ORIGINAL ARTICLE

Genome-Wide Association for Methamphetamine Dependence

Convergent Results From 2 Samples

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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

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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-

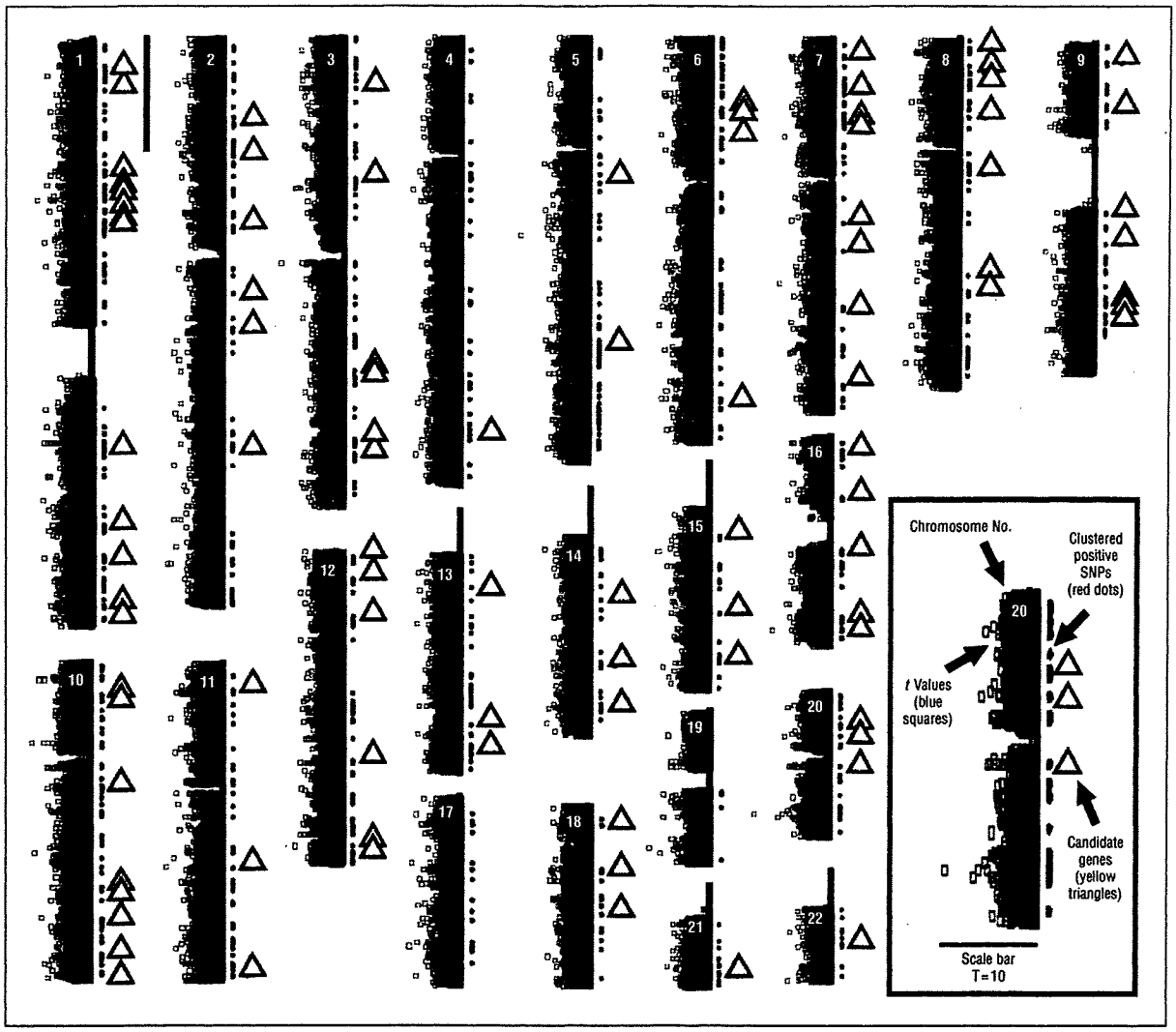

Figure. Cartoons of chromosomes 1 to 22. The blue squares to the left of the axis represent *t* values for the methamphetamine-dependent vs control allele frequency ratios mapped to the chromosomal position of each corresponding single-nucleotide polymorphism (SNP). The SNPs for which abuser-control 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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Animal models of depression in dopamine, serotonin, and norepinephrine transporter knockout mice: prominent effects of dopamine transporter deletions

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Antidepressant drugs produce therapeutic actions and many of their side effects via blockade of the plasma membrane transporters for serotonin (SERT/SLC6A2), norepinephrine (NET/SLC6A1), and dopamine (DAT/SLC6A3). Many antidepressants block several of these transporters; some are more selective. Mouse gene knockouts of these transporters provide interesting models for possible effects of chronic antidepressant treatments. To examine the role of monoamine transporters in models of depression DAT, NET, and SERT knockout (KO) mice and wild-type littermates were studied in the forced swim test (FST), the tail suspension test, and for sucrose consumption. To dissociate general activity from potential antidepressant effects three types of behavior were assessed in the FST: immobility, climbing, and swimming. In confirmation of earlier reports, both DAT KO and NET KO mice exhibited less immobility than wild-type littermates whereas SERT KO mice did not. Effects of DAT deletion were not simply because of hyperactivity, as decreased immobility was observed in DAT+/− mice that were not hyperactive as well as in DAT−/− mice that displayed profound hyperactivity. Climbing was increased, whereas swimming was almost eliminated in DAT−/− mice, and a modest but similar effect was seen in NET KO mice, which showed a modest decrease in locomotor activity. Combined increases in climbing and decreases in immobility are characteristic of FST results in antidepressant animal models, whereas selective effects on swimming are associated with the effects of stimulant drugs. Therefore, an effect on climbing is thought to more specifically reflect antidepressant effects, as has been observed in several other proposed animal models of reduced depressive phenotypes. A similar profile was observed in the tail suspension test, where DAT, NET, and SERT knockouts were all found to reduce immobility, but much greater effects were observed in DAT KO mice. However, to further determine whether these effects of DAT KO in animal models of depression may be because of the confounding effects of hyperactivity, mice were also assessed in a sucrose consumption test. Sucrose consumption was increased in DAT KO mice consistent with reduced anhedonia, and inconsistent with competitive hyperactivity; no increases were observed in SERT KO or NET KO mice. In summary, the effects of DAT KO in animal models of depression are larger than those produced by NET or SERT KO, and unlikely to be simply the result of the confounding effects of locomotor hyperactivity; thus, these data support reevaluation of the role that DAT expression could play in depression and the potential antidepressant effects of DAT blockade.
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Introduction

Inhibition of neurotransmitter reuptake by drugs acting at SERT, NET, and/or DAT can produce antidepressant effects (Skolnick *et al.*, 2003; Lucki and O'Leary, 2004), which, in part, has led to hypotheses that dysfunction of serotonin, norepinephrine, and/or dopamine systems might contribute to depression (Prange, 1964; Schildkraut, 1967; Akiskal and McKinney, 1973; van Praag, 1974; Willner, 1983a, b, c, 2000; Shelton, 2004; Dunlop and Nemeroff, 2007). Most drugs that are clinically effective antidepressants in humans produce reduced immobility in the rodent forced swim test (FST) (Porsolt, 1979). Modifications and extensions of the FST paradigm (Armario *et al.*, 1988; Cervo and Samanin, 1991; Detke *et al.*, 1995, 1997; Detke and Lucki; 1996) have sought to improve its specificity and predictive validity for human antidepressant efficacy (for review, see Cryan *et al.*, 2005a). These modified versions of the original test examine additional behavioral features and use slight modifications of the original apparatus that

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allow assessment of a broader behavioral repertoire. Such assessments may be especially important for studies of experimental manipulations that may alter both depressive behavior and general activity. In studies of Fawn Hooded rats that exhibit both hyperactivity (Hall *et al.*, 1998b) and reduced immobility in forced swim testing (Hall *et al.*, 1998a), for example, the examination of several behavioral endpoints allowed dissociation of hyperactivity from antidepressant-like effects. Such considerations might thus be especially useful in studies of rodents with genetic manipulations that could alter both general levels of motor activity and depressive-like symptoms, such as dopamine transporter knockout (KO) mice (Giros *et al.*, 1996; Sora *et al.*, 1998).

Monoamine transporter knockouts are potentially of interest in the study of antidepressant actions because they chronically elevate extracellular monoamine levels (Gainetdinov *et al.*, 1998; Xu *et al.*, 2000; Mathews *et al.*, 2004; Shen *et al.*, 2004) in a manner similar to antidepressant treatments (Abercrombie *et al.*, 1988; Nomikos *et al.*, 1990; Kreiss and Lucki, 1995). Effects of SERT, NET, and DAT KO mice in antidepressant models may thus be of relevance in understanding the mechanisms that underlie antidepressant effects. Decreased immobility, a correlate of antidepressant activity, has been observed in NET KO mice (Xu *et al.*, 2000) and DAT KO mice (Spielewoy *et al.*, 2000). No difference or increased immobility has been observed SERT KO mice depending on the background strain examined (Holmes *et al.*, 2002). All of these studies, however, have only examined immobility in the FST. Examination of other behaviors in this test (e.g. climbing and swimming) should help in clarifying the nature of these effects. In the case of DAT KO mice this is essential because DAT KO mice are hyperactive (Giros *et al.*, 1996; Sora *et al.*, 1998). In an earlier study of DAT KO mice in the FST (Spielewoy *et al.*, 2000) it was suggested that reductions in immobility were produced by increased 'swimming', but those authors did not in fact measure any other behavior in the test, nor differentiate between climbing and swimming. The nature of this behavior is rather important as it might reflect one of two possible effects, general activity or antidepressant activity.

As hyperactivity may be a confounding issue in the FST it is important to validate the findings of that model in other models of depression. Another model that has been extensively validated with a wide range of antidepressants is the tail suspension test (TST) (Cryan *et al.*, 2005b). SERT KO mice showed reduced immobility in the TST, which was dependent on the background strain against which the knockout was expressed (Holmes *et al.*, 2002), similar to observations in the FST. A similar profile has been observed in NET KO mice in the TST (Dziedzicka-Wasylewska *et al.*, 2006), but this has not yet been assessed in DAT KO mice. Thus, to clarify and extend these data, we now report examination of behavior in DAT, SERT, and NET KO mice using both the FST and the TST under identical experimental conditions so that comparisons may be made between the relative magnitude of the effects of each of these monoamine transporter gene knockouts.

The TST, as well as the FST, might, however, be interpreted as being open to the confounding effects of hyperactivity. To further address this issue we examined another model, sucrose consumption, which should not be open to the same type of locomotor confound. The sucrose consumption model assesses a different aspect of depressive behavior, anhedonia, and was originally used to assess the depressive effects of chronic mild stress (Papp *et al.*, 1991), but has since been used to assess the effects of several other models of depression (El Yacoubi *et al.*, 2003; Wintink *et al.*, 2003; Shumake *et al.*, 2005).

Methods

Subjects

The KO mice used in these experiments were from the DAT-SERT (Sora *et al.*, 2001) and NET-SERT (Hall *et al.*, 2002) double KO lines described previously which were produced from crossing the original DAT (Sora *et al.*, 1998), SERT (Bengel *et al.*, 1998), and NET (Wang *et al.*, 1999) single KO lines. The DAT mice used in these experiments from the DAT-SERT line were wild type (WT) for SERT, the SERT mice from the DAT-SERT line were WT for DAT and NET mice from the NET-SERT line were WT for SERT. For simplicity the second genotype, always +/+, is not included in the descriptions. These KO lines are therefore of a mixed C57BL/6J-129Sv background. Wild-type mice (+/+), heterozygote KO mice (+/−), and homozygote KO mice (−/−) were genotyped by PCR, using two internal primers, one targeted at the knockout insertion sequence and the other targeted at the WT gene, and one external primer, which generated two products identifying the WT and KO genes. The DAT and SERT transgenic knockout insertion sequences contained a neomycin gene sequence (NEO), whereas the NET KO contained a green fluorescent protein gene insert. PCR using TaKaRa DNA polymerase (Takara Bio, Shiga, Japan) was performed on DNA that was eluted from tail biopsies after digestion overnight in Protease K. For DAT genotyping the external primer (5′ AGT GTG TGC AGG GCA TGG TGT A 3′) and the WT primer (5′ TAG GCA CTG CTG ACG ATG ACT G 3′) produced a 500-bp band, whereas the external primer and the NEO primer (5′ CTC GTC GTG ACC CAT GGC GAT 3′) produced a 600-bp band. For SERT genotyping the external primer (5′ GCT CTC AGT CTT GTC TCC ATA AC 3′) and the WT primer (5′ TGC TGA CTG GAG TAC AGG CTA G 3′) produced a 620-bp band, whereas the external primer and the NEO

primer (5′ CTC GTC GTG ACC CAT GGC GAT 3′) produced a 800-bp band. For NET genotyping the external primer (5′ GCT CTG TCC CTG TGC TTC ACG 3′) and the WT primer (5′ TGA GGC CTA AGC TGG AGC TCG 3′) produced a 601-bp band, whereas the external primer and the green fluorescent protein primer (5′ CGG TGA ACA GCT CCT CGC CC 3′) produced a 470-bp band.

Forced swim test

This experiment used the FST (Porsolt *et al.*, 1977) modified and validated in a manner similar to that described earlier for rats (Hall *et al.*, 1998a). On two successive days, DAT, SERT, and NET KO mice (n = 8–10/genotype) were placed in 3-l cylindrical beakers (diameter 19 cm) that were filled to a depth of 14 cm with 25°C water. On the pretest day, mice were placed in the water for 15 min, towel-dried, placed under a warming lamp until completely dry, and then returned to their home cages. On the test day, the mice were placed into the water for 5 min and their behavior was recorded digitally. Their behavior was scored by observation of these recordings, blind to genotype. Durations of immobility, swimming, and climbing behaviors were measured using the TIMER behavioral scoring program (National Institutes of Health). 'Immobility' was defined as being stationary with only enough motion of the tail or forepaws to keep the head above water. The forepaws usually remained at the animal's sides. 'Swimming' was defined as active use of the forepaws with forward movement, in the center or along the sides of the cylinder, which did not involve lifting the paws above the surface of the water. The body was usually oriented parallel to the sides of the cylinder. 'Climbing' was defined as active pawing of the side of the cylinder, lifting the paws above the surface of the water. The body was oriented with the head toward the wall and the body oriented perpendicularly to the side of the cylinder. Inter-rater reliability estimates for these three measures were calculated by comparing the scores of two observers for 32 subjects. Reliability estimates for immobility ($r = 0.88$, $P < 0.001$), climbing ($r = 0.86$, $P < 0.001$), and swimming ($r = 0.81$, $P < 0.001$) were similar to the inter-rater reliability estimates obtained previously for rats (Hall *et al.*, 1998a).

As strain has been shown to affect responses to SERT and NET blockers in the FST (Lucki *et al.*, 2001; David *et al.*, 2003; Dulawa *et al.*, 2004), an additional experiment was performed to determine the sensitivity of WT mice from the DAT-SERT line to selective transporter blockers. The effects of pretreatment with saline (1 ml/100g IP), fluoxetine (30 mg/kg IP), desipramine (20 mg/kg IP), or GBR12909 (20 mg/kg IP), 30 min prior to the 5 min test on the second day were examined in WT mice (n = 8–9 per condition). In all other respects the experiment was identical to that described above.

Tail suspension test

In the TST DAT, SERT, and NET KO mice (n = 8–13/genotype) were suspended by the tail from a horizontal metal rod (8 mm diameter) for 5 min. The duration of immobility was measured by an observer with a stopwatch.

Sucrose consumption test

In the sucrose consumption test DAT KO (n = 7–11 per genotype), SERT KO (n = 7–13 per genotype), and NET KO (n = 6–10 per genotype), were placed in a sucrose consumption chamber (DM-8 lick counter, Columbus Instruments, Columbus, OH) each day with access to water and sucrose. The number of licks for sucrose and water were monitored for 30 min. In a preliminary experiment the volume consumed as well as the number of licks was monitored. The correlation between licks and volume was found to be highly significant ($r = 0.85$, $P < 0.01$); thus only the number of licks is presented. To account for differing weight of the subjects the number of licks is presented as licks/100 g body weight. The subjects were tested over 9 days, 3 days at each concentration of sucrose (0.7, 7, and 34% sucrose).

Locomotor activity

Earlier studies found that DAT−/− mice were profoundly hyperactive (Giros *et al.*, 1996), whereas locomotor activity was reduced in NET KO mice (Xu *et al.*, 2000). No basal differences in activity were found in SERT KO mice or in DAT +/− mice (Giros *et al.*, 1996; Bengel *et al.*, 1998; Xu *et al.*, 2000). Nonetheless, because differences in locomotor activity between genotypes might be thought to affect the paradigms described above, basal locomotion was assessed in DAT, SERT, and NET KO mice. For each gene knockout +/+, +/− and −/− mice (n = 9–11 per genotype) were assessed for locomotor activity for 1 h under novel conditions. Total distance traveled was measured in Optovarimax activity monitors (Columbus Instruments) under dark, sound-attenuated conditions using methods identical to our earlier publications with DAT KO, SERT KO, and DAT/SERT KO mice (Sora *et al.*, 1998, 2001).

Data analysis

The data were analyzed using analysis of variance (ANOVA) with the between-subjects factor of 'genotype', followed by Fisher's protected least significant difference (PLSD) for post-hoc comparisons where appropriate. For analysis of the sucrose consumption data, the additional within-subjects factors of 'sucrose' (sucrose vs. water) and 'concentration' (0.7, 7 and 34% sucrose) were included in the ANOVA. The data for the 3 days of testing at each concentration were averaged and the average values submitted to ANOVA. The effects of saline, fluoxetine, desipramine, and GBR12909 were compared by ANOVA followed by post-hoc comparison with Fisher's PLSD.

Results

Forced swim test

Deletion of the dopamine transporter gene profoundly affected FST behavior. The duration of immobility was decreased by DAT KO (Fig. 1a) so that DAT −/− mice exhibited virtually no immobility [$F(2,27) = 4.9$, $P < 0.02$]. These results were significantly different from those obtained from littermate DAT +/+ mice ($P < 0.05$). Immobility was also reduced in DAT −/− mice. Immobility in these mice was intermediate between values for DAT +/+ and DAT −/− mice, although post-hoc comparisons did not reach statistical significance. The duration of swimming was also greatly reduced in DAT KO mice (Fig. 1b) [$F(2,27) = 4.9$, $P < 0.02$]. DAT −/− mice exhibited virtually no swimming behavior. These differences achieved significance in comparison with DAT +/+ ($P < 0.05$) and DAT +/− mice ($P < 0.05$). The duration of swimming was unaltered in DAT +/− mice compared with DAT +/+ mice. DAT −/− mice exhibited climbing behavior for almost the entire period of testing. Six of the 10 DAT −/− subjects tested struggled to escape the cylinder for the entire session (Fig. 1c). The duration of climbing was greatly increased in DAT KO mice [$F(2,27) = 12.7$, $P < 0.001$]. In this case, as for immobility, DAT +/− mice exhibited behavior that was intermediate between DAT +/+ and DAT −/− mice. The duration of climbing was significantly greater in DAT −/− mice than in either DAT +/+ or DAT +/− mice ($P < 0.05$). DAT +/− mice also exhibited significantly more climbing than WT DAT +/+ mice ($P < 0.05$).

Deletion of the NET gene also reduced the duration of immobility in the FST [Fig. 1a; $F(2,27) = 4.9$, $P < 0.02$]. NET −/− mice exhibited substantially reduced immobility when compared with NET +/+ mice ($P < 0.05$). NET +/− mice did not differ significantly from WT mice. The duration of swimming was unaffected by NET KO [Fig. 1b; $F(2,24) = 0.1$, NS]. Although climbing showed a trend toward increase in NET −/− mice, this did not reach statistical significance [Fig. 1c; $F(2,24) = 2.1$, NS].

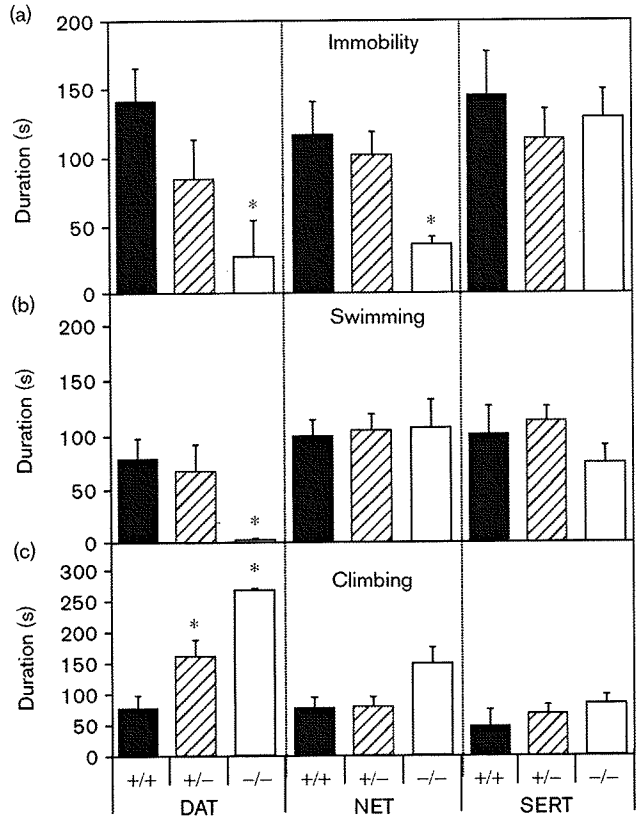
Deletion of the SERT gene had no effect on any FST behavior. Figure 1a-c shows that SERT KO failed to affect the duration of immobility [$F(2,25) = 0.3$, NS], the duration of swimming [$F(2,25) = 0.8$, NS], or the duration of climbing [$F(2,25) = 0.8$, NS].

In WT mice a significant difference was observed between drug treatment groups for immobility [Table 1; $F(3,28) = 10.2$, $P < 0.001$] and swimming followed by [Table 1; $F(3,28) = 5.4$, $P < 0.005$], but not for climbing [Table 1; $F(3,28) = 1.4$, NS]. Desipramine significantly reduced immobility ($P < 0.05$ vs. saline, Fisher's PLSD), and increased swimming ($P < 0.05$ vs. saline, Fisher's PLSD). Desipramine treatment also increased climbing but this difference was not statistically significant. In contrast, neither of the other two drugs, fluoxetine or GBR 12909, had any statistically significant effects, although a trend for GBR 12909 to decrease immobility and to increase climbing was observed.

Tail suspension test

Deletion of all three monoamine transporter genes decreased immobility in the TST (Fig. 2): DAT

Fig. 1



Behavior in the modified forced swim test in DAT KO, SERT KO, and NET KO mice. The duration of immobility (a), swimming (b), and climbing (c) in DAT KO, SERT KO, and NET KO mice (+/+, +/−, and −/− genotypes for each strain), expressed as mean ± the standard error of mean. *Significant difference from +/+ mice, Fisher's protected least significant difference.

Table 1 Effects of fluoxetine, desipramine, and GBR 12909 in the modified forced swim test in WT mice

	Behavioral measure		
Drug	Immobility	Swimming	Climbing
Saline	181.5 ± 13.4	105.9 ± 12.6	11.1 ± 7.6
Desipramine	69.1 ± 15.7[a]	193.5 ± 20.7[a]	34.8 ± 10.0
Fluoxetine	183.6 ± 17.7	100.1 ± 19.4	14.1 ± 5.2
GBR 12909	144.0 ± 19.6	121.0 ± 20.4	31.3 ± 14.7

The duration of immobility, swimming, and climbing in WT mice treated with saline, fluoxetine, desipramine, or GBR 12909 expressed as mean ± the standard error of mean.
WT, wild type.
[a]Significant difference from saline-treated mice, Fisher's protected least significant difference.

[$F(2,34) = 6.5$, $P < 0.01$], SERT [$F(2,31) = 3.5$, $P < 0.05$], and NET [$F(2,23) = 3.9$, $P < 0.05$]. In SERT KO mice, however, only homozygous mice exhibited a significant reduction in immobility ($P < 0.05$ Fisher's PLSD), whereas in DAT and NET KO mice both heterozygous and homozygous KO mice had reduced immobility compared with WT mice. The magnitude of the difference between +/+ and −/− mice differed between the knockout strains; the magnitude of the effect of a homozygous knockout was 74% in DAT KO mice, but only 51% in SERT KO and NET KO mice.

Sucrose consumption test

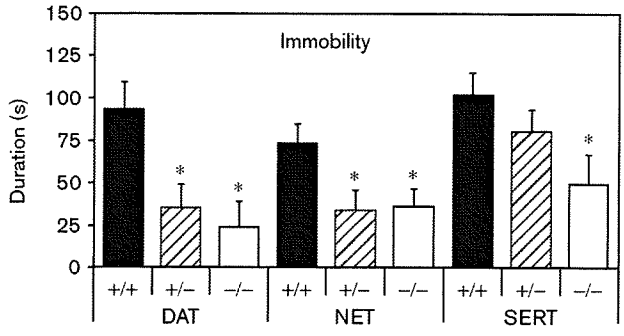
Deletion of the DAT gene increased sucrose consumption at all of the concentrations tested (Fig. 3a). An overall preference for sucrose over water was observed for all subjects [sucrose: $F(1,23) = 16.9$, $P < 0.001$]. DAT −/− mice had greater fluid consumption overall [genotype: $F(2,23) = 6.2$, $P < 0.01$], which was the result of greater consumption of sucrose, but not water at all concentrations tested [genotype × sucrose: $F(2,23) = 6.4$, $P < 0.01$], especially at higher concentrations [genotype × sucrose × concentration: $F(4,46) = 2.8$, $P < 0.05$]. DAT +/− mice were not different from WT mice.

Sucrose consumption was not affected by SERT KO (Fig. 3b) or NET KO (Fig. 3c). Although SERT KO mice showed a trend toward a reduction in sucrose consumption at the middle concentration, neither the main effect of genotype nor the interaction terms were significantly different in the ANOVA.

Locomotor activity

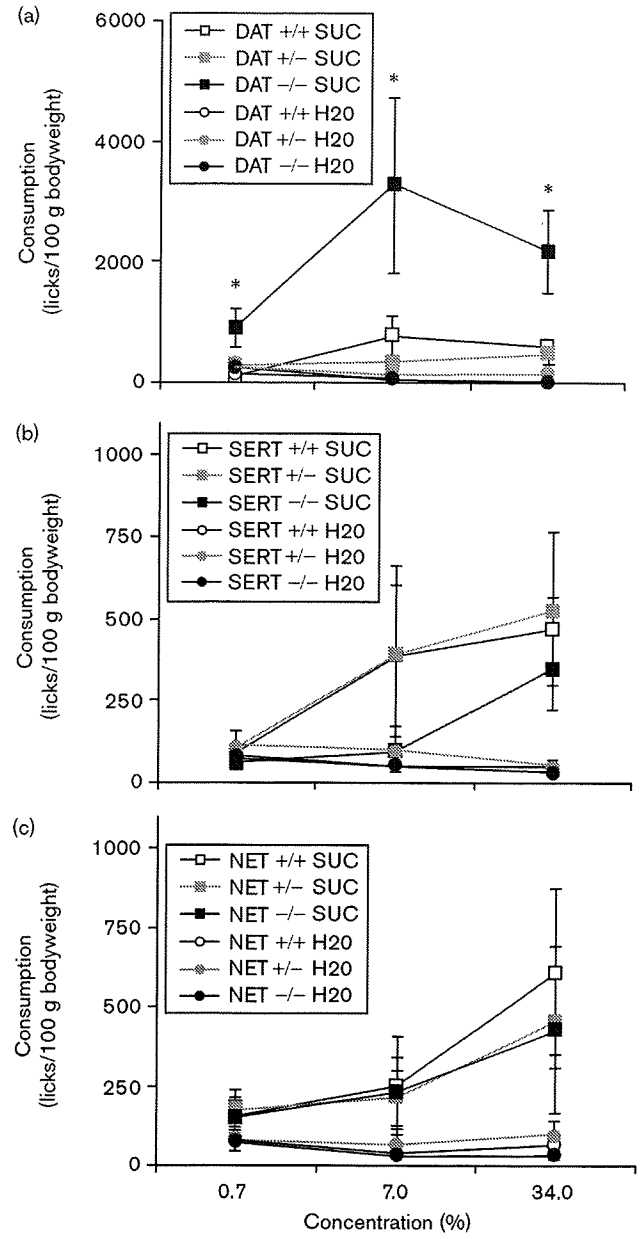
DAT −/− mice were profoundly hyperactive [Table 2; genotype: $F(2,27) = 14.4$, $P < 0.001$, $P < 0.05$ Fisher's PLSD], but there was no difference in activity between DAT +/− and DAT +/+ mice. No significant differences in baseline activity were observed in NET KO or SERT KO mice, although both had strong trends toward reduced locomotion [Table 2; NET: $F(2,26) = 3.2$, $P = 0.06$; SERT: $F(2,27) = 3.1$, $P = 0.06$].

Fig. 2



Immobility in the tail suspension test in DAT KO, SERT KO, and NET KO mice. The duration of immobility in DAT KO, SERT KO, and NET KO mice (+/+, +/−, and −/− genotypes for each strain) expressed as mean ± the standard error of mean. *Significant difference from +/+ mice, Fisher's protected least significant difference.

Fig. 3



Sucrose consumption in DAT, SERT and NET KO mice. Consumption of sucrose (0.7, 7, and 34%) and water in DAT (a), SERT (b), and NET (c) KO mice (+/+, +/− and −/− genotypes) expressed as average licks per 100 g body weight. The data are expressed as the mean ± the standard error of mean. *Significant difference from +/+ mice, Fisher's protected least significant difference.

Discussion

In this study, deletion of the DAT gene produced changes in behavior in three behavioral tests sensitive to

Table 2 Locomotor activity in DAT, SERT, and NET KO mice

	Genotype		
Knockout	+/+	+/−	−/−
DAT KO	12387 ± 1009	13140 ± 1365	32219 ± 4796[a]
NET KO	8536 ± 1025	6622 ± 825	5108 ± 1015
SERT KO	8273 ± 768	10847 ± 2103	6191 ± 564

Locomotor activity in +/+, +/−, and −/− genotypes of DAT, SERT, and NET KO mice. The data are expressed as the mean ± the standard error of mean.
[a]Significant difference from +/+ mice, Fisher's protected least significant difference.

antidepressant drugs. These effects were quite profound. In the FST and TST, homozygous DAT KO mice spent nearly the entire duration of the test struggling to escape whereas sucrose consumption was greatly enhanced, consistent with increased rewarding effects (e.g. reduced anhedonia). Contrary to what might be supposed, based on naive preconceptions of the relative importance of each of these neurotransmitter systems in depression and the mechanisms of the majority of commonly used antidepressant compounds, although similar effects were observed in NET KO mice in both the FST and TST and in SERT KO mice in the TST, these effects were less pronounced than those observed in DAT KO mice. Such a comparison must, however, be considered in light of potential interactions with genetic background, as discussed below, but suggests at the very least that under some circumstances DAT manipulations can have greater effects on antidepressant-like phenotypes than previously appreciated.

DAT knockout almost completely eliminated immobility in the FST, consistent with an earlier finding (Spielewoy *et al.*, 2000). That report suggested that increased immobility was associated with increased swimming, but those authors did not actually measure any other behavior; in particular, there was no attempt to dissociate general activity (swimming) from specific activity aimed at escaping from the cylinder (climbing). In this experiment both immobility and swimming behavior were almost completely eliminated in DAT KO mice, replaced with persistent and almost continuous climbing (e.g. escape attempts). As DAT KO mice are hyperactive (Giros *et al.*, 1996; Sora *et al.*, 1998), it might be supposed that differences in behavior in the FST between DAT +/+ and DAT −/− mice merely reflects this hyperactivity; indeed, this was the conclusion of the study mentioned earlier (Spielewoy *et al.*, 2000). Those authors interpreted the persistent attempts to escape of DAT KO mice in the FST as 'inappropriate' as opposed to the 'adaptive' strategy of immobility, in accordance with one interpretation of the paradigm (Borsini *et al.*, 1986). This interpretation is not generally accepted [see discussion of this subject in the review by Cryan *et al.* (2005a)]. To some extent the use of a single-exposure forced swim paradigm in the Spielewoy *et al.* (2000) study, compared with a two exposure-forced swim paradigm in this study might lead to different interpretations of the results, the former relating more to acute stress, and the latter to 'behavioral despair'. Of course, the type of paradigm used in this study should be more related to antidepressant actions, which was one of the conclusions of the review mentioned earlier (Cryan *et al.*, 2005a).

Returning to the issue of hyperactivity, several lines of evidence weigh against such a simple explanation of the effects of DAT KO in the FST. Reduced immobility and increased climbing were observed in both homozygous and heterozygous DAT KO mice, yet heterozygous DAT KO mice are not hyperactive (Table 2). Decreased immobility, although to a lesser extent, was also observed in NET KO mice, which are also not hyperactive (Table 2). In addition, decreased immobility in DAT KO mice was also observed in the TST, as was reduced immobility in both SERT KO and NET KO mice, consistent with earlier reports (Holmes *et al.*, 2002; Dziedzicka-Wasylewska *et al.*, 2006), and hyperactivity was not observed in either of those knockout strains.

In any case, the description of DAT KO mice as hyperactive is probably not an accurate depiction of their behavior. This is based upon the placement of DAT KO mice in a novel environment in which exploratory activity would be the dominant initial response tendency. More accurately, DAT KO, like amphetamine treatment (Evenden and Robbins, 1983), may increase the dominant response tendency. According to this view, escape behavior in TST and FST would be enhanced, and the present results would not be the result of hyperactivity *per se*. Behavior in a sucrose consumption test would not be confounded by locomotor hyperactivity in the same way as the other tests; indeed, pronounced locomotor hyperactivity should reduce sucrose consumption by producing a competing behavior. In contrast, DAT KO mice had increased consumption of sucrose when compared with WT mice. Increased sucrose consumption, particularly at low concentrations (e.g. 0.7% as in Fig. 3), has generally been taken to indicate differences in the hedonic properties of sucrose (Papp *et al.*, 1991; El Yacoubi *et al.*, 2003; Wintink *et al.*, 2003; Shumake *et al.*, 2005). This additional evidence further indicates that the effects of DAT KO in the FST and the TST are probably not the result of the confounding effects of hyperactivity. No differences were found in sucrose consumption in SERT or NET KO mice, consistent with the lack of effect of SERT KO in the FST, and somewhat reduced effects of NET KO in both tests.

The meaning of the relative lack of effect of NET KO and SERT KO in the present experiments must be interpreted with caution, however, as substantial strain differences have been reported in the response to antidepressants in both FST and TST (Liu and Gershenfeld, 2001, 2003; David *et al.*, 2003; Ripoll *et al.*,

2003; Dulawa *et al.*, 2004; Lucki and O'Leary, 2004; Crowley *et al.*, 2005). In particular, both C57BL/6 and 129Sv mice do not exhibit antidepressant-like effects in response to fluoxetine in the FST (Lucki *et al.*, 2001; Dulawa *et al.*, 2004), although both strains seem to be responsive to a wide range of SERT, NET, and DAT blockers in the TST (Ripoll *et al.*, 2003; Crowley *et al.*, 2005). In an interesting parallel to the present results, one study found that C57BL/6 mice were unresponsive to NET or SERT blockers in the FST, but did respond to GBR 12909 (David *et al.*, 2003). In the current experiments, mixed C57BL/6-129Sv background WT mice did respond to desipramine in the FST, but not fluoxetine, consistent with findings in C57BL/6 mice (Lucki *et al.*, 2001), so that the effects of SERT KO in the present experiments should be treated with particular caution.

Comparison of pharmacological antagonism of transporters and constituitive gene knockouts should, however, be treated with caution in any case. Results of tests performed on adult KO mice need to be potentially interpreted in light of the effects of absence of the gene during development as well as at the time of testing. In DAT KO mice, in particular, it might be supposed that differences in depressive phenotypes may largely reflect developmental consequences of the deletion. However, there is substantial evidence of the role of dopamine in depression and in depressive phenotypes in animal models of depression (Randrup *et al.*, 1975; Willner, 1983a,b,c, 2000; Dunlop and Nemeroff, 2007), much of it involving acute alterations in dopamine function. In humans the relatively selective DAT blocker, bupropion, and its metabolites, produce clinically effective antidepressant actions (Ascher *et al.*, 1995; Jefferson *et al.*, 2005; Volkow *et al.*, 2005). These findings are supported by numerous studies in animal models. Dopamine is also involved in anhedonia in the sucrose consumption model (Papp *et al.*, 1991), and in the FST dopamine agonists and selective dopamine reuptake blockers have antidepressant-like effects (Cooper *et al.*, 1980; Vaugeois *et al.*, 1996; Hemby *et al.*, 1997; Damaj *et al.*, 2004; Siuciak and Fujiwara, 2004; Basso *et al.*, 2005). In addition to reduced immobility, GBR 12909 selectively increased climbing behavior, but not swimming in the FST (Hemby *et al.*, 1997). Dopamine agonists also enhance the antidepressant-like effects of selective serotonin reuptake inhibitors (Renard *et al.*, 2001). The effects of DAT blockers are not the result of generally enhanced activity, as the effects of bupropion in the FST occur at doses that do not affect spontaneous locomotion (Cooper *et al.*, 1980; David *et al.*, 2003).

The present findings are consistent with earlier findings in DAT, SERT, and NET KO mice, while extending them in several respects. NET KO mice have been reported to have reduced immobility in the FST and TST (Xu *et al.*, 2000; Dziedzicka-Wasylewska *et al.*, 2006), as was observed in this study. The present data are also consistent with some earlier results using SERT KO mice, which were reported to have decreased immobility in the TST, but increased immobility in the FST when bred onto a 129S6 background, but did not display any differences in these tests when bred onto a C57BL/6J background (Holmes *et al.*, 2002). It is interesting to note that in the TST, the mixed background strain from this study had reduced immobility in the TST, similar to SERT KO mice on a 129S6 background in the Holmes *et al.* (2002) study, whereas no effect was observed in the FST, similar to C57BL/6J congenic mice in that study. This suggests somewhat different underlying genetic substrates for the TST and FST, consistent with some of the pharmacological findings in inbred strains discussed above.

In the FST antidepressants that act primarily at the serotonin transporter have been reported to increase swimming, whereas those that act primarily at the norepinephrine transporter have been reported to increase climbing (Detke *et al.*, 1995, 1997; Detke and Lucki, 1996; Page *et al.*, 1999; Cryan *et al.*, 2002, 2003). This was not found in desipramine-treated WT mice in this experiment. Nonetheless, it seems that changes in either type of active behavior and concomitant reductions in immobility may be associated with antidepressant-like effects. However, several models of depression, including those produced by withdrawal from amphetamine, ovarian hormone treatments that simulate pregnancy, and estrogen deficiencies caused by aromatase knockout, increase FST immobility and decrease climbing without affecting swimming (Galea *et al.*, 2001; Cryan *et al.*, 2003; Dalla *et al.*, 2004). These depressive-like effects are mirrored by the opposite, antidepressant-like effects of amphetamine administration, pregnancy, or estradiol: decreased immobility and increased climbing, in the absence of substantial changes in swimming (Galea *et al.*, 2001; Molina-Hernandez and Tellez-Alcantara, 2001; Cryan *et al.*, 2003). The pattern of antidepressant effects in these models is very similar to that observed in DAT KO mice, whereas the effects in models of depression are opposite to those observed in DAT KO mice.

The current results thus support a prominent role for DAT, and dopamine, in the mediation of antidepressant-like behavior, as assessed by the models used here. The striking magnitude of the effects of DAT KO, particularly in comparison with the more modest consequences or lack of consequences of NET or SERT KO, each support reevaluation of the clinically proven effects of DAT blockade on depression, and the role of the DAT gene or differences in DAT gene expression in individual differences in depression and responses to effective antidepressant treatments. The pronounced effects of background strain in both pharmacological and transgenic studies indicate that these very strong DAT effects may

be limited to certain genetic backgrounds, but the data suggest that, under these circumstances, DAT may have more importance than SERT or NET. It remains to be seen whether such profound and prominent effects may be observed for NET or SERT gene knockout when it is expressed on other genetic backgrounds in which SERT and NET blockers have more pronounced effects. Taken together, these data suggest that manipulations of DAT, SERT, and NET genes are highly dependent on genetic background and raise the possibility that this may influence both baseline depressive behavior and response to antidepressants acting selectively at each of these transporters.

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