-785	2004
Saito S, Ikeda M, Iwata N, Suzuki T, Kitajima T, Yamanouchi Y, Kinoshita Y, Takahashi N, Inada T, Ozaki N	No association was found between a functional SNP in ZDHHC8 and schizophrenia in a Japanese case-control population	Neurosci Lett	374	21-24	2005

Takano A, Uchiyama M, Kajimura N, Mishima K, Inoue Y, Kamei Y, Kitajima T, Shibui K, Katoh M, Watanabe T, Hashimotodani Y, Ozeki Y, Hori T, Yamada N, Toyoshima R, Ozaki N, Okawa M, Nagai K, Takahashi K, Isojima Y, Yamauchi T, Ebisawa T	A Missense Variation in Human Casein Kinase I Epsilon Gene that Induces Functional Alteration and Shows an Inverse Association with Circadian Rhythm Sleep Disorders.	Neuropsychopharmacology	29	1901-1909	2004
Ozaki N	Pharmacogenetics of antipsychotics.	Nagoya J Med Sci	67	1-7	2004
Okada M, Goldman D, Linnoila M, Iwata N, Ozaki N, Northup JK	Comparison of G-Protein Selectivity of Human 5-HT2C and 5-HT1A Receptors.	Ann N Y Acad Sci	1025	570-577	2004
Okada M, Northup JK, Ozaki N, Russell JT, Linnoila M, Goldman D	Modification of Human 5-HT2C Receptor Function by Cys23Ser, an Abundant, Naturally Occurring Amino Acid Substitution.	Mol Psychiatry	9	55-64	2004
Nokura K, Kanbayashi T, Ozeki T, Koga H, Zettsu T, Yamamoto H, Ozaki N, Shimizu T, Kawase T	Hypersomnia, asterixis and cataplexy in association with orexin A-reduced hypothalamic tumor.	J Neurol	251	1534-1535	2004
Koizumi H, Hashimoto K, Kumakiri C, Shimizu E, Sekine Y, Ozaki N, Inada T, Harano M, Komiyama T, Yamada M, Sora I, Ujike H, Takei N, Iyo M	Association between the glutathione S-transferase M1 gene deletion and female methamphetamine abusers.	Am J Med Genet B	126	43-45	2004
Kobayashi H, Ide S, Hasegawa J, Ujike H, Sekine Y, Ozaki N, Inada T, Harano M, Komiyama T, Yamada M, Iyo M, Shen HW, Ikeda K,	Study of Association between {alpha}-Synuclein Gene Polymorphism and Methamphetamine Psychosis/Dependence.	Ann N Y Acad Sci	1025	325-334	2004

Sora I					
Iwata N, Inada T, Harano M, Komiyama T, Yamada M, Sekine Y, Iyo M, Sora I, Ujike H, Ozaki N	No Association Is Found between the Candidate Genes of t-PA/Plasminogen System and Japanese Methamphetamine-Related Disorder: A Collaborative Study by the Japanese Genetics Initiative for Drug Abuse.	Ann N Y Acad Sci	1025	34-38	2004
Iwata N, Suzuki T, Ikeda M, Kitajima T, Yamanouchi Y, Inada T, Ozaki N	No Association With the Neuregulin 1 Haplotype to Japanese Schizophrenia.	Mol Psychiatry	9	126-127	2004
Itoh K, Hashimoto K, Shimizu E, Sekine Y, Ozaki N, Inada T, Harano M, Iwata N, Komiyama T, Yamada M, Sora I, Nakata K, Ujike H, Iyo M	Association study between brain-derived neurotrophic factor gene polymorphisms and methamphetamine abusers in Japan.	Am J Med Genet B Neuropsychiatr Genet	132B	70-73	2004
Inada T, Iijima Y, Uchida N, Maeda T, Iwashita S, Ozaki N, Harano M, Komiyama T, Yamada M, Sekine Y, Iyo M, Sora I, Ujike H	No Association Found between the Type 1 Sigma Receptor Gene Polymorphisms and Methamphetamine Abuse in the Japanese Population: A Collaborative Study by the Japanese Genetics Initiative for Drug Abuse.	Ann N Y Acad Sci	1025	27-33	2004
Ikeda M, Iwata N, Suzuki T, Kitajima T, Yamanouchi Y, Kinoshita Y, Inada T, Ozaki N	Association of AKT1 with schizophrenia confirmed in a Japanese population.	Biol Psychiatry	56	698-700	2004
Ide S, Kobayashi H, Tanaka K, Ujike H, Sekine Y, Ozaki N, Inada T, Harano M, Komiyama T, Yamada M, Iyo M, Ikeda K, Sora I	Gene polymorphisms of the mu opioid receptor in methamphetamine abusers.	Ann N Y Acad Sci	1025	316-324	2004
Hashimoto R, Yoshida M,	Association analysis of the	Journal of	111	217-222	2004

Ozaki N, Yamanouchi Y, Iwata N, Suzuki T, Kitajima T, Tatsumi M, Kamijima K, Kunugi H	G308A promoter polymorphism of the tumor necrosis factor alpha (TNF-α) gene in Japanese patients with schizophrenia.	Neural Transmission			
Harano M, Uchimura N, Abe H, Ishibashi M, Iida N, Yanagimoto K, Tanaka T, Maeda H, Sora I, Iyo M, Komiyama T, Yamada M, Sekine Y, Inada T, Ozaki N, Ujike H	A Polymorphism of DRD2 Gene and Brain Atrophy in Methamphetamine Psychosis.	Ann N Y Acad Sci	1025	307-315	2004
Deng X, Shibata H, Ninomiya H, Tashiro N, Iwata N, Ozaki N, Fukumaki Y	Association study of polymorphisms in the excitatory amino acid transporter 2 gene (SLC1A2) with schizophrenia.	BMC Psychiatry	4	21	2004
Matsumoto S, Sasaki T, Imamura A, Matsuo K, Kayashima T, Hashida A, Ono S, Tsujita T, Matsumto S, Nakane Y, Tokunaga K, Okazaki Y	HLA classI distribution in Japanese patients with schizophrenia.	Am J Med Genet	114	42-45	2002
Nishiyama T, Ikeda M, Iwata N, Suzuki T, Kitajima T, Yamanouchi Y, Sekine Y, Iyo M, Harano M, Komiyama T, Yamada M, Sora I, Ujike H, Inada T, Furukawa T, Ozaki N	Haplotype association between $GABA_A$ receptor gmma2 subunit gene (GABRG2) and methamphetamine use disorder.	Pharmacogenomics J			in press
Kinoshita Y, Suzuki T, Ikeda M, Kitajima T, Yamanouchi Y, Inada T, Yoneda H, Iwata N, Ozaki N	Association study of the calcineurin A-gamma subunit gene (PPP3CC) haplotype in Japanese schizophrenia.	Journal of Neural Transmission			in press
Hashimoto T, Hashimoto K, Matsuzawa D, Shimizu E, Sekine Y, Inada T, Ozaki N, Iwata N, Harano M,	A functional glutathione-S-transferase P1 polymorphism is associated with methamphetamine-induced	Am J Med Genet			in press

Komiyama T, Yamada M, Sora I, Ujike H, Iyo M	psychosis in Japanese population.				
Iwayama-Shigeno Y, Yamada K, Itokawa M, Toyota T, Meerabux JMA, Minabe Y, Mori N, Inada T, Yoshikawa T	Extended analyses support the association of a functional (GT)n polymorphism in the GRIN2A promoter with Japanese schizophrenia.	Neurosci Lett			in press
Suzuki A, Nakamura K, Sekine Y, Minabe Y, Takei N, Suzuki K, Iwata Y, Kawai M, Takebayashi K, Iyo M, Ozaki N, Inada T, Iwata N, Harano M, Komiyama T, Yamada M, Sora I, Ujike H, Mori N	An association study between COMT gene polymorphism and methamphetamine psychotic disorder.	Psychiatr Genet			in press
Ohtsuki T, Tanaka S, Ishiguro H, Noguchi E, Arinami T, Tanabe E, Yara K, Okubo T, Takahashi S, Matsuura M, Sakai T, Muto M, Kojima T, Matsushima E, Toru M, Inada T	Failure to find association between PRODH deletion and schizophrenia: population screening using simple PCR method.	Schizophr Res	67	111-113	2004
Takahashi N, Tomita K, Higuchi T, Inada T	The inter-rater reliability of the Montgomery-Asberg Depression Rating Scale (MADRS) using a Structured Interview Guide for Montgomery-Asberg Depression Scale (SIGMA).	Hum Psychopharmacol Clin Exp	19	187-192	2004
Iijima Y, Inada T, Ohtsuki T, Senoo H, Nakatani M, Arinami T	Association between chromogranin B gene polymorphisms and schizophrenia in the Japanese population.	Biol Psychiatry	56	10-17	2004
Ohtsuki T, Inada T, Arinami	Failure to confirm association	Mol	9	981-983	2004

T	between AKT1 haplotype and schizophrenia in a Japanese case-control population.	Psychiarty			
Hishimoto,A., Shirakawa, O. Nishiguchi, N., Aoyama, S., Ono, H., Hashimoto, T., Maeda, K.	Novel missense polymorphism in the Regulator of G-protein signaling 10 gene: analysis of association with schizophrenia	Psychiatry and Clinical Neurosciences	58(5)	579-81	2004
Ono, H., Shirakawa, O., Nushida,H., Ueno, Y., Maeda, K.	Association between Catechol-O-Methyltransferase Functional Polymorphism and Male Suicide Completers	Neuropsychopharmacology	29	1374-1377	2004
Miyasaka, K, Yoshida, Y, Matsushita, S, Higuchi, S, Shirakawa, O, Shimokata, H, Funakoshi, A.	Association of cholecystokinin-A receptor gene polymorphisms and panic disorder in Japanese.	Am J Med Genet.	127 B (1)	78-80	2004
Matsushita S, Suzuki K, Murayama M, Nishiguchi N, Hishimoto A, Takeda A, Shirakawa O, Higuchi S.	Serotonin transporter regulatory region polymorphism is associated with anorexia nervosa.	Am J Med Genet.	128 B (1)	114-117	2004
Fujimaru K., Imamura A., Tsujita T., Uraguchi M., Hashida A., Mori T., Matsumoto S., Okazaki Y., Nakane Y.	Minor Physical Anomalies in Japanese Patients with Schizophrenia.	Acta Medica Nagasakiensia	47	133-137	2002
Ono S, Imamura A, Hashida A, Matsumoto S, Tasaki S, Fujimaru K, Tsujita T, Hirayama R, Ozawa H, Okazaki Y	The relationship between neurotrophic factor-related genes and minor physical anomalies (MPAs) in Japanese patients with schizophrenia.	Am J Med Genet	130 B	55	2004
Imamura A, Jinno Y, Shimabukuro M, Hashida A, Matsumoto S, Ozawa H, Okazaki Y	Anticipation DNA methylation in schizophrenia.	Am J Med Genet	130 B	136	2004
Tasaki S, Imamura A, Hirayama R, Kikuchi T, Tsujita T, Okazaki Y, Ozawa H	Scanning of genomic differences in monozygotic twins discordant for schizophrenia, using CA repeat markers.	Am J Med Genet	130 B	147	2004

Human Molecular Genetics, 2004, Vol. 13, No. 21 **2699–2708**
doi:10.1093/hmg/ddh280
Advance Access published on September 2, 2004

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

Tadahiro Numakawa[1], Yuki Yagasaki[1], Tetsuya Ishimoto[2], Takeya Okada[1], Tatsuyo Suzuki[3], Nakao Iwata[3], Norio Ozaki[4], Takahisa Taguchi[2], Masahiko Tatsumi[5], Kunitoshi Kamijima[5], Richard E. Straub[6], Daniel R. Weinberger[6], Hiroshi Kunugi[1] and Ryota Hashimoto[1,*]

[1]Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashicho, Kodaira, Tokyo 187-8502, Japan, [2]Neuronics R.G. Special Division for Human Life Technology, National Institute of Advanced Industrial Science and Technology, 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan, [3]Department of Psychiatry, Fujita Health University School of Medicine, 1-98 Kutsukakechotagakugakubo, Toyoake, Aichi 470-1192, Japan, [4]Department of Psychiatry, Nagoya University Graduate School of Medicine, 65 Tsurumai, Chuoku, Nagoya, Aichi 466-8550, Japan, [5]Department of Psychiatry, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawaku, Tokyo 142-8666, Japan and [6]Clinical Brain Disorders Branch, National Institute of Mental Health, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892, USA

Received May 13, 2004; Revised and Accepted August 23, 2004

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

*To whom correspondence should be addressed. Tel: +81 423412711; Fax: +81 423461744; Email: rhashimo@ncnp.go.jp

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		*P*-value	Odds ratio (95% CI)
				Controls	Patients		
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		Haplotype	Haplotype frequency	
	Global	Individual		Controls	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

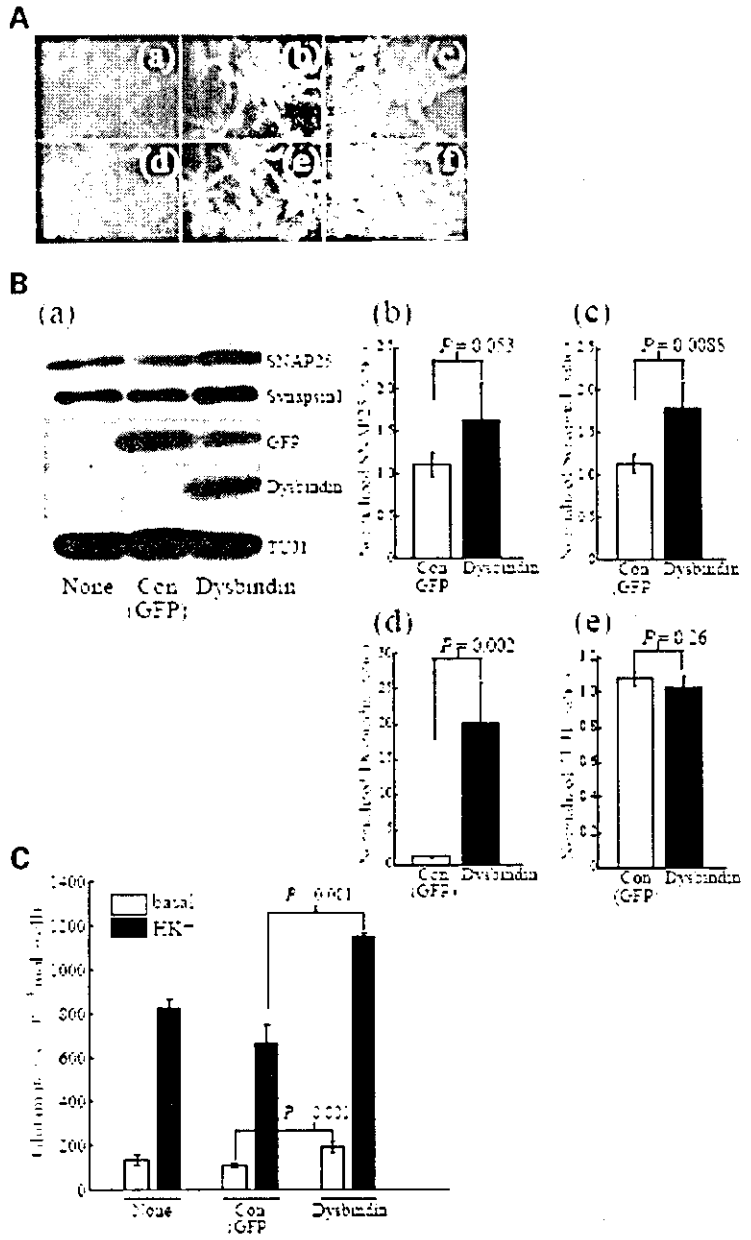

Figure 1. Dysbindin increases the expression of pre-synaptic proteins and glutamate release. (**A**) Double-staining of GFP and MAP2. Cortical cultures (6 days *in vitro*, DIV6) were prepared with viral infection of GFP only (a–c) or with viral infection of GFP and dysbindin (d–f) at DIV4. Images were 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

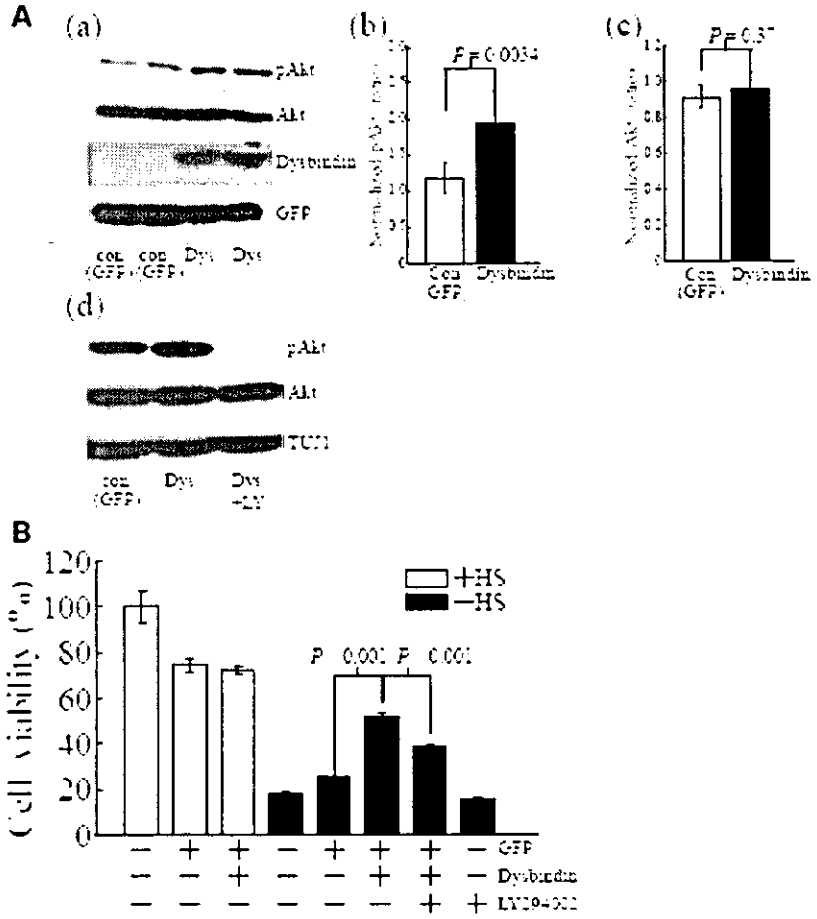

Figure 2. Dysbindin protects cortical neurons through PI3-kinase-Akt signaling. (**A**) (a) The activation of PI3-kinase pathway in dysbindin-overexpressing cultures. Cortical cultures after DIV4 were treated with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dys) for 48 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

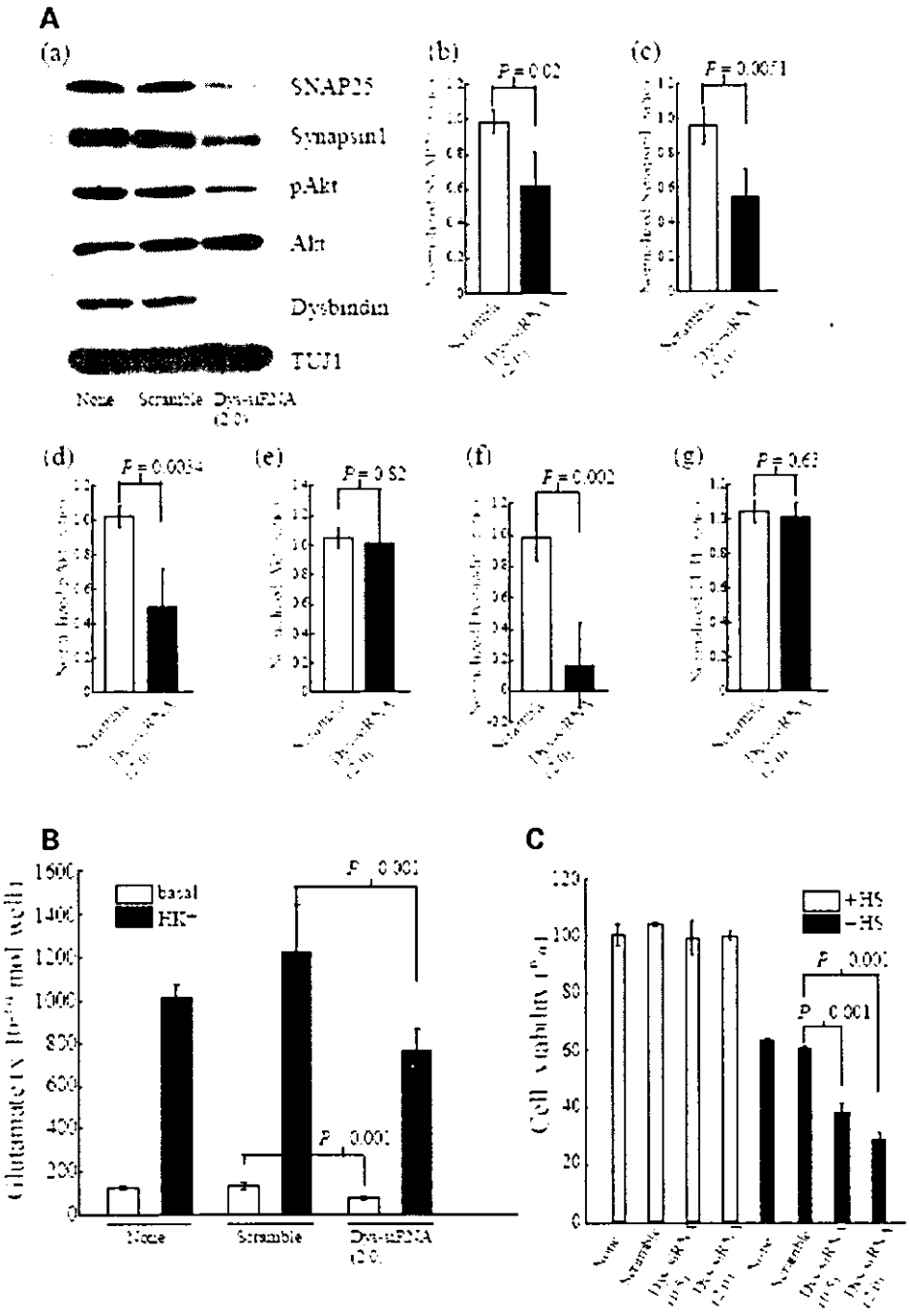

Figure 3. siRNA inhibition of endogenous dysbindin protein modulates protein expression, glutamate release and cell viability. (**A**) (a) Suppression of the pre-synaptic proteins and the phosphorylation of Akt in dysbindin-siRNA-transfected cultures. Cortical cultures after DIV4 were treated with siRNA 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 antiserum, 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 (QSDEEEVQVD-TALC: 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.

ACKNOWLEDGEMENTS

We wish to thank Dr Meiko Kawamura for kindly providing a bicistronic vector plasmid, and Tomoko Shizuno and Reiko Fujita for technical assistance. This work was supported by Grants-in-Aid from the Japanese Ministry of Health, Labor and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Uehara Memorial Foundation and Japan Foundation for Neuroscience and Mental Health.

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.,