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.** ©2007 Wiley-Liss, Inc.

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 $21 \times 31 \times 13$ cm, and the cages used for the isolation-housing condition were $17 \times 29 \times 13$ 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).

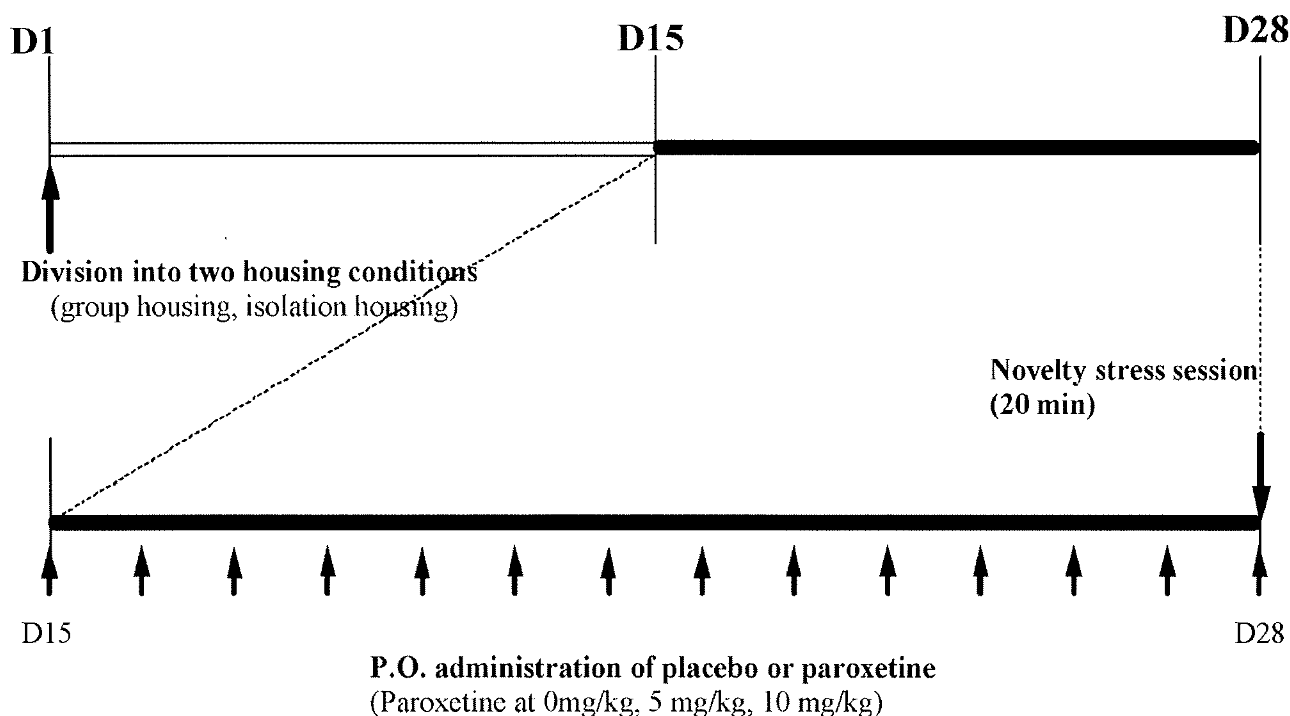


Fig. 1. Experimental time schedule. Mice were divided into 12 groups as described in Materials and Methods.

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.

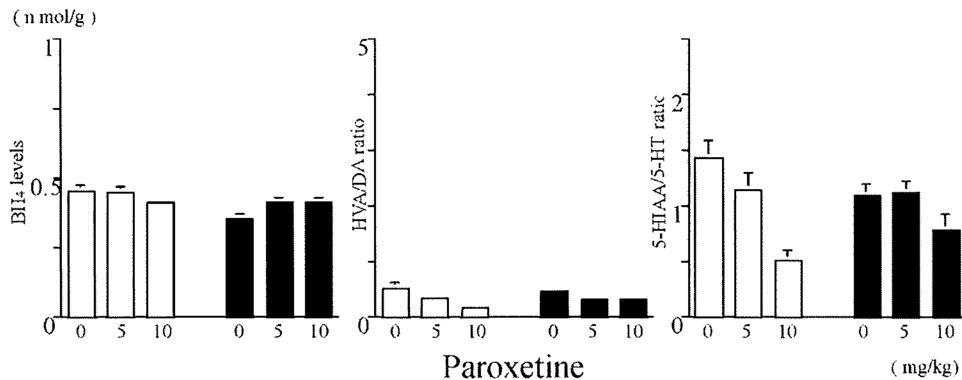
Midbrain

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-

A Group housing

BH4	HVA/DA	5-HIAA/5-HT
Novelty stress **	Novelty stress n.s.	Novelty stress n.s.
Paroxetine n.s.	Paroxetine	Paroxetine
	0 mg/kg vs. 5 mg/kg *	0 mg/kg vs. 10 mg/kg **
	0 mg/kg vs.10 mg/kg **	5 mg/kg vs. 10 mg/kg **



B Isolation housing

BH4	HVA/DA	5-HIAA/5-HT
Novelty stress **	Novelty stress n.s.	Novelty stress n.s.
Paroxetine	Paroxetine n.s.	Paroxetine
0 mg/kg vs. 5 mg/kg **		0 mg/kg vs. 10 mg/kg *
0 mg/kg vs.10 mg/kg **		

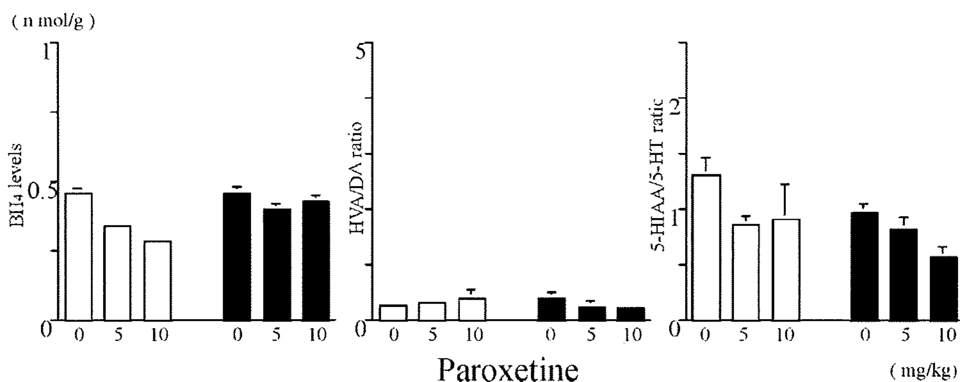
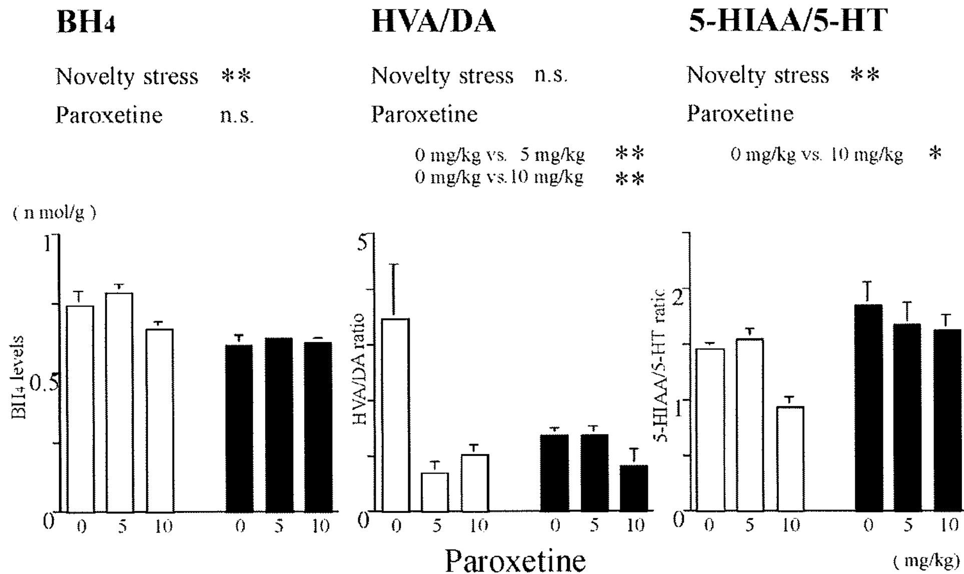


Fig. 2. Changes in BH_4 levels, and in HVA/DA and 5-HIAA/5-HT ratios in the prefrontal cortex elicited by novelty stress and by paroxetine. **A**: Group-housing condition ($n = 48$); (**B**) isolation-housing condition ($n = 48$). White bars, nonstress ($n = 48$); black bars, novelty stress ($n = 48$, $n = 96$ 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



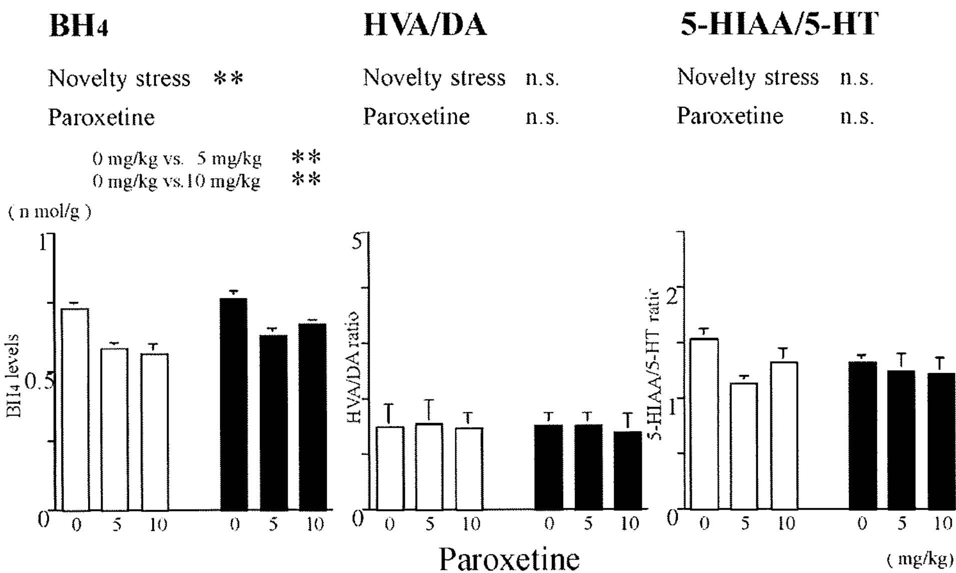


Fig. 3. Changes in BH_4 levels, and in HVA/DA and 5-HIAA/5-HT ratios in the midbrain elicited by novelty stress and by paroxetine. **A**: Group-housing condition ($n = 48$); (**B**) isolation-housing condition ($n = 48$). White bars, nonstress ($n = 48$); black bars, novelty stress ($n = 48$, $n = 96$ 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

by novelty stress. Further in vivo study using GTP-CH1 inhibitors will help to clarify the mechanisms of these BH_4 stress responses. Although 5-HT turnover was enhanced by novelty stress in the midbrain of the mice exposed to the group-housing condition, novelty stress did not change 5-HT turnover in the same region of the brain in the mice exposed to the isolation-housing condition. Thus, social isolation suppressed the increase in 5-HT turnover elicited by acute environmental stress in the midbrain.

Although the results indicating the influence of social isolation on changes in BH_4 levels elicited by novelty stress were somewhat different from those of our recent studies (Miura et al., 2005a), the discrepancy may have been because of differences in animal age and the stress protocol of the two studies. In the present study, mice were isolated at 9 weeks of age and reared for 28 days, whereas they were isolated at 7 weeks and reared for 35 days in our recent study (Miura et al., 2005a). Neurobiological development may therefore have been differed in the two studies. The novelty stress session consisted of one 20-min exposure in the present study, whereas two 10-min habituation sessions, followed by one 20-min true stress session, were used in our recent study. Thus, the present study utilized a more "acute" and "novel" stress procedure. We assume that the influence of "novelty" stress was more precisely detectable in the present study because of this revision of the protocol.

As mentioned above, social isolation altered the response to acute environmental stress, as determined by changes in BH_4 levels and 5-HT turnover. The BH_4 response to stress was enhanced in both regions of the brain examined, i.e., in the prefrontal cortex and midbrain, whereas the increased 5-HT turnover response to stress was suppressed in the midbrain. These results suggest that social isolation strengthens the BH_4 biosynthetic response to novelty stress, suppressing 5-HT release and reuptake in response to the stress. In other words, social isolation modified neuronal activity in response to the stress. Furthermore, social isolation altered the effects of paroxetine on BH_4 levels, as well as DA and 5-HT turnover. Social isolation shifted the main effects of paroxetine, which were to attenuate DA and 5-HT turnover, to a suppression of BH_4 elevation, which had been elicited by novelty stress. The underlying mechanism responsible for this shift remains unknown. However, the effects of paroxetine changed in such a manner that the expected changes (i.e., elevated BH_4 levels elicited by social isolation and novelty stress) were suppressed. These findings suggest that two environmental factors, namely, social isolation and novelty stress, which are known to be closely related to the etiology and pathophysiology of major depression, elicited modifications in neurochemical activity and shifted the effects of paroxetine, thus countering the neurochemical changes elicited by these factors. Investigation of the underlying mechanisms responsible for the modification of BH_4 levels by environmental factors will help clarify the neuropharmacological regulation of major depression by SSRIs.

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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.

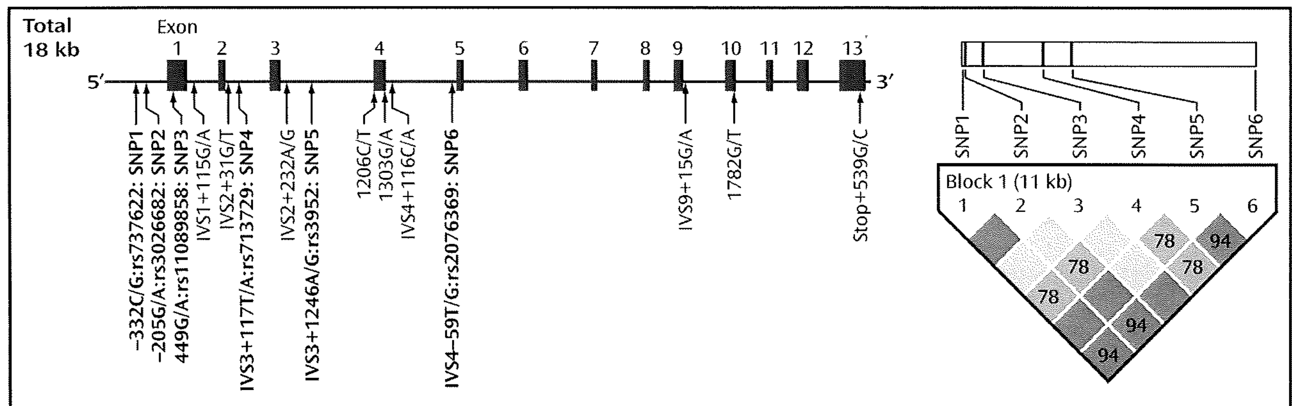
Identification of SNPs

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′-CGACGCGTCCGGACTCAATTAGCCACCT-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

FIGURE 2. Schematic Diagram of 5′-Upstream Region of the PICK1 Gene[a]

```
113581 ctgtccggactcaattagccacctaaggagagagtagggcggggcttccaccggccgtgg  SNP1:-332 C/G rs737622
113641 gatatgtggataatcatccttctgtatctttcttccatggctcctggggcagctggggaa
113701 gcaagctggatgggcctggccccatgctgccggatgaggtggatgcctggctgtggctct  SNP2:-205 G/A rs3026682
113761 gggagagccaacctcccccagggaacccactttacacaatagcagtggcagcagaggctg
113821 gcgaggagacaagattcggactctggggagcactgatagcatttcccgagcctcaggtac
113881 atgcggaccgtgaccctccctgggaccccaggggggctgctcctcaggactaaggaagga
113941 ggagggggtgtgagaaacctttcaccatataccatagaaagcatttacctcaatggcctt
                         ↑
114001 ggtttacatatggggaaactgaggcacataaagggaagggagcatgtccagtctgtcctt
114061 aatagcaagacccactgaatacacctctcctggctctctgtttagtgtttggacgttcaa
114121 agatccctagactaggcggcgggagtttcagggccacgatccagatcttacaccaactgt
114181 gtgtggccccgcacaaaatcactccccgctctttggcacttaagttggcgaaactgggat
114241 gggctgggacctcaaagggccattctagtaggggagtcacaggcccaggtggtgaagggg
114301 tgaaagggcatgatgtcttggggtttatagtccactgagcctcgccggaggtaaccccgg
114361 ctcagggatgctttcgttgccatggcaaccgccgggccggcgcggcccctgagtgcagc  SNP3:+449 G/A rs11089858
114421 tgaggaagctgggacaaaccctgcccttcccaagatggcggcggcggcagggcaaagggc
114481 ggggttagacgctgtcagcct...(exon1)...
114841 ggcctggagccccccctttgtacctagtaagaatcacctac...(intron 1)...
115021 ccggatccagttccccattcccctaccgagctgggcagttagccagcccactccaactct
115081 cggaaccatgtttgcagacttggattatgacatcgaagaggataaactgt...(exon2)...
```

[a] The numbers indicate the nucleotide positions cited from the NCBI database AL031587. A bold black arrow indicates the transcription start position we identified, which was 513 bp before the start position (114471) reported in the database. Blue characters indicate exons of PICK1, and the translation start codon, ATG, is orange. The positions of the three SNPs we identified are indicated in red.

distributions of SNP1, SNP2, and SNP5 were completely the same (Table 3). The allele frequencies and genotype distributions of SNP1, 3, 4, and 6 in methamphetamine abusers and comparison subjects are shown in Table 3. The genotype distributions were within the Hardy-Weinberg equilibrium.

We found significantly different frequencies between comparison subjects and methamphetamine abusers in SNP4 (Table 3). The frequency (88.7%) of carrying the T allele among the methamphetamine abusers was significantly higher (odds ratio=1.58, 95% confidence interval [CI]=1.06–2.34, $p<0.03$) than that of the comparison subjects (83.3%), and we also detected a different distribution of genotype ($p<0.03$). Positive associations were detected in the subgroup of those who experienced psychosis (alleles, $p=0.007$, odds ratio=1.79, 95% CI=1.17–2.74, genotype, $p<0.02$), transient-type psychosis (alleles, $p=0.01$, odds ratio=2.03, 95% CI=1.17–3.51, genotype, $p<0.03$), and psychosis with spontaneous relapse (alleles, $p=0.003$, odds ratio=2.61, 95% CI=1.35–5.07, genotype, $p=0.004$) and in abusers without polysubstance abuse (alleles, $p<0.03$, odds ratio=2.26, 95% CI=1.09–4.67, genotype, $p<0.04$) (Table 3). For SNP6, the frequency (48.7%) of the T allele among methamphetamine abusers who experienced psychosis with spontaneous relapse was significantly higher (odds ratio=1.62, 95% CI=1.19–2.35, $p<0.02$) than that of the comparison subjects (36.9%), and we also detected a different distribution of genotype ($p<0.02$) (Table 3). In contrast, no differences for SNP1, 2, 3, and 5 were detected between methamphetamine abusers and comparison subjects (Table 3).

TABLE 3. Genotypic and Allelic Distributions of the PICK1 Gene Polymorphisms in Comparison Subjects and Methamphetamine Abusers

Variable		Genotype							Allele				
		C/C		C/G		G/G			C		G		
SNP1[a] (rs737622)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	89	40.8	107	49.1	22	10.1		285	65.4	151	34.6	
Methamphetamine abusers	208	85	40.9	93	44.7	30	14.4	0.35	263	63.2	153	36.8	0.52
Psychosis	178	66	37.1	87	48.9	25	14.0	0.45	219	61.5	137	38.5	0.27
Transient	100	38	38.0	48	48.0	14	14.0	0.56	124	62.0	76	38.0	0.42
Prolonged	78	28	35.9	39	50.0	11	14.1	0.53	95	60.9	61	39.1	0.33
Spontaneous relapse													
Positive	77	32	41.6	33	42.9	12	15.6	0.37	97	63.0	57	37.0	0.62
Negative	118	48	40.7	55	46.6	15	12.7	0.73	151	64.0	85	36.0	0.74
Polysubstance abuse													
No	55	23	41.8	23	41.8	9	16.4	0.35	69	62.7	41	37.3	0.66
Yes	140	58	41.4	63	45.0	19	13.6	0.53	179	63.9	101	36.1	0.75
		G/G		G/A		A/A			G		A		
SNP3 (rs11089858)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	180	82.5	37	17.0	1	0.5		397	91.1	39	8.9	
Methamphetamine abusers	208	167	80.3	39	18.8	2	1.0	0.71	373	89.7	43	10.3	0.56
Psychosis	178	143	80.3	34	19.1	1	0.6	0.80	320	89.9	36	10.1	0.63
Transient	100	81	81.0	19	19.0	0	0.0	0.83	181	90.5	19	9.5	0.88
Prolonged	78	62	79.5	15	19.2	1	1.3	0.47	139	89.1	17	10.9	0.52
Spontaneous relapse													
Positive	77	64	83.1	13	16.9	0	0.0	1.00	141	91.6	13	8.4	1.00
Negative	118	94	79.7	23	19.5	1	0.8	0.65	211	89.4	25	10.5	0.49
Polysubstance abuse													
No	55	44	80.0	11	20.0	0	0.0	0.75	99	90.0	11	10.0	0.71
Yes	140	112	80.0	26	18.6	2	1.4	0.58	250	89.3	30	10.7	0.44
		T/T		T/A		A/A			T		A		
SNP4 (rs713729)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	150	68.8	63	28.9	5	2.3		363	83.3	73	16.7	
Methamphetamine abusers	208	166	79.8	37	17.8	5	2.4	<0.03	369	88.7	47	11.3	<0.03
Psychosis	178	145	81.5	30	16.9	3	1.7	<0.02	320	89.9	36	10.1	0.007
Transient	100	83	83.0	16	16.0	1	1.0	<0.03	182	91.0	18	9.0	0.01
Prolonged	78	62	79.5	14	17.9	2	2.5	0.14	138	88.5	18	11.5	0.15
Spontaneous relapse													
Positive	77	67	87.0	9	11.7	1	1.3	0.004	143	92.9	11	7.1	0.003
Negative	118	88	74.6	26	22.0	4	3.4	0.36	202	85.6	34	14.4	0.51
Polysubstance abuse													
No	55	47	85.5	7	12.7	1	1.8	<0.04	101	91.8	9	8.2	<0.03
Yes	140	109	77.9	28	20.0	3	2.1	0.16	246	87.9	34	12.1	0.11
		G/G		G/T		T/T			G		T		
SNP6 (rs2076369)	N	N	%	N	%	N	%	p[b]	N	%	N	%	p[b]
Comparison subjects	218	82	37.6	111	50.9	25	11.5		275	63.1	161	36.9	
Methamphetamine abusers	208	73	35.1	99	47.6	36	17.3	0.23	245	58.9	171	41.1	0.23
Psychosis	178	64	36.0	83	46.6	31	17.4	0.25	211	59.3	145	40.7	0.30
Transient	100	34	34.0	48	48.0	18	18.0	0.30	116	58.0	84	42.0	0.25
Prolonged	78	30	38.5	35	44.9	13	16.7	0.41	95	60.9	61	39.1	0.63
Spontaneous relapse													
Positive	77	21	27.3	37	48.1	19	24.7	<0.02	79	51.3	75	48.7	<0.02
Negative	118	46	37.9	56	47.5	16	13.6	0.77	148	62.7	88	37.3	0.93
Polysubstance abuse													
No	55	15	27.3	30	54.5	10	18.2	0.23	60	54.5	50	45.5	0.13
Yes	140	53	37.9	62	44.3	25	17.9	0.19	168	60.0	112	40.0	0.43

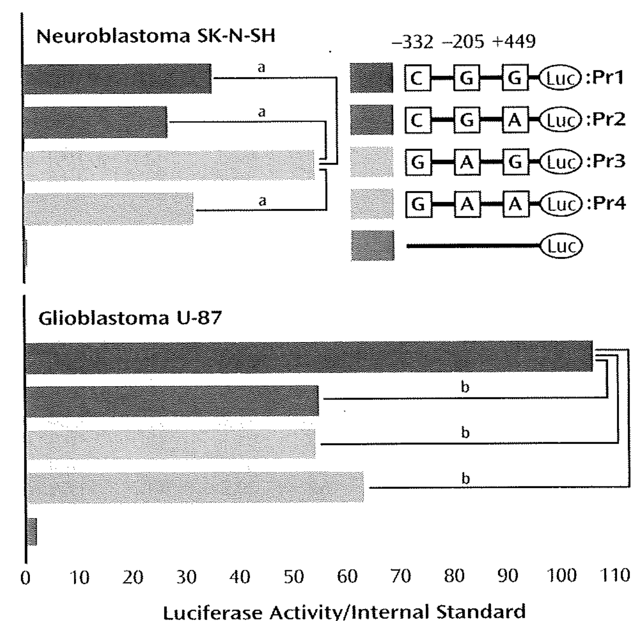
[a] The distributions of SNP2 (rs3026682) and 5 (rs3952) are the same as SNP1 (rs737622).
[b] Versus comparison subjects.

TABLE 4. Haplotype Analysis of Six Single Nucleotide Polymorphisms

Variable	Haplotype Analysis		
Overall			
Haplotype	Comparison Subjects (N=218)	Methamphetamine Abusers (N=208)	p
C-G-G-T-A-T	35.2%	33.7%	0.63
G-A-G-T-G-G	32.3%	32.3%	0.85
C-G-G-A-A-G	14.5%	9.2%	<0.02
C-G-A-T-A-G	8.3%	7.4%	0.66
C-G-G-T-A-G	5.5%	8.9%	<0.09
G-A-G-T-G-T	0.7%	3.5%	0.01
C-G-G-A-A-T	1.2%	1.7%	0.66
G-A-G-A-G-G	1.0%	0.4%	0.40
Methamphetamine abusers			
Haplotype	With Spontaneous Relapse (N=77)	Without Spontaneous Relapse (N=117)	p
C-G-G-T-A-T	42.3%	27.8%	0.001
G-A-G-T-G-G	32.1%	31.1%	0.86
C-G-G-A-A-G	4.5%	12.6%	<0.02
C-G-A-T-A-G	6.8%	6.3%	0.82
C-G-G-T-A-G	6.3%	11.8%	0.14
G-A-G-T-G-T	2.5%	4.9%	0.31
C-G-G-A-A-T	2.5%	1.3%	0.54

As shown in Figure 1, a strong linkage disequilibrium was observed in five of these six SNPs. Two haplotypes, C(SNP1)-G(SNP2)-G(SNP3)-A(SNP4)-A(SNP5)-G(SNP6) and G(SNP1)-A(SNP2)-G(SNP3)-T(SNP4)-G(SNP5)-T(SNP6), were significantly different between comparison subjects and methamphetamine abusers (Table 4). The frequency (9.2%) of the CGGAAG haplotype in the methamphetamine abusers was significantly lower (odds ratio=0.60, 95% CI=0.45–0.79, p<0.02) than that of the comparison subjects (14.5%), and the frequency (3.5%) of the GAGTGT haplotype in the methamphetamine abusers was significantly higher (odds ratio=5.2, 95% CI=2.27–11.6, p=0.01) than that (0.7%) of the comparison subjects (Table 4). Of interest, a haplotype analysis between methamphetamine abusers with and without spontaneous relapse of psychosis showed the significant difference in the most major haplotype (CGGTAT) as well as the CGGAAG type. The frequency (42.3%) of CGGTAT type in the methamphetamine abusers with spontaneous relapse was significantly higher (odds ratio=2.2, 95% CI=1.80–2.61, p=0.001) than that in those without spontaneous relapse (27.8%) (Table 4). As to the frequency of the CGGAGG type, the frequency (4.5%) in methamphetamine abusers with spontaneous relapse was significantly lower (odds ratio=0.33, 95% CI=0.23–0.47, p<0.02) than that in those without spontaneous relapse (Table 4).

FIGURE 3. Relative Luciferase Activity of the Four Haplotypes in SK-N-SH Cells (top) and U-87 Cells (bottom)[a]



[a] The phRL-TK vector used was a negative control. The pGL3 Basic vector, which does not contain any promoter sequences, was used as a negative control. Each value is shown as the mean for three independent experiments.
[b] p<0.001.

Transcriptional Effects of SNPs in the Promoter Region

The transcriptional effects of four promoter haplotypes on SK-N-SH cells and U-87 cells were also examined. As shown in Figure 3, the results for these two cell lines differed. For SK-N-SH cells, a substitution variant, Pr3 (G-332/A-205/A+449), showed significantly increased relative luciferase activity (1.54 for Pr3/Pr1, p<0.001, 2.03 for Pr3/Pr2, p<0.001, 1.74 for Pr3/Pr4, p<0.001). In contrast, for U-87 cells, every substitution showed significantly lower relative luciferase activity than that of the major type, Pr1 (C-332/G-205/G+449) (0.51 for Pr2/Pr1, p<0.001, 0.51 for Pr3/Pr1, p<0.001, 0.59 for Pr4/Pr1, p<0.001).

Discussion

The major findings of the present study were the discovery of an association between PICK1 gene polymorphisms and methamphetamine abusers and the identification of functional SNPs (SNP1 and SNP2) in the promoter region of the PICK1 gene. It was of great interest to find that SNP4 and SNP6 were significantly associated with methamphet-

amine abusers who experienced spontaneous relapse of psychosis. In addition, the haplotype analysis demonstrated that specific haplotypes, C(SNP1)G(SNP2)G(SNP3) A(SNP4)A(SNP5)G(SNP6) and GAGTGT, were significantly associated with methamphetamine abusers in general. Furthermore, we also found that the frequencies of major haplotypes CGGTAT and CGGAAG were significantly different between methamphetamine abusers with and without spontaneous relapse of psychosis. Spontaneous relapse of psychosis among methamphetamine abusers is known as "flashbacks," which are known to follow nonspecific stress, even after the consumption of methamphetamine has ceased and drug treatment has begun, and it appears that a psychotic state might be induced by excess dopaminergic activity (21, 22). Given the role of dopamine systems in the pathogenesis of methamphetamine psychosis, it is possible that a functional alteration of dopamine transporter may be caused by genetic variations in PICK1 and can lead to dysfunction of the dopamine system. Taken together, these results suggest that the CGG-TAT and CGGAAG haplotypes in the PICK1 gene are likely to be associated with the psychosis of methamphetamine abusers who experience spontaneous relapse. The different distributions of those two haplotypes between methamphetamine abusers with and without spontaneous relapse of psychosis also suggest the difference in genetic backgrounds between the two groups. In the present study, the group of subgroups was small. Because of the small size of subcategories, type I error cannot be ruled out. Therefore, further studies with a large group with subcategories would reveal the associations between the PICK1 gene and methamphetamine-induced psychosis.

In the 5′-upstream region of the PICK1 gene, we identified three SNPs (SNP1: –332 C/G, rs737622, SNP2: -205 G/A, rs3026682, and SNP3: 449G/A, rs11089858). A luciferase assay revealed the functional effects of these SNPs on transcriptional activities. Although the threshold scores were low, the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) predicted that the major transcription factors, including GATA1 (for SNP1, score 78.3) and AML-1a (for SNP2, score 83.7), bind to either position of SNPs in the PICK1 promoter position. Of course, it is likely that unidentified transcription factors may also be involved in the transcriptional process because we found that the levels of PICK1 expression could be altered by nucleotide substitutions of these SNPs in the promoter region. After consideration of the role of PICK1 in the proper targeting and surface clustering of dopamine transporter (16), it is possible that altered PICK1 expression might lead to altered dopamine transporter function in synaptic dopamine signal transmission, which would in turn influence the pathogenesis of methamphetamine abuse and related psychotic symptoms.

In this study, we found that transcriptional effects of SNPs in the promoter region of the PICK1 gene differed in SK-N-SH and U-87 cells. The nucleotide substitutions (C→G at –332 and G→A at –205) showed significantly increased luciferase activity in SK-N-SH cells (neuronal cells), whereas the substitutions (C→G at –332 and G→A at –205) showed significantly decreased luciferase activity in U-87 cells (glial cells). Although the mechanisms underlying the discrepancy in these two cell lines are currently unknown, these findings suggest that PICK1 expression could be affected in different ways by these SNPs in neuronal and glial cells. Fujii et al. (20) reported that a haplotype, T(rs713729)-A(rs3952)-T(rs2076369), revealed a statistically significant association with disorganized schizophrenia in methamphetamine abusers in relation to comparison subjects ($p<0.02$). The TAT haplotype, discussed by Fujii and coworkers, was found to correspond to C(rs737622: SNP1)-G(rs3026682: SNP2)-G(rs11089858: SNP3)-T(rs713729: SNP4)-A(rs3952: SNP5)-T(rs2076329: SNP6) in our study, and it was the most frequent haplotype in both comparison subjects and methamphetamine abusers. As discussed, the frequency (42.3%) of the CGG-TAT haplotype in methamphetamine abusers with spontaneous relapse was significantly higher ($p=0.001$) than that of those without spontaneous relapse (27.8%). These findings also suggest that methamphetamine abusers who experience a spontaneous relapse of methamphetamine psychosis might share a similar genetic susceptibility to schizophrenia.

It has been demonstrated that PICK1 interacts with other proteins, including AMPA receptors (14, 23) and metabotropic glutamate receptor 7 (mGluR7) (24, 25), which have been implicated in the pathophysiology of drug abuse as well as in schizophrenia (26–29). Thus, it seems that interactions of PICK1 with AMPA receptors and metabotropic glutamate receptors are likely to be involved in the pathogenesis of methamphetamine psychosis. Furthermore, Fujii et al. (20) identified PICK1 as a protein interactor with the D-serine synthesizing enzyme serine racemase in glial cells (30). After consideration of the role of D-serine in the pathophysiology of schizophrenia (31–35), it is likely that the interaction of PICK1 with serine racemase in glial cells may play a role in the pathophysiology of methamphetamine psychosis, although further studies will still be necessary.

In conclusion, the present findings revealed that PICK1 gene polymorphisms are associated with methamphetamine abusers, suggesting that the PICK1 gene plays a major role in a genetic susceptibility to methamphetamine psychosis.

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