Table 1 Primers Used for SSCP Analysis of the Human *CKIε* Gene

Exon number	Primer name	Sequence (5′–3′)	Fragment size	Annealing temperature (°C)
1	1F	CCA CGT CGC TGA CCC TCA TGT TCC	234	68
	1R	GCC CCT GGA GCC ACA TTC TGA CTT C		
2	2F	CAC ACG CCA GAT CTC AGA AAT GCT TAG TGG	266	63
	2R	CTG TGC TCA TGG CTG CCC ACC G		
3	3F	CTG CCT GCC TCT GAC CCC TGA C	264	63
	3R	GGC AGG AGG CAG GGC TGG TAT C		
4	4F	CTG CCT GGC CCA GAG TGC TAG GCA AG	335	68
	4R	AGT GGC CCC GGG TGC ACA CTG C		
5	5F	CCC AGA GGA TGA GTT AGG GGC CTG AGT G	306	68
	5R	GCC TCA CCT TTC CCT TAG ACA GTG CCT C		
6	6F	GTG GCT AGG ACA GTG CTG GCT GCA G	310	68
	6R	CCA GCT CAC TCT GGC CCT CTG AGT C		
7	7F	CTG GCC TCT GGG GCT GAC TGG TG	271	68
	7R	CTG AAC CCA GCC CAC TGC CTG AGT C		
8	8F	GAC TCA GGC AGT GGG CTG GGT TCA G	267	63
	8R	CTC AGT TCT GAG GCC CAG AGG GAC TG		
9	9F	ATC GCC AGC GGC TAA GGG ACT TGA C	241	63
	9R	CCC ACC CCT CCA CAA CAC ATT GGT C		

F or R, in the primer names indicate the forward or reverse orientation of each primer.

manufacturer, and detected by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). One of the PCR-amplified fragments in which a deletion was detected by direct sequence analysis was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and multiple isolates were sequenced on both strands. To determine the frequency of the S408N variant, all of the samples were amplified by PCR using 9F and 9R primers in Table 1 and subjected to either SSCP and/or denaturing high-performance liquid chromatography (DHPLC) analysis, followed by sequencing reactions as described above.

For DHPLC analysis, PCR products were denatured at 98°C for 30 s and 95°C for 7 min, followed by gradual reannealing from 95 to 15°C over 40 min. The crude PCR products (5–7 μl) were then injected into a DNASep column and separated through a 13.5–15.75% acetonitrile gradient at 61°C using a WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA).

Purification of Recombinant Proteins

The partial cDNAs encoding mouse PER1 (mPER1) (amino acids 547–799), rat PER2 (rPER2) (486–793), and mouse PER3 (mPER3) (367–880) fragments, which correspond to the CKIε-binding regions (Takano, A *et al*, unpublished observation), were subcloned into pGEX4T-3 or pGEX6P-1 vector (Pharmacia, Peapack, NJ, USA) for the production of glutathione-*S*-transferase (GST)-fused recombinant proteins. The partial fragments of PERs were used for *in vitro* kinase assay, because it is practically impossible to obtain enough amount of intact full-length PER proteins due to their instability when expressed in *Escherichia coli*. The PER fragments we used correspond to the CKIε-binding domains which contain the phosphorylation sites; therefore, they can be properly used for *in vitro* kinase assay of CKIεs to compare the kinase activity against PERs.

The S408N substitution was introduced into the rat CKIε (rCKIε) cDNA by site-directed mutagenesis using PCR, generating CKIε-S408N. The amino-acid sequence of rCKIε is identical to that of hCKIε, except for two amino acids. Neither of the two amino acids is a phosphoacceptor residue. The expression constructs encoding GST-fused wild-type rCKIε (GST-CKIε-WT) and CKIε-S408N (GST-CKIε-S408N) were prepared using pGEX4T-3 (for α-casein) or pGEX6P-1 (for GST-PERs) vector. *Escherichia coli* (*E. coli*) strain BL21 (DE3) was transformed with the expression plasmids and the fusion proteins expressed were purified with glutathione sepharose 4B (Pharmacia) according to the manufacturer's protocol. GST-CKIε proteins were easily degraded, therefore, for the use in kinetic analysis

against α-casein, the fusion proteins were further purified by immunoprecipitation with the specific antibody against the C-terminal end of rCKIε to remove the contamination of partially degraded recombinant rCKIε (Takano *et al*, 2000). To perform *in vitro* kinase assay using GST-fused PER fragments as substrates, GST tag was removed from GST-CKIε using PreScission protease (Amersham) to discriminate phosphorylated GST-PERs and autophosphorylated rCKIε on electrophoretic mobility.

In Vitro Kinase Assay and Kinetic Analysis

Kinase reactions were performed in buffer containing 45 mM Tris-HCl, pH 7.4, 9 mM $MgCl_2$, 0.9 mM β-mercaptoethanol, 40 μM ATP, 74 kBq of [γ-^{32}P], kinase and α-casein or GST-PER in a final volume of 20 μl. Approximately 40 ng of the immunoprecipitated GST-CKIε (for α-casein), 2 pmol of rCKIε (for GST-mPER1 and GST-rPER2), or 10 pmol of rCKIε (for GST-mPER3) was added to the reaction mixture. Varying concentrations of α-casein (0–100 μM), or 20 pmol of GST-mPER1, GST-rPER2, or GST-mPER3 protein, was used as a substrate. The amount of rCKIε, GST-CKIε, or GST-PER used in each reaction was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining using bovine serum albumin as a standard, revealing that the difference in the amount of rCKIε or GST-CKIε in each experiment was smaller than 7.3% of the wild type. The kinase reactions for α-casein were allowed to proceed at 37°C for 10 min, because the enzyme activity was linear with time for up to 20 min (data not shown). Reactions were terminated by addition of 20 μl SDS-PAGE sample buffer. A part of the reaction mixture was subjected to electrophoresis on 12% (for α-casein) or 7.5% (for GST-PERs) polyacrylamide gels, and [^{32}P] incorporation into the substrates was determined by a BAS-2000 image analyzer. When α-casein was used as a substrate, the data were presented as a double-reciprocal plot and V_{max} and K_m were obtained using computer software (Kaleida Graph, Abelbeck Software).

Statistical Analysis

Departure from Hardy–Weinberg equilibrium was tested using a χ^2 goodness-of-fit test. The allele and genotype frequencies were compared by means of Fisher's exact test. All *p*-values reported are two-tailed. Correction for multiple testing for the analyses in the previous studies was not performed since a considerable number of subjects were newly recruited for this study, which was conducted with a pre-established hypothesis (Perneger, 1998). Unpaired *t*-test was performed to compare the amounts of incorporated [^{32}P] into GST-PER by CKIε-WT and CKIε-S408N.

RESULTS

Using PCR-SSCP and subsequent sequencing of the PCR-amplified fragments, all of the coding exons and flanking exon–intron boundaries of the *CKIε* gene were screened for sequence variations. In an initial screen of 35 genomic DNA samples (17 of DSPS and 18 of N-24), three sequence variants were identified (Table 2). One single-nucleotide variation (51C>T) was located in exon 1, another variation (1223G>A) in exon 9, and one intronic deletion of 4 bp (77-63_77-60delGGCG) resided upstream of exon 2. The 1223G>A exonic variation predicted an amino-acid substitution, S408N. S408 is located in the C-terminal extension of the CKIε and is conserved in CKIεs of humans, hamsters, mice, rats, and *Xenopus laevis*, as well as in CKIδs of humans and rats. Previous studies demonstrated that the C-terminal extensions of mammalian CKIε (and CKIδ) can be autophosphorylated, inhibiting the kinase activity (Graves and Roach, 1995; Cegielska *et al*, 1998), and that S408 is one of the putative phosphoacceptor residues (Gietzen and Virshup, 1999). Therefore, the S408N variation is likely to eliminate one of the autophosphorylation sites, resulting in decreased autophosphorylation and increased enzyme activity. The 51C>T exonic variation resulted in synonymous substitution. Neither the 51C>T variation nor the intronic deletion (77-63_77-60delGGCG) appeared to affect known splice sites or to create better splice donor/acceptor consensus sequences, based on visual examination of the sequence context, so functional alterations appeared unlikely (Burset *et al*, 2000). Therefore, we focused on the S408N variation for further analysis.

The frequency of the S408N variation was analyzed in a total of 137 circadian rhythm sleep disorder patients and 138 control subjects. Allele and genotype distributions are shown in Tables 3 and 4. No significant deviation from Hardy–Weinberg equilibrium was detected for the variation either in patients or in controls. The distribution analysis resulted in the detection of an additional silent sequence variation (1263A>G) in the 3′-untranslated region of the

Table 2 Sequence Variations Identified in the Human *CKIε* Gene

DNA polymorphism	Location	Amino-acid substitution
51C>T	Exon 1	None
77-63_77-60delGGCG	Intron 1	None
1223G>A	Exon 9	S408N
1263A>G	Exon 9 (3′-untranslated region)	None

Variations were named basically according to den Dunnen and Antonarakis (2001). Nucleotide numbers refer to the human CKIε cDNA sequence (AB024597) with the A of the ATG start codon denoted as 1. The S408N variation was submitted to DDBJ (http://www.ddbj.nig.ac.jp/, Accession no. AB080742).

Table 3 Frequency of the S408N Variant in Patients and Controls

		Allele frequency		
	n	N408 (%)	S408 (%)	*p*-value
Control	276	34 (12.3)	242 (87.7)	
DSPS	196	12 (6.1)	184 (93.9)	0.028[a]
N-24	78	3 (3.8)	75 (96.2)	0.035[b]
DSPS/N-24	270	15 (5.6)	255 (94.4)	0.0067[c]

[a]Odds ratio (OR) = 0.46, 95% confidence interval (CI): 0.23–0.92.
[b]OR = 0.28, 95% CI: 0.085–0.95.
[c]OR = 0.42, 95% CI: 0.22–0.79.

Table 4 Genotype Distribution of the S408N Variant in Patients and Controls

		Genotype				
	n	N/N (%)	N/S (%)	S/S (%)	N/N+N/S (%)	*p*-value
Control	138	4 (2.9)	26 (18.8)	108 (78.3)	30 (21.7)	
DSPS	98	1 (1.0)	10 (10.2)	87 (88.8)	11 (11.2)	0.038[a]
N-24	39	0 (0)	3 (7.7)	36 (92.3)	3 (7.7)	0.061[b]
DSPS/N-24	135	1 (0.7)	13 (9.6)	121 (89.6)	14 (10.3)	0.013[c]

The frequency of the N408-allele carrier is shown as (N/N+N/S). Odds ratio (OR) and 95% confidence interval (CI) are for (N/N+N/S) *vs* S/S.
[a]OR = 0.46, 95% CI: 0.22–0.96.
[b]OR = 0.3, 95% CI: 0.086–1.04.
[c]OR = 0.42, 95% CI: 0.21–0.83.
Two of the DSPS subjects, who had siblings with N-24, were excluded from the combined DSPS/N-24 group to avoid an increase in the Type I error rate. Neither of the sibling pairs carried the S408N variation.

CKIε gene, which was located 40 bp downstream of S408N polymorphic site (Table 2). One of the DSPS patients and two of the N-24 patients were heterozygous for the 1263A > G variation, while it was not detected in the control individuals. However, the frequency of the 1263A > G variation was too low to establish whether the variation affects the development of DSPS and N-24.

The N408 allele was significantly less frequent in DSPS ($p = 0.028$) and in N-24 ($p = 0.035$) than in control subjects (Table 3). The frequency of the N408-allele carrier was also significantly lower in DSPS subjects ($p = 0.038$) compared to controls, while the difference in carrier frequency between N-24 subjects and controls showed a similar tendency but did not come to statistical significance ($p = 0.061$) (Table 4). N-24 patients often suffer from DSPS during the course of the illness (Kamgar-Parsi *et al*, 1983; Oren and Wehr, 1992; McArthur *et al*, 1996), and reportedly share some of the physiological characteristics of DSPS, such as prolonged interval between natural wake time and the core body temperature trough (Uchiyama *et al*, 2000) or melatonin midpoint (Shibui *et al*, 1999; Uchiyama *et al*, 2002). These observations led to an *a priori* prediction that DSPS and N-24 are essentially the same disorder expressed with different degrees of severity (Weitzman *et al*, 1981; Campbell *et al*, 1999; Regestein and Monk, 1995). Indeed, when DSPS and N-24 subjects were combined, highly significant inverse associations were found between the N408 variant and DSPS/N-24 in both allele frequency ($p = 0.0067$, odds ratio (OR) = 0.42, 95% confidence interval (CI): 0.22–0.79) and carrier frequency ($p = 0.013$, OR = 0.42, 95% CI: 0.21–0.83), suggesting that the N408 allele protects against the development of DSPS/N-24. Our sample size had a 78% power to detect this effect of the S408N allele at a significance level of $p = 0.05$.

We next considered whether the S408N variation induces a functional alteration in CKIε, as expected from the location of the substitution. To determine whether the N408 variation in CKIε affects kinase activity *in situ*, phosphorylation of PER1 was assayed in transfected COS-7 cells by pulse-chase analysis. COS-7 cells were co-transfected with expression plasmids encoding mPer1 and either wild-type rCKIε or rCKIε with the S408N substitution. The transfected cells were pulse-labeled with [^{35}S]methionine for 1 h and chased for 0–6 h. After the chase period, cells were lysed and mPER1 protein expressed in COS-7 cells was immunoprecipitated using anti-mPER1 antibody. The immunoprecipitates were electrophoresed, and [^{35}S]-labeled mPER1 was detected. rCKIε with the S408N substitution induced a more pronounced mobility shift and reduced mPER1 protein level at 6 h post-pulse, which was indistinguishable from the effects induced by wild-type rCKIε. These results indicate that, *in situ*, wild-type rCKIε and rCKIε with the S408N substitution induce similar levels of phosphorylation and subsequent instability of the mPER1 protein (data not shown).

Previous reports suggested that subsets of autophosphorylation sites in CKIε are dephosphorylated in HEK293 and NIH3T3 cells by endogenous phosphatases, thus activating CKIε activity (Gietzen and Virshup, 1999; Rivers *et al*, 1998). Therefore, it is possible that rCKIεs transfected into COS-7 cells are dephosphorylated, consequently masking the effect of the S408N substitution on kinase activity.

To test this hypothesis, we performed *in vitro* kinase assays of GST-CKIε with or without the S408N substitution, using α-casein as a substrate. Recombinant GST-CKIε proteins were expressed in *E. coli*, purified with glutathione sepharose 4B, and immunoprecipitated with the C-terminus-specific antibody for CKIε to remove partially degraded protein. The kinetic analysis was performed using varying substrate concentrations (0–100 μM). As expected, GST-CKIε-S408N exhibited higher kinase activity than GST-CKIε-WT (Figure 1a). The data were represented as a double-reciprocal plot (Figure 1b). GST-CKIε-S408N showed significantly increased V_{max} (181% of GST-CKIε-WT) and a slightly decreased apparent K_m (78% of GST-CKIε-WT) against casein. To investigate whether the S408N substitution in CKIε causes higher enzyme activity on endogenous clock components, *in vitro* kinase assays using GST-PERs as substrates were also performed. To distinguish the phosphorylated GST-PERs from autophosphorylated CKIεs, GST tags were removed from recombinant CKIεs. As shown in Figure 2, CKIε-S408N incorporated more [^{32}P] into PER1, PER2, and PER3 fragments, respectively, than CKIε-WT did.

DISCUSSION

CKIε is one of the seven isoforms of CKI, designated α, β, γ1–3, δ, and ε (Eide and Virshup, 2001). Activity of CKIε

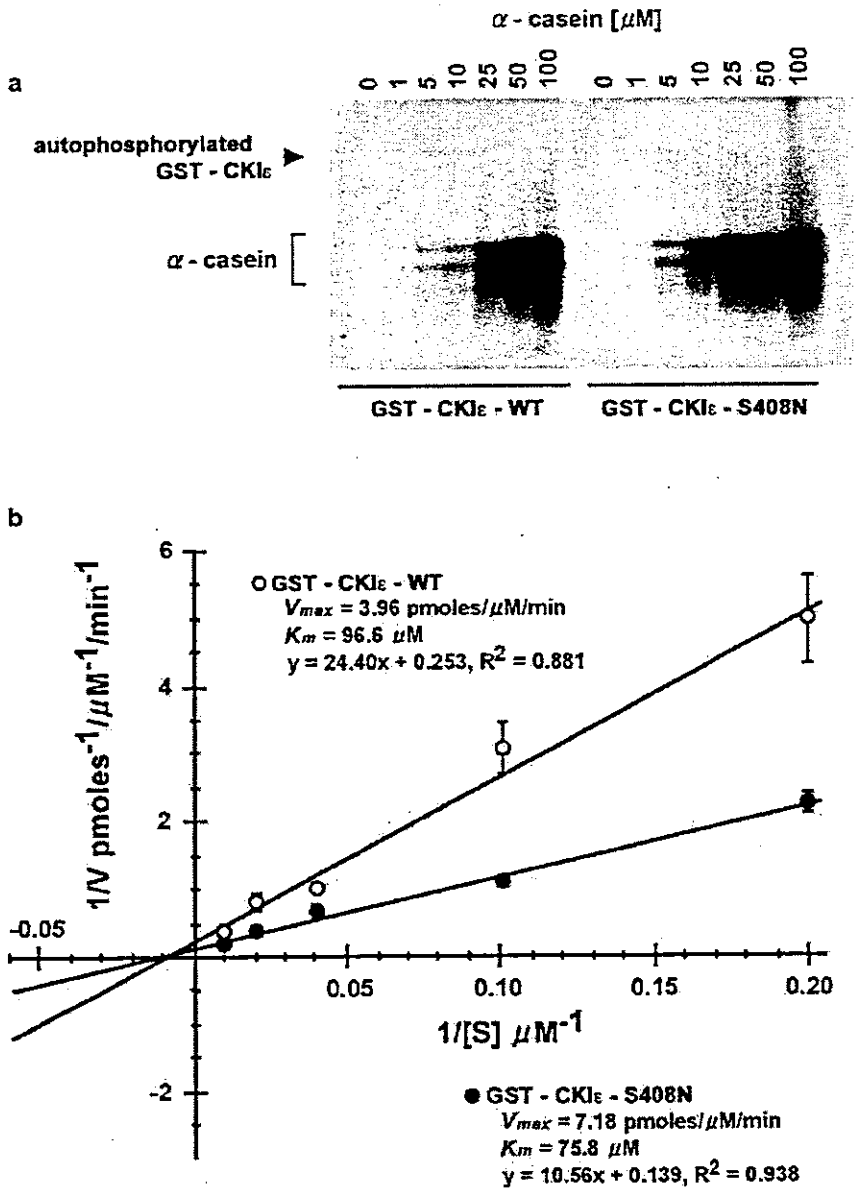

Figure 1 Kinetic analysis of recombinant GST-CKIε for α-casein. (a) Assays were performed with various concentrations of α-casein in the presence of GST-CKIε-WT or CKIε-S408N. Autophosphorylation of GST-CKIε-WT and CKIε-S408N that were only slightly visible in this figure were readily apparent when increased amounts of recombinant enzymes were used (data not shown). (b) Double-reciprocal plot of the data derived from the kinase assay performed with various concentrations of α-casein. Open and closed circles indicate the results for GST-CKIε-WT and CKIε-S408N, respectively. Calculated V_{max} and K_m of GST-CKIε-WT and CKIε-S408N are shown in the upper and the lower parts of the plot, respectively (means ± standard errors (SE) from three independent experiments).

(and the closely related CKIδ) is regulated in part by autophosphorylation of the C-terminal extension (Eide and Virshup, 2001; Graves and Roach, 1995; Cegielska *et al*, 1998). *In vitro*, CKIε is highly autophosphorylated, which inhibits enzyme activity (Gietzen and Virshup, 1999; Rivers *et al*, 1998). Both dephosphorylation by phosphatase treatment and removal of the C-terminal domain reactivate the kinase (Graves and Roach, 1995; Cegielska *et al*, 1998). In a site-directed mutagenesis study, eight amino acids in the C-terminal domain were identified as probable autophosphorylation sites, including serine-408 (Gietzen and Virshup, 1999). Therefore, the amino-acid change from serine-408 to asparagine (S408N) in CKIε, which was found in this study, is likely to eliminate one of the autophosphorylation sites, and is expected to reactivate part, but not all, of the kinase activity. Indeed, in our *in vitro* kinase assay with α-casein, recombinant GST-CKIε with the S408N substitution purified from *E. coli* exhibited a moderate (1.8-fold) elevation of specific activity compared to that of wild-type GST-CKIε, while a previous study showed that a mutant CKIε, in which all of the putative autophosphorylation sites are disrupted, was eight-fold more active than wild-type CKIε (Gietzen and Virshup, 1999). The moderate elevation of CKIε activity by S408N substitution was

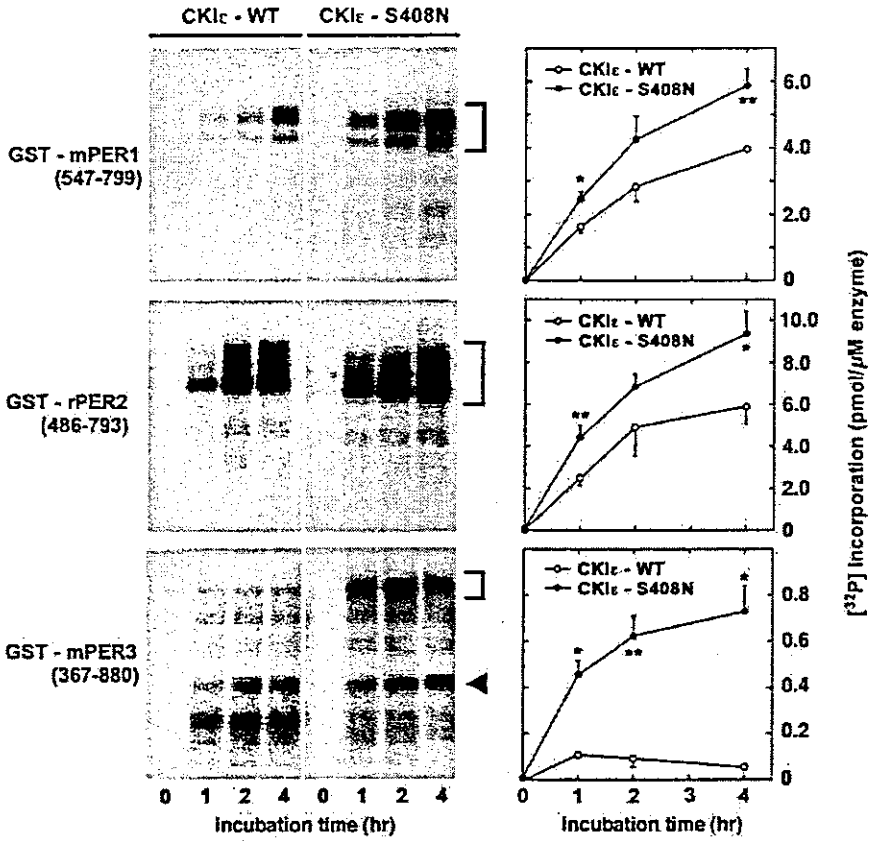

Figure 2 *In vitro* kinase assay of recombinant CKIε using GST-fused PER fragments as substrates. GST-mPER1 (amino acids 547–799) (top panels), GST-rPER2 (486–793) (middle panels), or GST-mPER3 (367–880) (bottom panels) fragment was incubated with recombinant CKIε-WT or CKIε-S408N for the indicated duration and analyzed by 7.5% polyacrylamide gels as described in Materials and methods. Representative autoradiograms are shown. Angle brackets indicate the phosphorylated GST-PER fragments. Arrowhead indicates autophosphorylated CKIε (left panels). Incorporated [^{32}P] was quantified and normalized to the total amount of kinase used (means ± SE from three to five independent experiments). Statistically significant differences in [^{32}P] incorporation induced by CKIε-WT and CKIε-S408N are shown by asterisks (*$p < 0.05$, **$p < 0.01$) (right panels).

identically observed in the *in vitro* kinase assay with each of the three subtypes of PER proteins, which are endogenous substrates for CKIε. It is intriguing that CKIε-S408N induced more phosphorylation of PER3 than CKIε-WT did, because we have previously reported that a *Per3* gene haplotype, which presumably alters PER3 protein phosphorylation, is significantly associated with DSPS (Ebisawa *et al*, 2001). However, it should be noted that we observed much less phosphorylation of PER3 compared with that of PER1 or PER2, which is consistent with the previous reports showing CKIε-induced phosphorylation of PER3 (Takano *et al*, 2000; Akashi *et al*, 2002) and unstable interaction of PER3 with CKIε in the absence of PER1 (Akashi *et al*, 2002; Lee *et al*, 2004). We could not find any elevation of enzyme activity in pulse-chase analysis *in situ*, presumably because of dephosphorylation by endogenous phosphatases as described in 'Results', or because the analysis was insufficiently sensitive to detect a moderate difference of activity.

The *tau* mutation in hamster *CKIε* decreases kinase activity by as much as eight-fold (Lowrey *et al*, 2000), whereas the S408N variant in h*CKIε* results in only 1.8-fold change (an increase) in the activity. This difference might explain the reason why the N408 allele of h*CKIε* induces a significant but modest effect (~2-fold reduction in the risk to develop DSPS/N-24), compared with the *tau* mutation in hamster *CKIε*, which causes a semidominant short-period phenotype (Ralph and Menaker, 1988).

Studies in flies and mammals suggest that CKIε binds to and phosphorylates PER proteins, leading to instability and intracellular relocalization of the PERs (Takano *et al*, 2000; Vielhaber *et al*, 2000; Keesler *et al*, 2000; Akashi *et al*, 2002). Mutant *CKIε* in the Syrian Golden hamster is deficient in PER phosphorylation (Lowrey *et al*, 2000). *Per2* S662G mutation in a reported familial ASPS cause hypophosphorylation by CKIε (Toh *et al*, 2001). In both cases, the PER protein(s) seems to undergo delayed degradation and accelerated accumulation, leading to hastened nuclear entry and shortened circadian period. In contrast, in flies with dbt^{L} or dbt^{ar} (long-period alleles of *dbt*, the Drosophila homolog of *CKIε*), it is likely that delayed phosphorylation and increased nuclear stability of PER protein slow the rate of PER elimination from the nucleus and lengthen circadian rhythm (Price *et al*, 1998; Rothenfluh *et al*, 2000). Therefore, hypophosphorylation of PER protein appears to cause different phenotypes depending on the subcellular localization of the stabilized PERs. hCKIε with an S408N substitution appears more active than wild type only when

the protein is autophosphorylated. A recent study suggests that the autophosphorylation level of CKIε, in neuroblastoma N2a cells, is dynamically regulated through transient dephosphorylation and subsequent phosphorylation, thus regulating the kinase activity (Liu *et al*, 2002). Additionally, in clock-relevant cells, CKIε intracellular localization is under circadian control (Lee *et al*, 2001); therefore, it is possible that a dynamic autophosphorylation/dephosphorylation cycle could differentially regulate CKIε activity at different subcellular locations in pacemaker cells. The S408N variation of h*CKIε* might alter circadian rhythmicity through increased phosphorylation and decreased stability of PER protein; the expected phenotypic consequences, however, would differ depending on the levels of CKIε autophosphorylation in each subcellular location. It will be of interest to investigate the autophosphorylation status of CKIε-S408N and to clarify its functional role in circadian clock machinery.

Although a significant inverse association was observed between the N408 variant and DSPS/N-24, 10.3% of the patients carried the N408 allele, indicating that DSPS/N-24 is genetically heterogeneous and multiple genes affect susceptibility to the development of DSPS/N-24.

The 1263A>G variation in the 3′-untranslated region of h*CKIε* was detected only in three of the rhythm disorder subjects, but not in controls. A larger sample size will be necessary to ascertain its relevance to DSPS/N-24.

Owing to the potential role of CKIε in the circadian rhythm, all of the coding exons in h*CKIε* gene were screened for variations in circadian rhythm sleep disorder patients and controls. We found a missense variation S408N, for the first time, which eliminates one of the putative autophosphorylation sites in hCKIε and confers 1.8-fold higher enzyme activity *in vitro*. There was a significant difference in the frequency of N408 allele between controls and DSPS or N-24, respectively, with an excess of N408 allele in controls. When considering the whole sample of circadian rhythm sleep disorders (DSPS/N-24), we found a highly significant inverse association between N408 allele and DSPS/N-24 ($p=0.0067$, OR = 0.42, 95% CI: 0.22–0.79). These results indicate that the N408 allele of the h*CKIε* gene is a marker for decreased risk of DSPS/N-24. S408N variation would also be useful to investigate other disorders related to disturbed circadian rhythm or interindividual differences of circadian rhythmicity in apparently normal subjects (Johansson *et al*, 2003). Our results will yield a new insight into the mechanism of DSPS/N-24 and raise a question in the role of CKIε autophosphorylation on mammalian clock functioning.

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INVITED REVIEW ARTICLE

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PHARMACOGENETICS OF ANTIPSYCHOATICS

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ABSTRACT

Although a number of antipsychotics have been introduced for the treatment of schizophrenia, inter-individual differences of in antipsychotic response and the number of refractory schizophrenic patients have become two of the most challenging problems in clinical psychiatry. Thus, the pharmacogenetics of antipsychotics have been aimed at providing genetic components of this inter-individual variability in antipsychotic response in order to establish an individually-based pharmacotherapy for schizophrenia and to elucidate the mechanism of antipsychotic response so as to solve the refractoriness of schizophrenia. Pharmacogenetics, which is defined as the science of pharmacological response and its modification by hereditary influence can be divided into two categories: the genetic background of pharmacokinetics, i.e. the absorption, distribution, tissue localization, biotransformation and excretion of drugs, and pharmacodynamics, i.e. the biochemical and physiological consequences of a drug and its mechanism of action. Pharmacokinetics of antipsychotics has been focused mainly on the association between genetic polymorphisms in CYP genes, including CYP2D6, and the metabolism of these drugs. Polymorphism in CYP2D6 enables a division of individuals within a given population into at least two groups, i.e. poor metabolizers (PMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs) of certain drugs. PMs have higher plasma concentrations of and more adverse effects from antipsychotics. UMs could be one of the important factors that induce treatment-refractoriness to antipsychotics. Genetic polymorphisms in serotonin and dopamine receptors that have a high affinity for antipsychotics have so far been extensively investigated in the pharmacodynamics of this type of drug. Not just one gene but multiple genes play a role in complex phenotypes, including the clinical response to medication. Thus, a multiple candidate genes approach has recently been adopted in the pharmacogenetics of antipsychotics. The new field of pharmacogenomics using DNA microarray analysis, which focuses on the genetic determinants of drug response at the level of the entire human genome, is important for development and prescription of safer and more effective individually-tailored antipsychotics.

Key Words: Antipsychotic, Genetics, Serotonin, Dopamine, Treatment response

INTRODUCTION

Since chlorpromazine was first introduced into clinical psychiatry, various kinds of antipsychotics have been developed and used for schizophrenia. Clinicians, however, still have considerable difficulty in choosing an appropriate antipsychotic for certain patients due to the inter-individual diversities of drug response. No definitive factor that can predict drug-response has yet been identified, although many researchers have tried to discover factors that can predict drug-response, e.g. demographics, and clinical features.

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Moreover, more than 40% of schizophrenic patients demonstrate varying levels of positive symptoms resistant to medication, even though a number of additional antipsychotics have been introduced. If treatment-failure is also defined to include remission of negative, cognitive, and mood symptoms in schizophrenia, the rate of poor response beomes considerably higher. On the other hand, it is essential to identify the mechanism of action of current antipsychotics in order to discover more effective drugs for refractory patients. However, the actual pharmacological mechanism underlying the clinical effects of antipsychotics have yet to be elucidated, although many hypotheses have been proposed.

Some treatment-resistant patients some of them cannot or will not take antipsychotics because of their side effects. Even worse, antipsychotics could cause fatal adverse effects such as malignant syndrome or agranulocytosis, or irreversible side effects such as tardive dyskinesia. Susceptibility to drug-induced side effects also shows inter-individual differences. Thus, indicators that can predict not only therapeutic efficacy but also adverse effects are urgently required in clinical psychiatry.

The importance of genetic factors among inter-individual diversities of drug response has been steadily clarified since the 1950s. As a result, such clarification has given rise to the new field of "pharmacogenetics", which is defined as the science of pharmacological response and its modification by hereditary influence. The consideration of drug-effects can be divided into two categories: pharmacokinetics and pharmacodynamics, both of which are subject to genetic influence. Although pharmacokinetic studies include the consideration of the absorption, distribution, tissue localization, biotransformation and excretion of drugs, the studies have so far focused mainly on the enzymes of metabolic clearance. Pharmacodynamics is the study of the biochemical and physiological consequences of a drug and its mechanism of action. In most phamacodynamics, the structures of receptors, ion-channels or carrier proteins have been investigated.

The genetic control of a drug-metabolizing enzyme ordinarily occurs via a single locus, whereas because of the complexity of receptor structures, often involving multiple units and proteins, phamacodynamics requires multiple genes and polymorphisms. The complexity of pharmacodynamics gave rise to the new field of pharmacogenomics, which focuses on the genetic determinants of drug response at the level of the entire human genome. Pharmacogenomics can be advanced by the progress of the Human Genome Project and by high-throughput genotyping that enables the creation of high-density single-nucleotide polymorphism (SNP) map.

In this article, the author will review the pharmacokinetics, pharmacodynamics, and pharmacogenomics of antipsychotics.

1. Pharmacokinetics of antipsychotics

Most antipsychotics are extensively metabolized by cytochrome (CYP) P450s that are members of a super-family of oxidative enzymes and that constitute a major system for the oxidative metabolism of therapeutic substances. Thus, the pharmacokinetics of antipsychotics has been focused mainly on the association between genetic polymorphisms in CYP genes and the metabolism of antipsychotic drugs. The CYP2D6 has been most extensively investigated in the field of psychiatry, since this enzyme is involved in the metabolism of many antipsychotics and has many genetic polymorphisms that influence the function of the enzyme. There are more than 70 variant alleles at the CYP2D6 gene locus, including the two most common variants, CYP2D6*4 and CYP2D6*45, encoding non-functional products.[1] Other variants that reduce activity, alter substrate specificity or increase activity have also been described. Compared with

efficient metabolizers (EM), poor metabolizers (PM) show no or reduced CYP2D6 activity by polymorphisms resulting in potentially increased concentrations of metabolized drugs. On the other hand, ultrarapid metabolizers (UM) that can be found in 1% of Caucasians often do not reach therapeutic concentrations and require an increased dose. Pronounced ethnic differences in the prevalence of both PM and UM have been reported; e.g., the frequency of PM is 5 to 10% among Caucasians, about 2% in Asians, and 7–8% in Africans.[2]

PMs have higher plasma concentrations of and suffer more adverse effects from antipsychotics. The incidence of the acute side effects of these drugs, including postural hypotension, excess sedation, or extrapyramidal symptoms, is disproportionately in PMs.[3] On the other hand, it is not clear whether the development of chronic side effects such as tardive dyskinesia is associated with a reduced metabolizing capacity of CYP2D6.

UMs have been reported at increased rates in select groups such as depressive inpatients, possibly due to the high percentage of insufficiently treated cases. Thus, UM can be one of the important factors that induce treatment-refractoriness in the field of psychiatry.

2. Pharmacodynamics of antipsychotics

All receptor and transporter genes for neurotransmitters as well as genes located down-stream of the intracellular signaling pathways can be considered candidate genes for the pharmacodynamics of antipsychotics. It is difficult to select a good candidate gene, since the true mechanism of therapeutic action of antipsychotics has not been clarified yet. However, association studies between genetic polymorphisms in neurotransmitter system and clinical drug-response have been carried out in order to investigate the potential involvement of a specific candidate gene in clinical response. These studies have adopted a candidate gene approach that uses a priori knowledge of drug profiles to identify genes relevant to drug-response. Furthermore, appropriate polymorphisms that influence the function of the gene-product or are merely markers have to be identified for potential candidate genes. So far, genetic polymorphisms in serotonin (5-HT) and dopamine (DA) systems have been extensively investigated in the pharmacodynamics of antipsychotics.

In addition to the selection of appropriate polymorphisms, it is also important to evaluate the clinical response in psychiatric pharmacodynamics, since there is still no biological marker to reflect the degree of severity in schizophrenia. Thus, it is essential to use a reliable and validated rating scale to evaluate clinical symptoms. The Positive and Negative Syndrome Scale (PANSS) or the Brief Psychotic Rating Scale (BPRS) for antispychotics have been used for clinical ratings in pharmacogenetic studies, although some studies have lacked any exact evaluation of clinical responses or definite protocols.

Clozapine is the only antipsychotic which has proven to be effective for treatment-resistant schizophrenia, although it may also induce the fatal adverse effect of agranulocytosis. In addition, the variety of clozapine responses is considerable, ranging from near total remission to little or no response. At present, no reliable means exist to predict who will experience a favorable clozapine response, and no pharmacological mechanism is yet known to explain the efficacy of clozapine for refractory schizophrenia. Thus, pharmacogenetic studies that address antipsychotic-response have focused primarily on clozapine and its variants in candidate genes of DA and 5-HT systems.

1) DA system (Table 1)

The first candidate gene examined with regard to clozapine response was the DA4 receptor

Table 1 Dopaminergic pharmacodynamic studies of antipsychotics

Variant	Influence on function	Findings of pharmacogenetics		Number of reports
		Medication	Result	
DRD4:				
48 bp VNTR	Clozapine-binding	clozapine	n.s.	5
		typical antipsychotics	n.s.	1
		typical antipsychotics	association	2
Val194Gly	Clozapine-binding	clozapine	n.s.	1
DRD3:				
Ser9Gly	Dopamine-binding	clozapine	n.s.	1
		clozapine	association	1
DRD2:				
Ser311Cys	cAMP synthesis	clozapine, typical antipsychotics	n.s.	2
-141Ins/Del	mRNA expression	clozapine	n.s.	3
		bromperidol,nemonapride	association	1
Taq 1 A	Receptor density	Nemonapride, haloperidol	association	2

gene (DRD4), because in addition to its high affinity for clozapine, the DA4 receptor is abundant in the prefrontal cortex, (a brain region thought to be related to the cognitive dysfunction of schizophrenia), and the DRD4 gene itself is highly polymorphic. Among polymorphisms in the DRD4, the 48 bp variable number of tandem repeats (VNTR) has been the most extensively investigated, since the VNTR was shown an in vitro study to influence the sodium chloride sensitivity of clozapine-binding and inhibition of c-AMP synthesis. However, this polymorphism did not seem to have any association with clozapine response.[4] On the other hand, multiple reports have shown an association between a typical antipsychotic response and the VNTR.

The DA3 receptor, which shares homologies with both the DA4 and DA2 receptors, has generated interest, since the DA3 receptor gene (DRDA3) has a known functional polymorphism, Ser9Gly, that influences dopamine binding. However, the association between the Ser9Gly and clozapine response remains controversial.[5]

The DA2 receptor is a major site of the action of conventional antipsychotics such as chlorpromazine and haloperidol, and of some atypical antipsychotics such as risperidone. One functional polymorphism (-141 Ins/Del) in the promoter region, as well as missense variants including Ser311Cer and an intronic variant (Taq 1 A), have been identified in the DA2 receptor gene (DRD2). The -141 Ins/Del polymorphism that influences the expression of the DRD2 was reported to be associated with anxiolytic and antidepressive effects during treatment with two conventional antipsychotics, bromperidol or nemonapride[6], although other studies failed to show any relation between that polymorphism and the clinical response to clozapine as well as other typical antipsychotics. Although theSer311Cer was shown to influence c-AMP synthesis, it has not been associated with clozapine or with a typical antipsychotic response. The Taq 1A that is located in the intron of DRD2 and has been reported to influence the density of the receptor was shown to have an association with the acute effects of a selective DA2 receptor antagonist, nemonapride, and haloperidol.[7]

2) 5-HT system (Table 2)

The 5-HT receptor genes have been regarded as good candidates for pharmacodynamic studies of antipsychotics, since 5-HT mediated mechanisms seem crucial to atypical antipsychotic drug action, including that of clozapine.

Among 14 human 5-HT receptors, the 5-HT2A receptor has received the most attention in

Table 2 Serotonergic pharmacodynamic studies of antipsychotics

Variant	Influence on function	Findings of pharmacogenetics		Number of reports
		Medication	Result	
HTR2A:				
T102C	None	clozapine	n.s.	5
		clozapine	association	3
		risperidone	association	1
-1438A/G	mRNA expression?	clozapine	association	2
Hys452Tyr	Ca ion response	clozapine	n.s.	2
		clozapine	association	2
HT2C:				
Cy23Ser	m-CPP binding	clozapine	n.s.	3
		clozapine	association	1
HTR6:				
C267T	None	clozapine	n.s.	1
		clozapine	association	1

the field of psychophamacogenetics, since it might be involved in the pathophysiogy of hallucination, and since most atypical antipsychotics have a relatively high affinity for the 5-HT2A receptor. An association between the silent polymorphism 102T/C in the 5-HT2A receptor gene (HTR2A) and clozapine has been reported[8], although those findings have not always been replicated.[9] However, a meta-analysis showed that the 102T/C is associated with clozapine response.[10] If this association is accurate, and if we consider that the 102T/C cannot influence the function of the receptor, the other variant that is in linkage disequilibrium with the 102T/C plays a genuine role in clozapine response. The -1438A/G in the promoter region of HTR2A is in linkage disequilibrium with the 102T/C and possibly influences the expression of HTR2A. Furthermore, the -1438A/G was shown to be associated with clozapine response.[11] Another polymorphism in HTR2A, Hys452Tyr[12] was shown to influence the intracellular signal transduction of the 5-HT2A receptor, as measured by Ca^{2+} mobilization induced by 5-HT stimulation. The 452Tyr was associated with both smaller peak amplitude in Ca^{2+} mobilization and a different time course of response, with slower peak latency and a longer half time in the Hys452Tyr heterozygote as compared to the His452His homozygote.[13] The Hys452Tyr was shown to be associated with clozapine response although some studies could not replicate the finding[9].

In addition to the 5-HT2A receptor, other 5-HT receptors, such as 5-HT 2C and 5-HT 6, have also been investigated in psychopharmacognetic studies because atypical antipsychotics also have high affinity for these receptors. The 5-HT2C receptor has been targeted for study based on the high densities of this receptor in brain regions implicated in both the pathophysiology of schizophrenia and the mechanism of action of clozapine as well as other atypical antipsychotics. The Ser23 in the 5-HT2C receptor gene influences m-chlorophenylpiperazine (m-CPP), a nonselective5-HT_{2C} agonist, binding, in comparison with Cys23. Therefore, the Ser23 may be constitutively more active and tends to be more desensitized.[14] The Cys23Ser was reported to have an association with clozapine response, although other studies could not replicate this association.[15] Although silent variant, C267T, in the 5-HT 6 receptor gene was shown to be marginally associated with clozapine response, that association also could not be replicated by any other studies.

3) Future direction: Multiple candidate genes

Not just one gene but multiple genes play a role in complex phenotypes, including the clinical response to medication. Arranz *et al.* published the most comprehensive study to date of a

pharmacogenetics screening strategy: a combination of 6 out of 19 candidate gene variants (in 5-HT2A, 2C, 5-HT transporter and Histamin 2 receptor genes) predicted response to clozapine with a prediction level of 76.9% (95.9 % sensitivity, 38.3 specificity).

We applied this approach combined with haplotype analysis to investigate the pharmacogetics of rispreridone, one of the most widely used atypical antipychotics. In our study multiple linear regressions were used to analyze the effects of these haplotypes/genotype of six candidate polymorphisms (HTR2A -1438G>A, 102T>C, H452Y; DRD2 -141delC, *Taq* I A; COMT V158M) on PANSS scale performance of risperidone treatment. Compared with patients who had Ins-A2/Ins-A2 diplotype (n=25), PANSS total scores of patients with Ins-A2/Del-A1 diplotype (n=10) showed 40% greater improved.[16]

3. Pharmacogonenomics

Since individual alleles may contribute only in a small degree to variable drug actions, phamacogenetic studies have not yet reached any definitive conclusion, as mentioned above. An alternative scenario is that it may be possible to identify collections of dozens, hundreds, or even thousands of SNPs that, taken together, might identify a patient as being at high or low risk for either beneficial or toxic drug effects. Thus, phamacogenomic studies encompass the sum of all genes, i.e., the genome, whereas the traditional pharmacogenetic approach relies on studying sequence variations in candidate genes suspected of affecting drug response.

First, progress in the human genome project has given rise to a new approach, i.e., phamacogenomic studies. Second, the high speed and specificity associated with newly emerging genomic technologies such as high-throughput DNA sequencing, gene mapping, and bioinformatics have enabled the search for relevant genes and their variants to include the entire genome.[17]

High-throughput technologies as wal are now being applied to the study of the genomic effects of antipsychotics. Profiling the expression patterns of genes in a target tissue reveals the mechanisms of drug action in a genomic context, and can serve to clarify interindividual differences in drug response that occur some time after the downstream of immediate drug effects in the body. For example, a recent DNA microarray analysis of clozapine-and haloperidol-treated rats identified a multiple differentially altered expression of the genes involved in synaptic function and in the regulation of intracellular Ca2+.[18] Transcript and protein profiling in patients could reveal an antipsychotic fingerprint for responsiveness or nonresponsiveness, as well as a signature motif that may be diagnostic of a specific phenotype. Similarly, antipsychotic-sensitive gene products could provide a new generation of pharmacological targets.

Current concepts in pharmacotherapy basically focuses large patient populations as a whole, in spite of the known inter-individual, genetically-based differences in drug response. In contrast, pharmacogenomics may help focus effective therapy on smaller patient subpopulations characterized by distinct genetic profiles although demonstrating the same disease phenotype. This strategy may also contribute to designing novel, more specific medications and to clarifying the mechanisms of the action of antipsychotics, thus furthering our knowledge of the pathophysiology of schizophrenia.

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

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

INTRODUCTION

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

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

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

RESULTS

Genetic association analysis

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

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

Functional analysis in dysbindin-overexpressing cultured neurons

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

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

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

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

Marker name	dbSNP ID	Polymorphism major/minor	Location	Minor allele frequency		*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

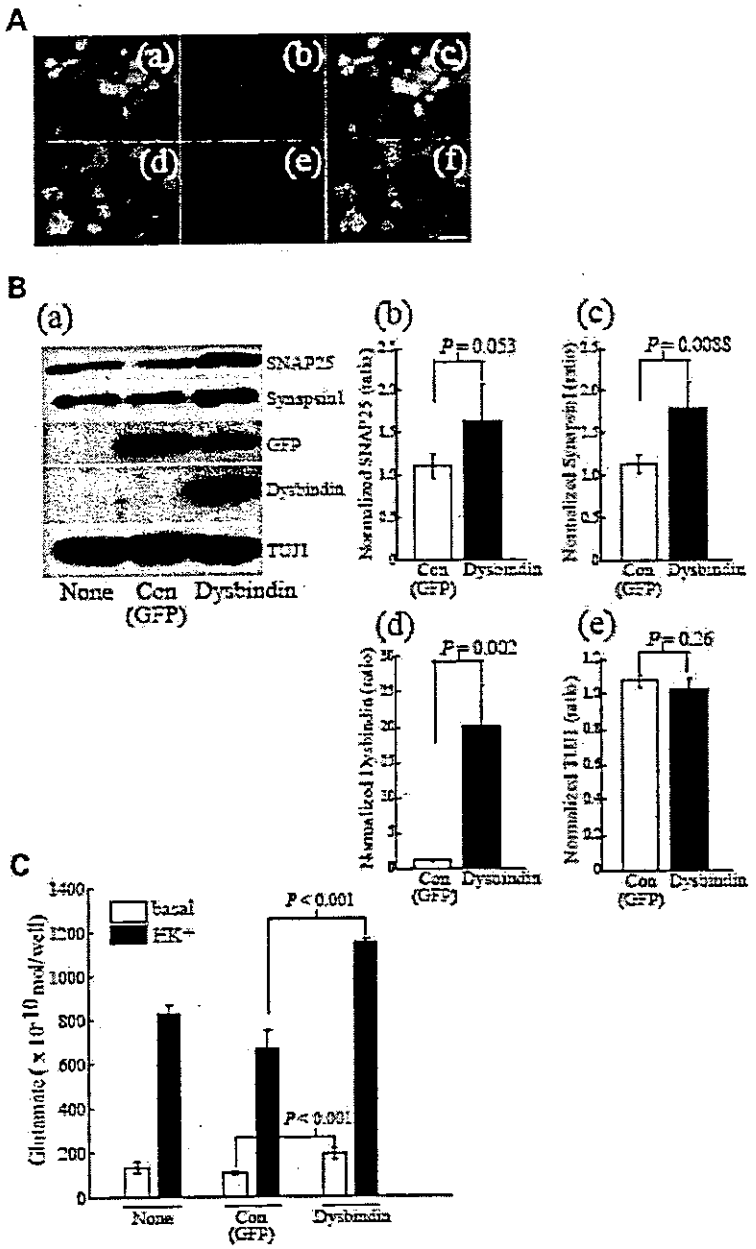

Figure 1. Dysbindin increases the expression of pre-synaptic proteins and glutamate release. (A) Double-staining of GFP and MAP2. Cortical cultures (6 days *in vitro*, DIV6) were prepared with viral infection of GFP only (a–c) or with viral infection of GFP and dysbindin (d–f) at DIV4. Images were obtained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

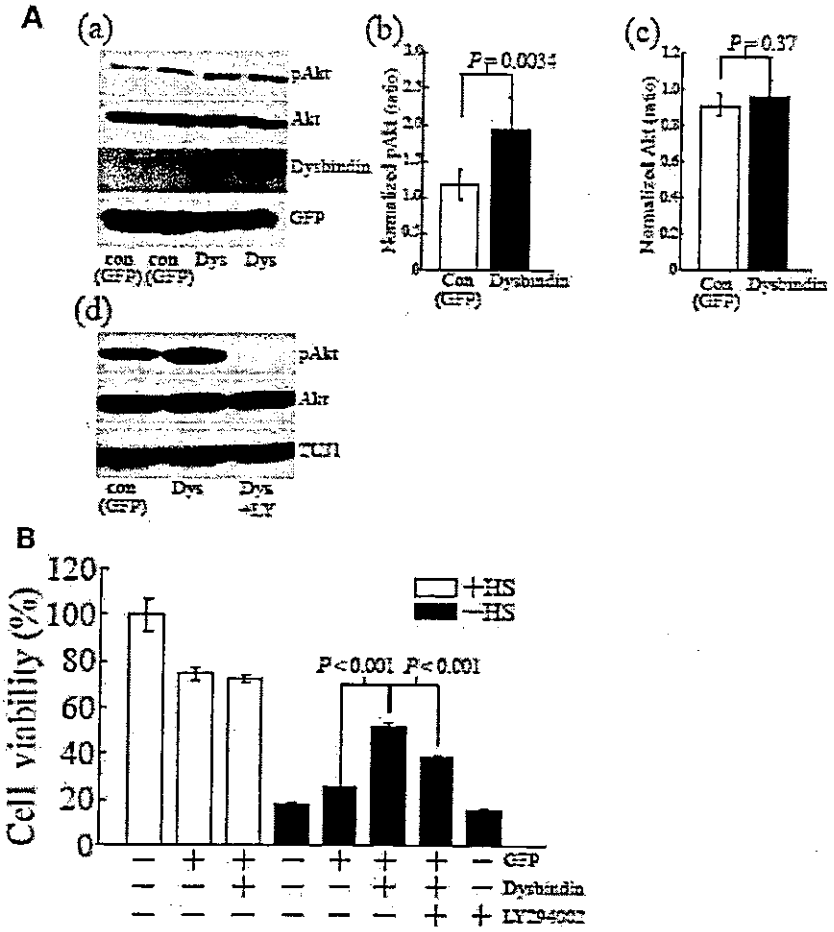

Figure 2. Dysbindin protects cortical neurons through PI3-kinase-Akt signaling. (**A**) (a) The activation of PI3-kinase pathway in dysbindin-overexpressing cultures. Cortical cultures after DIV4 were treated with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dys) for 48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

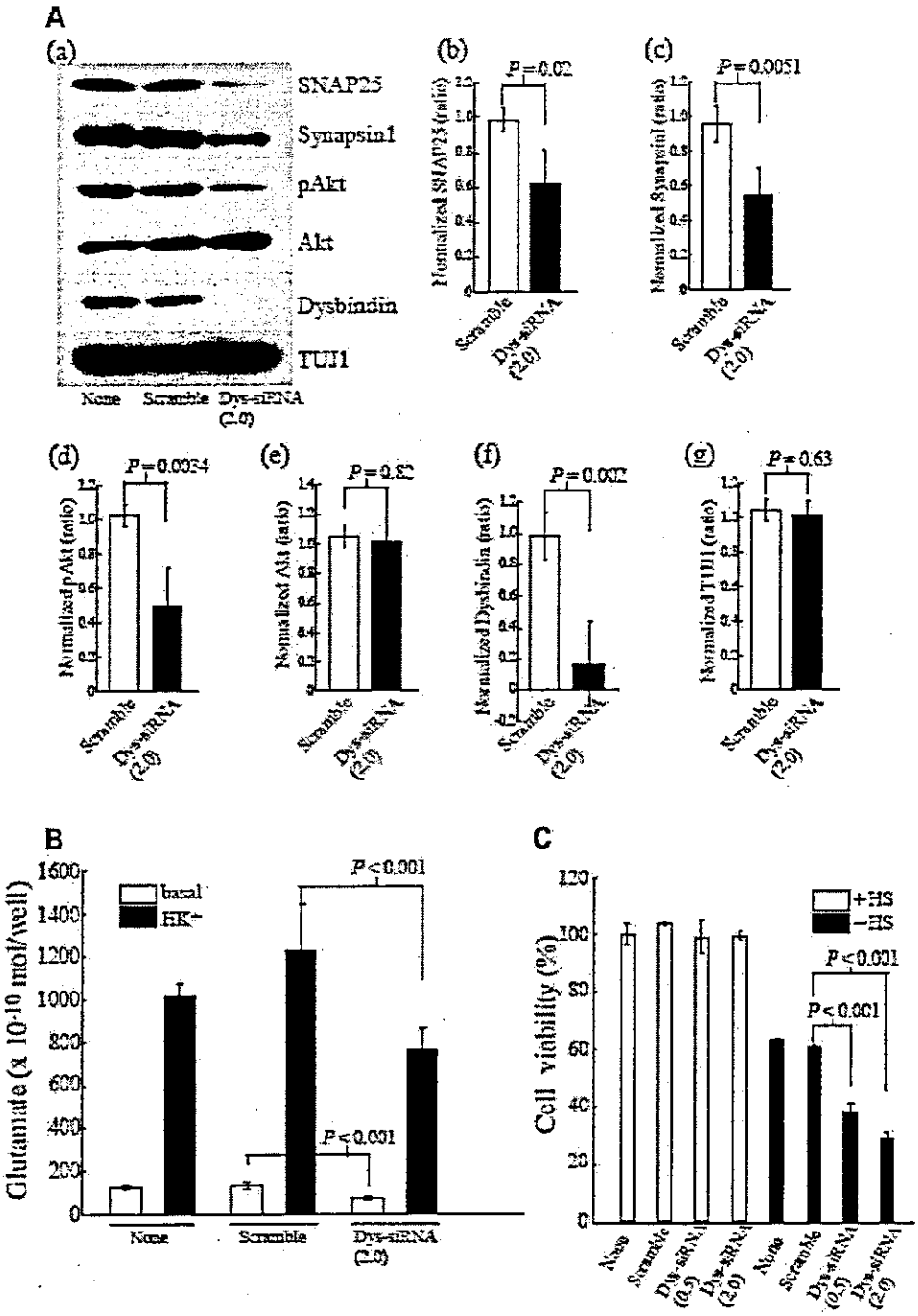

Figure 3. siRNA inhibition of endogenous dysbindin protein modulates protein expression, glutamate release and cell viability. (A) (a) Suppression of the pre-synaptic proteins and the phosphorylation of Akt in dysbindin-siRNA-transfected cultures. Cortical cultures after DIV4 were treated with siRNA 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 ($n = 8$).