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

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Genetic variation in dysbindin (DTNBP1: dystrobrevin-binding protein 1) has recently been shown to be associated with schizophrenia. The dysbindin gene is located at chromosome 6p22.3, one of the most promising susceptibility loci in schizophrenia linkage studies. We attempted to replicate this association in a Japanese sample of 670 patients with schizophrenia and 588 controls. We found a nominally significant association with schizophrenia for four single nucleotide polymorphisms and stronger evidence for association in a multi-marker haplotype analysis ($P = 0.00028$). We then explored functions of dysbindin protein in primary cortical neuronal culture. Overexpression of dysbindin induced the expression of two pre-synaptic proteins, SNAP25 and synapsin I, and increased extracellular basal glutamate levels and release of glutamate evoked by high potassium. Conversely, knockdown of endogenous dysbindin protein by small interfering RNA (siRNA) resulted in the reduction of pre-synaptic protein expression and glutamate release, suggesting that dysbindin might influence exocytotic glutamate release via upregulation of the molecules in pre-synaptic machinery. The overexpression of dysbindin increased phosphorylation of Akt protein and protected cortical neurons against 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
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 (χ² = 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
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...
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 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).
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 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 (Dys) 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).
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 xindy 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 schizophrenic 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 CACTAATCAAATGAAACAGCCCTT-3′, reverse primer 5′-CTCATCTGTATTAACTAGTGACATGGT-3′, probe 1 5′-VIC-TATAGCTATGATGTGTTTTAT-MGB-3′ and probe 2 5′-FAM-ATTAGCTATGATGTGTTTTAT-MGB-3′; P1635: forward primer 5′-GAATTTTTCCTGGTT-3′, reverse primer 5′-CTCCCTTCCG-3′, forward primer 5′-ACCCTAAACCC AAAAAAAGAAAAACA-3′, probe 1 5′-VIC-TAAAGGCC ATATATTACC-MGB-3′ and probe 2 5′-FAM-AGCCAG
TAAATTACC-MGB-3'; P1325: forward primer 5'-GATATG ACTCTTTATCCAGGTACAG-3', reverse primer 5'- GTTACTGCACAGCACTGTTAA-3', probe 1 5'-VIC -AATGGATGTGCTATTAGT-MGB-3' and probe 2 5'-FAM -ATGGATGTGCTATTAGT-MGB-3'; P1320: forward primer 5'-CAAATCCATCTTTATTTGACATGGGTTT-3', reverse primer 5'-TGGTGTCTATTTTATTCAATGTGTGTTCT -3', probe 1 5'-VIC-VIC-AACACACCAACAAACAG-MGB-3' and probe 2 5'-FAM-AAAAGCACAAATAACAAG-MGB-3'; P1763: forward primer 5'-GGCAGAAGCGATGATGAGA-3', reverse primer 5'-TGGGCTTTATGTGCTACTTCTAAA -3', probe 1 5'-VIC-TACCTGGATGTCAGC-MGB-3' and probe 2 5'-FAM-ACCTGCGTTGAC-MGB-3'; SNP: forward primer 5'-CTCTGTTATGGCACCCTACTGGTTT-3', reverse primer 5'-TAGGGCCTGGATGAGA-3', probe 1 5'-VIC-AAGCAGTTTACATCTACA-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 density of 5 x 10^5 per cm² on polyethylenimine-coated 12- or 24-well plates (4 and 2 cm² surface area/well, respectively; Corning, NY, USA) or cover glasses (Matsunami, Osaka, Japan) attached with 4% paraformaldehyde for 1 h at room temperature. Fluorescent images were captured by an inverted microscope (Axiovert 200, Zeiss) with a CCD camera (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.

**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 camera (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 Na3VO4, 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-SNAP2 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-synaptotagmin 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 (QSDDEEQVD-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
and controls were analyzed by the x-test of both siRNAs was performed using NeuroPORTER™ 1 SNPAlyse (DYNACOM, Yokohama, Japan). The presence of both siRNAs was confirmed by the expectation–maximization algorithm. Case–control haplotype analysis was performed by Students’ t-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 χ²-test with 1 df. P-values were calculated on the basis of 10 000 replications. Statistical analysis of neurobiological assays was performed by Students’ t-test. All P-values reported are two tailed. Statistical significance was defined at P < 0.05. To be conservative, Bonferroni corrections were applied for multiple comparisons, e.g. number of analyzed SNPs and haplotypes, although SNPs were in LD.

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