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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

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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

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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		*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

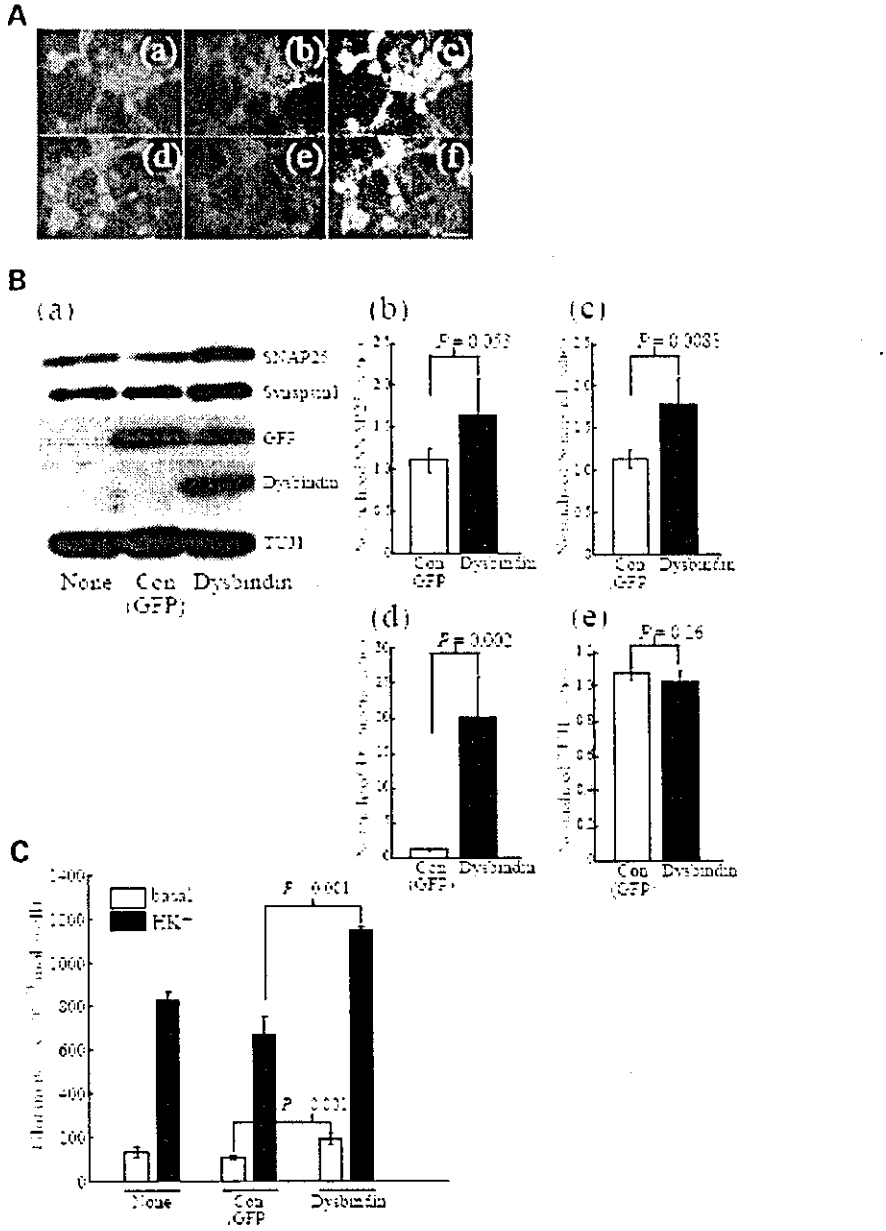

Figure 1. Dysbindin increases the expression of pre-synaptic proteins and glutamate release. (**A**) Double-staining of GFP and MAP2. Cortical cultures (6 days *in vitro*, DIV6) were prepared with viral infection of GFP only (a–c) or with viral infection of GFP and dysbindin (d–f) at DIV4. Images were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

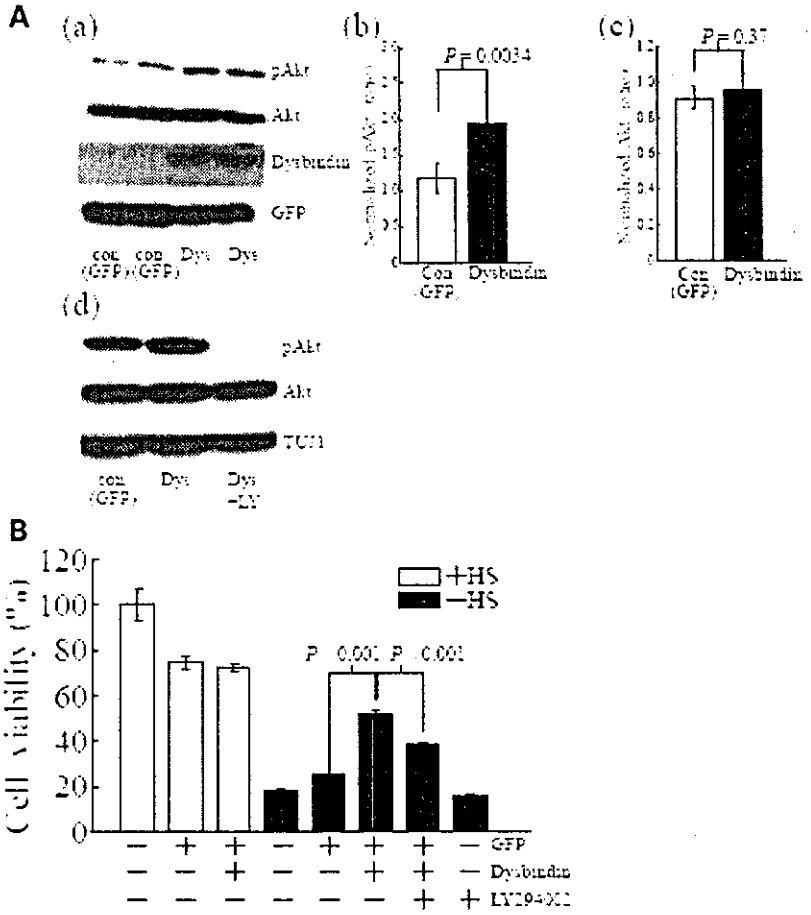

Figure 2. Dysbindin protects cortical neurons through PI3-kinase-Akt signaling. (**A**) (a) The activation of PI3-kinase pathway in dysbindin-overexpressing cultures. Cortical cultures after DIV4 were treated with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dys) for 48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

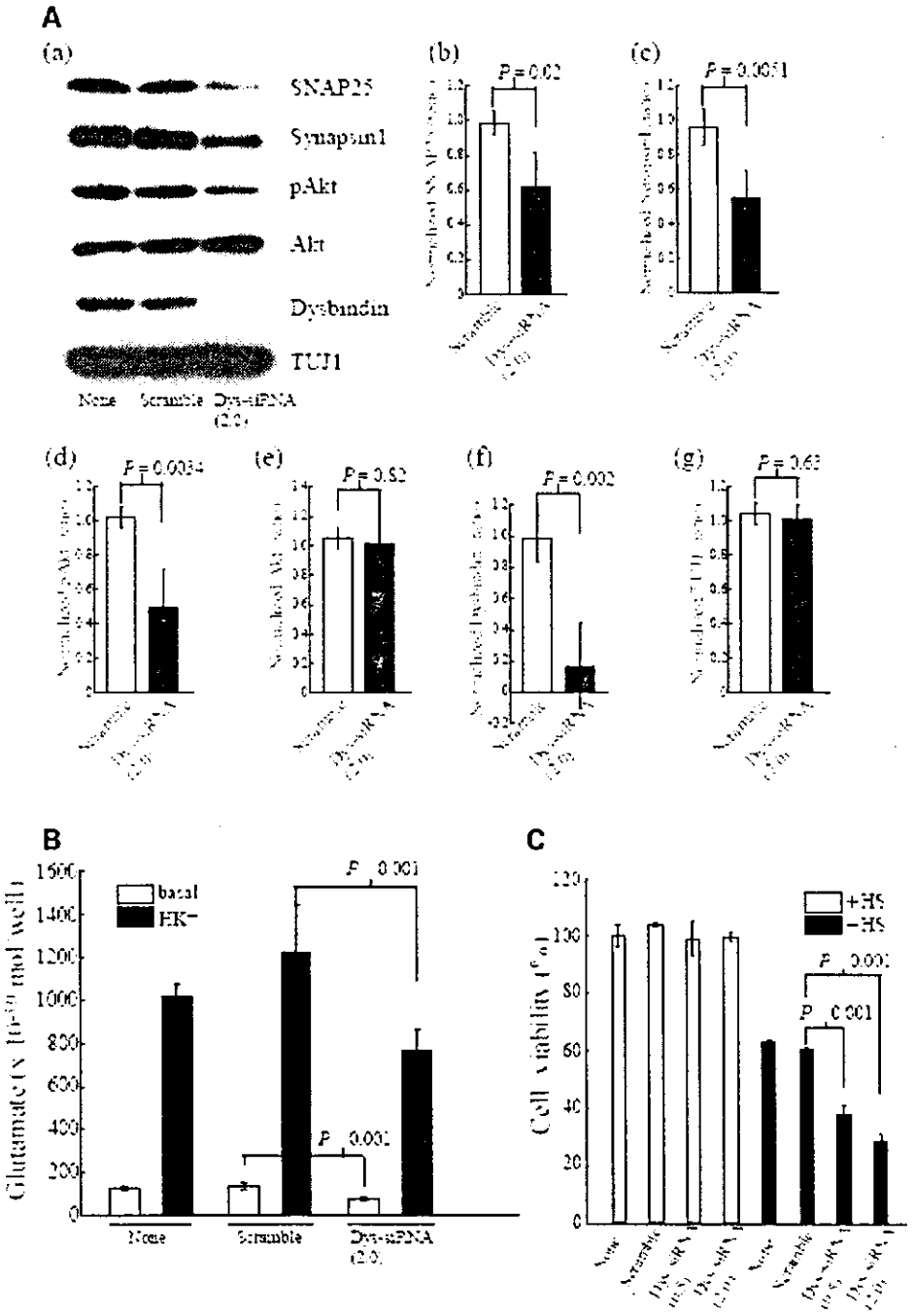

Figure 3. siRNA inhibition of endogenous dysbindin protein modulates protein expression, glutamate release and cell viability. **(A)** (a) Suppression of the pre-synaptic proteins and the phosphorylation of Akt in dysbindin-siRNA-transfected cultures. Cortical cultures after DIV4 were treated with siRNA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

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American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 129B:44–46 (2004)

Brief Research Communication

A Missense Polymorphism (S205L) of the Low-Affinity Neurotrophin Receptor $p75^{NTR}$ Gene Is Associated With Depressive Disorder and Attempted Suicide

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Several lines of evidence have implicated that neurotrophins play an important role in the pathophysiology of mood disorders. This study examined whether a common missense polymorphism (S205L) of a gene encoding the $p75^{NTR}$, the low-affinity receptor for neurotrophins, is associated with depressive disorder in a Japanese sample of 164 patients and the same number of controls matched for age and sex. There were significant differences in the genotype distribution and allele frequency between the cases and controls. The minor allele (L205) was significantly decreased in the patients than in the controls ($P < 0.05$, odds ratio 0.54, 95% CI 0.31–0.94), suggesting that this allele may have a protective effect against the development of major depression. Furthermore, this association was more strongly observed in the patients with a history of attempted suicide than those without such a history. Our results suggest that the S205L polymorphism of the $p75^{NTR}$ gene is involved in the pathogenesis of depressive disorder and suicidal behavior. © 2004 Wiley-Liss, Inc.

KEY WORDS: depression; $p75^{NTR}$; single nucleotide polymorphism (SNP); suicide; susceptibility

There is growing evidence for the involvement of neurotrophic factors in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents such as mood stabilizers and antidepressants (reviewed by Duman [2002]). Chronic electroconvulsive seizure or antidepressant drug treatments increase mRNA of brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, and its receptor trkB [Nibuya et al., 1995]. Lithium may also exert its neuroprotective effect through enhancing expression of BDNF and trkB [Hashimoto et al., 2002]. Furthermore, recent studies suggested the possible involvement of a missense polymorphism (V66M) of the *BDNF* gene giving susceptibility to bipolar disorder [Neves-Pereira et al., 2002; Sklar et al., 2002], although contradictive negative results have also been reported [Nakata et al., 2003].

In this context, the gene encoding the low-affinity receptor for neurotrophin family, $p75^{NTR}$ (alternatively referred to as nerve growth factor receptor (NGFR) or tumor necrosis factor receptor superfamily, member 16 (TNFRSF16)), is a good candidate for association analysis with mood disorders. To our knowledge, however, there is no study examining the possible association between the $p75^{NTR}$ gene and mood disorders. The $p75^{NTR}$ maps to chromosome 17q21-q22 [Huebner et al., 1986] and consisted of six exons and five introns [Sehgal et al., 1988]. We searched for polymorphisms in the $p75^{NTR}$ gene *in silico* and detected a common single nucleotide substitution (C727T; NCBI SNP ID: rs2072446) [Haga et al., 2002] in exon 4 giving rise to an amino acid change of serine to leucine at codon 205 (S205L) (amino acid numbering is according to NCBI protein data base accession NP_002498). In our search there was no other missense polymorphism that has been well validated in the $p75^{NTR}$ gene. Since this polymorphism may alter functions of the $p75^{NTR}$, we performed an association analysis between this polymorphism and major depressive disorder.

Subjects were 164 patients (59 males, mean age of 49.5 years [SD 12.7]) with major depressive disorder and 164 healthy controls matched for sex and age (59 males, 47.4 years [SD 9.5]). All the subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Diagnosis was made for each patient by a single psychiatrist (TM) according to American Psychiatric Association [1994] criteria on the basis of unstructured interviews and information from medical records. Among the 164 patients, 96 individuals (59%) had recurrent depressive episodes and the remainder had single episode. Eighty subjects (49%) had a history of admission to a psychiatric hospital and 46 (28%) had a history of attempted suicide. The mean age of onset was 42.2 years (SD 12.7). The controls were healthy volunteers recruited from hospital staffs. They were interviewed and those individuals who had current or past history of psychiatric treatment were not included in the study.

The study was performed in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees of Showa University School of Medicine and National Center of Neurology and Psychiatry, Japan.

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Genotyping for the S205L polymorphism was performed with polymerase chain reaction (PCR) amplification

Grant sponsor: Ministry of Health, Labor and Welfare (supported in part by the Health and Labor Science Research Grants for Psychiatric and Neurological Diseases and Mental Health (to HK)); Grant sponsor: Pharmacopsychiatry Research Foundation (to HK).

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Received 29 October 2003; Accepted 3 March 2004
DOI 10.1002/ajmg.b.30062

TABLE I. Genotype Distributions and Allele Frequencies of the S205L Polymorphism of the $p75^{NTR}$ Gene Among the Patients With Major Depressive Disorder and Controls

	Genotype distribution				Allele frequency		
	N	S/S	S/L	L/L	N	S	L
Patients							
Total	164	143 (87%)	21 (13%)	0 (0%)	328	307 (94%)	21 (6%)
With AS*	46	42 (91%)	4 (9%)	0 (0%)	92	88 (96%)	4 (4%)
Without AS*	118	101 (86%)	17 (14%)	0 (0%)	236	219 (93%)	17 (7%)
Controls	164	129 (79%)	33 (20%)	2 (1%)	328	291 (89%)	37 (11%)

*AS, attempted suicide.

encompassing the polymorphic site with primers of 5′-gctaaaagggaggagtgggggaag-3′ (forward) and 5′-ttcaggtcaaggtcacagcaaagtct-3′ (reverse). Thermal cycling was performed with an initial denaturing stage at 95°C for 10 min, 35 cycles of 95°C for 30 sec, 55°C for 20 sec, and 72°C for 30 sec, followed by the final extension at 72°C for 3 min. The polymorphic site gives rise to a restriction site of enzymes BanII, Bsp1286I, and EcoT38I. In the present study, the 386 base pair (bp) PCR products were cut with BanII into 289 and 97 bp for the S205 allele, but not for the L205 allele. Digested PCR products were visualized by 4% agarose gel electrophoresis with ethidium bromide staining. Genotype data were read blind to the case-control status.

Genotype distributions and allele frequencies of the S205L polymorphism among the patients and controls are shown in Table I. The genotype distributions for the two groups were both in Hardy–Weinberg equilibrium (data not shown). Since there were only two individuals homozygous for the minor allele (L205), individuals homozygous for this allele and those heterozygous were combined when we compared the genotype distribution between the patients and controls. There was a significant difference in the frequency of individuals carrying the L205 allele between the cases and controls ($\chi^2 = 4.2$, df = 1, $P < 0.05$, odds ratio 0.54, 95% CI 0.30–0.98). When allele frequencies were compared, the L205 allele was significantly reduced in the patients than in the controls ($\chi^2 = 4.8$, df = 1, $P < 0.05$, odds ratio 0.54, 95% CI 0.31–0.94).

Then we examined whether the polymorphism is related to suicide behavior. When the patients were dichotomized into two groups by the presence or absence of a history of attempted suicide, the frequency of the L205 allele was further decreased in individuals with a history of attempted suicide, compared to those without such a history (Table I). When the allele frequency was compared between patients with a history of attempted suicide (N = 46) and controls, the difference was almost significant ($\chi^2 = 3.9$, df = 1, $P < 0.05$, odds ratio 0.36, 95% CI 0.12–1.03). However, there was no significant difference in the allele frequency between the non-suicidal patients (N = 118) and controls ($\chi^2 = 2.6$, df = 1, $P = 0.11$, odds ratio 0.61, 95% CI 0.33–1.11). This disappearance of a significant difference in the allele frequency in spite of the larger number of subjects in the non-suicidal group, compared to the suicidal group, indicates a possibility that the effect of the S205L polymorphism of the $p75^{NTR}$ gene is more pronounced in depressive disorder with suicidal behavior.

The observed frequency for the minor allele (L205) in our control group (11%) was quite similar to that reported by Haga et al. [2002] (10%) estimated from 42 Japanese chromosomes. Thus the observed significant difference in the allele frequency between the cases and controls cannot be ascribed to an unusually higher frequency of the L205 allele in our control subjects.

Our results suggest that the minor allele (L205) has a protective effect against the development of depressive disorder and possibly, suicidal behavior. Alternatively, the major allele (S205) may have a risk-increasing effect on the development of such psychiatric conditions. However, there remains a possibility that other polymorphisms, which are in linkage dysequilibrium to the S205L polymorphism, are truly responsible for protective effect or giving susceptibility.

The $p75^{NTR}$ gene encodes a 427 amino acid protein containing a 28 amino acid signal peptide, an extracellular domain containing four 40 amino acid repeats with six cysteine residues at conserved positions followed by a serine/threonine-rich region, a single transmembrane domain, and a 155 amino acid cytoplasmic domain [Johnson et al., 1986]. The $p75^{NTR}$ binds all members of the neurotrophin family (nerve growth factor, BDNF, neurotrophin-3, and neurotrophin 4/5) in mammals [Rodriguez-Tebar et al., 1993]. Although main function of the $p75^{NTR}$ was thought to modulate the affinity and activity of tyrosine kinases (trkA, trkB, and trkC) that promote neuronal survival [Chao and Hempstead, 1995], the $p75^{NTR}$ has also been shown to play an important role in inducing apoptotic cell death mediated by a "death domain" in the cytoplasmic region, a domain common to other members of TNF receptor superfamily [Rabizadeh et al., 1993]. The S205L missense polymorphism changes one of the serine residues in the serine/threonine-rich region to a leucine residue. Although physiological roles of the serine/threonine-rich region are still unclear, this region possesses extensive O-linked glycosylation, suggesting a potential role in ligand binding and signaling [Chapman et al., 1996]. Furthermore, the $p75^{NTR}$ is heavily phosphorylated on serine residues by a protein kinase that has not been definitely identified [Grob et al., 1985; Taniuchi et al., 1986]. Thus it is likely that the S205L polymorphism, which substitutes serine residue (polar amino acid) with leucine (non-polar), alters the functions of $p75^{NTR}$.

A limitation in the present study might be that the obtained evidence for an association was weak (P-values of 0.05 level). Replication studies in larger sample sizes are clearly required. If our results are replicated in other samples, experiments elucidating the possible effects of the S205L amino acid substitution on the $p75^{NTR}$ protein function may serve to advance our understanding of the molecular mechanism of depression and may provide clues to production of new treatment of depression and suicide behavior.

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BRIEF REPORT

No Association Between the Val66Met Polymorphism of the Brain-Derived Neurotrophic Factor Gene and Bipolar Disorder in a Japanese Population: A Multicenter Study

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Background: *Two previous studies reported a significant association between a missense polymorphism (Val66Met) in the brain-derived neurotrophic factor (BDNF) gene and bipolar disorder; however, contradictory negative results have also been reported, necessitating further investigation.*

Methods: *We organized a multicenter study of a relatively large sample of 519 patients with bipolar disorder (according to DSM-IV criteria) and 588 control subjects matched for gender, age, and ethnicity (Japanese). Genotyping was done by polymerase chain reaction–based restriction fragment length polymorphism or direct sequencing.*

Results: *The genotype distributions and allele frequencies were similar among the patients and control subjects. Even if the possible relationships of the polymorphism with several clinical variables (i.e., bipolar I or II, presence of psychotic features, family history, and age of onset) were examined, no variable was related to the polymorphism.*

Conclusions: *The Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder, at least in a Japanese population.*

Key Words: Association study, bipolar disorder, brain-derived neurotrophic factor, genetics, single nucleotide polymorphism, susceptibility

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family and promotes the development, regeneration, survival, and maintenance of function of neurons (Maisonpierre et al 1991). It modulates synaptic plasticity and neurotransmitter release across multiple neurotransmitter systems, as well as the intracellular signal-transduction pathway (Thoenen 1995). Growing evidence has suggested important roles of BDNF in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents, such as mood stabilizers and antidepressants (reviewed by Duman 2002). In postmortem brains of patients with bipolar disorder, BDNF protein was reduced compared with control subjects (Knable et al 2004). Chronic electroconvulsive seizure or antidepressant drug treatments increase messenger ribonucleic acid of BDNF and its receptor, tyrosine kinase receptor B (Nibuya et al 1995). Lithium might also exert its neuroprotective effect through enhancing expression of BDNF and trkB (Hashimoto et al 2002).

The BDNF gene is, therefore, an attractive candidate gene that might cause susceptibility to bipolar disorder or influence the clinical phenotype of the illness. Indeed, at least two previous studies reported a significant association between a missense polymorphism (Val66Met; National Center for Biotechnology Information Database of Single Nucleotide Polymorphisms reference number rs6265) of the BDNF gene and bipolar disorder (Neves-Pereira et al 2002; Sklar et al 2002); however, contradictory negative results have also been reported (Hong et al 2003; Nakata et al 2003). One possible reason for this inconsistency is the lack of statistical power due to small sample size. To draw any conclusion with respect to this possible association, we organized a multicenter study in which six laboratories combined their data to ensure adequate statistical power.

Methods and Materials

Subjects

Six laboratories (National Institute of Mental Health, two laboratories of the Brain Science Institute, Showa University, Tokyo Women's Medical College, and Fujita Health University) collected deoxyribonucleic acid (DNA) samples from patients with bipolar disorder and healthy control subjects. Each institute provided DNA samples of patients and control subjects matched for gender, age, and geographic area, which yielded a combined sample of 519 patients with bipolar disorder (244 male) and 588 control subjects (287 male). Mean age (± SD) for the patients was 49.3 ± 14.3 years and for the control subjects was 48.4 ± 12.7 years. All the patients and control subjects were Japanese and biologically unrelated. Consensus diagnosis of bipolar disorder was made for each patient by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and medical records. Among the patients, 347 were diagnosed as bipolar I

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Received February 27, 2004; revised June 9, 2004; accepted June 16, 2004.

0006-3223/04/$30.00
doi:10.1016/j.biopsych.2004.06.017

Table 1. Genotype Distributions and Allele Frequencies for the Val66Met Polymorphism of the BDNF Gene Among the Patients with Bipolar Disorder and Control Subjects

	Genotype Distribution				Allele Frequency	
	n	Val/Val	Val/Met	Met/Met	*n*	Val	Met
Patients						
Total	519	188 (36.2)	239 (46.1)	92 (17.7)	1038	615 (59.2)	423 (40.8)
Bipolar I	347	123 (35.4)	166 (47.8)	58 (16.7)	694	412 (59.4)	282 (40.6)
Bipolar II	172	65 (37.8)	73 (42.4)	34 (19.8)	344	203 (59.0)	141 (41.0)
Control subjects	588	216 (36.7)	270 (45.9)	102 (17.3)	1176	702 (59.7)	474 (40.3)

Values in parentheses are percentages. Genotypewise comparisons: total patients vs. control subjects: χ^2 (2) = .0, p = .98; bipolar I vs. control subjects: χ^2 (2) = .3, p = .86; bipolar II vs. control subjects: χ^2 (2) = .8, p = .69. Allelewise comparisons: total patients vs. control subjects: χ^2 (1) = .0, p = .83; bipolar I vs. control subjects: χ^2 (1) = .0, p = .96; bipolar II vs. control subjects: χ^2 (1) = .0, p = .94.

and the remaining 172 as bipolar II. Control subjects were healthy volunteers who had no current or past contact with psychiatric services. The control subjects were recruited from the hospital staffs and their associates at each institution who showed good social functioning and reported themselves to be in good health. They were interviewed, and those individuals who had current or past contact with psychiatric services were excluded. Written informed consent for participation in the study was obtained from all subjects. The study protocol was approved by the institutional ethics committees.

Methods

Venous blood was drawn, and genomic DNA was extracted according to standard procedures. Genotyping was performed according to Neves-Pereira et al (2003). Briefly, the polymorphic site was amplified by polymerase chain reaction (PCR) and then digested with a restriction enzyme, Eco72I. The digested PCR products were visualized with gel electrophoresis and subsequent ethidium bromide staining. Genotyping for a portion of subjects was done by direct sequencing of PCR products encompassing the polymorphic site with an autosequencer (CEQ 8000; Beckman Coulter, Fullerton, California). Genotype data were read blind to the case–control status.

To examine the possible relationships of the Val66Met polymorphism with clinical variables, information on age of onset, family history, and presence of psychotic features (i.e., current or past episode with delusions or hallucinations) was obtained. We defined positive family history as having at least one first-degree relative with a history of contact with psychiatric services with a diagnosis of mood disorder or who was a suicide victim. Individuals with ambiguous clinical data were excluded from statistical analyses.

The presence of Hardy-Weinberg equilibrium for the genotype distributions in the patients and control subjects was examined with the χ^2 test for goodness of fit. The differences in the genotype and allele distributions between patients and control subjects were examined with the χ^2 test for independence. The possible relationships between the polymorphism and clinical variables were examined with the χ^2 test for independence or analysis of covariance (ANCOVA) within the patient group. All p values reported are two-tailed.

Results

Genotype and allele distributions of the Val66Met polymorphism in the patients and control subjects are shown in Table 1. The genotype distributions in the two groups were both in Hardy-Weinberg equilibrium [patients: $\chi^2(1) = 1.1$, $p = .29$; control subjects: $\chi^2(1) = 1.2$, $p = .27$]. The genotype and allele distributions for the patients were quite similar to those for the control group (see Table 1). The genotype and allele distributions of the patients with bipolar I and those with bipolar II were also similar.

When relationships between genotype and clinical variables were examined, genotype and allele distributions were not different according to presence of psychotic features (frequency of the Val66 allele for psychotic patients: .567; for nonpsychotic patients: .579) or family history (positive family history: .602; negative: .603). Age of onset was also similar, irrespective of the genotype (Val/Val: 35.3 ± 13.5 years; Val/Met: 37.7 ± 14.6 years; Met/Met: 36.3 ± 14.0 years). Even when ANCOVA controlling for age and gender was performed, there was no significant difference in age of onset across the three genotypic groups [$F(2) = .99$, $p = .37$].

Discussion

We tried to replicate the studies of Sklar et al (2002) and Neves-Pereira et al (2002), who found a significant association between the Val66Met polymorphism of the BDNF gene with bipolar disorder. They reported excess transmission of the Val66 allele to the patients in their family-based association studies. Contrary to these findings, the genotype and allele frequencies among the patients and control subjects were similar in our sample, which is in turn consistent with more recent studies (Hong et al 2003; Nakata et al 2003), suggesting that the Val66Met polymorphism of the BDNF gene is unrelated to the development of bipolar disorder in our sample. Because our study had adequate statistical power (more than 90% to detect an odds ratio of 1.33 or more in allelic association; power analysis was performed according to Armitage and Berry 1994), the potential type II error due to lack of statistical power is unlikely. One possible explanation for this inconsistency might be a differential effect of the polymorphism depending on ethnicity, given that the majority of the subjects of Sklar et al (2002) and Neves-Pereira et al (2002) were Caucasian, whereas those of Hong et al (2003), Nakata et al (2003), and in our study were Asian. Alternatively, the positive results of Sklar et al (2002) and Neves-Pereira et al (2002) might have arisen by chance.

Concerning the possible effect of the polymorphism on clinical features, Rybakowski et al (2003) reported an earlier age of onset in Val/Val than Val/Met genotype (27 years vs. 38 years) among patients with bipolar disorder. They also found that the performance in all domains of the Wisconsin Card Sorting Test was significantly better for bipolar patients with Val/Val than for those with Val/Met genotype, suggesting a role of the Val66Met polymorphism in prefrontal cognitive function in bipolar disorder. This accords with the findings of Egan et al (2003), who

reported that the Met66 allele was associated with lower activity-dependent secretion of BDNF and poorer human memory and hippocampal function; however, we could not find any significant effect of the genotype on clinical variables of age of onset, subtype (bipolar I or II), psychotic features, or family history. Hong et al (2003) also failed to find significant difference in age of onset or suicidal history across genotypic groups in their Chinese subjects with bipolar disorder.

In conclusion, our results, together with previous two studies (Hong et al 2003; Nakata et al 2003), suggest that the Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder at least in Asian populations; however, the possibility remains that other variants of the BDNF gene might be associated with bipolar disorder in Asian populations, which requires further investigation.

This work was supported in part by the Health and Labor Science Research Grants for Psychiatric and Neurologic Diseases and Mental Health from the Ministry of Health, Labor and Welfare (HK).

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Biological Psychiatry

Neuropsychobiology

Original Paper

Neuropsychobiology 2004;50:206–210
DOI: 10.1159/000079971

Analysis of Enhancer Activity of a Dinucleotide Repeat Polymorphism in the Neurotrophin-3 Gene and Its Association with Bipolar Disorder

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Key Words
Neurotrophin-3 · Dinucleotide repeat · Polymorphism · Enhancer activity · Luciferase assay · Association study · Bipolar disorder

Abstract
Growing evidence has implicated the possible involvement of neurotrophins in the pathogenesis of functional psychoses such as schizophrenia and bipolar disorder. Previous studies reported a significant association of a dinucleotide repeat polymorphism of the neurotrophin-3 (NTF3) gene with schizophrenia. The aims of the present study were to examine whether this polymorphism is associated with bipolar disorder and whether the polymorphic region has an enhancer/silencer effect on transcriptional activity in an allele-dependent manner. In an association analysis between the polymorphism and bipolar disorder in a Japanese sample of 88 patients and 98 controls matched for age, sex, and ethnicity, the distribution of alleles did not differ significantly between the two groups. pGL3-promoter luciferase reporter vectors containing the polymorphic region increased luciferase activity relative to empty pGL3-promoter vector in HeLa, IMR-32 (neuroblastoma) and Hs683 (glioma) cell lines; however, no significant difference was detected between alleles for either cell line. Our results suggest that the examined polymorphism has no major role in giving susceptibility to bipolar disorder. Although the polymorphic region may have an enhancer-like effect on transcriptional activity, we obtained no evidence for allele-dependent differential effects.

Introduction

Growing evidence has implicated the possible involvement of neurotrophins in the pathogenesis of functional psychoses such as schizophrenia [1] and bipolar disorder [2]. Neurotrophin-3 (NTF3) is a member of the neurotrophin family, which is highly expressed in the hippocampi of newborns and in immature neocortical regions of the fetus [3]. A previous study reported a significant association between schizophrenia and a dinucleotide repeat $(CA)_n$ polymorphism in the first intron of the NTF3 gene: the 'A3' allele, $(CA)_{23}$, was significantly more common in schizophrenics than in controls [4, 5]. Subsequently, sev-

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