ORIGINAL ARTICLE

ANKK1, *TTC12*, and *NCAM1* Polymorphisms and Heroin Dependence

Importance of Considering Drug Exposure

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Context: The genetic contribution to liability for opioid dependence is well established; identification of the responsible genes has proved challenging.

Objective: To examine association of 1430 candidate gene single-nucleotide polymorphisms (SNPs) with heroin dependence, reporting here only the 71 SNPs in the chromosome 11 gene cluster (*NCAM1*, *TTC12*, *ANKK1*, *DRD2*) that include the strongest observed associations.

Design: Case-control genetic association study that included 2 control groups (lacking an established optimal control group).

Setting: Semistructured psychiatric interviews.

Participants: A total of 1459 Australian cases ascertained from opioid replacement therapy clinics, 531 neighborhood controls ascertained from economically disadvantaged areas near opioid replacement therapy clinics, and 1495 unrelated Australian Twin Registry controls not dependent on alcohol or illicit drugs selected from a twin and family sample.

Main Outcome Measure: Lifetime heroin dependence.

Results: Comparison of cases with Australian Twin Registry controls found minimal evidence of association for all chromosome 11 cluster SNPs ($P \geq .01$); a similar comparison with neighborhood controls revealed greater differences ($P \geq 1.8 \times 10^{-4}$). Comparing cases (n = 1459) with the subgroup of neighborhood controls not dependent on illicit drugs (n = 340), 3 SNPs were significantly associated (correcting for multiple testing): *ANKK1* SNP rs877138 (most strongly associated; odds ratio = 1.59; 95% CI, 1.32-1.92; $P = 9.7 \times 10^{-7}$), *ANKK1* SNP rs4938013, and *TTC12* SNP rs7130431. A similar pattern of association was observed when comparing illicit drug–dependent (n = 191) and nondependent (n = 340) neighborhood controls, suggesting that liability likely extends to nonopioid illicit drug dependence. Aggregate heroin dependence risk associated with 2 SNPs, rs877138 and rs4492854 (located in *NCAM1*), varied more than 4-fold ($P = 2.7 \times 10^{-9}$ for the risk-associated linear trend).

Conclusions: Our results provide further evidence of association for chromosome 11 gene cluster SNPs with substance dependence, including extension of liability to illicit drug dependence. Our findings highlight the necessity of considering drug exposure history when selecting control groups for genetic investigations of illicit drug dependence.

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FAMILY AND TWIN STUDIES HAVE established that genetic factors are responsible for a substantial component of liability for opioid dependence.[1,2] However, identification of the genes associated with risk has proven challenging.[3] Opioid dependence is a complex trait for which many genes each likely account for a modest portion of liability. Thus far, no consistently replicated findings have emerged from genetic association studies focusing on opioid dependence. Most genetic association studies have had inadequate sample size[4-10] to detect modest effects, which, combined with publication bias for positive findings, increases the likelihood of type I error. Underpowered attempts at replication are also predisposed to type II error. Finally, inconsistency in findings across studies may result from differences in important aspects of experimental design.[11,12] This article examines a central component of study design, control group selection, in the context of a genetic association study of heroin dependence.

A number of issues are germane to determining the most appropriate control group for a genetic association study of heroin dependence. Genetic and environmental factors contribute to liability for heroin use[13,14] and, among users, for continued use and dependence.[14] Investigations in population-based twin samples have attempted to estimate the degree to which these influences are shared across the stages of this process for more commonly used substances.[15,16] However, owing to the low prevalence of opioid use, abuse, and dependence, these samples lack adequate power to conduct similar examinations.[17] Genes whose influence on dependence is not shared with risk for substance use would not be expected to display effects in the absence of substance exposure. Ascertainment of exposed, nondependent individuals is complicated by the relatively low prevalence of heroin use,[18-21] the lack of an identified population enriched with users who have survived the period of risk for developing dependence, the stigma associated with the drug,[18,19] and high rates of progression from use to dependence due to heroin's extreme addictivity. Heroin-dependent individuals are nearly always dependent on other drugs[22,23]; however, twin studies have produced widely varying estimates of the degree to which genetic and environmental risks for opioid dependence are shared with other substances.[1,2,14,24] Thus, it remains unclear whether those with a history of having used but not become dependent on other illicit drugs are an adequate proxy for heroin-exposed controls. Similarly, the potential for shared genetic risk with more common phenotypes may supersede the otherwise reasonable argument[25] that the use of an unassessed comparison group in genetic association studies of low-prevalence diseases results in only a mild reduction in power. These issues have left investigators conducting genetic association studies of heroin dependence without an obvious best choice for the most appropriate controls. In fact, they suggest that various alternative choices may be better suited depending on whether a gene's effects are specific to heroin dependence or shared with dependence on other drugs.

This article examines the association of single-nucleotide polymorphisms (SNPs) with heroin dependence in the Comorbidity and Trauma Study.[26,27] We compare a large Australian sample of heroin-dependent cases (n = 1459) receiving opioid replacement therapy (ORT) in New South Wales, Australia, with 2 control groups: (1) controls (n = 531) ascertained from economically disadvantaged neighborhoods in close proximity to ORT clinics who had little or no recreational opioid use lifetime (includes individuals dependent on alcohol and non-opioid illicit drugs and nondependent individuals with high rates of substance exposure); and (2) controls not meeting *DSM-IV* criteria for lifetime alcohol or illicit drug dependence (n = 1495 unrelated individuals) selected from a sample of twins and family members (nondependent with lower rates of drug exposure). The first stage of analyses[27] considered only 136 SNPs in opioid receptor genes. The second stage includes the remaining 1294 SNPs (1430 of 1536 total SNPs were retained for analyses after data cleaning). We report herein the most significant findings of these analyses involving association with SNPs in the chromosome 11 cluster of genes (neural cell adhesion molecule 1 [*NCAM1*; GenBank NM_000615.6], tetratricopeptide repeat domain 12 [*TTC12*; GenBank NM_017868.3], ankyrin repeat and kinase domain containing 1 [*ANKK1*; GenBank NM_178510.1], and dopamine receptor D_2 [*DRD2*; GenBank NM_000795.3]) for which a wealth of prior studies focusing on licit substance-related outcomes have reported similar associations. Our findings exemplify the importance of considering history of drug exposure in addition to drug dependence when selecting an appropriate control group.

METHODS

The Comorbidity and Trauma Study, a collaboration of investigators at Washington University School of Medicine, Queensland Institute of Medical Research, and National Drug and Alcohol Research Centre of the University of New South Wales, is a case-control genetic association study of heroin dependence. Details of data collection have been previously reported.[26,27] We again[27] include data here from pilot study participants (25 cases and 25 neighborhood controls) for whom protocols were identical and assessment comparable.

PARTICIPANTS

Cases recruited from ORT clinics in the greater Sydney, Australia, region were required to be aged 18 years or older, to understand English, and to have participated in ORT for opioid dependence. Participants reporting recent suicidal intent or current psychosis were excluded. Individuals recruited from geographic areas in proximity to ORT clinics, termed *neighborhood controls*, were excluded for recreational opioid use more than 5 times lifetime (data were included from 23 controls who denied opioid use >5 times at screening but reported greater use with no dependence symptoms at interview); other inclusion and exclusion criteria were identical to those for cases. Institutional review board approval was obtained from the University of New South Wales, Washington University School of Medicine, Queensland Institute of Medical Research, and area health service ethics committees governing participating clinics. Participants provided written informed consent and were reimbursed AU$50.00 for out-of-pocket expenses.

Concerns that comparisons with neighborhood controls might have inadequate power to detect effects on dependence that are shared with both drug exposure and dependence on other substances (eg, 191 [36.0%] of the 531 neighborhood controls were dependent on a nonopioid illicit drug; Supplemental Table 1, http://digitalcommons.wustl.edu/psychiatry) prompted a decision to genotype a second, more broadly unaffected control group of unrelated individuals selected from the large Australian Twin Registry (ATR), including twins and family members.[28] Inclusion criteria were institutional review board approval allowing genotyping of available DNA. Exclusion criteria were lifetime illicit drug or alcohol dependence at the prior interview. Non–nicotine-dependent individuals were preferentially selected; the prevalence of nicotine dependence (12.5%) in ATR controls is below that of the Australian population.

ASSESSMENT

Semistructured psychiatric diagnostic interviews were completed in person by cases and neighborhood controls; ATR controls had completed telephone interviews previously. Diagnostic sections on illicit drug and alcohol dependence were modified

from the Semistructured Assessment for the Genetics of Alcoholism–Australia[29]; the nicotine dependence section was modified from the Nicotine Addiction Genetics Study assessment.[30] The assessments provided *DSM-IV* lifetime diagnoses of opioid, cannabis, sedative, stimulant, cocaine, and alcohol abuse and dependence as well as nicotine dependence. Similar diagnoses were obtained for Comorbidity and Trauma Study pilot project participants via the World Health Organization Composite International Diagnostic Interview.[31]

MARKER SELECTION

The pairwise option of Tagger[32] (implemented in Haploview[33]) with a threshold of $r^2 \geq 0.8$ for most genes and $r^2 \geq 0.9$ for high-priority candidates (eg, opioid receptor genes) was used to select a custom set of 1536 SNPs. The set provided coverage of 72 candidate genes, 47 additional SNPs from prior reports, and 30 ancestry-informative markers.

GENOTYPING

Genotyping was performed on an Illumina BeadStation using GoldenGate technology.[34] Samples of DNA from CEPH trio 1334 obtained from the Coriell Cell Repository served as internal quality controls for clustering and reproducibility. Primary genotypic data analyses with Illumina BeadStudio software were followed by visual inspection and assessment of data quality and clustering.

STATISTICAL ANALYSIS

Data Cleaning

Details of data cleaning have been reported previously.[27] In brief, SNPs were excluded owing to genotyping failure (23 SNPs), call rate less than 95% (9 SNPs), minor allele frequency less than 2% (47 SNPs), and Hardy-Weinberg equilibrium deviations (27 SNPs); 1430 SNPs were retained for analyses (Supplemental Table 2 shows the complete list). The mean call rate for 1294 nonopioid receptor SNPs remaining after data cleaning exceeded 99.9%. Samples of DNA from 1506 cases, 538 neighborhood controls, and 1500 ATR controls were genotyped. Data from samples were excluded owing to genotyping failure (1 ATR control), phenotypic-genotypic sex mismatch (1 case, 2 neighborhood controls), duplication due to participation in the project multiple times (29 cases, 3 neighborhood controls—phenotypic data from the most recent, nonpilot study interview were retained), and cryptic relatedness with identity by descent greater than 0.5 (17 cases, 4 neighborhood controls, and 4 ATR controls—individuals with the higher project identifier were excluded). The sample used for analyses consisted of 1459 cases, 531 neighborhood controls, and 1495 ATR controls.

Admixture

Cases, neighborhood controls, and ATR controls all consisted primarily of individuals of European ancestry. The former 2 groups also included some individuals of Asian ancestry. Principal components (PC) analyses (PCAs) were conducted using the smartpca program in the Eigensoft version 3.0 statistical software package[35] to determine the appropriate admixture correction for each analysis. The kill r^2 setting of 0.8 was used to remove some SNPs in high linkage disequilibrium (LD) with others in the panel with data from 1123 of the 1430 SNPs retained for PCA. Because ancestry-informative markers were included in the PCA, Tracy-Widom statistics could not be used to determine the number of PCs. Separate PCA indicated that comparisons of cases with neighborhood controls required no inclusion of PCs as covariates, while that comparing cases with ATR controls found at least a trend-level significance for 4 PCs (for details and PC plots, see the article by Nelson et al[27]). For analyses that divided the neighborhood controls on the basis of lifetime licit and illicit drug dependence, a separate PCA run for each comparison found in each instance a single significant PC with the following *P* values: cases vs neighborhood controls not dependent on nicotine, alcohol, and illicit drugs, $P = .06$; cases vs neighborhood controls not dependent on alcohol and illicit drugs, $P = .06$; cases vs neighborhood controls not dependent on illicit drugs, $P = .02$; cases vs illicit drug–dependent neighborhood controls, $P = .02$; and illicit drug–dependent vs nondependent neighborhood controls, $P = .001$. Each of these analyses included a single PC as a covariate to control for admixture.

Association

Logistic regression analyses performed in PLINK software,[36] which included smartpca-derived PCs in models to control for admixture, examined the association between the log-additive effects of minor allele dosage and case status. We separately compared 1459 heroin-dependent cases (888 male, 571 female; mean [SD] age, 36.5 [8.6] years) with 531 neighborhood controls (235 male, 296 female; mean [SD] age, 34.7 [10.5] years) and 1495 ATR controls (972 male, 523 female; mean [SD] age, 45.0 [9.5] years). Because of uncertainty regarding the most appropriate control group for the current investigation and given within-group differences in the neighborhood controls observed in stage 1 analyses,[27] we compared heroin-dependent cases with subgroups of neighborhood controls who were not dependent on the following: (1) any illicit drugs (n = 340); (2) any illicit drugs or alcohol (n = 275); and (3) any illicit drugs, alcohol, or nicotine (n = 207). We also conducted a within-group comparison of neighborhood controls who were and were not illicit drug dependent. A conservative Bonferroni correction for multiple testing yielded a revised significance threshold of $P = 3.9 \times 10^{-6}$ (ie, .05/1430 SNPs/9 phenotypic comparisons: [1] cases vs neighborhood controls; [2] cases vs ATR controls; cases vs neighborhood controls not dependent on [3] illicit drugs, [4] illicit drugs or alcohol, and [5] illicit drugs, alcohol, or nicotine; cases vs neighborhood controls dependent on [6] illicit drugs, [7] illicit drugs or alcohol, and [8] illicit drugs, alcohol, or nicotine; and [9] neighborhood controls dependent on illicit drugs vs neighborhood controls not dependent on illicit drugs). We controlled for the allelic dosage of the most strongly associated SNP to examine whether a single signal adequately explained all observed chromosome 11 gene cluster associations. Consistent with prior reports that focused on haplotypes spanning these genes, we conducted analyses using SAS version 9.2 statistical software (SAS Institute, Inc) to characterize risk (ie, additive, dominant, or recessive) associated with each of the 2 SNPs for whom independent signals were found. To estimate their effects in tandem, we coded a risk level variable that was a sum of their effects. In doing so, we verified that risk associated with the alternative routes of obtaining the same risk level (eg, 1 copy of the rs877138 minor allele and the rs4492854 major allele vs 2 copies of the former and none of the latter) did not differ significantly.

RESULTS

The comparison of cases with ATR controls found *P* values greater than .01 for all SNPs in the chromosome 11 gene cluster (select SNPs [ie, primarily those more strongly

Table 1. Comparison of Select Single-Nucleotide Polymorphisms in 1459 Cases vs 1495 Australian Twin Registry Controls and 531 Neighborhood Controls Using Additive Models

Gene	SNP	SNP Location[a]	Minor Allele	*P* Value	
				Heroin-Dependent Cases vs ATR Controls[b]	Heroin-Dependent Cases vs Neighborhood Controls
NCAM1	rs4492854	112488744	G	.18	.007
	rs11214546	112611738	A	.05	.31
	rs587761	112615990	A	.55	.005
	rs2186798	112633271	C	.04	.06
TTC12	rs2303380	112705919	G	.19	.005
	rs10891536	112714638	G	.03	.01
	rs4938009	112736138	A	.03	.005
	rs719804	112739985	G	.08	.14
	rs7130431	112743433	A	.12	<.001
	rs12804573	112746936	G	.04	.001
ANKK1	rs877137	112761540	A	.12	.001
	rs877138	112761718	G	.04	<.001
	rs12360992	112768110	C	.25	.001
	rs4938013	112769680	A	.07	<.001
	rs2734849	112775370	G	.30	.002
	rs2734848	112775584	G	.02	.008
	rs1800497	112776038	T	.93	.12
DRD2	rs2234689	112783693	G	.03	.007
	rs1554929	112783974	A	.34	.001
	rs2440390	112792088	A	.17	.009
	rs1076563	112801119	A	.11	.003
	rs7125415	112815891	A	.01	.22

Abbreviations: ATR, Australian Twin Registry; SNP, single-nucleotide polymorphism.
[a] From NCBI build 37.2 (National Center for Biotechnology Information).
[b] Four principal components were included in the model for admixture correction.

associated but also including rs1800497, the Taq1A polymorphism] are shown in **Table 1**). The similar comparison of cases with neighborhood controls revealed greater intergroup differences; however, the minimum *P* value (1.8×10^{-4} for rs7130431) was not significant with adjustment for multiple testing (comparisons for additional genotyped SNPs are shown in Supplemental Table 3; unadjusted [for ethnicity] minor allelic frequencies for select and additional SNPs are shown in Supplemental Table 4 and Supplemental Table 5, respectively).

We next examined the effects of dividing neighborhood controls into subgroups based on their lifetime history of illicit and licit drug dependence (Table 1 shows a hierarchical breakdown). We found that the association signal became stronger as the criterion for exclusion of individuals with lifetime drug dependence was more narrowly defined (Supplemental Table 6). In the comparison between heroin-dependent cases and neighborhood controls not dependent on any illicit drugs (**Table 2**), significant association was found for 3 SNPs (rs877138, rs4938013, and rs7130431) in high LD (**Figure**) with rs877138, the most strongly associated SNP (odds ratio [OR] = 1.59; 95% CI, 1.32-1.92; $P = 9.7 \times 10^{-7}$). In contrast, for the comparison between cases and illicit drug–dependent neighborhood controls, only a single SNP reached even nominal significance. A within-neighborhood control group comparison of individuals with and without a lifetime history of illicit drug dependence found that rs877138 was again the most highly associated SNP ($P = 8.0 \times 10^{-4}$), indicating that liability attributable to this variant likely extends to risk for dependence on other illicit drugs.

To determine whether between-group differences in drug exposure may have contributed to the lack of association found with ATR controls, we compared ATR controls with neighborhood controls not dependent on illicit drugs. We found significantly greater lifetime use for all examined illicit drug categories in nondependent neighborhood controls; differences were most pronounced for cocaine and stimulants (**Table 3**). Extending the comparison of nondependent neighborhood controls vs ATR controls to use more than 11 times lifetime (not shown in Table 3), a similar pattern of between-group differences was observed: stimulants, 18.2% vs 1.4%, respectively (OR = 15.64; 95% CI, 9.38-26.08); any noncannabis illicit drug, 19.7% vs 3.0%, respectively (OR = 7.90; 95% CI, 5.30-11.78); and any illicit drug, 45.3% vs 15.7%, respectively (OR = 4.46; 95% CI, 3.45-5.76). In an assessment not used for ATR controls, 35.5% of nondependent neighborhood controls reported having seen someone use heroin and 28.4% reported having been offered heroin. These results provide strong evidence that the neighborhood controls not dependent on illicit drugs had substantially greater levels of lifetime drug use than the ATR controls. In addition, a surprisingly large proportion of these individuals had ready access to heroin.

Inclusion of rs877138 as a covariate in the comparison of heroin-dependent cases with neighborhood controls not dependent on illicit drugs yielded suggestive evidence ($P < 2.5 \times 10^{-3}$) of a second signal involving

Table 2. Comparison of Single-Nucleotide Polymorphisms Between Groups Using Additive Models

Gene	SNP	*P* Value[a]: Heroin-Dependent Cases (n = 1459) vs Neighborhood Controls Not Dependent on Illicit Drugs (n = 340)	Heroin-Dependent Cases (n = 1459) vs Illicit Drug–Dependent Neighborhood Controls (n = 191)	Within-Neighborhood Control Comparison of Those Not Dependent on Illicit Drugs (n = 340) vs Those Dependent (n = 191)	LD of SNP With rs877138, r^2
NCAM1	rs4492854	.002	.59	.13	0.00
	rs11214546	.33	.002	.003	0.00
	rs587761	.01	.15	.59	0.02
	rs2186798	.19	.16	.81	0.00
TTC12	rs2303380	.000035	.56	.003	0.70
	rs10891536	.0005	.88	.03	0.37
	rs4938009	.0005	.89	.02	0.35
	rs719804	.02	.47	.02	0.26
	rs7130431	.0000028[b]	.78	.005	0.91
	rs12804573	.00007	.53	.04	0.48
ANKK1	rs877137	.000024	.56	.02	0.51
	rs877138	.00000097[b]	.83	.001	1.00
	rs12360992	.000095	.45	.05	0.44
	rs4938013	.0000013[b]	.88	.001	0.89
	rs2734849	.00016	.48	.06	0.38
	rs2734848	.001	.93	.02	0.38
	rs1800497	.047	.92	.22	0.15
DRD2	rs2234689	.001	.93	.02	0.38
	rs1554929	.000066	.39	.06	0.45
	rs2440390	.00042	.83	.008	0.25
	rs1076563	.0002	.57	.05	0.27
	rs7125415	.12	.77	.32	0.05

Abbreviations: LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.
[a] One principal component was included in each model for admixture correction.
[b] Significant with correction for multiple testing.

rs4492854, an *NCAM1* SNP. Further analyses supported a dominant model for liability associated with this SNP's major allele (OR = 1.65; 95% CI, 1.26-2.17). An examination of aggregate risk associated with these 2 SNPs (ie, the number of rs877138 minor alleles [additive] plus the presence of a copy of the rs4492854 major allele) in data from cases and nondependent neighborhood controls found that risk varied more than 4-fold on the basis of these 2 SNPs (**Table 4**). The proportion of heroin-dependent individuals (shown as the column percentage) was observed to increase with this measure of aggregate risk; the *P* value for the risk-associated linear trend is 2.7×10^{-9}.

COMMENT

Our data provide strong evidence that *ANKK1* and *TTC12* SNPs are associated with dependence on heroin and other illicit drugs. These results are an important extension of previously reported associations that largely involved nicotine- or alcohol-related outcomes.[37-47] Our findings emphasize the necessity of considering both drug exposure and dependence history when selecting a control group for genetic association studies focusing on drug dependence.

Depending on the control group to which we compared heroin-dependent cases, the magnitude of observed associations varied markedly. The comparison of cases with ATR controls found scant evidence of association. Although *P* values exceeded .01 for all SNPs, rs877138 (*P* = .04) was among those nominally associated. Greater evidence of association was observed in the comparison of cases with neighborhood controls; however, no *P* value for any SNP was within an order of magnitude of the significance level required to correct for multiple testing. Our analyses that divided neighborhood controls into subgroups based on lifetime history of licit and illicit drug dependence found that the association signal became stronger as the exclusion was defined more narrowly to exclude only individuals with lifetime history of illicit drug dependence.

Three consistent findings emerged with this division. First, the comparison of heroin-dependent cases with nondependent neighborhood controls found 3 SNPs with *P* values significant after correction for multiple testing. Second, the comparison of cases with illicit drug–dependent neighborhood controls was remarkable for the complete lack of even nominally significant differences for *TTC12* and *ANKK1* SNPs. Third, the comparison of nondependent and illicit drug–dependent neighborhood controls found a pattern of association nearly identical to the comparison of the former with cases, with the smaller size of the latter sample limiting overall power. A post hoc comparison of nondependent neighborhood controls with a combined group of heroin-dependent cases and illicit drug–dependent controls found that significance improved incrementally for the association with

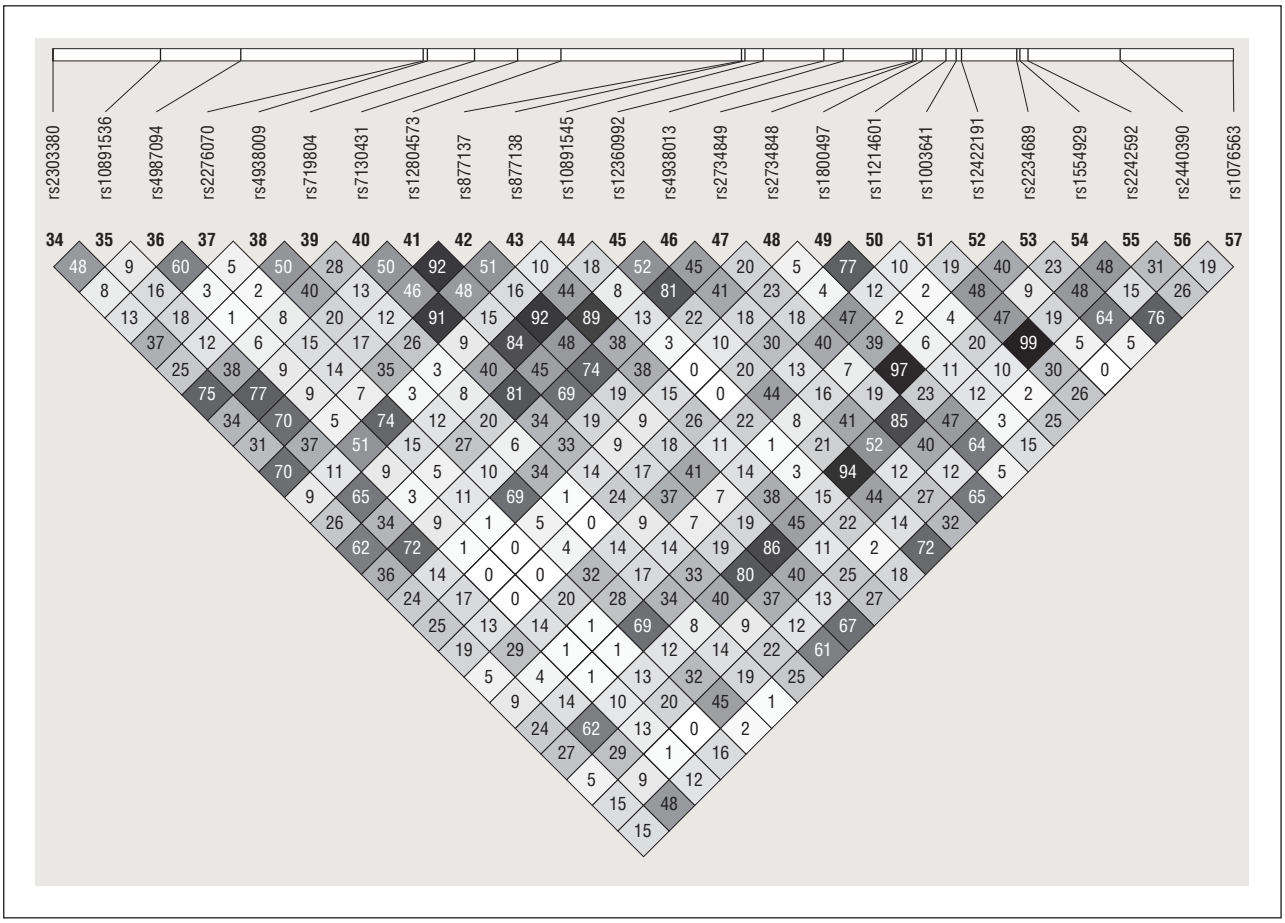

Figure. Linkage disequilibrium analysis of select *TTC12*, *ANKK1*, and *DRD2* single-nucleotide polymorphisms (r^2 values are shown).

Table 3. Comparison of 1495 Australian Twin Registry Controls With 340 Neighborhood Controls Not Dependent on Illicit Drugs

	Prevalence of Use by Control Group, %		
Illicit Drug	ATR	Nondependent Neighborhood	OR (95% CI)
Cannabis	44.4	71.1	4.22 (3.21-5.54)
Stimulant	6.8	44.4	10.91 (8.14-14.63)
Opioid	7.4	10.6	1.49 (1.00-2.22)
Sedative	3.5	12.9	4.12 (2.71-6.28)
Cocaine	2.3	25.9	14.57 (9.63-22.03)
Any	49.8	79.1	3.81 (2.88-5.05)
Any noncannabis	15.0	50.6	5.78 (4.48-7.46)

Abbreviations: ATR, Australian Twin Registry; OR, odds ratio.

rs877138 ($P = 6.4 \times 10^{-7}$). Our results provide strong evidence that a block of *ANKK1* and *TTC12* SNPs in high LD is associated with heroin and other illicit drug dependence. The large difference in the strength of association observed in the comparison with nondependent neighborhood controls (individuals with high exposure to illicit drugs, either via use or from residing in environments with widespread drug availability) vs ATR controls (individuals not dependent on alcohol or illicit drugs, with significantly lower illicit drug exposure) strongly suggests that this liability likely represents risk of dependence contingent on drug exposure. Our findings raise an intriguing possibility that nondependent highly substance-exposed controls might be particularly informative for attempts to identify polymorphisms associated with drug dependence liability.

The 3 SNPs (rs877138, rs4938013, and rs7130431) for which we observed significant association are located in a region spanning *ANKK1* and *TTC12*. High LD between SNPs in these genes prevents determination, without additional sequencing, of the gene primarily contributing to liability. For example, rs877138 is located in the 5' flanking region 2005 base pairs upstream from the first exon of *ANKK1* but is in complete LD with several intronic *TTC12* SNPs. Both rs4938013, an exonic *ANKK1* SNP resulting in a synonymous substitution, and rs7130431, an intronic *TTC12* SNP, are in high LD with rs877138 (respective r^2 values of 0.89 and 0.91), consistent with a single association signal. Although nominally significant association extends to the Taq1A polymorphism (rs1800497) and *DRD2* SNPs, analyses that controlled for allelic dose of rs877138 found no evidence of an independent signal involving this functional polymorphism or any other *ANKK1*, *TTC12*, or *DRD2* SNP. Since prior studies[6,11,48-52] that reported an association of rs1800497 with opioid dependence genotyped few or no additional *ANKK1* SNPs, the signal they observed may have resulted from similar LD. The only

Table 4. Comparison of 1459 Cases With 340 Neighborhood Controls Not Dependent on Illicit Drugs

Group	Risk Level, No. (%)[a]			
	0	1	2	3
Nondependent neighborhood controls[b]	47 (32.4)	168 (21.8)	107 (15.2)	18 (10.1)
Heroin-dependent cases[b]	98 (67.6)	603 (78.2)	598 (84.8)	160 (89.9)
OR (95% CI)	1 [Reference]	1.69 (1.15-2.49)	2.62 (1.75-3.93)	4.30 (2.36-7.84)

Abbreviation: OR, odds ratio.

[a] Risk level indicates the number of rs877138 minor alleles plus the presence of an rs4492854 major allele. Mantel-Haenszel test: $\chi^2_1 = 35.40$; $P = 2.7 \times 10^{-9}$ (linear trend).

[b] Number of individuals are shown with column percentage in parentheses.

association not attributable to LD in our sample involved rs4492854, an *NCAM1* SNP, for which risk was best explained by a dominant model. Owing to the complete lack of LD between rs877138 and rs4492854, we instead examined risk associated with these SNPs in tandem rather than the haplotype-based analyses used in prior investigations[37,39,41,43] and found greater than 4-fold variation in risk (OR = 4.30; 95% CI, 2.36-7.84) across combinations of these 2 SNPs.

Our results are broadly consistent with a literature[37-46] in which, led by the efforts of Gelernter, Kranzler, and colleagues, attention has shifted from *DRD2* to *ANKK1* and *TTC12* as the genes in this region most strongly associated with substance dependence. They initially conducted a family-based association study[37] of tobacco dependence in a largely polysubstance-dependent sample drawn from sets of European American and African American affected (cocaine or opioid dependent) sibling pairs. The strongest association in pooled African American and European American data included *ANKK1* and *TTC12* SNPs in a moderate to high LD block that overlaps our main findings (eg, their top hits included rs4938012 [$P = 8 \times 10^{-6}$] and rs4938013 [$P = 3 \times 10^{-5}$]). Of note, *DSM-IV* nicotine dependence is more highly correlated with other *DSM-IV* substance dependence diagnoses than is Fagerström score (for which they found much weaker association). Further analyses implicated a 4-SNP haplotype spanning *TTC12* and *ANKK1*. They later focused[39] on alcohol dependence in 2 European American samples, finding only nominally significant associations for individual SNPs; haplotype-based analyses observed significant associations centering on *TTC12*, *NCAM1*, and *ANKK1*. A subsequent examination[41] found that risk associated with most of these haplotypes was for comorbid alcohol and illicit drug dependence rather than alcohol dependence alone. In Collaborative Study on the Genetics of Alcoholism family data,[38] nominal associations for alcohol-related phenotypes were found in an overlapping *ANKK1* region (including both rs877138 and rs4938012). A Collaborative Study on the Genetics of Alcoholism genome-wide association study[44] found that rs10502172, a more upstream *TTC12* SNP, was nominally associated with alcohol dependence ($P = 7.0 \times 10^{-4}$). A Finnish population-based birth cohort study[45] reported strong association of SNPs within a haplotype block stretching from *TTC12* to *DRD2* (including rs877138; $P = .001$) with smoking at age 14 years; rs10502172 ($P = 9.1 \times 10^{-6}$) was most highly associated. These SNPs were more weakly associated with smoking at age 31 years. Overall, our association signal extensively overlaps those of reports focusing on nicotine- and alcohol-related phenotypes[37,38,45]; the weaker, independent association with the *NCAM1* SNP rs4492854 is consistent with prior reports.[39,41] Our investigation provides strong evidence of association of individual *ANKK1* and *TTC12* SNPs with heroin and other illicit drug dependence, replicating findings in a prior report.[41] Despite these converging findings, considerable variation in the intensity and location of association was found across reports.[37-39,41-45] Differences in examined phenotype likely contributed to this variance because alcohol- and nicotine-related outcomes differ but are significantly correlated.

The protein encoded by *ANKK1*, a member of the receptor interacting protein serine/threonine kinases, is believed to play a role in signal transduction that includes activation of transcription factors in response to environmental factors.[53] *ANKK1* is reportedly expressed in radial glia during development and in astrocytes within the adult brain.[54] *ANKK1* expression is upregulated by administration of apomorphine, a dopamine agonist, and is temporally associated with *DRD2* expression in developing mice.[53-55] These findings, coupled with the genes' proximity, have led investigators to posit that the protein encoded by *ANKK1* may play an important role in the alterations in dopaminergic signaling following drug exposure central to the addiction process.[53] *TTC12*, also expressed in the brain, contains a tetratricopeptide repeat structure known to facilitate protein-protein binding and for which effects on steroid hormone receptors have been reported.[56] Further investigation will be necessary to characterize more clearly the roles these genes may play in the pathophysiology of illicit drug dependence.

Several limitations must be considered when interpreting our findings. Because our cases were ascertained entirely from New South Wales ORT clinics, generalizability to samples of individuals not currently in treatment or from other areas will need to be demonstrated. Our primary findings emerged after dividing neighborhood controls into subsamples based on history of lifetime drug dependence; thus, replication in other similarly ascertained samples would provide important confirmation. It is possible that population stratification could have contributed to our findings. Cases and control groups primarily included individuals of European ancestry. Although we did observe ethnicity differences, the most substantial were between cases and ATR controls. We conducted PCA prior to each comparison

and, when appropriate, included PCs to control for population stratification. Post hoc comparisons of cases with nondependent neighborhood controls that included 4 PCs as covariates produced similar results. Another correlated phenotype (eg, a component of temperament) could be responsible for the current association findings and those previously reported. While this possibility is difficult to exclude, examinations that have incorporated aspects of temperament have produced mixed results.[40,47] Despite the considerably larger size of our sample (>3-fold larger than most prior association studies of heroin dependence), it is possible that we may have failed to detect significant associations because of limited power (ie, type II error). Similarly, the smaller size of the neighborhood control subsamples either could be limiting significant differences found in comparisons with cases or between subgroups or could be resulting in spurious associations (ie, type I error). Finally, the reductions in sample size produced by the more stringent exclusion criteria (ie, including alcohol or tobacco dependence along with illicit drug dependence) used for the neighborhood control subgroups may have contributed to the weaker observed associations by decreasing power.

In summary, we provide evidence that *ANKK1* and *TTC12* SNPs are strongly associated with substance dependence, substantially overlapping findings from other reports.[37-46] Our focus on illicit drug dependence is an important extension of scope beyond that of prior studies. Additional investigations (eg, deep sequencing) can characterize more definitively the polymorphisms most highly associated with heroin and other illicit drug dependence and determine the gene responsible for the observed association. Finally, our findings highlight the importance of considering substance exposure history when selecting the most appropriate control group for genetic investigations of substance dependence and raise an intriguing possibility that nondependent, highly substance-exposed controls might prove particularly informative.

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