

Genome-wide association study of a quantitative disordered gambling trait

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ABSTRACT

Disordered gambling is a moderately heritable trait, but the underlying genetic basis is largely unknown. We performed a genome-wide association study (GWAS) for disordered gambling using a quantitative factor score in 1312 twins from 894 Australian families. Association was conducted for 2 381 914 single-nucleotide polymorphisms (SNPs) using the family-based association test in Merlin followed by gene and pathway enrichment analyses. Although no SNP reached genome-wide significance, six achieved P -values $< 1 \times 10^{-5}$ with variants in three genes (*MTIX*, *ATXN1* and *VLDLR*) implicated in disordered gambling. Secondary case-control analyses found two SNPs on chromosome 9 (rs1106076 and rs12305135 near *VLDLR*) and rs10812227 near *FZD10* on chromosome 12 to be significantly associated with lifetime Diagnostic and Statistical Manual of Mental Disorders, fourth edition pathological gambling and South Oaks Gambling Screen classified probable pathological gambling status. Furthermore, several addiction-related pathways were enriched for SNPs associated with disordered gambling. Finally, gene-based analysis of 24 candidate genes for dopamine agonist-induced gambling in individuals with Parkinson's disease suggested an enrichment of SNPs associated with disordered gambling. We report the first GWAS of disordered gambling. While further replication is required, the identification of susceptibility loci and biological pathways will be important in characterizing the biological mechanisms that underpin disordered gambling.

Keywords association, canonical pathways, disordered gambling, enrichment analysis, genomewide, MERLIN, quantitative.

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INTRODUCTION

There is emerging evidence suggesting that the propensity to gamble is heritable (Slutske *et al.* 2009b). Whether it be purchasing a lottery ticket, betting on the outcome of a horse race or playing a slot machine, about half of the variation between people in the propensity to engage in a specific gambling activity can be attributed to genetic differences (Slutske *et al.* 2009b). The same is true of how much time or money one spends on gambling (Slutske *et al.* 2009b) as well as whether one develops problems as a consequence of one's gambling (Eisen *et al.* 1998; Slutske *et al.* 2010)—that is, disordered gambling (DG).

DG refers to the full continuum of problems related to excessive gambling that include pathological gambling disorder, for example, as defined by the American Psychiatric Association's Diagnostic and Statistical Manual of

Mental Disorders (American Psychiatric Association 1994). There have been only two major twin studies of DG. In the all-male US Vietnam Era Twin (VET) Registry, the lifetime rates of diagnosed DG disorder were significantly elevated among the monozygotic (MZ) (23%) and dizygotic (DZ) (10%) co-twins of men with DG, compared with the lifetime prevalence in the full sample (1.4%) (Eisen *et al.* 1998). Depending on the DG symptom cut-off imposed (from one or more to four or more symptoms), the heritability estimate ranged from 40 to 54% (Eisen *et al.* 1998). The other major twin study of DG was conducted with male, female and opposite-sex twin pairs recruited from the Australian Twin Registry (ATR) (Slutske *et al.* 2010). The heritability estimate of DG in the ATR study ranged from 40 to 58% depending on the symptom cut-off imposed and did not significantly differ for men and women. Additionally, there was no evidence

to suggest that there were sex differences in the specific genetic risk factors for DG. This study extended the findings of the previous VET registry study of men in the United States studied in the early 1990s to women and the heavier gambling culture of Australia.

There have been only a handful of published molecular genetic studies of DG to date (for a recent review, see Lobo & Kennedy 2009). All of the studies have been candidate gene association studies—there has not yet been a genome-wide linkage or association study of DG. The focus of most of the association studies has been one or more of the dopamine receptor genes (including *DRD1*, *DRD2*, *DRD3*, *DRD4* and *DRD5*) and the dopamine transporter gene (*DAT*), with at least one positive finding reported for *DRD1*, *DRD2* and *DRD4*. Other candidate genes that have been the focus of association studies of DG are the serotonin transporter gene, the monoamine oxidase A and B genes, and the tyrosine hydroxylase gene, with at least one positive finding reported for the serotonin transporter and monoamine oxidase A genes. Although the positive findings are intriguing, there have been no replicated results. It is difficult to draw any firm conclusions from so few molecular genetic studies of DG.

The association studies of DG to date have used a case-control design comparing patients that were affected with diagnosed DG disorder to unaffected controls (who were not diagnosed with DG disorder). The present investigation differs from the previous studies in four important ways: (1) participants were drawn from a community-based sample rather than from DG treatment programs; (2) the phenotype of interest was a quantitative continuous trait rather than a binary categorical DG diagnosis; (3) the use of single-nucleotide polymorphism (SNP) chips enabled us to search for loci across the entire genome rather than focusing on a selected number of candidate genes or a restricted set of markers; and (4) a genome-wide association analysis (GWAS) was supplemented with a follow-up analysis that focused on genes involved in putative biological pathways.

Use of a community-based sample may allow for greater generalizability of the results. Treatment-ascertained samples of individuals with DG are likely to be unrepresentative given the low rate of treatment-seeking for gambling disorders (~10–20% in the United States and Australia; Slutske, Blaszczyński & Martin 2009a). Furthermore, the most effective use of the information that can be provided by a community-based sample is through the use of a continuous, quantitative DG phenotype. This is because even though very few individuals in a community-based sample will exceed the diagnostic threshold for a DG diagnosis, many will provide valuable information about individual differences along the broader DG continuum. Previous studies that treated all individuals unaffected with DG disorder as

being equivalent may have had considerable heterogeneity in their samples of unaffected controls. Recognizing the variation below the diagnostic threshold can turn this potential liability into an asset. The use of a quantitative DG phenotype is also consistent with the increasing appreciation for the idea of DG, along with many other psychiatric disorders as reflecting a continuum of pathology (Slutske *et al.* 2011). Finally, the use of a quantitative DG phenotype has the advantage of increased statistical power to detect genetic associations (Evans 2009).

There have been a series of reports on the incidence of DG among individuals with Parkinson's disease and restless legs syndrome (for review, see Dagher & Robbins 2009). These individuals were being treated with a dopamine agonist medication (that typically demonstrate relative selectivity for dopamine D₃ receptors) in combination with or without levodopa (an amino acid precursor of dopamine that shows greater selectivity for dopamine D₁ and D₂ receptors), and whose DG usually resolved with the discontinuation of the dopamine agonist therapy. These correlational findings are supported by experimental evidence from studies of rats and humans demonstrating that administration of a dopamine D₂/D₃-selective receptor agonist (Johnson *et al.* 2011) or the administration of levodopa in the presence of the 4/7 *DRD4* genotype (Eisenegger *et al.* 2010) increases gambling-like behaviors in the laboratory. These pharmacologic findings provide important clues to a potential neurobiological pathway to DG and might prove to be useful in gene identification; this information was incorporated into the follow-up gene-enrichment analyses in the present study.

The aim of this study was to present the results of the first genome-wide association study of a quantitative DG trait using data collected from a large community-based cohort of 1312 Australian twins. We also report biological pathways associated with DG, which may provide new insights into the etiology of DG.

METHODS

Participants

The participants for this study were members of the national community-based ATR Cohort II (Slutske *et al.* 2009b). A structured telephone interview containing a thorough assessment of gambling behaviors was completed with 4764 ATR Cohort II members (individual response rate of 80%). The mean age of the participants was 37.7 years (range = 32–43) and 57.2% of the sample was female. For more details about sample characteristics, participation rates, potential sampling biases and zygosity determination, see Slutske *et al.* (2009b). Re-interviews were conducted with 166 participants

who had completed the baseline interview [follow-up interval $M = 3.4$ months, standard deviation (SD) = 1.4 months, range = 1.2–9.5 months], with an oversampling of participants who reported symptoms of DG, for the purpose of obtaining estimates of reliability of the study measures. Genotypic and phenotypic data were available for 1312 of the 4764 individuals who had completed the gambling assessment. Those individuals comprised 201 MZ twin pairs (117 female and 84 male), 214 DZ pairs (78 female, 49 male and 87 opposite sex), 50 unpaired MZ twins and 432 unpaired DZ twins from 894 families.

Genotyping and imputation

DNA samples used in the current study were part of several different projects focused primarily on the genetics of nicotine and alcohol addiction, collected in accordance with standard protocols and were drawn from 5 of 10 Illumina GWAS subsamples ($N = 19\,257$ individuals) genotyped from the Genetic Epidemiology laboratory at QIMR. DNA samples were genotyped using Illumina HumanCNV370-Quadv3 ($N = 966$) or Human610-Quadv1 ($N = 346$) SNP BeadChips. Standard quality control (QC) filters were applied and any SNP was removed unless it satisfied all of the following conditions: mean Illumina Beadstudio GenCall scores ≥ 0.7 , SNP call rate > 0.95 , minor allele frequencies (MAF) $\geq 1\%$, deviation from Hardy–Weinberg equilibrium (HWE) $> 10^{-6}$. Samples were also screened for ancestry outliers by using principal component analysis. As described elsewhere (Medland *et al.* 2009), a consensus SNP set ($N = 271\,069$) common to all BeadChips was imputed HapMap Centre d'Etude du Polymorphisme Humain (Utah Residents with Northern and Western European Ancestry) Phase I + II data (Release 22, Build 36) using Markov Chain Haplotyping (Li & Abecasis 2006), resulting in a dataset of 2 373 249 SNPs. Imputed SNPs were dropped if they had an Rsq imputation quality score < 0.3 , MAF $< 1\%$ or showed significant deviations from HWE $< 10^{-6}$ in the overall sample. Genotypes with Mendelian errors were set to missing. When only one individual from a MZ twin pair had been genotyped, the available genotype was used for both twins.

Measures

A quantitative DG phenotype was derived from four different indexes of gambling involvement and two different inventories of DG that were contained in the structured interview. Indicators of gambling involvement were included to provide a better characterization of the full DG continuum (i.e. non-diagnostic items might provide information about lower levels of the DG continuum that are not well differentiated by items used to establish a DG

diagnosis), and to therefore obtain a more normally distributed DG continuum.

Gambling involvement

Participants reported whether they had ever engaged in 11 different gambling activities (lottery, electronic gambling machines, instant scratch tickets, betting on horse or dog races, playing casino table games, keno, bingo, card games, betting on a sporting event, betting on games of skill, and Internet casino gambling) in their lifetime. Gambling versatility was a composite continuous indicator of the number of different activities in which the respondent had ever been engaged, which reflects the extensiveness or diversity of the respondent's gambling involvement. The internal consistency and test–retest reliabilities of the gambling versatility index were 0.68 and 0.73, respectively (Slutske *et al.* 2009b). Three categorical indices of the frequency of gambling involvement were also included in this study: ever gambled at least once a month for at least 6 months in a row (test–retest reliability, $\kappa = 0.69$), ever gambled at least once a week for at least 6 months in a row (test–retest reliability, $\kappa = 0.77$), and ever gambled at least daily for at least 2 weeks in a row (test–retest reliability, $\kappa = 0.46$).

Disordered gambling

Symptoms of DG were assessed using two different measures: the National Opinion Research Center Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) Screen for Gambling Problems (Gerstein *et al.* 1999) and the South Oaks Gambling Screen (SOGS; Lesieur & Blume 1987). The test–retest and internal consistency reliabilities of the 10-item DSM-IV lifetime symptom count (test–retest $r = 0.86$; coefficient alpha = 0.85) and the 20-item SOGS lifetime symptom count (test–retest $r = 0.86$; coefficient alpha = 0.81) were all high. Exploratory factor analyses provided strong and convincing evidence consistent with a single-factor model of DG for both the DSM-IV and the SOGS symptom sets (Slutske *et al.* 2011).

Data analysis

Development of a quantitative DG trait

Using the entire sample of 4764 respondents, a single factor was extracted from the four indexes of non-DG involvement, the 10 items from the DSM-IV symptom set and the 20 items from the SOGS using Mplus (Muthén & Muthén 1998–2004) (See Supporting Information Table S5). The factor score derived from this analysis was used as the quantitative DG phenotype used in the genetic analyses. In the entire sample, the mean, SD, skewness and kurtosis of the factor score were 0.06, 0.78, 0.38

and—0.31, respectively; in the subsample included in the GWAS analyses, they were 0.13, 0.78, 0.32 and—0.21, respectively. The quantitative DG trait was approximately normally distributed, and the mean scores of the genotyped subsample were slightly higher than those of the full twin sample. The heritability of the quantitative DG trait was 0.52 (95% confidence interval 0.36–0.69).

SNP-based genome-wide association analyses

The modal genotype at each SNP was tested for association with the quantitative DG trait using the family-based association test in Merlin (Chen & Abecasis 2007) (fastAssoc) that accounts for family relationships and zygosity. The additive genetic effect was computed by modeling the genotypic mean of the heterozygote (Aa) as the average of the two homozygotes (AA, aa). Correction for sex, age, age², sex*age and sex*age² was performed by fitting covariates in the regression model. Phenotypes were also adjusted for possible effects of population stratification in our sample by fitting the first 10 eigenvectors (PC1–10) from European-only principal components analysis of ancestry in the regression model (McEvoy et al. 2009). Association analyses of genotyped markers on the X chromosome were conducted using Minx (as implemented in Merlin). Because the imputation software did not support sex chromosomes, SNPs at the X chromosome are not imputed; the association analyses only included those SNPs that have been genotyped for at least 85% of the sample (N = 8666). We adopted a genome-wide significance level for the association between SNP and phenotype of 7.2×10^{-8} or smaller to correct for the total number of independent tests (Dudbridge & Gusnanto 2008).

Gene-based analyses

A gene-based test Versatile Gene-based Association Study (VEGAS), feasible for use with GWA data with related individuals (Liu et al. 2010), was conducted to determine whether any genes harbored an excess of SNPs with small *P*-values. In brief, this test explores association on a per-gene basis taking the *P*-values of all SNPs within 50 kb of each gene, as well as linkage disequilibrium (LD) and number of SNPs per gene into account. A *P*-value below $\alpha = 2.8 \times 10^{-6}$ was considered to be significant as the gene-based association test included 17 687 autosomal genes (0.05/17 687).

Enrichment analysis

We explored canonical pathway enrichment using Ingenuity Pathways Analysis (Ingenuity® Systems, <http://www.ingenuity.com>). The top 10 000 SNPs from the GWAS were mapped to genes and then assigned to

canonical pathways. For each pathway, a *P*-value (the likelihood that the association between the genes in the dataset and the canonical pathway is explained by chance alone using a right-tailed Fisher's exact test) and ratio (the number of molecules from the dataset that map to the pathway divided by the total number of molecules that map to the canonical pathway) were calculated. In WebGestalt (Duncan, Prodduturi & Zhang 2010), the top 10 000 GWAS SNPs were compared with the human genome reference set and assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment evaluation analysis using the hypergeometric test with the Benjamini-Hochberg test for multiple test adjustment. The significance cut-off was *P* = 0.05 and a minimum of 2 proteins were accepted per category. The resulting data are displayed as a 'Ratio of Enrichment' (the number of genes in the GWAS dataset present in the KEGG pathway divided by the expected number of genes in the KEGG pathway) compared with the entire human genome.

RESULTS

We conducted a genome-wide association study of a quantitative DG trait in 1312 individuals from 894 Australian families. The average age of the genotyped sample was 37.6 years (SD = 2.3) for males and 37.9 years (SD = 2.3) for females. Males obtained a slightly higher DG factor score; the average (\pm SD) and range of DG scores for men was 0.304 ± 0.794 (–1.343 to 3.204) and for women was 0.048 ± 0.728 (–1.343 to 2.753). The distribution of the quantitative DG factor score by sex and lifetime DSM-IV DG status is provided in Supporting Information Fig. S1.

The Manhattan plot of association *P*-values for 2 381 914 autosomal and X chromosome SNPs is shown in Fig. 1. While no SNP achieved genome-wide significance ($P < 7.2 \times 10^{-8}$), regions of suggestive association ($P < 1 \times 10^{-5}$) were observed on chromosome 6p23, 9p24, 12q24 and 16q13. The quantile–quantile plot (Q–Q plot) of the observed versus expected $\log_{10}(P\text{-value})$ from the association analysis is presented in Fig. 2. The genomic control λ (0.997) was close to 1.0, indicating that there was no evidence for inflation of the test statistics or a bias because of possible population stratification in the results (Bacanu, Devlin & Roeder 2000) and that the family-based association model had correctly accounted for relatedness.

The most significant SNPs are presented in Table 1, at a threshold of $P < 1 \times 10^{-5}$ after excluding redundant SNPs that are in high LD ($r^2 > 0.8$) with more significant SNPs. Three SNPs were located in two genes, *FLJ35024* and *ATXN1*, but none were exonic. The remaining SNPs were located downstream of *MT1X* and nearby (within 50 Kb) to *VLDLR* and *FZD10*. Regional association plots

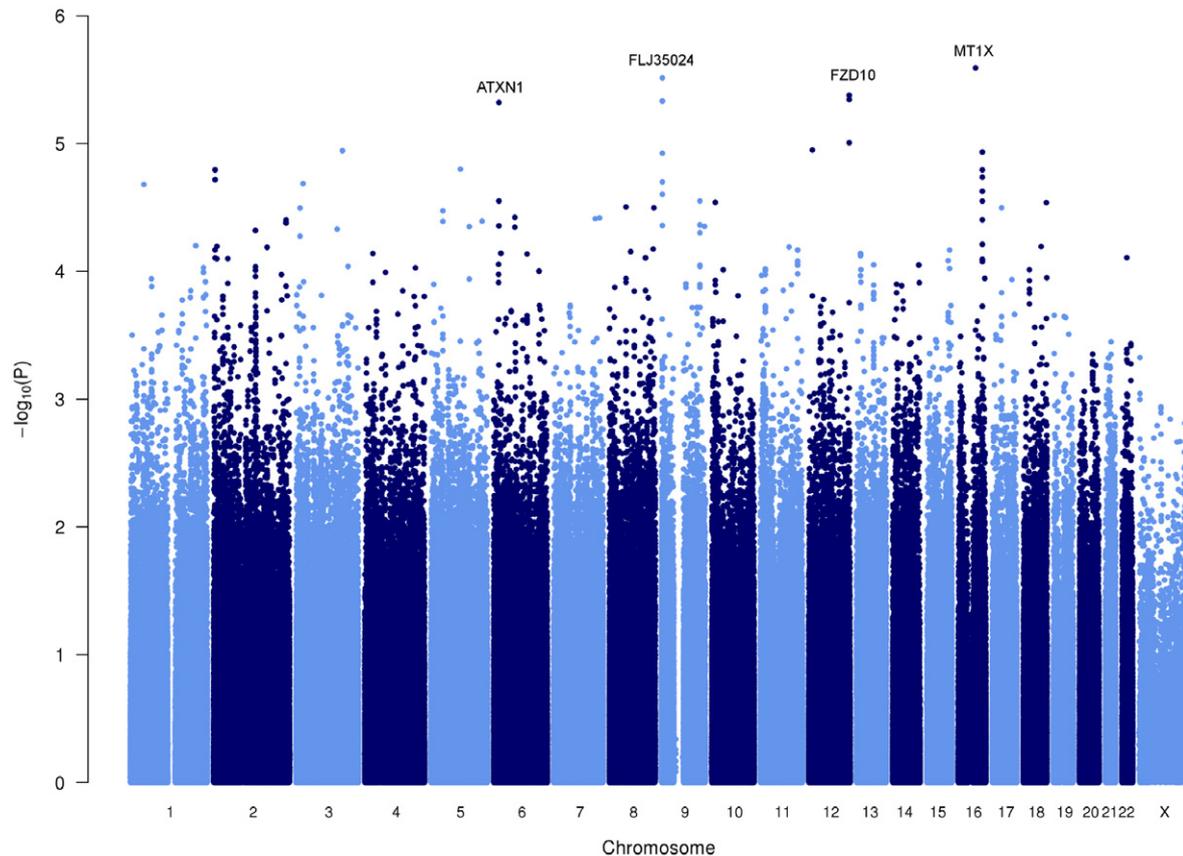


Figure 1 Manhattan plot for the quantitative disordered gambling factor score. The vertical axis shows the $-\log_{10}$ of the association P -values and the horizontal axis shows the whole autosomal genome divided into 22 autosomes and the X chromosome

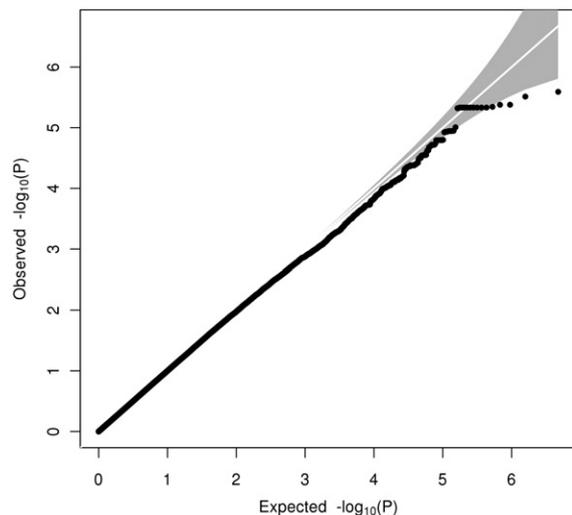


Figure 2 Quantile-quantile plot for the quantitative disordered gambling factor score (genomic inflation $\lambda=0.997$). The horizontal axis shows the $-\log_{10}$ of expected P -values of association from a 1 degrees of freedom chi-square distribution and the vertical axis shows the $-\log_{10}$ of P -values from the observed chi-square distribution. The shaded region represents the 95% confidence interval of the expected chi-square statistics under the null hypothesis of no association

for the six SNPs are provided in the Supporting Information Figs. S2–S7. While these SNPs explain almost 11% of the variance in DG, this result is most likely inflated because the estimate of the genetic effect explained by top SNPs in smaller samples, such as our study of 1312 twins, is significantly overestimated (the ‘winner’s curse’) (Zollner & Pritchard 2007). Secondary case-control analyses for the six SNPs were performed in a subsample of unrelated twins comprising (1) 31 lifetime DSM-IV pathological gamblers and 863 controls; (2) 44 SOGS classified probable pathological gamblers and 850 controls; (3) 101 SOGS classified problem gamblers or probable pathological gamblers and 793 controls; and (4) 425 individuals that met criteria for ≥ 1 (out of 30) DSM-IV or SOGS symptoms and 469 individuals that had 0 symptoms (see Supporting Information Table S1). The results for the first three analyses were similar where SNPs on chromosome 9 (rs1106076 and rs12305135 near *VLDLR*) and 12 (rs10812227 near *FZD10*) remained significant following 10 000 permutations. For example, DSM-IV pathological gamblers were ~ 2.5 times more likely to carry rs1106076 and rs12305135 C-alleles than controls (empirical $P=0.036$ and 0.027 , respectively) and 5.6-fold less likely to carry the

Table 1 Variants showing the strongest association with the quantitative disordered gambling factor score (independent markers with $P < 1 \times 10^{-5}$)

Chr	SNP	Position	SNPs in LD	Gene	Location	Nearby gene	AI	Freq	β	SE	R ² (%)	P _{corr}
16q13	rs8064100	55 282 674		MTIX	Downstream	MTIP, MTIH, MTIG, MTIE, MTIB, NUP93	A	0.582	-0.148	0.031	1.89	2.57E-06
9p24	rs12237653	2 541 654		FLJ35024	Intron	VLDLR	T	0.868	0.225	0.048	2.06	3.08E-06
12q24	rs11060736	129 196 888	2	FLJ35024	Intron	FZD10, FLJ31485	T	0.941	-0.285	0.062	1.60	4.19E-06
9p24	rs10812227	2 538 556	8	FLJ35024	Intron	VLDLR	C	0.868	0.221	0.048	1.98	4.67E-06
6p23	rs9383153	16 451 035		ATXN1	Intron	GMPR	A	0.948	0.331	0.072	1.91	4.79E-06
12q24	rs12305135	129 201 158		FZD10, FLJ31485		FZD10, FLJ31485	T	0.933	-0.259	0.059	1.49	9.87E-06

The LD between independent markers is $r^2 < 0.8$. SNPs in LD is the number of correlated SNPs with P -values $< 1 \times 10^{-5}$. Location is the physical location of the SNP within the gene. Nearby gene is the gene(s) closest to the SNP (cells are empty if no gene is within ± 50 kb). AI is the reference allele, and Freq is the frequency of the reference allele, β shows the mean increase in the disordered gambling factor score per added reference allele controlling for sex, age, age², sex*age, sex*age² and the first 10 eigenvectors (PC1–10) from European-only principal components analysis of ancestry. SE gives the standard error of the β -coefficient, R² represents the percentage of phenotypic variance explained by the SNP, and P_{corr} gives the P -value of association controlling for genomic inflation ($\lambda = 0.9972$).

LD, linkage disequilibrium; SE, standard error; SNP, single-nucleotide polymorphisms.

rs10812227 T-allele [odds ratio (OR): 0.18, 95% confidence interval (CI): 0.04–0.75, empirical $P = 0.0019$]. A different pattern of association was observed for the ' ≥ 1 DSM-IV/SOGS symptom' phenotype, where three SNPs (rs9383153 in *ATXN1*, rs8064100 downstream of *MTIX* and rs12305135 near *VLDLR*) remained significant following permutation analysis. The strongest evidence of association was with rs9383153 where cases were 2.4-fold less likely to carry the rs9383153 G-allele (OR: 0.42, 95% CI: 0.26–0.67, empirical $P = 0.0007$).

To complement our genome-wide association analysis, we performed genome-wide gene-based tests using VEGAS. The entire GWAS SNP dataset was assigned to genes according to their positions on the University of California Santa Cruz Genome Browser hg18 assembly, with gene boundaries defined as ± 50 Kb beyond the 5' and 3' untranslated regions. The gene-based analysis did not reveal significant results that survived correction for multiple testing ($P < 2.8 \times 10^{-6}$), with the smallest empirical P -value being 0.00028 for *PNMA1*. We list the 50 genes most associated with the quantitative DG factor score in Supporting Information Table S2. Gene names, ranks and P -values, as well as the top SNP for each gene, are provided. The most notable result was the third ranked *CDKRAP2*, a gene associated with three traits measuring an individual's level of response to alcohol (an endophenotype for alcohol use disorders) (Joslyn *et al.* 2010).

We then tested a candidate gene set derived from a candidate gene study for pathological gambling by Comings *et al.* (2001) and literature on dopamine agonist-induced DG. Supporting Information Fig. S8 illustrates the interactions between dopamine, dopamine agonists (cabergoline, levodopa, pergolide and pramipexole) and the proteins encoded by the 24 candidate genes. Gene-based results are shown in Table 2, with *ADRA2C* and *CREB1* achieving P -values < 0.05 but no gene was associated with $P < 0.0021$ (0.05/24). However, the average gene ranking for the 24 genes was 6645. In R, we randomly sampled sets of 24 genes 10 000 times and empirically observed that an average ranking of 6645 was higher than expected by chance ($P = 0.0165$). As shown in the Q–Q plot in Supporting Information Fig. S9, the distributions of P -values for this candidate gene set (2160 SNPs) was enriched for association with DG. First, the shape of the Q–Q plot suggests that there is an inflation of weak associations with SNPs of small effect that do not reach genome-wide significance in the full GWAS SNP because of power constraints and second, enrichment is reflected by the high λ -value of 1.503 compared with 0.997 in the full GWAS analysis.

Next, we tested whether genes with the strongest association signals were enriched within canonical or biological pathways. We performed canonical pathway

Table 2 VEGAS gene rankings and *P*-values of 24 candidate genes for disordered gambling

Gene rank	Position	Symbol	Gene	Test statistics for disordered gambling candidate genes					Test statistics for the SNP most strongly associated within each candidate gene				
				Size (bp)	#SNPs	<i>P</i> -value	SNP	<i>P</i>	Allele	β	SE		
8456	22q11.23	ADORA2A	Adenosine A2a receptor	14 796	77	0.532	rs8141793	0.05375	G	0.133	0.069		
257	4p16.3	ADRA2C	Adrenergic, alpha-2C-, receptor	1 958	29	0.015	rs11725040	0.03658	C	-0.068	0.032		
4993	22q11.21	COMT	Catechol-O-methyltransferase	27 222	130	0.305	rs2531716	0.01827	T	-0.087	0.037		
370	2q34	CREB1	Cyclic adenosine monophosphate responsive element-binding protein 1	68 885	91	0.022	rs12998817	0.00233	C	-0.096	0.032		
9588	7p11	DDC	Dopa decarboxylase (aromatic L-amino acid decarboxylase)	107 021	251	0.596	rs10235371	0.01231	C	0.14	0.056		
4869	5q34	DRD1	Dopamine receptor D1	3 489	136	0.309	rs251937	0.0132	T	0.086	0.035		
3309	11q22	DRD2	Dopamine receptor D2	65 685	197	0.244	rs17529477	0.00546	G	0.103	0.037		
6526	3q13.3	DRD3	Dopamine receptor D3	50 343	143	0.387	rs7620955	0.03214	C	-0.074	0.034		
17202	11p15.5	DRD4	Dopamine receptor D4	3 399	46	0.982	rs6598007	0.09252	C	0.232	0.138		
8007	4p16.1	DRD5	Dopamine receptor D5	2 376	88	0.516	rs1519094	0.05932	C	0.15	0.079		
8020	14q24.3	FOS	FBJ murine osteosarcoma viral oncogene homolog	3 382	53	0.521	rs6574222	0.05985	G	-0.072	0.038		
8522	9q34.3	GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	29 600	18	0.519	rs12238250	0.05361	A	0.159	0.082		
5052	12p12	GRIN2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	418 613	659	0.312	rs10772723	0.00169	C	-0.1	0.032		
11842	5q11.2	HTR1A	5-hydroxytryptamine (serotonin) receptor 1A	1 269	41	0.693	rs13361335	0.1444	T	0.073	0.05		
7210	13q14	HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	62 663	212	0.451	rs2094591	0.0405	A	0.071	0.035		
1408	2q36.3	HTR2B	5-hydroxytryptamine (serotonin) receptor 2B	16 870	45	0.087	rs13424110	0.03594	C	0.122	0.058		
7891	9q34.11	NCST1	Neuronal calcium sensor 1	64 727	160	0.481	rs2240913	0.01547	A	0.081	0.033		
7830	14q24.3	PSEN1	Presenilin 1	83 931	135	0.477	rs362353	0.04441	A	0.144	0.071		
15404	8p21.3	SLC18A1	Solute carrier family 18 (vesicular monoamine), member 1	38 346	147	0.173	rs2410639	0.01301	G	-0.202	0.081		
2765	10q25	SLC18A2	Solute carrier family 18 (vesicular monoamine), member 2	36 380	158	0.477	rs363241	0.01835	T	0.079	0.033		
10164	5p15.3	SLC6A3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	52 629	114	0.617	rs7732456	0.02884	A	0.254	0.116		
7034	17q11.2	SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	37 810	75	0.464	rs2020941	0.02856	C	-0.32	0.146		
1905	11p15.5	TH	Tyrosine hydroxylase	7 877	111	0.116	rs2070762	0.00121	G	-0.104	0.032		
859	12q15	TPH2	Tryptophan hydroxylase 2	93 596	215	0.053	rs11179002	0.00698	C	-0.099	0.037		

Bold type indicates the candidate genes that yielded a *P*-value < 0.05. #SNPs is the number of SNPs in each gene (± 50 Kb beyond the 5' and 3'UTRs), *P* gives the *P*-value of association for the SNP. Allele is the reference allele, β shows the mean increase in the disordered gambling factor score per added reference allele controlling for sex, age, age², sex*age, sex*age² and the first 10 eigenvectors (PC1–10) from European-only principal components analysis of ancestry. SE gives the standard error of the β -coefficient.

SE, standard error; SNP, single-nucleotide polymorphisms; UTR, untranslated region.

Table 3 Enrichment of Ingenuity canonical pathways for disordered gambling

Rank	Canonical pathway	P-value	Ratio	Genes
1	Neuropathic pain signaling in dorsal horn neurons	0.000023	0.18	GRIN2B,CAMK1D,GRM1,ITPR2,ITPR1,GRIA4,PRKAG1,GRM5,TACR1,GRM7,CAMK2D,PRKAR2B,PRKAG2,PLCB1,KCNQ3,PRKCH,PRKD1,PRKCA,PRKCB
2	Synaptic long-term potentiation	0.000023	0.17	PPP1R14C,GRIN2B,GRM1,PPP1R1A,ITPR2,CACNA1C,ITPR1,GRIA4,PRKAG1,GRM5,GRM7,CAMK2D,PRKAR2B,PRKAG2,PLCB1,PRKCH,PRKD1,PRKCA,PRKCB
3	Synaptic long-term depression	0.000076	0.14	GUCY1A3,GRM1,ITPR2,GRID2,RYR2,GNAI1,ITPR1,GRIA4,GRM7,GRM5,PRKG1,LCAT,PPP2R2B,RYR3,IGF1R,PLCB1,PRKCH,PRKD1,PRKCA,PRKCB
4	CREB signaling in Neurons	0.0021	0.10	GRIN2B,ADCY2,GRM1,ITPR2,GRID2,GNAI1,ITPR1,GRIA4,GNG5,PRKAG1,GRM7,GRM5,PRKAR2B,CAMK2D,PRKAG2,PLCB1,PRKCH,PRKD1,GRIK1,PRKCA,PRKCB
5	Breast cancer regulation by stathmin1	0.0030	0.11	PPP1R14C,ADCY2,CAMK1D,ITPR2,ARHGEF7,GNAI1,ITPR1,TUBA1B,PRKAG1,GNG5,PRKAR2B,CAMK2D,TUBA1A,ARHGEF10,PPP2R2B,PRKAG2,PLCB1,PRKCH,ARHGEF3,PRKD1,PRKCA,PRKCB
6	GNRH signaling	0.0049	0.11	ADCY2,ITPR2,MAP3K1,GNAI1,ITPR1,MAP3K4,PRKAG1,CAMK2D,PRKAR2B,PRKAG2,PLCB1,PRKCH,PRKD1,PRKCB,EGFR,PRKCA
7	α -Adrenergic signaling	0.0049	0.12	ADCY2,ITPR2,GNAI1,ITPR1,GNG5,PRKAG1,PRKAR2B,PRKAG2,PRKCH,ADRA1A,PRKD1,PRKCA,PRKCB
8	Hepatic cholestasis	0.0049	0.10	ADCY2,MAP3K4,IL1R1,PRKAG1,IL1F9,PRKAR2B,IL1RL2,ABCC1,PRKAG2,PRKCH,INSR,SLCO1B1,HNF4A,IRAK4,PRKD1,PRKCB,PRKCA
9	Axonal guidance signaling	0.0056	0.08	LRRC4C,ADAM22,EPHB2,NFATC3,ARHGEF7,ROBO1,PRKAG1,EFNA5,ABLIM3,DCC,UNC5D,PLCB1,PRKD1,GLIS1,PRKCA,SEMA5A,GNAI1,NFATC1,SLIT2,TUBA1B,BMP5,PLXND1,GNG5,DOCK1,PRKAR2B,TUBA1A,GLIS2,NTRK3,SEMA6D,PRKAG2,PRKCH,PDGFD,BMP6,PRKCB
10	Glutamate receptor signaling	0.0058	0.15	GRM5,GRM7,GRIN2B,SLC1A6,GRM1,GRID2,SLC1A2,GRIA4,GNG5,GRIK1
11	Melatonin signaling	0.0062	0.14	PRKAR2B,CAMK2D,RORA,GNAI1,PRKAG2,PLCB1,PRKCH,PRKAG1,PRKD1,PRKCA,PRKCB
64	Dopamine receptor signaling	0.275	0.08	PPP1R14C,TH,ADCY2,PRKAR2B,PPP2R2B,PRKAG2,PRKAG1
164	Parkinson's signaling	0.951	0.06	PARK2

Enrichment analysis was performed in Ingenuity Pathway Analysis using the top 10 000 SNPs from the GWAS. The 15 most enriched canonical pathways are listed, as well as the dopamine receptor signaling and Parkinson's signaling candidate pathways. *P-value* is the *P-value* of enrichment adjusted for multiple testing using the Benjamini–Hochberg test. *Ratio* is the number of genes in the GWAS dataset compared with the total number of genes in the pathway. *Genes* is the list of pathway genes present in the GWAS dataset.

CREB, adenosine 3'5' cyclic monophosphate response element binding protein; GNRH, gonadotrophin-releasing hormone; GWAS, Genome-Wide Association Study; SNP, single-nucleotide polymorphisms.

analyses including the top 10 000 SNPs (*P-values* < 0.0047) using Ingenuity Pathway Analysis. The most significant pathways (*P* < 0.01) and the dopamine receptor signaling and Parkinson's signaling pathways are summarized in Table 3. Three pathways [synaptic long-term potentiation, synaptic long-term depression, gonadotrophin-releasing hormone (GNRH) signaling] had been previously identified by Li, Mao & Wei (2008) to be enriched for substance addiction-related genes, with the synaptic long-term depression and GNRH signaling pathways common to cocaine, alcohol, opioid and nicotine addiction. It should be noted that many of the genes in these three pathways overlap (see Supporting Information Table S3). Supporting Information Table S4 shows the results of secondary KEGG pathway enrichment analyses, respectively, using WebGestalt.

Five of the 10 most enriched KEGG pathways listed overlapped the canonical pathways summarized in Table 3. A sixth KEGG pathway (gap junction) was common to all four addictive substances investigated by Li *et al.* (2008).

DISCUSSION

We performed the first genome-wide association analysis of DG reported to date in a community-based sample of 1312 Australian twins from 894 families using a quantitative factor score derived from four indexes of gambling involvement and symptoms of DG assessed by the National Opinion Research Center DSM-IV Screen for Gambling Problems and SOGS. We did not detect genome-wide significant SNPs and the Q–Q plot (Fig. 2)

shows that the distribution of observed associations closely follows that expected under the null hypothesis of no association. Our finding is consistent with the majority of GWAS of addictive behavior in larger European ancestry samples that have failed to identify genome-wide significant variants of large effect for alcoholism (Heath *et al.* 2011; for review, see Treutlein & Rietschel 2011), cigarette smoking behaviors (Liu *et al.* 2009; Vink *et al.* 2009; Uhl *et al.* 2010), nicotine dependence (Bierut *et al.* 2007) and heroin dependence (for review, see Treutlein & Rietschel 2011).

Four of six independent SNPs showing suggestive association with the quantitative DG score ($P < 1 \times 10^{-5}$) were located in or nearby genes that appear to be theoretically relevant to DG. The top GWAS hit (rs8064100) is located downstream of metallothionein 1X (*MT1X*) and nearby to a cluster of five metallothionein 1 (*MT1*) gene family members. *MT1* expression occurs primarily in astrocytes and to a lesser degree in neurons (Xie *et al.* 2004). *MT1X* has been previously reported to play a role in addiction, including alcohol and opioid dependence (Li *et al.* 2008), while *MT1* expression is associated with anxiety (Czibere *et al.* 2011) and a neuroprotective role in 3,4-methylenedioxymethamphetamine-induced toxicity to dopamine neurons in mice (Xie *et al.* 2004). Furthermore, *MT1* expression in rats is induced throughout the brain by physical stress and by dopamine in neurons (Gasull *et al.* 1994). In our gene-based test the *MT1X* gene had a *P*-value of 0.0046 (ranked 77).

The variant rs9383153 was located in the seventh intron of *ATXN1* (ataxin 1), previously known as *SCA1* (spinocerebellar ataxia type 1 protein), on chromosome 6p23. *ATXN1* plays a key role in spinocerebellar ataxia, type 1, a neurodegenerative disease characterized by loss of motor coordination (ataxia), dysarthria and mild cognitive impairment (Zoghbi & Orr 2009). There is also evidence that *ATXN1* is involved in alcohol dependence where *ATXN1* expression is down-regulated in post-mortem frontal and motor cortices of human alcoholics (Mayfield *et al.* 2002) and in successful smoking cessation (Uhl *et al.* 2009). *ATXN1* has also been shown to occupy the dopamine 2 receptor (*DRD2*) promoter *in vivo* and to regulate *DRD2* gene expression in Purkinje cells of the cerebellum in mice (Hearst *et al.* 2010). In our gene-based test the *ATXN1* gene had a *P*-value of 0.182 (ranked 2910).

Two SNPs (rs12237653 and rs10812227) were located upstream within 50 Kb of *VLDLR*, the very low density lipoprotein receptor gene on chromosome 9p24. *VLDLR* is a receptor for Reelin and the Reelin-*VLDLR*/ApoER2 signaling pathway controls cortical neuronal migration in early development and modulates synaptic plasticity, memory and learning in the adult brain (Herz & Chen 2006). Furthermore, the signaling pathway has

been postulated to contribute to the pathophysiology of schizophrenia (Suzuki *et al.* 2008), bipolar and unipolar depression (Barr, Fish & Markou 2007).

We next analyzed the GWAS data using a systems biology strategy. First we performed the VEGAS gene-based test to determine whether any genes harbored an excess of variants with weak associations. The gene-enrichment analysis did not show any study-wide significant gene hits for DG. We therefore proceeded to the pathway analysis, which is able to mine a wider range of association results by testing for over-representation of genes within pre-defined pathways without restricting the search to 'significant' SNPs. This led to identification of 11 enriched canonical pathways listed in Table 3 for DG. Three pathways (synaptic long-term potentiation, synaptic long-term depression and GNRH signaling) have been previously implicated in aspects of alcohol and nicotine dependence and addiction to opioids and cocaine. The enriched KEGG gap junction pathway has been identified as a common molecular pathway for all four drugs of abuse (Supporting Information Table S4).

The observation that DG and substance dependence share enriched pathways is supported by a proposed change to the fifth revision of the DSM where pathological gambling will be included as a non-substance (or behavioral) addiction in a section labeled 'Addiction and Related Disorders' (O'Brien 2011). Brain imaging and neurochemical studies have shown that gambling activates the same reward system in the brain as addictive drugs with problem casino gamblers showing increases in dopamine, a key 'reward' neurotransmitter in the brain (Holden 2010). It is hypothesized that different substances of abuse (and hypothetically by extension, behavioral addictions) act on different receptors yet activate common downstream signaling cascades and events (Ron & Jurd 2005), and that there may be common neurochemical substrates and neuronal circuits for pathological gambling and addiction (Mutschler *et al.* 2010).

Synaptic long-term depression and long-term potentiation are the activity-dependent weakening and strengthening of synaptic transmission, respectively, and are essential for synaptic plasticity. Synaptic plasticity underlies neural adaptation to substances of abuse and is thought to be important in the development and maintenance of addictions (for review, see Kauer & Malenka 2007). GNRH controls the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Elevated levels of gonadotropins (LH and FSH) are reported in male chronic alcoholics (Heinz *et al.* 1995).

Turning to other enriched pathways, the axonal guidance-signaling pathway is critical for neurodevelopment and has also been implicated in neuroadaptive responses elicited by addictive drugs (Jassen *et al.* 2006). Guanine nucleotide-binding protein-coupled receptors

are a large protein family that regulates physiological responses to a spectrum of biologically active substances such as dopamine and glutamate (Gainetdinov *et al.* 2004). The glutamate receptor-signaling pathway has been shown to play a role in pathological gambling. Two small pharmacotherapy studies of glutamate-modulating agents (N-acetyl cysteine and memantine) resulted in treated pathological gamblers showing reduced Yale Brown Obsessive Compulsive Scale Modified for Pathological Gambling scores (Grant, Kim & Odlug 2007) as well as reduced hours spent gambling per week and money spent gambling (Grant *et al.* 2010). Interestingly, while there seems to be no relationship between our most enriched pathway, neuropathic pain signaling in dorsal horn neurons and addiction, another study also identified this pathway as significantly overrepresented (ranked sixth) among addiction-related genes (Sun & Zhao 2010).

A number of limitations must be kept in mind in considering the present results. First is the comparatively small sample size. Second, it is clear that larger sample sizes are needed to achieve the power required to detect common variants of smaller effect and that the reported size of effects for the six top GWAS SNPs are most certainly overestimates. Power simulations in Merlin showed that our sample had 80% power and 92% power to detect genome-wide significant SNPs explaining 3 and 3.5% of variance in DG, respectively. Third, the gene- and pathway-enrichment analyses are gene-centric and do not include markers located in intergenic regions of the genome. A final feature, which could either be considered a strength or a limitation, is recruitment of subjects from the general population rather than a clinical source. The severity of DG will be less among a population-based sample, but most gambling-related problems occur in the large number of people who are only moderately affected.

In summary, we report results from the first GWAS of DG using a quantitative factor score. We identified three loci (*ATXN1* and two intergenic loci located near *MTIX* and *VLDLR*) for DG with highly significant evidence of association ($P < 1 \times 10^{-5}$). These loci are novel with respect to DG and will complement the findings of candidate gene studies in the literature. We also report biological pathways enriched in DG that have been previously associated with substance addiction. Our findings offer the potential for new insights into the etiology of DG and will serve as a resource for replication in other studies to clarify the potential role of variants in these genes and pathways with DG.

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All authors report no biomedical financial interest or potential conflicts of interest.

Author Contributions

WSS and NGM were responsible for the study concept. WSS provided phenotypic expertise. NGM, ACH, PAFM and GWM contributed to the genotypic data acquisition. PAL, GZ and NGM provided genotypic analysis expertise. Data were analyzed by PAL. PAL, WSS and NGM assisted with interpretation of findings. PAL and WSS drafted the manuscript. All authors approved final version for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Secondary case-control analyses of the top SNPs for the quantitative disordered gambling trait (DG).

Table S2. The 50 genes most strongly associated with the quantitative disordered gambling factor score.

Table S3. Gene overlap between the enriched Ingenuity canonical pathways for disordered gambling

Table S4. Enrichment of KEGG pathways for disordered gambling.

Table S5. Derivation of the quantitative disordered gambling trait based on extraction of a single factor from a confirmatory factor analysis of data obtained from 4764 Australian adults.

Figure S1. Distribution of the disordered gambling factor score (DG) in 1312 Australian twins.

Figure S2. Regional association plot for chromosome 16 (rs8064100).

Figure S3. Regional association plot for chromosome 9 (rs12237653).

Figure S4. Regional association plot for chromosome 12 (rs11060736).

Figure S5. Regional association plot for chromosome 9 (rs1081227).

Figure S6. Regional association plot for chromosome 6 (rs9383153).

Figure S7. Regional association plot for chromosome 12 (rs12305135).

Figure S8. Connectivity diagram illustrating the interactions between proteins encoded by 24 candidate genes for disordered gambling and dopamine agonist (cabergoline, pergolide, pramipexole, levodopa [L-dopa]) induced disordered gambling.

Figure S9. Quantile–Quantile (Q–Q) plot for 2159 SNPs located within 24 candidate genes for disordered gambling (genomic inflation $\lambda = 1.503$).

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