reward vs mnemonic components of addictions, for example.[20] Increasing our ability to determine which constellation of genetic and environmental factors plays a role in the methamphetamine dependence of each affected individual should improve "personalized" targeting of treatment and prevention efforts to those most likely to benefit from them.

Genome-wide association (GWA) can help to elucidate chromosomal regions and genes that contain allelic variants that predispose to substance abuse. This approach does not require family member participation. It gains power as densities of genomic markers increase.[21-24] Association identifies smaller chromosomal regions than linkage-based approaches. Genome-wide association fosters pooling strategies that preserve confidentiality and reduce costs, including those that we have previously validated.[25-28] This approach provides ample genomic controls that can minimize the chances of unintended ethnic mismatches between disease and control samples (eg, stratification). The large numbers of assessments that are key components of GWA do mandate careful use of statistical approaches that correct for multiple comparisons and studies in multiple independent samples, such as those that we now report.

We thus now describe GWA in 2 samples of methamphetamine-dependent and control individuals. These studies test the a priori hypothesis that marker allele frequency differences between methamphetamine-dependent and control individuals will help us to identify genes whose alleles predispose to development of dependence on methamphetamine. Sample 1 contrasts (1) Han Chinese methamphetamine-dependent individuals from the Taipei region of Taiwan with (2) age- and sex-matched Han Chinese Taiwanese control individuals free from any histories of abuse or dependence on any legal or illegal addictive substance. Sample 2 contrasts (1) Japanese methamphetamine-dependent individuals with (2) age- and sex-matched Japanese control individuals free from any histories of abuse or dependence on any legal or illegal addictive substance. We used standard statistical approaches to document the power that these samples provided to identify genetic influences of different magnitudes. We identified striking convergence of the data from sample 1 and sample 2, in ways that are never attained by chance in many Monte Carlo simulation trials. We discuss the convergence that these data provide with recently reported GWA studies of related phenotypes that include polysubstance abuse, nicotine dependence, alcohol dependence, success in quitting smoking, and individual differences in memory. To our knowledge, these results provide the first replicated GWA study that identifies "methamphetamine dependence" genes.

METHODS

RESEARCH VOLUNTEERS

Sample 1

Subjects recruited in Taipei provided informed consent for genetic studies under protocols approved by ethics committees at the respective institutions: 30% were female and the mean (SD) age was 32.5 (10) years. One hundred forty individuals were diagnosed independently by each of 2 psychiatrists based on interviews, review of records, and Chinese versions of the Diagnostic Interview for Genetic Studies[29] and the Family Interview for Genetic Studies[30] using *DSM-IV* criteria.[31] These individuals were of ethnic Han Chinese origin and older than 17 years, reported methamphetamine use more than 20 times per year (unless they described well-documented methamphetamine psychosis), and denied histories of psychosis either prior to methamphetamine use or in relation to other psychedelic drugs. Most reported use of at least 1 other addictive substance. Two hundred forty Han Chinese controls, who were matched for sex and age, were older than 17 years, and denied either illegal drug use or psychotic symptoms to psychiatric interviewers, were recruited in Taipei from hospital and pharmacy staffs, blood donation centers, and an electric company.

Sample 2

Subjects who were born and resided in the northern Kyushu, Setouchi, Chiba, Tokai, or Kanto regions of Japan provided informed consent for genetic studies under protocols approved by ethics committees at the respective institutions. Twenty-one percent of subjects were female and the mean (SD) age was 39.9 (13) years. One hundred methamphetamine-dependent subjects were inpatients or outpatients of psychiatric hospitals in these regions that participate in the Japanese Genetics Initiative for Drug Abuse[32-45] and met *International Statistical Classification of Diseases, 10th Revision, Diagnostic Criteria for Research*[46] criteria F15.2 and F15.5 for methamphetamine dependence in independent diagnoses made by each of 2 trained psychiatrists based on interviews and review of records. Ninety-one percent revealed histories of methamphetamine psychosis, 89% used methamphetamine intravenously, 62% also abused organic solvents, and most abused at least 1 other substance. Subjects who displayed clinical diagnoses of schizophrenia, other psychotic disorders, or organic mental syndromes were excluded. Controls were 100 age-, sex-, and geographically matched staff recruited at the same institutions, who denied use of any illegal substance, abuse or dependence on any legal substance, any psychotic psychiatric illness, or any family history of substance dependence or psychotic psychiatric illness during interviews with trained psychiatrists.

DNA PREPARATION AND ASSESSMENT OF ALLELE FREQUENCIES

Genomic DNA was prepared from blood,[28,32,47,48] quantitated,[28,32] and combined into pools representing 20 individuals of the same ethnicity and phenotype. Relative allele frequencies were assessed using Affymetrix (Santa Clara, California) microarrays.

Hybridization probes were prepared from the genomic DNA pools (as described in the Affymetrix GeneChip Mapping Assay manual), with precautions to avoid contamination that included dedicated preparation rooms and hoods. Briefly, 50 ng of pooled genomic DNA was digested by *Xba*I or *Hind*III (100K) or by *Sty*I or *Nsp*I (500K), ligated to appropriate adaptors, and amplified using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California) with a 3-minute 94°C hot start; 30 cycles of 30 seconds at 94°C, 45 seconds at 60°C, and 60 seconds at 68°C (100K) or 15 seconds at 68°C (500K); and a final 7-minute 68°C extension. Polymerase chain reaction (PCR) products were purified (MinElute 96 UF kits; Qiagen, Valencia, California) and quantitated. Forty micrograms of PCR product were digested for 35 minutes at 37°C with 0.04-unit/μL deoxyribonuclease I to produce 30- to 100–base pair fragments, which were end-labeled using terminal deoxynucleotidyl trans-

ferase and biotinylated dideoxynucleotides and hybridized to the appropriate 100K (*Xba*I or *Hind*III arrays) or 500K (*Sty*I or *Nsp*I arrays) array (early-access Centurion and commercial Mendel array sets; Affymetrix). Arrays were stained and washed as described in the Affymetrix GeneChip Mapping Assay manual using immunopure strepavidin (Pierce, Milwaukee, Wisconsin), biotinylated antistreptavidin antibody (Vector Labs, Burlingame, California), and R-phycoerythrin strepavidin (Molecular Probes, Eugene, Oregon). Arrays were scanned and fluorescence intensities were quantitated using an Affymetrix array scanner, as described previously.[28] Estimates for "genomic coverage" for these marker densities were almost 0.8 (sample 1) and almost 0.9 (sample 2).[49]

Chromosomal positions for each single-nucleotide polymorphism (SNP) were sought using NCBI (build 36.1; National Center for Biotechnology Information, Bethesda, Maryland) and NetAffyx (Affymetrix) data. Allele frequencies for each SNP in each DNA pool were assessed based on hybridization intensity signals from 4 arrays, allowing assessment of hybridization to the 20 (100K arrays) or 12 (500K arrays) "perfect match" cells on each array that were complementary to the PCR products from alleles "A" and "B" for each diallelic SNP on sense and antisense strands. We eliminated (1) SNPs with minor allele frequencies less than 0.02 determined using Affymetrix data; (2) SNPs on sex chromosomes; and (3) SNPs whose chromosomal positions could not be adequately determined. We thus analyzed data from the remaining 371 820 and 466 883 SNPs (for sample 1 and sample 2, respectively) in detail. Each array was analyzed, as described previously,[28] subtracting background values, normalizing to the highest values noted on the array, averaging the hybridization intensities from the array cells that corresponded to the perfect match "A" and "B" cells, calculating "A/B ratios" by dividing average normalized A values by average normalized B values, performing arctangent transformations to aid combination of data from arrays hybridized and scanned on different days, and determining the average arctan value for each SNP from the 4 replicate arrays. This approach is thus based on hybridization intensity data from Affymetrix scanners rather than relative allele score (RAS) or k corrections derived from RAS scores.[50,51]

The analyses presented in this work use standard methods for correcting hybridization values for each perfect match feature based on chip-to-chip differences in background fluorescence and in total fluorescence intensity. These approaches have generated good, approximately 0.95, correlations between individually genotyped and pooled-genotype values in extensive validation experiments.[32,52] Other approaches to analysis of pooling-based GWA studies have focused on the RAS measurements that derive from Affymetrix software to generate k correction scores for each SNP that attempt to correct for probe × probe variation (ie, that induced by, or consistent with, differential hybridization effects).[50,51] In studies that have used these corrections, correlations between individually and pooled genotyped SNP allelic frequencies can equal or exceed those that we have observed in validation experiments.[53,54] However, RAS scores have been used less and less as the genotype-calling algorithms for successive generations of Affymetrix arrays have improved their accuracy. Initial RAS scores are based in part on data from mismatch cells, which have again been eliminated from successive generations of Affymetrix arrays because of their inconsistent effects on accuracy. The k corrections based on RAS scores that are generated in different laboratories produce differing results.[55] Further, we have found that substantial numbers of the array features that provide information for the RAS scores are saturated under conditions used to conduct individual genotyping (Q-R I., D.W., and G.R.U., unpublished data, 2005), leading us to use smaller amounts of input DNA and hybridization probes for the pooled assays reported herein. The k corrections may prove to be useful for experiments in which saturation is controlled carefully and where data from heterozygote control individuals are generated in the same experiments and in the same laboratories as the pooling data. However, in the present analysis, this adds to the variation that we already parse as quantified by replicate pools (ie, biological haplotype replication), applications of different chips to the same pool (ie, chip-oriented technical replication), and different samples altogether (ie, overall association replication).

ANALYSES

We compared data for all the pools from methamphetamine-dependent individuals with all of the pools from control individuals separately for sample 1 and sample 2, as previously described.[28] A *t* statistic for the differences between abusers and controls was generated, as described previously,[29] for each SNP for each sample. For each sample, we focused on "nominally positive" SNPs that displayed *t* statistics with $P < .05$ for abuser-control differences. We first sought evidence for clustering of the nominally positive SNPs from each sample. We focused on chromosomal regions in which at least 3 of these nominally positive SNPs, assessed by at least 2 different array types, lay within 25 kilobases (kb) of each other. We term these clustered nominally positive SNPs *clustered positive SNPs* and focus our analyses on regions in which they lie. The degree of clustering within each single sample provides a technical control (eg, assurance that there are haplotypes that occur at different frequencies in dependent vs control samples) that could result from stochastic differences in haplotypes as well as differences related to the methamphetamine-dependence phenotype.

To seek the SNPs within the strongest positive support from both data sets, we sought convergence between data from sample 1 and sample 2 (**Table**).[56] Analyses focused on genes identified by clustered positive results from both samples, rather than on individual SNPs whose informativeness might differ between samples 1 and 2. Clustering of positive results in the same gene in each of 2 independent samples is unlikely to represent purely stochastic effects for most genes and is thus likely to reflect differences related to dependence on methamphetamine (and/or to dependence on addictive substances in general).

Monte Carlo simulation trials assessed the significance of the results in ways that correct for the number of repeated comparisons made herein, as described previously.[28] These empirical statistical approaches do not require assumptions about the underlying distribution of the data sets, as do statistical approaches such as analysis of variance, and allow correction for the hundreds of thousands of repeated comparisons in ways that would provide difficulties for repeated analyses of variance. For each trial, a randomly selected set of SNPs from the current data set was assessed to see if it provided results equal to or greater than the results that we actually observed (eg, to see how frequently randomly selected sets of 15 565 SNPs from sample 1 and 25 538 SNPs from sample 2 contained nominally positive SNPs that lie clustered within 25 kb of each other on the chromosomes, see "Results" section). The number of trials for which the randomly selected SNPs displayed the same features of observed results was then tallied to generate an empirical *P* value. These simulations thus corrected for the number of repeated comparisons made in these analyses, an important consideration in evaluating this large association genome scanning data set. We used a similar approach to assess the likelihood that the convergences between the current data and data obtained from other samples might occur by chance.

To seek possible generalization of these results, we sought locations where the clustered positive data from both sample 1 and sample 2 lie at chromosomal positions near clustered positive results from studies that compared allelic frequencies in

Table. Selected "Methamphetamine Dependence" Genes Identified by Clustered Positive Results From Both Sample 1 and Sample 2[a]

Gene	Class	Description	SNPs[b]	*P* Value[c]
SGCZ	CAM	Sarcoglycan, zeta	3, 20	<.00001
DAF/CD55	ENZ	Decay-accelerating factor for complement system	1, 4	<.00001
ACSL6	ENZ	Acyl-CoA synthetase long-chain family member 6	9, 5	<.00001
FKBP15	ENZ	FKBP15	4, 4	<.00001
PDE6C	ENZ	cGMP phosphodiesterase 6C α'	4, 7	<.00001
POU5F1	TF	POU-domain 5 transcription factor 1	1, 5	<.00001
SH3MD4	PROT	SH3 multiple domains 4	9, 7	<.00001
RALY	RNA	Autoantigenic RNA binding protein	5, 3	<.00001
PRKG1	ENZ	cGMP-dependent protein kinase I	14, 5	.00001
LARGE	ENZ	Like-glycosyltransferase	11, 3	.00001
PCOLCE2	STR	Procollagen C endopeptidase enhancer 2	3, 2	.00001
MOSC2	ENZ	MOCO sulphurase C-terminal domain containing 2	4, 5	.00002
ZNF423	TF	Zinc finger protein 423	5, 4	.00002
MAP2K5	ENZ	Mitogen-activated protein kinase kinase 5	5, 3	.00003
USP48	PROT	Ubiquitin-specific peptidase 48	3, 2	.00003
SMYD3	TF	SET MYND domain containing 3	7, 5	.00007
CCHCR1	REC	Coiled-coil α-helical rod protein 1	2, 4	.00009
LRRN6C	CAM	Leucine-rich repeat neuronal 6C	4, 13	.00010
CENPC2	STR	Centromere protein C2	2, 3	.00012
RAPGEF5	REC	Rap guanine nucleotide exchange factor 5	4, 1	.00016
SERPINA5	ENZ	Serpin peptidase inhibitor A 5	4, 1	.00018
PRDM2	TF	PR domain containing 2 with ZNF domain	6, 3	.00022
ASTN2	CAM	Astrotactin 2	12, 3	.00037
TM7SF4	PROT	Transmembrane 7 superfamily member 4	2, 3	.00037
TRPM3	CHAN	Transient receptor potential cation channel, subfamily M, member 3	4, 10	.00039
RGS17	ENZ	Regulator of G-protein signaling 17	4, 3	.00047
COL28A1	STR	Collagen, type XXVIII, alpha 1	4, 3	.00047
MOSC1	ENZ	MOCO sulphurase C-terminal domain containing 1	5, 1	.00048
PDE4B	ENZ	Phosphodiesterase 4B	8, 4	.00049
AOAH	ENZ	Acyloxyacyl hydrolase	3, 4	.00049
PDE4D	ENZ	Phosphodiesterase 4D	6, 6	.00057
ZNF659	TF	Zinc finger protein 659	6, 9	.00060
NRG1	CAM	Neuregulin 1	5, 3	.00064
HS3ST4	ENZ	Heparan sulfate (glucosamine) 3-O-sulfotransferase 4	3, 7	.00064
MYO5B	STR	Myosin 5B	4, 11	.00065
PSD3	REC	Pleckstrin and sec7 domain containing 3	3, 15	.00078
AK5	ENZ	Adenylate kinase 5	6, 3	.00080
CUBN	REC	Cubilin	6, 6	.00085
FHIT	ENZ	Fragile histidine triad gene	8, 20	.00088

Abbreviations: Acyl-CoA, acyl coenzyme A; CAM, cell adhesion molecule; cGMP, cyclic guanine monophosphate; CHA, channels; DIS, disease associated; ENZ, enzymes; PROT, protein processing; REC, receptors (combining single TM, 7 TM, and ligand-gated channel families); RNA/DNA, RNA/DNA handling or modification; SNP, single-nucleotide polymorphism; STR, structural proteins; TF, transcriptional regulation; TRANSP, transporter.

[a] Each gene listed here contains at least 5 clustered positive SNPs with *P* < .05 from sample 1 and/or sample 2, has a function that can be inferred, and displays a Monte Carlo *P* value < .001. Genes are grouped by the class of the function to which they appear to contribute: CAM, ENZ, STR, TF, PROT, REC, RNA/DNA, TRANSP, CHA, and DIS. The Monte Carlo *P* value represents probabilities of chance discovery of clustered nominally positive SNPs in segments of randomly selected genes that sum to the same size as the true gene identified in the present work. Genes listed in this Table are selected because their Monte Carlo *P* values are < .001 and/or because they are identified in other samples in ways that are discussed in the text (see eTable [available at http://www.archgenpsychiatry.com] for full table, in which correction for 109 repeated comparisons would require *P* < .0004 for significance).

[b] Numbers of clustered nominally positive SNPs from samples 1 and 2 that lie within the gene's exons or 10-kilobase flanks.

[c] Monte Carlo *P* value for the number of nominally significant SNPs lying within a gene region of the same size.

polysubstance abusers vs controls,[32] alcohol-dependent individuals vs controls,[37] nicotine-dependent individuals vs nondependent smokers,[38] individuals successful in quitting smoking vs those unsuccessful,[39] and individuals with better or poorer scores in memory testing[35] (Table).

To provide controls for the alternative possibilities that the results obtained herein could come from (1) occult racial/ethnic stratification or (2) assay noise, we compared the clustered positive SNPs from sample 1 and from sample 2 with SNPs that displayed the largest allele frequency differences between (1) European American vs African American control individuals, as previously described[32]; (2) HapMap Japanese (JPT) and Han Chinese (HCB) samples; and (3) SNPs that displayed the largest variances from array to array, as previously described.[32]

To assess the statistical power of our analysis, we used the program PS version 2.1.31[60] with (1) $\alpha = .05$, (2) sample sizes equal to the numbers of pools from the current data set, (3) mean abuser-control differences of 0.05 and 0.1, and (4) standard deviations from the SNPs that provided the largest differences between control and abuser population means from the current data set. We also present data from the Genetic Power Calculator

Power Calculations

There is no single standard for calculation of the power of GWA; we have thus presented calculations based on allele frequency differences in the body of this article. An alternative approach,

the Genetic Power Calculator, assumes substantial additional information about the genetic architecture and marker frequencies for the disorder being studied and is adapted to use with allele frequency information from individual genotyping. Using a reasonable set of assumptions about the genetic architecture and linkage disequilibrium between markers and disease alleles, we obtained powers of 0.63 and 0.4 for samples 1 and 2 from this approach.[31]

Alternative Means for Analyzing GWA Data

The experiments presented herein compare (1) disease/nondisease pools (a group factor); (2) multiple case and control pools (a within–disease group factor); (3) for each pool, multiple chip assays (a within-pool factor); and (4) sample 1 vs sample 2 results. While there is no single consensus for how to treat issues raised by so many multiple comparisons, there is also no reason to assume that there is such underlying normality of the data that parametric tests, or tests that make assumptions about underlying distributions of the data (eg, analysis of variance), should be used. Monte Carlo approaches used herein provide empirical statistical values that are based on the data sets that are actually generated in these experiments and provide tests for most of the hypotheses. In previous work, these results have correlated reasonably well with those from permutation and false discovery rate tests.[32]

Use of Detailed Linkage Disequilibrium Data From HapMap Samples as a Proxy for the Detailed Linkage Disequilibrium for the Present Samples

While general patterns of linkage disequilibrium are readily inferred from HapMap data, the detailed patterns of linkage disequilibrium from a number of samples that we have previously investigated have differed, often significantly, from those in HapMap samples. Use of HapMap data as a primary basis for calculation of linkage disequilibrium in the present samples complicates the Monte Carlo simulation paradigms that we used. We have thus used chromosomal distances as a primary metric in ways that allow crisp Monte Carlo simulations for the SNPs that are well localized and eliminated data from SNPs that are not well localized.

RESULTS

A number of features of the genotyping data support the validity of the approach used herein.[32] From sample 1, 371 820 SNPs (of 489 922 on 2 array types) and, from sample 2, 466 614 SNPs (of 609 431 on 4 array types) lie on chromosomes 1 to 22 and displayed minor allele frequencies of 0.02 or less. In the data from samples 1 and 2, 368 811 SNPs overlapped. Pooled genotyping for these SNPs displays features that support modest variability. Mean SEMs for the differences among the 4 replicate measurements of each DNA pool were ±0.040 and 0.038 for samples 1 and 2. The SEMs for pool-to-pool differences were ±0.025 and 0.029. Power calculations that used the observed variability from these samples, $\alpha = .05$, and the observed within-group standard deviations document 0.92 and 1 and 0.7 and 0.99 power to detect 5% and 10% differences in mean abuser vs control allele frequencies in samples 1 and 2, respectively.

A number of SNPs displayed nominally significant allele frequency differences between methamphetamine-dependent vs control individuals. In samples 1 and 2, 15 565 and 25 538 SNPs displayed t values with $P < .05$ (**Figure**). We term these SNPs *nominally positive SNPs*; since these P values are not corrected for multiple comparisons, these data do not allow us to distinguish these values from chance.

We obtained results that differed from those expected by chance; however, when we evaluated the extent to which 3 or more of these nominally positive SNPs "cluster" together with 25 kb or more separating them, 846 clusters contained 3749 of the 15 569 nominally positive SNPs from sample 1 and 1787 clusters contained 8388 of the 25 538 nominally positive SNPs from sample 2. Such clustering is found in no Monte Carlo trial of how frequently randomly selected sets of either 15 565 SNPs from sample 1 or 25 538 SNPs from sample 2 lie clustered within 25 kb of each other. With correction for the multiple comparisons made herein, the empirical P value for clustering of nominally positive SNPs is thus $< .00001$ for both samples 1 and 2. This degree of clustering within each single sample provides a control for the fact that we identified bona fide haplotypes that occur at different frequencies in the pools constructed from methamphetamine-dependent vs control samples. Stochastic differences in the frequencies at which these haplotypes occurred in our methamphetamine-dependent vs control samples that are independent of the addiction phenotype could conceivably contribute to some of the clustering in each individual sample, however.

We obtained evidence for replication and results that could not be expected by chance alone when we evaluated the genes that were identified by clustered nominally positive results from both sample 1 and sample 2 (Table and eTable, available at http://www.archgenpsychiatry.com). The degree of convergent identification of genes by data from each of these 2 samples was never observed by chance in any of 100 000 Monte Carlo simulation trials ($P < .00001$). The clustering of positive results in the same genes in both samples is thus very unlikely to represent stochastic effects. We term the genes identified in 2 samples in this way *"methamphetamine dependence" genes*. We use this term in quotation marks because variants in at least some of these genes are also likely to alter vulnerabilities to addictions for other substances (see later). The Monte Carlo P values assigned to each gene in the Table identify the probabilities that random segments of genes that have the same size as the true gene identified in each of these 2 samples would display at least the numbers of nominally positive SNPs actually identified in the true gene (see correction for multiple comparisons in the Table legend).

These "methamphetamine dependence" genes displayed convergence with genes identified by (1) clustered positive results from 639 000 SNP GWA studies of polysubstance abuse in National Institute on Drug Abuse European American and African American samples,[32] (2) nominally positive SNPs from 100 000 GWA studies of alcohol dependence,[35-38] and (3) nominally positive SNPs in comparisons of nicotine-dependent vs nondependent smokers (Table).[38] Data from samples 1 and 2 converge with these previously reported data sets, with Monte Carlo P values of (1) .0412, (2) .0016, and (3) .0003, re-

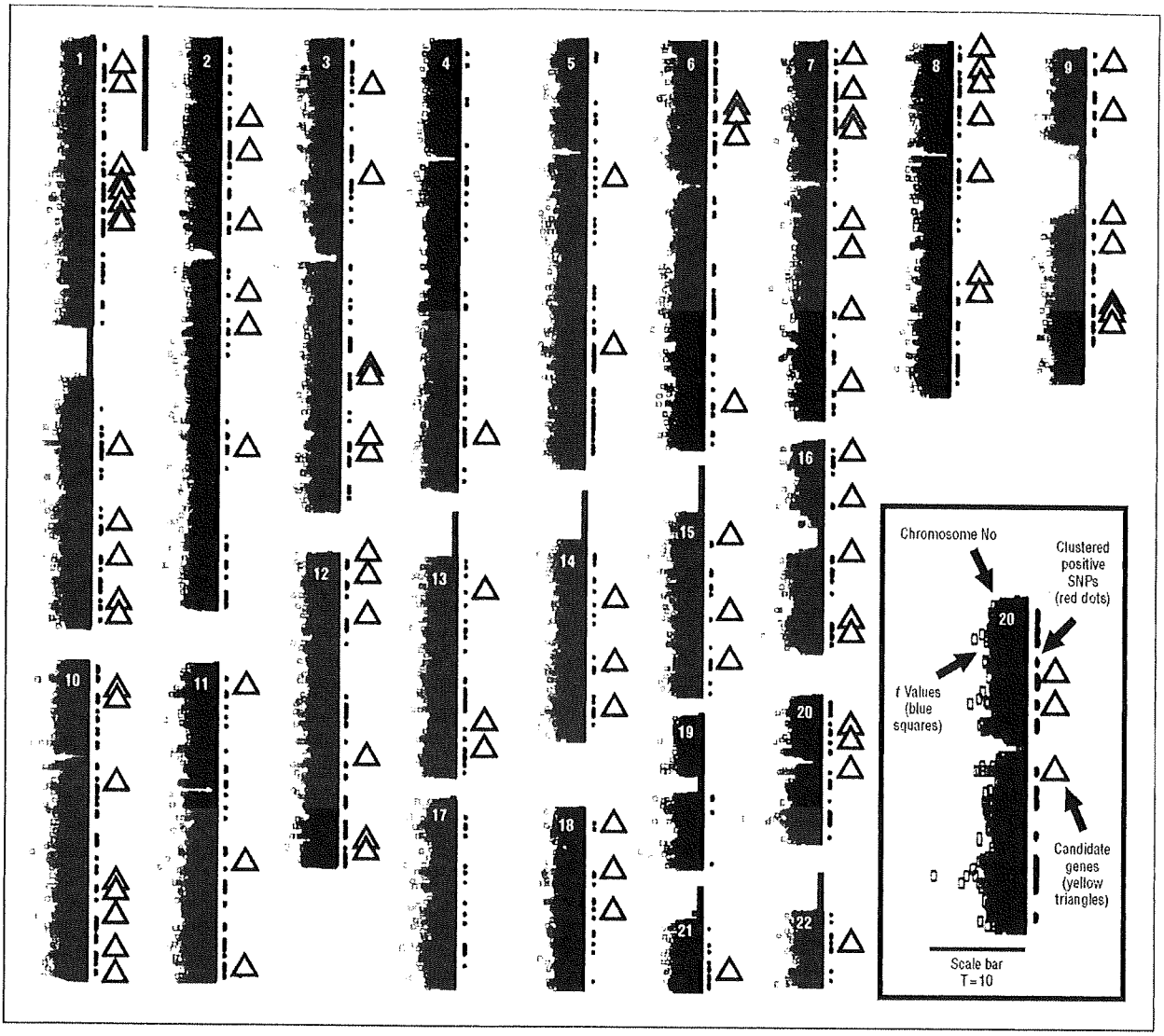

Figure. Cartoons of chromosomes 1 to 22. The blue squares to the left of the axis represent *t* values for the methamphetamine-dependent vs control allele frequency ratios mapped to the chromosomal position of each corresponding single-nucleotide polymorphism (SNP). The SNPs for which abuser-control differences display *P* values ≤.05 and that pass the clustering criteria of 3 outlier SNPs from 2 array types with less than a 25-kilobase inter-SNP distance are marked with red dots to the right of the axis. Clustered SNPs in genes with convergent evidence from both sample 1 and sample 2 are marked by yellow triangles to the right of the axis. The scale bar represents 50 Mb, with chromosomal positions based on National Center for Biotechnology Information (Bethesda, Maryland) MAPVIEWER coordinates and supplemental data from NetAffyx. The chromosomes are ordered in rows from left to right by chromosome number.

spectively. These analyses both correct for the multiple comparisons made and provide substantial additional support for many of the genes identified herein.

A number of the reproducibly positive genes identified in the current study are also identified by clustered positive results from 500 000 GWA studies of European American smokers who were successful vs unsuccessful in abstaining from smoking during clinical trials for smoking cessation ($P=.002$[59] and $P<.00001$ [G.R.U., unpublished data, 2007]).

The large differences between these observed results and chance clustering makes it highly unlikely that most of the clustered positive SNPs resulted from misgenotyping, for which there should be no reason that results should cluster. The SNPs that displayed clustered positive results in the current study failed to overlap appreciably more than expected by chance with the SNPs that displayed the largest variances from array to array (391 SNPs identified vs 386 expected by chance). Many of the positive SNPs in this report were thus likely to cluster since they lie near and display linkage disequilibrium with functional variants that contribute to individual differences in vulnerability to methamphetamine dependence. Convergence with observations made in other samples supports this idea and suggests that some of the functional variants that were identified by these clustered positive SNPs are likely to contribute to vulnerability to addictions to other substances as well as to methamphetamine.

There is also no evidence that most of the SNPs identified herein were found because of occult racial/ethnic stratification between methamphetamine-dependent and control groups. There was no significant overlap between clustered reproducibly positive SNPs from samples 1 and 2 with the SNPs that provided the largest racial/

ethnic differences from comparisons between European American and African American controls or between Japanese and Chinese HapMap samples (though 523 and 737 of the outlier SNPs from samples 1 and 2 do lie in the top 2.5% of the SNPs that distinguish JPT [Japanese from Tokyo] from CHB [Han Chinese from Beijing] HapMap samples, when 389 and 532 would be expected by chance; $.06 > P > .05$).

COMMENT

This report identifies chromosomal regions that are likely to contain allelic variants that alter vulnerability to methamphetamine dependence. The validity of these observations is supported by the clustering of nominally positive SNPs and from the convergence of data from 2 independent samples. The clustered positive markers from this work identify "methamphetamine dependence" genes whose products are involved in cell adhesion, enzymatic, transcriptional regulation, and other processes. The classes of genes identified and convergence with results from other GWA studies point toward substantial roles for individual differences in mnemonic, as well as rewarding, brain systems and individual differences in vulnerability to methamphetamine dependence.[20]

The reliability and validity of the current approach are supported by many lines of evidence. These include data for clinical assessments made by multiple observers, the reliability and validity of the microarray-based genotyping approaches used herein,[32,52,57,62] the extent to which the markers that displayed nominally positive differences between abusers and controls clustered together in specific chromosomal regions, the extent to which observations made in these 2 samples converge with each other, and the extent to which these results converge with those from other studies that compare dependent vs control individuals. We have also confirmed many of the results from these approaches using individual genotyping (A. Hishimoto, MD, PhD, T.D., and G.R.U., unpublished data, 2007).

Modeling studies indicate that the experimental designs used herein have significant statistical power to detect modest differences in allelic frequencies between methamphetamine-dependent individuals and controls. Nonetheless, there remains the likelihood of both false-positive and false-negative results. Power calculations indicate that our current approach will fail to identify 1% and 38% of the alleles that actually have 10% and 5% abuser vs control differences, respectively, in both samples; other calculations support higher false-negative rates (see "Power Calculations" subsection). As always, larger samples would help to reduce these false-negative results. However, independent of the separate statistical considerations for each population studied herein, the degree of replication and convergence between the 2 samples and with other drug-abusing populations provides additional confidence in results obtained.

Monte Carlo analyses indicate that we never, by chance, could identify a group of SNPs as large as the group in the Table that (1) display nominally significant P values, (2) cluster together in groups of 3 or more within small chromosomal regions, and (3) provide replication so that clustered nominally positive SNPs from comparisons in sample 1 fit with the clustered nominally positive SNPs in comparisons from sample 2. These statistical arguments are buttressed by technical convergence. Each of the clusters of nominally positive SNPs identified herein contain positive SNPs that are independently identified on at least 2 array types, each determined in quadruplicate.

In addition to the overall statistical confidence in the set of the genes identified herein, a number of these "methamphetamine dependence" genes overlap with genes identified in other GWA studies of addiction vulnerability and related phenotypes. More than half of the 23 cell adhesion genes identified in the current work are identified by prior GWA studies of polysubstance and alcohol dependence (8 genes), nicotine dependence (1 gene), memory (1 gene), and/or smoking cessation success (4 genes) in samples collected in the United States and Australia from individuals of self-reported European and African ancestries. Clustered positive markers in *DAB1* thus also distinguish those successful in quitting smoking vs those unsuccessful; *CLSTN2* (OMIM *611323) markers also identify success in quitting smoking and individual differences in memory; *NRXN1* markers also identify vulnerability to nicotine dependence among smokers; markers in *CRIM1*, *CSMD1*, *SGCZ*, *PTPRD*, and *LRRN6C* identify vulnerability to polysubstance use and to alcohol dependence; and markers in *CDH13* (OMIM *601364) and *DSCAM* (OMIM *602523) identify vulnerability to polysubstance use, alcohol dependence, and success in quitting smoking. These molecules join neurexin 3,[52,63] *NrCAM*,[64] and *PTPRB* (H. Ishiguro, MD, PhD, and G.R.U., unpublished data, 2007) and other cell adhesion molecule genes that display addict vs control associations in at least 3 different samples. Such results support careful use of *"methamphetamine dependence" genes* to describe genes likely to contain variants that predispose to methamphetamine dependence rather than to describe gene variants that predispose to vulnerability to only this drug.

Enzyme genes that are identified herein and also by repeated substance abuse GWA studies include *DAF1/CD55*, *FHIT*, *PDE4D*, and *PRKG1* (OMIM *176894). The putative transcription factor *ZNF423* is also identified by comparisons between those successful and unsuccessful in smoking cessation.

The channel gene *RYR3*, the transporter gene *XKR4*, the gene for RNA processing *A2BP1* (OMIM *605104), and the structural genes *ELMO1*, *SORCS1*, and *TACC2* are also identified by clustered positive results from repeated comparisons between substance-dependent and control samples. Markers at *A2BP1* also distinguish smokers who are successful vs unsuccessful in quitting.

The genes that contain markers whose frequencies distinguish the methamphetamine-dependent vs control subjects in the present report and also distinguish dependent vs nondependent subjects and those successful vs unsuccessful in quitting smoking represent an especially interesting group. These genes include *CDH13*, *DSCAM*, *PRKG1*, and *A2BP1*. Cadherin 13 is a glycosyl phosphatidylinositol–anchored cell adhesion molecule that is expressed in neurons in brain regions that are known to have a role in addiction, including the hippo-

campus, frontal cortex, and ventral midbrain.[65] *CDH13* can inhibit neurite extension from select neuron populations[65,66] and activate a number of signaling pathways.[67-70] It is thus a strong candidate for roles in brain mechanisms important for both developing and quitting addictions.

DSCAM is a single transmembrane domain cell adhesion molecule with immunoglobulin and fibronectin domains that is expressed strongly in the brain[71,72] and hippocampus in ways that are required for appropriate neuronal connections to form in memory-associated circuits in model organisms.[73,74] Flies with altered *Dscam* expression display alterations in memories of both rewarded and punished behaviors.[74]

PRKG1 is expressed in the brain and hippocampus and other neurons.[75,76] Nitric oxide dramatically modulates brain cyclic guanine monophosphate systems; *PRKG1* thus provides a major target for the products of nitric oxide synthases. Mnemonic and addictive functions can each be altered by changes in cyclic guanine monophosphate–dependent protein kinase and/or nitric oxide synthases.[77-79]

The *A2BP1* gene is highly expressed in neurons in brain regions that include the hippocampus.[80] *A2BP1* binds to a UGCAUG splicing enhancer element found 3′ to a substantial number of neuron-specific exons and thus acts as a specific regulator of the splicing processes that form mature messenger RNAs.[81] *A2BP1* itself contains a number of splicing variants that are likely to alter its functions.

Identifying *CLSTN2* markers in the present repeated comparisons between methamphetamine-dependent vs control subjects in repeated comparisons of success in quitting smoking and in relation to individual differences in memory is also interesting. *CLSTN2* is well positioned to provide calcium-dependent cell-adhesion functions in brain regions that include the hippocampus and in the postsynaptic densities where it is highly expressed. The identification of this and other genes whose variants are good candidates to contribute to mnemonic aspects of addiction support the view that substantial components of the individual difference in vulnerability to dependence on addictive substances relate to individual differences in mnemonic systems.[20]

The convergence between the genes identified by these samples and by genes identified in previous GWA studies for dependence on other legal and illegal addictive substances supports roles for allelic variants that are well represented in chromosomes from African, European, and Asian racial/ethnic groups.[32,57] Genes identified by these methamphetamine-dependence studies, but not as strongly by any of these other GWA comparisons, are also of interest. Neuregulin 1 is a strong candidate gene for vulnerability to schizophrenia in Icelandic and related populations.[82-84] Conceivably, variants in neuregulin 1 might even provide a generalized vulnerability to psychosis that could manifest itself in the presence of either methamphetamine or other risk factors for schizophrenia.

It is important to consider limitations of this convergent replicated GWA data for methamphetamine dependence. (1) The sample sizes available for this work provide moderate power to detect gene variants related to methamphetamine dependence in each sample. False-negative results are likely since we required positive data from each of the 2 samples. The likelihood of false negatives is also increased since we required positive results from several SNPs from at least 2 array types that cluster within small chromosomal regions, making it easier to miss modest association signals within small genes that contain few SNPs or genes whose SNPs lie on only 1 array type. (2) We focused only on data from autosomal regions herein. This focus allowed us to combine data from male and female subjects but may have neglected potentially important contributions from genes on sex chromosomes. (3) Differences in allele frequencies in different populations could explain why some genes were strongly associated with methamphetamine dependence in the Asian samples studied herein but not as strongly with related substance-dependence phenotypes studied in European American or African American samples. (4) Many of the subjects for this work came to clinical attention because of methamphetamine psychosis. They might thus not be totally representative of all methamphetamine-dependent individuals. (5) While each of these individuals was methamphetamine dependent, many also reported use of additional addictive substances, such as inhalants. These clinical considerations, as well as the overlap between the "methamphetamine dependence" genes identified herein and the genes identified in other GWA work, support the idea that many, but not all, of these loci are likely to contain allelic variants that provide a more general vulnerability to addictive substances. While we term these genes *"methamphetamine dependence" genes* to denote the fact that variants in these genes are likely to alter vulnerability to developing dependence on this substance, we use the term in quotation marks to denote the probability that many of these allelic variants may predispose individuals to dependence on other addictive substances as well. (6) None of the controls for this study reported any significant use of methamphetamine. The genes identified herein thus could influence vulnerabilities to initiation of methamphetamine use, persistence of this use, and/or the transition from persistent use to methamphetamine dependence. (7) The current report uses only one of a number of current approaches to analysis of data from GWA. Additional discussion of the limits of techniques for identifying polygenic influences in complex disorders and traits can be found elsewhere.[85-87] Despite these cautions, however, the replicated positive results that we document herein and the failure of control experiments to support alternative hypotheses do provide substantial confidence in roles for most of the genes reported.

The current data, and results of classic genetic studies, thus support polygenic influences on vulnerability to methamphetamine dependence from genes that, as a group, are highly unlikely to represent chance observations. *P* values for individual genes, based on the data from the current work, suggest that some of these genes are very strongly supported and some more modestly supported by these current data. Genes identified by both the current results and by data from other related reports appear especially worthy of further evaluation. Taken together, the data point toward the likelihood that brains of individuals who are most vulnerable to this addiction are likely to differ in a number of ways from those of in-

dividuals who are least vulnerable. Understanding these differences in increasing detail should aid us in improving understanding, prevention, and treatments for methamphetamine dependence.

Submitted for Publication: May 8, 2007; final revision received September 14, 2007; accepted September 17, 2007.

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Financial Disclosure: None reported.

Funding/Support: Financial support was received from National Institutes of Health Intramural Program (National Institute on Drug Abuse), Department of Health and Human Services, and the Taiwanese and Japanese Ministries for Science and Technology and the participating Japanese and Taiwanese institutions.

Additional Information: The eTable is available at http://www.archgenpsychiatry.com.

Additional Contributions: We acknowledge the invaluable assistance of the subjects and their families.

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American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 147B:54–58 (2008)

The Glycine Transporter 1 Gene (*GLYT1*) Is Associated With Methamphetamine-Use Disorder

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Glycine transporter (GlyT)-1 plays a pivotal role in maintaining the glycine level at the glutamatergic synapse. Glycine is an allosteric agonist of *N*-methyl-D-aspartate (NMDA) receptors. Because activation of NMDA receptors is an essential step for induction of methamphetamine dependence and psychosis, differences in the functioning of GlyT-1 due to genetic variants of the *GlyT-1* gene (*GLYT1*) may influence susceptibility. A case-control genetic association study of the *GLYT1* gene examined 204 patients with methamphetamine-use disorder and 210 healthy controls. We examined three single nucleotide polymorphisms (SNPs), SNP1, IVS3 + 411C > T, rs2486001; SNP2, 1056G > A, rs2248829; and SNP3, IVS11 + 22G > A, rs2248632, of the *GLYT1* gene and found that SNP1 showed a significant association in both genotype ($P = 0.0086$) and allele ($P = 0.0019$) with methamphetamine-use disorder. The T-G haplotype at SNP1 and SNP2 was a significant risk factor for the disorder ($P = 0.000039$, odds ratio: 2.04). The present findings indicate that genetic variation of the *GLYT1* gene may contribute to individual vulnerability to methamphetamine dependence and psychosis.

KEY WORDS: substance dependence; glycine transporter (GlyT); association study

Please cite this article as follows: Morita Y, Ujike H, Tanaka Y, Kishimoto M, Okahisa Y, Kotaka T, Harano M, Inada T, Komiyama T, Hori T, Yamada M, Sekine Y, Iwata N, Iyo M, Sora I, Ozaki N, Kuroda S. 2008. The Glycine Transporter 1 Gene (*GLYT1*) Is Associated With Methamphetamine-Use Disorder. Am J Med Genet Part B 147B:54–58.

INTRODUCTION

Abuse of methamphetamine induces a strong psychological dependence, and further consumption produces highly psychotic states, such as auditory hallucinations and persecutory delusions [Tatetsu, 1963; Ujike and Sato, 2004]. Conditioned place preference (CPP) and behavioral sensitization induced by methamphetamine treatment in rodents have been recognized as animal models of methamphetamine dependence and psychosis. Many lines of experimental evidence have shown that A10 dopamine neurons in the ventral tegmentum area (VTA) projecting into the accumbens, amygdala, and prefrontal cortex play central roles in the induction of CPP and sensitization to methamphetamine [Kalivas and Stewart, 1991; McBride et al., 1999; Ujike, 2002]. It was also demonstrated that activation of glutamate neurons in the prefrontal cortex projecting into the accumbens, amygdala, and VTA and activation of *N*-methyl-D-aspartate (NMDA) receptors are essential to the development of CPP and sensitization [Wolf, 1998]. For example, bilateral lesions of the prefrontal cortex prevented the induction of sensitization to psychostimulants [Wolf et al., 1995], and systemic treatment or intra-VTA injection of NMDA antagonists also prevented it [Karler et al., 1989; Vezina and Queen, 2000]. NMDA receptors are multimeric protein complexes that are activated by glutamate binding to the NR2 subunit [Laube et al., 1997]. Glycine also activates the NMDA receptors by binding to allosteric sites of the NR1 subunit [Johnson and Ascher, 1987]. Recent studies indicated that glycine concentrations around NMDA receptors in the forebrain are efficiently regulated and maintained at a subsaturated level by glycine transporters (GlyTs), which belong to a superfamily of 12 transmembrane Na^+/Cl^--dependent transporters [Sato et al., 1995]. GlyT antagonists, e.g., *N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)] propylsarcosine (NFPS), inhibited glycine uptake [Harsing et al., 2003]

Grant sponsor: Zikei Institute of Psychiatry (Okayama, Japan); Grant sponsor: Ministry of Health, Labour and Welfare of Japan; Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received 9 November 2006; Accepted 13 April 2007

DOI 10.1002/ajmg.b.30565

and robustly enhanced the NMDA receptor functions [Bergeron et al., 1998; Kinney et al., 2003]. GlyTs are divided into two subtypes, GlyT-1 and GlyT-2 encoded by *GLYT1* (SLC6A9, MIM 601019) and *GLYT2* (SLC6A5, MIM 604159), respectively. GlyT-1 is known to be predominantly expressed in glial cells in the central nervous system, especially the frontal cortex, and hippocampus [Borowsky et al., 1993; Zafra et al., 1995]. However, GlyT-1 was recently reported in neurons as well [Cubelos et al., 2005], where it is closely associated with NMDA receptors and regulates the extracellular glycine concentration at synapses [Smith et al., 1992]. GlyT-2 is mainly expressed in the spinal cord and brainstem, where it belongs to the family of Na^+/Cl^--dependent plasma membrane transporters [Liu et al., 1993], and is colocalized with inhibitory glycine receptors [Gomeza et al., 2003]. Therefore, it is possible that altered function of GlyT due to genetic variants of the *GLYT1*, but not the *GLYT2* gene, may affect individual susceptibility to methamphetamine-use disorder. We investigated the association between the *GLYT1* gene polymorphisms and the disorder by a case-control study.

MATERIALS AND METHODS

Subjects

The subjects were 204 patients with methamphetamine-use disorder (167 males and 37 females; mean age, 37.4 years; SD 11.9 years) who met the ICD-10-DCR criteria (F15.2) and who were outpatients or inpatients in psychiatric hospitals of the Japanese Genetics Initiative for Drug Abuse (JGIDA), and 210 age-, gender-, and geographical origin-matched normal controls (163 males and 47 females; mean age, 36.5 years; SD 10.6 years), who were mostly medical staff members without a past individual or family history of drug dependence or major psychotic disorders. Patients who had a comorbidity of any other major psychiatric disorders, e.g., schizophrenia and bipolar disorder, were excluded. Assessment for the diagnosis of methamphetamine-use disorder and controls were performed by trained psychiatrists on the basis of all available information, including hospital notes. These assessments were done by unstructured interview.

The patients started methamphetamine abuse at 21.0 ± 5.5 years. As to multi-substance abuse status, 37.2% patients concurrently abused other illicit drugs besides methamphetamine. Cannabinoids were most frequently abused (34.0%), and followed by LSD (14.1%), cocaine (13.1%), opioids (12%), and hypnotics (9.9%). More than 60% patients abused methamphetamine solely, but about half of them had a past history of organic solvent abuse in their teenage. One hundred eighty-eight patients have or had the comorbidity of methamphetamine psychosis (F15.50). More details about the backgrounds of the patients have been published elsewhere [Ujike et al., 2003]. After the study was described, written informed consent was obtained from all participants. This study was approved by the Ethics Committee at each institute of the JGIDA.

Genotyping

The genomic DNA was extracted from peripheral leukocytes using the standard phenol/$CHCl_3$ method. Over 100 single nucleotide polymorphisms (SNPs) spanning the *GLYT1* gene were listed in the NCBI SNP database and the International HapMap project data. Since there was no non-synonymous SNP in the gene which should have potential physiological effects, we selected SNPs which fulfilled the following conditions: (1) those allele frequencies in a Japanese population were already known by the database and their minor allele frequencies were over 10%, (2) they were recognized by inexpensive restriction enzymes, (3) they were not located in deep introns, (4) they were located apart from each other to cover the entirety of the gene. Finally, we selected three SNPs, rs2486001 (IVS3 + 411C > T, SNP1), rs2248829 (1056G > A, SNP2), rs2248632 (IVS11 + 22G > A, SNP3). The three SNPs of the *GLYT1* gene were individually amplified by polymerase chain reaction (PCR) using the primers listed in Table I. PCR was carried out in a total volume of 15 μl with 3% dimethyl sulfoxide and 0.75 units of Taq DNA polymerase in the reaction mixture. Initial denaturation was performed for 5 min at 95°C; 35 cycles were then performed (30 sec of denaturing at 95°C, 30 sec of annealing at the appropriate temperature, and 30 sec of extension at 72°C), followed by a final extension at 72°C for 5 min. The PCR products were then analyzed on 3.0% agarose gels after digestion with Eco47I (SNP1), HincII (SNP2), and MnlI (SNP3), respectively (Table I). Genotyping of SNP1, SNP2, and SNP3 were confirmed by direct sequencing of a part of the samples. To ensure the positive association for SNP1, we confirmed the genotypes of all samples by a different PCR mismatch primer set to produce a recognition site for a different restriction enzyme, Eco0109I (Table I).

Statistical Analysis

Deviation of the genotype counts from Hardy–Weinberg equilibrium was tested using a chi-square goodness-of-fit test. The statistical significance of difference was assessed by a chi-square test (genotype comparison) or log likelihood ratio test (allele comparison) at a significance level of 0.05. The pairwise linkage disequilibrium (LD) and haplotype frequencies were estimated by the EH algorithm using the SNPalyze program (Dynacom Co., Mobara-shi, Chiba, Japan).

RESULTS

Both genotype and allele frequency distributions of patients with methamphetamine-use disorder and control subjects are shown in Table II. The genotype distributions of SNP1, 2, and 3 of patients and controls did not deviate significantly from Hardy–Weinberg equilibrium. We found significant differences in the frequency of genotypes and alleles of SNP1 (genotype, $\chi^2 = 9.52$, $P = 0.0086$; allele, $G = 9.66$, $P = 0.0019$) and SNP2 (genotype, $\chi^2 = 6.04$, $P = 0.048$; allele, $G = 4.14$, $P = 0.042$) between patients with methamphetamine-use disorder and control subjects. SNP3 showed no significant differences in allele or genotype between groups. After Bonferroni correction, the genotype and allele frequencies of SNP1, but not SNP2, remained significant. The odds ratio of the T allele of SNP1 for methamphetamine-use disorder was 1.69 (95% confidence interval: 1.22–2.36).

We calculated the pairwise LD between SNP1, SNP2, and SNP3. D′ (absolute value) and r^2 for pairwise LD are shown in Table III. High LD was detected between SNP1 and SNP2 ($D' = 0.76$, $r^2 = 0.064$), SNP1 and SNP3 ($D' = 0.92$, $r^2 = 0.087$), and SNP2 and SNP3 ($D' = 0.87$, $r^2 = 0.70$).

Because SNP1, SNP2, and SNP3 were shown to be located on the same LD block, the global haplotypic association was analyzed for every combination of the three SNPs (Table IV). Haplotypes consisting of SNP1–SNP2, SNP2–SNP3, and SNP1–SNP2–SNP3 showed significant association with patients with methamphetamine-use disorder. Among them, the haplotype comprising SNP1–SNP2 showed the smallest P value ($P = 0.000011$). The frequency of each haplotype consisting of SNP1–SNP2 is shown in Table V. The T-G haplotype of SNP1–SNP2 showed a significant excess in patients with methamphetamine-use disorder over control subjects ($P = 0.000039$), indicating that this haplotype was a risk factor for methamphetamine-use disorder, with an odds ratio of 2.04 (95% confidence interval, 1.45–2.86). These associations were still significant even after Bonferroni correction.

TABLE I. PCR Primers for Single Nucleotide Polymorphisms in the Glycine Transporter-1 (*GLYT1*) Gene

SNP		Primer sequence	Product size (bp)	Annealing temp. (°C)	Restriction enzyme
SNP1 (IVS3 + 411C > T, rs2486001)	Forward-1	5′-CACCCCCAGCCTGTCTTTCAAC-3′	122	61	Eco471
	Reverse-1	5′-CCCCTAAATGGTTCAGGGATGT-3′			
	Forward-2	5′-AGCCTGGGCTGAGGCACACCAC-3′	188	65	Eco01091
	Reverse-2	5′-TTCTATTCCCTGGGGTTCAGCA-3′			
SNP2 (1056G > A, rs2248829)	Forward	5′-TGGGCAGAGGCAGGCACCTC-3′	141	61	HincII
	Reverse	5′-GAGTGACCCTGGAGGGAGCCGTTGA-3′			
SNP3 (IVS11 + 22G > A, rs2248632)	Forward	5′-AGAAGAGGGGTGGTGGGAATCC-3′	131	63	MnlI
	Reverse	5′-TACGGTGAGCACTCGAGCGTCC-3′			

TABLE II. Genotype and Allele Frequencies of SNPs of the *GLYT1* gene

Group	N	Genotype			*P*	Corrected *P*	Allele		*P*	Corrected *P*
SNP1 (rs2486001)		C/C (%)	C/T (%)	T/T (%)			C (%)	T (%)		
Control	210	139 (66.2)	63 (30.0)	8 (3.8)			341 (81.0)	79 (19.0)		
METH-use disorder	204	106 (51.9)	82 (40.2)	16 (7.8)	0.0086	0.026	294 (72.1)	114 (27.9)	0.0019	0.0057
SNP2 (rs2248829)		G/G (%)	G/A (%)	A/A (%)			G (%)	A (%)		
Control	210	110 (52.4)	76 (36.2)	24 (11.4)			296 (70.5)	124 (29.5)		
METH-use disorder	204	119 (56.7)	75 (35.7)	10 (4.8)	0.048	—	313 (76.7)	95 (23.3)	0.042	—
SNP3 (rs2248632)		G/G (%)	G/A (%)	A/A (%)			G (%)	A (%)		
Control	210	113 (53.8)	81 (38.6)	16 (7.6)			307 (73.1)	113 (26.9)		
METH-use disorder	204	122 (58.1)	70 (33.3)	12 (5.7)	0.45	—	287 (76.3)	89 (23.7)	0.20	—

METH, methamphetamine.

TABLE III. Pairwise Linkage Disequilibrium Estimations Between Single Nucleotide Polymorphisms (SNPs) in the *GLYT1* Gene

	SNP1	SNP2
SNP2	$D' = 0.76$ $r^2 = 0.064$	
SNP3	$D' = 0.92$ $r^2 = 0.087$	$D' = 0.87$ $r^2 = 0.70$

TABLE V. Estimated Haplotype Frequencies for Patients With Methamphetamine-Use Disorder and Controls

SNP	Haplotype	METH	CON	*P* value
SNP1–2	C-G	0.48	0.54	0.080
	C-A	0.24	0.27	0.30
	T-G	0.28	0.16	0.000039
	T-A	0.00	0.027	—

DISCUSSION

We found that the T allele of SNP1 (IVS3 + 411C > T, rs2486001) and the T-G haplotype consisting of SNP1 and SNP2 (1056G > A, rs2248829) of the *GLYT1* gene showed a substantially significant association with methamphetamine-use disorder (allele $P = 0.0018$, haplotype $P = 0.000039$). The T-G haplotype of the gene approximately doubles the risk of predisposition to methamphetamine-use disorder.

GlyTs strictly maintain glycine concentrations in the vicinity of NMDA receptors. Glycine binds to glycine sites on the NR1 subunit of NMDA receptors and activates NMDA receptor signaling. Because the glycine concentration is set low by GlyTs, glycine sites are subsaturated in the physiological condition [Sato et al., 1995]. An increase of glycine concentration due to a glycine diet or administration of a glycine transport inhibitor, NFPS, enhanced the NMDA receptor function in vitro [Bergeron et al., 1998; Martina et al., 2004] and in vivo [Chen et al., 2003]. Heterozygous *GLYT1* gene knockout mice showed enhancement of NMDA receptor function [Gabernet et al., 2005]. Therefore, increases and decreases in glycine induce stronger and weaker NMDA receptor neurotransmission. Because many lines of experimental evidence have shown that the glutamatergic system and NMDA receptor signaling in the brain play pivotal roles in the development of substance dependence on psychostimulants including amphetamine, methamphetamine, and cocaine [Cervo and Samanin, 1995; Bespalov, 1996; Kim and Jang, 1997; Wolf, 1998], it is possible that modulation of glycine sites or GlyTs also affects substance dependence. Induction of amphetamine-induced CPP in rodents was prevented by a glycine site antagonist L-701,324 (7-chloro-4-hydroxy-3-(2-phenoxy)phenyl-2(1H)-quinolone) [Mead and Stephens, 1999], and a glycine site partial agonist ACPC (1-aminocyclopropanecarboxylic acid), which disturbs the effects of endogenous glycine [Papp et al., 2002]. Combined with our findings, variants of the *GLYT1* gene may affect susceptibility to methamphetamine dependence by modulating NMDA receptor function.

NMDA receptor signaling is also considered to be involved in psychotic disorders. Phencyclidine and ketamine, noncompetitive antagonists of NMDA receptors, produce a psychotic state in healthy subjects and exacerbate symptoms in schizophrenics [Javitt and Zukin, 1991; Breier et al., 1997], and hypofunction of NMDA receptors is assumed to be a possible pathophysiology of schizophrenia. Mice with reduced expression of NR1 and the ε1 subunit of NMDA receptors due to genetic manipulation showed abnormal phenotypes similar to those observed in animal models of schizophrenia [Mohn et al., 1999; Miyamoto et al., 2001]. Recent human genetic studies also revealed a significant association of the *GRIN1* and *GRIN2A* genes encoding the NR1 and NR2A subunits of NMDA receptors, respectively, with susceptibility to schizophrenia [Itokawa et al., 2003; Zhao et al., 2006]. In addition, the GlyT inhibitors SSR504734 (2-chloro-*N*-[(*S*)-phenyl[(2*S*)-piperidin-2-yl]methyl]-3-trifluoromethyl benzamide) and NFPS produced antipsychotic profiles in experimental paradigms [Javitt et al., 2004; Depoortere et al., 2005], and heterozygous *GLYT1* knockout mice had reversed amphetamine-induced disruption of prepulse inhibition, one of the physiological phenotypes of psychosis [Tsai et al., 2004]. Because the majority of patients with methamphetamine-use disorder examined in the present study had a comorbid diagnosis of methamphetamine psychosis, it is possible that the *GLYT1* gene may be involved in liability to psychotic symptoms. We re-analyzed the data and found that methamphetamine psychosis is also significantly associated with SNP1 of the *GLYT1* gene (data not shown). Hence, the present findings may indicate that genetic variants of the *GLYT1* gene could predict a risk of comorbidity of psychosis after repeated methamphetamine abuse.

The coding sequence of the human *GLYT1* gene is divided into 14 exons (based on NM 1024845) distributed over a region of 133 kb, most of which (exons 2–14) cluster within 7 kb [Adams et al., 1995]. SNP1 (IVS3 + 411C > T) is located in intron 3, and SNP2 (1056G > A) is located in exon 7, but is synonymous. Usually, these SNPs are considered nonfunctional. However, recent advances in molecular genetics show intronic RNAs can actually be processed to smaller RNAs, snoRNAs, or miRNAs, which control various levels of gene expression in physiology and development, including transcription, RNA splicing, editing, translation, and turnover [Mattick and Makunin, 2006]. It is possible that SNP1 affects the *GLYT1* gene expression and results in susceptibility to methamphetamine-use disorders. Alternatively, other functional mutations in the *GLYT1* gene with LD with SNP1 or locating on the T-G haplotype of SNP1–SNP2 may be involved in an increased genetic risk for methamphetamine-use disorder because SNP1 was shown to be in an LD block extending at least from intron 3 to intron 11 in our Japanese samples. HapMap project data of a Japanese population (Rel 21a) showed a larger LD block from intron 1 to intorn 12. *GLYT1* is expressed as three splice forms by alternative splicing in 5′-regions, GLYT1a, GLYT1b, and GLYT1c [Borowsky et al., 1993; Zafra et al., 1995]. It is possible that unexamined polymorphisms in intron 1 in LD with SNP1 may affect alternative splicing of the *GLYT1* mRNA and result in susceptibility to methamphetamine-use disorders.

TABLE IV. Multi-Loci Association Analyses of the *GLYT1* Gene

SNP ID	1SNP	2SNP	3SNP
SNP1 (C > T)	0.0019		
		0.000011	
SNP2 (G > A)	0.043		0.000050
		0.0040	
SNP3 (G > A)	0.20		

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The Dysbindin Gene (*DTNBP1*) Is Associated with Methamphetamine Psychosis

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Background: The dysbindin (*DTNBP1* [dystrobrevin-binding protein 1]) gene has repeatedly been shown to be associated with schizophrenia across diverse populations. One study also showed that risk haplotypes were shared with a bipolar disorder subgroup with psychotic episodes, but not with all cases. *DTNBP1* may confer susceptibility to psychotic symptoms in various psychiatric disorders besides schizophrenia.

Methods: Methamphetamine psychosis, the psychotic symptoms of which are close to those observed in schizophrenia, was investigated through a case (n = 197)–control (n = 243) association analyses of *DTNBP1*.

Results: *DTNBP1* showed significant associations with methamphetamine psychosis at polymorphisms of P1635 (rs3213207, $p = .00003$) and SNPA (rs2619538, $p = .049$) and the three-locus haplotype of P1655 (rs2619539)-P1635-SNPA (permutation $p = .0005$). The C-A-A haplotype, which was identical to the protective haplotype previously reported for schizophrenia and psychotic bipolar disorders, was a protective factor ($p = .0013$, odds ratio [OR] = .62, 95% confidence interval [CI] .51–.77) for methamphetamine psychosis. The C-G-T haplotype was a risk for methamphetamine psychosis ($p = .0012$, OR = 14.9, 95% CI 3.5–64.2).

Conclusions: Our genetic evidence suggests that *DTNBP1* is involved in psychotic liability not only for schizophrenia but also for other psychotic disorders, including substance-induced psychosis.

Key Words: Akt1, *DTNBP1*, dysbindin, methamphetamine psychosis, substance dependence

A genetic variation of the dystrobrevin-binding protein 1 (*DTNBP1*) gene has recently been shown to be associated with schizophrenia in several independent studies. Straub *et al.* (1) revealed original evidence for a positive genetic association between schizophrenia and variants in a gene on 6p22.3, dysbindin (*DTNBP1*), which is located within one of several promising loci revealed by a genomewide linkage scan. Many replication studies showed consistent findings in different populations, for example, German (2), Irish (3), Chinese (4), Swedish/German/Polish (5), UK/Irish (5), Bulgarian (6), American (7), Scottish/Chinese (8), and Japanese (9), although the significantly associated alleles and haplotypes were not always consistent among populations. Two postmortem studies also revealed that dysbindin protein or its mRNA level was reduced in the dorsolateral prefrontal cortex and in presynaptic glutamatergic terminals of the hippocampus of schizophrenia patients (10,11). These findings suggest that the dysbindin is involved in the pathogenesis of schizophrenia.

Recently, Raybould *et al.* (12) examined three loci of the *DTNBP1* gene in a large sample of patients with bipolar disorder, another endogenous psychosis, in UK Caucasians, and found that the *DTNBP1* gene was not associated with all cases of bipolar disorder but was associated with a subgroup of bipolar disorder characterized by the complication of psychotic features during episodes. The risk and protective haplotype were identical to those found in their previous schizophrenia study (13). Therefore, they speculated that the *DTNBP1* genetic variation influences susceptibility to schizophrenia and bipolar psychosis across the Kraepelinian dichotomy.

Abuse of large amounts of methamphetamine for long periods easily produces psychotic symptoms, such as delusions of reference, persecution, and poisoning, as well as auditory and visual hallucinations (14–16). Further consumption of methamphetamine may result in severe psychosis, liability to relapse with reconsumption of methamphetamine or psychological stress, and a gradually worsening prognosis. Clinical similarities between methamphetamine psychosis and schizophrenia in a cross-section of clinical features have been noted; these include auditory hallucination and delusion, the longitudinal process of progressive exacerbation with acute relapses, relatively good response to neuroleptics, and enduring vulnerability to relapse to stressors, especially in the paranoid type of schizophrenia. Indeed, methamphetamine psychosis has long been considered a pharmacologic model of schizophrenia (17,18), and shared molecular mechanisms could be involved in these psychotic disorders. Based on this rationale, it is possible that the *DTNBP1*

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Received January 6, 2007; revised February 17, 2007; accepted March 14, 2007.

0006-3223/08/$34.00
doi:10.1016/j.biopsych.2007.03.019

BIOL PSYCHIATRY 2008;63:191–196

gene may influence susceptibility to substance-induced psychoses in the same manner that influence susceptibility to schizophrenia and bipolar psychosis disorders. To examine this hypothesis, we investigated the association between *DTNBP1* and methamphetamine psychosis in a case–control analyses.

Methods and Materials

Subjects

The subjects consisted of 197 patients (162 male, 35 female; mean age ± SD, 38.1 ± 12.6) with methamphetamine psychosis (MAP) and 243 age-, gender-, and geographic-origin-matched healthy control subjects (193 male, 50 female; mean age ± SD, 37.2 ± 12.0) who had no individual or family history of drug dependence or major psychotic disorders such as schizophrenia and bipolar disorders. All the subjects were unrelated Japanese who were born and lived in relatively restricted areas of Japan. All patients were outpatients or inpatients in psychiatric hospitals of the Japanese Genetics Initiative for Drug Abuse (JGIDA). Consensus diagnoses of methamphetamine psychosis were made by two trained psychiatrists according to the ICD-10 criteria on the basis of unstructured interviews and medical records. All healthy control subjects were also psychiatrically screened based on unstructured interviews. The study protocol and purpose were explained to all subjects participating in the study, and written informed consent was obtained from all subjects. This study was approved by the Ethics Committee of each participating institute of JGIDA.

The patients with methamphetamine psychosis were divided into subgroups according to three clinical phenotypes that may indirectly indicate the severity of and liability to psychosis:

1. *Latency to onset of psychotic state after initial methamphetamine consumption:* Median latency was 3 years; 99 (54.4%) of patients developed psychotic symptoms within 3 years of the first methamphetamine abuse, and 83 (45.6%) patients did after 3 or more years.
2. *Duration of the psychotic state after therapy:* Methamphetamine-induced psychosis (transient type) will usually subside within 10 days to 1 month following discontinuance of consumption and beginning of pharmacologic therapy with antipsychotics such as haloperidol or risperidone. Some patients show sustained (longer than 1 month) psychotic symptoms (prolonged type), however, regardless of detoxification from methamphetamine and adequate antipsychotic therapy (16,19). In our study, 107 (56.6%) patients showed the transient type of psychosis, and 82 (43.4%) patients showed the prolonged type of psychosis.
3. *Complication of spontaneous psychosis:* Once methamphetamine psychosis has developed, some remitted patients may experience spontaneous relapse due to nonspecific stresses, such as severe fatigue or life problems, without consumption of methamphetamine. The observation period for the presence or absence of spontaneous relapse was at least 1 year and averaged 12.3 ± 11.1 years. Eighty-three patients (42.8%) experienced spontaneous relapse, and 111 (57.2%) did not.

As to multisubstance abuse status, 37.2% patients concurrently abused other illicit drugs in addition to methamphetamine. Cannabinoids were most frequently abused (34.0%), followed by LSD (14.1%), cocaine (13.1%), opioids (12%), and hypnotics (9.9%). More than 60% of patients abused only methamphetamine, but about half had a past history of organic solvent abuse in their teenage years. All clinical data were obtained from interviews with patients and their families. Urine examination was not applied.

DNA Analysis

We genotyped the three single nucleotide polymorphisms (SNPs), P1655 (rs26619539), P1635 (rs3213207), and SNPA (rs2619538) of the *DTNBP1* gene that were examined previously by O'Donovan's group and were shown to have a significant association with both schizophrenia and psychotic bipolar disorders (12,13). They showed in the schizophrenia study that these three locus haplotypes showed the most significant results among 26 significantly associated haplotypes constructed by combinations of 9 SNPs of *DTNBP*. P1655 and P1635 were two of the markers that had provided the most significant results in the study by Straub *et al.* (1), and SNPA was reported to be significantly associated with schizophrenia in a Japanese population (9).

The genomic DNA was extracted from peripheral leukocytes using the phenolchloroform method. Genotyping was performed by the polymerase chain reaction (PCR)–restriction fragment length polymorphism method. Each polymorphic site was amplified by PCR in a volume of 15 mL containing 3% dimethyl sulfoxide and .75 units of Taq DNA polymerase (Promega, Japan) using a unique primer set (P1655 [mismatch]; 5′-ATCAGGCAAAATGATGTACTGC-3′, 5′-GCCTTTTAAATAATCCTATTAGCTATGAGAGT-3′, P1635; 5′-CTTTATGCAATAAGTATTCCTG-3′, 5′-GTATACCCTGTTTTAAGCAGAC-3′, SNPA; 5′-CCTGTTTCTCAACTTAGTACAC-3′, 5′-CCTTTATCTTATTTAACTCCTG-3′). PCR reaction was performed under the following conditions: 95°C for 5 min, then 35 denaturing cycles of 30 sec each at 95°C, 1 min of annealing at the appropriate temperature, and 30 sec of extension, and final elongation at 72°C for 10 min. The PCR products were digested with the corresponding restriction enzyme for each polymorphism, *Hin*fI for P1655, *Bse*NI for P1635, and *Cai*I for SNPA, and then electrophoresed on 3.0% agarose gels and stained with ethidium bromide. All genotyping was performed in a blinded fashion, with the control and case samples mixed randomly. Part of the genotyping of P1655, P1635, and SNPA was confirmed by direct sequencing and a TaqMan SNP genotyping assay (C_16036968_10), respectively.

Statistical Analysis

Statistical analysis of association was performed using SNPAlyze software (Dynacom, Mobara City, Chiba, Japan). Deviation from Hardy–Weinberg equilibrium and the case–control study were tested using the χ^2 test. Linkage disequilibrium (LD) was tested using the χ^2 test, and D' and r^2 values were made the index in the authorization of LD. Case–control haplotype analysis was performed by the permutation method, and permutation p values were calculated based on 100,000 replications.

GenBank/EMBL Accession Numbers

Genome; NC_000006.10, NT_007592.14, MIM; 607145.

Results

The genotype distribution and allele frequencies for each polymorphism of patients with methamphetamine psychosis and control subjects are shown in Table 1. The genotype distributions of patients and control subjects did not deviate from the Hardy–Weinberg equilibrium at any of the three SNPs. We found a significant difference between patients and control subjects in the frequencies of the genotype or allele at P1635 and SNPA of

Table 1. Genotype and Allele Distribution of Three Single Nucleotide Polymorphisms of the *DTNBP1* Gene in Control Subjects and Patients with Methamphetamine (MAP) Psychosis

			Genotype				Allele		
		N	C/C	C/G	G/G	*p*	C	G	*p*
P1655	rs2619539								
Control		240	118 (49.2)	107 (44.6)	15 (6.2)		343 (71.5)	137 (28.5)	
MAP Psychosis		190	78 (41.0)	94 (49.5)	18 (9.5)	.17	250 (65.8)	130 (34.2)	.076
P1635	rs3213207		A/A	A/G	G/G		A	G	
Control		243	239 (98.4)	4 (1.6)	0 (.0)		482 (99.2)	4 (.8)	
MAP Psychosis		197	175 (88.8)	22 (11.2)	0 (.0)	.000025	372 (94.4)	22 (5.6)	.000030
SNPA	rs2619538		A/A	A/T	T/T		A	T	
Control		232	225 (97.0)	7 (3.0)	0 (.0)		457 (98.5)	7 (1.5)	
MAP Psychosis		197	182 (92.4)	15 (7.6)	0 (.0)	.046	379 (96.2)	15 (3.8)	.049

Numbers in parentheses indicate percentages.

the *DTNBP1* gene (P1635: genotype, $\chi^2 = 17.74$, $df = 1$, $p = .000025$; allele $\chi^2 = 17.20$, $df = 1$, $p = .000030$; SNPA: genotype $\chi^2 = 4.63$, $df = 1$, $p = .046$; allele $\chi^2 = 4.51$, $df = 1$, $p = .049$). The minor alleles of P1635 and SNPA, G and T alleles, respectively, were in excess in methamphetamine psychosis when compared with control subjects. To avoid a type I error due to multiple comparison, the Bonferroni correction was applied to the results. The G allele of P1635 was still significantly more frequent in the methamphetamine psychosis patients than in control subjects, but SNPA was not significantly different after correction. P1655 did not show significant differences in distribution of allele and genotype between groups.

Comparison between subgroups of the patients according to clinical phenotypes showed a significant difference in allelic and genotypic distribution of P1635 between the two subgroups divided by duration of psychotic state after therapy, transient and prolonged types (Table 2). The frequency of the minor allele G of P1635 was only 0.8% in control subjects, whereas it was 3.3% in patients with transient psychosis and 8.5% in patients with prolonged psychosis ($p = .027$, compared with transient psychosis). After Bonferroni correction, this was not significant. The other clinical phenotypes, psychosis latency and spontaneous relapse, were not associated with any SNP examined.

Estimation of the pairwise LD between the three SNPs of the *DTNBP1* gene using the D′ and r^2 values as an index showed that P1655, P1635, and SNPA have strong LD (D′ ranging between 0.65 and 1.0) with each other (Table 3). We then analyzed the three-marker haplotypes (Table 4) and found significant differences in patients and control subjects at P1655-P1635-SNPA ($\chi^2 = 27.8$, $df = 6$, global permutation $p = .0005$).

Table 2. Association of the *DTNBP1* Gene with Subgroups of Patients Divided by Clinical Phenotypes

		Genotype				Allele		
	N	C/C	C/G	G/G	*p*	C	G	*p*
P1655								
Latency to Onset of Psychosis, <3Y	96	35 (36.5)	50 (52.1)	11 (11.4)		120 (62.5)	72 (37.5)	
Latency to Onset of Psychosis, ≥3Y	79	36 (45.6)	37 (46.8)	6 (7.6)	.41	109 (69.0)	49 (31.0)	.20
Transient MAP Psychosis	103	44 (42.7)	50 (48.6)	9 (8.7)		138 (67.0)	68 (33.0)	
Prolonged MAP Psychosis	79	29 (36.7)	42 (53.2)	8 (10.1)	.71	100 (63.3)	58 (36.7)	.46
Spontaneous Relapse; No	108	41 (38.0)	54 (50.0)	13 (12.0)		136 (63.0)	80 (37.0)	
Spontaneous Relapse; Yes	77	34 (44.1)	38 (49.4)	5 (6.5)	.40	106 (68.8)	48 (31.2)	.24
P1635		A/A	A/G	G/G		A	G	
Latency to Onset of Psychosis, <3Y	99	89 (89.9)	10 (10.1)	0 (.0)		188 (94.9)	10 (5.1)	
Latency to Onset of Psychosis, ≥3Y	81	71 (87.7)	10 (12.3)	0 (.0)	.63	152 (93.8)	10 (6.2)	.64
Transient MAP Psychosis	107	100 (93.5)	7 (6.5)	0 (.0)		207 (96.7)	7 (3.3)	
Prolonged MAP Psychosis	82	68 (82.9)	14 (17.1)	0 (.0)	.022	150 (91.5)	14 (8.5)	.027
Spontaneous Relapse; No	111	98 (88.3)	13 (11.7)	0 (.0)		209 (94.1)	13 (5.9)	
Spontaneous Relapse; Yes	82	73 (89.0)	9 (11.0)	0 (.0)	.87	155 (94.5)	9 (5.5)	.88
SNPA		A/A	A/T	T/T		A	T	
Latency to Onset of Psychosis, <3Y	99	91 (91.9)	8 (8.1)	0 (.0)		190 (96.0)	8 (4.0)	
Latency to Onset of Psychosis, ≥3Y	82	75 (91.5)	7 (8.5)	0 (.0)	.91	157 (95.7)	7 (4.3)	.910
Transient MAP Psychosis	108	102 (94.4)	6 (5.6)	0 (.0)		210 (97.2)	6 (2.8)	
Prolonged MAP Psychosis	82	73 (89.0)	9 (11.0)	0 (.0)	.170	155 (94.5)	9 (5.5)	.18
Spontaneous Relapse; No	110	104 (94.5)	6 (5.5)	0 (.0)		214 (97.3)	6 (2.7)	
Spontaneous Relapse; Yes	82	74 (90.2)	8 (9.8)	0 (.0)	.26	156 (95.1)	8 (4.9)	.27

Number in parentheses indicate percentages.

Table 3. Pairwise Linkage Disequilibrium Between Single Nucleotide Polymorphisms of the *DTNBP1* Gene

	P1655	P1635	SNPA
P1655		.9643	1.0000
P1635	.0128		.6519
SNPA	.0114	.3522	

Right upper and left lower diagonal showed D′ and r^2 values, respectively.

The estimated haplotype frequency of C-A-A of P1655-P1635-SNPA was significantly lower in patients with methamphetamine psychosis than in control subjects (p = .0013). Conversely, the C-G-T haplotype was significantly higher in patients than in control subjects (p = .0012). Permutation p values of these haplotypes remained significant even after Bonferroni correction. Odds ratios were .62 (95% confidence interval [CI] .51–.77) and 14.9 (95% CI 3.5–64.2), respectively, indicating that the C-A-A haplotype protected against development of methamphetamine psychosis. On the other hand, the C-G-T haplotype was a significant risk factor for development of methamphetamine psychosis.

Discussion

We found a significant association between the *DTNBP1* gene and methamphetamine psychosis in individual marker and haplotype-based case–control analyses. The G allele of P1635 was shown to be a risk factors for methamphetamine psychosis. Numakawa *et al.* (9) reported that the G allele of P1635 was a risk factor for schizophrenia in Japanese; other reports have shown that it was also overtransmitted in Irish (1) but not in German schizophrenia (2). We also found that the G allele of P1635 was in excess in a subgroup showing a prolonged psychotic state, indicating that the allele was a risk for a worse prognosis of psychosis or refractoriness to antipsychotic therapy in patients with methamphetamine psychosis. The T allele of SNPA also showed a nominally significant risk for methamphetamine psychosis. Although it did not remain significant after multiple comparison correction, one study of schizophrenia showed that it was a significant risk (9), whereas another did not (13). The most striking findings in our study were that analyses of a haploype constructed by P1655-P1635-SNPA of the *DTNBP1* gene revealed a strong association with methamphetamine psychosis (p = .0005). The C-A-A haplotype was significantly more common in control subjects than patients with methamphetamine psychosis (p = .0013), implying a substantial protective factor given the odds ratio of .62. The protective haplotype found in our study of methamphetamine psychosis was identical with that previously reported in studies of schizophrenia and psychotic bipolar disorders (12,13). This evidence may indicate that the C-A-A haplotype of *DTNBP1* reduces the liability of individuals who suffer from endogenous psychoses or substance abuse to complications of psychotic symptoms such as delusions and hallucinations. Another possibility should be also considered, however; the C-A-A haplotype may be associated with methamphetamine dependence but not methamphetamine psychosis because all the patients examined in our study suffered not only from methamphetamine psychosis but also dependence. Accordingly, these hypotheses should be examined in other psychotic disorders—for example, psychotic depression, organic psychoses, and cocaine paranoia—as well as in other dependence disorders. In contrast, the C-G-T haplotype was a significant risk for development of methamphetamine psychosis. The frequency of the C-G-T haplotype was small at about 3% in methamphetamine psychosis but almost absent in control subjects, resulting in a strong risk and an odds ratio of 14.9. This haplotype was absent in the UK/Irish studies. In these studies, the C-A-T haplotype was a risk for both schizophrenia and psychotic bipolar disorder; however, this haplotype was rare (<1%) in Japanese samples and was not a significant factor for methamphetamine psychosis. In addition, the UK/Irish studies showed the G-G-T haplotype was as rare as 3% in control subjects but completely absent in schizophrenia, indicating a potent protective factor against schizophrenia. Again, this haplotype was absent in our samples. Such inconsistencies between the present study and UK/Irish studies indicate that the influence of genetic variation of *DTNBP1* on susceptibility to psychiatric disorders differs among the three distinct disorders (i.e., methamphetamine psychosis, schizophrenia, and psychotic bipolar disorder), although the protective C-A-A haplotype was common to all of them. In addition, population differences in SNP frequencies may also affect results. For example, the minor allele frequency of SNPA was .02, which was consistent with another Japanese study (9), but UK/Irish samples showed a frequency of .45 (13). The P1655 frequency was .28 in our samples, which was similar to another Japanese sample (.31) but different from Caucasian samples (.47 in Straub's study [1] and .49 in Williams's study [13]).

The relationship between abnormal dysbindin function and methamphetamine psychosis is unclear. The *DTNBP1* gene encodes a 40-Kd coiled-coil-containing protein that binds to β-dystrobrevin to form dystrophin-associated protein complex (DPC), which is found in postsynaptic densities of the brain (20). *DTNBP1*, however, is particularly expressed in certain axon terminals, notably, mossy fiber synaptic terminals in the cerebellum and hippocampus independent of DPC (20). Talbot *et al.* (10) found that patients with schizophrenia displayed a presynaptic DTNBP1 reduction in the hippocampus, and an inversely correlated increase in vesicular glutamate transporter-1 occurred in the same schizophrenia cases, suggesting a relationship between glutamatergic neurotransmission and DTNBP1. Evidence in vitro showed that overexpression of DTNBP1-enhanced glutamine release accompanied by an increase of presynaptic machinery SNAP25 and synapsin 1 and a knockdown of DTNBP1 by siRNA-reduced glutamate release. Reduced expression of DTNBP1 in schizophrenic brains may result in hypofunction of the glutamatergic system in the brain, which has been promising hypothesis for the pathophysiology of schizophrenia (21,22). Based on the clinical similarity between methamphetamine psychosis and schizophrenia, it has been assumed that shared neural mechanisms, not only dopamine systems but also gluta-

Table 4. Haplotype Frequencies of the *DTNBP1* Gene of Control Subjects and Methamphetamine (MAP) Psychosis

Haplotype P1655-P1635-SNPA	Controls Frequency	MAP Psychosis Frequency	Permutation p
C-A-A	.7101	.6046	.0013
G-A-A	.2741	.3315	.076
C-G-T	.0022	.0318	.0012
C-G-A	.0023	.0178	.11
C-A-T	.0073	.0055	.83
G-G-A	0	.0089	.15
G-A-T	.0039	0	.18

Haplotype analysis was performed by the permutation method. The global permutation p value was .0005.

mate systems, may be involved in the two psychotic disorders. Many lines of evidence from experimental studies using behavioral sensitization by repeated psychostimulant treatment, which has been recognized as an animal model of methamphetamine psychosis (18), showed pivotal roles of N-methyl-D-aspartate (NMDA) receptors and glutamate systems in the development of behavioral sensitization. Thus repeated administration of amphetamine or cocaine produces behavioral sensitization with enhanced efflux of glutamate in the ventral tegmental area (VTA) and accumbens, which are key brain structures for sensitization phenomena (23,24). NMDA receptor antagonists, including the noncompetitive antagonist MK-801, prevent behavioral sensitization to amphetamines when administered systemically or microinjected into the VTA (25–28). In contrast, phencyclidine, another NMDA antagonist, exacerbates amphetamine-induced abnormal behaviors and a hyperdopaminergic state in the prefrontal cortex and striatum (29–31). Amphetamines can also directly inhibit the NMDA receptor complex (32). Although the roles of NMDA receptors and glutamatergic systems in animal models of methamphetamine psychosis seem to be complex, our findings may indicate that variants of *DTNBP1* affect susceptibility to methamphetamine psychosis by implication of glutamatergic neurotransmission. In addition, DTNBP1 was shown to enhance phosphorylation of AKT protein by PI3-kinase and protect against neuronal cell death. Impaired PI3-kinase-Akt signaling and a genetic association with the *AKT1* gene were found in schizophrenia (20,33,34). Previously, we also found a significant association of the *AKT1* haplotype with the same patients of methamphetamine psychosis (35). It is possible that DTNBP1 confers susceptibility to methamphetamine psychosis via the PI3-kinase-Akt signaling cascade. In vitro evidence of interaction between dysbindin and dopamine system was recently reported. Kumamoto *et al.* (36) found that mRNA of disbindin expressed in the mouse substantia nigra, that suppression of dysbindin expression in PC 12 cells resulted in an increase of dopamine release, and that overexpression of dysbindin produced a tendency to decrease dopamine release. This finding suggests that dysbindin dysfunction may induce susceptibility to methamphetamine psychosis through interaction with dopamine systems.

Alternatively, the effect of DTNBP1 on cognitive ability should be considered. In an analysis of the phenotype–haplotype relationship, Williams *et al.* (13) found that the C-A-A protective haplotype was significantly associated only with higher educational attainment. A longitudinal study of childhood and adolescent antecedents of drug and alcohol problems in adulthood showed that, for both males and females, educational attainment was directly associated with a reduced risk for substance use problems (37). In this respect, higher educational attainment due to carrying the C-A-A haplotype might be involved in a reduced risk for methamphetamine psychosis, and the phenotype of higher educational attainment might be a common protective factor in methamphetamine psychosis and schizophrenia. Further studies are required to confirm this possibility.

Although our results remained significant after Bonferroni correction, it is possible that this was a chance finding resulting from reduced power due to small sample size. Analysis showed, however, that our sample size for the three SNPs had powers of .9994, 1.0000, and .9594 to detect an effect size (w = .1892, .5388, and .1263, respectively), with a significance level of .05 to detect significant associations in allelic analysis between control subjects and subjects with methamphetamine psychosis. Our total sample size is therefore large enough statistically, and it is unlikely that our positive findings results from reduced power. When methamphetamine psychosis patients are divided into subgroups according to clinical phenotypes, however, the statistical power may be reduced. It is possible that a rare haplotype C-G-T as a risk for methamphetamine psychosis may result from a chance fluctuation. In addition, a false-positive association owing to population stratification could not be excluded in this study despite careful matching of control subjects and patients. Our findings should be confirmed in larger samples and in different populations.

This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a grant from the Ministry of Health, Labor, and Welfare of Japan; and from the Zikei Institute of Psychiatry (Okayama, Japan).

The authors have no conflicts of interest to declare.

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