nent abusers in the community as a whole but may provide some support for the high occurrence of psychopathologic symptoms observed in this study. In Japan, most methamphetamine abusers take the substance intravenously,[8] whereas in a study[66] from the United States, approximately 90% of methamphetamine abusers had no history of intravenous or intramuscular injection of methamphetamine. Furthermore, a study by Domier and colleagues[66] revealed that among recently abstinent methamphetamine abusers who had discontinued its use for several months, the injecting abusers had a significantly higher incidence of psychopathologic symptoms than the noninjecting abusers. These results suggest that in Japan, the intravenous intake of methamphetamine could predispose its abusers to persistent psychiatric problems, even after the cessation of methamphetamine use. Nevertheless, it remains an important and unresolved issue whether a reduction in serotonin transporter could be expected to occur in abusers with no psychopathologic signs or symptoms. To verify our findings that methamphetamine abuse is linked to a reduction in brain serotonin transporters, which in turn underlies persistent psychopathologic symptoms, additional studies that also incorporate a group of methamphetamine abusers with no apparent psychopathologic problems are required.

Wilson and colleagues[67] examined serotonin concentrations in postmortem tissue samples from human brains with a history of long-term methamphetamine abuse, although they did not study serotonin transporters per se. They concluded that there were no substantial alterations in serotonin concentrations in the global brain except in the medial prefrontal cortex (Brodmann area 11: a reduction of 56% compared with controls) and in the orbitofrontal cortex (Brodmann area 12: a reduction of 61% compared with controls). These results seem to contradict our observation of reductions in serotonin transporters in widely distributed brain regions. The discrepancy between the results of that postmortem study and those of present study is puzzling. However, one possible explanation for this discrepancy could be related to differences in the pattern and amount of drug use between the samples.[1,16,17] In Western countries, methamphetamine abusers often use other drugs, mainly cocaine or cannabis[68-70]; however, no information is provided with respect to this issue in the study by Wilson and colleagues.[67] Because methamphetamine is more likely to produce neurotoxic effects in serotonergic neurons than either cocaine or cannabis,[71,72] methamphetamine abusers who use this drug only could have experienced more severe damage to serotonergic neurons than abusers who simultaneously use other drugs, such as cocaine or cannabis. Furthermore, similar to most methamphetamine abusers in Japan, those in this study intravenously injected the substance. The intravenous intake may further potentiate the neurotoxic effects of methamphetamine.

To our knowledge, this is the first study to demonstrate a severe and long-lasting reduction in the density of the serotonin transporter in the living brains of methamphetamine abusers. The observed decrease in serotonin transporter density was also found to be associated with elevated levels of aggression. The present findings, combined with the results of previous animal studies, suggest that those who abuse methamphetamine may be at substantial risk for severe serotonin neuronal damage in the brain, potentially leading to persistently elevated aggression, even in those in a currently abstinent state.

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For details about this new policy see the editorials by DeAngelis et al in the September 8, 2004 (2004;292:1363-1364) and June 15, 2005(2005;293:2927-2929) issues of *JAMA*.

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Genetic variant of prodynorphin gene is risk factor for methamphetamine dependence

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Abstract

Previous studies have indicated that genetic factors substantially affect development of substance use disorders, including methamphetamine dependence. Prodynorphin (PDYN) is an opioid peptide precursor that yields dynorphins, endogenous κ opioid-receptor agonists that play important roles in substance abuse. A physiologically active polymorphism of 1–4 repeats of a 68-bp element in the promoter region of the PDYN gene has been identified. We analyzed this polymorphism of the PDYN gene by a case-control association study in 143 patients with methamphetamine dependence and 209 healthy controls in the Japanese population. A 3- or 4-repeat allele in the PDYN gene promoter was found significantly more frequently in patients with methamphetamine dependence than in controls ($\chi^2 = 9.45$, $p = 0.0021$). A 3- or 4-repeat allele in the PDYN gene promoter, which was shown to produce significantly higher transcription activity of the PDYN gene than a 1- or 2-repeat allele, is a genetic risk factor for development of methamphetamine dependence (odds ratio: 1.83, 95% CI = 1.24–2.68).

Keywords: Addiction; Psychostimulant; Genetics; Methamphetamine; Opioid receptor

Psychoactive substance use disorder is a serious problem worldwide. Methamphetamine, one of the amphetamines, has been the most commonly abused drug in Japan since World War II [13,19,24]. Genetic factors have been indicated to be important and relatively strong contributors to the risk for psychoactive substance use disorder. Intensive family, twin and adoption studies have indicated that genetic factors substantially influence the vulnerability to psychoactive substance use disorder, including those to psychostimulants such as amphetamines and cocaine [2,9,20,25]. However, only a few genetic factors were found to associate significantly with vulnerability to psychostimulant dependence [11,23]. Understanding of the precise involvement of the genetic mechanisms inducing psychostimulant substance use disorder, including methamphetamine dependence, remains elusive.

Prodynorphin (PDYN) belongs to the family of opioid peptide precursor proteins that yields α- and β-neoendorphin, dynorphin A- and dynorphin B-related peptides [8]. There are several lines

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of evidence that dynorphin peptides interact with psychostimulants. Administration of cocaine and amphetamine increases prodynorphin mRNA and dynorphin peptides in the nucleus accumbens and dorsal striatum in rats via D1 dopamine receptor activation due to enhanced release of dopamine [15]. Dynorphin peptides are relatively selective endogenous agonists for the κ opioid receptors [3], and activation of κ opioid receptors located on dopamine terminals results in a decrease of dopamine release. Activation of κ receptors by MR2033, a benzomorphan κ agonist, is associated behaviorally with an aversive syndrome in humans, e.g., dysphoria, anhedonia and fatigue [12]. Therefore, dynorphin systems may play a role in the emotional-motivational aspects of withdrawal from psychostimulant consumption. Zimprich et al. [26] identified a functional polymorphism in the PDYN gene promoter, 1–4 repeats of a 68-bp element containing one binding site per repeat for the transcription factor AP-1. Upon activation of the AP-1 complex, a 3- or 4-repeat allele was associated with a significant increase (about 50%) in gene expression in a chloramphenicol acetyltransferase reporter gene assay, whereas a 1- or 2-repeat allele could not be stimulated over basal conditions [26]. Recently, Chen et al. [4] reported that this functional polymorphism in the PDYN gene promoter might be associated with protection against cocaine dependence or abuse in the American population. Considering that both cocaine and methamphetamine are psychostimulants and have similar mechanisms of action in the central nervous system, we hypothesized that a 68-bp repeat polymorphism in the PDYN gene promoter might contribute to the vulnerability to methamphetamine dependence. In this study, we examined the allelic frequencies and genotypic distributions of this polymorphism in unrelated individuals with a primary diagnosis of methamphetamine dependence and healthy controls.

Participants in this study included 143 unrelated patients with methamphetamine dependence (114 males and 29 females; mean age, 35.5 ± 11.3 years) who were outpatients or inpatients of psychiatric hospitals of the Japanese Genetics Initiative for Drug Abuse (JGIDA). All participants were Japanese, born and living in restricted regions of Japan. Diagnoses were made using the international statistical classification of the disease, revision 10, diagnostic criteria. Two trained psychiatrists assessed these diagnoses on the basis of all available information, including hospital notes. Control subjects included 209 age-, gender- and region-matched healthy volunteers (162 males and 47 females, mean age 36.5 ± 10.6 years), recruited primarily from medical staff. Only unaffected subjects who had no known personal or family history of major psychiatric diseases, including alcoholism, were included in this study. The patients were divided into subgroups by two clinical features: multiple substance use status and age at initial consumption of methamphetamine. Thirty-nine patients consumed only methamphetamine (methamphetamine dependence without multiple substance abuse), 95 consumed not only methamphetamine but also other substances, including volatile solvents, cocaine and hypnotics (methamphetamine dependence with multiple substance abuse), and 9 were not clear about substance use except for methamphetamine. Seventy-three patients were less than 20 years old when they started using methamphetamine, and 66 were 20 or older. This study was performed after obtaining approval from the ethics committees of each institution of JGIDA, and all subjects provided written informed consent for the use of their DNA samples for this research. The genomic DNA was extracted from peripheral leukocytes using standard procedures. Polymerase chain reaction (PCR) and agarose gel electrophoresis were performed to identify genotype polymorphism of the prodynorphin gene containing the 68-bp repeat element (accession number: X02536). PCR was carried out according to the method reported by Chen et al. with minor modification [4]. PCR products were subsequently electrophoresed on 2.0% agarose gels and stained with gel star (Takara, Japan) to visualize DNAs. We confirmed genotyping reliability in sixteen samples by DNA sequencing using a Big Dye Terminator Cycle Sequencing kit and ABI PRISM 3100 DNA Sequencer (Applied Biosystems, Foster City, CA). The presence of Hardy–Weinberg equilibrium was tested using a chi-square goodness-of-fit test. To evaluate the statistical significance of differences in genotype distributions and allele frequencies between controls and patients and between clinical subgroups of patients, the T4 value of the Monte Carlo method as implemented in CLUMP [14] was applied. Bonferroni correction for multiple testing was carried out to exclude type I errors.

We observed four different lengths of PCR products amplified in the promoter region of the human PDYN gene in a Japanese population. Sequencing confirmed that the different lengths were due to a 68-bp repeat element occurring either as a single element or as a tandem repeat two, three or four times. This finding is consistent with that of the Caucasian population in Zimplich's study. These alleles were designated alleles 1–4 according to the number of tandem repeats [26]. The distributions of genotypic and allelic frequencies of patients with methamphetamine dependence and controls are shown in Table 1. These distributions in controls and patients were within the Hardy–Weinberg equilibrium (controls: $p = 0.48$, patients: $p = 0.87$). The genotypic and allelic frequencies of the promoter region of the PDYN gene were significantly different between controls and patients with methamphetamine dependence (genotypes: $\chi^2 = 16.8, p = 0.0010$; alleles: $\chi^2 = 11.1$, $p = 0.0025$; Table 1). Based on the physiological significance of the gene expression, alleles were divided into two groups, alleles 1–2 and alleles 3–4, which were called L and H alleles, respectively, because H alleles produced significantly greater expression of the PDYN than L alleles; this resulted in three genotypes, L/L, L/H and H/H. Using this dichotomy, the genotypic distribution of the promoter region of the PDYN gene polymorphism was significantly different between controls and patients ($\chi^2 = 11.9$, d.f. = 2, $p = 0.0018$), and H alleles were significantly more frequent in patients with methamphetamine dependence than controls ($\chi^2 = 9.45$, d.f. = 1, $p = 0.0021$, odds ratio 1.83, 95% CI = 1.24–2.68, Table 2). Even after the Bonferroni correction, differences in the frequencies of the alleles and genotypes between controls and patients remained significant ($p < 0.05$). In subcategory groups defined by clinical features, no significant differences were found in the frequencies of genotypes or

Table 1
Genotype and allele frequencies of the polymorphism in the promoter region of the prodynorphin gene

	N	Genotypes										p value
		1/1	1/2	1/3	1/4	2/2	2/3	2/4	3/3	3/4	4/4	
METH-dependence (%)	143	0 (0.0)	7 (4.9)	5 (3.5)	0 (0.0)	78 (54.5)	41 (28.7)	2 (1.4)	10 (7.0)	0 (0.0)	0 (0.0)	
Control (%)	209	1 (0.5)	12 (5.7)	2 (1.0)	0 (0.0)	136 (65.1)	53 (25.4)	4 (1.9)	0 (0.0)	1 (0.5)	0 (0.0)	0.001

	N	Alleles				p value
		1	2	3	4	
METH-dependence (%)	286	12 (4.2)	206 (72.0)	66 (23.1)	2 (0.7)	
Control (%)	418	16 (3.8)	341 (81.6)	56 (13.4)	5 (1.2)	0.003

The prodynorphin gene promoter has a 68-bp repeated element occurring either as a single element or as a tandem repeated element two, three, or four times. These alleles are called alleles 1–4 according to the number of tandem repeats. Patients with metha.

Table 2
Genotype and allele frequencies of the polymorphism in the prodynorphin gene promoter

	N	Genotypes			p value	N	Alleles		p value
		L/L	H/L	H/H			L	H	
METH-dependence (%)	143	85 (59.4)	48 (33.6)	10 (7.0)		286	218 (76.2)	68 (23.8)	
Control (%)	209	149 (71.3)	59 (28.2)	1 (0.5)	0	418	357 (85.4)	61 (14.6)	0

Based on physiological significance of gene expression, the alleles of the prodynorphin gene promoter are divided into two groups, alleles 1 and 2 and alleles 3 and 4, which are called as L and H alleles, respectively.

alleles between methamphetamine dependence with and without multiple substance abuse status, or between patients beginning methamphetamine consumption at the age of less than 20 and at 20 and over (Table 3).

The present study demonstrated that a certain genetic variant of the promoter region of the PDYN gene contributed to the individual variation in vulnerability to methamphetamine dependence, but not to clinical phenotypes of dependence such as multiple substance use status or age at the beginning of methamphetamine use. We identified four kinds of alleles, 1–4 copies of a 68-bp tandem repeat, of the polymorphism in the promoter region of the PDYN gene. H alleles (containing 3 or 4 repeats) of the PDYN gene were significantly more frequent in patients with methamphetamine dependence than in controls. These results suggested that the H allele is a genetic risk factor for vulnerability to methamphetamine dependence. The odds ratio for H alleles was 1.83 (95% CI = 1.24–2.68), which indicates moderate potency as a genetic risk.

The major central action of methamphetamine is enhancement of dopamine release from axon terminals, which in turn robustly increases dopamine concentrations in the synaptic clefts. The enhancement of dopamine release in the mesolimbic region including the nucleus accumbens is considered to account for the mood-elevating (euphoric) and reinforcing effects [22]. Stimulation of D1 dopamine receptors by increased dopamine leads to activation of the PDYN gene expression and to release of dynorphin peptides [5,6,17]. Following repeated administration of methamphetamine, levels of PDYN gene expression and dynorphin peptides increase significantly in the dorsal and ventral striatum [7,10,16]. Dynorphin peptides stimulate κ opioid receptors located on dopamine terminals and decrease dopamine release [15], which produces dysphoria [1,12]. Accordingly, the

Table 3
Clinical subgroups of patients with methamphetamine dependece

Multiple-substance abuse	N	Genotypes			p value	N	Alleles		p value
		L/L	L/H	H/H			L	H	
No (%)	39	21 (53.8)	16 (41.0)	2 (5.1)		78	58 (74.4)	20 (25.6)	
Yes (%)	95	56 (58.9)	31 (32.6)	8 (8.4)	0.65	190	143 (75.3)	47 (24.7)	0.88

Age at initial methamphetamine consumption	N	Genotypes			p value	N	Alleles		p value
		L/L	L/H	H/H			L	H	
<20 Y (%)	73	42 (57.5)	26 (35.6)	5 (6.8)		146	110 (75.3)	36 (24.7)	
≥ 20 Y (%)	66	40 (60.6)	21 (31.8)	5 (7.6)	0.93	132	101 (76.5)	31 (23.5)	0.82

increase in dynorphin peptides produced by chronic methamphetamine administration may inhibit dopamine release and contribute to the emotional and motivational effects of methamphetamine withdrawal. H alleles were shown to have significantly higher promoter activity of the PDYN gene than L alleles (containing 1 or 2 repeats) [26]. It is possible that dynorphin feedback against methamphetamine-induced dopamine release is greater in patients with an H allele of the PDYN gene than those with an L allele. Therefore, methamphetamine abusers with an H allele of the PDYN gene may consume more frequent and higher doses of methamphetamine to avoid dysphoric withdrawal induced by intense dynorphin-negative feedback, and may finally result in heavier metamphetamine dependence than those with an L allele.

Recently, Chen et al. [4] reported that an allelic variation in the promoter region of the PDYN gene is associated with individual differences in vulnerability to cocaine dependence or abuse ($p = 0.042$) and suggested that the H allele of the gene might contribute to protection from and decrease individual vulnerability to cocaine dependence or abuse. Cocaine belongs to the class of psychostimulants and shares a similar pharmacological profile with methamphetamine, which enhances dopamine release in the striatum and accumbens. However, findings of the present study and the study of Chen et al. indicate that the roles of variants of the PDYN gene may be opposite in cocaine dependence and methamphetamine dependence. There is a report that amphetamines and cocaine induce similar but not identical effects on expression of the PDYN gene. Turchan et al. [21] investigated the influence of acutely and chronically administered cocaine and amphetamine on biosynthesis of PDYN in the rat amygdala, which is one of the key structures for the development of substance dependence. They found that the level of PDYN mRNA was significantly increased in the amygdala after a single injection of cocaine, but not of amphetamine. In contrast, repeated injections of amphetamine significantly increased the PDYN mRNA level in the amygdale, but repeated injections of cocaine did not. These temporal differences in acute and chronic effects of cocaine and amphetamines on expression of the PDYN gene may result from different roles of the H allele of the PDYN gene as protective and risk factors for cocaine and methamphetamine dependencies, respectively. Thus, a single administration of cocaine to individuals with the H allele may increase the level of PDYN gene expression and dynorphin peptides more than to those with the L allele. Increased dynorphin induced by acute cocaine administration may produce a dysphoric effect by stimulating κ opioid receptors rather than a euphoric effect at the initial consumption. Therefore, individuals with the H allele of the PDYN gene may not want or like cocaine because of its less euphoric and more dysphoric effects, with the result that it may be hard to continue consuming cocaine and develop dependence. On the other hand, a single dose of methamphetamine does not increase the level of PDYN gene expression. Individuals with the H allele, as well as those with the L allele, can obtain euphoric and reinforcing effects from methamphetamine because dynorphin-negative feedback is not initiated. The reinforcing effect due to acute methamphetamine consumption can lead to repeated consumption of methamphetamine. Repeated methamphetamine administration in individuals with the H allele of the PDYN gene may increase the level of PDYN gene expression and dynorphin release more than it does in those with L allele. Then, increased dynorphin may gradually produce a dysphoric effect by stimulation of κ opioid receptors and activation of negative feedback against dopamine activity, and may force those with H allele to crave methamphetamine to compensate for the reduction of dopaminergic stimulation. Carrying the H allele may promote development of methamphetamine dependence more easily than carrying the L allele. Accordingly, the H allele may be a genetic risk factor for methamphetamine dependence and a negative risk factor for cocaine dependence, respectively.

Alternatively, the difference in roles of the polymorphism of the PDYN gene in substance dependence may due to population differences between Japanese and Americans. The present study showed that genotype and allele distributions of the PDYN gene polymorphisms in a Japanese population differ considerably from those in the Caucasian population reported by Zimprich et al. [26]. Frequencies of alleles 1, 2, 3 and 4 were 3.8, 81.6, 13.4 and 2.8%, respectively, in Japanese, and they were 2.7, 32.0, 63.5 and 1.8%, respectively, in Caucasians. Allele 2 and L alleles were common in the Japanese population whereas allele 3 and H alleles were common in the Caucasian population [18,26]. The subjects examined in Chen's study consisted of three ethnicities, European American, African American and Hispanic American [4]. Although the relative distribution of the PDYN gene alleles differs among the three American populations, predominance of the H allele is common in all of them. These data suggest that the roles of genetic variants of the PDYN gene in drug dependence may differ between Japanese and American populations.

The present study may have some limitations. The sample size may not be large enough to eliminate type I and II errors. Statistical power analyses by G· Power program (v 2.1.2) considering an alpha value of 0.05 showed that the present sample size has 0.99 statistical power to detect significant differences in allele (d.f. = 7, $w = 0.372$), genotype (d.f. = 3, $w = 0.239$), allele of dichotomy (d.f. = 1, $w = 0.216$) and genotype of dichotomy (d.f. = 2, $w = 0.304$). As judged by the statistical power, the present sample size was estimated to have been sufficient to reveal any statistically significant differences. However, with regard to the subcategories of patients, the powers were only 0.55 and 0.063 to detect genotype difference in multi-substance abuse status (d.f. = 2, $w = 0.204$) and age at initial methamphetamine consumption (d.f. = 2, $w = 0.081$), respectively, indicating the possibility of type II errors. Secondly, the effect of population stratification must be taken into account. Indeed, there was no 3/3 genotype in 209 controls whereas 10 cases of the 3/3 genotype were observed in 143 patients. Because the frequency of allele 3 in controls was 0.134, the mathematically expected value of the 3/3 genotype cases should be 3.7, which may indicate possible stratification. However, genotype distribution of the control group did not deviate from the Hardy–Weinberg equilibrium. In addition, all the subjects were unrelated Japanese, born and living in a restricted area of Japan, and were carefully matched for age, gender and geography. Therefore, it is unlikely that our positive results are due to population stratification.

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