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

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

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

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

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

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

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

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

## Acknowledgments

We thank the patients and healthy volunteers who participated in our investigation. This study was supported in part by research grants from the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labor, and Welfare of Japan.

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J Pharmacol Sci 103, 175 – 180 (2007)

*Journal of Pharmacological Sciences*


*Full Paper*

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

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*Received September 6, 2006; Accepted December 5, 2006*

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

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

## Introduction

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

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

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

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Published online in J-STAGE: February 14, 2007
doi: 10.1254/jphs.FP0061021

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

## Materials and Methods

*Animals*

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

*Olfactory bulbectomy*

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

*The hole-board test*

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

*Analysis of the concentrations of monoamines and their metabolites*

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

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

*Data analyses*

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

*Drugs*

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

## Results

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

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

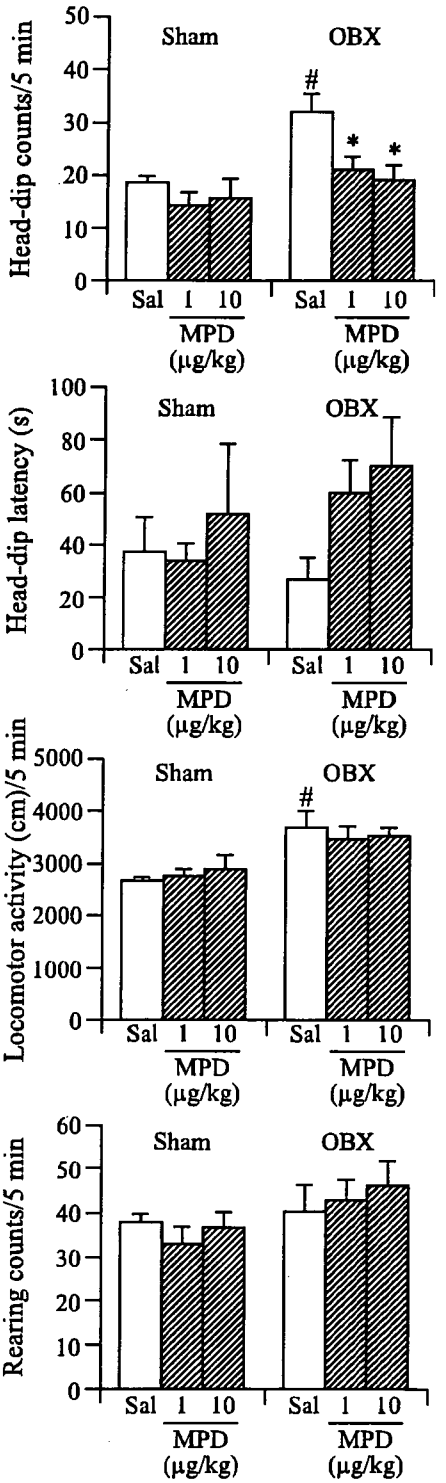


**Fig. 1.** Effects of methylphenidate on the exploratory behavior observed in the sham-operated and OBX mice during the hole-board test. Methylphenidate (MPD; 1 and 10 $\mu$g/kg, respectively) or saline was injected s.c. 30 min prior to the measurement of the exploratory behavior. Each column represents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

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

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

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

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

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

## Discussion

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

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

It has been reported that the decrease in the amounts

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

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

## Acknowledgments

The authors would like to thank Mr. M. Akitaya and Ms. M. Irie for their technical assistance.

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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.

*(Am J Psychiatry 2007; 164:1105–1114)*

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

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

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

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

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

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

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

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

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

## Materials and Methods

### Subjects

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

### Background of Methamphetamine Abusers

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

### Prognosis of Psychosis

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

### Spontaneous Relapse

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

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

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

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

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

### *Polysubstance Abuse*

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

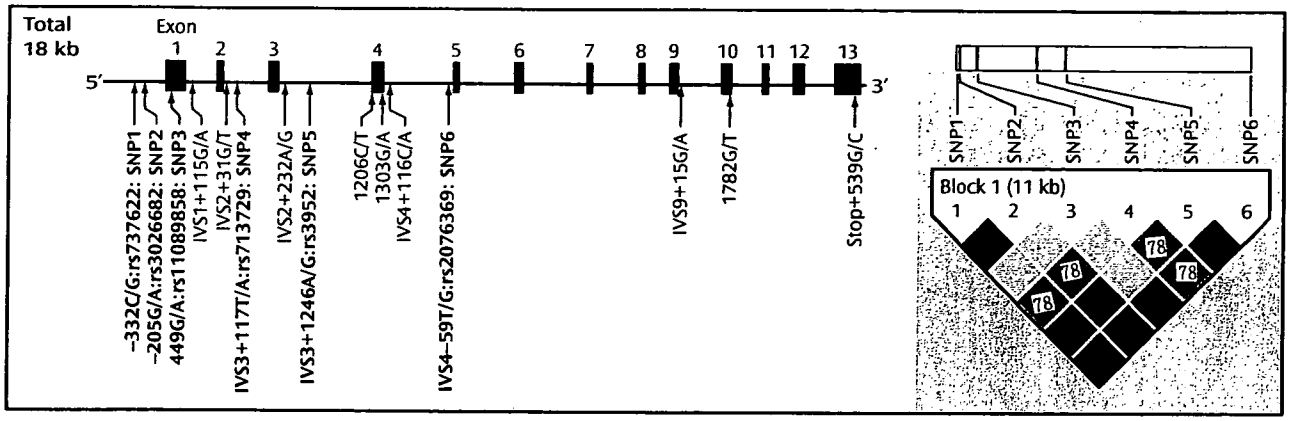
### *Identification of SNPs*

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

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

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

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




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

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

### *Genotyping of Identified SNPs*

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

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

### *Dual-Luciferase Gene Reporter Assays*

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

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

### *Statistical Analysis*

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

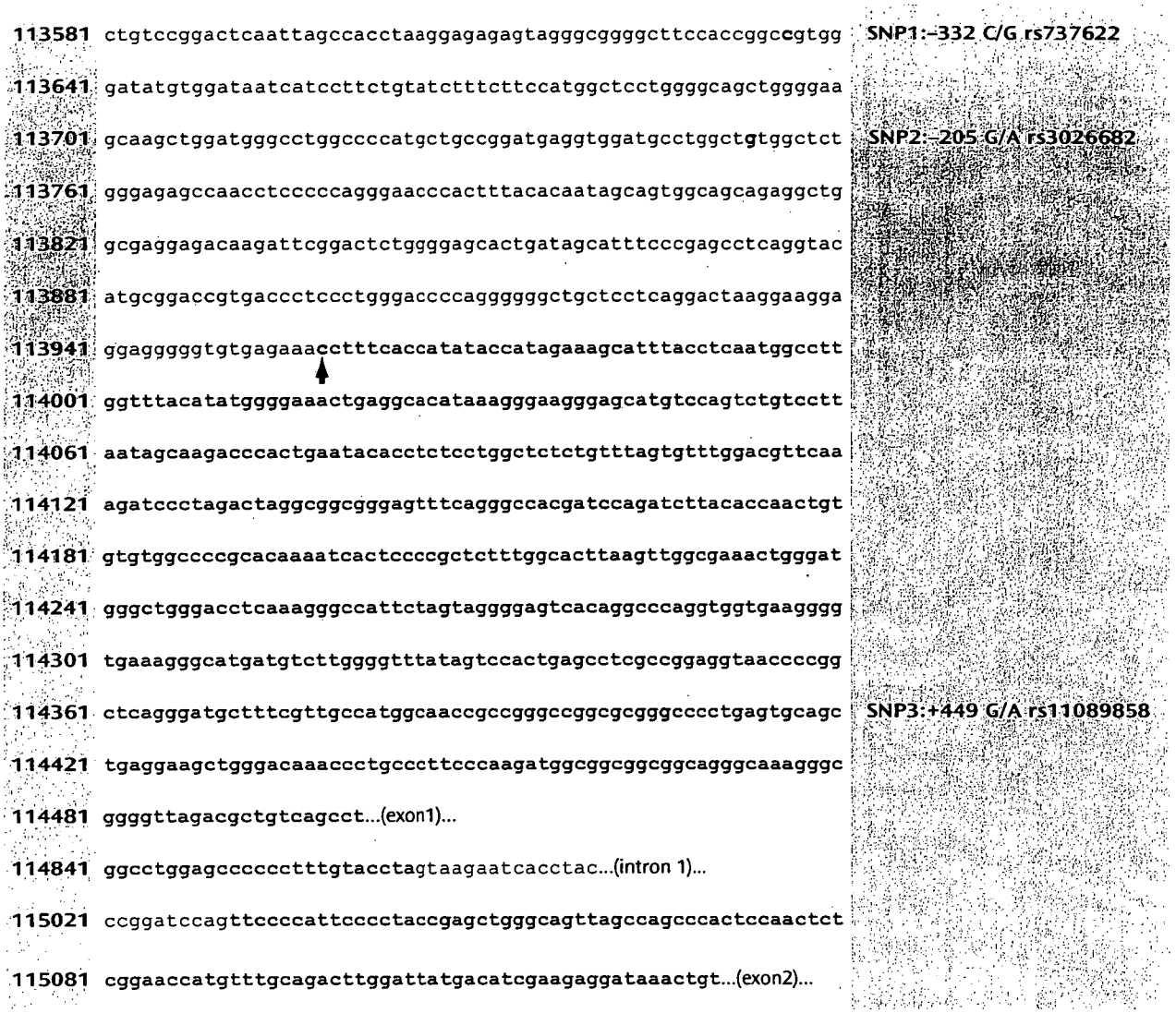
## Results

### *Identification of SNPs and Association Studies*

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

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

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




[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 |

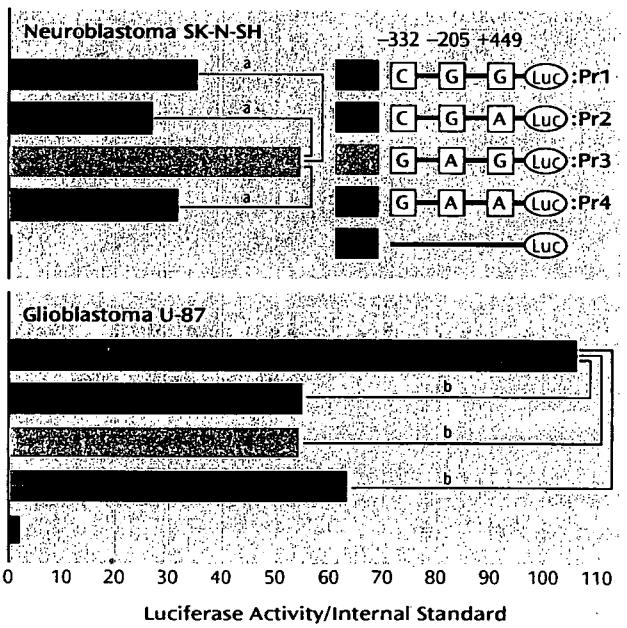
[a] The distributions of SNP2 (rs3026682) and 5 (rs3952) are the same as SNP1 (rs737622).
[b] Versus comparison subjects.

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 CGGTAT 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 CGGTAT 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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All authors report no competing interests.

Supported in part by a grant for psychiatric and neurological diseases and mental health from the Ministry of Health, Labor and Welfare (Dr. Hashimoto) and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (Dr. Hashimoto).

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human psychopharmacology
Hum. Psychopharmacol Clin Exp 2008; 23: 121–128.
Published online 20 November 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/hup.907

# Influence of the tyrosine hydroxylase val81met polymorphism and catechol-O-methyltransferase val158met polymorphism on the antidepressant effect of milnacipran

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**Objective** Genetic polymorphisms of the noradrenergic pathway can be factors to predict the effect of antidepressants when their pharmacological mechanisms of action include the noradrenergic system. The purpose of the present study was to determine whether the tyrosine hydroxylase (TH) val81met and catechol-O-methyltransferase (COMT) val158met polymorphisms are associated with the antidepressant effect of milnacipran, a serotonin/noradrenaline reuptake inhibitor.
**Method** Eighty-one Japanese patients with major depressive disorder were treated with milnacipran for 6 weeks. Severity of depression was assessed with the Montgomery and Åsberg Depression Rating Scale (MADRS). Assessments were carried out at baseline and at 1, 2, 4 and 6 weeks of treatment. The method of polymerase chain reaction was used to determine allelic variants.
**Results** The met/met genotype of the COMT val158met polymorphism was associated with a significantly faster therapeutic effect of milnacipran in the MADRS score during this study. No influence of the TH val81met polymorphism on the antidepressant effect of milnacipran was detected.
**Conclusion** These results suggest that the COMT val158met polymorphism in part determines the antidepressant effect of milnacipran. Copyright # 2007 John Wiley & Sons, Ltd.

key words— catechol-O-methyltransferase; major depressive disorder; milnacipran; polymorphism; tyrosine hydroxylase

## INTRODUCTION

Individual genetic differences of monoaminergic pathways can have an impact on the effect of antidepressant agents, though the exact mechanism of their action is still unclear. Several lines of evidence have suggested the relationship between genetic polymorphisms of the serotonergic pathway, especially those of the 5-hydroxytriptamine transporter (5-HTT), and the antidepressant effect of selective serotonin reuptake inhibitors (SSRIs) (Binder and Holsboer, 2006).

Genetic polymorphisms of the noradrenergic pathway as well as serotonergic pathway could also affect the effect of antidepressants, especially when their pharmacological mechanisms of action include the noradrenergic system. Tyrosine hydroxylase (TH) is

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Received 7 June 2007
Accepted 3 October 2007

the initial and rate-limiting enzyme in the biosynthesis of catecholamine neurotransmitters including noradrenaline. The TH val81met polymorphism in exon 2 (Ludecke and Bartholome, 1995) is located in the amino-terminal regulatory domain of the tetrameric enzyme. The regulatory region is reported to have an inhibiting effect on the enzymatic function (Kumer and Vrana, 1996). Catechol-O-methyltransferase (COMT) is an important enzyme involved in degradation of catecholamine neurotransmitters including noradrenaline. The COMT val158met polymorphism located in exon 4 (Lotta et al., 1995) was reported to be associated with variation in COMT enzyme activity (Lachman et al., 1996).

No pharmacogenetic study addressed the relationship between the TH val81met polymorphism and antidepressant response. Only two studies investigated the relationship between the COMT val158met polymorphism and antidepressant response to SSRIs and mirtazapine. One reported its no overall effect on the antidepressant response to SSRIs (Arias et al., 2006), and the other reported its significant effect on the antidepressant response to mirtazapine but not paroxetine (Szegedi et al., 2005).

So far, there has been no study investigating the relationship between the TH val81met polymorphism, the COMT val158met polymorphism and the antidepressant response to serotonin noradrenaline reuptake inhibitors (SNRIs), although noradrenergic genetic factors could be one of the most plausible candidates for pharmacogenetic analysis of SNRIs. The class of SNRIs now comprises of three medications: venlafaxine, duloxetine and milnacipran. Among SNRIs, venlafaxine has a high affinity for the 5-HTT but not the noradrenaline transporter. Duloxetine has a more balanced affinity but is still more selective for the 5-HTT. Milnacipran is the most balanced and may even be slightly more noradrenergic than serotonergic (Stahl et al., 2005). Thus, the authors investigated whether the above two noradrenergic polymorphisms affect the antidepressant effect of milnacipran.

## SUBJECTS AND METHODS

### Subjects

For the present study, one subject treated with milnacipran was added to those in our previous study (Yoshida et al., 2007). Detailed inclusion criteria have been described previously (Yoshida et al., 2007). In brief, the subjects were Japanese patients who fulfilled DSM-IV criteria for a diagnosis of major depressive disorder and whose scores on the Montgomery Åsberg Depression Rating Scale (MADRS) (Montgomery and Åsberg, 1979) were 21 or higher. Patients with other axis I and II disorders determined by clinical interview and those with severe nonpsychiatric medical disorders were excluded. The patients were 25–69 years of age (mean age ( SD) ¼ 51.1 12.3) and had been free of psychotropic drugs at least 14 days before entry into the study. After complete description of the study to the subjects, written informed consent was obtained. This study was approved by the Ethical Committee of Akita University School of Medicine and Nagoya University Graduate School of Medicine. The clinical characteristics of the patients are shown in Table 1. There was no significant difference between responders and nonresponders in regard to sex, age, number of previous episodes and presence of melancholia.

### Milnacipran treatment

Milnacipran was administered twice daily (the same dose after dinner and at bedtime) for 6 weeks. The initial total daily dose was 50 mg/day, and after a week it was increased to 100 mg/day. Patients with insomnia were prescribed 0.25 or 0.5 mg of brotizolam, a benzodiazepine sedative hypnotic, at bedtime. No other psychotropic drugs were permitted during the study. Of 98 enrolled patients, 10 did not complete the study: five patients because of side effects, one patient because of severe insomnia and four patients without explanation. Of the 88 patients who completed the 6-week study, seven patients were excluded from the

Table 1. Clinical characteristics of the patients (responders and nonresponders)

| | Responders (n¼ 51) | Nonresponders (n¼ 30) | | p |
|---|---|---|---|---|
| Sex (male/female) | 20/31 | 9/21 | $x^2$¼ 0.70 | 0.40[a] |
| Age (year) ( SD) | 50.7 12.4 | 51.8 12.2 | t¼ 0.41 | 0.68[b] |
| No. of previous episodes ( SD) | 0.47 1.3 | 0.23 0.6 | t¼ 0.97 | 0.34[b] |
| Melancholia (p/ ) | 16/35 | 9/21 | $x^2$¼ 0.017 | 0.90[a] |

[a]Analysis performed with the use of the $x^2$ test.
[b]Analysis performed with the use of the unpaired t-test.


Hum. Psychopharmacol Clin Exp 2008; 23: 121–128.
DOI: 10.1002/hup