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ORIGINAL INVESTIGATION

Effects of milnacipran and fluvoxamine on hyperemotional behaviors and the loss of tryptophan hydroxylase-positive cells in olfactory bulbectomized rats

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Abstract
Rationale It has been reported that many of the behavioral and serotonergic neuronal changes observed in olfactory bulbectomy (OBX) were improved by subchronic administration of a variety of antidepressants.
Objective We examined the effects of subchronic treatment with milnacipran, a dual serotonin and noradrenaline reuptake inhibitors (SNRIs) and fluvoxamine, selective serotonin reuptake inhibitors (SSRI) in the OBX-induced hyperemotional behaviors and tryptophan hydroxylase (TPH), rate-limiting enzyme of 5-HT.
Materials and methods The olfactory bulbs were removed by suction. Drugs were administered p.o. once daily for 8 days beginning 14 days post-surgery. The hyperemotionality behaviors of OBX rats were measured by rating scale and in the elevated plus-maze test.
Results OBX rats, after milnacipran or fluvoxamine treatment, showed significant decrease in the score of hyperemotional responses on 7th day as compared with vehicle-treated OBX rats. In addition, milnacipran and fluvoxamine in OBX rats respectively produced a significant increase in the percentage of time spent in and number of entries into open arms in the elevated plus maze test. Furthermore, when 5-HTnergic neuronal function was examined using antibodies against tryptophan hydroxylase (TPH) following the behavioral tests, fluvoxamine significantly reversed the loss of TPH-positive cells produced by OBX in the dorsal raphe.
Conclusion We demonstrated that chronic treatment with milnacipran or fluvoxamine was effective to improve both the hyperemotional behavior and the loss of TPH-positive cells seen in OBX rats.

Keywords Olfactory bulbectomy · Milnacipran · Fluvoxamine · Tryptophan hydroxylase

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Introduction

The following behavioral abnormalities have been observed after bilateral olfactory bulbectomy: stress-induced increase in locomotor activity and increases in various measures of irritability or hyperemotional responses to given stimuli (Thorne and Rowles 1988; Redmond et al. 1997; Okuyama et al. 1999; Ho et al. 2001, 2004; Saitoh et al. 2003; Chaki et al. 2004). It has been reported that OBX-induced behavioral abnormalities are reversed by chronic adminis-

tration of a wide range of clinically effective antidepressant drugs, including selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs; see review Kelly et al. 1997; Song and Leonard 2005). These reports suggest that impairment of the central serotonergic system may play an important role in mediating the behavioral changes observed after OBX. In addition, it was reported that the lower 5-HT synthesis in the dorsal raphe, which contains cell bodies of 5-HTnergic neurons were observed in the OBX rats and that this reduction was reversed by chronic treatment with SSRI citalopram (Watanabe et al. 2003; Hasegawa et al. 2005). An imbalance in the serotonergic system is specifically observed in the OBX syndrome, as there have been consistent observations of abnormal 5-HT concentration, synthesis and receptor expression throughout the limbic system (Lumia et al. 1992; Watanabe et al. 2003). Indeed, many investigators demonstrated that behavioral and neurochemical changes induced by OBX were attenuated by chronic (but not acute) antidepressant treatment (see review Song and Leonard 2005). Thus, it has been suggested that the OBX procedure is a useful model for detecting antidepressant activity.

Previously, the OBX procedure increased the time spent in the closed arm of a plus-maze, and these behavioral changes were significantly reversed by treatment with antidepressants and anxiolytic agents (Yamaguchi et al. 2002). Similarly, OBX rats exhibited hyperemotional responses to given stimuli and a decrease in the time spent in the open arm of a plus-maze; these behavioral changes were reduced by subchronic treatment with the antidepressant desipramine for 7 days (Saitoh et al. 2003). Thus, it was suggested that the evaluation of hyperemotional behaviors and the performance on a plus-maze in OBX rats may provide a suitable model for evaluating antidepressants (Yamaguchi et al. 2002; Saitoh et al. 2003, 2006).

On the other hand, the concentration of TPH in the frontal cortex, which is a terminal area in the serotonergic neuron, was elevated in the OBX rats, and these changes were reversed by the chronic treatment with tricyclic antidepressants imipramine (Grecksch et al. 1997). Tryptophan hydroxylase (TPH) can influence the efficacy of serotonergic transmission and provide the major means of regulating 5-HT presynaptic homeostasis. Previously, it has been reported that the changes in TPH activity, rate-limiting enzyme of 5-HT, led to corresponding alterations in the amount of 5-HT released into the synaptic space (Gartside et al. 1992). However, the influence of olfactory bulbectomy and the subchronic SSRI and/or SNRIs treatment on TPH activity in the raphe area, which contains the cell bodies of serotonergic neuron, is unknown.

In the present study, we chose two primary antidepressants with distinct targets: fluvoxamine, a selective serotonin reuptake inhibitors (SSRI), and milnacipran, a dual serotonin and noradrenaline reuptake inhibitors (SNRIs). Initially, we examined the effects of subchronic treatment with milnacipran and fluvoxamine on hyperemotional behaviors in OBX rats. Furthermore, to assess the influence of olfactory bulbectomy and the subchronic milnacipran and/or fluvoxamine treatment on presynaptic serotonergic transmission, we examined the influence of TPH-positive cells in dorsal and median raphe using antibodies against TPH following the behavioral tests in OBX rats.

Materials and methods

Animals

Male 8-week-old (200–240 g) Wistar rats (Charles River Japan, Kanagawa, Japan) were used. They had free access to food and water in an animal room that was maintained at 22±1°C with a 12-h light–dark cycle (light automatically on at 8:00 A.M.). This study was carried out in accordance with the Declaration of Helsinki and the guide for the care and use of laboratory animals of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture.

Olfactory bulbectomy-induced hyperemotionality in rats

Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed in a stereotactic apparatus (ASI Instruments, USA). The olfactory bulbs were removed by suction. Postoperatively, animals were housed in single cages. Fourteen days post-surgery, hyperemotionality was measured using a modification of the procedure described by Brady and Nauta (1955) and Shibata et al. (1984). Hyperemotionality of rats was measured by scoring the responses to the following stimuli: (1) Bite response: Bite response is scored by a rod presented 4–5 cm in front of the snout, (2) startle response: Startle response to a stream of air directed at the dorsum was scored. The air was delivered using 5-ml syringe, (3) struggle response: Struggle response was scored by handling with a gloved hand (struggle response), and (4) fight response: Fight response was scored by the tail pinching with a forceps. The rat tail is gently held from the back of a rat using mosquito forceps. The trained researcher performed these operations. These responses were graded as follows: 0, no reaction; 1, slight; 2, moderate; 3, marked; or 4, extreme response. For each emotional response, audible vocalization were also scored and graded as follows, 0, no vocalization; 1, occasional vocalization; or 2, marked vocalization. The vocal score was added to each emotional response score. All animals in each group were observed in 1 day. The score for each

animal in emotional response was given within 5 min. The observers were blind with respect to the drug treatment. Only rats that exhibited hyperemotionality (score, >14) were selected for further study. Ninety-five rats as OBX rats were selected from 110 rats.

Elevated plus-maze test

The plus-maze (Neuroscience, Tokyo, Japan) consisted of a black Plexiglas apparatus with two open arms, 50×10 cm, and two closed arms, 50×10×50 cm, with an open roof; these arms are arranged such that the two open arms are opposite to each other (Pellow et al. 1985). Two opposing arms are delimited by vertical walls (closed arms), whereas the other two opposing arms have unprotected edges (open arms). The maze was elevated 70 cm above the ground and placed in indirect light. At the beginning of the 5-min test session, each rat was placed in the central platform area and faced one of the open arms. Several classical parameters were monitored during the session, including the following: (1) open arm duration, i.e., the total amount of time spent by the rat in an open arm; (2) closed arm duration, i.e., the total amount of time spent by the rat in a closed arm; (3) open arm entries, i.e., the total number of entries with all four paws into the open unprotected arms; (4) closed arm entries, i.e., the total number of entries with all four paws into the closed protected arms; and (5) open arm frequencies, i.e., the ratio of cumulative time spent in the open arms to the total time expressed as a percentage of the time spent in the open arms. The ratio of the cumulative number of visits to the open arms to the total number of visits was expressed as the percentage of open arm entries. The total number of visits and the cumulative time spent were then determined automatically on a monitor through a video camera system. All of the data were stored in a personal computer and analyzed using analytical software (Comp ACT HBS, Muromachi Kikai, Japan). This protocol was based on that previously described (Saitoh et al. 2003).

Immunohistochemistry

Two hours after the last administration (8th day), the animals performed the elevated plus maze test. Immediately after the elevated plus-maze tests, animals were anesthetized with pentobarbital (130 mg/kg; Sigma, St. Quentin, France) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) after the elevated plus-maze tests. Brains were removed, postfixed, and cryoprotected. Immunohistochemistry was performed on free-floating cryomicrotome-cut sections (20 μm thick) encompassing the entire dorsal raphe and median raphe. After incubation in 3% H_2O_2/20% methanol followed by 0.2% Triton X-100 and 2% bovine serum albumin in 0.1 phosphate buffered saline, the sections were stained overnight at 4°C using a polyclonal antibody against tryptophan hydroxylase (1:1,000; Pel Freez, Rogers, AR, USA) for serotonergic neurons. Sections were then treated with secondary antibodies (Vectastain; Vector Laboratory, Burlingame, CA, USA) and subsequently incubated with avidin-biotinylated horseradish peroxidase complex. The peroxidase was revealed by incubation with 0.05% 3,3′-diaminobenzidine tetrahydrochloride containing 0.015% hydrogen peroxide. For Nissl cell counts, TPH sections were counterstained with cresyl violet. All sections for a given marker were stained simultaneously for all animals using the same solutions.

Data analysis

The data are expressed as means±SE. The statistical significance of differences between groups was assessed with one-way analysis of variance (ANOVA) followed by the Steel test (emotional responses score data) and Dunnett test (elevated plus maze test data, immunohistochemistry data). Analyses were made using StatView statistical software SAS system Ver. 6.12 (SAS Institute, Cary, NC, USA).

Drugs

The drugs used in the present study were milnacipran (Toledomin® Tablets25;Asahikasei Pharmacorporation, Japan) and fluvoxamine (DEPROMEL® Tablets25; MEIJISEIKA, Tokyo, Japan). The dose used in the present study was calculated on the basis of the free base and was referred to the previous reports. (milnacipran: Redmond et al. 1999; Mochizuki et al. 2002, fluvoxamine: van Riezen and Leonard 1990). The tablets were crushed and the resulting sludge, including the inset filler, made up on carboxymethyl cellulose (CMC). Antidepressants agents were dissolved suspended in 0.5% sodium CMC. Each drug was injected p.o. in a volume of 1.0 ml/kg body weight. Rats were weighted every day. In chronic administration, the antidepressants (milnacipran treatment group: sham/vehicle; n=8, OBX/vehicle; n=8, OBX/milnacipran 3 mg/kg; n=8, OBX/milnacipran 10 mg/kg; n=8, fluvoxamine treatment group: OBX/vehicle; n=7; OBX/fluvoxamine 10 mg/kg; n=8; OBX/fluvoxamine 30 mg/kg; n=7) were administered p.o. once daily for a total of 8 days. On the 7th day after the 14-day post surgical period, emotional responses were measured before and 120 min after drug administration. On the 8th day, OBX rats received antidepressants 120 min before the elevated plus-maze test.

Results

The changes of the olfactory bulbectomy-induced hyperemotionality in rats after subchronic treatment with milnacipran and fluvoxamine

There were no changes in body weight gain of each group of animals over the course of the 8 days of treatment. There was no difference in body weight between vehicle and antidepressants-treated OBX rats group on any day treatment.

As shown in Fig. 1a, total emotional responses in OBX rats were significantly increased at 14 days post-surgery compared to those in sham-operated rats. The total scores of emotional responses in OBX rats were not significantly affected by vehicle after subchronic administration on 1st, 3rd, 5th and 7th day. OBX rats, after milnacipran (3 mg/kg, p.o.) treatment, showed the significant decrease in the score of emotional responses on 7th day. In addition, the higher dose of milnacipran (10 mg/kg, p.o.) significantly reduced these emotional responses of OBX rats 2 h after administration on the 3rd and 7th day (Fig. 1a). OBX rats, after fluvoxamine (10 and 30 mg/kg, p.o.) treatment, showed significant decrease in the score of emotional responses on the 7th day (Fig. 1b).

Respective emotional responses (bite, startle, struggle, fight response) in OBX rats on the 7th day were shown in Fig. 2. Vehicle-treated OBX rats clearly showed a significant increase in score of the bite, startle, struggle, and fight response as compared with sham rats (Fig. 2a). Milnacipran (10 mg/kg, p.o.) completely reversed all hyperemotionality responses in OBX rats after subchronic administration (Fig. 2a). Fluvoxamine (10 and 30 mg/kg, p.o.) significantly reduced the score of both the struggle and the fight responses compared to those in vehicle-treated OBX rats after subchronic administration (Fig. 2b). In contrast, fluvoxamine reversed the score of neither the bite nor the startle responses in OBX rats (Fig. 2b).

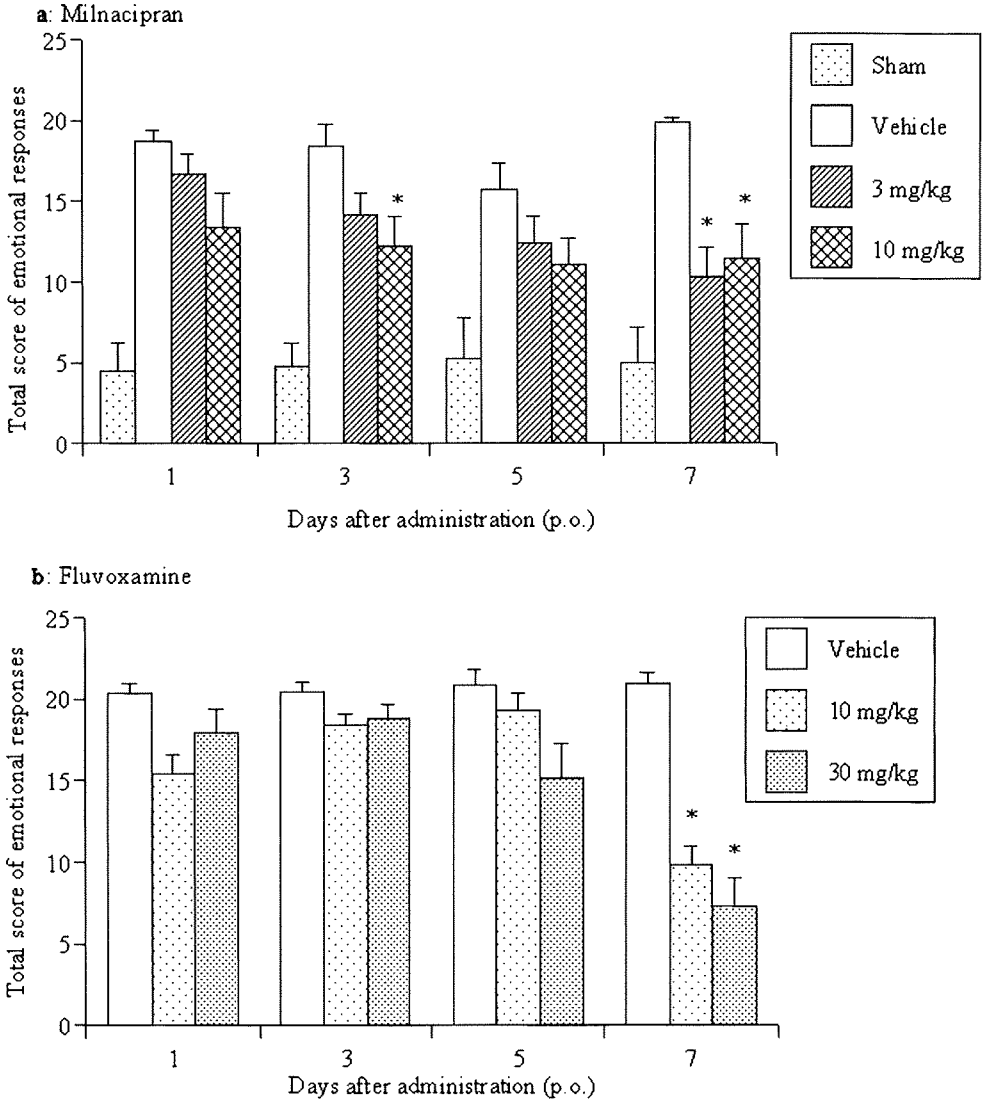

Fig. 1 The changes of OBX-induced hyperemotionality in rats after subchronic treatment with milnacipran (**a**) and fluvoxamine (**b**). Drugs were administered chronically once daily for 7 days. The total hyperemotionality score were counted 2 h after administration of antidepressants on 1st, 3rd, 5th and 7th day. Data represent means with SE. *$P<0.05$ vs vehicle (0.5% MC)-treated OBX rats

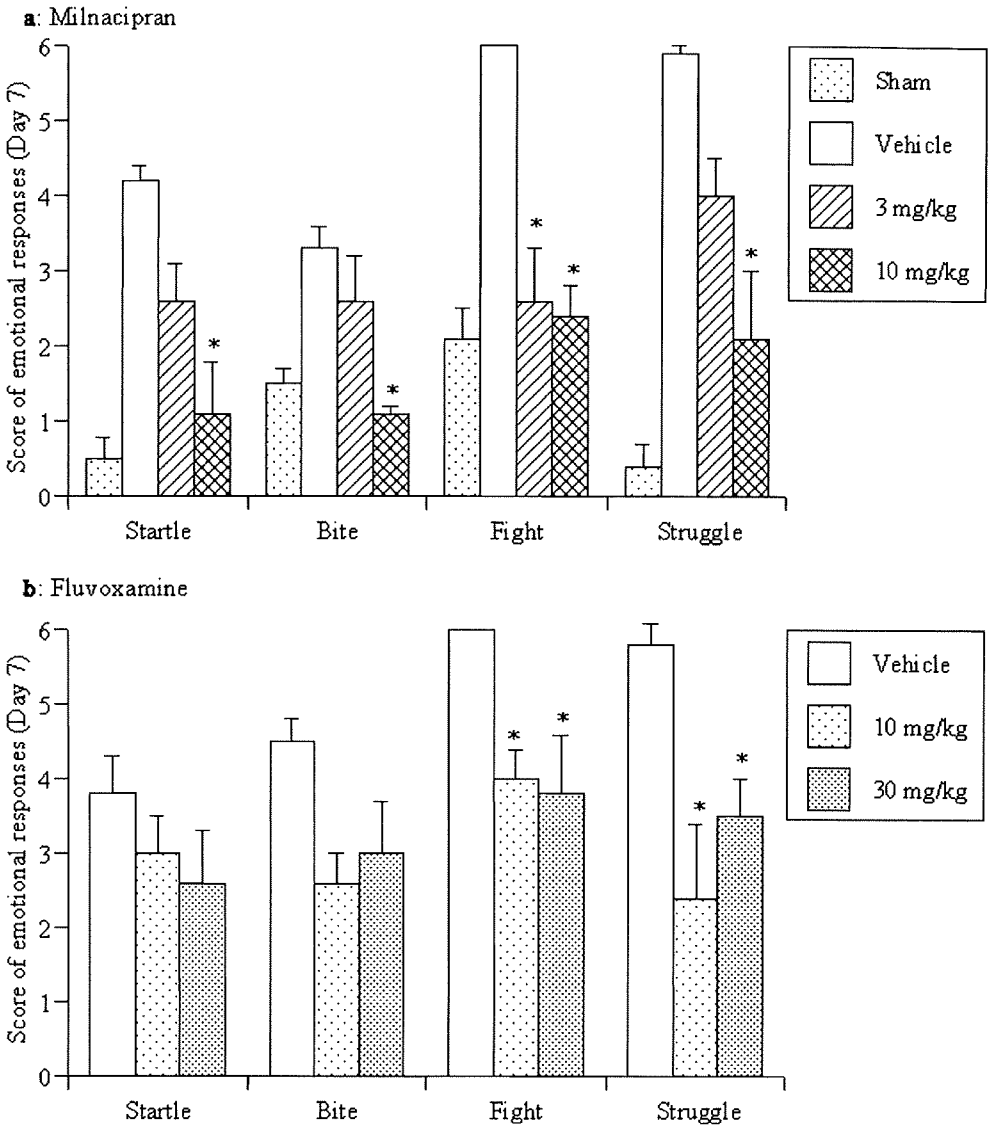

Fig. 2 Effects of chronic treatment with milnacipran (**a**) and fluvoxamine (**b**) on the score of bite, startle, struggle, and fight emotional response of OBX rats. Drugs were administered chronically once daily for 7 days. The respective emotional responses were measured 2 h after administration of antidepressants on 7th day. Data represent means with SE. #$P<0.05$ vs sham-lesioned rats. *$P<0.05$ vs vehicle (0.5% MC)-treated OBX rats

Effects of subchronic treatment with milnacipran and fluvoxamine on the elevated plus-maze test in OBX rats

The percentage of time spent in and entries into the open arms in sham rats respectively showed the 13.4±2.3% and 17.1±2.8%. On the other hand, the percentage of time spent in and entries into the open arms was decreased in OBX rats that had been treated with vehicle (Fig. 3a,b). Milnacipran (10 mg/kg, p.o.) significantly increased the percentage of time spent in the open arms in OBX rats as compared with vehicle-treated OBX rats (Fig. 3a). On the other hand, fluvoxamine (30 mg/kg, p.o.) significantly increased the percentage of entries into open arms (Fig. 3b). The number of total arm entries in OBX rats was significantly increased by treatment with milnacipran (10 mg/kg) as compared with vehicle-treated OBX rats (Table 1). However, the number of total entries in both milnacipran (10 mg/kg)- and fluvoxamine (30 mg/kg)-treated OBX rats was of the same levels as those in sham rats (Table 1).

Effects of subchronic treatment with milnacipran and fluvoxamine on the number of tryptophan hydroxylase-positive cells in dorsal or median raphe of OBX rats

Most of the tryptophan hydroxylase (TPH)-positive cells are located in the dorsal raphe rather than the median raphe of the midbrain (Fig. 4a,b). The OBX procedure significantly decreased TPH-positive cells in the dorsal raphe (−52%) of vehicle-treated rats relative to sham-operated control rats (Fig. 4a,c). Subchronic treatment with fluvoxamine (10 mg/kg) significantly reversed the decrease in TPH-positive cells produced by OBX in the dorsal raphe (Fig. 4a,c). Milnacipran (10 mg/kg) also reversed the decrease in TPH-positive cells produced by OBX in the dorsal raphe, but this effect was not significant (Fig. 4a,c).

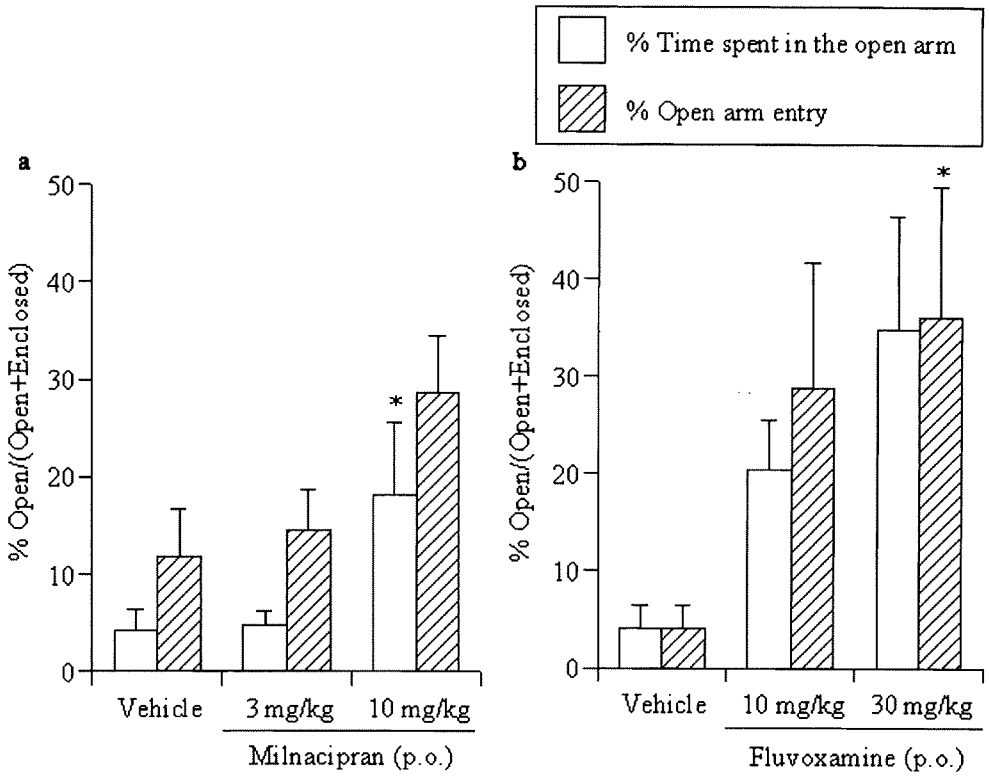

Fig. 3 Effects of chronic treatment with antidepressants on the percentage of time spent (*open column*) the percentages of entries into (*hated column*) in the elevated plus-maze test in OBX rats. Drugs were administered chronically once daily for 8 days. The elevated plus-maze test was performed 2 h after the final (8th day) administration. Data represent means with SE. *$P<0.05$ vs vehicle (0.5% MC)-treated OBX rats

There was no significant difference in the effects on TPH immunoreactivity in the median raphe of OBX rats between vehicle- and antidepressants-treated animals (Fig. 4a,c).

Discussion

In the present study, we observed the characteristics of hyperemotionality in rats 2 weeks after OBX. Furthermore, the OBX procedure was associated with a significant decrease in the percentages of time spent in and entries into open arms in the elevated plus-maze test. These results are consistent with those reported previously (Shibata et al. 1984; Yamaguchi et al. 2002; Saitoh et al. 2003).

Table 1 Effects of chronic treatment with milnacipran and fluvoxamine on the total number of arm entries in the elevated plus-maze test in OBX rats

Group	Total arm entry counts
Sham	26.1±2.7
OBX/vehicle	5.6±1.6
OBX/milnacipran 3 mg/kg	13.0±2.2
OBX/milnacipran 10 mg/kg	21.0±5.1*
OBX/vehicle	11.9±4.8
OBX/fluvoxamine 10 mg/kg	20.5±5.0
OBX/fluvoxamine 30 mg/kg	34.7±11.7

*$P<0.05$ vs OBX/vehicle-treated group

Many investigators have demonstrated that the various changes in behavior observed in OBX rats can be useful for evaluating the activity of antidepressants when the antidepressants are chronically administered (Kelly et al. 1997). In the present study, we also clearly found that all hyperemotional behavior (bite, startle, struggle, and fight response) was completely reversed in OBX rats that had been subchronically treated with milnacipran at a dose of 3 and 10 mg/kg (p.o.) when the antidepressants were administered for 7 days beginning 2 weeks after OBX. Furthermore, we demonstrated that the chronic treatment with milnacipran showed the greater inhibition of OBX-induced hyperemotionality than single treatment. Similarly, fluvoxamine (10, 30 mg/kg, p.o.) also significantly reduced the total hyperemotional score in OBX rats. The dose used in the present study was based on previous reports. (milnacipran: Redmond et al. 1999; Mochizuki et al. 2002, fluvoxamine: van Riezen and Leonard 1990). However, the scores of struggle and fight, but not bite and startle, response were significantly reduced by milnacipran but not fluvoxamine. These results led us to suggest that the hyperemotional behaviors observed in bite and startle response correlate with the activation of noradrenergic neurons. Based on these results, we propose that differences in the effects on the noradrenergic and/or serotonergic system might be implicated in the reduction of OBX-induced hyperemotional behavior.

There is some information available which suggests that SNRIs may offer therapeutic advantages. Specifically,

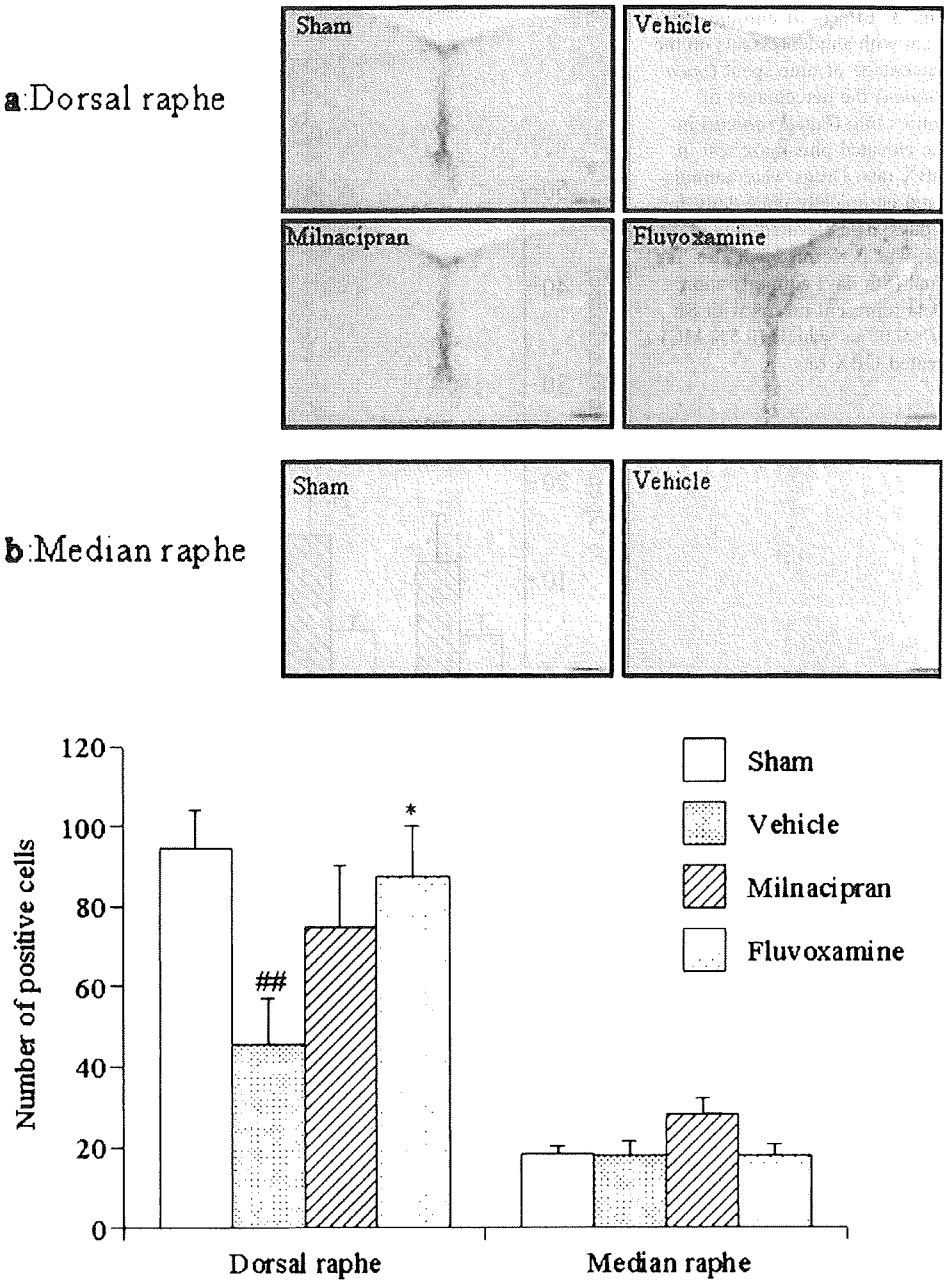

Fig. 4 Effects of chronic treatment with milnacipran and fluvoxamine on the number of tryptophan hydroxylase (*TPH*)-positive cells in dorsal or median raphe of OBX rats. Drugs (10 g/kg, p.o.) were administered chronically once daily for 8 days. TH immunostaining of sections through the dorsal (**a**) and median (**b**) raphe prepared from sham- or antidepressant-treated OBX rats. Most of the TPH-positive cells are located in the dorsal raphe of the midbrain (**a**). The results are the mean with SEM number of TPH-positive cells in dorsal or median raphe ($n=7$–11). *Scale bars* 1.0 μm. ##$P<0.01$ vs sham-lesioned rats. *$P<0.05$ vs vehicle (0.5% MC)-treated OBX rats

among the known SNRIs, venlafaxine and milnacipran have been approved as generalized anxiolytics as well as antidepressants (Meoni et al. 2001; Fukuchi and Kanemoto 2000). Fukuchi and Kanemoto (2002) reported that milnacipran is preferred compared to SSRI for the treatment of depressed patients with agitation. In the present study, only subchronic treatment with milnacipran had a potent effect on startle and bite responses in OBX rats. Thus, it is possible that OBX-induced hyperemotional behavior might resemble psychomotor agitation, a diagnostic criterion for depression.

Contrary to our results, Wieronska et al. (2001) reported that OBX rats produced the decreased level of anxiety in the elevated plus maze. Furthermore, it was reported that subchronic treatment with SNRIs venlafaxine reversed the OBX-induced hyperactivity in the open field apparatus, whereas there are no significant effects on the performance in the elevated plus maze (McGrath and Norman 1998). However, we found that milnacipran reversed the performance in the elevated plus maze as well as hyperemotional score in OBX rats. The detailed reasons for these discrepancies are not clear. However, they housed OBX rats in groups in cages after post-surgery, whereas we singly housed OBX rats. On the other hand, it was reported that whereas milnacipran blocks 5-HT and NE reuptake with equal affinity, venlafaxine has a 30-fold selectivity for 5-HT (Stahl et al. 2006). These differences between the affinity to

5-HT and NE reuptake site and housing might have a connection with the performance in the elevated plus maze. Our results showed that milnacipran significantly increased the number of entries on the elevated plus maze in OBX rats. Furthermore, milnacipran showed the greater effect on hyperemotional behaviors in OBX rats on the 7th day. Thus, we speculated that the performance in the elevated plus maze in OBX rats might reflect, at least in part, emotionality behaviors.

Previously, it has been reported that there was a decrease in the concentration of 5-HT in several brain areas of OBX rats (Kelly et al. 1997; Song and Leonard 2005). Furthermore, the changes in 5-HT contents were reversed following chronic treatment with antidepressant (Song and Leonard 1997). In the present study, we found histological evidence that OBX procedure significantly decreased TPH-positive cells in the dorsal raphe, but not the median raphe, compared with sham-operated rats. Indeed, this result indicated the loss of cell bodies on presynaptic 5-HTnergic neurons in the dorsal raphe. These results were consistent with previous report that abnormalities in the 5-HTnergic system were most apparent in the dorsal raphe of OBX rats (Redmond et al. 1997) and strongly supported the previous report that OBX was decreased the basal extra cellular levels in the basolateral amygdala in microdialysis study (Van der Stelt et al. 2005). Furthermore, the present results indicated that the loss of TPH-positive cells in the dorsal raphe in OBX rats was dramatically reversed by subchronic treatment with fluvoxamine. Milnacipran also produced a recovery, albeit non-significant, of the OBX-induced loss of TPH-positive cells. Recently, it was reported that the higher 5-HT synthesis in terminal areas of 5-HTnergic neurons and the lower 5-HT synthesis in cell bodies of 5-HTnergic neurons were observed in the OBX rats using autoradiographic study (Watanabe et al. 2003; Hasegawa et al. 2005). Furthermore, Hasegawa et al. (2005) reported that the lower 5-HT synthesis in dorsal raphe was reversed by the chronic treatment with SSRI citalopram. It has been shown that TPH may be regulated by 5-HT_{1A} autoinhibitory receptors (Bohmaker et al. 1992; Esteban et al. 1999; Okazawa et al. 1999). Thus, it was suggested that 5-HT synthesis is modulated through 5-HT_{1A} receptors. On the other hand, behavioral studies demonstrated that 5HT_{1A} receptor responsiveness was increased after OBX (Kelly and Leonard 1994). On the other hand, electrophysiological experiments suggest that the SSRI cause the desensitization of 5-HT_{1A} receptors in the cell bodies (Blier et al. 1990). Based on these results, we suggest that the chronic antidepressants treatment in the OBX rats might reverse the loss of TPH-positive cells due to the desensitization of cell body 5-HT_{1A} auto-receptors in the dorsal raphe. Thus, the functional changes in 5-HTnergic function in the dorsal raphe by subchronic treatment with antidepressants may contribute to the abnormal behaviors in OBX rats.

The present results suggest that OBX-induced hyperemotional behaviors in rats, which classified into struggle and fight response, might be more effectively reversed by subchronic treatment with milnacipran and fluvoxamine. On the other hand, the decreased time spent and entries on the open arm in OBX rats were reversed by subchronic treatment with both milnacipran and fluvoxamine. In addition, subchronic treatment with fluvoxamine significantly reversed the decrease in TPH-positive cells produced by OBX in the dorsal raphe. Based on these results, we propose that the hyperemotionality behaviors and the loss of TPH-positive cells seen in OBX rats might make them a useful animal model for evaluating the characteristics of chronic treatment with SSRI and/or SNRIs.

In conclusion, we demonstrated that chronic treatment with milnacipran or fluvoxamine was effective to improve both the hyperemotional behavior and the loss of TPH-positive cells in OBX rats.

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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.

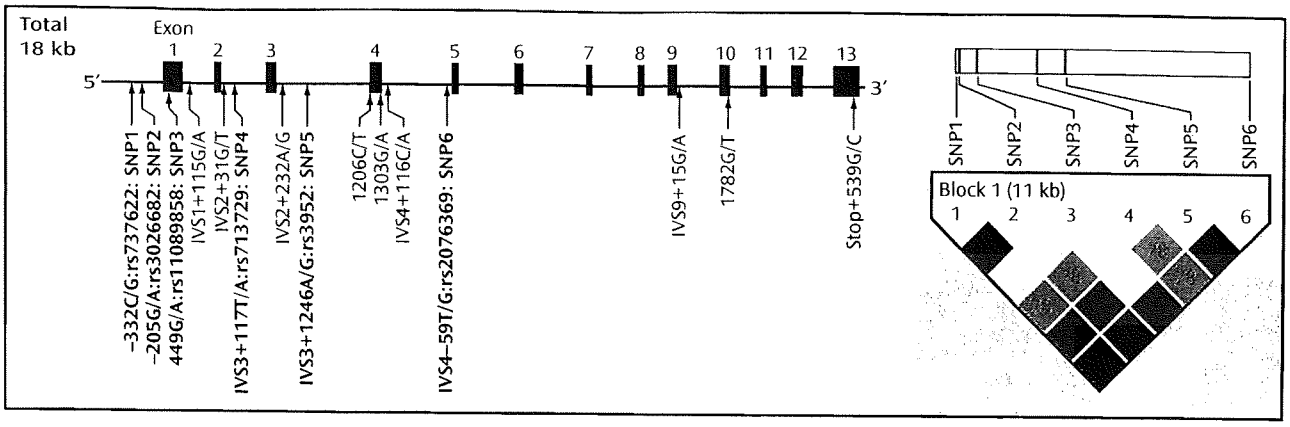
Identification of SNPs

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′-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.

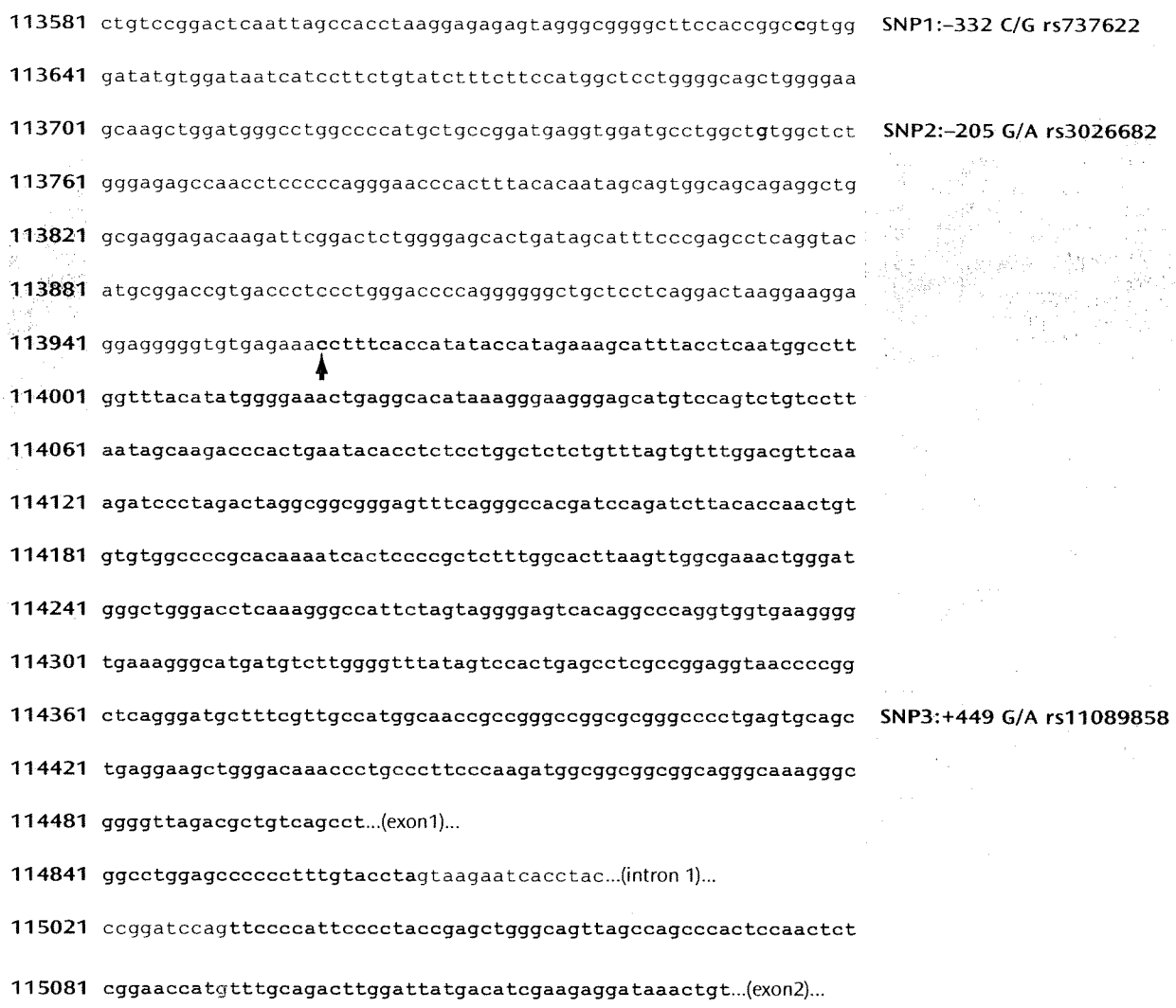
Results

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

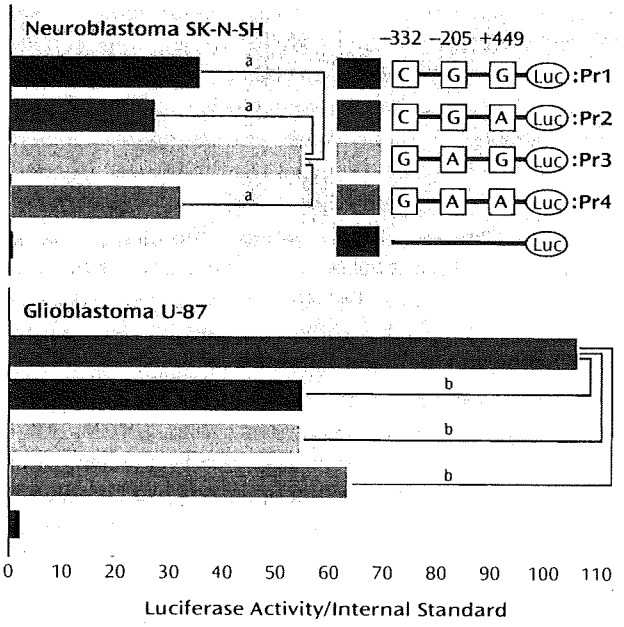
[a] The distributions of SNP2 (rs3026682) and 5 (rs3952) are the same as SNP1 (rs737622).
[b] Versus comparison subjects.

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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Full Paper

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

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

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

Introduction

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

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

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

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