ORIGINAL ARTICLE

Genome-Wide Association for Methamphetamine Dependence

Convergent Results From 2 Samples

George R. Uhl, MD, PhD; Tomas Drgon, PhD; Qing-Rong Liu, PhD; Catherine Johnson, MSc; Donna Walther, MSc; Tokutaro Komiyama, MD; Mutsuo Harano, MD; Yoshimoto Sekine, MD, PhD; Toshiya Inada, MD; Norio Ozaki, MD, PhD; Masaomi Iyo, MD, PhD; Nakao Iwata, MD, PhD; Mitsuhiko Yamada, MD; Ichiro Sora, MD, PhD; Chih-Ken Chen, MD, PhD; Hsing-Cheng Liu, MD, PhD; Hiroshi Ujike; Shih-Ku Lin, MD

Context: We can improve understanding of human methamphetamine dependence, and possibly our abilities to prevent and treat this devastating disorder, by identifying genes whose allelic variants predispose to methamphetamine dependence.

Objective: To find "methamphetamine dependence" genes identified by each of 2 genome-wide association (GWA) studies of independent samples of methamphetamine-dependent individuals and matched controls.

Design: Replicated GWA results in each of 2 case-control studies.

Setting: Japan and Taiwan.

Participants: Individuals with methamphetamine dependence and matched control subjects free from psychiatric, substance abuse, or substance dependence diagnoses (N=580).

Main Outcome Measures: "Methamphetamine dependence" genes that were reproducibly identified by clusters of nominally positive single-nucleotide polymorphisms (SNPs) in both samples in ways that were unlikely to represent chance observations, based on Monte Carlo simulations that corrected for multiple comparisons, and subsets of "methamphetamine dependence" genes that were also identified by GWA studies of dependence on other addictive substances, success in quitting smoking, and memory.

Results: Genes identified by clustered nominally positive SNPs from both samples were unlikely to represent chance observations (Monte Carlo $P<.00001$). Variants in these "methamphetamine dependence" genes are likely to alter cell adhesion, enzymatic functions, transcription, cell structure, and DNA, RNA, and/or protein handling or modification. Cell adhesion genes *CSMD1* and *CDH13* displayed the largest numbers of clustered nominally positive SNPs. "Methamphetamine dependence" genes overlapped, to extents much greater than chance, with genes identified in GWA studies of dependence on other addictive substances, success in quitting smoking, and memory (Monte Carlo P range $<.04$ to $<.00001$).

Conclusion: These data support polygenic contributions to methamphetamine dependence from genes that include those whose variants contribute to dependence on several addictive substances, success in quitting smoking, and mnemonic processes.

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METHAMPHETAMINE abuse is a growing problem in many regions of the United States and a long-standing concern in Taiwan and Japan. Elucidating which genetic variants enhance individuals' vulnerability should increase our understanding of methamphetamine dependence.

Recent reviews suggest that addictive substance dependence is likely to display a polygenic genetic architecture.[1-3] Psychostimulant dependence displays strong familial and genetic influences in family and twin studies.[4-18] Individual differences in vulnerability to methamphetamine are thus likely to display substantial genetic determinants. Since much of the genetic vulnerability to stimulant abuse overlaps with the genetics of vulnerability to other classes of addictive substances, it is likely that methamphetamine dependence displays such genetic overlaps as well.[13-16,19] However, there is no evidence that any single gene's variants mediate a large portion of vulnerability to psychostimulant dependence.

Identifying the genes that harbor allelic variants that contribute to human individual differences in vulnerabilities to methamphetamine dependence will help us to understand processes that underlie human addictions. We may improve understanding of the relative contributions of variants in the brain systems that underlie

Author Affiliations are listed at the end of this article.

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.L., 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,[28] 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,[57] nicotine-dependent individuals vs nondependent smokers,[58] individuals successful in quitting smoking vs those unsuccessful,[59] and individuals with better or poorer scores in memory testing[55] (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.[61]

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,[57,58] and (3) nominally positive SNPs in comparisons of nicotine-dependent vs nondependent smokers (Table).[58] 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-

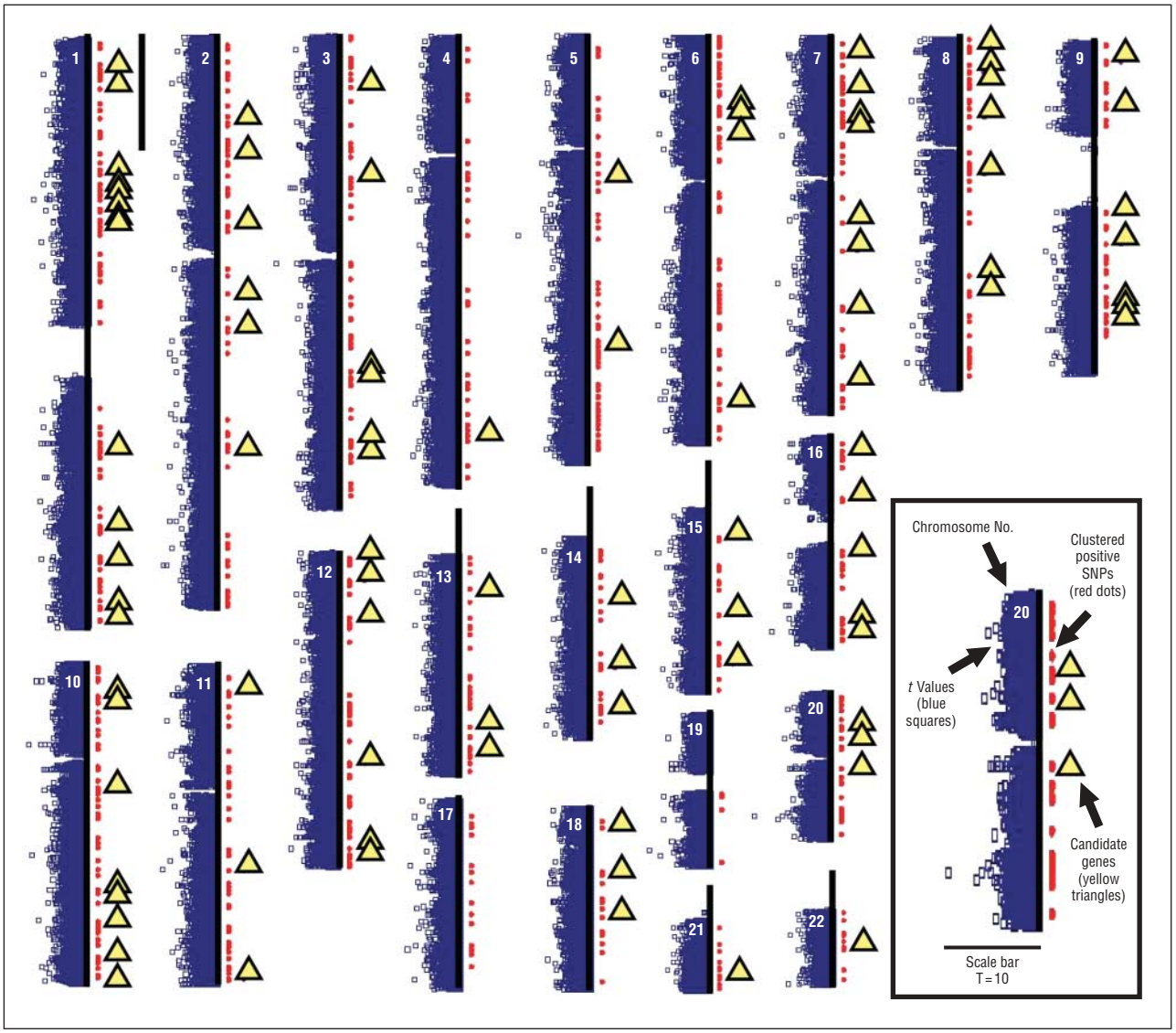

Figure. Cartoons of chromosomes 1 to 22. The blue squares to the left of the axis represent *t* values for the methamphetamine-dependent vs control allele frequency ratios mapped to the chromosomal position of each corresponding single-nucleotide polymorphism (SNP). The SNPs for which abuser-control 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 *DAF/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.

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Author Affiliations: Molecular Neurobiology Branch, National Institutes of Health Intramural Program, Department of Health and Human Services, Baltimore, Maryland (Drs Uhl, Drgon, and Q-R. Liu and Mss Johnson and Walther); Division of Psychiatry, National Center Hospital for Mental, Nervous, and Muscular Disorders, National Center of Neurology and Psychiatry, Tokyo, Japan (Dr Komiyama); Department of Neuropsychiatry, Kurume University School of Medicine, Kurume, Japan (Dr Harano); Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan (Dr Sekine); Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan (Drs Inada and Ozaki); Department of Psychiatry, Teikyo University School of Medicine, Chiba Medical Center (Dr Inada), and Department of Psychiatry, Graduate School of Medicine, Chiba University (Dr Iyo), Chiba, Japan; Department of Psychiatry, Fujita Health University School of Medicine, Aichi, Japan (Dr Iwata); Department of Psychiatry, Showa University Northern Yokohama Hospital, Yokohama, Japan (Dr Yamada); Department of Biological Psychiatry, Tohoku University Graduate School of Medicine, Sendai, Japan (Dr Sora); Japanese Genetics Initiative on Drug Abuse (Drs Komiyama, Harano, Sekine, Inada, Ozaki, Iyo, Iwata, Yamada, Sora and Ujike); Department of Psychiatry, Chang Gung Memorial Hospital, Keelung, Taiwan (Dr Chen); Department of Psychiatry, Taipei City Hospital, and Taipei City Psychiatric Center, Taipei, Taiwan, Republic of China (Drs H-C. Liu and Lin); Department of Neuropsychiatry, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Japan (Dr Ujike).

Correspondence: George R. Uhl, MD, PhD, Molecular Neurobiology, 333 Cassell Dr, Ste 3510, Baltimore, MD 21224 (guhl@intra.nida.nih.gov).

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