Table 2. Genotype and allele frequencies of the C −116G polymorphism of the X box-binding protein 1 (*XBP1*) gene and response for lithium treatment

Response for lithium treatment	Allele frequency		χ^2		Genotype distribution			MH		χ^2	
	C	G	*p* value	OR (95% CI)	C/C	C/G	G/G	*p* value	C/C, C/G	G/G	*p* value	OR (95% CI)
Responders (43)	35 (40.7%)	51 (59.3%)	0.054	2.18 (0.96–3.03)	5 (11.6%)	25 (58.1%)	13 (30.2%)	0.038	30 (69.8%)	13 (30.2%)	0.037	3.00 (1.05–8.58)
Non-responders (23)	11 (23.9%)	35 (76.1%)			1 (4.3%)	9 (39.1%)	13 (56.5%)		10 (43.5%)	13 (56.5%)		
Total patients (66)	46 (34.8%)	86 (65.2%)			6 (9.1%)	34 (51.5%)	26 (39.4%)		40 (60.6%)	26 (39.4%)		

OR, Odds ratio; CI, confidence interval; MH, Mantel–Haenszel.

Table 3. Genotype of the −116C/G polymorphism of the *XBP1* gene and response for valproate treatment in lithium non-responders

Response for valproate treatment	Genotype distribution			χ^2
	C/C	C/G	G/G	*p* value
Responders (7)	1 (14.3%)	2 (28.6%)	4 (57.1%)	0.53
Non-responders (8)	0 (0%)	3 (37.5%)	5 (62.5%)	
Total patients (15)	1 (6.7%)	5 (33.3%)	9 (60.0%)	

proliferation (Chen et al., 2000; Hallcher and Sherman, 1980; Hashimoto et al., 2002a, b; 2003; Klein and Melton, 1996). Recently, it has been reported that chronic lithium treatment increased 78-kDa glucose-regulated protein (GRP78), a molecular chaperone of the heat shock protein 70 family, and showed cyto-protective effects in rat PC12 cells (Hiroi et al., 2005). In this regard, one of therapeutic actions of lithium might be associated with reducing ER stress, including signal transduction by *XBP1*. Although there was no direct evidence suggesting that *XBP1* is involved in the pathway of action of lithium, the −116C allele of the *XBP1* gene may contribute to reduce ER stress more effectively by lithium treatment.

Considering the action of valproate in cells with the −116G allele, it is possible that BPD patients with the −116G allele respond to valproate treatment better than those with the −116C allele. Therefore, we investigated the association between valproate response and the −116C/G polymorphism in non-responders to lithium treatment using the same criteria as for lithium response. However, we did not find any association in our small sample. It has been reported that lithium is effective for classical mania, while valproate is effective for both classical and irritable mania (Swann et al., 2002). In this context, valproate is likely to have a wider treatment spectrum than lithium, which may explain our finding. To clarify the association between the −116C/G polymorphism and treatment response to valproate, an independent and larger sample should be investigated.

After Kakiuchi et al. (2003) showed that the −116G allele was a risk factor of BPD in a Japanese sample, there have been two negative studies investigating American and European samples (Cichon et al., 2004), and a Chinese sample (Hou et al., 2004). Among our sample, the allele frequency of the −116G allele in

patients (0.65) was closer to that in controls (0.64) than that in BPD patients (0.71) in Kakiuchi et al.'s report (2003), although both subjects were of the same ethnicity (Japanese). To conclude whether the −116C/G contributes to the genetic risk factor for BPD in the Japanese population, larger number of BPD patients of Japanese origin should be examined.

On the other hand, two positive association studies between the −116G allele and schizophrenia have been reported (Chen et al., 2004; Kakiuchi et al., 2004). It has been reported that schizophrenia and BPD share several susceptibility loci such as 22q12 where the *XBP1* gene is located (Badner and Gershon, 2002). Therefore, these studies concerning schizophrenia might help to identify a shared pathogenesis of these two mental disorders.

To our knowledge, this is the first report indicating that long-term lithium treatment was more effective in BPD patients with the −116C allele on the promoter region of the *XBP1* gene than in those without the −116C allele. The mechanism of lithium response in the C allele-carrier patients is still unknown, however, it may be related to other mechanisms than dysregulation of ER stress response caused by the −116G allele. The limitations of the current study are retrospective design and small sample size. The association between the −116C/G polymorphism and clinical efficacy of mood stabilizers should be further investigated in a prospective study with a larger sample.

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Statement of Interest

None.

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Effect of antipsychotic drugs on DISC1 and dysbindin expression in mouse frontal cortex and hippocampus

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Summary. Altered expression of Disrupted-In-Schizophrenia-1 (DISC1) and dysbindin (DTNBP1), susceptibility genes for schizophrenia, in schizophrenic brain has been reported; however, the possible effect of antipsychotics on the expression levels of these genes has not yet been studied. We measured the mRNA expression levels of these genes in frontal cortex and hippocampus of mice chronically treated with typical and atypical antipsychotics by a real-time quantitative RT-PCR method. We found that atypical antipsychotics, olanzapine and risperidone, in a clinically relevant dose increased DISC1 expression levels in frontal cortex, while a typical antipsychotic, haloperidol, did not. No significant effect on dysbindin expression levels was observed in either brain region. These data suggest that prior evidence of decreased expression of dysbindin in postmortem brain of schizophrenics is not likely to be a simple artifact of antemortem drug treatment. Our results also suggest a potential role of DISC1 in the therapeutic mechanisms of certain atypical antipsychotics.

Keywords: Antipsychotic, DISC1, dysbindin, schizophrenia, gene expression.

Introduction

Schizophrenia is a common neuropsychiatric disorder affecting 0.5–1% of the general population worldwide. The pathophysiology of schizophrenia is still unclear; however, this disease is highly heritable (Owen et al., 2004). Several genes, e.g. Disrupted-In-Schizophrenia 1 (DISC1), dysbindin, catechol-O-methyltransferase, neuregulin 1, the regulator of G-protein signaling-4, GRM3 and G72 have been proposed as susceptibility genes for schizophrenia (Harrison and Weinberger, 2005).

The DISC1 gene has initially been identified at the breakpoint of a balanced translocation (1;11) (q42.1;q14.3), which segregates with schizophrenia and related psychiatric

disorders in a large Scottish family (Millar et al., 2000). Genetic association and linkage studies have also suggested that the DISC1 gene may be implicated in schizophrenia in independent populations (Ekelund et al., 2001, 2004; Hennah et al., 2003; Hodgkinson et al., 2004; Callicott et al., 2005). The function of DISC1 is still unclear, however, increasing evidence suggests a role in cytoskeletal organization, as DISC1 interacting proteins are associated with the components of microtubule and actin (Millar et al., 2003; Miyoshi et al., 2003; Morris et al., 2003b; Ozeki et al., 2003). Expression analysis of DISC1 using lymphocytes from patients in a balanced translocation family revealed that patients with the breakpoint expressed lower expression of DISC1 compared with controls, suggesting that lower levels of DISC1 might be related to the pathogenesis of schizophrenia (James et al., 2004). Further recent evidence implicates DISC1 in transcription regulation (Sawamura et al., 2005).

A significant association between schizophrenia and genetic variation in dysbindin has been reported in various populations from Ireland, Wales, Germany/Hungary/Israel, Sweden, Bulgaria, United States, China, and Japan (Straub et al., 2002; Schwab et al., 2003; Tang et al., 2003; Van Den Bogaert et al., 2003; van den Oord et al., 2003; Funke et al., 2004; Kirov et al., 2004; Numakawa et al., 2004; Williams et al., 2004). One study, which failed to replicate a positive association based on single SNPs in an Irish population, was subsequently positive using a haplotype strategy (Morris et al., 2003a). Dysbindin is a binding partner of alpha- and beta-dystrobrevins, which are parts of the dystrophin-associated protein complex (Benson et al., 2001), and is a component of the biogenesis of lysosome-related organelles complex 1, which regulates trafficking to lysosome-related organelles (Li et al., 2003). Recently, dysbindin has been reported to play roles in glutamate release and in cell models of neuroprotection, which have also been hypothesized to be related to the pathophysiology of schizophrenia (Numakawa et al., 2004).

Abnormal expression of DISC1 and dysbindin in schizophrenic brain has been reported. The expression ratio of an isoform of DISC1 was increased within the nuclear fraction extracted from orbitofrontal cortex of brains from patients with schizophrenia and also major depression (Sawamura et al., 2005) and the mRNA levels of DISC1 tended to be increased in hippocampus in patients with schizophrenia (Lipska et al., 2004). The expression levels of dysbindin mRNA and protein were reduced in the prefrontal cortex and hippocampus in schizophrenic brain (McClintock et al., 2003; Talbot et al., 2004; Weickert et al., 2004). In studies of schizophrenic postmortem brain, patients have received antipsychotic medication at various times in their lives, including in most cases around the time of death, while control subjects do not. Thus, possible effects of antipsychotics on gene expression are an important potential confounder when interpreting results of postmortem tissue studies of schizophrenic cases. Here, we examined for a possible effect of chronic administration of typical and atypical antipsychotics on the mRNA expression levels of DISC1 and dysbindin in mouse frontal cortex and hippocampus.

Materials and methods

Drug preparation

Haloperidol, risperidone and clozapine were purchased from Sigma-Aldrich (Tokyo, Japan). Olanzapine was a gift from Eli Lilly and Company Lilly Corporate Center (Greenfield, IN). Haloperidol was dissolved in glacial acetic acid solution, diluted with saline up to 1 ml with adjustment to pH 5.5 with 1 N sodium hydroxide, and brought to a final concentration of 0.005 or 0.1 mg/ml. Clozapine was dissolved in glacial acetic acid solution, diluted with saline up to 1 ml with adjustment to pH 5.5 with 8 N sodium hydroxide, and brought to a final concentration of 0.05 or 1 mg/ml. Olanzapine and risperidone were dissolved in 1 N acetic acid solution, diluted

with saline up to 1 ml with adjustment to pH 5.5 with 1 N sodium hydroxide, and brought to a final concentration of 0.004 or 1 mg/ml (olanzapine) and 0.0025 or 0.075 mg/ml (risperidone), respectively.

Animals and drug treatment

Male C57BL/6J mice (CLEA, Japan) weighing 20–25 g received once-daily injections intraperitoneally (i.p.) for 21 days with haloperidol (clinical dose: 0.05 mg/kg; high dose: 1 mg/kg), olanzapine (clinical dose: 0.04 mg/kg; high dose: 10 mg/kg), risperidone (clinical dose: 0.025 mg/kg; high dose: 0.75 mg/kg), clozapine (clinical dose: 0.5 mg/kg; high dose: 10 mg/kg), or vehicle (0.1 N acetic acid in saline). This dose regimen was chosen to simulate the therapeutic range of doses given to patients (Kapur et al., 2000), and was shown to be effective in several behavioral and biochemical studies (Lipska et al., 2001; Parikh et al., 2004). Haloperidol is a typical (conventional) antipsychotic, whereas the others are termed atypical antipsychotics, which are associated with fewer motor side effects and possibly greater efficacy. Animals were sacrificed 20 hr after the final injection. Brain regions were removed, frozen in liquid nitrogen, and stored at −80°C. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience, Japan.

RNA extraction, DNAse treatment and reverse transcriptase reaction

Tissues from frontal cortex or hippocampus were homogenized in 4 mol/L guanidinium isothiocyanate (containing 25 nmol/L sodium citrate, pH 7.5, and 1% 2-mercaptoethanol), and total RNA was isolated by a standard phenol-chloroform extraction. The yield of total RNA determined by the absorbance at 260 nm and the quality of total RNA was also analyzed using agarose gel electrophoresis.

Total RNA was treated with DNase for removal of contaminating genomic DNA using DNase Treatment & Removal Reagents (Ambion, Austin, TX), according to the manufacturer's protocol. Total RNA (3.3 μg) treated with DNase was used in 50 μl of reverse transcriptase reaction to synthesize cDNA, by using a SuperScriptII First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Briefly, total RNA (3.3 μg) was denatured with 1 mM of dNTP and 6 ng/μl of random primers at 65°C for 5 min. After addition of RT buffer, dithiothreitol (10 mM in final concentration), RNAsin Plus RNase Inhibitor (40 units) and SuperScriptII RT (200 units), the reaction mixture was incubated at 25°C for 10 min, at 42°C for 40 min, and at 70°C for 15 min. RNAse H (2 units) was added to the reaction mixture and then incubated at 37°C for 20 min.

Real-time quantitative PCR

The TaqMan® Endogenous Controls (Applied Biosystems, Foster City, CA) were used for measurements of house keeping genes, β-actin (Mm00607939_s1) and GAPDH (Mm99999915_q1). TaqMan® Gene Expression Assays (Applied Biosystems) were used for DISC1 (Mm00533313_m1) and dysbindin (Mm00458743_m1) genes. Both TaqMan assay kits included optimized concentrations of primers and probes to detect the target gene expression. The levels of mRNA expression of these genes were measured by a real-time quantitative RT-PCR using an ABI Prism 7900 sequence detection system with 384-well format (Applied Biosystems), described previously (Hashimoto et al., 2004). Briefly, each 20 μl PCR reaction mixture contained 6 μl of cDNA, 0.5 μl of TaqMan assay kit and 10 μl of TaqMan Universal PCR Mastermix (Applied Biosystems). PCR cycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR data were obtained with the Sequence Detector Software (SDS version 2.1, Applied Biosystems) and quantified by a standard curve method. Standard curves were prepared using serial dilutions (1:4) of pooled cDNA from total RNA derived from whole brain of three mice.

Statistical analysis

An analysis of variance (ANOVA) was used to compare gene expression levels between drug treatment groups with SPSS 11.0J for Windows (SPSS Japan Inc, Tokyo, Japan). Bonferroni post hoc comparisons were performed when applicable. Statistical significance was defined at $p < 0.05$.

Results

The expression levels of the two standard "housekeeping" genes, β-actin and GAPDH in frontal cortex and hippocampus of control mice and mice treated with typical or atypical antipsychotics for three weeks in clinical or high dose are shown in Table 1. The expression levels of both genes in frontal cortex and hippocampus were not significantly influenced by drug treatments at clinical dosing (all p values >0.4, ANOVA), however, there was a significant drug treatment effect on expression of the two house keeping genes

Table 1. Expression analysis of house keeping genes in frontal cortex and hippocampus in clinical and high dose

Drugs		Clinical dose		High dose			
		Frontal cortex (n)	Hippocampus (n)	Frontal cortex (n)	*p* value	Hippocampus (n)	*p* value
VEH	β-actin	100.0 ± 36.4 (19)	100.0 ± 33.1 (19)	100.0 ± 36.4 (19)		100.0 ± 33.1 (19)	
	GAPDH	100.0 ± 22.8 (19)	100.0 ± 26.6 (19)	100.0 ± 22.8 (19)		100.0 ± 26.6 (19)	
HPD	β-actin	105.4 ± 33.0 (10)	91.5 ± 16.7 (10)	72.2 ± 22.6 (12)	NS	102.8 ± 39.3 (12)	NS
	GAPDH	95.8 ± 15.9 (10)	96.7 ± 24.5 (10)	86.1 ± 15.5 (12)	NS	112.8 ± 29.7 (12)	NS
OZP	β-actin	139.4 ± 34.8 (10)	90.3 ± 40.4 (10)	67.4 ± 19.4 (12)	0.023	65.8 ± 22.1 (12)	0.041
	GAPDH	118.3 ± 22.8 (10)	89.4 ± 26.3 (10)	73.5 ± 11.3 (12)	0.002	77.5 ± 19.9 (12)	NS
RPD	β-actin	99.2 ± 32.7 (10)	83.4 ± 16.8 (10)	75.6 ± 24.8 (11)	NS	75.0 ± 33.1 (11)	NS
	GAPDH	92.3 ± 24.9 (10)	93.0 ± 30.4 (10)	88.0 ± 20.9 (11)	NS	94.7 ± 34.9 (11)	NS
CZP	β-actin	105.7 ± 40.9 (9)	88.1 ± 27.3 (9)	67.2 ± 25.1 (11)	0.027	84.5 ± 22.8 (12)	NS
	GAPDH	93.1 ± 36.3 (9)	85.6 ± 20.3 (9)	72.9 ± 14.0 (11)	0.002	90.3 ± 22.7 (12)	NS

VEH vehicle, *HPD* haloperidol, *OZP* olanzapine, *RPD* risperidone, *CZP* clozapine, *NS* not significant, *n* number of animals used. Data are the means ± SD. Post hoc *p* values compared with VEH are shown

at high dosing (frontal cortex: β-actin, $F_{4, 60} = 3.97$, $p = 0.006$, GAPDH, $F_{4, 60} = 5.73$, $p = 0.001$; hippocampus: β-actin, $F_{4, 61} = 3.42$, $p = 0.014$, GAPDH, $F_{4, 61} = 2.79$, $p = 0.034$). Post hoc analysis revealed that the expression levels of β-actin and/or GAPDH were significantly decreased in mice received clozapine or olanzapine in high dose. Body weight loss or lower level of body weight gain after three weeks of drug administration was also observed in clozapine or olanzapine treated mice in high dose compared with control mice (body weights change ± standard deviation for clozapine: -0.73 ± 0.51 g, $p = 0.00005$; olanzapine: 0.67 ± 0.81 g, $p = 0.083$, control: 1.57 ± 1.62 g), while no significant difference was observed at the clinical dose (clozapine: 2.5 ± 1.02 g, $p = 0.13$; olanzapine: 2.31 ± 0.88 g, $p = 0.19$; control: 1.57 ± 1.62 g). These results suggest that olanzapine and clozapine treatment in high dose might affect the general health of mice, which could result in the altered expression levels of house keeping genes. Thus, we focused on possible effects on the gene expression levels of DISC1 and dysbindin at the clinical dose only.

The expression levels of DISC1 mRNA normalized by β-actin and GAPDH (to reduce effects of possible mRNA degradation not detectable by electrophoresis and possible variations in RT efficiency) in frontal cortex of mice administrated with a typical antipsychotic (haloperidol) or atypical antipsychotics (olanzapine, risperidone, clozapine) at the clinical dose are shown in Fig. 1. Analysis of the DISC1 expression demonstrated significant effects of drug treatments (normalized by β-actin, $F_{4, 53} = 6.41$, $p < 0.001$, or GAPDH, $F_{4, 53} = 5.25$, $p = 0.001$). Post hoc analysis revealed that DISC1 expression levels were increased by treatments with atypical antipsychotics, olanzapine (normalized by β-actin: 36%, $p = 0.0029$; or GAPDH: 64%, $p = 0.016$) and risperidone (normalized by β-actin: 39%, $p = 0.0077$; or GAPDH: 55%, $p = 0.0031$)

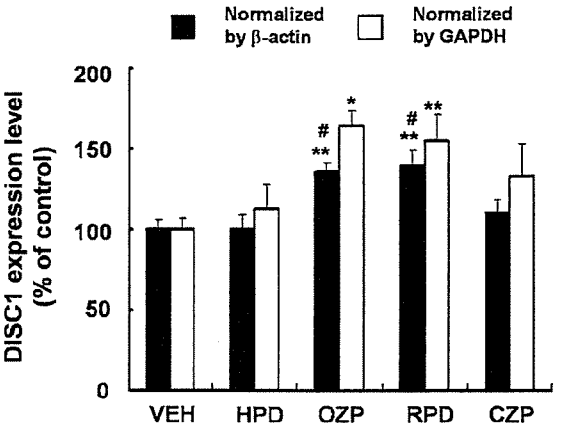

Fig. 1. Relative expression levels of DISC1 in frontal cortex in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9). $*p < 0.05$, $**p < 0.01$, compared with the control group. $\#p < 0.05$, compared with the haloperidol treated group

compared with the control group. No significant difference of DISC1 expression levels was observed after treatment with the typical antipsychotic (haloperidol). Elevated expression levels of the DISC1 gene normalized by β-actin were also found in olanzapine (36%, $p = 0.013$) and risperidone (39%, $p = 0.028$) treatment groups compared with haloperidol. Similar trends were obtained after normalization with GAPDH (olanzapine: 45%, $p = 0.095$; risperidone: 37%, $p = 0.30$). Treatment with clozapine tended to increase the expression levels of the DISC1 gene compared with control group, although they did not reach statistical significance.

The expression levels of DISC1 mRNA normalized by β-actin and GAPDH in hippocampus of mice administrated with a typical antipsychotic or atypical antipsychotics at the clinical dose are shown in Fig. 2. Analysis of the DISC1 expression in hippocampus

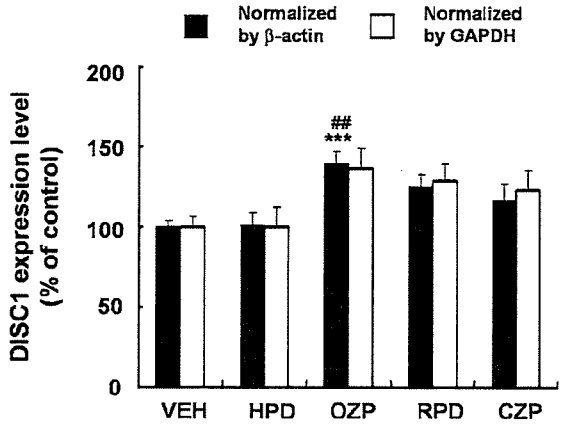

Fig. 2. Relative expression levels of DISC1 in hippocampus in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9). ***$p < 0.001$, compared with the control group. ##$p < 0.01$, compared with the haloperidol treated group

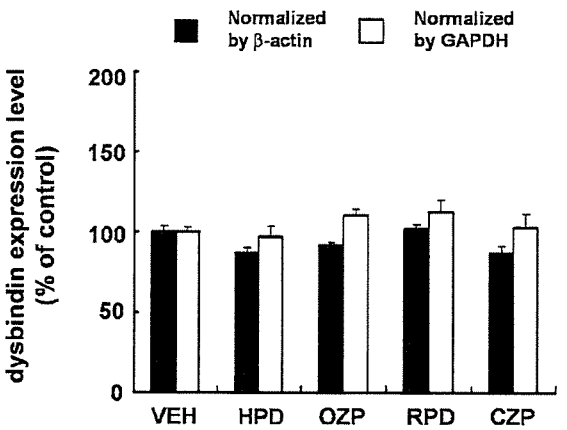

Fig. 3. Relative expression levels of dysbindin in frontal cortex in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9)

demonstrated significant effects of drug treatments (normalized by β-actin, $F_{4,53} = 6.09$, $p < 0.001$, or GAPDH, $F_{4,53} = 2.82$, $p = 0.034$). In post hoc analysis, DISC1 expression levels normalized by β-actin were significantly increased by the atypical antipsychotic, olanzapine, compared with control (39%, $p = 0.0006$) or haloperidol (29%, $p = 0.0054$) and similar trend was observed in risperidone compared with control (25%, $p = 0.079$). On the other hand, a slight increase of DISC1 expression was also found when normalizing by GAPDH (olanzapine vs control: 37%, $p = 0.094$; olanzapine vs haloperidol: 29%, $p = 0.23$; risperidone vs control: 29%, $p = 0.39$), which did not reach statistical significance. No effect of haloperidol or clozapine treatment was found in either normalization. These findings suggest that the mRNA expression levels of the DISC1 gene are increased by the chronic administration of some atypical antipsychotics in frontal cortex and possibly in hippocampus.

The expression levels of dysbindin mRNA normalized by β-actin and GAPDH in frontal cortex and hippocampus of mice administered treatment with a typical antipsychotic or atypical antipsychotics at the clinical dose are shown in Figs. 3 and 4. Dysbindin gene expression normalized by either β-actin or GAPDH in frontal cortex or hippocampus did not significantly differ between the treatment groups (frontal cortex: GAPDH, $F_{4,53} = 1.45$, $p = 0.23$; hippocampus: β-actin, $F_{4,53} = 0.64$, $p = 0.64$, GAPDH, $F_{4,53} = 0.46$, $p = 0.77$), except for that in frontal cortex normalized by β-actin ($F_{4,53} = 3.68$, $p = 0.01$). However, post hoc analysis demonstrated no significant difference in dysbindin expression in frontal cortex normalized by β-actin in any of the drug treatments, although there were trends towards slightly decreased expression of dysbindin in mice

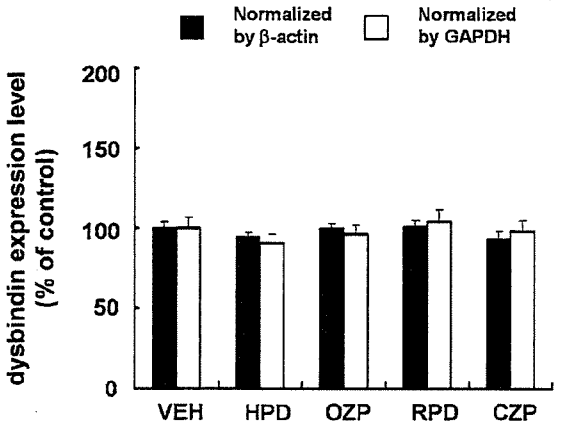

Fig. 4. Relative expression levels of dysbindin in hippocampus in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD ($n=10$), OZP ($n=10$), RPD ($n=10$) or CZP ($n=9$)

treated with haloperidol, compared with control (14%, $p=0.074$) and in mice treated with risperidone (16%, $p=0.094$). These data suggest that administration of typical and atypical antipsychotics do not have a consistent influence on mRNA expression levels of the dysbindin gene in frontal cortex or in hippocampus.

Discussion

In this study, we have measured mRNA expression levels of two susceptibility genes for schizophrenia, DISC1 and dysbindin, in frontal cortex and hippocampus using a real-time quantitative RT-PCR in mice treated chronically with typical or atypical antipsychotics. We found preliminary evidence that the expression levels of DISC1 may be altered by treatment with the atypical agents in frontal cortex and possibly in hippocampus and that the expression levels of dysbindin may not be changed under these conditions. Upregulation of DISC1 mRNA in frontal cortex by olanzapine and risperidone was observed in both normalizations by β-actin and GAPDH, however, that in hippocampus by olanzapine was found only in normalization by β-actin. As DISC1 has been shown to interact with actin (Miyoshi et al., 2003), it is possible that the DISC1 mRNA expression level normalized by β-actin in hippocampus may be somehow affected by the interaction. Upregulation of DISC1 mRNA in hippocampus by atypical antipsychotics appears to be marginal while that in frontal cortex is more apparent. As DISC1 expression is dominant in hippocampus compared with frontal cortex (Miyoshi et al., 2003), there is a possibility that this differential expression of DISC1 might affect the degree of the upregulation of DISC1 mRNA by the atypical antipsychotics.

Specifically, there was an increase of DISC1 expression levels after treatment with olanzapine and risperidone and possibly with clozapine in a simulated clinical dose in frontal cortex. As consistent results were obtained from normalization of the DISC1 expression by two house keeping genes, our findings would seem to be robust at least in comparison to results that might have been based on using only one control gene. However, it should be noted that there were some effects of antipsychotics on housekeeping gene expression, though largely nonsignificant. It is conceivable that some of the effect on our measures of DISC1 expression could be exaggerated by these effects on our control genes, as significant effects of drug treatments on the raw expression levels of DISC1 (non-normalized) were not observed in either frontal cortex or hippocampus (data not shown). Our data raise the possibility that DISC1 may be involved in the treatment of schizophrenia. However, as our study did not include the measurement of DISC1 proteins, or expression in other brain regions, or of treatment with other psychotropic drugs, further work is necessary to clarify whether

changes in DISC1 mRNA impact on protein expression and are specific for brain regions and psychotropic drugs. It also should be noted that we measured expression only of the common transcript for both of these genes. It is not currently known whether schizophrenia involves alternate processing of these genes into disease related transcripts or isoforms and we cannot rule out that treatment may impact on variable splicing or processing of these genes.

A balanced translocation in the DISC1 gene segregates with schizophrenia and other major psychiatric illnesses in a Scottish family (Millar et al., 2000). However, little is known about how the translocation affects the expression and/or function of the DISC1 gene. DISC1 protein expression in lymphoblasts derived from the family member with the translocation was observed to be decreased but the mutant truncated form of DISC1, which should be produced by the translocation, was not found (James et al., 2004). It is unknown whether the expression of DISC1 in brains of the family members is altered or not, however, this observation in peripheral cells suggested that the translocation might decrease the expression of DISC1. Alternatively, mutant truncated form of DISC1, which has been shown to play a role in inhibiting neurite outgrowth (Ozeki et al., 2003), might down-regulate the DISC1 protein expression and/or function. These findings suggest that reduced expression of DISC1 in brain might be expected in schizophrenic brain if DISC1 is involved in the pathogenesis of schizophrenia. On the other hand, gross expression levels of DISC1 protein have not been found to be changed in frontal cortex in patients with schizophrenia (Sawamura et al., 2005) and expression levels of DISC1 mRNA tended to be increased in hippocampus of schizophrenia patients (Lipska et al., 2004). Our data suggest that increased expression of DISC1 mRNA may be, at least in part, related to treatment with some atypical antipsychotics.

Evidence that dysbindin is associated with schizophrenia is now quite strong, although no functional mutation in dysbindin gene has yet been identified. Recent postmortem studies have found decreased expression of dysbindin mRNA and protein in hippocampus and frontal cortex in schizophrenic patients (McClintock et al., 2003; Talbot et al., 2004; Weickert et al., 2004). In contrast to our data with DISC1, we found no consistent pattern of altered dysbindin expression in hippocampus and frontal cortex following antipsychotic treatment.

Knowledge about protein functions of DISC1 and dysbindin is insufficient, however, we discuss a possibility how these genes affect the mechanisms of schizophrenia. As DISC1 has a prominent role in the neurite extension and its expression is developmentally regulated (Ozeki et al., 2003), upregulation of DISC1 could support the maturation of dendritic spine, which is believed to be affected in schizophrenia. As dysbindin promotes glutamate release in neuronal culture (Numakawa et al., 2004), reduced expression of dysbindin in schizophrenic brain could be relevant to glutamatergic dysfunction, which has been implicated in the pathophysiology of schizophrenia.

In summary, our findings offer preliminary evidence that altered expression of DISC1 may be caused by certain antipsychotic drugs, suggesting a role for DISC1 in therapeutic actions of these drugs. Additional studies are warranted to examine DISC1 and dysbindin expression, including western blotting analysis, in situ hybridization, immunohistochemistry, and the effect of other psychotropic drugs.

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Possible association between nonsynonymous polymorphisms of the anaplastic lymphoma kinase (ALK) gene and schizophrenia in a Japanese population

Short Communication

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Summary. We examined, for the first time, the possible association between schizophrenia and the anaplastic lymphoma kinase (ALK) gene which plays an important role in neurodevelopment. When two nonsynonymous polymorphisms (Arg1491Lys and Glu1529Asp) were examined, there were significant differences in genotype and allele distributions between patients and controls. Individuals homozygous for the minor allele (1491Lys–1529Asp) were more common in patients than in controls ($p = 0.0064$, odds ratio 2.4, 95% CI 1.3–4.6). These results suggest that genetic variations of the ALK gene might confer susceptibility to schizophrenia.

Keywords: Schizophrenia, anaplastic lymphoma kinase (ALK), single nucleotide polymorphism (SNP), association, susceptibility.

Introduction

Growing evidence has suggested that alterations of neurotrophic factors may be involved in the morphological, cytoarchitectural and neurobiochemical abnormalities in the brain of schizophrenic patients (Thome et al., 1998; Durany and Thome, 2004). Anaplastic lymphoma kinase (ALK) was originally identified as an oncogene activated in anaplastic large cell lymphomas with chromosomal translocation t (2;5) (Morris et al., 1994; Shiota et al., 1994). Subsequent cloning of the ALK gene revealed that it encodes a receptor-type protein tyrosine kinase (RTK) of the insulin receptor family (Iwahara et al., 1997; Morris et al., 1997). Neurotrophic factors exert their effects through binding to RTKs and play an important role in neurodevelopment such as

differentiation, proliferation, survival, and synaptic formation. Indeed, ALK was found to be a receptor for heparin-binding growth factors, midkine (Stoica et al., 2002) and pleiotrophin (Stoica et al., 2001). Midkine and pleiotrophin show approximately 50% identity in amino acid sequence and share the same genomic organization. These proteins play an important role in early neurogenesis, neurite outgrowth, nerve cell migration, and neuroprotection (reviewed by Kadomatsu and Muramatsu, 2004). Of note, a recent study reported alterations in serum midkine levels in patients with schizophrenia (Shimizu et al., 2003).

ALK is expressed almost exclusively in perinatal neural cells. In the central nervous system, it is highly expressed in diencephalons, midbrain, and the ventral half of the spinal cord. After birth, its expression decreases; however, it persists to be expressed in some regions such as the thalamus, olfactory bulb, and midbrain (Iwahara et al., 1997). These brain regions have been implicated in the pathophysiology of schizophrenia (e.g., Moberg and Turetsky, 2003; Clinton and Meador-Woodruff, 2004). The ALK gene is, therefore, a good candidate gene for association analysis with schizophrenia. To our knowledge, however, there is no study examining the possible association between the ALK gene and schizophrenia. The ALK gene maps to chromosome 2p23 (Morris et al., 1994). We searched for nonsynonymous single nucleotide polymorphisms (SNPs) in the ALK gene *in silico* and found only 2 common SNPs which have been well validated: a nucleotide substitution (G>A: NCBI SNP ID rs1881420) resulting in an amino acid change of Arg1491Lys (amino acid numbering is according to NCBI protein data base accession NP_004295) and G>C (rs1881421) resulting in Glu1529Asp. Since these polymorphisms may alter functions of ALK protein, we performed an association study between these polymorphisms and schizophrenia.

Materials and methods

Subjects

Subjects were 300 patients with schizophrenia (154 males, mean age of 45.3 years [SD 14.3]) and 308 healthy controls (140 males, 39.8 years [SD 11.5]). All subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association, 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from hospital staffs and their associates. They were interviewed and those individuals who had current or past history of psychiatric treatment were not enrolled in the study.

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

Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. The index SNPs (rs1881420 and rs1881421) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, as described previously (Hashimoto et al., 2004, 2005). Primers and probes for detection of the SNPs were as follows: 5′-TTCTCTCAGTCCAACCCTCCTT-3′ (forward primer), 5′-CTGGTGGGCTTGTTTCTGGAT-3′ (reverse primer), 5′-VIC-TTGCACAAGGTCCAC-MGB-3′ (probe 1), and 5′-FAM-TGCACAGGGTCCAC-MGB-3′ (probe 2) for rs1881420; 5′-AGAGAAACCC ACCAAAAAGAATAATCCT-3′ (forward primer), 5′-GTTAGGTGGGACAGTACAGCTT-3′ (reverse primer), 5′-VIC-CAGGTTACCCCTGTCGTGT-MGB-3′ (probe 1), and 5′-FAM-CAGGTTACCCCTCTCGTGT-MGB-3′ (probe 2) for rs1881421. Thermal cycling for polymerase chain reaction (PCR) were 1 cycle at 95°C for 10 minutes followed by 50 cycles of 92°C for 15 seconds and 60°C for 1 minute. Genotype data were read blind to the case-control status.

Statistical analysis

The presence of Hardy-Weinberg equilibrium was examined by using the χ^2 test for goodness of fit.

Table 1. Genotype distributions and allele frequencies of the Glu1529Asp polymorphism of the ALK gene (rs1881421) in patients with schizophrenia and controls

	Genotype distribution				Allele frequency		
	N	Glu/Glu	Glu/Asp	Asp/Asp	N	Glu	Asp
Patients	300	141 (47%)	128 (43%)	31 (10%)	600	410 (68%)	190 (32%)
Controls	308	171 (55%)	123 (40%)	14 (5%)	616	465 (75%)	151 (25%)

Genotype and allele distributions were compared between patients and controls by using the χ^2 test for independence. All p-values reported are two-tailed.

Results

Nearly all the subjects except for three (99.5%) had the same genotype for the two SNPs of rs1881420 and rs1881421, i.e., genotypes of G/G, G/A, and A/A in the former corresponded to those of G/G, G/C, and C/C in the latter. Thus, we show results of statistical analyses for the SNP rs1881421 (Glu1529Asp) only. Genotype distributions and allele frequencies in patients and controls are shown in Table 1. The genotype distribution was not significantly deviated from Hardy-Weinberg equilibrium for patients and controls (patients: $\chi^2 = 0.1$, $df = 1$, $p = 0.81$; controls: $\chi^2 = 1.9$, $df = 1$, $p = 0.16$). There was a significant difference in the overall genotype distribution between patients and controls ($\chi^2 = 9.3$, $df = 2$, $p = 0.0095$). Individuals homozygous for the minor allele (1529Asp) was significantly more common in patients than in controls ($\chi^2 = 7.4$, $df = 1$, $p = 0.0064$, odds ratio 2.4, 95% CI 1.3–4.6). When allele frequencies were compared, the 1529Asp allele was significantly more frequent in patients than in controls ($\chi^2 = 7.7$, $df = 1$, $p = 0.0055$, odds ratio 1.4, 95% CI 1.1–1.8).

Discussion

We examined, for the first time, the possible association between schizophrenia and the anaplastic lymphoma kinase (ALK) gene which plays an important role in neurodevelopment such as early neurogenesis, neurite outgrowth, nerve cell migration, and neuroprotection. We found that the minor allele (1529Asp) of the Glu1529Asp polymorphism (rs1881421) and homozygosity for this allele were significantly more common in patients with schizophrenia than in controls. Since nearly all the subjects had the same genotype for the other SNP, Arg1491Lys (rs1881420), the risk alleles constitute a haplotype 1491Lys–1529Asp. Thus, our results suggest that the 1491Lys–1529Asp haplotype or its homozygosity may confer susceptibility to schizophrenia. However, we do not know whether these nonsynonymous polymorphisms do alter functions of the ALK protein to give susceptibility to schizophrenia. Accordingly, there remains a possibility that other polymorphisms, which are in linkage disequilibrium to these polymorphisms, are truly responsible for giving susceptibility.

The ALK gene encodes a 1620 amino acid protein containing a putative 26 amino acid signal peptide, an extracellular domain of 1004 amino acid after signal peptide cleavage, a transmembrane domain of 28 hydrophobic amino acids, a juxtamembrane segment of 64 amino acids, a catalytic domain (protein tyrosine kinase domain) of 254 amino acids, followed by the carboxyl-terminal tail of 244 amino acids (Morris et al., 1997). The Arg1491Lys and Glu1529Asp residues lie close to a NPTY motif (residue 1504–1507) in the carboxyl-terminal tail (Morris et al., 1997). Such motifs mediate the interaction of RTKs with signaling substrates such as the insulin receptor substrate-1 and Src homology

and collagen proteins through the substrates's phosphotyrosine binding (PTB) domain (van der Geer and Pawson, 1995). It is possible that amino acid changes of Arg1491Lys and Glu1529Asp may alter protein structure and affect functions (e.g., binding to these substrates).

ALK is a receptor-type protein kinase (RTK) that is expressed preferentially in neurons of the central and peripheral nervous systems at late embryonic stages (Iwahara et al., 1997; Morris et al., 1997). Neurotrophic factors exert their effects through binding to RTKs, and ALK is a receptor for heparin-binding growth factors, midkine and pleiotrophin (Stoica et al., 2001, 2002). Thus it is likely that ALK play an important role in neurodevelopment such as differentiation, proliferation, survival, neurite outgrowth and synaptic formation, and alterations of ALK functions may result in vulnerability to developing schizophrenia, which accords with the neurotrophic factor theory of schizophrenia (Thome et al., 1998; Durany and Thome, 2004). Indeed, alterations in other neurotrophic factors such as brain-derived neurotrophic factors (BDNF) and neurotrophin-3 have been implicated in schizophrenia (e.g., Durany et al., 2001; Nanko et al., 2003; Hattori et al., 2002).

A limitation in the present study might be that the obtained evidence for association was not very strong (p-values of <0.01 level in a single sample). Replication studies in independent samples are required. If our results are replicated, experiments elucidating the possible effects of the amino acid substitutions (Arg1491Lys and Glu1529Asp) on the ALK protein functions may serve to advance our understanding of the molecular mechanisms of schizophrenia and may provide clues to production of new treatment of the illness.

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ORIGINAL ARTICLE

A complex polymorphic region in the brain-derived neurotrophic factor (BDNF) gene confers susceptibility to bipolar disorder and affects transcriptional activity

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Previous studies have suggested that genetic variations in the brain-derived neurotrophic factor (BDNF) gene may be associated with several neuropsychiatric diseases including bipolar disorder. The present study examined a microsatellite polymorphism located approximately 1.0 kb upstream of the translation initiation site of the BDNF gene for novel sequence variations, association with bipolar disorder, and effects on transcriptional activity. Detailed sequencing analysis revealed that this polymorphism is not a simple dinucleotide repeat, but it is highly polymorphic with a complex structure containing three types of dinucleotide repeats, insertion/deletion, and nucleotide substitutions that gives rise to a total of 23 novel allelic variants. We obtained evidence supporting the association between this polymorphic region (designated as BDNF-linked complex polymorphic region (BDNF-LCPR)) and bipolar disorder. One of the major alleles ('A1' allele) was significantly more common in patients than in controls (odds ratio 2.8, 95% confidential interval 1.5–5.3, $P=0.001$). Furthermore, a luciferase reporter gene assay in rat primary cultured neurons suggests that this risk allele (A1) has a lower-transcription activity, compared to the other alleles. Our results suggest that the BDNF-LCPR is a functional variation that confers susceptibility to bipolar disorder and affects transcriptional activity of the BDNF gene.
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Keywords: association study; brain-derived neurotrophic factor (BDNF); bipolar disorder; polymorphism; susceptibility; transcriptional activity

Introduction

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophic factor family and promotes the development, regeneration, survival and maintenance of function of neurons.[1] It modulates synaptic plasticity and neurotransmitter release across multiple neurotransmitter systems, as well as the intracellular signal-transduction pathway.[2] BDNF has been implicated in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents such as mood stabilizers and antidepressants.[3] BDNF protein was reduced in postmortem brains of patients with bipolar disorder, compared to controls.[4] Chronic electroconvulsive seizure and antidepressant drug treatments increase mRNA of BDNF and its receptor trkB.[5] Lithium may also exert its neuroprotective effect through enhancing expression of BDNF and trkB.[6]

The BDNF gene is, therefore, an attractive candidate gene which may give susceptibility to bipolar disorder.[7] In accordance with this, at least three previous studies reported a significant association between the Val66Met polymorphism (NCBI dbSNP rs6265) of the BDNF gene and bipolar disorder in Caucasian populations.[8–10] In these studies, the Val66 allele was consistently found to have a risk-increasing effect on the development of bipolar disorder. However, this association was not replicated in other Caucasian[11–13] or Asian populations including ours.[14–16]

Another polymorphism of the BDNF gene that has been well studied as to the possible association with neuropsychiatric diseases is the 'GT repeat' located approximately 1.0 kb upstream of the translation initiation site of the gene.[17] With respect to the possible effect on mood disorders, a significant

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linkage disequilibrium with bipolar disorder[8] and a significant association with childhood onset mood disorder[18] have been reported in Caucasian populations, although one study failed to find such an association with bipolar disorder.[13] Furthermore, there is some evidence suggesting that this polymorphism plays a role in the pathogenesis of schizophrenia.[13,19,20] However, there is no study that examined whether this polymorphism has functional effects. Since micro- and minisatellite polymorphisms even located in intron have been shown to play a role in the expression of many genes,[21] it might be intriguing to examine whether this microsatellite polymorphism of the BDNF gene is associated with transcriptional activity in an allele-dependent manner.

The aim of the present study was to examine this microsatellite polymorphism (designated here as BDNF-linked complex polymorphic region (BDNF-LCPR) due to its complex structure) for novel sequence variations, association with bipolar disorder, and effects on transcriptional activity.

Materials and methods

Subjects

Subjects were 153 patients with bipolar disorder (71 males) and the same number of controls (71 males), matched for age, sex, ethnicity, and geographical area. These subjects, who were recruited from Showa University Hospital and Shiga University of Medical Science Hospital, Japan, were previously genotyped for the Val66Met polymorphism of the BDNF gene, yielding a result of no significant association.[16] Mean age (standard deviation (s.d.)) in the patients was 47.8 (s.d. 15.3) years and that in the controls 47.1 (11.0). All the patients and controls were biologically unrelated Japanese. Consensus diagnosis of bipolar disorder was made for each patient by at least two experienced psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (DSM-IV),[22] based on unstructured interviews and medical records. Among the patients, 94 individuals (61%) were diagnosed with bipolar I and the remaining 59 with bipolar II disorder. Patients who had one or more comorbid axis I disorders were excluded. The mean age of onset and number of episodes were 37.8 (s.d. 15.2) years and 3.9 (1.4) times, respectively. Thirty-four patients (22.2%) had at least one episode with psychotic features. Sixty-seven patients (43.8%) had a family history of major psychiatric illness (mood disorders or schizophrenia spectrum disorders) within their second-degree relatives. The controls were screened with a semi-structured interview and those individuals who had current or past contact to psychiatric services were excluded. In addition, those individuals who had a family history of major psychiatric illness or those who had a current or past history of regular use of psychotropic medication, including hypnotics, were excluded from the control group. After description of the study, written informed consent for the participation of the study was obtained from every subject. The study protocol was approved by ethics committee of each institution.

Sequence analysis

Venous blood was drawn and genomic DNA was extracted according to standard procedures. To determine accurate DNA sequences for the BDNF-LCPR, we cloned this polymorphic region and performed direct sequencing. An approximately 400 base-pair (bp) DNA fragment encompassing the polymorphic region was amplified by polymerase chain reaction (PCR) with primers of *Hind*III-tagged BDNF-LCPR-F1 and *Hind*III-tagged BDNF-LCPR-R1 (see Table 1 and Figure 1a). The purified PCR products were ligated into the *Hind*III site of the pBluescriptII SK (+) vector (Toyobo, Tokyo, Japan). The vector was transformed into *Escherichia coli*, DH5α and incubated. For sequencing, PCR amplification was performed with primers of GTTGTAAAACGACGGCCA GTG (Universal primer) and GGAAACAGCTATGAC CATG (Reverse primer). At least four clones were examined for each individual. Direct sequencing was performed with the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

Cloning and sequencing analysis described above suggested that the polymorphic region is not a simple

Table 1 Primer sequences for sequencing the BDNF-linked complex polymorphic region (BDNF-LCPR)

Primer No.	*Primer Name*	*UCSC Chromosome11 5′→3′*	*Numbers in Fig. 1 5′→3′*	*Primer Sequence 5′→3′*
1	BDNF-LCPR-F1	27637949→27637930	139→158	TAGAGCAACCCTCTGGCAAA
2	BDNF-LCPR-R1	27637545→27637567	543→521	TGTCATGAAAACAATGTGTCTGG
3	BDNF-LCPR-F2	27637844→27637822	244→266	CCAAAATGTGTAAAACACCACTC
4	B-BDNF-LCPR-F2	27637844→27637822	244→266	**Biotin**-CCAAAATGTGTAAAACACCACTC
5	BDNF-LCPR-R2	27637715→27637741	373→347	GAAAGCTCAACTTTTCTTTTTACTAGA
6	B-BDNF-LCPR-R2	27637715→27637741	373→347	**Biotin**-GAAAGCTCAACTTTTCTTTTTACTAGA
7	BDNF-LCPR-F3	27637810→27637791	278→297	AGTAGGATAAACTCAGAGCG
8	BDNF-LCPR-R3	27637730→27637749	358→339	CTTTTTACTAGAGATGTTCT
9	Reverse		1→19	GGAAACAGCTATGACCATG
10	Unversal		641→621	GTTGTAAAACGACGGCCAGTG