psychotropic drugs were permitted during the study. Of 96 enrolled patients, ten did not complete the study; five patients because of side effects, one patient because of severe insomnia and four patients without explanation. Of the 86 patients who completed the 6-week study, six patients were excluded from the current analysis because plasma samples revealed very low milnacipran concentrations, indicative of poor compliance. Patients who completed the study included 52 women and 28 men, 49 outpatients and 31 inpatients, and ranged from 25 to 69 years of age (mean age = 51.4 ± 12.2 (±SD)).

Fluvoxamine treatment

Fluvoxamine was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day. The daily dose was increased to 100 mg/day after a week and was increased to 200 mg/day after another week. Concomitant administration of psychotropic drugs was restricted as in the milnacipran study. Of 66 enrolled patients, nine did not complete the study; four patients because of side effects and five patients without explanation. Of the 57 patients who completed the 6-week study, three patients were excluded from the current analysis because plasma samples revealed very low fluvoxamine concentrations, indicative of poor compliance. Patients who completed the study included 32 women and 22 men, 43 outpatients and 11 inpatients, and ranged from 24 to 69 years of age (mean age = 51.2 ± 13.2 (±SD)).

Data collection

Depression symptom severity was assessed with the use of the MADRS. Assessments were conducted at baseline and at 1, 2, 4 and 6 weeks after initiation of antidepressant treatment. A clinical response was defined as a 50% or greater decrease in the baseline MADRS score. Clinical remission was defined as a final MADRS score less than ten (Hawley *et al.*, 2002). Collection of blood samples was performed 12 hours after drug administration at bedtime, 4 weeks after initiation of each antidepressant treatment.

Genotyping

The BDNF G196A polymorphism was determined by a minor modification of the method of Tsai *et al.* (2003). The BDNF C132T polymorphism was determined by a minor modification of the method of Szekeres *et al.* (2003). Primers and enzymes used in this study were the same as previous studies; the conditions of the polymerase chain reaction and the chemical reagents were adjusted to our instruments.

Quantification of plasma milnacipran/fluvoxamine concentration

Plasma concentrations of milnacipran were measured with high performance liquid chromatography (HPLC). Details of the method have been described previously (Higuchi *et al.*, 2003). Plasma concentrations of fluvoxamine were measured with HPLC. Details of the method have been described previously (Ohkubo *et al.*, 2003). Genotyping and measurement of plasma concentrations were performed by laboratory personnel blind to the identity and clinical antidepressant effect of the patients. Moreover, clinicians were unaware of the genotyping results and the plasma milnacipran concentrations of each patient.

Statistical analysis

Differences in patient characteristics were analysed with the use of the unpaired t-test or chi-square test where appropriate. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures analysis of variance (ANOVA), with genotype and time as factors. When significant interaction between factors was observed, contrasts were used to enable comparisons between each two of the three genotype groups. Differences in the MADRS scores at each evaluation point were examined with the one-way factorial ANOVA followed by the Fisher's PLSD test. Genotype deviation from Hardy-Weinberg equilibrium was evaluated by the chi-square test. Genotype distribution and allele frequencies were analysed with the use of the chi-square test. Plasma concentrations of milnacipran or fluvoxamine were analysed with the use of one-way factorial ANOVA in each genotype group; an unpaired t-test was then used to analyse differences between groups who were or were not responsive to milnacipran or fluvoxamine. Statistical analysis was performed using StatView version 5.0 (SAS Institute Inc., Cary, NC), except the two-way repeated measures ANOVA with contrasts was performed using SuperANOVA version 1.11 (Abacus Concepts, Inc., Berkeley, CA). Power analysis was performed with the use of G·Power (Buchner *et al.*, 1996). All tests were two-tailed; alpha was set at 0.05.

Results

Minor allele frequencies for the C132T polymorphism were very low and similar to those reported by Kunugi *et al.* (2001) and Szekeres *et al.* (2003); 5.0% in the patients treated with milnacipran and 3.7% in those treated with fluvoxamine. Therefore, only the G196A polymorphism was included in the statistical analysis. The observed genotype frequencies of the G196A polymorphism were within the distribution expected according to the Hardy-Weinberg equilibrium. As the authors reported that response to fluvoxamine was associated with allelic variations of the 5-hydroxytriptamine transporter gene-linked polymorphic region (5-HTTLPR) (Yoshida *et al.*, 2002) and response to milnacipran was associated with those of the norepinephrine transporter T-182C and G1287A polymorphisms (Yoshida *et al.*, 2004), it was necessary to confirm these polymorphisms to be controlled. The genotype distribution of these genotypes was not significantly different among the *G/G*, *G/A* and *A/A* genotype groups of the BDNF G196A polymorphism (data not shown).

Fig. 1 shows the MADRS scores over time in relation to the BDNF G196A polymorphism for all subjects receiving fluvoxamine or milnacipran. There was no significant difference in baseline MADRS scores among each genotype group. Two-way repeated

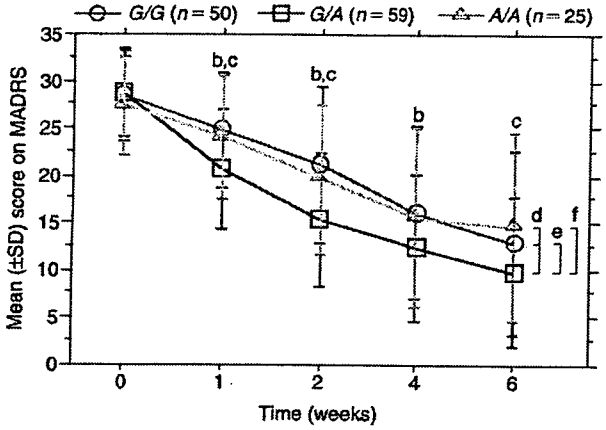

Figure 1 MADRS scores during 6 weeks of milnacipran/fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents te mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.
[b] Significant differences at each point between the *G/A* and *G/G* groups ($p = 0.0009$ at week 1, $p = 0.0001$ at week 2 and $p = 0.025$ at week 4).
[c] Significant difference at each point between the *G/A* and *A/A* groups ($p = 0.032$ at week 1, $p = 0.019$ at week 2 and 0.029 at week 6).
[d] Significant geneotype × time interaction among all three genotype groups ($F = 3.64$, $df = 8$, $p = 0.0004$).
[e] Signicant genotype × time interaction between the *G/A* and *G/G* groups ($F = 5.21$, $df = 4$, $p = 0.0004$)
[f] Significant genotype × time interaction between the *G/A/* and *A/A*: groups ($F = 3.99$, $df = 4$, $p = 0.0034$).

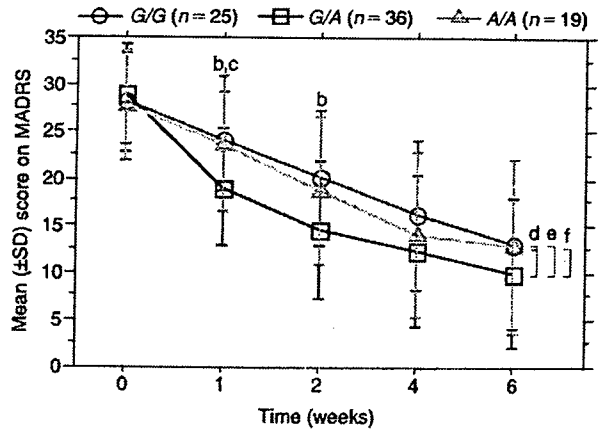

Figure 2 MADRS scores during 6 weeks of milnacipran treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.
[b] Significant difference at each point between the *G/A* and *G/G* groups ($p = 0.0031$ at week 1 and $p = 0.0056$ at week 2).
[c] Significant difference between the *G/A* and *A/A* groups ($p = 0.011$ at week 1).
[d] Significant genotype × time interaction among all three genotype groups ($F = 2.30$, $df = 8$, $p = 0.021$).
[e] Significant genotype × time interaction between the *G/A* and *G/G* groups ($F = 3.54$, $df = 4$, $p = 0.0077$).
[f] Significant genotype x time interaction between the *G/A* and *A/A* groups ($F = 2.56$, $df = 4$, $p = 0.039$).

measures ANOVA including all three genotype groups indicated a significant genotype × time interaction. Contrast analysis indicated a significant genotype × time interaction between the *G/A* and *G/G* genotype groups. The MADRS score of the *G/A* genotype group was significantly lower than that of the G/G genotype group at 1, 2 and 4 weeks. Contrast analysis indicated a significant genotype x time interaction between the *G/A* and *A/A* groups. The MADRS score of the *G/A* genotype group was significantly lower than that of the *A/A* group at 1, 2 and 6 weeks. Contrast analysis indicated no significant genotype × time interaction between the *G/G* and *A/A* genotype groups ($F = 0.99$, $df = 4$, $p = 0.41$). There was no significant difference in the MADRS score at any evaluation point between the *G/G* and *A/A* genotype groups. When milnacipran- and fluvoxamine-treated subjects were analysed independently, the *G/A* genotype group showed greater reduction of MADRS scores than other genotype groups, irrespective of which antidepressant was administered (Figs. 2 and 3). Mean plasma concentrations of milnacipran were 92.3 ± 50.4 (±SD) ng/ml, 88.1 ± 31.1 ng/ml and 91.7 ± 36.2 ng/ml for the *G/G*, *G/A* and *A/A* genotype groups, respectively. There was no significant difference among the groups ($F = 0.99$, $df = 2, 77$, $p = 0.90$). Mean plasma concentrations of fluvoxamine were 169.1 ± 174.7 (±SD) ng/ml, 155.1 ± 118.6 ng/ml and 94.8 ± 35.3 ng/ml for the *G/G*, *G/A* and *A/A* genotype groups respectively. There was no significant difference among the groups ($F = 0.65$, $df = 2, 51$, $p = 0.53$).

Table 2 shows the genotype distribution and allele frequencies of responders and non-responders for all subjects receiving milnacipran or fluvoxamine. The proportion of responders was higher in *G/A* subjects than in subjects of other genotypes, but it did not reach a significant difference. There was no significant difference in the allele frequencies between responders and non-responders. The proportion of responders was non-significantly higher in *G/A* subjects than in subjects of other genotypes, irrespective of which antidepressants were administered (Table 3 and Table 4). When remitters and non-responders were compared, there was also no significant difference in the genotype distribution ($\chi^2 = 2.53$,

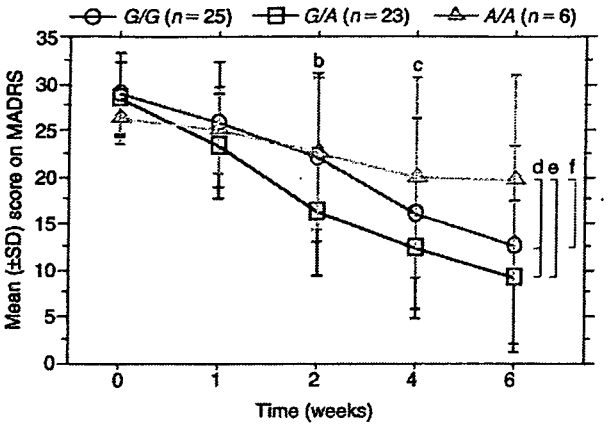

Figure 3 MADRS scores during 6 weeks of fluvoxamine treatment in three BDNF G196A genotype groups[a]

[a] Each point represents the mean score ± SD. Differences in the MADRS scores during this study were examined with the use of two-way repeated-measures ANOVA with contrasts. Differences in the MADRS scores at each evaluation point were examined with the use of one-way factorial ANOVA followed by Fisher's PLSD test.

[b] Significant difference between the *G/A* and *G/G* groups ($p = 0.015$ at week 2).

[c] Significant difference between the *G/A* and *A/A* groups ($p = 0.024$ at week 6).

[d] Significant genotype × time interaction among all three genotype groups ($F = 2.83$, $df = 8$, $p = 0.0053$).

[e] Significant genotype × time interaction between the *G/A* and *A/A* groups ($F = 4.55$, $df = 4$, $p = 0.0015$).

[f] Significant genotype × time interaction between the *G/G* and *A/A* groups ($F = 2.77$, $df = 4$, $p = 0.029$).

$df = 2$, $p = 0.12$ for the milnacipran treatment, $\chi^2 = 3.25$, $df = 2$, $p = 0.20$ for the fluvoxamine treatment and $\chi^2 = 4.26$, $df = 2$, $p = 0.12$ for both treatments) and genotype frequencies ($\chi^2 = 2.53$, $df = 1$, $p = 0.52$ for the milnacipran treatment, $\chi^2 = 0.64$, $df = 1$, $p = 0.64$ for the fluvoxamine treatment and $\chi^2 = 4.26$, $df = 1$, $p = 0.63$ for both treatments) (data not shown).

The plasma concentrations of milnacipran or fluvoxamine were not significantly different between responders and nonresponders, as shown in our previous studies (Yoshida *et al.*, 2002; Yoshida *et al.*, 2004).

This study of both milnacipran and fluvoxamine had a power of 0.16 to detect a small effect, 0.88 to detect a medium effect and 0.99 to detect a large effect in the genotype distribution ($n = 134$). For the allele frequency analysis ($n = 268$), this study had a power of 0.37 to detect a small effect, 0.99 to detect a medium effect and 0.99 to detect a large effect. In the power analysis, effect size conventions were determined according to the method of Buchner *et al.* (1996) as follows: small effect size = 0.10, medium effect size = 0.30 and large effect size = 0.50 (alpha = 0.05).

Discussion

The present study revealed that the BDNF G196A polymorphism affected the efficacy of both milnacipran and fluvoxamine. The *G/A* genotype of this polymorphism was associated with a significantly better therapeutic effect in the MADRS scores during this study, although the difference in final therapeutic response was not significant between the *G/A* and other genotype groups.

The results of this study are not well explained by the findings by Egan *et al.* (2003). Their human study showed that the *A* allele was associated with poorer episodic memory, abnormal hippocampal activation as determined by functional magnetic resonance imaging (fMRI), and lower hippocampal n-acetyl aspartate levels as assayed by MRI spectroscopy. According to their expression study, high concentrations of KCl induced detectable release of *G*-BDNF, whereas the activity-dependent release of *A*-BDNF was severely reduced and sometimes not detectable. Thus, the presence of the *G* allele is related to appropriate hippocampal function, neuronal function and activity-dependent BDNF release. In consideration of these findings, it is difficult to interpret the present results.

However, several aspects should be considered before trying to interpret our study based on the findings by Egan *et al.* (2003). First, the behavioural and mood abnormalities associated with major depressive disorder appear to result from disturbances mainly in the temporolimbic-frontal-caudate network (Drevets, 1999; Czeh *et al.*, 2001), although several lines of research support the notion that the hippocampus is also an important

Table 2 Genotype distribution and allele frequencies in responders and non-responders (milnacipran/fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	29 (34.1%)	43 (50.6%)	13 (15.3%)	101 (59.4%)	69 (40.6%)
Non-responder	21 (42.9%)	16 (32.6%)	12 (24.5%)	58 (59.2%)	40 (40.8%)

[a] Analysis performed with the use of the χ^2 test.

[b] No significant difference between responders and nonresponders ($\chi^2 = 1.32$, $df = 2$, $p = 0.12$).

[c] No significant difference between responders and nonresponders ($\chi^2 = 0.001$, $df = 1$, $p = 0.97$).

Table 3 Genotype distribution and allele frequencies in responders and non-responders (milnacipran treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	13 (26.0%)	26 (52.0%)	11 (22.0%)	52 (52.0%)	48 (48.0%)
Non-responder	12 (40.0%)	10 (33.3%)	8 (26.7%)	34 (56.7%)	26 (43.3%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and non-responders ($\chi^2 = 2.80$, df = 2, $p = 0.25$).
[c] No significant difference between responders and non-responders ($\chi^2 = 0.030$, df = 1, $p = 0.57$).

Table 4 Genotype distribution and allele frequencies in responders and non-responders (fluvoxamine treatment)[a]

	Genotype distribution[b]			Allele frequency[c]	
	G/G	*G/A*	*A/A*	*G*	*A*
Responder	16 (45.7%)	17 (48.6%)	2 (5.7%)	49 (70.0%)	21 (30.0%)
Non-responder	9 (47.4%)	6 (31.6%)	4 (21.0%)	24 (63.2%)	14 (36.8%)

[a] Analysis performed with the use of the χ^2 test.
[b] No significant difference between responders and nonresponders ($\chi^2 = 3.45$, df = 2, $p = 0.18$).
[c] No significant difference between responders and nonresponders ($\chi^2 = 0.53$, df = 1, $p = 0.47$).

region in the pathophysiology of major depressive disorder (Campbell and Macqueen, 2004). Therefore, the functional effect of the BDNF G196A polymorphism on the temporolimbic-frontal-caudate network is necessary to understand the present results adequately. However, such information is extremely limited; to our knowledge, there have been no reports investigating this issue using functional brain imaging, such as fMRI or positron emission tomography.

Only one cognitive study (Foltynie *et al.*, 2005) investigated the effect of the BDNF G196A polymorphism on performance of planning ability in Parkinson's disease using the Tower of London (TOL) task, a test of working memory (Robbins, 1996). The TOL task is reported to increase relative regional cerebral blood flow in the dorsolateral prefrontal cortex, lateral premotor cortex, rostral anterior cingulate cortex and dorsal caudate nucleus (Dagher *et al.*, 1999). Foltynie *et al.* (2005) revealed that the *A* allele of the BDNF G196A polymorphism was associated with better performance at the TOL task. This result is inconsistent with the results by Egan *et al.* (2003), who reported that the presence of the *A* allele was associated with impaired function in the hippocampus. The exact mechanism underlying this discrepancy is unclear. The study by Foltynie *et al.* (2005) was performed in Parkinson's disease not in major depressive disorder. However, it is possible that the functional effects of the BDNF G196A polymorphism differ among areas of the brain in major depressive disorder, and this regional difference in the temporolimbic-frontal-caudate network and the hippocampus may contribute to the better antidepressant effect in patients with the *G/A* genotype.

Additionally, some other studies indicated that subjects heterozygous for the BDNF G196A polymorphism have significant differences in expression of dichotomous or quantitative phenotypes than those homozygous for either allele. Momose *et al.* (2002) reported that homozygosity of the BDNF G196A polymorphism was more frequent in patients with Parkinson's disease. This finding suggests that the *G/A* genotype is less susceptible to Parkinson's disease than other genotypes. Tsai *et al.* (2003) reported a trend to a higher percentage change of the total Hamilton Depression Rating score for heterozygote patients in comparison to homozygote patients after fluoxetine treatment for 4 weeks. Their results are consistent with those of the present study and suggest that the *G/A* genotype is related to a favourable antidepressant effect. Besides the possible regionally different effects of the BDNF G196A polymorphism on brain function, another possibility is that the polymorphism may be in linkage disequilibrium with an as yet unidentified functional polymorphism with a molecular heterotic effect (Comings and MacMurray, 2000).

One major limitation of this study is the relatively small number of subjects, especially in the fluvoxamine arm. A second limitation is the relatively small end point treatment differences. These limitations may increase the possibility of a false positive and make it difficult to conclude that the BDNF G196A polymorphism is the common genetic factor for prediction of the antidepressant effect of both milnacipran and fluvoxamine. Further studies with a larger number of subjects are needed not only to confirm the results of this study but also to investigate the interaction of many genes, including the BDNF gene, on the mechanisms of antidepressant action.

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References

Buchner A, Faul F, Erdfelder E (1996) G·Power: A priori, post-hoc, and compromise power analyses for the Macintosh (Version 2.1.1) [Computer program]. University of Trier, Trier, Germany

Burke M J, Preskorn S H (1999) Therapeutic drug monitoring of antidepressants: cost implications and relevance to clinical practice. Clin Pharmacokinet *37*: 147–165

Campbell S, Macqueen G (2004) The role of the hippocampus in the pathophysiology of major depression. J Psychiatry Neurosci *29*: 417–426

Charlier C, Pinto E, Ansseau M, Plomteux G (2002) Venlafaxine: the relationship between dose, plasma concentration and clinical response in depressive patients. J Psychopharmacol *16*: 369–372

Comings D E, MacMurray J P (2000) Molecular heterosis: a review. Mol Genet Metab *71*: 19–31

Czeh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, Bartolomucci A, Fuchs E (2001) Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. Proc Natl Acad Sci U S A *98*: 12796–12801

Dagher A, Owen A M, Boecker H, Brooks D J (1999) Mapping the network for planning: a correlational PET activation study with the Tower of London task. Brain *122* (Pt 10): 1973–1987

Drevets W C (1999) Prefrontal cortical-amygdalar metabolism in major depression. Ann N Y Acad Sci *877*: 614–637

Egan M F, Kojima M, Callicott J H, Goldberg T E, Kolachana B S, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger D R (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell *112*: 257–269

Foltynie T, Lewis S G, Goldberg T E, Blackwell A D, Kolachana B S, Weinberger D R, Robbins T W, Barker R A (2005) The BDNF Val66Met polymorphism has a gender specific influence on planning ability in Parkinson's disease. J Neurol *252*: 833–838

Hawley C J, Gale T M, Sivakumaran T (2002) Defining remission by cut off score on the MADRS: selecting the optimal value. J Affect Disord *72*: 177–184

Higuchi H, Yoshida K, Takahashi H, Naito S, Kamata M, Ito K, Sato K, Tsukamoto K, Shimizu T, Nakanishi M, Hishikawa Y (2003) Milnacipran plasma levels and antidepressant response in Japanese major depressive patients. Hum Psychopharmacol *18*: 255–259

Hong C J, Huo S J, Yen F C, Tung C L, Pan G M, Tsai S J (2003) Association study of a brain-derived neurotrophic-factor genetic polymorphism and mood disorders, age of onset and suicidal behavior. Neuropsychobiology *48*: 186–189

Kunugi H, Ueki A, Otsuka M, Isse K, Hirasawa H, Kato N, Nabika T, Kobayashi S, Nanko S (2001) A novel polymorphism of the brain-derived neurotrophic factor (BDNF) gene associated with late-onset Alzheimer's disease. Mol Psychiatry *6*: 83–86

Malhotra A K, Murphy G M Jr, Kennedy J L (2004) Pharmacogenetics of psychotropic drug response. Am J Psychiatry *161*: 780–796

Momose Y, Murata M, Kobayashi K, Tachikawa M, Nakabayashi Y, Kanazawa I, Toda T (2002) Association studies of multiple candidate genes for Parkinson's disease using single nucleotide polymorphisms. Ann Neurol *51*: 133–136

Montgomery S A, Åsberg M (1979) A new depression scale designed to be sensitive to change. Br J Psychiatry *134*: 382–389

Nakata K, Ujike H, Sakai A, Uchida N, Nomura A, Imamura T, Katsu T, Tanaka Y, Hamamura T, Kuroda S (2003) Association study of the brain-derived neurotrophic factor (BDNF) gene with bipolar disorder. Neurosci Lett *337*: 17–20

Ohkubo T, Shimoyama R, Otani K, Yoshida K, Higuchi H, Shimizu T (2003) High-performance liquid chromatographic determination of fluvoxamine and fluvoxamino acid in human plasma. Anal Sci *19*: 859–864

Okamoto H, Shino Y, Hashimoto K, Kumakiri C, Shimizu E, Shirasawa H, Iyo M (2003) Dynamic changes in AP-1 transcription factor DNA binding activity in rat brain following administration of antidepressant amitriptyline and brain-derived neurotrophic factor. Neuropharmacology *45*: 251–259

Perry P J, Pfohl B M, Holstad S G (1987) The relationship between antidepressant response and tricyclic antidepressant plasma concentrations. A retrospective analysis of the literature using logistic regression analysis. Clin Pharmacokinet *13*: 381–392

Robbins T W (1996) Dissociating executive functions of the prefrontal cortex. Philos Trans R Soc Lond B Biol Sci *351*: 1463–1470; discussion 1470–1471

Russo-Neustadt A, Beard R C, Cotman C W (1999) Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. Neuropsychopharmacology *21*: 679–682

Shirayama Y, Chen A C, Nakagawa S, Russell D S, Duman R S (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J Neurosci *22*: 3251–3261

Smith M A, Makino S, Kvetnansky R, Post R M (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci *15*: 1768–1777

Stahl S M (2000) Blue genes and the monoamine hypothesis of depression. J Clin Psychiatry *61*: 77–78

Szekeres G, Juhasz A, Rimanoczy A, Keri S, Janka Z (2003) The C270T polymorphism of the brain-derived neurotrophic factor gene is associated with schizophrenia. Schizophr Res *65*: 15–18

Tsai S J, Cheng C Y, Yu Y W, Chen T J, Hong C J (2003) Association study of a brain-derived neurotrophic-factor genetic polymorphism and major depressive disorders, symptomatology, and antidepressant response. Am J Med Genet B Neuropsychiatr Genet *123*: 19–22

Yoshida K, Takahashi H, Higuchi H, Kamata M, Ito K, Sato K, Naito S, Shimizu T, Itoh K, Inoue K, Suzuki T, Nemeroff C B (2004) Prediction of antidepressant response to milnacipran by norepinephrine transporter gene polymorphisms. Am J Psychiatry *161*: 1575–1580

Yoshida K, Ito K, Sato K, Takahashi H, Kamata M, Higuchi H, Shimizu T, Itoh K, Inoue K, Tezuka T, Suzuki T, Ohkubo T, Sugawara K, Otani K (2002) Influence of the serotonin transporter gene-linked polymorphic region on the antidepressant response to fluvoxamine in Japanese depressed patients. Prog Neuropsychopharmacol Biol Psychiatry *26*: 383–386

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Association Study between Vesicle-Associated Membrane Protein 2 Gene Polymorphisms and Fluvoxamine Response in Japanese Major Depressive Patients

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Key Words
Depressive disorder · Fluvoxamine response · Haplotype analysis · Vesicle-associated membrane protein 2

Abstract
Background: Vesicle-associated membrane protein 2 (VAMP2) is a key component of the synaptic vesicle docking/fusion machinery and its mRNA reportedly increases in the frontal cortex of rats following chronic antidepressant and electroconvulsive treatment. VAMP2 is therefore thought to be involved in the mechanism of action of antidepressants and may alter their efficacy. The purpose of this study was to investigate whether the *VAMP2* gene is associated with clinical responses to a specific antidepressant, fluvoxamine. ***Methods:*** A total of 106 patients with major depressive disorder were given fluvoxamine (50–200 mg/day) for 8 weeks and assessed for severity of depression using the Semi-Structured Interview Guide of the Hamilton Depressive Scale (SIGH-D; 17 items) at 0 and 8 weeks. We defined a clinical response as more than a 50% reduction in baseline SIGH-D within 8 weeks, and defined clinical remission as a SIGH-D score of less than 7 at 8 weeks. Genotyping was performed by PCR-RFLP. ***Results:*** Analysis of haplotype tagging single nucleotide polymorphisms as well as haplotype analysis did not reveal any significant associations. ***Conclusion:*** Our results suggest that the *VAMP2* gene is unlikely to play a major role in the efficacy of fluvoxamine.

Introduction

The selective serotonin reuptake inhibitors (SSRIs) are one of the first line drugs for treatment of major depressive disorder. Unfortunately, approximately 2 weeks are required for the onset of clinical effect, and only 60% of major depressive patients show a complete response to antidepressant treatment [1]. It is difficult for clinicians to predict which patients will respond to which drug based on clinical or biological features, although genetic factors are believed to play a major role in the variety of responses to treatment [2]. Many pharmacogenetic studies of the SSRI response have been undertaken, most of which have con-

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Table 1. Clinical characteristics of the patients in both definition groups

	Total patients	Males	Females	Age, years	Baseline SIGH-D	Fluvoxamine dose at 8 weeks mg/day	Previous episodes
Overall	106	49	57	43.2 ± 16.0	20.1 ± 6.5	122.0 ± 40.9	1.37 ± 0.56
Clinical response group[1]							
Responders	59	30	29	45.3 ± 16.6	21.5 ± 6.5	116.8 ± 43.4	1.34 ± 0.50
Nonresponders	47	19	28	41.0 ± 15.1	18.2 ± 5.9	128.9 ± 36.8	1.41 ± 0.59
p value		0.285		0.216	0.008	0.167	0.584
Clinical remission group[2]							
Remission	47	24	23	43.6 ± 14.7	19.2 ± 5.6	107.5 ± 43.2	1.34 ± 0.52
Nonremission	59	25	34	43.0 ± 17.0	20.7 ± 7.0	133.3 ± 35.5	1.39 ± 0.70
p value		0.373		0.694	0.23	0.002	0.698

Values for age, baseline SIGH-D, fluvoxamine dose and previous episodes are expressed as mean ± SD.
[1] Clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score.
[2] Clinical remission was defined as a final SIGH-D score of less than 7.

centrated on mutations in the genes coding for pathways in the serotonergic systems [3–6]. However, no consistent pattern of results has been established [7]. Recently, researchers conducting SSRI pharmacogenetic studies have shifted their emphasis to genes other than those involved in serotonin concentration or receptor function [8, 9].

Vesicle-associated membrane protein 2 (VAMP2), which is located on synaptic vesicle membranes, is a key component of the regulated secretory pathway at nerve terminals and, along with syntaxin 1 and SNAP-25, forms the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex [10–13]. Messenger RNA of *VAMP2* was recently reported to increase in the frontal cortex of rats following chronic antidepressant and electroconvulsive treatment [14]. Therefore, neuron secretory behavior alteration due to *VAMP2* gene expression was hypothesized to play a role in the mechanism of action of antidepressants and possibly to alter the clinical response of treated patients. In the present study, we investigated the effects of *VAMP2* gene polymorphisms on antidepressant responses to fluvoxamine in Japanese patients.

Materials and Methods

Subjects and Treatments

This study included 106 Japanese patients (49 males and 57 females; mean age ± standard deviation, 43.2 ± 16.0) who fulfilled the DSM-IV criteria for the diagnosis of major depressive disorder and whose scores on the 17 items of the Semi-Structured Interview Guide of the Hamilton Depressive Scale (SIGH-D) [15] were 12 or higher, indicative of their being in at least moderate depression [16]. In addition to these patients, 96 healthy volunteers, including unrelated medical staff and medical students, were recruited for linkage disequilibrium (LD) mapping. Patients with other axis I disorders, including schizophrenia, dementia, substance abuse, panic disorder, obsessive-compulsive disorder and generalized anxiety disorder, and those with axis II disorders diagnosed by the DSM-IV criteria were excluded by clinical interviews. All patients were free of psychotropic drugs in the past at least 1 month before beginning the study. After explaining the study to the subjects, written informed consent was obtained from each one of them. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University.

Fluvoxamine was administered two or three times a day for 8 weeks. The initial total dose was 50–100 mg/day and was gradually increased depending on the patient's condition. Patients with insomnia and severe anxiety were prescribed benzodiazepine drugs appropriately, but no other psychotropic drugs were allowed during the study.

Data Collection

Severity of depression symptoms was assessed using the 17 SIGH-D items. Assessments were conducted at baseline and 8 weeks after initiating antidepressant treatment. A single evaluator performed all the ratings for a single patient. A clinical response was defined as a 50% or greater decrease in the baseline SIGH-D score, and clinical remission was defined as a final SIGH-D score of 7 or less. The clinical characteristics of the patients, classified according to these definitions, are shown in table 1.

Single Nucleotide Polymorphism Selection and Genotyping

Genomic DNA was extracted from the peripheral blood of all subjects. For LD mapping, we selected single nucleotide polymorphisms (SNPs; rs8067606, rs1061032, rs2278637) distributed equally throughout the gene based on data from dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/; fig. 1). These SNPs were first genotyped in the 96 control samples to avoid redundant genotyping, and then LD blocks were determined with reasonable criteria based on 95% confidence intervals on the D′ values using Haploview v3.2 software (http://www.broad.mit.edu/mpg/haploview/). Next, haplotype-tagging SNPs (htSNPs) were selected within each LD block to provide 90% haplotype coverage. Finally, we genotyped each selected htSNP in the depressive samples using PCR-RFLP. Detailed protocols for the PCR-RFLP method are available upon request.

Statistical Analysis

Genotyping deviation from Hardy-Weinberg equilibrium was evaluated by a χ^2 test using the Haploview software. Marker-trait allele/genotype-wise association was evaluated using a conventional χ^2 test (SPSS version 11.0J, Tokyo, Japan) and haplotype-wise was evaluated using COCAPHASE software (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/). The COCAPHASE program performs log-likelihood ratio tests under a log-linear model for global p values. To estimate the haplotype frequencies of htSNPs, we used the expectation-maximization algorithm. Global p values were calculated for haplotype-wise analyses, and we also performed exploratory analysis of the possible correlations between response or remission, fluvoxamine treatment, and several clinical factors by logistic regression (SPSS). In these analyses, response classification was set as the dependent variable, and gender, age at the time of recruitment, fluvoxamine dose at 8 weeks, SIGH-D total score at the baseline, and htSNP genotypes were set as the independent variables. The significance level for all statistical tests was $p < 0.05$.

Results

After three SNPs were genotyped to evaluate the LD in the control samples, one LD block was defined and two SNPs (rs1061032 and rs8067606) were selected as the htSNPs for this gene (fig. 1). Genotyping these two htSNPs in all the depressive samples revealed that all the respective genotypic frequencies of these SNPs were in accordance with Hardy-Weinberg equilibrium.

In the allele/genotype-wise association analysis, the frequencies of each htSNP were not significantly different between fluvoxamine responders and nonresponders, or between remitters and nonremitters (table 2). In the haplotype-wise association analysis, we found no significant associations between fluvoxamine responders and nonresponders, or between remitters and nonremitters (table 3).

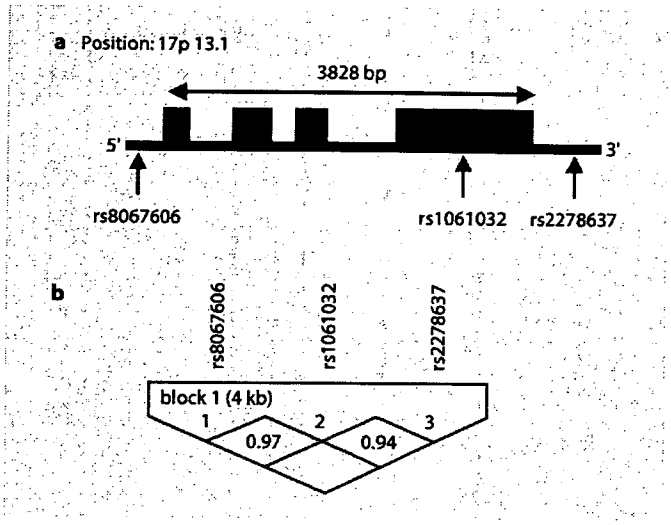

Fig. 1. Genomic structure and pairwise LD of *VAMP2*. **a** Genomic structure of VAMP2 and SNPs used in association analyses and LD mapping. The vertical bars represent exons of VAMP2, and the numbers under the arrows represent SNP IDs. **b** Pairwise LD of VAMP2 in the 96 controls. Each block was defined by a solid spine of the LD using Haploview v3.2. The numbers in the polygons represent D′, and the blank space represents complete LD.

In our exploratory logistic regression analysis, none of the htSNP genotypes correlated with clinical responses or remission (data not shown).

Discussion

In this study, the polymorphisms and estimated haplotypes of *VAMP2* were not associated with response or remission in fluvoxamine-treated Japanese depressive subjects. The exploratory logistic regression analysis revealed that none of the htSNP genotypes could serve as a predictor for clinical response or remission.

VAMP2 forms the SNARE complex at the presynapse, and SNARE proteins are central to the membrane fusion machinery. Membrane fusion is regulated by a Ca^{2+} sensor, and synaptotagmin is believed to function as a Ca^{2+} sensor, not only binding to the membrane in a Ca^{2+}-dependent manner but also interacting with SNARE complexes [17]. As VAMP2 interacts with various proteins that are essential for vesicular transport and/or fusion, other genes interacting with VAMP2 might play important roles in the response to antidepressants. We must therefore consider gene-gene interactions between *VAMP2* and these other genes [18].

Table 2. Genotype and allele distributions of *VAMP2* SNPs in both definition groups

Definition	SNP number	Sample	Genotype			p value	Allele		p value
			W/W	W/M	M/M		W	M	
Clinical response group	rs1061032	responders	18	28	13		64	54	
		nonresponders	17	25	5	0.297	59	35	0.262
	rs8067606	responders	18	27	14		63	55	
		nonresponders	15	25	7	0.434	55	39	0.488
Clinical remission group	rs1061032	remission	13	24	10		50	44	
		nonremission	22	29	8	0.434	73	45	0.395
	rs8067606	remission	13	24	10		50	44	
		nonremission	20	28	11	0.784	68	50	0.578

W = Wild-type allele; M = mutant allele.

Table 3. Haplotype distribution of *VAMP2* in both definition groups

Definition	Sample	Haplotype frequency				Global p value
		A^1–A^2	A–C^3	G^4–A	G–C	
Clinical response group	responders	0.361	0.053	0.011	0.573	
	nonresponders	0.449	0.017	0.008	0.525	
	p value	0.197	0.14	0.87	0.475	0.339
Clinical remission group	remission	0.372	0.051	0.008	0.567	
	nonremission	0.457	0.01	0.01	0.521	
	p value	0.213	0.083	0.868	0.498	0.256

[1]Minor allele of rs8067606.
[2]Minor allele of rs1061032.
[3]Major allele of rs1061032.
[4]Major allele of rs8067606.

Two limitations in this paper deserve mentioning. First, our sample size was not large enough to deny the type II error. If the relative risk is set at 1.5, a total of 150 samples would be needed to obtain over 80% statistical power. Second, we did not examine the patients' plasma concentrations of fluvoxamine. The daily fluvoxamine dosage was higher in nonremitted subjects than in those in remission, though this should be self-evident for a study design incorporating fixed-flexible dosing. However, these effects should be minimal because no correlation between plasma fluvoxamine concentration and clinical response has been reported [19]. The small but significant difference in baseline SIGH-D scores between responders and nonresponders might also have affected the results; however, the baseline SIGH-D scores could not predict clinical response in the exploratory logistic regression analysis.

In this pharmacogenetic study of fluvoxamine, our results suggest that the *VAMP2* gene is not a predictor of antidepressant efficacy in Japanese depressive patients. We must further investigate the role of the *VAMP2* gene in both the mechanisms of action of antidepressants and the pathophysiology of depression.

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References

1 Dalery J, Honig A: Fluvoxamine versus fluoxetine in major depressive episode: a double-blind randomised comparison. Hum Psychopharmacol 2003;18:379–384.

2 Malhotra AK, Murphy GM Jr, Kennedy JL: Pharmacogenetics of psychotropic drug response. Am J Psychiatry 2004;161:780–796.

3 Smeraldi E, Zanardi R, Benedetti F, Di Bella D, Perez J, Catalano M: Polymorphism within the promoter of the serotonin transporter gene and antidepressant efficacy of fluvoxamine. Mol Psychiatry 1998;3:508–511.

4 Yoshida K, Ito K, Sato K, Takahashi H, Kamata M, Higuchi H, Shimizu T, Itoh K, Inoue K, Tezuka T, Suzuki T, Ohkubo T, Sugawara K, Otani K: Influence of the serotonin transporter gene-linked polymorphic region on the antidepressant response to fluvoxamine in Japanese depressed patients. Prog Neuropsychopharmacol Biol Psychiatry 2002;26:383–386.

5 Minov C, Baghai TC, Schule C, Zwanzger P, Schwarz MJ, Zill P, Rupprecht R, Bondy B: Serotonin-2A-receptor and -transporter polymorphisms: lack of association in patients with major depression. Neurosci Lett 2001;303:119–122.

6 Sato K, Yoshida K, Takahashi H, Ito K, Kamata M, Higuchi H, Shimizu T, Itoh K, Inoue K, Tezuka T, Suzuki T, Ohkubo T, Sugawara K, Otani K: Association between -1438G/A promoter polymorphism in the 5-HT(2A) receptor gene and fluvoxamine response in Japanese patients with major depressive disorder. Neuropsychobiology 2002;46:136–140.

7 Serretti A, Artioli P: The pharmacogenomics of selective serotonin reuptake inhibitors. Pharmacogenomics J 2004;4:233–244.

8 Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Putz B, Papiol S, Seaman S, Lucae S, Kohli MA, Nickel T, Kunzel HE, Fuchs B, Majer M, Pfennig A, Kern N, Brunner J, Modell S, Baghai T, Deiml T, Zill P, Bondy B, Rupprecht R, Messer T, Kohnlein O, Dabitz H, Bruckl T, Muller N, Pfister H, Lieb R, Mueller JC, Lohmussaar E, Strom TM, Bettecken T, Meitinger T, Uhr M, Rein T, Holsboer F, Muller-Myhsok B: Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. Nat Genet 2004;36:1319–1325.

9 Zill P, Baghai TC, Zwanzger P, Schule C, Minov C, Riedel M, Neumeier K, Rupprecht R, Bondy B: Evidence for an association between a G-protein beta3-gene variant with depression and response to antidepressant treatment. Neuroreport 2000;11:1893–1897.

10 Trimble WS, Cowan DM, Scheller RH: VAMP-1: a synaptic vesicle-associated integral membrane protein. Proc Natl Acad Sci U S A 1988;85:4538–4542.

11 Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, Wilson MC: The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol 1989;109:3039–3052.

12 Bennett MK, Calakos N, Scheller RH: Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 1992;257:255–259.

13 Weis WI, Scheller RH: Membrane fusion. SNARE the rod, coil the complex. Nature 1998;395:328–329.

14 Yamada M, Takahashi K, Tsunoda M, Nishioka G, Kudo K, Ohata H, Kamijima K, Higuchi T, Momose K: Differential expression of VAMP2/synaptobrevin-2 after antidepressant and electroconvulsive treatment in rat frontal cortex. Pharmacogenomics J 2002;2:377–382.

15 Williams JB: A structured interview guide for the Hamilton Depression Rating Scale. Arch Gen Psychiatry 1988;45:742–747.

16 Peveler R, Kendrick T: Selective serotonin reuptake inhibitors: THREAD trial may show way forward. BMJ 2005;330:420–421.

17 Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Sudhof TC: Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 1994;79:717–727.

18 Motsinger AA, Lee SL, Mellick G, Ritchie MD: GPNN: Power studies and applications of a neural network method for detecting gene-gene interactions in studies of human disease. BMC Bioinformatics 2006;7:39.

19 Kasper S, Dotsch M, Kick H, Vieira A, Moller HJ: Plasma concentrations of fluvoxamine and maprotiline in major depression: implications on therapeutic efficacy and side effects. Eur Neuropsychopharmacol 1993;3:13–21.

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Full Paper

Effects of Methylphenidate on the Hyperemotional Behavior in Olfactory Bulbectomized Mice by Using the Hole-Board Test

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Abstract. The most consistent behavioral changes caused by olfactory bulbectomy are hyperemotional responses such as hyperactivity in a novel environment. However, the changes in the emotional behavior of mice after undergoing olfactory bulbectomy have not yet been described in detail. The effects of methylphenidate on the hyperemotional behavior of olfactory bulbectomized (OBX) mice were examined by using the hole-board test. Mice (4-week-old) were subjected to olfactory bulbectomy, and the behavioral test was performed 2 weeks after surgery. OBX mice showed a significant increase in the number of head-dips as compared to the sham-operated mice. This increase was significantly decreased after treatment with methylphenidate (10 μg/kg, s.c.). The norepinephrine (NE) turnover ratio in the frontal cortex in OBX mice was significantly less than that in the sham-operated mice. However, the decreased NE ratio in OBX mice normalized after treatment with methylphenidate. Our results suggest that the increased head-dipping behavior in OBX mice might reflect an impulsive-like behavior. In addition, we proposed that the improvement in the noradrenergic abnormalities in the frontal cortex due to methylphenidate treatment may play a key role in the improvement of impulsive-like behaviors observed in OBX mice.

Keywords: olfactory bulbectomy, hole-board test, methylphenidate, norepinephrine

Introduction

Removal of the main olfactory bulbs in rats has been shown to alter the neuronal function of the brain areas involved with emotion regulation, resulting in maladaptive behavioral patterns that are similar to the symptoms observed in patients with depression. The most consistent behavioral changes caused by olfactory bulbectomy are hyperemotional responses such as hyperactivity in a novel environment. Although many studies have demonstrated hyperemotional responses in olfactory bulbectomized (OBX) rats (for a review, see ref. 1), changes in the emotional behavior of mice after olfactory bulbectomy have not yet been described in detail.

Attention deficit hyperactivity disorder (ADHD) is a common behavioral disorder in children and is characterized by elevated and age-inappropriate levels of motor activity, impulsiveness, distractibility, and inattention (2). Methylphenidate is one of the most widely prescribed drugs for the treatment of ADHD (3). It was reported that methylphenidate blocks dopamine (DA) and norepinephrine (NE) transporters, thereby enhancing catecholamine neurotransmission (4, 5). However, the etiology of ADHD and/or the detailed mechanisms of methylphenidate are not well understood.

The hole-board test has been recognized as a useful tool for objectively estimating the various emotional states of animals in response to an exposure to an unfamiliar environment (6, 7). Thus, we hypothesized that methylphenidate may be able to reduce olfactory bulbectomy-induced hyperemotional behaviors. In order to test this hypothesis, we investigated the effect of olfactory bulbectomy on the emotional behaviors of

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mice by using an automatic hole-board apparatus. In addition, we examined the effects of methylphenidate on the olfactory bulbectomy-induced hyperemotional behaviors of mice. Furthermore, we examined the changes in the amounts of DA and NE and their metabolites in the frontal cortex of the OBX mice after treatment with methylphenidate.

Materials and Methods

Animals

The experiments were conducted using 4-week-old male ICR mice (Tokyo Laboratory Animals Science, Tokyo) that weighed 18 – 23 g. They had free access to food and water and were housed in an animal room that was maintained at 24 ± 1°C under a 12-h light-dark cycle. This study was carried out in accordance with the Declaration of Helsinki and the guidelines for the use of laboratory animals of Hoshi University, a university accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Olfactory bulbectomy

In the present study, we subjected 4-week-old mice to olfactory bulbectomy, and a behavioral test was performed 2 weeks after the surgery; this was because the hole-board test is generally performed in 6-week-old mice (8). The mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) diluted in 0.9% saline. The part of the skull covering the bulbs was exposed by making a skin incision, and a burr hole was drilled (2.0 mm prior to the bregma, 1 mm lateral to the midline); both the olfactory bulbs were removed by suction through this hole. Sham operations were performed in an identical manner, but the skull and bulbs were left intact. After completing the behavioral experiments, the mice were decapitated, and the results of the bulbectomy were visually inspected. The data obtained from the animals that had undergone incomplete removal of the bulbs or showed frontal cortex damage were discarded. After the olfactory bulbs were lesioned, the sham-operated and OBX mice were immediately housed in individual Plexiglas cages (15 × 10 × 12.5 cm) for 14 days.

The hole-board test

The results of the hole-board test were automatically determined as described previously (7). The hole-board apparatus comprised a gray wooden box (50 × 50 × 50 cm) with four 3-cm-diameter holes that were equally spaced on the floor, and the box was placed in indirect light (40 lux). An infrared beam sensor was installed on the wall to detect the number of rearing and head-dipping behaviors and the latency to the first head-dip. Other behavioral parameters such as the locus and distance of movement [total locomotor activity (cm)] of mice were recorded by an overhead color CCD camera. The heads of the mice were painted yellow, and the color CCD camera followed the center of gravity. Data from the CCD camera were collected by using a custom-designed interface (CAT-10; Muromachi Kikai, Tokyo) in the form of a reflection signal. The head-dipping behaviors were double checked by using an infrared beam sensor and an overhead color CCD camera. All data were analyzed and stored in a personal computer equipped with analytical software (Comp ACT HBS, Muromachi Kikai). After the 14-day postsurgical period, the emotional response was measured using the hole-board test. Drugs were administered 30 min before the test was conducted. For the hole-board experiments, each animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min. The total locomotor activity, number of rearing and head-dipping behaviors, and latency to the first head-dip were recorded automatically. Each mouse was used only once. The floor in the hole-board apparatus was cleaned with a paper towel after each trial. The experiments were performed between 1300 and 1700.

Analysis of the concentrations of monoamines and their metabolites

The concentrations of DA, NE, and their major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3MT), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), and normetanephrine (NM), were determined by high-performance liquid chromatography (HPLC). After the behavioral experiments were completed, the mice were decapitated. After removing their brains, the success of the bulbectomy was evaluated, and the frontal cortices were quickly dissected and placed on an ice-cold glass plate. These brain tissues were stored at −80°C until use. The tissues were homogenized in 300 μl of 0.2 M perchloric acid containing 100 μM EDTA (2 Na) and 100 ng isoproterenol that was used as an internal standard. In order to remove the proteins completely, the homogenates were placed in cold water for 30 min. They were then centrifuged at 14,500 × g for 15 min at 0°C and the supernatants were obtained. The pH values of solutions were adjusted to 3.0 by 1 M sodium acetate, and these solutions were used as the samples. Sample solutions of 20 μl were analyzed by HPLC with electrochemical detection. The electrochemical detector (ECD-300; Eicom Co., Kyoto) was equipped with a graphite electrode (WE-3G, Eicom Co.) that was used at a voltage setting of 750 mV versus an Ag/AgCl reference

electrode. The mobile phase comprised 0.1 M sodium acetate/0.1 M citric acid buffer (pH 3.5) containing 17% methanol, sodium 1-octanesulfonate, and EDTA (2 Na). The monoamines were separated using a C-18 column (150 mm × 3.0 mm reverse phase column, Eicompak SC-5ODS; Eicom Co.). The flow rate in the mobile phase was maintained at 0.5 ml/min at a column temperature of 25°C.

Data analyses

The data were each expressed as a mean ± S.E.M. The statistical significance of differences was assessed by two-way analysis of variance (ANOVA, surgery × drug manipulation). Individual group comparisons were made using Tukey-Kramer's post hoc test. Analyses were made using the statistical software StatView ver. 5.0 (SAS Institute, Inc., Cary, NC, USA). P values of less than 0.05 were considered to be significant.

Drugs

The methylphenidate used in this study was from Sigma Chemical Co., St. Louis, MO, USA; it was dissolved in saline and injected 30 min prior to the hole-board test.

Results

Figure 1 shows the effects of methylphenidate on the exploratory behaviors of the OBX mice 30 min after s.c. administration in the hole-board test. Two-way factorial ANOVA revealed the significant effects of the head-dip counts for surgery × drug interaction [$F(2, 44) = 4.008$, $P<0.05$]. Post hoc analysis showed that OBX mice showed a significant increase in head-dip counts as compared to the sham-operated mice. Furthermore, methylphenidate (1, 10 μg/kg) produced a significant decrease in the head-dip counts in the OBX mice; the count decreased to the same levels as that observed in the sham-operated mice. In addition, two-way factorial ANOVA revealed the main significant effects of the locomotor activity for the surgery, but not drug or surgery × drug interaction [$F(1, 44) = 5.408$, $P<0.05$]. These results revealed that OBX mice showed a significant increase in locomotor activity as compared to the sham-operated mice. On the other hand, there were no significant effects on the head-dip latency and rearing counts between sham-operated and OBX mice.

The effects of olfactory bulbectomy and methylphenidate treatment on the concentrations of DA, NE, and their metabolites in the mouse frontal cortex are listed in Table 1. Two-way factorial ANOVA revealed the main significant effects of the amount of NE for the surgery [$F(1, 41) = 35.972$, $P<0.05$]. On the other

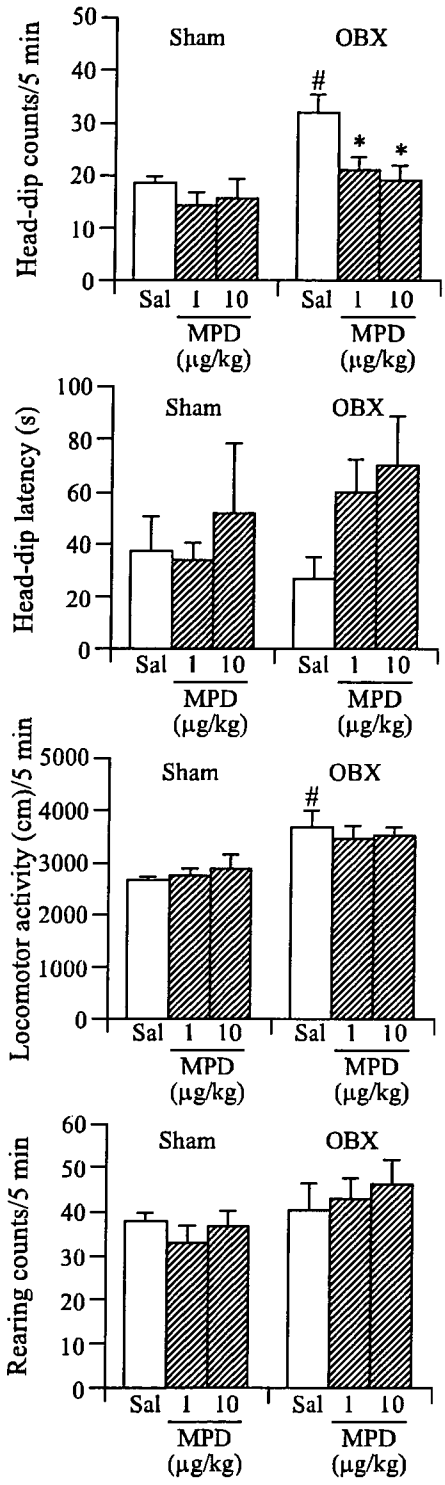

Fig. 1. Effects of methylphenidate on the exploratory behavior observed in the sham-operated and OBX mice during the hole-board test. Methylphenidate (MPD; 1 and 10 μg/kg, respectively) or saline was injected s.c. 30 min prior to the measurement of the exploratory behavior. Each column represents the mean with an S.E.M. of 9 to 11 mice. The statistical significance of the differences between the groups was assessed using two-way analysis of variance (ANOVA) followed by the Tukey-Kramer's post hoc test. $^{\#}P<0.05$ *vs* saline (Sal)-treated sham-operated mice. $^{*}P<0.05$ *vs* saline (Sal)-treated OBX mice.

Table 1. The effects of OBX and treatment with methylphenidate on the concentrations of DA, NE, and their metabolites in mouse frontal cortex

Group	Dose	(ng/mg wet tissue)			NE ratio
		NE	MHPG	NM	<(MHPG + NM) / NE>
Sham	Saline	0.786 ± 0.045	0.396 ± 0.019	0.012 ± 0.002	0.545 ± 0.030
	MPD	0.764 ± 0.027	0.380 ± 0.016	0.012 ± 0.001	0.518 ± 0.019
OBX	Saline	0.576 ± 0.022#	0.230 ± 0.015#	0.014 ± 0.001	0.425 ± 0.025#
	MPD	0.568 ± 0.044#	0.288 ± 0.022	0.015 ± 0.002	0.542 ± 0.028*

Group	Dose	(ng/mg wet tissue)				DA ratio
		DA	DOPAC	HVA	3MT	<(DOPAC + HVA + 3MT) / DA>
Sham	Saline	0.832 ± 0.196	0.082 ± 0.009	0.172 ± 0.013	0.029 ± 0.004	0.430 ± 0.077
	MPD	0.858 ± 0.097	0.090 ± 0.007	0.179 ± 0.007	0.029 ± 0.002	0.394 ± 0.042
OBX	Saline	0.798 ± 0.110	0.061 ± 0.004#	0.186 ± 0.011	0.020 ± 0.002#	0.518 ± 0.167
	MPD	0.731 ± 0.073	0.058 ± 0.005#	0.157 ± 0.012	0.020 ± 0.001#	0.334 ± 0.024

Values are expressed as nanograms per milligram of brain tissue. Each value represents the mean with S.E.M. of 10 – 13 mice. Methylphenidate (MPD, 10 mg/kg) or saline was injected s.c. 30 min prior to the measurement of exploratory behavior. After the behavioral experiments were complete, mice were decapitated. #$P<0.05$ *vs* saline-treated sham-operated mice, *$P<0.05$ *vs* saline-treated OBX mice.

hand, olfactory bulbectomy significantly decreased the amount of MHPG (41.9%), the major metabolite of NE [surgery × drug interaction: $F(1, 41) = 4.203$, $P<0.05$]. Furthermore, the NE ratio [(MHPG + NM) /NE] in the frontal cortex in the OBX mice was also significantly less than that observed in the sham-operated mice [surgery × drug interaction: $F(1, 41) = 6.792$, $P<0.05$]. In addition, treatment with methylphenidate (10 μg/kg, s.c.) significantly reversed the decrease in the NE ratio in the OBX mice. Two-way factorial ANOVA revealed the significant major effects of the surgery, but not drug or surgery × drug interaction, with regard to the DA metabolites DOPAC and 3MT [DOPAC: $F(1, 41) = 19.928$, $P<0.05$; 3MT: $F(1, 41) = 11.861$, $P<0.05$].

Discussion

In the present study, we observed the characteristics of hyperemotionality in mice 2 weeks after olfactory bulbectomy. The number of head-dips and locomotor activity in the OBX mice were significantly greater than those in the sham-operated mice. The most consistent behavioral changes caused by olfactory bulbectomy are hyperemotional responses such as hyperactivity in a novel environment (for a review, see ref. 1). Recently, Zueger et al. (9) reported that OBX mice exhibited hyperactivity in the open field. The hyperactivity of OBX animals is not due to the impairment of olfaction (10). Furthermore, the hyperactivity of OBX rodents is believed to reflect an increased responsiveness to the aversive nature of the task (11) because the benzodiazepines and the selective serotonin reuptake inhibitors normalize the hyperactivation induced by OBX (12). Thus, the present results of the OBX mice were consistent with those in previous reports.

Several animal models of hyperactivity and the effect of methylphenidate on hyperactivity have been described by using such animal models. For example, it has been shown that mice lacking the dopamine transporter gene (13) and rats with neonatal 6-hydroxydopamine-lesions in the DA system in the forebrain (14) were hyperactive as compared to the control animals. In addition, it has been demonstrated that these behavioral changes were reduced by the administration of methylphenidate (13, 14). Furthermore, it was reported that the spontaneously hypertensive rat is also hyperactive and these hyperactive behaviors were reduced by the administration of methylphenidate (15). Indeed, it has been recognized that a good animal model for ADHD should have the key features of hyperactivity and/or motor impulsiveness (16). The present study indicated that OBX mice exhibit increased locomotor activity and head-dip counts as compared with the sham-operated mice. Furthermore, we observed that methylphenidate selectively suppressed the increased head-dipping behavior in the OBX mice; it had no effect on the head-dipping behavior in the sham-operated mice. The head-dipping behavior is a more sensitive marker than locomotion to assess the emotion of animals (8). Therefore, it can be speculated that methylphenidate at this dose range selectively affects the number of head-dipping. Based on these results, we hypothesize that the increased head-dipping behavior observed in the OBX mice might indicate an impulsive-like behavior.

It has been reported that the decrease in the amounts

of tissue NE and/or DA and their metabolites in OBX animal models was most apparent in the frontal cortex (17, 18). Similarly, we observed that the levels of NE and its metabolite MHPG in the frontal cortex were significantly decreased in the OBX mice. The levels of the DA metabolites, namely, DOPAC and 3MT, in the frontal cortex were also decreased in these mice. The neurons of the olfactory bulb widely distribute to the brain including cortical areas and limbic nuclei (1). Therefore, the frontal cortex may be regulated by olfactory neurons with direct and indirect neuronal networks, and OBX might affect the synthesis and/or metabolism rate(s) of catecholamine in the cortical areas. However, the details are still unclear and will be resolved in future investigations. It has been reported that neuropsychological and imaging studies indicate that the prefrontal cortex functions are weaker in patients with ADHD; this substantially contributes to the development of ADHD symptoms (19). Furthermore, it was suggested that impulsiveness and/or distractibility as well as inattention increases due to disturbances in catecholaminergic neurotransmission, with particular emphasis on norepinephrine (20 – 22). Thus, it has been proposed that prefrontal cortical norepinephrine might play a key role in the transmission of impulses. In the present study, we demonstrated that when OBX mice treated with methylphenidate completely decreased the head-dip counts to the levels observed in the sham-operated mice, methylphenidate normalized the decrease in the NE turnover ratio in the frontal cortex of the OBX mice. It has been reported that methylphenidate is a preferred inhibitor of NE/DA transport. However, Andrews and Lavin (23) recently demonstrated that methylphenidate increases the frontocortical excitability in noradrenergic neurons. These findings suggest that the decrease in the levels of NE or its metabolites in the frontal cortex, rather than in the levels of DA or its metabolites, may be important for the induction of the head-dipping behavior in OBX mice. Although the details are still unclear, it can be speculated that the OBX sensitizes the effect of methylphenidate due to the noradrenaline deficiency in the frontal cortex and leads to the specific increase in noradrenaline metabolism in the synaptic cleft. Our results indicate that the hyperactivity in OBX mice is unlikely to depend on the DA system in the frontal cortex. However, we can not exclude the possibility that the dopaminergic dysfunction in the other regions such as nucleus accumbens and/or striatum is associated with the impulsive-like behavior of OBX mice. Further studies are needed to resolve this issue completely.

In conclusion, our results suggest that the increase in the head-dipping behavior in OBX mice might indicate an impulsive-like behavior. In addition, we proposed that the improvement of the noradrenergic abnormalities in the frontal cortex due to treatment with methylphenidate may play a key role in the improvement of the impulsive-like behavior observed in the OBX mice. It has been noted that impulsive behaviors are observed in psychiatric disorders such as the bipolar disorder, ADHD, and personality disorders. Thus, it is possible that the increased head-dipping behavior observed in OBX mice can be used as a useful index for the impulsive-like behavior in psychiatric disorders, particularly in the case of ADHD.

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References

1 Song C, Leonard BE. The olfactory bulbectomised rat as a model of depression. Neurosci Biobehav Rev. 2005;29:627–647.
2 Biederman J. Attention-deficit/hyperactivity disorder: a selective overview. Biol Psychiatry. 2005;57:1215–1220.
3 Barkley RA. A review of stimulant drug research with hyperactive children. J Child Psychol Psychiatry. 1977;18:137–165.
4 Gatley SJ, Pan D, Chen R, Chaturvedi G, Ding YS. Affinities of methylphenidate derivatives for dopamine, norepinephrine and serotonin transporters. Life Sci. 1996;58:231–239.
5 Kuczenski R, Segal DS. Effects of methylphenidate on extracellular dopamine, serotonin, and norepinephrine: comparison with amphetamine. J Neurochem. 1997;68:2032–2037.
6 Kamei J, Matsunawa Y, Miyata S, Tanaka S, Saitoh A. Effects of nociceptin on the exploratory behavior of mice in the hole-board test. Eur J Pharmacol. 2004;489:77–87.
7 Saitoh A, Yamada M, Yamada M, Kobayashi S, Hirose N, Honda K, et al. ROCK inhibition produces anxiety-related behaviors in mice. Psychopharmacology. 2006;188:1–11.
8 Takeda H, Tsuji M, Matsumiya T. Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. Eur J Pharmacol. 1998;350:21–29.
9 Zueger M, Urani A, Chourbaji S, Zacher C, Roche M, Harkin A, et al. Olfactory bulbectomy in mice induces alterations in exploratory behavior. Neurosci Lett. 2005;374:142–146.
10 Mar A, Spreekmeester E, Rochford J. Antidepressants preferentially enhance habituation to novelty in the olfactory bulbectomized rat. Psychopharmacology. 2000;150:52–60.
11 Kelly JP, Wrynn AS, Leonard BE. The olfactory bulbectomized rat as a model of depression: an update. Pharmacol Ther. 1997;74:299–316.
12 Mar A, Spreekmeester E, Rochford J. Fluoxetine-induced increases in open-field habituation in the olfactory bulbectomized rat depend on test aversiveness but not on anxiety. Pharmacol Biochem Behav. 2002;73:703–712.
13 Gainetdinov RR, Jones SR, Caron MG. Functional hyperdopaminergia in dopamine transporter knock-out mice. Biol Psychiatry. 1999;46:303–311.

14 Davids E, Zhang K, Tarazi FI, Baldessarini RJ. Stereoselective effects of methylphenidate on motor hyperactivity in juvenile rats induced by neonatal 6-hydroxydopamine lesioning. Psychopharmacology. 2002;160:92–98.
15 Sagvolden T. Behavioral validation of the spontaneously hypertensive rat (SHR) as an animal model of attention-deficit/hyperactivity disorder (AD/HD). Neurosci Biobehav Rev. 2000;24:31–39.
16 Solanto MV. Clinical psychopharmacology of AD/HD: implications for animal models. Neurosci Biobehav Rev. 2000;24:27–30.
17 Jancsar SM, Leonard BE. Changes in neurotransmitter metabolism following olfactory bulbectomy in the rat. Prog Neuropsychopharmacol Biol Psychiatry. 1984;8:263–269.
18 Redmond AM, Kelly JP, Leonard BE. Behavioural and neurochemical effects of dizocilpine in the olfactory bulbectomized rat model of depression. Pharmacol Biochem Behav. 1997;58:355–359.
19 Arnsten AF, Li BM. Neurobiology of executive functions: catecholamine influence on prefrontal cortical functions. Biol Psychiatry. 2005;57:1377–1384.
20 Pliszka SR, McCracken JT, Maas JW. Catecholamines in attention-deficit hyperactivity disorder: current perspectives. J Am Acad Child Adolesc Psychiatry. 1996;35:264–272.
21 Arnsten AF, Steere JC, Hunt RD. The contribution of alpha 2-noradrenergic mechanisms of prefrontal cortical cognitive function. Potential significance for attention-deficit hyperactivity disorder. Arch Gen Psychiatry. 1996;53:448–455.
22 Biederman J, Spencer T. Attention-deficit/hyperactivity disorder (ADHD) as a noradrenergic disorder. Biol Psychiatry. 1999;46:1234–1242.
23 Andrews GD, Lavin A. Methylphenidate increases cortical excitability via activation of alpha-2 noradrenergic receptors. Neuropsychopharmacology. 2006;31:594–601.

Article

Identification of Functional Polymorphisms in the Promoter Region of the Human PICK1 Gene and Their Association With Methamphetamine Psychosis

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Objective: Protein interacting with C-kinase-1 (PICK1) plays a role in the targeting and clustering of dopamine transporter, which is the primary target site for the abused drug methamphetamine. Based on the interaction of PICK1 with dopamine transporter, it is of particular interest to investigate the association between the PICK1 gene and methamphetamine abusers.

Method: The authors studied the association between PICK1 gene polymorphisms and methamphetamine abusers in a Japanese group. Two hundred and eight methamphetamine abusers and 218 healthy comparison subjects were enrolled in the study. Furthermore, the authors also examined the effects of single nucleotide polymorphisms (SNPs) in the promoter and 5′-untranslated region on transcription levels of PICK1.

Results: The authors identified four highly frequent SNPs, rs737622 (–332 C/G) and rs3026682 (–205 G/A) in the promoter region and rs713729 (T/A) in intron3 and rs2076369 (T/G) in intron4. Of these SNPs, rs713729 was significantly associated with methamphetamine abusers in general, and rs713729 and rs2076369 were significantly associated with those with spontaneous relapse of psychosis. Furthermore, haplotype analysis revealed that specific haplotypes of these SNPs were associated with methamphetamine abusers. A gene reporter assay revealed that the two SNPs in the promoter region significantly altered transcriptional activity.

Conclusions: Our findings suggest that the PICK1 gene may be implicated in the susceptibility to spontaneous relapse of methamphetamine psychosis and that, as an intracellular adapter protein, PICK1 may play a role in the pathophysiology of methamphetamine psychosis.

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Methamphetamine is one of the most widely used illicit drugs, and its abuse continues to be a growing problem worldwide. Accumulating evidence has suggested that genetic factors play a role in vulnerability to methamphetamine abuse and the psychiatric symptoms related to methamphetamine abuse (1–5). The principal target for the action of methamphetamine is the dopamine transporter, which removes dopamine from the extracellular space at the synapse and thereby controls dopamine signals (6, 7). Both the activity and the surface availability of the dopamine transporter are believed to be tightly regulated by different cellular mechanisms, the best characterized being modulation by protein kinase C activation (8, 9). Recent positron emission tomography (PET) studies of methamphetamine abusers have demonstrated that the density of dopamine transporter is significantly low in the caudate/putamen of methamphetamine abusers (10, 11), suggesting that the long-term use of methamphetamine leads to damage of dopaminergic neurons in the human brain. Of interest, the variable number of tandem repeats polymorphism of the human dopamine transporter gene has been shown to be a risk factor for a prognosis of prolonged-type methamphetamine psychosis (12).

A protein interacting with C kinase (PICK1), one of the PSD95/disk-large/ZO-1 (PDZ) domain-containing synaptic proteins, was originally identified by a yeast two-hybrid system on the basis of its interaction with protein ki-

This article is featured in this month's AJP **Audio** and is discussed in an editorial by Dr. McMahon on p. 999.

TABLE 1. Demographic and Clinical Characteristics of Comparison Subjects and Methamphetamine Abusers

Variable	Comparison Subjects			Methamphetamine Abusers			p
	N			N			
Sex (men/women)	175/43			169/39			0.81[a]
Prognosis of psychosis				178			
Transition type				100			
Prolonged type				78			
Spontaneous relapse							
Positive				77			
Negative				118			
Polysubstance abuse							
No				55			
Yes				140			
	Mean	SD	Range	Mean	SD	Range	p
Age (years)	39.0	12.3	19–73	36.9	11.3	18–69	0.29[b]

[a] Chi-square test.
[b] t test.

nase C alpha (13, 14). PICK1 plays a role in the targeting and, when serving as a scaffold, in the localization of synaptic membrane proteins such as the dopamine transporter (15). PICK1 interacts with dopamine transporter through the PDZ domain of PICK1 and the last three residues of the carboxyl terminal of dopamine transporter (16). Thus, it is likely that the interaction of PICK1 with dopamine transporter results in a clustering of dopamine transporter on the cell surface and a subsequent enhancement of dopamine transporter uptake activity due to an increase in plasma membrane dopamine transporter density in mammalian cells and dopamine neurons in culture.

The PICK1 gene has been mapped to chromosome 22q13.1, a region thought to contain a gene for schizophrenia (17). It is well known that methamphetamine psychosis is similar to the psychosis associated with schizophrenia (18). In a case-control study, Hong et al. (19) reported that the PICK1 gene was associated with schizophrenia in the Taiwanese population. Furthermore, in a case-control association study with well-characterized Japanese subjects, Fujii et al. (20) reported an association of the PICK1 gene with schizophrenia, which is more prominent in people with the disorganized type of schizophrenia. Taken together, these findings point to the possibility of an association between the PICK1 gene and methamphetamine psychosis.

The present study was undertaken to examine the association between PICK1 gene polymorphisms and methamphetamine abuse. Using a gene reporter assay, we also investigated the effects of the single nucleotide polymorphisms (SNPs) in the promoter and 5′-untranslated regions on the levels of PICK1 transcription.

Materials and Methods

Subjects

The subjects were 208 patients (169 men and 39 women, ages: mean=36.9 years, SD=11.3, age range=18–69) with methamphetamine dependence and a psychotic disorder meeting the ICD-10-DCR criteria (F15.2 and F15.5) who were outpatients or inpatients of psychiatric hospitals affiliated with the Japanese Genetics Initiative for Drug Abuse and 218 age-, gender-, and geographical origin-matched normal comparison subjects (175 men and 43 women, age: mean=39.0 years, SD=12.3, age range=19–73) with no past history and no family history of drug dependence or psychotic disorders (Table 1). The age of the normal subjects did not differ from that of the methamphetamine abusers (Table 1). The research was performed after approval was obtained from the ethics committees of each institute of the Japanese Genetics Initiative for Drug Abuse, and all subjects provided written informed consent for the use of their DNA samples as part of this study.

Background of Methamphetamine Abusers

Diagnoses were made by two trained psychiatrists based on interviews and available information, including hospital records. Subjects were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder, or an organic mental syndrome. All subjects were Japanese and were born and living in restricted areas of Japan, including northern Kyushu, Setouchi, Chukyo, Tokai, and Kanto. The patients were divided into subgroups by characteristic clinical features (Table 1).

Prognosis of Psychosis

The prognosis of methamphetamine psychosis varied among patients, some of whom showed continued psychotic symptoms, even after methamphetamine discontinuance, as previously reported (21, 22). Accordingly, the patients were categorized by prognosis into two groups, a transient type and a prolonged type, based on the duration of the psychotic state after methamphetamine discontinuance. The transient type is defined as those whose symptoms improved within 1 month, and the prolonged type is those whose psychosis continued for more than 1 month after methamphetamine discontinuance and the start of treatment *with neuroleptics*. In this study, there were 100 transient type and 78 prolonged type patients with methamphetamine psychosis (Table 1). One of the issues in categorizing was the difficulty in distinguishing patients who coincidently developed schizophrenia. Therefore, we excluded cases in which the predominant symptoms were of the negative and/or disorganized type in order to maintain the homogeneity of the subgroup.

Spontaneous Relapse

It has been well documented that once methamphetamine psychosis has developed, patients in a state of remission are susceptible to spontaneous relapse without reconsumption of methamphetamine (21, 22). It has thus been postulated that a sensitization phenomenon induced by the repeated consumption of methamphetamine develops in the brain of patients

TABLE 2. Polymerase Chain Reaction Primers Used to Search for Single Nucleotide Polymorphisms (SNPs) in 5′ Upstream Region and Exons of the PICK1 Gene and for Genotyping of SNP1-6

Region	Primer Sequences Forward (5′-3′)	Reverse (5′-3′)	Product size (bp)
5′-upstream-1	CACAATGTGGCTGGCAAGA	CCCCCCCTCCTTCCTTAGT	498
5′-upstream-2	CTCTGGGGAGCACTGATAGC	AGACACATGCCCTTTCACC	478
5′-upstream-3	GGGCCATTCTAGTAGGGGAGT	CAATCCCTGCAGACAATCCT	368
5′-upstream-4	GGGAAGGGAAGGATTATTGTCTGC	CAAGTGCCTAAATGCCAACGCC	395
Exon 2	GAGGGGTGGCGTTGGCATTTA	CACTGCTCCATCTGCTTTGCT	441
Exon 3	CAGTGGAGCCCCTCAGGAGTTTTAG	CAGGTGGTCAGAAAGCCCCTCTG	341
Exon 4	GAGCAGAGGGTAGAGTGGAAGAGG	ACAAGGAAGGGGGCGGTGAG	358
Exon 5	AGGAGTCTCAGTCCAGAACAGTCTTG	TTGGTCAGAGGTCAGAGCCCAC	301
Exon 6	CTCCCTGTGCATGGAGGTAAGG	TGGTGACTTCTCAGTTCCACGG	317
Exon 7	TGACCTCCCCTCTTCTTTGA	ATTTTGTAGGCTGGCATTCC	189
Exon 8	GGTTGGGTCGGACTGAGCTTTTAC	AGCTTTGGGGGATGCCATTACC	256
Exon 9	GCTTCTCCCCAACAAACCCCTG	CTCCAGCATACGACCTTCTCTGC	295
Exon 10	AGTCCACCAACAAGGGTGACGC	AGCATGGCTGACTGAAGTGGGG	263
Exon 11	GCCAGCCTCTCCTGCTGCGT	CCAGGAACGAGAGTCCAGCC	204
Exon 12	AGGTCTCAGGAATGAAGAACAGCC	TTTCCCACCTCTGAAATGGAGAG	288
Exon 13-1	GAGAGTCTCCTCCCTGAGGC	CTCCTTCCTAAGGCAGGTCC	729
Exon 13-2	AGAGGGAGAGCTTGGTCTCTGGACC	AAGGAGGGTCTGAAGCCACTGCGAC	358
SNP[a]	Primer or probe sequences forward primer (5′-3′) or probe 1 (5′-3′)	Reverse primer (5′-3′) or probe 2 (5′-3′)	Product size (bp)
SNP1 (rs737622)	TCCGGACTCAATTAGCCACCTA; probe 1: VIC-CATATC-CCACGGCCGGT-MGB	GCCATGGAAGAAAGATACAGAAGGA; probe 2: FAM-CATATCCCACCGCCGGT-MGB	98
SNP2 (rs3026682)	CTGCCGGATGAGGTGGAT; probe 1: VIC-CTGGCTGTG-GCTCT-MGB	GCTGCCACTGCTATTGTGTAAAG; probe 2: FAM-CCTGGCTATGGCTCT-MGB	86
SNP3 (rs11089858)	GGCTCAGGGATGCTTTCGTT; probe 1: VIC-CGCGGGC-CCCTGA-MGB	GGGTTTGTCCCAGCTTCCT; probe 2: FAM-CGCG-GACCCCTGA-MGB	83
SNP4 (rs713729)	CCAGTACT GTCCCTGCCTCT	TAAGTGCCGAGAAGGAAAAA	235
SNP5 (rs3952)	GGTCTTGCTTCTGCTCACAGT; probe 1: VIC-CCTCCT-TCATGAGCC-MGB	GGTCACAGGAGGCCGAAT; probe 2: FAM-CCTCCT-TCGTGAGCC-MGB	58
SNP6 (rs2076369)	CCAAATTGTTGGGATTACAGGT	GCTCTGACCAGCTTACCAATGT	220

[a] TaqMan 5′-exonuclease allelic discrimination assay was used for the genotyping of SNP1-3 and 5, and direct sequencing was used for the genotyping of SNP4 and 6.

with methamphetamine psychosis, which provides a neural basis for an enhanced susceptibility to relapse. Therefore, the patients in this study were divided into two groups according to the presence or absence of spontaneous relapse. In this study, 77 patients underwent a spontaneous relapse, and 118 did not (Table 1).

Polysubstance Abuse

The patients were divided according to polysubstance abuse status; 55 patients had abused only the drug methamphetamine in their lifetime, and 140 patients had abused both methamphetamine and other drugs in the present or past. After methamphetamine abuse, organic solvents and marijuana were the most frequently used substances. Cocaine and heroin were rarely abused in this group of subjects.

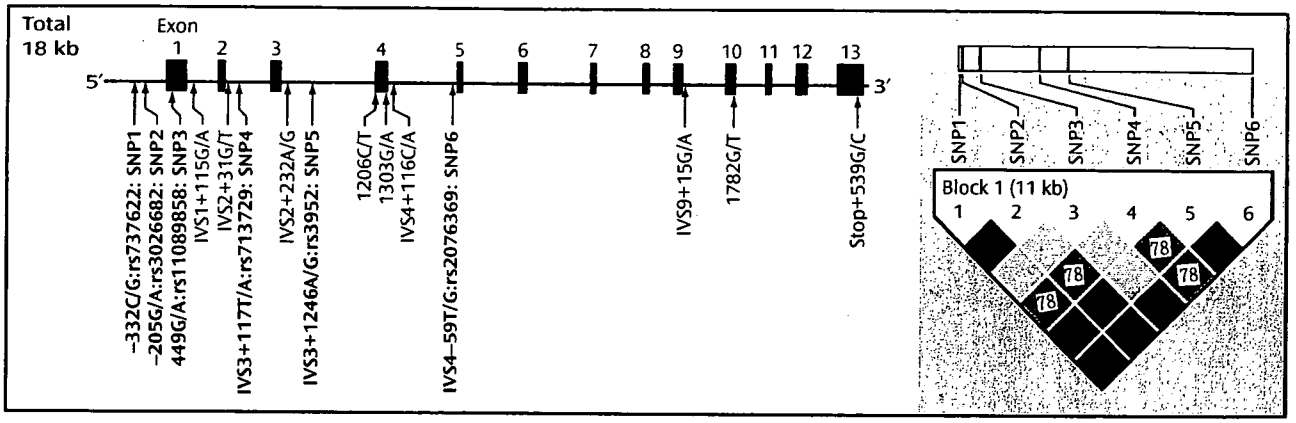
Identification of SNPs

The association between the SNPs of the PICK1 gene and schizophrenia has been reported by two groups. Hong et al. (19) reported a case-control study of the PICK1 gene polymorphism (rs3952) and schizophrenia patients in a Chinese sample. In a Japanese sample, Fujii et al. (20) demonstrated an association between two SNPs (rs713729 and rs2076369) of the PICK1 gene and schizophrenia. However, it remained unclear whether highly common SNPs exist in the 5′-upstream region and the exons of the PICK1 gene in the Japanese population. Therefore, we searched for SNPs in the 5′-upstream region and in all 13 exons with the flanking intronic region of the PICK1 gene using a direct sequencing method. We designed a total of 34 primers for polymerase chain reactions (Table 2) based on information about the PICK1 gene obtained from a public database (the PICK1 gene sequence was assigned as a portion of AL031587, May 18, 2005, i.e., as protein kinase C alpha binding protein; http://www.ncbi.nlm.nih.gov/). Amplification was carried out with an initial denaturation at 95°C for 1 minute, followed by 40 cycles at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 40 seconds, with a final extension at 72°C for 5 minutes. The sequencing reaction was performed on an ABI 310 genetic analyzer (PE Biosystems, Foster City, Calif.) following the manufacturer's protocol.

For the screening of the 5′-upstream region, pairs of polymerase chain reaction primers were designed to amplify 368–498-bp fragments in approximately 1000 bp of the 5′-upstream region (Table 2). To determine the transcription start position, we used a large-insert cDNA library made from human fetal brain (Clontech Laboratories, Inc., Mountain View, Calif.). Based on SMART technology (Clontech), the cDNA library contains high-fidelity full-length transcripts. We performed polymerase chain reactions with 5′-sequencing primer supplied by the manufacturer and the 5′-3R primer we designed in our laboratory (Table 2). By using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.), the polymerase chain reaction product was cloned into TA plasmids according to the manufacturer's instructions. Then the inserted 5′-upstream region was direct-sequenced with sequencing primers provided with the TA cloning kit.

For all polymerase chain reaction products, we first analyzed the sequences of the 32 comparison subjects, and we identified three SNPs in the 5′-upstream region and 11 SNPs in the exons and their flanking intronic regions (Figure 1). Of these 14 SNPs, minor allele frequencies of two SNPs in the 5′-upstream region and two SNPs in introns 3 and 4 were more than 10%. By referring to the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/), we confirmed that two of these SNPs in the 5′-upstream region were rs737622 (SNP1) and rs3026682 (SNP2) (Figure 1). Although none of the SNPs was described as highly frequent in all exons observed, we found that rs713729 (SNP4) in intron 3 and rs2076369 (SNP6) in intron 4 were highly frequent; these re-

FIGURE 1. Genomic Structure and Location of Polymorphic Sites of the PICK1 Gene[a]



[a] The rectangles and horizontal lines represent exons and introns, respectively. Of these single nucleotide polymorphisms (SNPs), six (SNPs 1–6, indicated in boldface) were highly frequent. The haplotype block structure with linkage disequilibrium parameters D′ is shown in the right hand panel. The D′ values were calculated from comparison groups.

sults are in good agreement with those of a previous study (20) (Figure 1).

Genotyping of Identified SNPs

To investigate the putative association between PICK1 gene polymorphisms and methamphetamine abuse, we selected the following SNPs for genotyping: rs737622 (C/G: SNP1), rs3026682 (G/A: SNP2), rs110898858 (G/A: SNP3), rs713729 (T/A: SNP4), and rs2076369 (T/G: SNP6). To compare the present results with those of previous reports (19, 20), we also selected rs3952 (A/G: SNP5) for genotyping. For four of these SNPs, i.e., SNP1, 2, 3, and 4, genotyping was performed by TaqMan 5′-exonuclease allelic discrimination assay in accordance with the manufacturer's protocol. The primers and probes used for these SNPs are shown in Table 2.

For SNP4 (rs713729) and SNP6 (rs2076369), genotyping was performed by direct sequencing, and the primers used for polymerase chain reactions are shown in Table 2.

Dual-Luciferase Gene Reporter Assays

Reporter plasmids containing the rs737622 (-332C/G: SNP1), rs3026682 (-205G/A: SNP2), and rs11089858 (449G/A: SNP3) polymorphic sites were constructed, and 1039-bp fragments (from −373 to +666, Figure 2) were amplified from the genomic DNAs with the identified genotypes as templates. The polymerase chain reaction primers were as follows: forward, 5′-CGACGCGTC-CGGACTCAATTAGCCACCT-3′ (including a MluI site) and reverse, 5′-CGCTCGAGTCGGAACCAAGAACGAGAAC-3′ (including an XhoI site). The polymerase chain reaction products of four haplotypes (C-332/G-205/G+449: Pr1, C-332/G-205/A+449: Pr2, G-332/A-205/A+449: Pr3, and G-332/A-205/A+449: Pr4) were cloned into the pGL-3 Basic Plasmid (Promega Corporation, Madison, Wis.). The inserted sequences were confirmed with direct sequencing by using an ABI 310 genetic analyzer (PE Biosystems, Foster City, Calif.) according to the manufacturer's protocol.

Two cell lines, human neuroblastoma SK-N-SH and human glioblastoma U-87, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Luciferase reporter plasmids containing the four haplotypes were transiently transfected into these cells by using the TransFast lipofection reagent (Promega Corporation, Madison, Wis.). The renilla luciferase expression plasmid phRL-TK was cotransfected as an internal standard. After 48 hours, the cells were harvested, and the luciferase reporter activity was measured by using a TD-20/20 luminometer and a Dual-Luciferase Assay Kit (Promega Corporation, Madison, Wis.). All experiments were repeated at least three times.

Statistical Analysis

Allele and genotype frequencies were calculated, and the differences between groups were evaluated with Fisher's exact test. Case-control haplotype analysis was performed by the maximum-likelihood method by using SNPAlyse (DYNACOM, Yokohama, Japan, http://www.dynacom.co.jp/); p values of haplotypes were obtained by 1000-fold permutation to correct for bias due to multiple tests. For the luciferase assay, one-way analysis of variance (ANOVA) followed by post hoc Bonferroni tests were performed for comparison of relative luciferase activity among four types of inserted vectors. The analysis was performed with SPSS software (SPSS version 12.0J, Tokyo). All statistically significant p values were set at <0.05.

Results

Identification of SNPs and Association Studies

In searching the transcription start position, we found that exon 1 turned out to stretch beyond the position reported in the public database (Figure 2). Namely, we found that the transcription start position was at 113958, which is 513 bp before the start position (114471) reported in AL031587 (http://www.ncbi.nlm.nih.gov/).

We searched for the SNPs in the PICK1 gene, including the promoter region approximately 500 bp ahead of the transcription start position, the entire 5′-untranslated sequence from the translation start position in exon 2, and all 13 exons and their neighboring sequences. In this study, we found 14 SNPs in the PICK1 gene (Figure 1). Of these SNPs, rs737662 (-332C/G: SNP1), rs3026682 (-205G/A: SNP2), rs11089858 (449 G/A: SNP3), rs713729 (IVS3+117T/A: SNP4), and rs2076369 (IVS4-59T/G: SNP6) were found to be highly frequent (the minor allele >10%) (Figure 1). Subsequent genotyping was performed for these five SNPs (SNP1, 2, 3, 4, and 6) and rs3952 (IVS3+1246A/G: SNP5). Both the genotype and the allele