Study of Association between α-Synuclein Gene Polymorphism and Methamphetamine Psychosis/Dependence

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ABSTRACT: **Methamphetamine (MAP) dissipates proton gradients across the membranes of synaptic vesicles, enhances cytoplasmic dopamine (DA) concentrations, and causes calcium-independent, nonvesicular DA release into synapses. MAP is taken into the cytosol by the dopamine transporter (DAT) on the synaptic terminals of DA neurons, and endogenous DA is concurrently released through the transporter by carrier exchange mechanisms, resulting in a robust increase in DA concentration in the synaptic clefts. The enhanced DA release through DAT by MAP is the main mechanism for the reinforcing ef-**

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fects of MAP. The complexes of α-synuclein and DAT facilitate membrane clustering of the DAT, thereby accelerating DA uptake *in vitro*. α-Synuclein has been shown to be overexpressed in the midbrain DA neurons of chronic cocaine abusers. The present study was performed to study the association between the α-synuclein gene polymorphisms and MAP psychosis/dependence in Japanese population. Since the T10A7 polymorphic site at the 5′ end of the noncoding exon 1′ in the α-synuclein gene is highly polymorphic, we analyzed the noncoding exon 1′ and intron 1, including this polymorphic site by sequencing. We confirmed four single nucleotide polymorphisms (SNPs) within 1.38 kbp of the T10A7 polymorphic site. No significant difference was found in genotype or allele frequencies in the T10A7 polymorphic site between MAP psychotic/dependent and control subjects. We found significant association between three SNPs in the vicinity of this polymorphic site in intron 1 and MAP psychosis/dependence in female subjects, but not in males. These results suggest an association of the α-synuclein gene polymorphisms with MAP psychosis/dependence in our female subjects. Further analyses are necessary to clarify the gender difference, by using a larger sample size and/or different ethnic groups, as well as functional variations in the α-synuclein gene.

KEYWORDS: **methamphetamine; dopamine transporter; α-synuclein; mesolimbic dopaminergic pathway**

INTRODUCTION

α-Synuclein is a major component of nigral Lewy bodies in Parkinson's disease.[1,2] α-Synuclein is a soluble presynaptic protein and is abundant in neurons,[3] but its function is yet to be elucidated. Lee and colleagues found that complexes of α-synuclein and dopamine transporter (DAT) facilitate membrane clustering of the DAT, thereby accelerating dopamine (DA) uptake *in vitro*.[4] Excess α-synuclein potentiates production of reactive oxygen species by DA, which may cause cell death.[5–8] Modulation of DA transmission by α-synuclein is probably involved with neurodegenerative and neuropsychiatric disorders such as drug dependence.

The mesolimbic dopaminergic pathway has an important role in addiction to psychostimulants and reinforcement. [^{3}H]-WIN 35428 binding sites, which reflect DAT protein amount and/or function, were increased in postmortem brains of cocaine abusers.[9] Mash and colleagues found overexpression of α-synuclein protein in DA neurons in cocaine abusers.[10] These findings provide further support for the involvement of α-synuclein in regulating dopaminergic neurons.[9,10] Methamphetamine (MAP) dissipates proton gradients across the membranes of synaptic vesicles, enhances cytoplasmic DA concentrations, and causes calcium-independent, nonvesicular DA release into synapses. MAP is taken into cytosol by DAT on the synaptic terminals of DA neurons, and endogenous DA is concurrently released through the transporter by carrier exchange mechanisms, resulting in a robust increase of DA concentration in the synaptic clefts. The enhanced DA release through DAT by MAP is the main mechanism for the reinforcing effects of MAP.[11,12] It has been reported that long-term MAP abuse induced development of psychosis. These findings suggested the importance of α-synuclein on MAP abusers and prompted us to study the association between the α-synuclein gene and MAP psychosis/dependence in Japa-

nese population. A highly polymorphic sequence variation (T10A7) has been reported at the 5′ end of the noncoding exon 1′ of α-synuclein gene.[13] In the present study, we have investigated whether the polymorphic sites in the noncoding exon 1′ and intron 1, including T10A7, are associated with MAP psychosis/dependence in Japan.

MATERIALS AND METHODS

Subjects

This study was performed following approval from the ethics committees of each institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA); all subjects provided written informed consent for the use of their DNA samples for this research. The subjects were 170 unrelated patients with MAP-dependence disorder meeting ICD-10-DCR criteria (F15.2 and F15.5), who were outpatients or inpatients of psychiatric hospitals of JGIDA, and also 161 geographical origin-matched healthy controls, mostly medical staff who had no past or family history of drug dependence or psychotic disorders. Patients were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder, or an organic mental syndrome. All subjects were Japanese, born and living in certain areas of Japan, including northern Kyusyu, Setouchi, Chukyou, Toukai, and Kantou. Blood samples were drawn for DNA extraction from 170 patients (male 138, female 32) and 161 controls (male 83, female 78). The mean age of the patients was 37.6 ± 12.0 years (male: 39.5 ± 12.0 years; female: 29.4 ± 7.4 years). The mean age of the controls was 38.6 ± 12.0 years (male: 38.2 ± 11.1 years; female: 39.1 ± 12.9 years). Genomic DNA was extracted from peripheral blood by the phenol/chloroform method.

Defining Variation with the α-Synuclein Gene

The 5′ end of the noncoding exon 1′ in the α-synuclein gene (accession no. AF163864) was amplified by PCR, and the products were sequenced in both directions using BigDye terminators (Applied Biosystems). Amplification primer pairs were 11F: CAT CTC CCA TCC ATC TTG GC and 12F: AGA AGC TCT GAC AAA TCA GCG GTG. The PCR product was 1.38 kbp and was sequenced using four primers (11F, 11R: AAA TCT GTC TGC CCG CTC TC, 12F, 12R: ACC CGG TGT TCT CCA GGA TTT CCA). Genotyping and sequencing were performed on an ABI3100 Genetic Analyzer (Applied Biosystems). The position numbers of polymorphic variants are quoted with respect to the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) consortium database.

Statistical Analysis

Data for each locus were used to estimate allele and genotype frequencies and to test for Hardy-Weinberg equilibrium (HWE), using the chi-squared method or the Arlequin program available from http://anthropologie.unige.ch/arlequin.[14] The allele and genotype frequencies of patients and control groups were compared using the chi-squared method and the Monte Carlo type CLUMP analysis program.[15]

RESULTS

Our subjects were 170 MAP psychotic/dependent patients and 161 controls. DNA samples from 16 of the patients were sequenced in 1.38 kbp around the T10A7 polymorphic site at the 5′ end of the noncoding exon 1′ of the α-synuclein gene. We confirmed four SNPs (rs#1372520, 3756063, 2870027, 3756059) in these patients in addition to the T10A7 polymorphic site. All these four SNPs were in intron 1. The genotype and allele frequencies of these four SNPs were all in Hardy-Weinberg equilibrium (HWE), indicating no sample bias in our case and control samples. These four SNPs showed no association in genotypic or allelic analysis according to the chi-squared test (TABLE 1a). We found four allelic variations in the T10A7 polymorphic site (TABLE 2a). The genotype frequencies of the MAP psychotic/dependent group and control group were in HWE (control $P = .73$, MAP $P = .77$). Genotype frequencies were compared using the CLUMP analysis program and showed no association ($P = .677$, T1 = 4.00). Allele frequencies also showed no association based on the chi-squared test ($P = .622$, chi-squared = 1.77).

Since there were many more MAP psychotic/dependent males than females, we analyzed the associations in each gender. In males, there was no difference in the four SNPs and the T10A7 polymorphic site between patients and control samples (TABLES 1b and 2b). In females, genotype frequencies were significant in rs#1372520 ($P = .03$), rs#3756063 ($P = .03$), and rs#3756059 ($P = .03$) (TABLES 1c and 2c).

DISCUSSION

We have analyzed the sequence variation (T10A7) at the 5′ end of the noncoding exon 1′ in the α-synuclein gene and found no significant difference in genotype or allele frequencies between MAP psychotic/dependent subjects and controls. We confirmed four SNPs in intron 1 and found a significant difference of genotype in three SNPs in MAP psychotic/dependent females, but not in males. Association in the T10A7 polymorphism was first studied by Autere and colleagues. They found no statistically significant differences in Parkinson's disease patients in Finland.[13] Since the T10A7 polymorphic site has many variations, this site is thought to be a good marker for an association study of the α-synuclein gene. Our results at this site do not suggest any role for the α-synuclein gene in MAP psychosis/dependence. We nevertheless found significant association between three SNPs in the vicinity of this polymorphic site and MAP psychosis/dependence in female subjects, though not males. The reason for this gender difference is not clear, although recent evidence suggests women and men differ in their progression to dependence and abuse. In preclinical and clinical studies, it has been suggested that ovarian hormones, particularly estrogen, are involved in gender differences in drug abuse.[16] Koizumi and colleagues also found a correlation between glutathione S-transferase M1 gene deletion and MAP abuse by females (Koizumi and Iyo, unpublished data). The data in our study should be carefully treated, as the samples were divided into two groups by gender. The significance was corrected to $P = .025$ by Bonferroni corrections, and the P value of these sites was .03, suggesting weak association.

The functional alterations caused by these SNPs are not clear in the present study, but there are several possibilities. First, the SNPs or relating linkage disequilibrium

TABLE 1. Genotype and allele distribution of α-synuclein gene SNPs in patient and control groups

SNP	Group	*N*	Genotype (Percent)			*P*	Allele (Percent)		*P*
a. Male and Female									
rs#1372520			GG	GA	AA		G	A	
	Control	161	141 (88)	18 (11)	2 (1)		300 (93)	22 (7)	
	MAP	170	142 (84)	27 (16)	1 (1)	.389	311 (92)	29 (5)	.502
rs#3756063			CC	CG	GG		C	G	
	Control	161	2 (1)	18 (11)	141 (88)		22 (7)	300 (93)	
	MAP	170	1 (1)	29 (17)	140 (82)	.263	31 (9)	309 (91)	.348
rs#2870027			GG	GA	AA		G	A	
	Control	161	55 (34)	75 (47)	31 (19)		185 (58)	137 (43)	
	MAP	170	60 (35)	88 (52)	22 (13)	.281	208 (61)	132 (39)	.371
rs#3756059			CC	CT	TT		C	T	
	Control	161	2 (1)	18 (11)	141 (88)		22 (7)	300 (93)	
	MAP	170	1 (1)	28 (17)	141 (83)	.323	30 (9)	310 (91)	.420
b. Male									
rs#1372520			GG	GA	AA		G	A	
	Control	83	71 (86)	11 (13)	1 (1)		153 (92)	13 (8)	
	MAP	138	119 (86)	18 (13)	1 (1)	.932	256 (93)	20 (7)	1.000
rs#3756063			CC	CG	GG		C	G	
	Control	83	1 (1)	11 (13)	71 (86)		13 (8)	153 (92)	
	MAP	138	1 (1)	20 (14)	117 (84)	.909	22 (8)	254 (92)	.888
rs#2870027			GG	GA	AA		G	A	
	Control	83	27 (32)	40 (48)	16 (19)		94 (57)	72 (43)	
	MAP	138	46 (33)	76 (55)	16 (12)	.274	168 (61)	108 (39)	.435
rs#3756059			CC	CT	TT		C	T	
	Control	83	1 (1)	11 (13)	71 (86)		13 (8)	153 (92)	
	MAP	138	1 (1)	19 (14)	118 (86)	.932	21 (8)	255 (92)	.920

TABLE 1. (*continued*) **Genotype and allele distribution of α-synuclein gene SNPs in patient and control groups**

SNP	Group	*N*	Genotype (Percent)			*P*	Allele (Percent)		*P*
c. Female									
rs#1372520			GG	GA	AA		G	A	
	Control	78	70 (90)	7 (9)	1 (1)		147 (94)	9 (6)	
	MAP	32	23 (72)	9 (28)	0 (0)	.030	55 (86)	9 (14)	.077
rs#3756063			CC	CG	GG		C	G	
	Control	78	1 (1)	7 (9)	70 (90)		9 (6)	147 (94)	
	MAP	32	0 (0)	9 (28)	23 (72)	.030	9 (14)	55 (86)	.077
rs#2870027			GG	GA	AA		G	A	
	Control	78	28 (36)	35 (45)	15 (19)		91 (58)	65 (42)	
	MAP	32	14 (44)	12 (38)	6 (19)	.719	40 (63)	24 (38)	.671
rs#3756059			CC	CT	TT		C	T	
	Control	78	1 (1)	7 (9)	70 (90)		9 (6)	147 (94)	
	MAP	32	0 (0)	9 (28)	23 (72)	.030	9 (14)	55 (86)	.077

TABLE 2a. Genotype and allele frequencies of the T10A7 polymorphism of the α-synuclein gene in MAP psychosis/dependence: male and female

Subjects	*N*	Genotype (Percent)						
		T10A7/ T10A7	T10A8/ T10A8	T11A6/ T11A6	T10A7/ T10A8	T10A7/ T11A6	T10A8/ T11A6	T11A6/ T12A5
Control	161	18 (11)	7 (4)	31 (19)	30 (19)	46 (29)	29 (18)	0 (0)
MAP	170	20 (12)	10 (6)	22 (13)	29 (17)	53 (31)	35 (21)	1 (1)
		Allele (Percent)						
		T10A7	T10A8	T11A6	T12A5			
Control	322	112 (35)	73 (23)	137 (43)	0 (0)			
MAP	340	122 (36)	84 (25)	133 (39)	1 (0)			

NOTE: *N*, number of genotypes and alleles in MAP psychotic/dependent subjects and controls. MAP and control genotypes of both sexes were in Hardy-Weinberg equilibrium (control $P = .73$, MAP $P = .77$). CLUMP analysis for genotype frequencies: $P = .677$ (T1 = 4.00). Chi-squared test for allele frequencies: $P = .622$ (chi-squared = 1.77).

TABLE 2b. Genotype and allele frequencies of the T10A7 polymorphism of the α-synuclein gene in MAP psychosis/dependence: male

Subjects	*N*	Genotype (Percent)						
		T10A7/ T10A7	T10A8/ T10A8	T11A6/ T11A6	T10A7/ T10A8	T10A7/ T11A6	T10A8/ T11A6	T11A6/ T12A5
Control	83	7 (8)	4 (5)	16 (19)	16 (19)	23 (28)	17 (20)	0 (0)
MAP	138	14 (10)	9 (7)	16 (12)	22 (16)	45 (33)	31 (22)	1 (1)
		Allele (Percent)						
		T10A7	T10A8	T11A6	T12A5			
Control	166	53 (32)	41 (25)	72 (43)	0 (0)			
MAP	276	95 (34)	71 (26)	109 (39)	1 (0)			

NOTE: *N*, number of genotypes and alleles in MAP psychotic/dependent subjects and controls. MAP and control genotypes of males were in Hardy-Weinberg equilibrium (control $P = .82$, MAP $P = .34$). CLUMP analysis for genotype frequencies: $P = .682$ (T1 = 3.96). Chi-squared test for allele frequencies: $P = .748$ (chi-squared = 1.22).

TABLE 2c. Genotype and allele frequencies of the T10A7 polymorphism of the α-synuclein gene in MAP psychosis/dependence: female

Subjects	*N*	Genotype (Percent)						
		T10A7/ T10A7	T10A8/ T10A8	T11A6/ T11A6	T10A7/ T10A8	T10A7/ T11A6	T10A8/ T11A6	T11A6/ T12A5
Control	78	11 (14)	3 (4)	15 (19)	14 (18)	23 (29)	12 (15)	0 (0)
MAP	32	6 (19)	1 (3)	6 (19)	7 (22)	8 (25)	4 (13)	0 (0)
		Allele (Percent)						
		T10A7	T10A8	T11A6	T12A5			
Control	156	59 (38)	32 (21)	65 (42)	0 (0)			
MAP	64	27 (42)	13 (20)	24 (38)	0 (0)			

NOTE: *N*, number of genotypes and alleles in MAP psychotic/dependent subjects and controls. MAP and control genotypes of females were in Hardy-Weinberg equilibrium (control $P = .87$, MAP $P = .33$). CLUMP analysis for genotype frequencies: $P = .991$ (T1 = 0.82). Chi-squared test for allele frequencies: $P = .936$ (chi-squared = 0.42).

positions may change DAT and α-synuclein complex formation. MAP is taken into cytosol by DAT on the synaptic terminals of DA neurons, and endogenous DA is concurrently released through the transporter by carrier exchange mechanisms.

α-Synuclein forms functional protein–protein complexes, thereby modifying dopaminergic neurotransmission.[4] Overexpression of α-synuclein in mice increased the density of the DAT.[17] Mutation of the α-synuclein gene may affect complex formation with DAT, modulating dopaminergic neurotransmission. Modulated expression from the mutated α-synuclein gene may then alter the development of MAP psychosis/dependence.

As a second possibility, the SNPs or relating linkage disequilibrium positions may change the transcriptional expression level. Several positron emission tomography studies found that DAT in the caudate/putamen of MAP abusers was significantly reduced.[18,19] Some patients showed a lasting reduction of DAT for several months after detoxication. Sekine and colleagues also showed reduction of DAT in the caudate/putamen, and also in the nucleus accumbens and prefrontal cortex of MAP dependents.[19] Elevated DA concentration in the synaptic clefts is removed rapidly by reuptake through DAT. Reduced DAT density in MAP dependence may delay DA clearance and contribute to the persistence of a hyperdopaminergic state. Cocaine potentiates dopaminergic neurotransmission in a different way from MAP, binding to the DAT, blocking neurotransmitter uptake, and giving rise to marked elevations in synaptic DA. It has been reported that chronic cocaine abuse increases α-synuclein levels in midbrain DA neurons.[10] α-Synuclein levels in the DA cell groups of the substantia nigra/ventral tegmental complex were elevated threefold in chronic cocaine users compared with normal age-matched subjects. These results suggest that overexpression of α-synuclein may occur as a protective response to changes in DA turnover. Since the three SNPs were in intron 1, it is possible that these variants contribute to changes in expression of the α-synuclein gene.

In conclusion, our findings suggest a weak association of the α-synuclein gene with MAP psychosis/dependence in our female samples. Further work is necessary to clarify the gender difference, using a larger sample size and/or different ethnic groups of MAP psychotic/dependent subjects as well as functional variations in the α-synuclein gene.

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No Association Is Found between the Candidate Genes of t-PA/Plasminogen System and Japanese Methamphetamine-Related Disorder

A Collaborative Study by the Japanese Genetics Initiative for Drug Abuse

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ABSTRACT: **In the central nervous system, tissue-plasminogen activator (t-PA)/plasmin system is involved in long-term synaptic plasticity and remodeling, and participates in rewarding effects of methamphetamine (MAP), by acutely regulating MAP-induced dopamine release in the nucleus accumbens. The aim of this study was to examine the relationships between the patients with MAP abusers/psychosis and the t-PA/plasminogen system genes. Subjects comprised 185 MAP abusers and 288 healthy controls. Four polymorphisms in the t-PA,**

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plasminogen activator inhibitor, and plasminogen genes were examined in the present study. No significant differences were observed in each polymorphism between healthy controls and MAP abusers/psychosis. This study suggests that t-PA/plasminogen system is unlikely to be a major contributor to the substance abuse liability and/or the development of MAP psychosis.

KEYWORDS: **tissue-plasminogen activator; plasminogen activator inhibitor; plasminogen; methamphetamine; drug dependence; polymorphisms; association study**

INTRODUCTION

In the central nervous system, tissue-plasminogen activator (t-PA)/plasmin system is involved in long-term synaptic plasticity and remodeling, and participates in rewarding effects of methamphetamine (MAP), by acutely regulating MAP-induced dopamine release in the nucleus accumbens.[1] The acute effect of morphine on t-PA expression was diminished by repeated administration. The function of the t-PA/plasmin system in regulating dopamine release as well as rewarding effects of MAP indicates that the pathophysiology of MAP-related disorder could be involved in t-PA/plasminogen system in human.

Genetic factors have been reported to be involved in the susceptibility to drug dependence and the manifestation of drug-induced psychosis from the results of family, twin, and adoption studies.[2,3] Use of MAP is in epidemic proportions among psychostimulants in Japan. MAP induces a strong psychological dependence and its repeated consumption is known to frequently result in the development of psychiatric symptoms that resemble the paranoid type of schizophrenia.[4]

Therefore, it can be hypothesized that the genes involved in the t-PA/plasminogen system are important candidate genes for schizophrenia and psychostimulant-induced psychosis. In this study, the relationships between three genes of the t-PA/plasminogen system and MAP abusers/psychosis were examined in the Japanese population. The Alu insertion/deletion polymorphism of the human t-PA gene (PLAT),[5] the 4G/5G insertion/deletion polymorphism of the human plasimonogen activator inhibitor gene (PAI-1),[6] and two single nucleotide polymorphism genes of the human plasminogen gene (PLG) were genotyped for study by our association. This study was carried out as one of the collaborative studies by the Japanese Genetics Initiative for Drug Abuse (JGIDA) organized to facilitate the sample collection of MAP abusers/psychosis for investigating the genetic factors related to the substance abuse liability and the characteristics of MAP psychosis.

SUBJECTS AND METHODS

Ethical Considerations

This study was initiated after the approval of the ethical committee in each attending institution of JGIDA. Written informed consent was obtained from all participating patients.

Subjects

The subjects consisted of 185 unrelated patients with MAP dependence and psychotic disorder who met the ICD-10-DCR criteria (F15.2 and F15.5) and who were outpatients or inpatients of psychiatric hospitals of JGIDA; and 288 age-, gender- and geographical origin–matched unrelated healthy controls mostly consisted of medical staffs who had no past history and no family history of drug dependence or psychotic disorders. All subjects were Japanese, born and living in the western area of the main island of Japan.

Genomic Procedure

Genomic DNA was extracted from the leukocyte nuclei of peripheral blood. Genotyping was carried out according to the standard protocol, slightly modified from the published methods.[5,6]

Statistics

The presence of Hardy-Weinberg equilibrium was tested using a chi-squared goodness-of-fit test. Comparisons of allele and genotype frequencies were carried out using the chi-squared test for 2×2 and 2×3 contingency tables. The significance level and significant trend level were defined when the P value was less than 0.05 and 0.1, respectively. For the PLG gene, the haplotype association test was carried out using the SAS/Genetics program.[7,8]

RESULTS

All genotype distributions were not significantly different from those expected according to the Hardy-Weinberg equilibrium in any group examined in the present study.

The genotypic distribution and the allelic frequency of four polymorphisms—that is, Alu insertion/deletion in PLAT, 4G/5G insertion/deletion in PAI-1, and two polymorphisms (rs3757017 and rs14224) in PLG between healthy controls and the patients with MAP abusers/psychosis are shown in TABLE 1. No significant differences were observed in both polymorphisms between healthy controls and MAP abusers/psychosis.

For PLG, we performed haplotype case controls study; however, there was no difference in haplotype frequency among patients and controls.

DISCUSSION

Using the results of fundamental research, we typed each tag polymorphism of three candidate genes of the t-PA/plasminogen system; we were not, however, able to find significant association with any candidate genes and MAP-related patients. Based on the common variants–common disease hypothesis,[9] our results showed that there are no high-frequency disease variants at a range in linkage disequibrium where we typed in the present study.[10] However, complex genetics disorders might

TABLE 1. Genotype, allele, and haplotype frequencies of the PLAT, PAI-1, and PLG polymorphism in MAP-related disorders and healthy controls

a) PLAT

		Genotype			Allele		Frequency
	N	D/D	D/I	I/I	D	I	(I)
Controls	288	77	146	65	300	276	0.48
MAP	185	48	96	41	192	178	0.48

b) PAI-1

		Genotype			Allele		Frequency
	N	4G/4G	4G/5G	5G/5G	4G	5G	(5G)
Controls	288	115	138	35	368	208	0.36
MAP	184	87	80	17	254	114	0.31

c) PLG

		Haplotype					
	N	D/D	D/I	I/I	D	$?^2$	Exact P
Controls	288	0.48	0.09	0.01	0.42		
MAP	184	0.47	0.11	0.01	0.41	1.11	0.75

be caused by combinations of many small effect genes, for example, by genetic heterogeneity. We did not search for genetic variants, including low-frequency variants.[11] Further resequencing is needed to confirm that unknown rare variants in linkage disequilibrium might be a real disease gene.

The present results did not show any association between t-PA/plasminogen system candidate genes and the patients with MAP abusers/psychosis. Although the t-PA/plasminogen system plays an important role in the possibility of developing the dependence to MAP and other psychostimulants such as morphine and cocaine, further investigation is needed to explore other clinical samples and/or other candidate genes which might be related to the t-PA/plasminogen system.

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Comparison of G-Protein Selectivity of Human 5-HT_{2C} and 5-HT_{1A} Receptors

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ABSTRACT: **We compared the ability of human 5-HT_{2C} and 5-HT_{1A} receptors to couple to selected G proteins expressed in insect Sf9 cells through simultaneous infection with recombinant baculoviruses. We also examined the coupling of G proteins to these same receptors in membranes derived from the Sf9 cells using *in situ* reconstitution with purified G proteins. Our data show that unoccupied 5-HT_{2C} and 5-HT_{1A} receptors can attain an activated conformation that is stabilized by interaction with specific G proteins. While high-affinity agonist binding to the 5-HT_{2C} receptor was increased to a greater extent by $G\alpha_q$ than by $G\alpha_{i2}$, the high-affinity agonist binding to the 5-HT_{1A} receptor was preferentially enhanced by $G\alpha_{i2}$ coexpression. When the two 5-HT receptors were expressed in cells also expressing G proteins, both 5-HT_{2C} and 5-HT_{1A} receptors appear to activate $G\alpha_{i2}$ in preference to $G\alpha_q$. In contrast, *in situ* reconstitution data show that 5-HT_{2C} receptors robustly activate $G\alpha_q$ and marginally activate $G\alpha_o$ or $G\alpha_i$, whereas 5-HT_{1A} receptors only marginally activate $G\alpha_q$ and robustly activate $G\alpha_o$ and $G\alpha_i$. These results suggest that the**

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ABBREVIATIONS USED: 5-HT, 5-hydroxytryptamine (serotonin); G protein, guanine nucleotide–binding regulatory protein; GPCRs, G-protein–coupled receptors; Sf9, *Spodoptera frugiperda* 9; GTPγS, guanosine 5′-*o*-(3-thiotriphosphate); 8OH-PIPAT, *R*(+)-*trans*-8-hydroxy-2-[*N*-*n*-propyl-*N*-(3′-iodo-2′-propenyl) amino]tetralin; *p*-MPPI, 4-(2′-methoxyphenyl)-1-[2′-[*N*-(2″-pyridinyl)-*p*-iodobenzamido]ethyl]piperazine; EGTA, ethylene glycol–bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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overexpression of receptor and potential G-protein coupling partners in Sf9 cells may lead to erroneous conclusions as to the signaling selectivity of receptors.

KEYWORDS: **serotonin receptor; G protein; serotonin; Sf9 cells**

INTRODUCTION

A single serotonin (5-HT) receptor subtype can elicit multiple signaling outputs[1] and may yield different responses when heterologously expressed in different cell types. Such apparent coupling promiscuity is at least partially explained by cross talk between second messenger pathways[2,3] and by the fact that not only Gα subunits, but also Gβγ subunits regulate effector systems.[4,5] Thus, it is often the case that evidence is obtained for the interaction of a single receptor with several different G proteins.[6]

Receptor/G-protein coupling specificity can be examined by coexpression in Sf9 insect cells of the receptor with distinct G-protein subunits through simultaneous infection with the appropriate recombinant baculoviruses.[7] Successful 5-HT receptor/G-protein interaction is characterized by high-affinity agonist binding[8,9] and receptor-catalyzed activation of G proteins, as measured by agonist-stimulated [^{35}S]-GTPγS binding to G proteins.[7,10,11]

It has been widely accepted that the 5-HT_{2C} receptor is positively coupled to phospholipase C through G_q protein,[10,11] and several studies have established that this receptor is positively coupled to phospholipase D through G13 protein.[12,13] On the other hand, it is well known that 5-HT_{1A} is negatively coupled to adenylyl cyclase through the G_i subfamily of G proteins, such as $G\alpha_i$, $G\alpha_o$, and $G\alpha_z$.[7]

In the present study, using the Sf9 cell/baculovirus system, we compared the G-protein selectivity of the human 5-HT_{2C} and 5-HT_{1A} receptors coexpressed with $G\alpha_i$ and $G\alpha_q$ subunits. We have also examined this by *in situ* reconstitution of the Sf9 expressed receptors with purified fractions of these G proteins. Our data show a major discrepancy between the results obtained with the two methods and suggest the possibility of promiscuous G-protein coupling in the Sf9 coexpression system due either to a superoptimal level of expression or to variation in posttranslational modification or protein-protein modulation of G-protein subunits or 5-HT receptors in Sf9 cells.

MATERIALS AND METHODS

Materials

Baculovirus containing recombinant DNA encoding mouse $G\alpha_q$ was kindly provided by T. Kozasa and A. Gilman at Southwestern Medical Center, Dallas. The baculovirus for rat $G\alpha_{i2}$ was the gift of J. Garrison (University of Virginia). Baculoviruses encoding $G\beta_1$, $G\gamma_1$, and $G\gamma_2$ have been previously reported.[14] Recombinant baculoviruses of the 5-HT_{2C} or 5-HT_{1A} receptors were constructed in our laboratory by respectively subcloning the EcoRI fragment of the cDNA of 5-HT_{2C} and 5-HT_{1A} receptor into pVL1393. Sf9 cells were transfected with a combination of the recom-

binant transfer vector and linearized AcMNPV DNA. Baculovirus containing recombinant DNA was isolated using standard techniques.

Coexpression of 5-HT$_{2C}$ or 5-HT$_{1A}$ Receptors with G-Protein Subunits in Sf9 Cells and Membrane Preparation

Sf9 cells were maintained in suspension culture in TNM-FH media containing 10% fetal calf serum, 0.1% pluronic F-68, and 0.01 mg/mL gentamycin at 29°C. For expression, cells were subcultured in monolayer and grown to 60% confluency, at which time they were infected with one or more recombinant viruses. For analyses of ligand binding, cells were harvested 48–60 h after infection. The infected cells were homogenized and centrifuged at 30,000*g* for 20 min. Crude membranes were washed twice and resuspended in solution A (10 mM MOPS, pH 7.5, 1 mM EGTA, 12% sucrose) at a protein concentration of ~100–200 μg/mL and stored at –70°C.

To evaluate expression levels of the receptors, membranes expressing 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors were incubated for 20 min at 30°C in solution B (50 mM MOPS, pH 7.5, 100 mM NaCl, 1 mM EDTA, 3 mM $MgSO_4$, 1 mM DTT, 3 mg/mL BSA) with [^{3}H]-mesulergine and [^{125}I]-*p*-MPPI, respectively. Mianserin and 5-HT were used to measure nonspecific binding of 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors, respectively. B_{max} values were determined by Scatchard transformation.

In Situ *Reconstitution of 5-HT$_{2C}$ and 5-HT$_{1A}$ Receptors with G-Protein Subunits*

The P_2 pellet of the 5-HT$_{2C}$ or 5-HT$_{1A}$ receptors was resuspended in solution A (10 mM MOPS, pH 7.5, 1 mM EGTA, 100 μM AEBSF) containing 7 M urea. After incubation in 7 M urea for 30 min on ice, the extracted membranes were diluted to 4 M urea with solution A, pelleted, and washed once with solution A. The final pellet was resuspended in solution A with 12% sucrose, and aliquots were frozen and stored at –80°C. The number of binding sites was determined by a saturation-binding assay using [^{3}H]-mesulergine (for the 5-HT$_{2C}$ receptor) or [^{125}I]-*p*-MPPI (for the 5-HT$_{1A}$ receptor).

Squid retinal $G\alpha_q$ was purified from squid photoreceptors as described by Okada *et al.*[11] Bovine brain $G\alpha_i$ and $G\alpha_o$ as well as bovine brain $G\beta\gamma$ were purified as described previously.[15]

GTPγS Binding Assay

The receptor-catalyzed binding of GTPγS to Gα was determined with the addition of 10 μM GDP to compete for uncatalyzed GTPγ[^{35}S] binding. Receptor-containing membranes were mixed with G-protein subunits with or without the ligand (agonist or inverse agonist) on ice in a total volume of 50 μL. The reactions contained 10 μM GDP and ~2 nM [^{35}S]-GTPγS in solution B. Reactions were incubated at 30°C, terminated by adding 2 mL of ice-cold solution C (20 mM Tris/HCl, pH 8.0, 25 mM $MgCl_2$, 100 mM NaCl), and filtered over nitrocellulose membranes on a vacuum manifold. The filters were washed four times with 2 mL each of ice-cold solution C and dried. The radioactivity on the filters was determined in a Wallac 1219 liquid scintillation spectrometer.

Analysis of Data and Curve Fitting

All results shown are representative of data obtained in two or more separate experiments. All curves presented were best fits to a simple exponential model for progress curves or a single binding model for saturation isotherms using the program "Grafit4".

RESULTS AND DISCUSSION

Expression of 5-HT$_{2C}$ and 5-HT$_{1A}$ Receptors in Sf9 Cells

We evaluated the expression of 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors in Sf9 cells by the binding of the partial inverse agonists, [^{3}H]-mesulergine (5-HT$_{2C}$) and [^{125}I]-*p*-MPPI (5-HT$_{1A}$), to P_2 membranes prepared from baculovirus infected cells. As shown in FIGURE 1A and B, high levels of expression of both receptors were obtained 48 h after baculovirus infection. We have routinely obtained from 50 to 200 pmol/mg membrane protein of antagonist binding sites for these two receptors. In the absence of coinfection with G-protein–encoding viruses, both the 5-HT$_{2C}$ and the 5-HT$_{1A}$ receptors display a single low-affinity displacement of antagonist binding by 5-HT (data not shown), consistent with homogeneous populations of receptors uncoupled from G proteins.

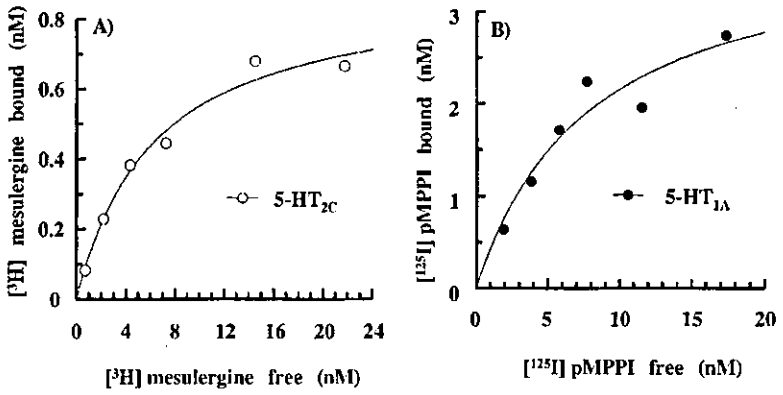

FIGURE 1. Binding of [^{3}H]-mesulergine (A) and [^{125}I]-*p*-MPPI (B) in membranes from Sf9 cells coexpressing 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors, respectively. Saturation experiments using [^{3}H]-mesulergine and [^{125}I]-*p*-MPPI were performed on membranes prepared from Sf9 cells following infection with recombinant baculoviruses encoding human 5-HT$_{2C}$ and 5-HT$_{1A}$ receptors, respectively. The figure represents data obtained in one of several experiments. Each point in the figure represents the mean of triplicate determinations.

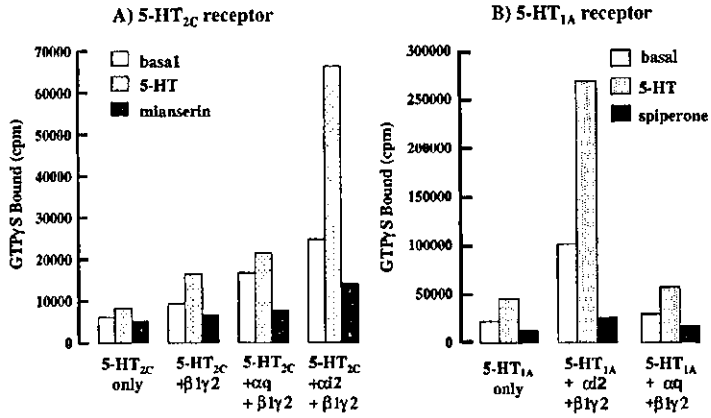

FIGURE 2. 5-HT_{2C} **(A)** and 5-HT_{1A} **(B)** receptor-promoted binding of GTPγS to Gα subunits. Membranes were prepared from Sf9 cells expressing recombinant proteins and then incubated with [^{35}S]-GTPγS. The GTPγS binding reactions proceeded for 10 min at 30°C. Mianserin (10 μM) and spiperone (10 μM) were used as the inverse agonists for the 5-HT_{2C} and 5-HT_{1A} receptors, respectively.

Functional Coupling of 5-HT_{2C} and 5-HT_{1A} Receptors with G-Protein Heterotrimers in Sf9 Cells

We have previously reported on the restoration of the G-protein interaction with 5-HT_{2C} receptors by *in situ* reconstitution with G_q.[10] We wished to examine the G-protein selectivity of the 5-HT_{2C} and 5-HT_{1A} receptor subtypes by the coinfection strategy of Barr and Manning.[7] This latter strategy allows for the examination of a variety of G proteins that may not be readily expressed and purified to homogeneity as is required for the *in situ* reconstitution approach. These experiments produced the startling results shown in FIGURE 2A. Whereas our previous findings indicated a nearly absolute specificity for G_q for the rat 5-HT_{2C} receptor, coinfection of Sf9 cells with the human 5-HT_{2C} receptor and G-protein subunits yields a greater amount of G-protein activation for the $G\alpha_{i2}$ protein than for $G\alpha_q$ (FIG. 2A). This finding is at odds with results on the ability of G proteins to restore high-affinity agonist binding for [^{125}I]-DOI in the same membranes. In this analysis, the $G\alpha_q$ protein increased the ratio of [^{125}I]-DOI to [^{3}H]-mesulergine binding to a greater extent than did $G\alpha_{i2}$ (data not shown).

The G-protein–coupling behavior of the human 5-HT_{1A} receptor in these studies in Sf9 cells is more consistent with the known signaling pathways for this receptor. Membranes from Sf9 cells coinfected with the 5-HT_{1A} receptor and $G\alpha_{i2}$ display a far greater 5-HT-stimulated binding of GTPγS than those coinfected with $G\alpha_q$ (FIG. 2B). Similarly, $G\alpha_{i2}$ enhanced the ratio of the agonist [^{125}I]-8OH-PIPAT to antagonist [^{125}I]-*p*-MPPI to a far greater extent than found with $G\alpha_q$ (data not shown).