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

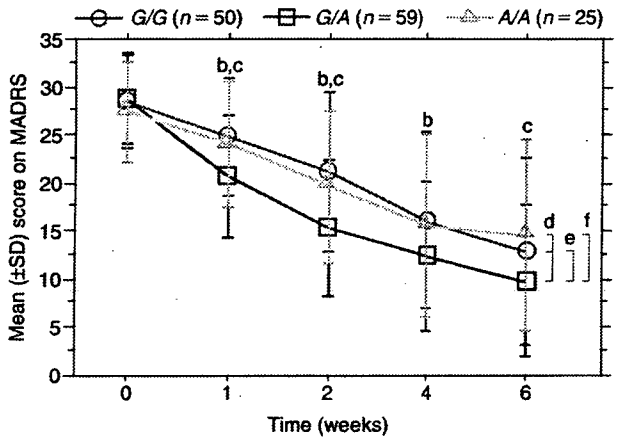

Figure 1 MADRS scores during 6 weeks of milnacipran/fluvoxamine treatment in three BDNF G196A genotype groups[a]

[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 ($F = 3.99$, $df = 4$, $p = 0.0034$).

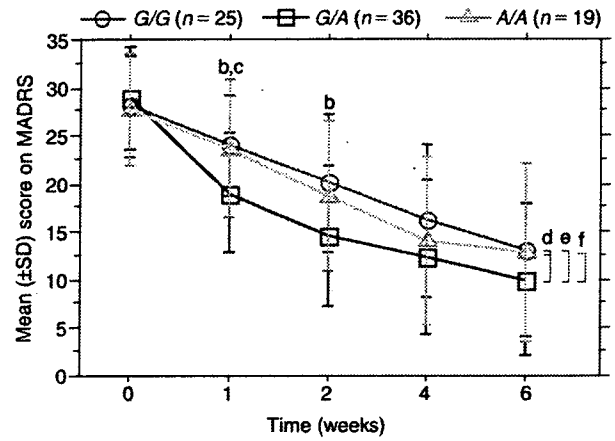

Figure 2 MADRS scores during 6 weeks of milnacipran treatment in three BDNF G196A genotype groups[a]

[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 ($\chi^2 = 2.53$,

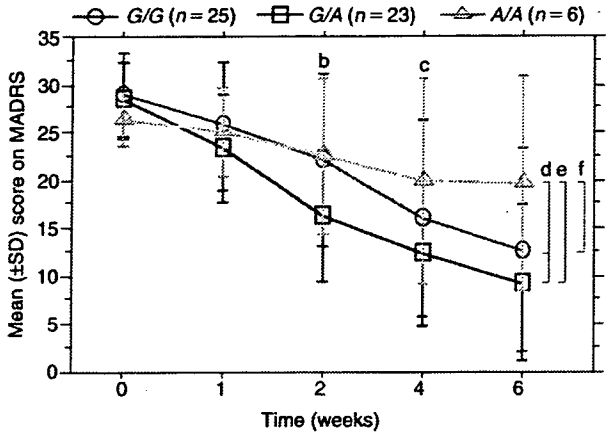

Figure 3 MADRS scores during 6 weeks of fluvoxamine treatment in three BDNF G196A genotype groups[a]

[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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Association Study between *Apolipoprotein L* and Schizophrenia by Exhaustive and Rule-Based Combination Analysis for Identification of Multilocus Interactions

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Several single marker association and haplotypic analyses have been performed to identify susceptible genes for various common diseases, but these approaches using candidate genes did not provide accurate and consistent evidence in each analysis. This inconsistency is partly due to the fact that the common diseases are caused by complex interactions among various genetic factors. Therefore, in this study, to evaluate exhaustive genotype or allele combinations, we applied the binomial and random permutation test (BRP) proposed by Tomita *et al.* [IPSJ Digital Courier, 2, 691–709 (2006)] for the association analysis between an *Apolipoprotein L* gene cluster and schizophrenia. Using the seven selected representative single nucleotide polymorphisms (SNPs) based on the results of linkage disequilibrium evaluation, we analyzed 845 schizophrenic patients and 707 healthy controls, and investigated the validation of risk and protective factors with two randomly divided data sets. A comparative study of a method for analyzing the interactions was performed by conventional methods. Even if all the tested methods were used for analysis, the risk factor with a high significance was not commonly selected from both independent data sets. However, the significant interactions for the protective factor against disease development were commonly obtained from both data sets by BRP analysis. In conclusion, although it is considered that the causality of schizophrenia is too complex to identify a susceptible interaction using a small sample size, it was suggested that the healthy controls tend to have the same combination of certain alleles or genotypes for protection from disease development when BRP as a new exhaustive combination analytical method was used.

[**Key words:** complex genetic diseases, gene–gene interaction, single nucleotide polymorphism, binomial and random permutation test]

The HapMap project has provided valuable information on linkage disequilibrium (LD) in a particular population for elucidating genetic risk factors for common disorders (1). To date, the identification of genetic risk factors for common disorders has been successful only to a certain extent; for example, *APOE* for Alzheimer's disease (2), *NOD2* and 5q31 for inflammatory bowel disease (3), and *NRG1* for schizophrenia (4). However, these candidate genes do not provide accurate and consistent evidence in each case (*i.e.*, for review of *NRG1* [5]). Since these disorders are considered to be complex and caused by complex interactions between various genetic factors, the single marker association and haplotypic analyses cannot clarify the possibility of gene–gene interactions. Therefore, we should consider gene–gene interactions. Such interactions alter or increase the risk of complex genetic diseases in addition to the independent effects of the genes involved in such diseases, because such interactions can modify transcription or translation levels either directly or protein products indirectly (6).

Although a multidimensional approach requires the development of statistical methods that would enable us to handle multiple variable loci in different combinations, it is difficult to detect the interactions of candidate genes by traditional parametric statistical methods and case-control studies. This matter arises because it is difficult to predict complex relationships in analytical space with very few or no data points and to establish a highly dimensional prediction model from a small sample size.

To date, several analytical approaches have been proposed for gene–gene interactions (6), including logistic re-

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gression (LR) (7–9), multifactor dimensionality reduction (MDR) (10–12), artificial neural network (ANN) (13–16), S-sum statistic (17–19), and classification and regression tree (CART) (20–22). The methods that enable the statistical evaluation of one rule comprising a combination of certain alleles and genotypes with respect to both risk and protective factors (23) have been scarcely proposed to detect an interaction between genes and predict the development of a complex disease. The most important cause and effect relationship among the combinations seems to be considered as the marked rule in which the existing ratio between a case and a control is mostly biased among all rules.

In this study, we introduced a new alternative approach based on one rule and an exhaustive combination analysis to extract causal interactions with minimum errors; the approach, which is the binomial and random permutation test (BRP) (24) method, enables the automatic estimation of dominant or recessive models with respect to any exhaustive combinations and the selection of any risk and protective factor candidates composed of genotype or allele combinations. Model estimation was performed using a binomial test (25). In our method, the random permutation test (26–28) was additionally included to adjust multiple testing problems. Using the proposed method, we considered that the gene–gene interactions in the *Apolipoprotein L* (*APOL*) gene cluster are genetic susceptibility factors for schizophrenia in a Japanese population.

MATERIALS AND METHODS

Subjects, psychiatric assessment, and SNP data In this study, 96 healthy controls were recruited for the evaluation of linkage disequilibrium (LD). All subjects were unrelated to each other and were ethnically Japanese. In this study, the subjects with schizophrenia have been referred to as case subjects and the healthy controls were referred to as control subjects.

The psychiatric assessment of each subject was performed, as described in our previous paper (29). After describing the study, a written informed consent was obtained from each subject. This study was approved by the Ethnics Committee at Nagoya University and Fujita Health University. Each SNP was detected using the established method based on PCR-RFLP and TaqMan assay. None of the subjects had any missing SNP data.

The SNPs used in the evaluation of LD are listed in Table 1. LD refers to the fact that particular alleles at nearby sites can co-occur on the same haplotype more often than is expected by chance. The entire chromosome can be partitioned into high-LD regions interspersed by low-LD regions. The high-LD regions are usually called haplotype blocks and the low-LD regions are referred to as recom-

TABLE 1. Single nucleotide polymorphisms (SNPs) in linkage disequilibrium (LD) mapping and association analysis of all data and first- and second-set samples

All data SNP[a]	Genotypic association[c]										Allelic association[c]			
	MAF[b]	N		M/M		M/m		m/m		*P*-value[d]	m		*P*-value[d]
	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON		SCZ	CON	
*SNPVI-1	0.26	845	707	499	448	302	226	44	33	0.222	0.23	0.21	0.104
SNPVI-2	0.17												
SNPV-I	0.29												
*SNPV-2	0.46	845	707	271	249	400	335	174	123	0.203	0.44	0.41	0.0754
SNPIII-1	0.3												
*SNPIII-2	0.32	845	707	346	314	398	300	101	93	0.183	0.36	0.34	0.510
*SNPIV-1	0.13	845	707	619	519	210	169	16	19	0.544	0.14	0.15	0.801
*SNPIV-2	0.14	845	707	614	525	214	163	17	19	0.426	0.15	0.14	0.717
SNPIV-3	0.076												
SNPII-1	0.087												
*SNPI-1	0.13	845	707	585	484	236	207	24	16	0.678	0.17	0.17	0.942
*SNPI-2	0.42	845	707	307	260	406	330	132	117	0.827	0.40	0.40	0.891
First-set samples													
SNPVI-1		375	352	216	213	136	119	23	20	0.727	0.24	0.22	0.450
SNPV-2		375	352	109	118	182	166	84	68	0.359	0.47	0.43	0.149
SNPIII-2		375	352	150	163	187	150	38	39	0.143	0.35	0.32	0.280
SNPIV-1		375	352	289	260	80	81	6	11	0.319	0.12	0.15	0.186
SNPIV-2		375	352	285	258	84	85	6	9	0.543	0.13	0.15	0.310
SNPI-1		375	352	250	236	113	109	12	7	0.587	0.18	0.17	0.693
SNPI-2		375	352	126	132	195	159	54	61	0.174	0.40	0.40	0.850
Second-set samples													
SNPVI-1		470	355	283	235	166	107	21	13	0.211	0.22	0.19	0.0917
SNPV-2		470	355	162	131	218	169	90	55	0.379	0.42	0.39	0.213
SNPIII-2		470	355	196	151	211	150	63	54	0.663	0.36	0.36	0.838
SNPIV-1		470	355	330	259	130	88	10	8	0.651	0.16	0.15	0.466
SNPIV-2		470	355	329	267	130	78	11	10	0.171	0.16	0.14	0.184
SNPI-1		470	355	335	248	123	98	12	9	0.899	0.16	0.16	0.701
SNPI-2		470	355	181	128	211	171	78	56	0.645	0.39	0.40	0.737

[a] SNPs denoted by an asterisk in all data indicate representative SNPs.
[b] MAF = minor allele frequency of 96 samples. MAF is the frequency of minor allele m, which is determined by counting two alternative alleles within a sample.
[c] N, Number of subjects; M, major allele; m, minor allele; SCZ, schizophrenics; and CON, controls.
[d] *P* value was calculated using the χ^2 test.

TABLE 2. Two-locus haplotype frequencies for the evaluation of linkage disequilibrium (LD)

		Locus 2	
		Allele M	Allele m
Locus 1	Allele M	a	b
	Allele m	c	d

bination hotspots. Within a haplotype block, there is little or no recombination that occurs and the SNPs are highly correlated. Consequently, a small subset of SNPs (called representative SNPs or tag SNPs) is sufficient to capture the haplotype pattern of the block. Thus, in order to obtain SNPs to provide the enough information required for combination analysis, we first performed one representative SNP selection in one LD block. The D′ value is one of the measures for assessing the strength of LD ranging from 0 (no disequilibrium) to 1 (complete disequilibrium). The criterion of an LD block is to be a region in which all pairwise D′ values are not lower than 0.8, using the Genotype2LDblock v0.2 software (30). The estimation of relative two-locus haplotype frequencies is performed using Table 2. The frequencies of haplotypes MM, Mm, mM and mm in loci 1 and 2 are defined as the values a, b, c, and d, respectively. D is defined as (ad)−(bc) ranging from $D_{max} = \min\{(a+b)(b+d), (c+d)(a+c)\}$ to $D_{min} = \max\{-(a+b)(a+c), -(c+d)(b+d)\}$. D′ is defined as D/D_{max} for $D>0$ and as D/D_{min} for $D<0$ to change D to D′ ranging from 0 to 1. From each LD block, we selected representative SNPs (denoted by an asterisk in Table 1) with the highest minor allele frequencies (MAFs). MAF is (c+d) and (b+d) in loci 1 and 2, respectively. The higher the MAF is, the more information the SNP has, compared with the other SNPs in the same LD block for identifying factors susceptible to diseases because of subject variation.

SNP association analysis To assess the association of an SNP with a disease, the χ^2 test based on genotypic and allelic association analyses was performed in all data and in the first- and second-set samples divided randomly, as shown in Table 1. The number of subjects (N) belonging to each category composed of phenotypes (schizophrenia or control) and genotypes or alleles, and the P value calculated using the χ^2 test are shown in Table 1. We employed this validation test using two independent data sets (referred to as the first- and second-set samples), because commonly selected significant factors might have the potential as factors susceptible to schizophrenia. Therefore, these two data sets indicate a relationship between modeling and test data sets, which are generally used for validation analysis. The extents of genotypic and allelic associations were measured from the P value calculated using the χ^2 test in the 2 (case or control)×3 (genotype; M/M, M/m and m/m) and 2 (case or control)×2 (allelic association, M or m) tables, respectively.

BRP analysis The concept of the BRP test (24) is based on the evaluation of the interactions between several factors by statistically assessing the extent of bias in the number of case or control subjects belonging to one rule comprising a combination of certain alleles or genotypes. A rule table constructed to analyze two SNPs (SNPs A and B) with a dominant model and a recessive model, respectively is shown in Fig. 1. One cell in Fig. 1 corresponds to one rule; thus, there are four and eight rules in the cases of two and three SNP combinations, respectively. For example, in rule no. 1 in Fig. 1, one of the rules in using the two-SNP combination, subjects with the genotype AA of SNP A and the B allele of SNP B belong to the rule. In each rule, the extent of bias in the number of case or control subjects (the ratio between them) was assessed using the BRP test mentioned below. We focused the rule with statistically biased ratios between the case and control subjects in either the first- or second-set samples. Thus, the rules consisting of more

		SNP A	
		AA	Aa + aa
SNP B	BB + Bb	$N_{case,1}/N_{control,1}$	$N_{case,2}/N_{control,2}$
	bb	$N_{case,3}/N_{control,3}$	$N_{case,4}/N_{control,4}$

FIG. 1. Rule table using combination of two single nucleotide polymorphisms (SNPs). $N_{case,i}$ and $N_{control,i}$ represent the numbers of case and control subjects, respectively, belonging to rule no. i. The rule circled by a dotted line denotes subjects with the genotype AA of SNP A and the B allele of SNP B. This rule is regarded as rule no. 1.

case subjects are regarded as risk factor candidates (RFCs) and the rules with control subjects are regarded as protective factor candidates (PFCs). In addition, if the rules have statistically biased ratios between case and control subjects in both data sets, they are defined as risk or protective factors.

We applied the binomial test (25) to the combination analysis and selected RFCs or PFCs. The binominal test is used for the evaluation of the potentiality of a risk or protective factor. The P value of the binomial test for evaluating the existing ratio between the case and control subjects is calculated using the binomial distribution as

$$f(N_{case,i}) = \frac{n!}{N_{case,i}!(n-N_{case,i})!} p^{N_{case,i}}(1-p)^{n-N_{case,i}} \quad (1)$$

where n is the sum of the observed $N_{case,i}$ and $N_{control,i}$ existing in rule i. The probability p represents $N_{case}/(N_{case}+N_{control})$, where N_{case} and $N_{control}$ represent the total numbers of cases and controls analyzed in the combination. The null hypothesis ($N_{case,i}/N_{case} \le N_{control,i}/N_{control}$) in the case of selecting RFCs is tested by computing the sum (P value) of all $f(N_{case,i})$ that are equal to or lesser than that for the observed $N_{case,i}$ (one-tailed test) (24, 25).

In addition, to adjust for the multiple testing problems caused by a simultaneous significance test, a random permutation test (26–28) was performed in this method. The procedure of the BRP analysis is outlined in Fig. 2 and has two steps. This procedure with two steps was repeated twice in the selection of RFCs and PFCs. The exhaustive combinations of loci were analyzed in this method; therefore, when using g SNPs, the number of combinations N_{comb} is given as

$$N_{comb} = \sum_{i=1}^{g} {}_gC_i \quad (2)$$

In step 1, the most efficacious genotype combination in each SNP combination is determined as follows. In the case of the two-SNP combination, there are 16 P values calculated using the binomial test in four genotype combinations: dominant-dominant, dominant-recessive, recessive-dominant and recessive-recessive (each combination has four rules). By comparing these P values under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$, the combination of dominant and recessive is determined when the lowest P value is obtained in the exhaustive genotype combinations. (In the case of selecting PFCs, the condition used is $N_{case,i}/N_{case} < N_{control,i}/N_{control}$.) N_{case} and $N_{control}$ represent the numbers of case and control subjects analyzed in the combination, respectively. Next, the P values in the acquired genotype combination are used in the selection of RFCs or PFCs (step 2). In this study, since the method of SNP analysis using dominant

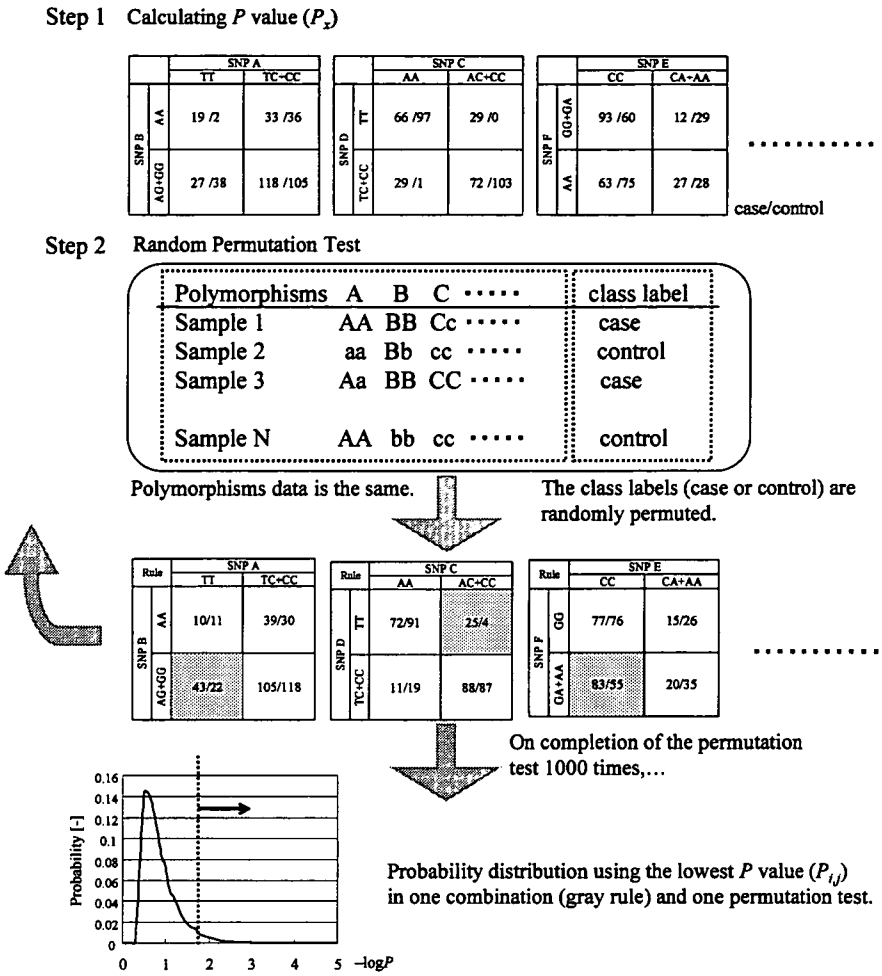

FIG. 2. Selection procedure for risk factor candidates (RFCs) using binomial and random permutation test (BRP) in combination of two polymorphisms. The analysis procedure has been divided into two steps. In step 1, the P values in all rules (under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$) are calculated for the genotype combinations of the dominant and recessive defined by the following condition. The combination of dominant and recessive is determined when the P value in one of the rules under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$ is the lowest among the P values in exhaustive genotype combinations. In step 2, to select the RFCs, the statistical significance of the rule in each combination is assigned to the P value ($P^{ran}(P_x)$) that is calculated using the P value (P_x) derived from step 1 by modeling the null distribution; the P value comprising the null distribution is the lowest ($P_{i,j}$ in Eq. 3) under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$ in each combination (gray rule) by the random permutation test. In the random permutation test, the signal of each subject (case or control) is randomized to avoid a change in the number of subjects contained in the rule; in the present study, the number of permutations was 1000 times. RFCs were inferred at the $P^{ran}(P_x)$ obtained using the distribution and was calculated to be smaller than 0.05 ($P^{ran}(P_x)<0.05$).

and recessive concepts appears practical for the application of various phenotypes (such as diseases), the heterozygote is combined with either of the homozygotes as described above. The dominant model is determined by comparing the Aa plus aa genotypes with the AA genotype, and the recessive model is determined by comparing the aa genotype with the AA plus Aa genotypes.

In step 2, to select RFCs (or PFCs), the statistical significance of the rule in each combination is denoted by the P value ($P^{ran}(P_x)$) calculated using the P value derived from step 1 by modeling the null distribution; the P value comprising the null distribution is the lowest under the condition $N_{case,i}/N_{case} > N_{control,i}/N_{control}$ in each combination using the random permutation test (26–28). (In the case of selecting PFCs, the condition used is $N_{case,i}/N_{case} < N_{control,i}/N_{control}$.) This leads to the development of a procedure for determining the ratio between the case and control subjects, which is statistically significant when compared with the null hypothesis of the ratio in randomly labeled data. The null hypothesis indicates that given a particular rule (r), the conditional probability of a label (y) being case (+1) and that of a label (y) being control (−1) are equal as

$$H_0:\ p(y=+1|r) = p(y=-1|r)$$

In the random permutation test, the label of each subject (case or control) is randomized to inhibit a change in the number of subjects contained in the rule. In essence, we can examine how well the rule of correctly labeled data in each combination explains the extent of risk (or protection) in comparison with the rule of randomly labeled data. The significance of the rule is $P^{ran}(P_x)$ (Eq. 3), which is a percentage of random rules (27).

$$P^{ran}(P_x) = \frac{1}{T_1 \times T_2} \sum_{i=1}^{T_1} \sum_{j=1}^{T_2} \theta(P_x - P_{i,j}) \qquad (3)$$

Here, $\theta(z)=1$ if $z \geq 0$ and 0 otherwise. $P_{i,j}$ is the lowest P value of the rule obtained using randomly labeled data calculated using the binomial test in one combination and one permutation test. P_x is the P value of the rule obtained using correctly labeled data calculated using the binomial test in step 1. In other words, $P^{ran}(P_x)$ is

the P value of P_x in the null distribution. T_1 and T_2 are the numbers of permutations and combinations, respectively. T_1 is 1000 times in this study. T_2, for example, in two-SNP combinations using seven SNPs is ${}_7C_2=21$, because in the random permutation test, the combination of dominant and recessive is already determined using correctly labeled data as mentioned above. In this study, RFCs and PFCs are inferred at the $P^{ran}(P_x)$ level using the distribution calculated using the random permutation test and are found to be less than 0.05 ($P^{ran}(P_x)<0.05$). The BRP software is available at http://www.nubio.nagoya-u.ac.jp/proc/english/indexe.htm.

In addition, our proposed BRP method was compared with MDR and S-sum statistic, because these two methods enable the evaluation of interactions between SNPs using the P value based on the results of the random permutation test. The null distribution used for calculating the P value is different among these methods. MDR (11) enables the evaluation of ratios between case and control subjects in all rules in one combination of SNPs and the calculation of the testing accuracies of exhaustive combinations (Eq. 2) in the 10-fold cross validation. MDR was also assessed from the cross-validation consistency and P value computed by comparing its (accuracy or consistency) value with the empirical distribution (random permutation test) (11). The null distribution is determined using the testing accuracy in randomly labeled data. In S-sum statistic (17), SNPs were added to the model stepwise according to their S-value ranked highest ($S=\Sigma_i(t_i \times u_i)$, where t_i is the χ^2 value that enables the evaluation of allelic association in the 2×2 table and u_i is the Hardy-Weinberg disequilibrium for association in the ith SNP), that is, their contribution to the disease risk. SNPs reducing the P value estimated using the permutation test in each sum to a minimum provides information regarding the significant SNP combination and the number of SNPs in the analyzed SNPs. The null distribution is determined using the S value in randomly labeled data (17).

RESULTS

Interaction analysis of complex genetic diseases using BRP First, the association between the isolated SNPs and schizophrenia was assessed from the P value calculated using the χ^2 test with respect to the genotypic and allelic data. As shown in Table 1, there was no association between the isolated SNPs and schizophrenia in the genotypic and allelic analyses. Therefore, we focused on the analysis of SNP combinations. To validate risk or protective factor candidates (RFCs or PFCs), the BRP analysis (24) was performed by dividing the original data to two data sets (first- and second-set samples) randomly. In Fig. 3, rules (up to a three-SNP combination) with a higher control subject rate ($N_{control,i}/(N_{case,i}+N_{control,i})$) than that of population data were plotted in the more than 0 area on the $-\log P_b$ axis, whereas those with a higher case subject rate ($N_{case,i}/(N_{case,i}+N_{control,i})$) than that of population data were plotted in the less than 0 area on the $-\log(1/P_b)$ axis. P_b was calculated with the binomial test. The black dot in Fig. 3 represents the RFC or PFC rule in which $P^{ran}(P_x)$ is smaller than 0.05 in both data sets. Although the validation of the RFC could not be found out in this sample size, several rules for the control showed the same tendency of the P value between both data sets, on the basis of the rules plotted in the more than 0 area on the $-\log P_b$ axis in both data sets. In addition, four identical PFCs (protective factors) were obtained, as shown in Fig. 3. As shown in Table 3 and Fig. 4, the protective factors were obtained by combining the GG genotypes of SNP VI-1 and SNP V-2. Because the ratio between the case and control subjects in the protective factor comprising these two genotypes patterns is statistically significant in both data sets divided randomly, the evidence indicating that the distribution of subjects who have this genotype combination in the present data might be the same as that of population data was obtained in the present sample size by assessing the ratio in one rule using BRP.

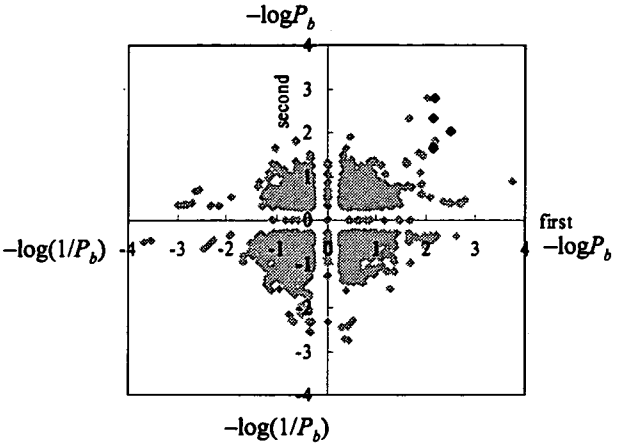

FIG. 3. Relationship of P value in same rule between first- and second-set samples in up to three single nucleotide polymorphism (SNP) combinations. Rules with a higher control subject rate than that of population data were plotted in the more than 0 area on the $-\log P_b$ axis, whereas those with a higher case subject rate than that of population data were plotted in the less than 0 area on the $-\log(1/P_b)$ axis. P_b was calculated with the binomial test. The black dot represents the rule in which the P value ($P^{ran}(P_x)$) is smaller than 0.05 in both data sets.

TABLE 3. Protective factors against disease development obtained in first- and second-set samples by BRP analysis

SNP			P^{ran} [a]	
			First set	Second set
VI-1	V-2		0.0104	0.0442
VI-1	V-2	IV-1	0.0195	0.0065
VI-1	V-2	IV-2	0.0201	0.0194
VI-1	V-2	I-1	0.0083	0.0417

[a] The P value was calculated with the binomial and random permutation test (BRP). The P values of the protective factors were smaller than 0.05 ($P^{ran}(P_x)<0.05$) in both data set samples.

Comparative study of interaction analysis with BRP and screening signal SNPs Next, in order to investigate the performance of BRP in the gene–gene interaction analysis, we compared BRP with MDR and S-sum statistic that enable the evaluation of a gene–gene interaction using the P value based on the results of the random permutation test. In Table 4, with the best models in each data set evaluated on the basis of the testing accuracy, cross-validation consistency and P value in each number of input variables, the same significant interactions were not observed by MDR. An interaction effect was not observed in the S-sum statistic analysis using the P value (0.285 and 0.792 in the first- and second-set samples, respectively).

DISCUSSION

Schizophrenia is a neurodevelopmental disorder and one

First-set samples

SNP V-2	SNP VI-1: GG	SNP VI-1: GA + AA
GG	61 / 86 (P_b = 0.0072)	48 / 32
GC + CC	155 / 127	111 / 107

case / control

Second-set samples

SNP V-2	SNP VI-1: GG	SNP VI-1: GA + AA
GG	94 / 95 (P_b = 0.0223)	68 / 36
GC + CC	189 / 140	119 / 84

case / control

FIG. 4. Protective factor (gray cell) in both first- and second-set samples. P_b was calculated with the binomial test.

of the common diseases with an estimated heritability of 80%. Chromosome 22q11–q13 (OMIM: #600850 SCZD4) is one of the most probable schizophrenia susceptibility regions because the microdeletions of the 22q11 chromosome are reported to be associated with schizophrenia (31); furthermore, the two independent meta-analyses of linkage studies reveal the suggested linkage in this region (32, 33).

The APOL proteins belong to a group of high-density lipoproteins (HDL), and all 6 *APOL* genes (*APOL1–6*) are located near each other on the 22q12 chromosome. A recent postmortem study using the candidate gene cDNA array showed the upregulations of *APOL1*, *APOL2*, and *APOL4* in the prefrontal cortex of schizophrenic patients (34).

The most recent association analysis using the DNA pooling method showed no association of this gene cluster with schizophrenia in Irish patients (35). Moreover, the single marker association analysis (*i.e.*, allelic and genotypic analyses), as well as haplotypic association analysis, could not confirm the possibility of gene–gene interactions. In the present study, we performed the BRP analysis (24) to evaluate the association of the *APOL* gene cluster with schizophrenia (interaction) in a Japanese population by focusing on the analysis of SNP combinations that should not be within the same LD block. By the combination analysis using BRP, we considered that the interactions among SNPs in the *APOL* gene cluster are genetic susceptibility factors for schizophrenia.

In this validation analysis, although the same risk factor could not be selected in the two independent data sets, a combination of the GG genotypes of SNP VI-1 and SNP V-2 was selected as a significant protective factor against disease development in both data sets (Fig. 4). This result indicates the possibility that the distribution of subjects who have this genotype combination in the present data might be the same as that of population data because of the concordance between the two independent data sets. However, this significant protective factor was not detected in the MDR and S-sum statistic analyses, because these approaches do not enable the evaluation of the ratio between the case and control subjects in one rule. The ratio in rules, except risk or protective factors, seems to indicate the same tendency as that in randomly labeled data and these rules can be ex-

TABLE 4. Results of multifactor dimensionality reduction (MDR) analysis in first- and second-set samples

Number of input variable[a]	VI-1	V-2	III-2	IV-1	IV-2	I-1	I-2	Training accuracy[b]	Testing accuracy[b]	*P* value[c]	Cross validation consistency[d]
First-set samples											
1			*[e]					0.537	0.488	0.992	5/10
2	*	*						0.558	0.502	0.950	5/10
3	*	*					*	0.586	0.479	0.998	5/10
4	*	*	*				*	0.625	0.490	0.987	8/10
5	*	*	*		*		*	0.670	0.518	0.806	8/10
6	*	*	*		*	*	*	0.715	0.491	0.983	10/10
7	*	*	*	*	*	*	*	0.749	0.506	0.921	10/10
Second-set samples											
1	*							0.570	0.560	0.889	6/10
2	*		*					0.576	0.543	1.000	2/10
3	*	*		*				0.597	0.539	1.000	6/10
4	*	*	*			*		0.628	0.505	1.000	3/10
5	*	*	*		*		*	0.664	0.510	1.000	4/10
6	*	*	*		*	*	*	0.705	0.509	1.000	8/10
7	*	*	*	*	*	*	*	0.740	0.519	1.000	10/10

[a] The model with the lowest prediction error and highest cross validation consistency was selected for each of the input variables considered.
[b] Ratio of correct classifications to total number of instances classified through 10-fold cross validation within training or testing data set.
[c] An empirical *P* value for the result was determined using 1000-fold random permutation test strategies.
[d] Frequency of times in particular cross-validated run for which given input variable combination was selected as best model.
[e] SNP was used as the input variable in the model.

cluded using BRP based on the random permutation test. Because MDR enables the simultaneous evaluation of the ratios in all rules and the testing accuracy (11), it might miss the significant rule. In S-sum statistics, the χ^2 values of allelic association and Hardy-Weinberg disequilibrium in each SNP are added simply (17); thus, the ratio obtained by combining several SNPs is not considered. It is suggested that this rule selected using BRP could be the sole protective factor against disease development with a statistical significance and is a preventable factor for schizophrenia discussed below. There are 336 subjects with this genotype corresponding to this rule in this study. These 336 subjects consist of 155 cases and 181 controls, and correspond to 25.6% of all the controls. Although the number of subjects corresponding to the rule is small, the odd ratio is significantly high (1.5).

The conventional argument is that the identification of susceptible genes leads to the discovery of new therapeutics and diagnoses. However, the occurrence of healthy individuals who can prevent disease development, despite the presence of genetic and environmental risks, increases the possibility that protective alleles or genotypes (protective factors) maintain good health (36). These protective factors probably prevent the development of disease effectively and safely. This finding seems to be the same as the following example; people who are active in immune response can avoid a viral infection, which is generally desirable. Therefore, even if subjects have the same genotype or allele combination corresponding to a risk factor, some of these subjects are considered controls because a protective factor functions. On the other hand, it is likely that there are important and inevitable protective factors; if the protective factor has been destroyed or inactivated, a disease will develop at a high probability. By using BRP that enables the evaluation of all rules exhaustively, this protective factor was selected in both independent data sets. Thus, in complex genetic diseases, such as schizophrenia, the evidence indicating that healthy control subjects tend to have the same combinations of certain alleles and genotypes was obtained using BRP. With respect to the risk factor, the causality of schizophrenia is too complex to identify a susceptible interaction using a small sample size because there might be many development patterns and differs on an individual basis.

Thus, we could not find any risk factor which can explain the biological mechanism of disease development in all patients. Owing to this result, it is likely that there might be more biological routes for disease, namely, it is considered that there might be the effects of confounding factors (such as age, sex, environmental factors and other genes) for disease development except seven SNPs analyzed in this study. In selecting risk factors, because the effects of these susceptible confounding factors might differ between independent data sets, a common risk factor might not be selected despite its significance in either data set. However, in selecting protective factors, because the effects of these factors might be small between data sets compared with the case of selecting risk factors, a protective factor might be selected using a comparatively small sample size.

For achieving a high power in selecting the protective factor, the BRP analysis has three characteristic features: (i) exhaustive combination analysis, (ii) the automatic assessment of the dominant or recessive model, and (iii) the statistical evaluation of the ratio between the case and control subjects in one rule comprising genotype or allele combinations using the binomial and random permutation tests. The first feature ensures the analysis of all possible combinations and thus helps in finding the synergistic interaction effects required for the development of a complex genetic disease. Using the second feature, the data in high dimensions obtained by combining three genotype patterns can be transformed to those in low dimensions using dominant-recessive combinations. Furthermore, from this information, important evidence on the biological aspects and the extent of risk in one rule determined from the P value calculated with BRP, which is a one-dimensional analysis (risk or non risk), might be obtained. The third feature enables us to determine how well the rule of correctly labeled data in each combination explains the extent of risk or protection compared with the rule of randomly labeled data; thus, statistically significant risk or protective factors can be obtained. Consequently, BRP can be a more effective tool than MDR and S-sum statistic because of the three features mentioned above. To clarify the pathophysiology of complex genetic diseases or the mechanism of treatment response, it is very important to identify the protective factor comprising a combination of certain alleles and genotypes, as well as the risk factor.

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Suppressive Effect of Paroxetine, a Selective Serotonin Uptake Inhibitor, on Tetrahydrobiopterin Levels and Dopamine as Well as Serotonin Turnover in the Mesoprefrontal System of Mice

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KEY WORDS tetrahydrobiopterin; dopamine; serotonin; social isolation; novelty stress

ABSTRACT Tetrahydrobiopterin (BH_4) is a coenzyme of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), which are rate-limiting enzymes of monoamine biosynthesis. According to the monoamine hypothesis of depression, antidepressants will restore the function of the brain monoaminergic system and the BH_4 concentration. In the present study, we investigated the effect of paroxetine, a selective serotonin reuptake inhibitor (SSRI), on the BH_4 levels and dopamine (DA) and serotonin (5-HT) turnover in the mesoprefrontal system, incorporating two risk factors of depression, social isolation and acute environmental change. Male ddY mice (8W) were divided into two housing groups, i.e., group-housing (eight animals per cage; 28 days), and isolation-housing (one per cage; 28 days), being p.o.-administered paroxetine (5 or 10 mg/kg; days 15–28), and exposed to a 20-min novelty stress (day 28). The levels of BH_4, DA, homovanilic acid (HVA), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) were measured in the prefrontal cortex and midbrain. In both the regions, novelty stress significantly increased BH_4 levels under the isolation-housing condition, whereas these levels were decreased under the group-housing condition. Thus, social isolation altered the neurochemical response to novelty stress. Paroxetine significantly decreased BH_4 levels under the isolation-housing condition, whereas decreased HVA/DA and 5-HIAA/5-HT ratios were observed under the group-housing condition. Thus, social isolation may have influenced the suppressive effects of paroxetine on BH_4 levels as well as exerted an influence on DA and 5-HT turnover. We replicated our recent findings that SSRI, fluvoxamine, suppressed BH_4 levels, as well as DA and 5-HT turnover in the mouse mesoprefrontal system. **Synapse 61:698–706, 2007.**

INTRODUCTION

Major depression is a prevalent human psychiatric disorder that reduces the psychological activity of patients who exhibit depressive mood, loss of interest or pleasure, and/or psychomotor agitation or retardation. Major depression often precludes such patients from access to job-related and social support, and occasionally they commit suicide. Because of the huge socioeconomic loss elicited by major depression, it has become one of the primary target diseases worldwide in terms of the search for treatment and/or cure. The monoamine hypothesis has played an important role in the investigation of the etiology and pathophysiology of human major depression. This hypothesis is primarily based on reports of impaired norepinephrine and serotonin (5-HT) neural transmission (Delgado, 2000; Hirschfeld, 2000; Leonard, 2000; Smith et al., 1997). Pharmacological studies of almost all clinically effective antidepressants have supported

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this hypothesis. In the past decade, 5-HT transporter (5-HTT) function has been the main focus of investigation of the etiology and pathophysiology of major depression. Thus, selective serotonin reuptake inhibitors (SSRIs), a class of antidepressants that produce clinical effects by selectively inhibiting 5-HTT function, have been examined in great detail. However, the pathophysiology of major depression has yet to be elucidated.

To investigate the etiology and pathophysiology of major depression, interactions between environmental factors and genetic factors, i.e., gene–environment interactions, have become increasingly important (Lesch, 2004). Both adverse life events and lack of social support have been shown to exert an influence on the onset of major depression in genetically susceptible persons (Kendler et al., 1993; Paykel 1994). Thus, we hypothesized that an animal model including these environmental factors would help identify altered brain monoamine system activity. In recent years, we have investigated the influence of social isolation and novelty stress on rat brain monoamine turnover (Miura et al., 2002a,b). Furthermore, we have studied the influence of fluvoxamine, an SSRI, on this animal model (Miura et al., 2004, 2005a,b).

(6R)-5,6,7,8-tetrahydrobiopterin (BH_4) is a coenzyme of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), which are the rate-limiting enzymes of monoamine biosynthesis. Because some studies have reported that antidepressants suppressed TH (Nestler et al., 1990) and TPH (Lapierre et al., 1983) activity, it was considered possible that antidepressant-induced suppression may be related to changes in BH_4 levels. Thus, investigation of the relationship between changes in the activities of these enzymes (TH, TPH) and BH_4 levels induced by antidepressants are expected to help clarify the pathophysiology of human depression. We recently reported the suppressive effects of fluvoxamine on BH_4 levels and monoamine turnover (Miura et al., 2004, 2005a). We now propose that changes in brain BH_4 levels play an important role in the pathophysiology of depression, and that antidepressants modulate these changes.

In the present study, we further investigated the effects of another SSRI, paroxetine, using our animal model of social isolation and novelty stress. Mesocorticolimbic DA projections (A8, A10) originating from the ventral tegmental area of the midbrain (Cooper et al., 2003) have been shown to play an important role in a reward system, i.e., in motivating behavior (Kupferman and Schwartz, 1995). We therefore selected two regions of focus, the prefrontal cortex and the midbrain. The aim of the present study was to examine the effects of paroxetine on BH_4 levels, as well as on DA and 5-HT turnover in the mesoprefrontal region, and to clarify the effects of paroxetine on a novel animal model that simulates two of the major environmental risk factors associated with human depression (Miura et al., 2002a,b, 2004, 2005a,b).

MATERIALS AND METHODS

Animals

A total of 96 male ddY mice were used in the present experiments. At 8 weeks of age, the mice were transported from a breeding company to our experimental animal center. After a 1 week habituation period, the mice, all of which had previously been housed in groups (eight per cage), were divided into two different groups according to one of two housing conditions, i.e., group housing (eight per cage; $n = 48$) or isolation housing (one per cage; $n = 48$; Fig. 1). After being assigned to one of the two housing conditions, the mice were reared for 28 days (Fig. 1). The animals were further separated into two groups: in the stress group ($n = 48$), the animals were exposed to a 20-min novelty stress on day 28; and in the nonstress group ($n = 48$), the animals were not exposed to the novelty stress (Fig. 1). In the third and fourth weeks (days 15–28), the mice were p.o.-administered either placebo (1% methyl cellulose) or low- (5 mg/kg) or high-dose (10 mg/kg) paroxetine once per day (Fig. 1). Then, the mice were further divided into three groups as follows: controls (0 mg/kg, $n = 32$), low-dose group (5 mg/kg, $n = 32$), and high-dose (10 mg/kg, $n = 32$; Fig. 1) group. Finally, by combining the above conditions, the mice were divided into 12 groups: group housing, nonstress, paroxetine 0 mg/kg ($n = 8$); group housing, nonstress, paroxetine 5 mg/kg ($n = 8$); group housing, nonstress, paroxetine 10 mg/kg ($n = 8$); group housing, stress, paroxetine 0 mg/kg ($n = 8$); group housing, stress, paroxetine 5 mg/kg ($n = 8$); group housing, stress, paroxetine 10 mg/kg ($n = 8$); isolation housing, nonstress, paroxetine 0 mg/kg ($n = 8$); isolation housing, nonstress, paroxetine 5 mg/kg ($n = 8$); isolation housing, nonstress, paroxetine 10 mg/kg ($n = 8$); isolation housing, stress, paroxetine 0 mg/kg ($n = 8$); isolation housing, stress, paroxetine 5 mg/kg ($n = 8$); isolation housing, stress, paroxetine 10 mg/kg ($n = 8$).

The cages used for the group-housing condition were 21 × 31 × 13 cm, and the cages used for the isolation-housing condition were 17 × 29 × 13 cm. Cage exchange was performed two times a week in the case of the group-housing group, whereas this was performed once per week in the case of the isolation-housing group. Food and water were provided ad libitum. The animals were kept on a 12-h light/dark cycle, and room temperature was maintained at 21–23°C. All efforts were made to minimize both the number of animals used and the degree of their suffering. All of the experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

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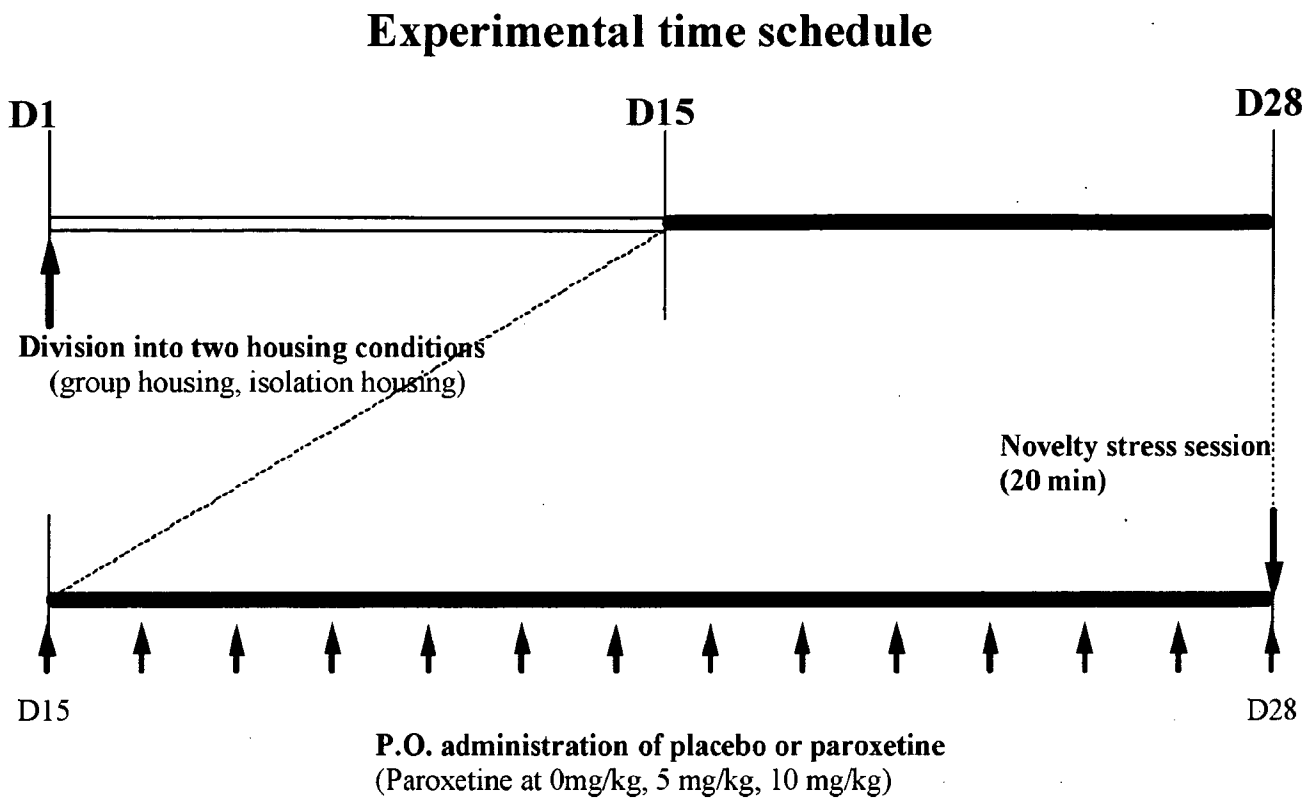

Fig. 1. Experimental time schedule. Mice were divided into 12 groups as described in Materials and Methods.

Paroxetine administration

Glaxo Smith Kline (UK) kindly donated the paroxetine, which was suspended in 1% methyl cellulose.

Novelty stress test

In the stress group, 20-min novelty stress sessions were performed on day 28 (i.e., the animals were placed into a transparent plastic box (28 × 35 × 30 cm^3) that they had not yet experienced). The novelty stress test was performed in a room that was kept dark.

Sample preparation

Mice in the stress group were sacrificed by decapitation immediately after the 20-min stress session, whereas mice in the nonstress group were decapitated without exposure to stress. The brains were removed and the prefrontal cortex and midbrain were dissected out as quickly as possible on glass plates over ice. The samples were weighed and treated with 1000 μl of an ice-cold 0.2 M perchloric acid solution containing 0.2 mM sodium pyrosulfite, 0.01% EDTA-2Na, and 0.5 μM isoproterenol (ISO) as an internal standard per 100 mg of wet tissue. The solution was sonicated and then centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was filtered through a Millipore HV filter (0.45 μm pore size) and then subjected to both high-performance liquid chromatography (HPLC) with the electrochemical detection (ECD) of monoamines (DA, 5-HT) and their metabolites (homovanilic acid, HVA; 5-hydroxyindoleacetic acid, 5-HIAA), and HPLC with the fluorimetric detection (FD) of BH_4.

HPLC-ECD determination of brain levels of monoamines and their metabolites

The levels of DA, HVA, 5-HT, and 5-HIAA in the brain extracts were measured by HPLC with ECD. The system employed for HPLC-ECD consisted of a CMA/200 autosampler (CMA/Microdialysis AB, Stockholm, Sweden), a micro LC pump (BAS, West Lafayette, IN), an LC-4C ECD (BAS), a Bio-Phase ODS-4 51-6034 column (4.0 × 110 mm; BAS), a CR-6A recorder (Shimadzu, Kyoto, Japan), an LC-26A vacuum degasser (BAS), and a CTO-10A column heater set at 35°C (Shimadzu). The mobile-phase solution consisted of 0.1 M tartaric acid-0.1 M sodium acetate buffer, pH 3.2, containing 0.5 mM EDTA-2Na, 555 μM sodium 1-octane sulfonate, and 5% acetonitrile. The flow rate was 700 μl/min. The concentration of each compound was calculated by comparison with both the internal and the external standards.

HPLC-FD by postcolumn sodium nitrite oxidation for the determination of brain levels of BH_4

Tani and Ohno (1993) developed a method for the direct measurement of BH_4, the active form of biopterin, and we used this method to measure BH_4 levels in the present study. BH_4 (SIGMA) was stored in 0.1 M HCl (20 mM), and was prepared in 0.01 M HCl as an external standard (0.25 μM) immediately before sample injection. This system consisted of two LC-10AD pumps (Shimadzu), a CMA/200 autosampler, a Cosmosil 5C18 column (4.6 × 250 mm), a CR-6A recorder (Shimadzu), an LC-26A vacuum degasser, and a PF-10A FD (Shimadzu). The excitation wavelength was 350 nm, and the emission wavelength was 440 nm. The temperature of the reaction coil was set at 80°C using a column heater. The concentration of BH_4 was calculated by comparison with an external standard. The mobile phase was 0.1 M sodium phosphate buffer (pH 2.9) containing 5% methanol, 3 mM sodium 1-octane sulfonate, 0.1 mM EDTA-2Na, and 0.1 mM ascorbic acid (to prevent oxidation). The flow rate was 1.0 ml/min. Reduced pterins were oxidized by $NaNO_2$ (5 mM; flow rate: 1.0 ml/min) in the reaction coil (80°C).

Statistical analyses

To examine differences in the levels of BH_4 and in the ratios of HVA/DA and 5-HIAA/5-HT, three-way MANOVA (Wilks's lambda) for housing condition, novelty stress, and paroxetine was conducted on dependent measures in each brain region. Further analyses were performed to consider the interactions. Under each housing condition, i.e., group-housing and isolation-housing, two-way MANOVA (Wilks's lambda) for novelty stress and paroxetine was conducted on dependent measures in each brain region, followed by the Tukey–Kramer test.

RESULTS

Prefrontal cortex

Three-way MANOVA (Wilks's lambda) for housing condition, novelty stress, and paroxetine was conducted to determine BH_4 levels and to determine the HVA/DA and 5-HIAA/5-HT ratios. Although housing condition (F (3, 82) = 2.644, P = 0.0546) and novelty stress (F (3, 82) = 1.522, P = 0.2150) did not significantly influence the dependent measures, paroxetine (F (6, 164) = 7.351, $P < 0.0001$) significantly changed these measures. The interactions between housing condition and novelty stress (F (3, 82) = 11.796, $P < 0.0001$), housing condition and paroxetine (F (6, 164) = 6.102, $P < 0.0001$), and novelty stress and paroxetine (F (6, 164) = 4.540, P = 0.0003) were significant. The interaction among housing condition, novelty stress, and paroxetine (F (6, 164) = 1.902, P = 0.0834) was not significant.

In the group-housing condition, two-way MANOVA for novelty stress and paroxetine was conducted on the dependent measures. Both novelty stress (F (3, 40) = 3.172, P = 0.0345) and paroxetine (F (6, 80) = 6.083, $P < 0.0001$) significantly altered the dependent measures. The interaction between novelty stress and paroxetine was also significant (F (6, 80) = 2.491, P = 0.0292). The post hoc test revealed that novelty stress significantly decreased BH_4 levels ($P < 0.01$, Fig. 2A). In addition, paroxetine significantly decreased the HVA/DA (0 vs. 5 mg/kg, $P < 0.05$; 0 vs. 10 mg/kg, $P < 0.01$) and 5-HIAA/5-HT (0 vs. 10 mg/kg, $P < 0.01$; 5 vs. 10 mg/kg, $P < 0.01$) ratios (Fig. 2A). Under the isolation-housing condition, two-way MANOVA for novelty stress and paroxetine was conducted on the dependent measures; both novelty stress (F (3, 40) = 9.802, $P < 0.0001$) and paroxetine (F (6, 80) = 6.624, $P < 0.0001$) significantly altered these measures. The interaction between novelty stress and paroxetine was also significant (F (6, 80) = 3.734, P = 0.0025). The post hoc test revealed that novelty stress significantly increased BH_4 levels ($P < 0.01$, Fig. 2B). Paroxetine significantly decreased BH_4 (0 vs. 5 mg/kg, $P < 0.01$; 0 vs. 10 mg/kg, $P < 0.01$) levels and the 5-HIAA/5-HT (0 vs. 10 mg/kg, $P < 0.05$) ratio (Fig. 2B).

Thus, under the group-housing condition, novelty stress was found to decrease BH_4 levels, and paroxetine was shown to reduce DA and 5-HT turnover. Under the isolation-housing condition, novelty stress was found to increase BH_4 levels, and paroxetine was shown to decrease BH_4 levels and 5-HT turnover.

Midbrain

Three-way MANOVA (Wilks's lambda) for housing condition, novelty stress, and paroxetine was conducted to determine the BH_4 levels as well as the HVA/DA and 5-HIAA/5-HT ratios. Housing condition (F (3, 82) = 3.138, P = 0.0297), novelty stress (F (3, 82) = 3.184, P = 0.0281), and paroxetine (F (6, 164) = 5.408, $P < 0.0001$) significantly altered these measures. The interactions between housing condition and novelty stress (F (3, 82) = 13.398, $P < 0.0001$), housing condition and paroxetine (F (6, 164) = 5.571, $P < 0.0001$), and novelty stress and paroxetine (F (6, 164) = 2.333, P = 0.0345) were all significant. The interaction among housing condition, novelty stress, and paroxetine (F (6, 164) = 2.054, P = 0.0614) was not significant.

In the group-housing condition, two-way MANOVA for novelty stress and paroxetine was conducted on dependent measures. Novelty stress (F (3, 40) = 10.094, $P < 0.0001$) and paroxetine (F (6, 80) = 4.800, P = 0.0003) significantly altered the dependent meas-

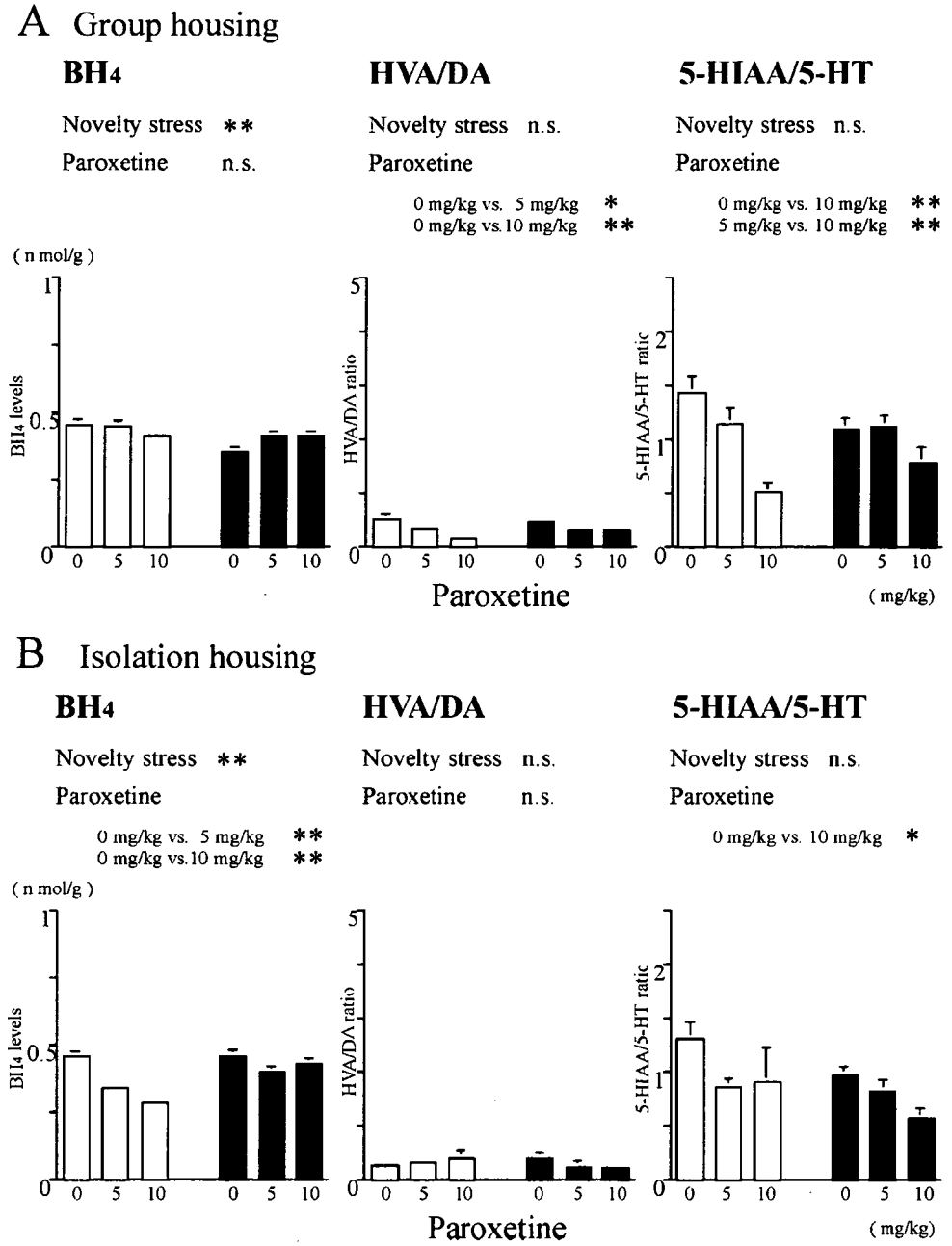

Fig. 2. Changes in BH_4 levels, and in HVA/DA and 5-HIAA/5-HT ratios in the prefrontal cortex elicited by novelty stress and by paroxetine. **A**: Group-housing condition (n = 48); (**B**) isolation-housing condition (n = 48). White bars, nonstress (n = 48); black bars, novelty stress (n = 48, n = 96 total). Paroxetine: 0, 0 mg/kg (n = 32); 5, 5 mg/kg (n = 32); 10, 10 mg/kg (n = 32, n = 96 total). Each bar indicates the final group division. The number of animals used for each group was eight. Values are shown as the mean ± SEM. Asterisks indicate the results of the Tukey–Kramer test for novelty stress and paroxetine under each housing and stress condition: * $P < 0.05$, ** $P < 0.01$, n.s. not significant.

ures. The interaction between novelty stress and paroxetine was significant (F (6, 80) = 3.229, P = 0.0068). The post hoc test revealed that novelty stress significantly decreased BH_4 ($P < 0.01$) levels, whereas it increased the 5-HIAA/5-HT ($P < 0.01$) ratio (Fig. 3A). Paroxetine significantly decreased the HVA/DA (0 vs. 5 mg/kg, $P < 0.01$; 0 vs. 10 mg/kg, $P < 0.01$) and 5-HIAA/5-HT (0 vs. 10 mg/kg, $P < 0.05$) ratios (Fig. 3A). In the isolation-housing condition, two-way MANOVA for novelty stress and paroxetine was conducted on dependent measures. Novelty stress (F (3, 40) = 3.786, P = 0.0176) and paroxetine (F (6, 80) = 5.579, $P < 0.0001$) significantly altered these measures, whereas the interaction between novelty stress and paroxetine was not significant (F (6, 80) = 0.757, P = 0.6057). The post hoc test revealed that novelty

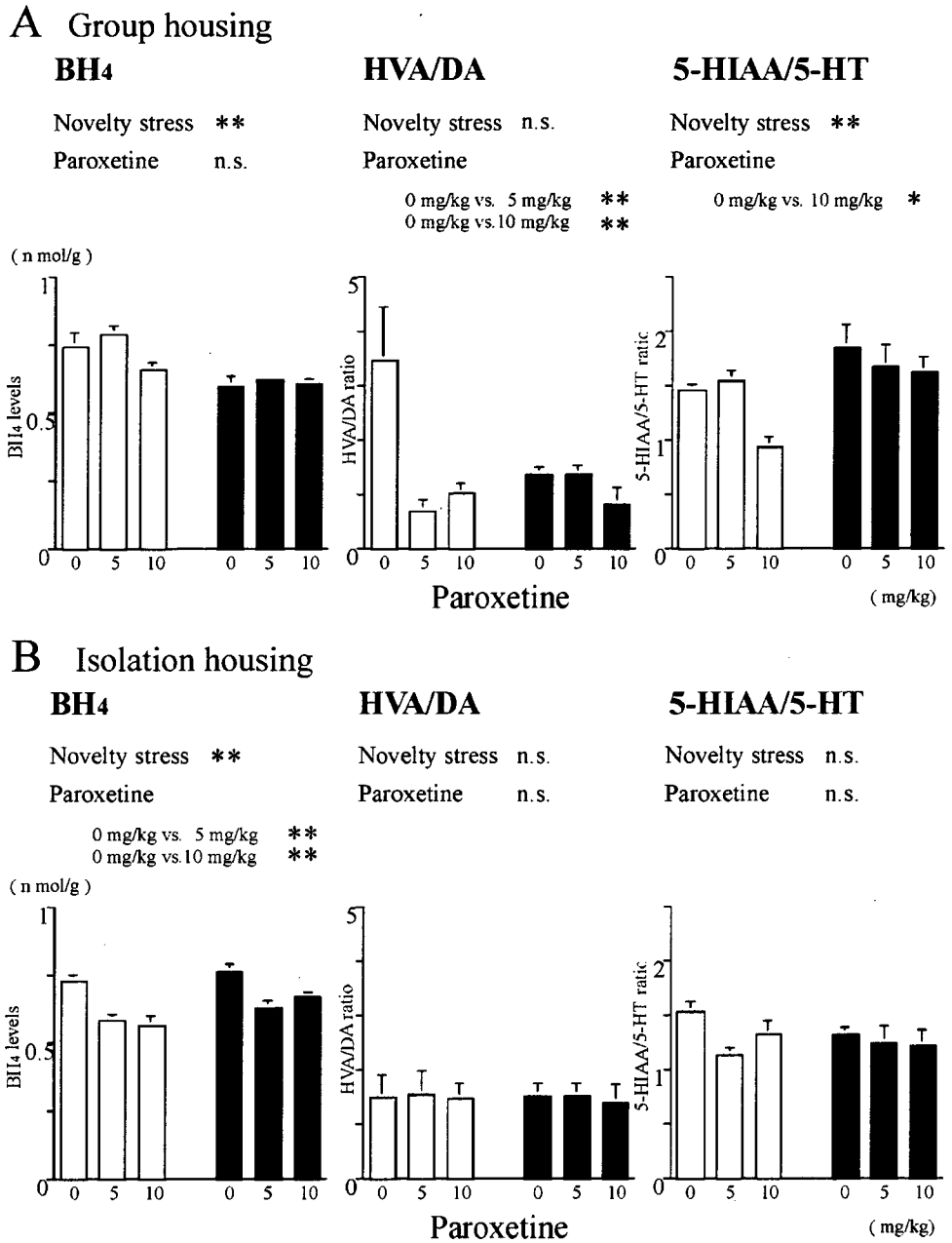

Fig. 3. Changes in BH_4 levels, and in HVA/DA and 5-HIAA/5-HT ratios in the midbrain elicited by novelty stress and by paroxetine. **A**: Group-housing condition ($n = 48$); (**B**) isolation-housing condition ($n = 48$). White bars, nonstress ($n = 48$); black bars, novelty stress ($n = 48$, $n = 96$ total). Paroxetine: 0, 0 mg/kg ($n = 32$); 5, 5 mg/kg ($n = 32$); 10, 10 mg/kg ($n = 32$, $n = 96$ total). Each bar indicates the final group division. The number of animals used for each group was eight. Values are shown as the mean ± SEM. Asterisks indicate the results of the Tukey–Kramer test for novelty stress and paroxetine under each housing and stress condition: * $P < 0.05$, ** $P < 0.01$, n.s. not significant.

stress significantly increased BH_4 ($P < 0.01$, Fig. 3B) levels. Moreover, paroxetine significantly reduced BH_4 (0 vs. 5 mg/kg, $P < 0.01$; 0 vs. 10 mg/kg, $P < 0.01$; Fig. 3B) levels.

Thus, under the group-housing condition, novelty stress decreased BH_4 levels and increased 5-HT turnover, and the administration of paroxetine reduced DA and 5-HT turnover. Under the isolation-housing condition, novelty stress increased BH_4 levels, and the administration of paroxetine reduced the level of BH_4.

DISCUSSION

The monoamine hypothesis has been one of the most convincing explanations of the etiology and pathophysiology of major depression, and pharmaco-

logical studies of almost all clinically effective antidepressants have supported this hypothesis. SSRIs are thought to exhibit their clinical effects by inhibiting 5-HTT function. Once 5-HT molecules are released from the nerve terminal, they remain in the synaptic cleft by inhibition of the reuptake activity of 5HTT, and thus a higher synaptic 5-HT concentration induces adaptive changes in pre- and postsynaptic 5-HT receptors, resulting in turn in improved signal transduction. In contrast, the adaptive changes in the biosynthesis of monoamines elicited by SSRIs have remained controversial. One study reported an increase in TPH activity by sertraline and fluoxetine (Kim et al., 2002), although another study reported no significant changes in TPH activity by fluoxetine (Zhou et al., 2006). Nevertheless, repeated electroconvulsive therapy (ECT), an alternative treatment for depression, has been shown to increase the activities of TH, TPH, and GTP cyclohydrolase 1 (GTP-CH1), and levels of BH_4 (Hossain et al., 1992; Weiner et al., 1991). To clarify the effects of SSRIs on the central nervous system in major depression, we examined the effects of paroxetine on BH_4 levels and DA and 5-HT turnover in our novel animal model that simulates two of the major environmental risk factors of human depression, i.e., social isolation and novelty stress (Miura et al., 2002a, b, 2004, 2005a).

Social isolation changed the effects of paroxetine on BH_4 levels and monoamine turnover. In the isolation-housing group, paroxetine suppressed the increase in BH_4 levels elicited by novelty stress, whereas paroxetine did not change BH_4 levels in the group-housing group in either brain region under investigation. In mice exposed to the group-housing condition, paroxetine suppressed DA and 5-HT turnover, whereas it did not alter monoamine turnover in the isolation-housing group, with the exception of a decrease in 5-HT turnover in the prefrontal cortex. In other words, social isolation enhanced the BH_4 response to acute environmental stress, and appeared to preferentially induce BH_4 inhibitory effects of paroxetine over effects on DA and 5-HT turnover.

Our recent studies revealed decreases in BH_4 levels as well as decreases in DA and 5-HT turnover elicited by fluvoxamine, an SSRI, in the mouse brain (Miura et al., 2004, 2005a, b). In the present study, we found that paroxetine, another SSRI, also decreased BH_4 levels, as well as DA and 5-HT turnover in the mouse mesoprefrontal system. Thus, we replicated our recent findings using another SSRI. Because paroxetine inhibited 5HTT function, as determined by the reuptake of 5-HT in the synaptic cleft, paroxetine suppressed 5-HT turnover. This explanation is consistent with our results. Nevertheless, the question remains: how did paroxetine suppress BH_4 levels and DA turnover? The mechanism related to decreases in BH_4 levels remains to be clarified. We considered two possible explanations for the suppressive effects of paroxetine on DA turnover, although both of these explanations remain at the level of speculation. The first explanation involves the regulation of DA neuron activity by the innervation of 5-HT neurons. The 5-HT innervations of the DA system are thought to attenuate the activity of DA neurons, and thus paroxetine may have potentiated the attenuation by increasing the level of 5-HT (Di Mascio et al., 1998; Dong et al., 1999). The second possibility is that paroxetine may have suppressed TH activity via the decrease in BH_4 levels, and thereby suppressed DA biosynthesis. A study using 6-pyruvoyltetrahydropterin synthase-knockout mice (i.e., mice in which the second step of BH_4 biosynthesis is blocked) showed that the suppression of TH activity in the brain did not affect TPH activity (Sumi-Ichinose et al., 2001). In a study by Flatmark (2000), TH activity was highly dependent on the intracellular concentration of BH_4. Thus, paroxetine-induced BH_4 suppression may be related to the decrease in DA biosynthesis and turnover.

In the present study, we found elevated BH_4 levels elicited by novelty stress under the social isolation condition. Animal models using a stress protocol have shown neurochemical changes in the brain. One review noted that intracellular concentrations of BH_4, which are mainly determined by GTP-CH1, probably regulate the activity of TH and TPH (Nagatsu and Ichinose, 1999). Thus, the increase in BH_4 levels elicited by novelty stress may have been related to the activities of TH and TPH. Previous studies have shown that physiological stress increased levels of DA, BH_4, TH, and GTP-CH1 (Kim et al., 2005), and elevated brain TH and TPH activity (Boadle-Biber et al., 1989; Chamas et al., 1999; Serova et al., 1998). Physiological stress is known to induce GTP-CH1 (van Amsterdam and Opperhuizen, 1999). Thus, novelty stress may have elevated GTP-CH1 activity, increased the BH_4 concentration, and differentially regulated TH and TPH activity in each brain region.

Further, we observed that changes in BH_4 level elicited by novelty stress converted the direction according to housing condition. Under the isolation-housing condition, novelty stress significantly increased BH_4 levels, whereas the stress significantly decreased BH_4 levels under the group-housing condition in both regions, the prefrontal cortex and the midbrain. These results suggest that social isolation alters the response to acute environmental stress and in particular enhances the biosynthesis of BH_4. Social isolation was also found to change monoamine turnover in response to novelty stress. Although the mechanisms of BH_4 elevation elicited by novelty stress and the effect of isolation housing on this type of elevation remain unknown, our results suggest that isolation housing enhanced the elevation of BH_4 levels elicited