Norepinephrine system

Norepinephrine systems have been the least associated with the rewarding effects of psychostimulants of the three main monoamine systems, at least in recent years. However, as discussed in the preceding, NET KO affects several psychostimulant-induced behaviors, including psychostimulant reward. Although not extensively investigated, there is accumulating evidence for involvement of norepinephrine systems in several psychostimulant responses from recent transgenic studies. At least a part of the impetus for examining particular norepinephrine receptors comes from evidence that these receptors modulate somatodendritic DA function.[167]

Cocaine

Transgenic mice that lack the enzyme that synthesizes norepinephrine, DA β-hydroxylase (DBH), are hypersensitive to the locomotor-stimulant effects of cocaine.[168] There was also a leftward shift in the dose–response curve for cocaine CPP, with a greater CPP observed in DBH KO mice at a cocaine dosage of 5 mg/kg, as well as a pronounced cocaine conditioned place aversion at 20 mg/kg cocaine. These authors suggested that this change in responsiveness was due to profound adaptive changes in DA systems, including substantially reduced presynaptic dopaminergic responses and postsynaptic receptor supersensitivity caused by increased numbers of both D_1 and D_2 receptors in the high-affinity state. Furthermore, these effects were observed in the striatum, but not the prefrontal cortex. The reason for these differences is uncertain, as is the degree to which the adaptations may be driven, or prevented in the prefrontal cortex, by DA release from norepinephrine synapses. Another report suggested that DBH KO eliminated the rewarding effects of cocaine in the CPP paradigm,[169] but this might be due to the dose–effect relationship noted earlier. The specific receptors involved in these effects is uncertain and will remain so until more noradrenergic receptor subtypes have been investigated, but initial evidence implicates the α_{1b} receptor. Oral cocaine consumption was reduced by α_{1b} KO, and there were substantial decreases in the locomotor-stimulant effects of cocaine as well as locomotor sensitization.[170]

Surprisingly, few studies have addressed the aversive effects of cocaine, which are often presumed to involve noradrenergic mechanisms. However, a recent report has found that the aversive effects of cocaine are eliminated in DBH KO mice.[171] With regard to lethal or toxic effects, again, not much has been done, but DBH KO had no effect on cocaine-induced seizures.[172]

Amphetamines

DBH KO mice are hypersensitive to the locomotor-stimulant effects of AMPH and exhibited a leftward shift in the dose–response curve for AMPH, including exhibiting stereotypical behavior at much lower doses of AMPH than is observed in WT mice.[173] However, this may have resulted from alterations in DA receptor function because these mice were less sensitive to a D_1 agonist and more sensitive to a D_2 agonist. Sensitization of AMPH responses was unaltered in these mice. Indeed, norepinephrine may have a more general modulating effect upon dopaminergic function and the effects of psychostimulants. Recent pharmacological studies have suggested that stimulation of the α_{1b} receptor increases psychostimulant effects, whereas stimulation of the α_2 adrenergic receptor inhibits those effects.[174] This supposition has been supported by transgenic studies. α_{1b} KO produces substantial decreases in the locomotor stimulant effects of AMPH as well as sensitization of those responses,[170] whereas the locomotor-stimulant effects of AMPH are enhanced in α_{2c} KO mice and reduced by transgenic overexpression of the α_{2c} receptor.[175] Consistent with the evidence for the involvement of both SERT- and NET-mediated responses underlying the retention of CPP in DAT KO mice,[18] there is evidence in α_{1b} KO mice for compensatory involvement of 5-HT systems. In α_{1b} KO mice a 5-HT_{2A} antagonist blocked the locomotor-stimulant effects of AMPH and the sensitization of those effects.[176] Under normal circumstances these two receptors have been suggested to be mutually inhibitory, even though they are individually behaviorally activating, and one mechanism of sensitization has been suggested to be the decoupling of these receptors producing increased DA activity.[177]

Again, little work has been done on the role of noradrenergic system genes in other psychostimulant effects. Adrenergic receptors may contribute to the effect of AMPH on sensorimotor gating in the PPI model. Consistent with some of the other effects discussed earlier, α_{2A} KO mice have increased PPI-disrupting effects of AMPH.[178] α_{2A} KO also alters the effects of MDMA on temperature.[179] Finally, with regard to adverse effects of amphetamines, elimination of norepinephrine in DBH KO mice increases the effect of METH on DA release, oxidative stress, and neurotoxicity.[180]

Discussion

From the data presented here it is clear that there is accumulating evidence from transgenic, and especially gene KO studies, for the role of monoaminergic transporter and receptor genes in the actions of psychostimulants, and by implication addiction. This review has been limited in two major ways: to discussion of the effects of transgenic manipulations of monoamine transporter and receptor genes and to the effects of psychostimulants. There is substantial evidence that monoamine gene manipulations also affect the actions of addictive drugs that do not act directly through monoamine transporters or receptors, such as morphine and ethanol, and similarly, there is a substantial body of work demonstrating that transgenic manipulations of genes other than those discussed here affect the actions of psychostimulants. However, what is evident from the transgenic work discussed here is that there is a complex emerging picture of interactive gene effects, even when considering just the monoaminergic genes, that is important in determining the effects of psychostimulants.

An additional point that has been substantially sidestepped in this review is the relationship of these transgenic "models" to human addiction. One of the conclusions that has become most evident in recent genomewide association studies of addiction[181–187] is that the genes that underlie addiction in humans seem to rather rarely include the classes of genes discussed here, monoamine transporters and receptors. Instead, the allelic variation in the actual human population that seems to underlie addiction involves a higher proportion of other classes of genes, including many involved in signal transduction and synaptic plasticity.[188] This realization will be important for developing animal models of addiction, and as the sophistication of these approaches develops, for modeling the specific allelic variants that may underlie human addiction.

This is not to say that the extensive studies discussed here have not contributed a great deal to the study of addiction. First, these transgenic models indicate genes that may be involved in addiction in humans (this may or may not be the case depending upon the actual allelic variation that exists in these genes in humans). Second, they indicate genes that, when manipulated, produce substantial changes in observable phenotypes that are relevant for addition, and the many diverse actions of psychostimulants, and may therefore contribute to the development of addiction therapeutics. Thus, in these ways the use of transgenic techniques has substantially improved our understanding of addiction genetics and provides insight into the polygenic determination of drug addiction phenotypes in ways that would not be possible with other methods. The complex picture that has emerged from this research fits with recent polygenic descriptions of genetic influences on human addiction developed from genomewide association studies, and no matter how much we may desire simple answers to our questions, we must accept the complex reality that is evident in these data.

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Conflicts of interest

The authors declare no conflicts of interest.

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Article

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Subjects

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

Background of Methamphetamine Abusers

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

Prognosis of Psychosis

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

Spontaneous Relapse

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

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

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

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

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

Polysubstance Abuse

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

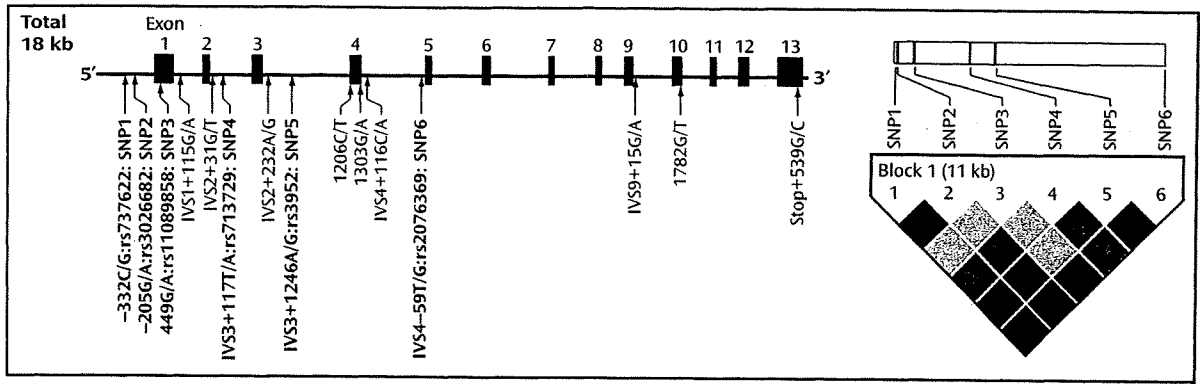
Identification of SNPs

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

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

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

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



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

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

Genotyping of Identified SNPs

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

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

Dual-Luciferase Gene Reporter Assays

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

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

Statistical Analysis

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

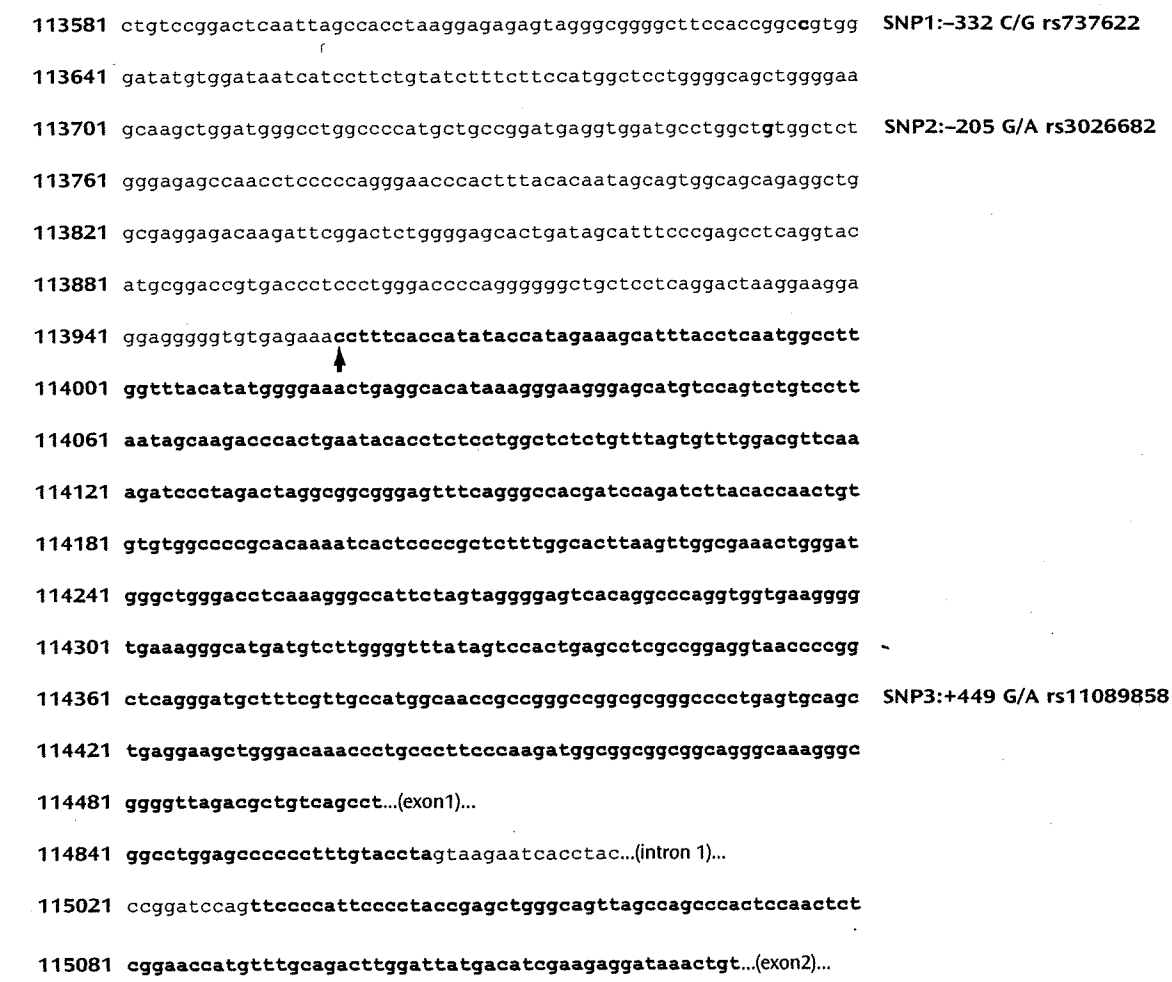
Results

Identification of SNPs and Association Studies

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

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

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



[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

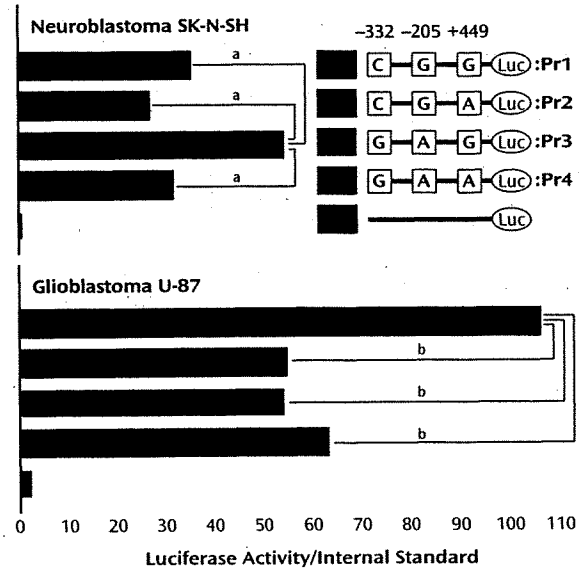
Variable	Haplotype Analysis		
Overall			
Haplotype	Comparison Subjects (N=218)	Methamphetamine Abusers (N=208)	p
C-G-G-T-A-T	35.2%	33.7%	0.63
G-A-G-T-G-G	32.3%	32.3%	0.85
C-G-G-A-A-G	14.5%	9.2%	<0.02
C-G-A-T-A-G	8.3%	7.4%	0.66
C-G-G-T-A-G	5.5%	8.9%	<0.09
G-A-G-T-G-T	0.7%	3.5%	0.01
C-G-G-A-A-T	1.2%	1.7%	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).

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).

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.

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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ORIGINAL ARTICLES

Protective Effects of Minocycline on the Reduction of Dopamine Transporters in the Striatum After Administration of Methamphetamine: A Positron Emission Tomography Study in Conscious Monkeys

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Background: Positron emission tomography (PET) studies of methamphetamine (METH) abusers suggest that psychotic symptoms of METH abusers may be attributable to the reduction of dopamine transporters (DAT) in the human brain. However, there are currently no particular pharmacological treatments for the wide range of symptoms associated with METH abuse.

Methods: Using a PET study in conscious monkeys, we investigated whether the second generation antibiotic minocycline could protect against the reduction of DAT in monkeys treated with METH (2 mg/kg × 3, 3-hour intervals).

Results: Pretreatment and subsequent administration of minocycline significantly attenuated the reduction of DAT in the striatum of monkeys treated with METH. Furthermore, posttreatment and subsequent administration of minocycline also significantly attenuated the reduction of DAT. In contrast, repeated administration of minocycline alone did not alter the density of DAT in the striatum of monkeys treated with METH.

Conclusions: Our findings suggest that minocycline protects against METH-induced neurotoxicity in the monkey brain. Therefore, minocycline is likely to be a promising therapeutic agent for the treatment of several symptoms associated with METH use in humans.

Key Words: Dopamine transporter, methamphetamine, minocycline, monkey brain, neurotoxicity, positron emission tomography

Methamphetamine (METH) abuse has become a major public health problem worldwide, as demonstrated by increases in the number of emergency room visits, substance abuse treatment episodes, and arrests attributable to METH manufacture and abuse. However, there are currently no particular pharmacological treatments for the wide range of symptoms associated with METH abuse (National Institute on Drug Abuse 2002). Multiple lines of evidence indicate that dopamine (DA) plays a key role in a variety of motivated behaviors associated with abused drugs, including METH (Nestler 2001, 2002; Pierce and Kumaresan 2006). In addition, it is well known that METH elevates extraneuronal DA concentrations through its actions on the plasma membrane DA transporter (DAT) (Davidson et al 2001; Hanson et al 2004; Mortensen and Amara 2003).

Positron emission tomography (PET) studies of METH users have demonstrated that the reduction of DAT in the striatum is associated with motor and cognitive impairment (Volkow et al 2001) and that the reduction of DAT is also associated with the duration of METH use and the severity of psychiatric symptoms (Sekine et al 2001, 2003). These findings suggest that psychotic symptoms of METH users may be attributable to the reduction of DAT in the brain. Furthermore, it has been demonstrated that the densities of DAT in the striatum are significantly decreased in the postmortem brains of chronic METH users (Wilson et al 1996). Thus, although METH-induced neurotoxicity in the dopaminergic terminals is well documented, the precise mechanism underlying METH-induced neurotoxicity remains unknown (Cadet et al 2003). In addition, chronic METH users show severe structural and functional deficits in areas of the brain associated with emotion, especially depression and anxiety, as well as memory (London et al 2004). From the point of view of developing novel pharmacological interventions for the treatment or prevention of METH abuse, it is necessary to develop therapeutic drugs to protect against the reduction of DAT in the brain associated with METH use.

Minocycline is a second-generation tetracycline that has been in use for over 30 years. This drug easily crosses the blood-brain barrier and has powerful neuroprotective properties in several models of neurological diseases, including Parkinson disease, Huntington disease, amyotrophic lateral sclerosis (ALS), and ischemic stroke (Blum et al 2004; Domercq and Matute 2004; Stirling et al 2005; Yong et al 2004). For example, the impressive therapeutic effects of minocycline have been demonstrated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of Parkinson's disease (Du et al 2001; Wu et al 2002). Minocycline mitigates both the demise of nigrostriatal dopaminergic neurons and the formation of nitrotyrosine produced by MPTP (Wu et al 2002). In addition, minocycline not only prevents MPTP-induced activation of microglia but also the formation of mature interleukin-1β and the activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and induction of nitric oxide synthase (iNOS), three key microglial-derived cytotoxic mediators (Wu et al 2002). Thus, it is likely that a blockade of microglial activation by minocycline plays a role in the neuroprotective actions of this drug (Tikka et al 2001; Wu et al 2002; Zhu et al 2002). On the other hand, direct neuronal

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protection by minocycline has been documented and probably involves the preservation of mitochondrial integrity and cytochrome c, followed by the suppression of caspase-dependent as well as caspase-independent cell death (Chen et al 2000; Wang et al 2003). Taken together, these findings indicate that minocycline holds great promise as a therapeutic drug for the treatment of human neurodegenerative diseases (Blum et al 2004; Domercq and Matute 2004; Stirling et al 2005; Yong et al 2004).

The current study was conducted to investigate whether minocycline could protect against the reduction of DAT in monkeys treated with METH. For this purpose, we used PET imaging in conscious monkeys.

Methods and Materials

Subjects

Ten young-adult male rhesus monkeys (*Macaca mulatta*) weighing from 4 kg to 6 kg were used for PET measurements. Monkeys were maintained and handled in accordance with the recommendations of the US National Institutes of Health and also the guidelines of the Central Research Laboratory, Hamamatsu Photonics (Hamamatsu, Shizuoka, Japan). The monkeys were trained to sit on a chair by means of twice-weekly training sessions over the course of 3 months. The magnetic resonance images (MRI) of all monkeys were obtained with a Toshiba MRT-50A/II (.5T) (Toshiba Medical Systems Corporation, Tokyo, Japan) under anesthesia with pentobarbital. The stereotactic coordinates of PET and MRI were adjusted based on the orbitomeatal (OM) line with monkeys secured in a specially designed head holder (Takechi et al 1994). At least 1 month before the PET study, an acrylic plate, with which the monkey was fixed to the monkey chair, was attached to the head under pentobarbital anesthesia as described previously (Onoe et al 1994).

Drug Administration

Methamphetamine hydrochloride (Dainippon Pharmaceuticals Ltd., Osaka, Japan) was administered intramuscularly (2 mg/kg as a salt, three times at 3-hour intervals) into each monkey using the previously reported method (Hashimoto et al 2004) with slight modifications. This dose regimen of METH closely approximates the binge use of METH by some humans (20 to 40 mg every 2 to 3 hours) (Konuma 1994).

In the first trial, which consisted of minocycline pretreatment, METH administration, and subsequent minocycline administration, subjects (n = 3) received minocycline (Wako Pure Chemicals Ltd., Tokyo, Japan; 200 mg, subcutaneous [SC], 0800 hours) or vehicle (physiological saline 1 mL, SC, 0800 hours) as a control condition 30 minutes before administration of METH and a subsequent administration of minocycline (200 mg, SC, twice daily [b.i.d.], 0800 and 2000 hours) or vehicle according to the method reported previously with slight modifications (Diguet et al 2004) (Figure 1A). In the second trial, which consisted of METH administration, minocycline posttreatment, and subsequent minocycline administration, subjects (n = 2) received minocycline (200 mg, SC, 2000 hours) 30 minutes after the final administration of METH, followed by subsequent administration of minocycline (200 mg, SC, b.i.d., 0800 and 2000 hours) or vehicle. In both experiments, the subsequent administration of minocycline was performed for 6 consecutive days (Figure 1B; day 2 to day 7). In the third trial, to examine the effect of minocycline alone on the DAT in the monkey brain, subjects (n = 2) received minocycline (200 mg, SC, b.i.d, 0800 and 2000 hours) for 7 days (Figure 1C).

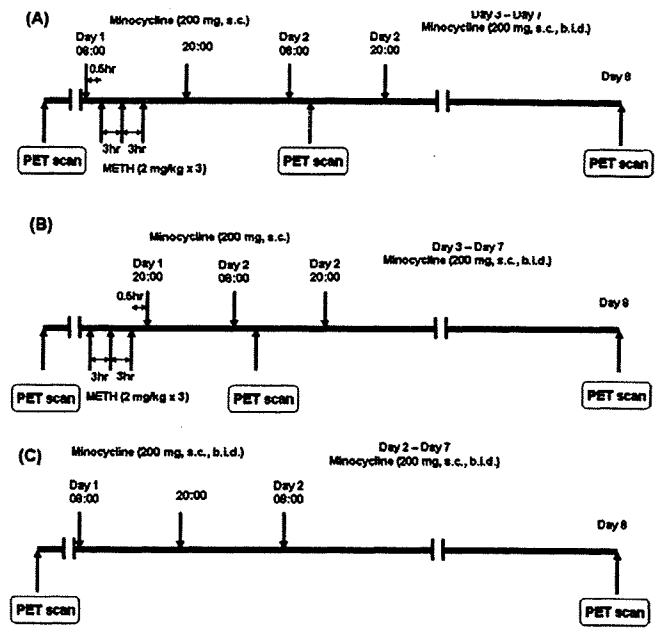

Figure 1. Treatment schedule of METH and/or minocycline in the monkeys. **(A)** Minocycline (200 mg, SC, 0800 hours) or vehicle (saline; 1 mL) was administered to monkeys (n = 3). Thirty minutes after injection, METH (2 mg/kg × 3, 3-hour intervals) was administered to the subjects (day 1). Then, minocycline (200 mg, SC, 2000 hours) or vehicle (saline; 1 mL) was administered to the monkeys (day 1). Minocycline (200 mg, b.i.d., 0800 and 2000 hours) was administered daily for 6 consecutive days (day 2 to day 7). **(B)** METH (2 mg/kg × 3, 3-hour intervals) was administered to the monkeys (n = 2). Thirty minutes after injection, minocycline (200 mg, SC, 2000 hours) was administered to the subjects (day 1). Then, minocycline (200 mg, b.i.d., SC, 0800 and 2000 hours) was administered daily for 6 consecutive days (day 2 to day 7). **(C)** Minocycline (200 mg, SC, 0800 and 2000 hours) was administered to the monkeys (n = 2) (day 1 to day 7). METH, methamphetamine; SC, subcutaneous; b.i.d., twice daily.

Synthesis of [^{11}C]-Labeled Compounds

Carbon-11 (^{11}C) was produced by ^{14}N (p,α)^{11}C nuclear reaction using a cyclotron (HM-18; Sumitomo Heavy Industries, Tokyo, Japan) at the Hamamatsu Photonics PET Center and obtained as [^{11}C]CO2, which was converted to [^{11}C]methyl iodide. [^{11}C]2β-carbomethoxy-3β-(4-fluorophenyl)tropane (β-CFT) (for DAT) and [^{11}C]SCH 23390 (for DA D_1 receptors) were synthesized as previously reported (Harada et al 2002; Tsukada et al 2001). The radiochemical and chemical purities of labeled compounds were greater than 98% and 99%, respectively. After analysis for identification, the solution was passed through a .22-μm pore size filter before intravenous administration to the monkeys.

PET Scans

Positron emission tomography data were collected before (control) and at 1 day (day 2) and 7 days (day 8) after the repeated administration of METH or METH/minocycline. In the trial in which minocycline was administered alone, PET data were collected before (control) and 7 days (day 8) after the repeated administration of minocycline. Data were collected on a high-resolution PET scanner (SHR-7700; Hamamatsu Photonics K. K., Hamamatsu, Japan) with transaxial resolution of 2.6-mm full-width at half-maximum (FWHM) and a center-to-center distance of 3.6 mm (Watanabe et al 1997). The PET camera allowed 31 imaging slices to be recorded simultaneously. After an overnight fast, animals were fixed to the monkey chair with

stereotactic coordinates aligned parallel to the OM line. A cannula was implanted in the posterior tibial vein of the monkey for administration of [^{11}C]-labeled compounds. The [^{11}C]β-CFT or [^{11}C]SCH 23390 was injected through the posterior tibial vein cannula. For [^{11}C]SCH 23390, a PET scan was performed for 64 minutes with 6 time frames at 10-second intervals, 6 time frames at 30-second intervals, 12 time frames at 1-minute intervals, and 16 time frames at 3-minute intervals. For [^{11}C]β-CFT, additional scans of nine time frames at 3-minute intervals were done to collect data for 91 minutes total. After completion of the first scan with [^{11}C]β-CFT, scans with [^{11}C]SCH 23390 were continuously performed at 3-hour intervals. Due to the very short half-life of ^{11}C (20.4 minutes), a time lag of at least 3 hours between scans provided a sufficient decay time of radioactivity in monkeys (approximately 1/400 of the injected dose). Therefore, the level of radioactivity associated with the previous injection of labeled compound did not interfere with the next scan.

Data Analysis and Statistical Analysis

For quantitative analysis, time-activity curves of radioactivity in the cerebellum, used as an input function because of the much lower density of dopamine receptors and DAT (Creese et al 1975; Kaufman and Madras 1993), and each region of interest (ROI) were fitted to a two-compartment model using the least-squares fitting method to estimate the kinetic parameters, and the binding potential in each ROI was calculated as described previously (Hashimoto et al 2004; Lammertsma and Hume 1996). The differences between the control (pre-METH) monkeys and METH-treated (post-METH) monkeys were determined using a paired two-tailed t test. The data on effects of minocycline were analyzed using an unpaired two-tailed t test. Significance was set at $p < .05$.

Results

Positron emission tomography studies using [^{11}C]β-CFT (for DAT) or [^{11}C]SCH 23390 (for DA D_1 receptor) were performed before (control) and at 1 day (day 2) and 7 days (day 8) after repeated administration of METH (2 mg/kg × 3, 3-hour intervals). High accumulation of radioactivity in the striatum after intravenous administration of [^{11}C]β-CFT or [^{11}C]SCH 23390 was detected in the control monkeys (Figure 2). Repeated administration of METH (2 mg/kg × 3, 3-hour intervals) significantly (t = 10.27, p = .009) decreased the binding of [^{11}C]SCH 23390 to DA D_1 receptors at 1 day (day 2) after administration of METH (Figure 2 and Table 1), although the difference (less than 10%) was small. However, the binding of [^{11}C]SCH 23390 in the striatum was recovered to control levels at 7 days (day 8) after administration of METH (Figure 2 and Table 1). In contrast, repeated administration of METH (2 mg/kg × 3, 3-hour intervals) markedly decreased the binding of [^{11}C]β-CFT (for DAT) in the striatum at 1 day (day 2; t = 16.82, p = .004) or 7 days (day 8; t = 28.60, p = .001) after administration of METH (Figure 2 and Table 1).

First, we examined the effect of pretreatment and subsequent administration of minocycline (200 mg b.i.d.) on the reduction of DAT in the striatum after repeated administration of METH (2 mg/kg × 3, 3-hour intervals) (Figure 1A). Pretreatment (30 minutes before administration of METH) and subsequent administration of minocycline (200 mg b.i.d.) (Figure 1A) significantly (day 2: t = 12.18, $p < .001$; day 8: t = 22.97, $p < .001$) attenuated the reduction of DAT in the striatum after administration of METH as compared with that of the striatum of monkeys treated with METH alone (Figure 2 and Table 1).

Second, we examined the effect of posttreatment and subsequent administration of minocycline (200 mg b.i.d.) on the reduction of DAT in the striatum after repeated administration of METH (2 mg/kg × 3, 3-hour intervals) (Figure 1B). Interestingly, posttreatment (30 minutes after the final administration of METH) and subsequent administration of minocycline (200 mg b.i.d.) significantly (day 2: t = 4.33, p = .023; day 8: t = 13.69, p = .001) attenuated the reduction of DAT in the striatum of monkeys treated with METH (Figure 2 and Table 1).

Finally, we examined the effects of minocycline alone on the binding potential of [^{11}C]β-CFT binding in the monkey striatum (Figure 1C). Repeated administration of minocycline (200 mg b.i.d. for 7 days) did not alter the binding potential of [^{11}C]β-CFT binding in the monkey striatum (Figure 2 and Table 1), suggesting that minocycline alone does not affect DAT in the monkey striatum.

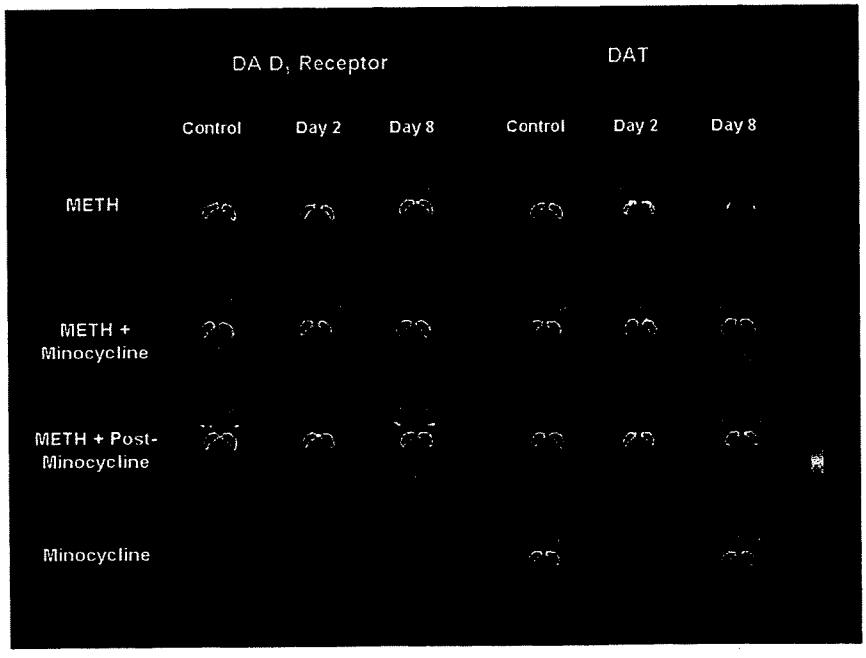

Figure 2. Representative PET images of [^{11}C]SCH 23390 binding (for DA D_1 receptor) and [^{11}C]β-CFT binding (for DAT) in the brains of monkeys. PET images of [^{11}C]SCH 23390 and [^{11}C]β-CFT in the brains of monkeys were obtained before (control) and at 1 day (day 2) and 7 days (day 8) after the repeated administration of METH (2 mg/kg × 3, 3-hour intervals). The PET image of [^{11}C]SCH 23390 was generated by the summation of data from 37 to 64 minutes after injection. PET images for [^{11}C]β-CFT were generated by the summation of data from 61 to 91 minutes after injection. PET, positron emission tomography; [^{11}C]SCH 23390, ; DA, dopamine; [^{11}C]β-CFT, [^{11}C]2β-carbomethoxy-3β-(4-fluorophenyl)tropane; DAT, dopamine transporter; METH, methamphetamine.

Table 1. Effects of Minocycline on the Binding Potential of DA D_1 Receptors and DAT in the Monkey Striatum After Administration of METH

Groups	Control Study	Day 2	Day 8
DA DI Receptors			
METH (n = 3)	100.0 ± 12.69	93.90 ± 1.00[b]	98.52 ± 16.91
METH + Preminocycline (n = 3)	100.0 ± 19.55	91.78 ± 3.00[a]	96.67 ± 5.25
METH + Postminocycline (n = 2)	100.0 ± 1.69	91.91 ± 2.39	108.5 ± 6.09
Minocycline (n = 2)	ND	ND	ND
DAT			
METH (n = 3)	100.0 ± 8.36	39.51 ± 6.20[b]	40.0 ± 3.54[b]
METH + Preminocycline (n = 3)	100.0 ± 11.28	88.66 ± 3.16[a,e]	89.99 ± 1.03[b,e]
METH + Postminocycline (n = 2)	100.0 ± 2.89	65.28 ± 7.20[c]	78.05 ± 1.10[a,d]
Minocycline (n = 2)	100.0 ± 3.96	ND	97.45 ± 5.30

PET studies were performed before (control) and at 1 day (day 2) and 7 days (day 8) after the repeated administration of METH (2 mg/kg × 3, 3-hour intervals). The binding potential of each monkey at control study was expressed as 100%. Values are the mean ± SD of three or two monkeys.

DA, dopamine; DAT, dopamine transporter; METH, methamphetamine; PET, positron emission tomography; ND, not determined.

[a] $p < .05$ as compared with control (paired t test).
[b] $p < .01$ as compared with control (paired t test).
[c] $p < .05$ as compared with METH group (unpaired t test).
[d] $p < .01$ as compared with METH group (unpaired t test).
[e] $p < .001$ as compared with METH group (unpaired t test).

Discussion

The major finding of the present study was that minocycline showed protective effects against METH-induced neurotoxicity in the monkey striatum. It is noteworthy that posttreatment with minocycline (30 minutes after the final administration of METH) followed by subsequent administration of minocycline conferred neuroprotection against METH-induced neurotoxicity in the monkey striatum without minocycline pretreatment. Recently, we found that posttreatment with minocycline (2 hours after the final administration of METH) and the subsequent administration of minocycline significantly attenuated the METH-induced neurotoxicity in the mouse striatum (Zhang et al, in press). In addition, we found that repeated administration of minocycline alone did not affect the density of DAT in the striatum of mice (Zhang et al, in press) and monkeys (this study). Taking these results together, minocycline is likely to be a useful therapeutic drug for treatment of METH-induced neurotoxicity in the human brain.

A recent study demonstrated that activation of microglia might contribute to METH-induced neurotoxicity in the striatum (Thomas et al 2004). It has also been reported that minocycline inhibits microglial activation and neurotoxicity in the striatum of MPTP-treated mice (Wu et al 2002), suggesting that microglia-related inflammatory events play a role in MPTP-induced neurotoxicity and that minocycline may be a valuable neuroprotective agent for the treatment of Parkinson disease (Wu et al 2002). Recently, we found that minocycline significantly attenuated the METH-induced neurotoxicity in the mouse striatum and that minocycline significantly attenuated microglial activation in the mouse striatum by repeated administration of METH (Zhang et al, in press). Therefore, it is likely that minocycline may, at least in part, attenuate METH-induced neurotoxicity in the monkey striatum via the inhibition of microglial activation.

Accumulating evidence suggests that METH induces neuronal apoptosis by activating the mitochondrial cell death pathway, and mitochondrial dysfunction may play a role in METH-induced neurotoxicity (Deng et al 2002; Jayanthi et al 2004). Administration of METH was shown to cause the gradual appearance of cytochrome c in the cytosol of the mouse striatum, and these changes were countered by marked decreases in cytochrome c in the mitochondrial fraction (Jayanthi et al 2004). It has been shown that minocycline blocks the release of the proapoptotic factors cytochrome c and apoptosis-inducing factor (Domercq and Matute 2004; Wang et al 2003; Zhu et al 2002). Furthermore, it has been demonstrated that minocycline delayed mortality and/or progression in mouse models of Huntington disease (Chen et al 2000) and ALS (Zhu et al 2002), presumably by inhibiting caspase-3 expression and cytochrome c release, suggesting that the primary target of minocycline is cytochrome c release (Chen et al 2000; Zhu et al 2002). Taken together, these results suggest that a blockade of the release of cytochrome c by minocycline might, at least in part, contribute to its neuroprotective activity in the context of METH-induced neurotoxicity in the striatum, although further studies will be needed to determine the precise mechanisms of minocycline on METH-induced neurotoxicity.

In clinical trials, minocycline was well tolerated, and no side effects or negative interactions with other simultaneously administered drugs were observed (Blum et al 2004; Domercq and Matute 2004; Smith and Leyden 2005; Yong et al 2004). Minocycline is an antibiotic that possesses superior penetration through the blood-brain barrier (Aronson 1980; Barza et al 1975; Zhang et al, in press), and the bioavailability of minocycline is very high in humans (Kelly and Kanegis 1967). Therefore, the present study suggests that minocycline might serve as a promising therapeutic drug for prevention of the long-term effects associated with METH abuse in humans.

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