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ORIGINAL ARTICLE

Poor replication of candidate genes for major depressive disorder using genome-wide association data

FJ Bosker[1,8], CA Hartman[1,8], IM Nolte[2,8], BP Prins[2], P Terpstra[2], D Posthuma[3,4], T van Veen[5], G Willemsen[3], RH DeRijk[5], EJ de Geus[3], WJ Hoogendijk[6], PF Sullivan[7], BW Penninx[1,5,6], DI Boomsma[3], H Snieder[2,8] and WA Nolen[1,8]

[1]*Department of Psychiatry, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands;* [2]*Unit of Genetic Epidemiology and Bioinformatics, Department of Epidemiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands;* [3]*Department of Biological Psychology, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands;* [4]*Department of Medical and Functional Genomics, VU University Medical Center, Amsterdam, The Netherlands;* [5]*Department of Psychiatry, Leiden University Medical Center, Leiden, The Netherlands;* [6]*Department of Psychiatry, VU University Medical Center, Amsterdam, The Netherlands and* [7]*Department of Genetics, University of North Carolina, Chapel Hill, NC, USA*

Data from the Genetic Association Information Network (GAIN) genome-wide association study (GWAS) in major depressive disorder (MDD) were used to explore previously reported candidate gene and single-nucleotide polymorphism (SNP) associations in MDD. A systematic literature search of candidate genes associated with MDD in case–control studies was performed before the results of the GAIN MDD study became available. Measured and imputed candidate SNPs and genes were tested in the GAIN MDD study encompassing 1738 cases and 1802 controls. Imputation was used to increase the number of SNPs from the GWAS and to improve coverage of SNPs in the candidate genes selected. Tests were carried out for individual SNPs and the entire gene using different statistical approaches, with permutation analysis as the final arbiter. In all, 78 papers reporting on 57 genes were identified, from which 92 SNPs could be mapped. In the GAIN MDD study, two SNPs were associated with MDD: *C5orf20* (rs12520799; $P = 0.038$; odds ratio (OR) AT = 1.10, 95% CI 0.95–1.29; OR TT = 1.21, 95% confidence interval (CI) 1.01–1.47) and *NPY* (rs16139; $P = 0.034$; OR C allele = 0.73, 95% CI 0.55–0.97), constituting a direct replication of previously identified SNPs. At the gene level, *TNF* (rs76917; OR T = 1.35, 95% CI 1.13–1.63; $P = 0.0034$) was identified as the only gene for which the association with MDD remained significant after correction for multiple testing. For SLC6A2 (norepinephrine transporter (NET)) significantly more SNPs (19 out of 100; $P = 0.039$) than expected were associated while accounting for the linkage disequilibrium (LD) structure. Thus, we found support for involvement in MDD for only four genes. However, given the number of candidate SNPs and genes that were tested, even these significant may well be false positives. The poor replication may point to publication bias and false-positive findings in previous candidate gene studies, and may also be related to heterogeneity of the MDD phenotype as well as contextual genetic or environmental factors.
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Introduction

Major depressive disorder (MDD) is a multi-factorial disease, with both genetic and environmental factors likely to have a role in its etiology. The Netherlands Study of Depression and Anxiety (NESDA; www.nesda.nl) and the Netherlands Twin Registry (NTR; www.tweelingenregister.org) took part in the Genetic Association Information Network (GAIN; http://www.fnih.org/GAIN) project to enable a genome-wide association study (GWAS) using a 600K Perlegen chip (Perlegen Sciences, Mountain View, CA, USA).[1] Within the GAIN MDD study,[2] 1862 participants with a diagnosis of MDD and 1860 controls at low liability for MDD were selected for genome-wide genotyping.

A GWAS approach allows a hypothesis-free search for potential new susceptibility genes. The downside of a GWAS is that a strict statistical adjustment for the large number of single-nucleotide polymorphisms

Correspondence: Dr FJ Bosker, Department of Psychiatry, University Medical Center Groningen, University of Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands.
E-mail: f.j.bosker@psy.umcg.nl
[8]These authors contributed equally to this work.
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(SNPs; in the GAIN MDD study 435 291 SNPs) is required before associations can be considered significant on a genome-wide level,[3] and replication of such findings in independent cohorts is mandatory to exclude false-positive findings.[4]

Another potential use of a GWAS is to use the results for a large-scale replication study of previous candidate gene studies. Application of such previous knowledge within the context of a GWAS allows less stringent significance thresholds than those for the hypothesis-free GWAS analysis.[5,6] So far, the role of candidate genes in MDD has been the subject of many association studies. Unfortunately, there is little consistency between candidate gene studies for multifactorial diseases such as MDD (see, for example, Hirschhorn *et al*.[7] and Munafo[8]).

In the current study we attempted to replicate significant findings from previous candidate gene studies in MDD. To this end we conducted a systematic review of the literature and selected those genes that were reported to be significantly associated with MDD at least once. The GWAS data from the GAIN MDD study were used to screen all the identified candidate genes in two ways: (1) for association with the specific SNPs reported in the literature; and (2) for association with any of the SNPs (genotyped or imputed) from the Perlegen chip within the identified genes.

Materials and methods

Selection of studies reporting on candidate genes

To prevent any bias from the results of the GAIN MDD study, we identified candidate genes for MDD before the results from the GAIN MDD study became available. Therefore, a so-called 'enhanced search' was performed in Medline through PubMed on 1 September 2007 using the following search terms: ((('genes' (TIAB) NOT Medline(SB])) OR 'genes' (MeSH terms) OR gene (text word)) OR ('genes'(MeSH terms) OR genes (text word)) OR snp (all fields) OR ('single nucleotide polymorphism' (text word OR 'polymorphism, single nucleotide' (MeSH terms) OR snps (text word)) OR ('genetic polymorphism' (text word) OR 'polymorphism, genetic' (MeSH terms) OR polymorphism (text word)) OR polymorphisms (all fields) AND ('major depressive disorder' (text word) OR 'depressive disorder, major' (MeSH Terms) OR major depression (text word)) AND 'humans' (MeSH terms). This resulted in 641 hits. We additionally scrutinized the reference list of the meta-analysis of genetic studies on MDD by Lopez-Leon *et al*.[9] that appeared online on 16 October 2007, shortly after the end date of our search, resulting in an additional 39 hits of possibly relevant papers. These researchers used somewhat broader search terms than we did, and their search ran until June 2007; therefore, as a final check, we searched the literature using their search terms for the period June 2007 to September 2007 not covered in their paper. This yielded an additional 110 hits. Of all these papers we retrieved the abstracts, and if considered relevant, the full paper.

In the next step, we selected all papers fulfilling the following five inclusion criteria: (1) The study had to be a candidate gene case–control association study. Linkage and fine-mapping studies were excluded. (2) The primary diagnosis of the patients in the candidate gene study had to be MDD to enable comparison with the GAIN MDD study. Therefore, we excluded studies involving patients: (i) with a depressive episode in the course of bipolar disorder, (ii) with a primary psychotic disorder such as schizophrenia and a secondary depression, (iii) with a seasonal affective disorder not being MDD with a seasonal pattern, (iv) with a primary anxiety disorder (that is, panic disorder, agoraphobia or social phobia) or obsessive compulsive disorder and a secondary depression and (v) with MDD plus an additional specific comorbid disorder or condition, for example, MDD plus alcoholism. However, we allowed subgroups within MDD, for example, MDD in women or in men, recurrent MDD or early-onset MDD. (3) The sample of a specific study consisted of at least 30 patients with MDD and 30 healthy controls. (4) The findings on the association with MDD of any variant within the candidate gene (either a SNP, a microsatellite marker or a haplotype) had reached a statistical significance at the level of $P<0.05$. (5) Finally, the genetic association had to be with the diagnosis of MDD and not with other aspects such as associated personality features (for example, neuroticism) or factors related to treatment response. This resulted in 78 papers.

In order not to miss potential true-positive findings, we did not exclude candidate genes with associated markers that had low allele frequencies or that deviated from Hardy–Weinberg equilibrium. Two investigators (FB and CH) independently made a selection from the initial list of papers using the above-mentioned criteria. When both reached consensus, the paper was included or excluded; in case of disagreement, consensus was obtained with assistance of two other authors (WN and HS). As a final step, one author (IN) double checked whether all selected papers fulfilled the aforementioned selection criteria 1–5. Figure 1 shows a flowchart of how we retrieved the 78 papers for the present study.

Bioinformatic tools

For many SNPs no reference SNP identification number (rs-id) was given in the original papers, but codes based on position (for example, 677C/T in *MTHFR* or Tyr129Ser in *HTR3B*) or even own codes (for example, s1-s5 in *AVPR1B*) were given. To retrieve rs-ids for these SNPs, we used searches in PubMed or in the SNP database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/SNP/) using the 'Geneview' option in conjunction with NCBI's MapViewer with the human genome assembly build 37.1 (http://www.ncbi.nlm.nih.gov/projects/mapview/). Six SNPs

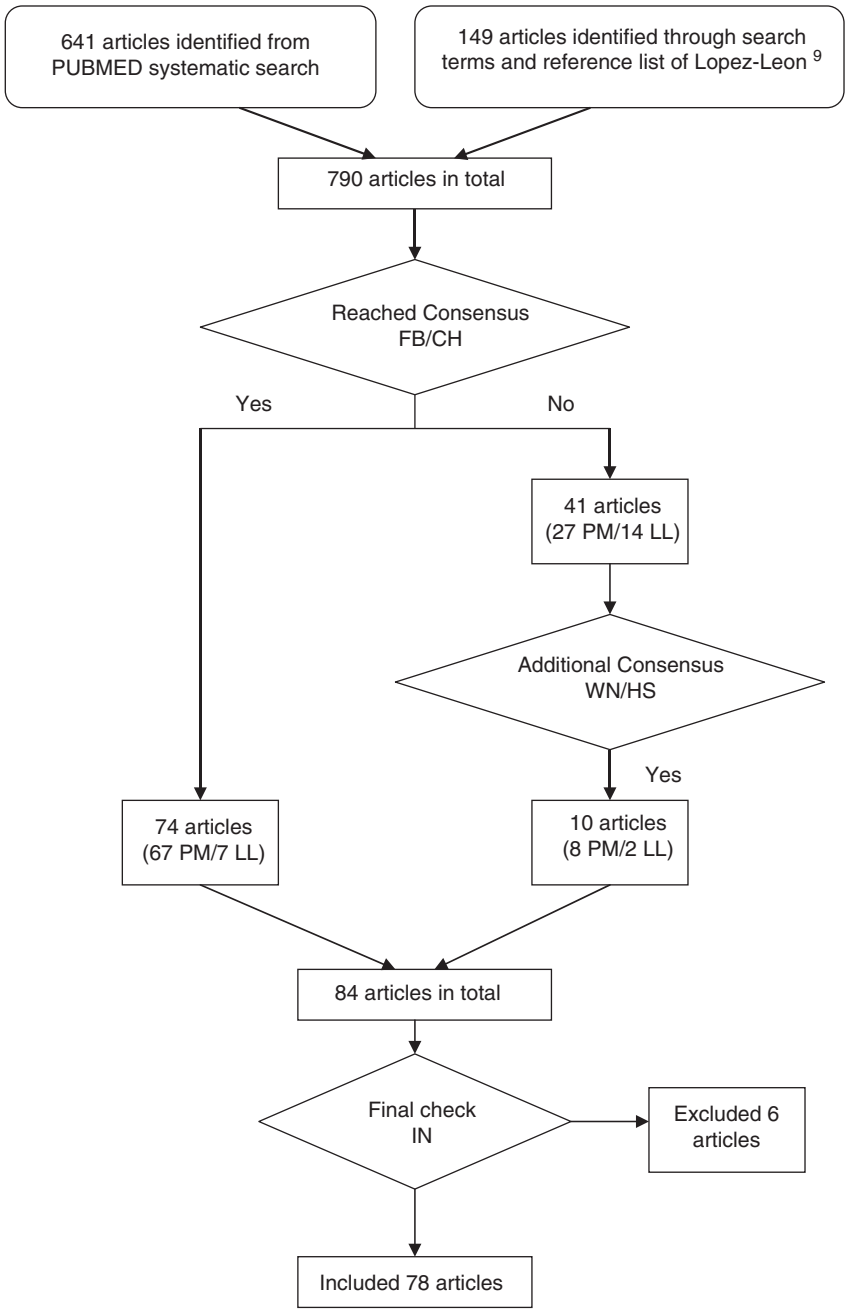

Figure 1 Selection procedure of studies reporting on candidate genes. Abbreviations: LL, Lopez-Leon; PM, PubMed.

remained that could not be easily found in this way. We contacted the corresponding authors of the papers and used NCBI's Primer-Blast and SNP-Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to map these SNPs using the provided primer sequences.

Sample

The 1862 MDD cases included in the GAIN MDD study were mainly from NESDA, a longitudinal cohort study designed to be representative of individuals with depressive and/or anxiety disorders[10] and were recruited from mental health-care organizations ($N=785$), primary care ($N=603$) and community samples ($N=314$). Additional cases came from the NTR ($N=160$). Regardless of recruitment setting, similar inclusion and exclusion criteria were used to select MDD cases. Inclusion criteria were a lifetime diagnosis of MDD according to DSM-IV (Diagnostic and Statistical Manual, Fourth Edition)[11] as diagnosed through the Composite International Diagnostic Interview (CIDI Version 2.1.[12]), age 18–65 years and self-reported western European ancestry. People who were not fluent in Dutch and those with a primary diagnosis of a psychotic disorder, obsessive compulsive disorder, bipolar disorder or severe alcohol or substance use disorder were excluded.

Most of the 1860 control subjects were from the NTR ($N=1{,}703$) and additional controls from NESDA ($N=157$). Longitudinal phenotyping in NTR included assessment of depressive symptoms (through multiple instruments), anxiety and neuroticism. Inclusion required no report of MDD at any measurement occasion and never scoring high (>0.5 s.d.) on a general factor score based on combined measures of neuroticism, anxiety and depressive symptoms. Finally, controls and their parents were required to have been born in the Netherlands or Western Europe. Only one control per family was selected. NESDA controls had no lifetime diagnosis of MDD or an anxiety disorder as assessed by the CIDI and reported low depressive symptoms at baseline. For more details, see Boomsma *et al*.[2]

Genotyping
Perlegen Sciences performed all genotyping according to strict standard operating procedures. DNA samples from cases and controls were randomly assigned to plates, shipped to Perlegen and identified only by barcode. High-density oligonucleotide arrays were used yielding 599 164 SNPs. Eight SNPs with duplicate numbers were deleted and 73 mitochondrial SNPs were removed for later analysis. From the remaining 599 083 SNPs on the Perlegen chip, 435 291 passed quality control tests. A total of 280 subjects were excluded because of various quality control issues, leaving 1738 cases (93.4%) and 1802 controls (96.9%) in the final analysis data set. For more details see Boomsma *et al*.[2] and Sullivan *et al*.[4]

Imputation
Not all SNPs selected from the literature were present on the genotyping array. On the basis of the linkage disequilibrium (LD) structure between SNPs we followed an imputation procedure to predict non-genotyped SNPs using the HapMap CEU data (release 22, build 36) as the reference database. The imputation was performed by IMPUTE version 0.3.2 using the default settings and the recommended number 11418 for the effective population size of Caucasians.[13] In this way we extended the genome-wide autosomal SNP data set from 427 049 to 2 467 430 SNPs. For our candidate genes this meant an extension from 851 to 4955 SNPs. However, the quality of the imputation was low for 85 SNPs (SNPTEST proper_info <0.5). These SNPs were excluded leaving 4870 SNPs for analysis. No SNP had a minor allele frequency of $<1\%$.

Association test
The association between MDD and the autosomal SNP data was tested using a frequentist case–control test provided in the software package SNPTEST version 1.1.4 using allele dosages with sex as a covariate to adjust for the slight imbalance in the percentage of females between cases (69.6%) and controls (62%),[2,4] and the 'proper' option to account for the uncertainty of the genotypes that were imputed.[13] In addition, 7988 genotyped SNPs on the X chromosome were analyzed in PLINK version 1.03[14] using logistic regression with sex as a covariate. SNPs on the Y chromosome ($n=15$) and SNPs mapped to ambiguous locations ($n=239$) were not analyzed.

Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the significant candidate SNPs according to the disease model from the original article using counted or estimated numbers for genotyped or imputed SNPs, respectively. To establish true replication, we checked whether the effect was for the same allele and in the same direction.

Permutation procedure
To facilitate interpretation of the significance of our findings for each SNP, we calculated three *P*-values by permutation: (1) a crude uncorrected significance, (2) a gene-wide significance (corrected for all SNPs in the gene) and (3) an overall significance (corrected for all SNPs in all selected genes). The crude uncorrected *P*-value was determined as the fraction of permutations that yielded a *P*-value that was smaller than the observed one. This *P*-value was determined to validate the permutation procedure as it should be similar to the *P*-value calculated by SNPTEST or PLINK. The gene-wide significance of a particular SNP was defined as the fraction of permutations that the *P*-value of any SNP in the candidate gene concerned was smaller than the one observed for that SNP. This procedure corrects the *P*-value for testing multiple SNPs within a gene. On the basis of the rationale that each selected gene is a candidate for MDD (hypothesis driven), no correction for all SNPs in the entire gene set is necessary. Nonetheless, we also calculated an overall significance (that is, corrected for all SNPs in all candidate genes) by determining an overall *P*-value for each SNP as the fraction of permutations for which any of the SNPs in any of the candidate genes had a *P*-value smaller than the observed one. For each of the three *P*-values we conducted 10 000 permutations. Case–control status was randomly permuted within males and females separately, hence leaving the number of affected males and females intact.

In addition to the three SNP-specific *P*-values, we also determined whether the number of SNPs within a candidate gene with an original *P*-value of <0.05 based on SNPTEST or PLINK (see above) was higher than expected. For each permutation the number of SNPs in each candidate gene that was significant at $P<0.05$ was recorded. The fraction of permutations with a higher number of significant SNPs than originally observed determined the significance of the number of significant SNPs of that candidate gene. By permuting case–control status the LD structure of the genes is preserved; that is, the resulting significance is corrected for possible high correlation between SNPs. The number of permutations for this test was 10 000 as well.

Results

Literature search

The 78 papers that resulted from our systematic literature search reported 115 statistically significant differences between MDD cases and healthy controls in 57 genes: for 96 SNPs, 7 microsatellite markers (that is, length polymorphisms), 11 haplotypes and one protein polymorphism in the haptoglobin gene[15] (Table 1). Twenty-nine SNPs were identified by an rs-id, whereas 67 SNPs were only specified by a location code, restriction enzyme or author-designed code. For 64 of these 67 SNPs, rs-ids could be obtained, whereas two rs-ids were not found (246G/A in *CCKAR*[16] and –7054C/A in *DRD2*[16]). In addition, for SNP 1463G/A in *TPH2*, others have tried to replicate the association, but the SNP seemed to be nonexistent.[17,18] Thus, we could map 93 SNPs.

For the seven microsatellite markers, no LD data with SNPs are known and hence we cannot determine whether these are covered by any of the available SNPs in the corresponding genes. Among them is the 5-HTTLPR 44-bp deletion in the promoter region of the serotonin transporter gene *SLC6A4* (*SERT*), which has attracted considerable attention in various previous candidate gene studies for MDD.

Association in the GAIN MDD data with specific SNPs from the literature

Of the 93 selected SNPs in the 57 candidate genes, 61 were either present ($n=18$) on the Perlegen array or could be imputed ($n=43$). Four additional SNPs could be tagged by other available SNPs (Table 2). Two of these were not genotyped in the Centre d'Etude du Polymorphisme Humain, Utah (CEU) population of the HapMap Phase2 (release 22, build 36) project, but were available in the Japanese individuals from Tokyo (JPT) and Chinese Han individuals from Beijing (CHB) populations, in which they showed high LD ($r^2=0.97$ and 1.00, respectively) with at least one other available SNP. The two other SNPs were genotyped in the CEU population and could be tagged by available SNPs with $r^2=1$, but were for unreported reasons not included in the HapMap reference file used for the imputation procedure as provided on the website of the imputation software package IMPUTE (https://mathgen.stats.ox.ac.uk/impute/impute_v0.5.html). A total of 28 SNPs were neither genotyped directly nor imputed nor could be tagged, and hence were not available for replication.

We investigated which of the 65 available or tagged SNPs could be confirmed in our data (Table 2). SNPs rs12520799 in *C5orf20* (*DCNP1*) ($P=0.038$; OR AT = 1.10, 95% CI 0.95–1.29; OR TT = 1.21, 95% CI 1.01–1.47) and rs16139 in *NPY* ($P=0.034$; OR C allele = 0.73, 95% CI 0.55–0.97) replicated the reported effect in the literature. In addition, three SNPs in the *ACE* gene selected from the literature, which were in strong LD with each other ($r^2=0.78$–1.00), were also significant in the GAIN MDD sample (rs4333: $P=0.029$; rs4329: $P=0.030$; rs4461142: $P=0.036$). However these three SNPs showed effects in the opposite direction compared with previous results[19] (TC Baghai, personal communication). Hence, these SNPs do not represent a true replication.

Candidate genes from the literature in the GAIN MDD study

As in different populations different SNPs might have a role, we also studied all SNPs present on the genotyping array or available through imputation in an area from 5-kb upstream to 5-kb downstream of each selected candidate gene to cover the promoter and 3′ untranslated region, respectively. Of the 57 genes, 49 were covered by one or more SNPs that were present on the Perlegen chip. For another six genes no genotyped SNPs were available but imputed ones were. Neither genotyped nor imputed SNPs were available for two genes (*AVPR1B* and *CHRFAM7A*). In total, 4870 SNPs, of which 820 were genotyped and 4019 were imputed, covered the 55 candidate genes ranging from 1 SNP for *AR* to 642 SNPs for *PDE11A* (Figure 2). We noted a significant correlation of 0.75 between gene size and the number of genotyped SNPs (including genes on the X chromosome) and a correlation of 0.96 between gene size and total number of SNPs (excluding genes on the X chromosome, as SNPs on the X chromosome were not imputed).

For 28 of the 55 genes, one or more SNPs were found to be different between MDD cases and healthy controls in the GAIN MDD GWA scan at a significance level of $P<0.05$ (Table 3). The remaining 27 genes were not associated with MDD as none of the SNPs reached $P<0.05$. The smallest *P*-value was observed for SNP rs769178 in the *TNF* gene region ($P=0.00029$; OR T allele = 1.35, 95% CI 1.13–1.63). The minor allele T was observed in 8.0% of the MDD cases and 6.1% of the controls.

With genes covered by a large number of SNP markers, the expected minimal *P*-value will decrease purely as a result of chance alone as a function of the number of SNPs. Thus, we used gene-wide *P*-values from the permutation procedure that corrects for the number of SNPs within a candidate gene and only SNP rs769178 in *TNF* remained significant ($P=0.0034$; Figure 3 and Table 3). The second strongest associated SNP, which was observed in *DISC1* (rs7533169: *P*-value = 0.0025), became non-significant after this correction (gene-wide *P*-value = 0.28), because in this gene there were 491 SNPs and apparently the small *P*-value was observed purely based on chance. In addition to the gene-wide significance, we also determined overall significance per SNP accounting for testing 4870 SNPs in 55 candidate genes. In that case none of the SNPs remained significant, not even the *TNF* SNP rs769178 (overall $P=0.33$).

The significance levels per SNP are one way of testing the true value of the selected candidate genes. If many SNPs in a candidate gene are associated, this

Table 1 Candidate genes and associated polymorphisms or haplotypes based on the systematic literature search

Gene	*HUGO name*	*Chromosome position*	*Gene size (kb)*	*Variant*	*rs-id*	*Refs*	N *cases*	N *controls*	P *allele*	*OR allele*	P *genot.*	*OR genot.*	P *multi-marker*	*OR*
MTHFR	MTHFR	1p36.3	20.3	677C/T	rs1801133	[34]	32	419	0.005	2.8				
						[35]	100	89			0.03	1.90		
5-HT1A	HTR1A	5q11.2-q13	1.3	−1019C/G	rs6295	[36]	129	134	0.0006	1.84[a]	0.0017	het:1.54 hom:3.75[a]		
5-HT2A	HTR2A	13q14-q21	62.7	−1438A/G	rs6311	[37]	71	157	0.018	0.62	0.039	0.47		
						[38]	189	148	0.007	1.52	0.005			
				102T/C	rs6313	[39]	120	131	0.03		0.01	2.4		
5-HT2C	HTR2C	Xq24	326.1	68G/C = Cys23Ser	rs6318	[16]	177	160	0.02	1.84[a]				
						[40]	513	901	0.006	n.a.				
5-HT3B	HTR3B	11q23.1	41.7	Tyr129Ser (3B06)	rs1176744	[41]	47	90			0.027	n.a.		
				Ala154Ala (3B08)	rs2276305	[41]	47	90	0.015	2.27	0.011	n.a.		
				IVS6 + 72A > G (3B10)	rs2276307	[41]	47	90			0.023	n.a.		
				IVS6 + 161C > G (3B11)	rs2276308	[41]	47	90			0.031	n.a.		
				3B04 + 3B06 + 3B08 + 3B09 + 3B10 + 3B11 + 3B12	rs1176746 + rs1176744 + rs2276305 + rs3782025	[41]	47	90					0.002	2.11
ACE	ACE	17q23.3	20.5	dbSNP4295	rs4295	[19]	642	608			0.0011	rec:1.87[a]		
						[42]	92	92	< 0.0001		0.001		0.0001	n.a.
				dbSNP4309	rs4309	[42]	92	92	0.03	1.59				
				rs2286572[b]	rs2286572	[19]	642	608			< 0.05	n.a.		
				rs4968779[b]	rs4968779	[19]	642	608			< 0.05	n.a.		
				rs4291	rs4291	[19]	642	608			0.00076	rec:1.91[a]		
				rs4277405	rs4277405	[19]	642	608			< 0.05	n.a.		
				rs4305	rs4305	[19]	642	608			< 0.01	n.a.		
				rs4311	rs4311	[19]	642	608			< 0.05	n.a.		
				rs4329	rs4329	[19]	642	608			= 0.05	n.a.		
				rs4333	rs4333	[19]	642	608			< 0.05	n.a.		
				rs4461142	rs4461142	[19]	642	608			< 0.05	n.a.		
				rs867640	rs867640	[19]	642	608			< 0.05	n.a.		
FACL4	ACSL4	Xq22.3-q23	92.1	C/T in intron 1	rs1324805	[43]	198	229	0.003	1.5 (F) 1.9 (M)				
AR	AR	Xq11.2-q12	180.2	microsatellite	NA	[44]	71	83	0.003	0.41[a]				
AT1R	AGTR1	3q21-q25	45.1	1166A/C	rs5186	[45]	132	132			0.036	het:0.66 hom:1.61[a]		
APOE	APOE	19q13.2	3.6	epsilon2	rs429358 + rs7412	[46]	42	73	0.04	0.36[a]				
						[47]	273	429			< 0.0005	het:0.17 hom:0.03[a]		
AVPR1B	AVPR1B	1q32	7.7	AVPR1b-s3	rs33976516	[48]	89	88	0.026	n.a.	0.014	n.a.		
				AVPR1b-s5	rs33933482	[48]	89	89	0.043	n.a.				
				AVPR1b-s1 + s2 + s3 + s4 + s5	rs33976516 + rs33933482	[48]	89	88					0.002	0.52
						[48]	89	89					0.0003	0.28

Table 1 Continued

Gene	*HUGO name*	*Chromosome position*	*Gene size (kb)*	*Variant*	*rs-id*	*Refs*	N *cases*	N *controls*	P *allele*	*OR allele*	P *genot.*	*OR genot.*	P *multi-marker*	*OR*
BDNF	BDNF	11p13	66.9	Val66Met	rs6265	[49]	110	171	0.001		0.003	2.49		
						[50]	284	331	0.005	1.7				
				(GT)n (microsatellite)	NA	[51]	465	1097	0.028	n.a.				
BCR	BCR	22q11.23	137.7	rs2267012	rs2267012	[52]	329	351	0.0097	1.33				
				rs3761418	rs3761418	[52]	329	351	0.012	1.32				
				rs2267013	rs2267013	[52]	329	351	0.044	1.27				
				rs2267015	rs2267015	[52]	329	351	0.023	1.30				
				rs2156921	rs2156921	[52]	329	351	0.031	1.29				
				rs2213172	rs2213172	[52]	329	351	0.031	1.28				
COMT	COMT	22q11.21	27.2	Val158Met	rs4680	[53]	120	628	0.009	1.44				
				−287A > G	rs2075507	[54]	30	467	0.046	1.79				
				−287A > G + rs737865 + Val158Met + rs165599	rs2075507 + rs737865	[54]	30	467					0.0378	2.12
MCP1	CCL2	17q11.2-q12	1.9	−2518G/A	rs1024611	[55]	90	114	0.004	1.83	0.027	1.87		
CCKAR	CCKAR	4p15.1-p15.2	9.0	246G/A	rs number unknown	[16]	177	160	0.006	2.58[a]				
				−128G/T-246G/A-−608G/A-1266T/C	rs1799723-rs?-−rs1800856-rs1800857	[16]	177	160					0.002	6.68[a]
CHRM2	CHRM2	7q31-q35	148.4	1890A/T	rs8191992	[56]	126	304			0.001	het:0.44 hom:0.47[a]		
a7nAChR	CHRFAM7A	15q13.1	32.4	2 bp deletion	rs10581632	[57]	72	103	0.037	n.a.	0.027	2.14		
CRH-BP	CRHBP	5q11.2-q13.3	16.6	CRH-BPs02	rs7728378	[58]	89	88	0.042		0.031			
				CRH-BPs11	rs1875999	[58]	89	88	0.048		0.043			
				CRH-BPs17 + s02 + s14 + s11	rs3811939 + rs7728378	[58]	89	88					0.00014	2.21
CRHR1	CRHR1	17q12-q22	51.5	rs242939	rs242939	[59]	206	195	0.0008	2.07[a]	0.0002	het:2.45 hom:1.18[a]		
CRHR2	CRHR2	7p15.1	29.3	1047G/A	rs2240403	[60]	89	89	0.04	2.14[a]	0.03	het:1.21 hom:2.06[a]		
CYP2C9	CYP2C9	10q24	50.7	CYP2C9*3	rs1057910	[61]	70	142	< 0.05	2.03				
						[62]	70	138	< 0.01	2.61				
DCNP1	C5orf20	5q31.1	3.1	rs12520799	rs12520799	[23]	431	433	0.003	2.01[a]				
DISC1	DISC1	1q42.1	414.5	Ser704Cys (SNP12)	rs821616	[63]	373	717	0.005	1.46				
				SNP1	rs6541281	[63]	373	717	0.048	0.75				
DRD1	DRD1	5q35.1	3.5	−2102C/A	rs10063995	[16]	177	160	0.008	3.69[a]				
DRD2	DRD2	11q23	65.6	−7054C/A	rs number unknown	[16]	177	160	0.03	0.53[a]				
DRD3	DRD3	3q13.3	50.3	Bal I	rs6280	[64]	36	38	0.014	2.32[a]	0.027	3.00[a]		

Table 1 Continued

Gene	*HUGO name*	*Chromosome position*	*Gene size (kb)*	*Variant*	*rs-id*	*Refs*	N *cases*	N *controls*	P *allele*	*OR allele*	P *genot.*	*OR genot.*	P *multi-marker*	*OR*
DRD4	DRD4	11p15.5	3.4	48 bp polymorphism	NA	[31]	49	100	0.011 (4)	0.43[a]	0.007 (4/4)	0.36[a]		
									0.041 (5)	2.79[a]	0.035 (4/5)	2.99[a]		
						[65]	126	471			0.004	2.09		
ER-alpha	ESR1	6q25.1	295.7	PvuII	rs2234693	[66]	89	126	0.004	1.76[a]	0.01	het:1.63 hom:3.83[a]		
				XbaI	rs9340799	[66]	89	126	0.02	n.a.				
ER-beta	ESR2	14q23.2	111.5	microsatellite	NA	[67]	102	150	<0.005	0.30[a]	<0.0005	het:0.40 hom:0.14[a]		
GPR50	GPR50	Xq28	4.9	Val606Ile	rs13440581	[68]	226	562	0.040	1.30[a]				
						[68]	136	260	0.0096	1.49[a]				
				Δ502-505	in complete LD with rs561077	[68]	226	562	0.011	1.39[a]				
						[68]	136	260	0.0064	1.51[a]				
GABA3	GABRA3	Xq28	284.2	microsatellite	NA	[69]	128	81	<0.0001	0.14				
GMIP	GMIP	19p11-p12	14.2	-525G/A (SNP1)	rs3794996	[70]	164	164	0.039	1.66				
				rs2043293 (SNP2)	rs2043293	[70]	59	59	0.0086	1.46				
				rs2304129 (SNP3)	rs2304129	[70]	59	59	0.0086	1.50				
				rs880090 (SNP4)	rs880090	[70]	59	59	0.015	1.46				
Gbeta3	GNB3	12p13	7.2	825C/T	rs5443	[71]	184	158			0.001	2.21		
						[72]	106	133	0.012	2.14				
						[73]	78	111	0.004	1.67	0.008	n.a.		
HP	HP	16q22.1	6.4	genotyping based on phenotypes	NA	[15]	72	200	0.04	0.68[a]	0.01	rec:0.45[a]		
cPLA2-α	PLA2G4A	1q25	160.1	BanI	rs10798059	[74]	62	117	0.007	1.83	0.017	3.40		
CD-MPR	M6PR	12p13	9.3	C/T in 3'UTR of exon 7	rs3318	[75]	193	259	0.014	1.96				
MAOA	MAOA	Xp11.3	90.7	uVNTR1 (microsatellite)	NA	[76]	73	68			0.029	dom:0.47[a]		
						[77]	228	213	<0.001					
						[77]	133	110	0.009	2.39	0.008	n.a.		
						[77]	95	103	0.015	1.97				
				EcoRV2	rs1137070	[78]	78	104	0.01	2.3				
				uVNTR1 + EcoRV2	NA	[78]	78	104					0.008	2.5
p75NTR	NGFR	17q21-q22	19.7	S205 L	rs2072446	[79]	164	164	<0.05	0.54				
NPY	NPY	7p15.1	7.7	−399T/C	rs16147	[24]	48	140	0.03	1.69[a]				
				1128T/C (Leu7Pro)	rs16139	[24]	51	140	0.009	0.00[a,c]				
GR	NR3C1	5q31.3	157.6	BclI	rs41423247	[80]	170	374			0.03	1.8		
				ER22/23EK	rs6189 + rs6190	[80]	183	497	0.043	1.98				
				R23K	rs6190	[81]	134	181	0.02	n.a.	0.02	n.a.		
				−3211T/C (NR3C1-1)	rs10482605	[81]	180	173	0.02	n.a.	0.02	n.a.		
				−3211T/C + R23K	rs10482605 + rs6190	[81]	134	181					0.01	4.58
						[81]	180	173					0.02	n.a.
PAM	PAM	5q14-q21	163.9	dbSNP13340364 G > A	rs13340364	[42]	92	92	0.04	0.31	0.03			
PDE10A	PDE10A	6q26	331.1	rs717602	rs717602	[82]	284	331	0.009	n.a.				
				rs220818	rs220818	[82]	284	331	0.01	n.a.				
				rs676389	rs676389	[82]	284	331	0.03	n.a.				

Table 1 Continued

Gene	*HUGO name*	*Chromosome position*	*Gene size (kb)*	*Variant*	*rs-id*	*Refs*	N *cases*	N *controls*	P *allele*	*OR allele*	P *genot.*	*OR genot.*	P *multi-marker*	*OR*
PDE11A	PDE11A	2q31.2	485.1	rs3770018	rs3770018	[82]	284	331	0.0005	2.1				
PDE2A	PDE2A	11q13.4	98.2	rs370013	rs370013	[82]	284	331	0.01					
PDE5A	PDE5A	4q25-q27	134.4	rs3775845	rs3775845	[82]	284	331	0.007					
PDE6C	PDE6C	10q24	53.1	rs650058	rs650058	[82]	284	331	0.01					
				rs701865	rs701865	[82]	284	331	0.03					
PDE9A	PDE9A	21q22.3	121.8	rs729861	rs729861	[82]	284	331	0.0006	0.6				
P2RX7	P2RX7	12q24	53.2	rs2230912	rs2230912	[22]	1000	1029	0.0019	1.30				
										1.40				
NET	SLC6A2	16q12.2	47.1	-182T/C	rs2242446	[25]	145	164			0.02	het:0.99 hom:0.33[a]		
						[83]	112	136			0.019	1.86		
SERT	SLC6A4	17q11.1-q12	37.5	5-HTTLPR (44 bp del/ins)	NA	[61]	70	142	<0.05	2.03				
						[71]	184	158	0.01	1.82				
						[84]	68	68	0.021	1.81	0.025	n.a.		
						[85]	466	836	0.007	1.26	0.015	1.5		
						[86]	262	475	0.05	1.24				
						[87]	184	360	0.01	1.40[a]	0.009	1.39[a]		
						[88]	95	156			0.04	2.6		
						[89]	53	103			0.008	het:2.75 hom:0.87[a]		
				VNTR STin2	rs57098334	[90]	39	193	<0.02	6.51[a]	<0.004	6.95[a]		
						[91]	33	362	0.011	3.73[a]				
						[92]	71	99			<0.05	rec:2.50[a]		
						[93]	114	120	0.04	0.68				
						[94]	119	346	0.005	4.44	0.001	n.a.		
				5-HTTLPR + VNTR STin2	NA	[95]	74	84					0.0069	2.53
NK1R	TACR1	2p13.1	150.0	dbSNP13013430 C>T	rs13013430	[42]	92	92	0.04	0.64				
LBP-1C	TFCP2	12q13	78.0	2236G/A	rs13463	[96]	180	225	0.016	0.43				
TPH	TPH1	11p15.3-p14	19.8	IVS7 + 218A/C	rs1800532	[97]	91	139			0.032	het:2.43 hom:1.39[a]		
				SNP1	rs4537731	[98]	228	253			0.019	het:0.59 hom:1.01[a]		
				SNP2	rs684302	[98]	228	253			0.0119	het:0.78 hom:1.54[a]		
				SNP5	rs1799913	[98]	228	253	0.0013	1.52[a]	0.0035	het:1.39 hom:2.50[a]		
				SNP1 + SNP2 + SNP3 + SNP4 + SNP5 + SNP6	rs4537731 + rs684302 + + rs211105 + rs1800532 + rs1799913 + rs7933505	[98]	228	253					<0.00001	2.11[a]

Table 1 Continued

Gene	*HUGO name*	*Chromosome position*	*Gene size (kb)*	*Variant*	*rs-id*	*Refs*	N *cases*	N *controls*	P *allele*	*OR allele*	P *genot.*	*OR genot.*	P *multi-marker*	*OR*
TPH2	TPH2	12q21.1	93.6	SNPB	rs1843809	[99]	300	265	0.0496	1.38				
				SNPE	rs1386494	[99]	300	265	0.0012	0.6				
				SNPB + SNPE + SNPD	rs1843809 + rs1386494 + + rs1386495	[99]	300	265					< 0.0001	n.a
				SNP4 = C_245410	rs10784941	[100]	135	315	0.01	0.62[a]				
				SNP7 = C_15836061	rs2171363	[100]	135	315	0.001	0.51[a]				
				SNP12 = C_8376042	rs1386486	[100]	135	315	0.01	0.67[a]				
				1463G/A	unconfirmed variant	[17]	87	219	< 0.001	8.31[a]				
TNF-alpha	TNF	6p21.3	2.8	308G/A	rs1800629	[26]	108	125	0.0125	2.24	0.024			
WFS1	WFS1	4p16	33.4	684C/G = R228R	rs7672995	[101]	177	160	0.007	1.56[a]				
				1023C/T = F341F	rs56072215	[101]	177	160	0.02	0.55[a]				
				1185C/T = V395V	rs1801206	[101]	177	160	0.01	1.56[a]				
				2206G/A = G736S	rs number unknown	[101]	177	160	0.04	0.57[a]				
				2565G/A = S855S	rs1046316	[101]	177	160	0.04	0.71[a]				

Abbreviations: dom, dominant; F, female; GAIN, Genetic Association Information Network; GWAS, genome-wide association study; het, heterozygotes; hom, homozygotes; M, male; MDD, major depressive disorder; OR, odds ratio; rec, recessive; rs-id, reference SNP identification number; SNP, single-nucleotide polymorphism.
Sample sizes (*N* cases/*N* controls) as well as *P*-values for allelic (*P* allele), genotypic (*P* genotype) and/or multimarker tests (*P* multimarker) are shown. ORs are given whenever possible.
[a]OR was not reported in corresponding article, but could be estimated from genotype counts or frequencies.
[b]SNP is located more than 5 kb away from gene and is not included in our analyses.
[c]Allele was not observed in cases, which resulted in OR = 0.00.

Table 2 Significance of candidate SNPs for MDD identified from the literature within the GAIN MDD GWAS

Gene	*SNP*	P-*value*	*Genotyped or imputed*
ACE	rs4295	0.36	Genotyped
ACE	rs4305	0.11	Imputed
ACE	rs4309	0.057	Imputed
ACE	rs4311	0.21	Imputed
ACE	rs4329	**0.030**	Imputed
ACE	rs4333	**0.029**	Imputed
ACE	rs4461142	**0.036**	Imputed
ACE	rs867640	0.39	Imputed
BCR	rs2156921	0.76	Imputed
BCR	rs2213172[a]	0.73	Genotyped
BCR	rs2267012[b]	0.92	Imputed
BCR	rs2267013	0.68	Imputed
BCR	rs2267015	0.89	Imputed
BCR	rs3761418	0.96	Imputed
BDNF	rs6265	0.22	Genotyped
C5orf20	rs12520799	**0.038**	Genotyped
CCL2	rs1024611	0.50	Imputed
CHRM2	rs8191992	0.13	Imputed
COMT	rs4680	0.55	Genotyped
CRHBP	rs1875999	0.68	Imputed
CRHBP	rs7728378	0.40	Imputed
CRHR1	rs242939	0.22	Imputed
CRHR2	rs2240403	0.86	Genotyped
CYP2C9	rs1057910	0.46	Genotyped
DISC1	rs6541281	0.93	Imputed
DISC1	rs821616	0.58	Genotyped
DRD3	rs6280	0.87	Genotyped
ESR1	rs2234693	0.75	Imputed
ESR1	rs9340799	0.82	Imputed
GMIP	rs2043293	1.00	Imputed
GMIP	rs2304129	0.46	Imputed
GMIP	rs3794996	0.23	Imputed
GMIP	rs880090	0.58	Imputed
GNB3	rs5443[c]	0.70	Imputed
HTR2A	rs6311	0.57	Imputed
HTR2A	rs6313	0.52	Genotyped
HTR2C	rs6318	0.092	Genotyped
HTR3B	rs1176744	0.50	Genotyped
HTR3B	rs2276307	0.62	Imputed
HTR3B	rs2276308[d]	0.65	Imputed
MAOA	rs1137070	0.72	Genotyped
MTHFR	rs1801133	0.074	Genotyped
NGFR	rs2072446	0.52	Imputed
NPY	rs16139	**0.034**	Genotyped
NPY	rs16147	0.82	Imputed
P2RX7	rs2230912	0.42	Genotyped
PAM	rs13340364	0.78	Imputed
PDE10A	rs220818	0.60	Imputed
PDE10A	rs676389	0.48	Imputed
PDE10A	rs717602	0.78	Imputed
PDE11A	rs3770018	0.58	Imputed
PDE5A	rs3775845	0.41	Imputed
PDE6C	rs650058	0.36	Genotyped
PDE6C	rs701865	0.84	Genotyped
SLC6A2	rs2242446	0.37	Imputed
TACR1	rs13013430	0.056	Genotyped
TNF	rs1800629	0.29	Imputed
TPH1	rs1800532	0.066	Imputed
TPH1	rs4537731	0.11	Imputed
TPH1	rs684302	0.071	Imputed
TPH2	rs1386486	0.26	Imputed
TPH2	rs1386494	0.61	Imputed
TPH2	rs1843809	0.69	Imputed
TPH2	rs2171363	0.58	Imputed
WFS1	rs1801206	0.18	Imputed

Abbreviations: GAIN, Genetic Association Information Network; GWAS, genome-wide association study; MDD, major depressive disorder; SNP, single-nucleotide polymorphism.
[a]Tagged by rs877590 ($r^2 = 0.97$).
[b]Tagged by rs2267010 ($r^2 = 1$).
[c]Tagged by rs2301339 ($r^2 = 1$).
[d]Tagged by rs12270070 ($r^2 = 1$).

could also indicate potential involvement of a gene in the disorder under study.[20] We noticed, for instance, that in the norepinephrine transporter *SLC6A2* (*NET*), 19 of the 100 SNPs were significant at a *P* level of < 0.05 (Table 3). The permutation procedure that tested whether the number of SNPs that were significant at 0.05 was larger than expected, revealed that this finding for *SLC6A2* was indeed significant ($P = 0.039$). Figure 4 shows that the 19 SNPs are scattered in the right half of the gene; 17 of them lie in three independent haplotype blocks and the other 2 SNPs are not in LD with the haplotype blocks or with each other. For none of the other candidate genes this permutation test revealed a significant result.

Discussion

Several genome-wide linkage studies of MDD have been published (reviewed in Boomsma *et al*.[2]), but the GAIN MDD study is among the first GWAS in MDD.[4,21] We used these GWAS data as a large-scale replication of previously reported significant findings from candidate gene studies in MDD. To this end, we first conducted a systematic review of the literature and identified a total of 57 genes for which a significant association with MDD has been reported at least once. Fifty-five of these genes could be included in our replication study, with either genotyped or imputed SNPs available from the GWAS data. With a sample size (1738 cases and 1802 controls) by far exceeding all previous candidate gene studies (with a mean sample size of 164 MDD cases and 252 controls, and only 1 study with >1000 MDD cases and controls),[22] we found minimal support for involvement in MDD for only 4 out of 55 candidate genes: *C5orf20* ($P = 0.038$), *NPY* ($P = 0.034$), *TNF* ($P = 0.0034$) and *SLC6A2* ($P = 0.039$). Replication of these genes was based on three different statistical approaches. First, the involvement of *C5orf20* (rs12520799) and *NPY* (rs16139) constituted a direct replication at SNP level of previously identified SNPs associated with MDD. Second, studying the selected candidate genes for all SNPs present on

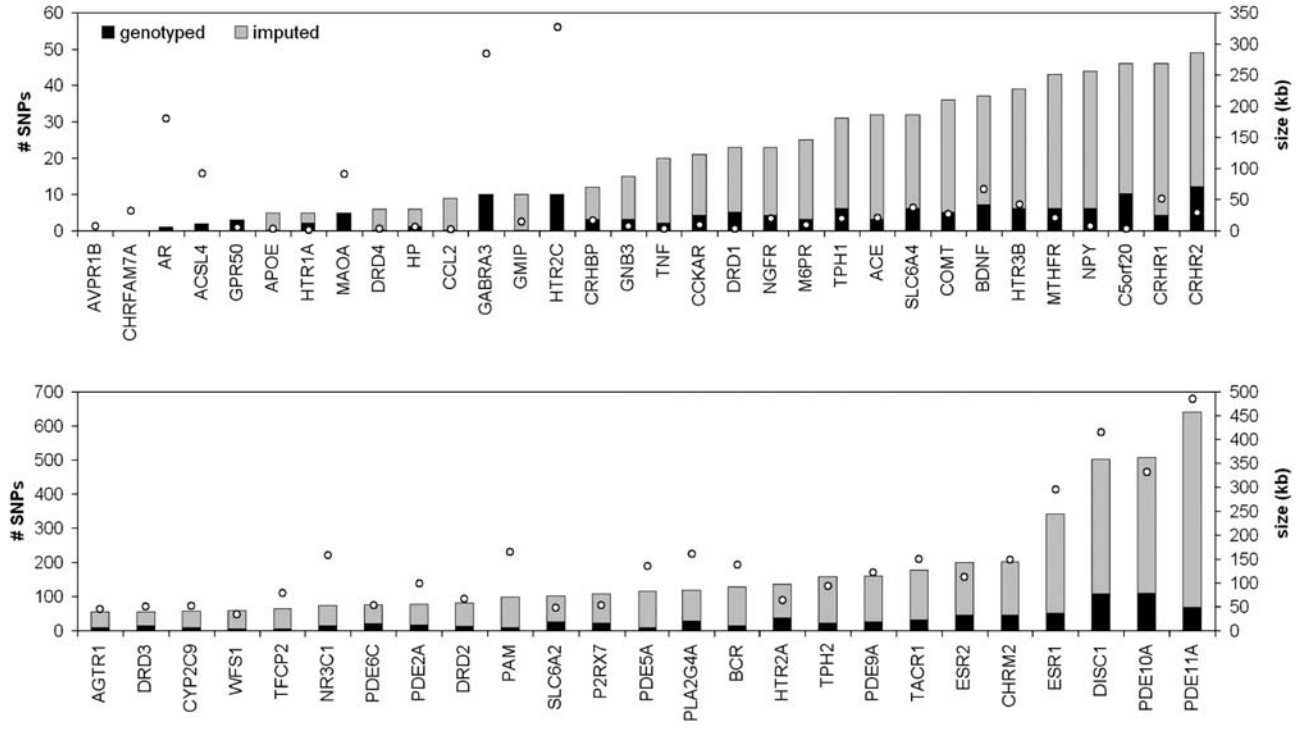

Figure 2 Coverage by genotyped (black bar) and imputed (grey bar) SNPs and size (white circles) of the 57 selected candidate genes (above: genes with $\leqslant 50$ SNPs; below: genes with >50 SNPs).

the genotyping array, *TNF* (rs76917) was identified as the only gene for which the association with MDD remained gene-wide significant. Third, the potential involvement of *SLC6A2* (*NET*) in MDD was derived on the basis of a statistically significant number of SNPs (that is, 19 out of 100) associated with MDD within this gene, which could not be explained solely by high LD between the SNPs. Note that the previous evidence in the selected studies for the involvement of *C5orf20*, *NPY*, *TNF* and *SLC6A2* in MDD did not stand out as particularly strong; that is, ORs were mostly in the moderate range (minimum OR = 0.33 and maximum OR = 2.41), and none of the *P*-values in these studies was smaller than $P < 0.001$, not even for the largest study on C5orf20 with a total sample size of 864. NPY was the one exception in which the allele was present in 6.3% of the controls but not in the patients, thus suggesting a strong effect (OR = 0). However, this was based on only 51 patients.[23–26]

An important question is why so few candidate genes were replicated by our GWAS, whereas the sample size of our study was so much larger than any of the 78 selected candidate gene studies. One possible—and in our opinion most likely—explanation is publication bias; positive results have a better chance of being published than negative results. This would also imply that many previously reported positive findings were actually false-positive findings (type 1 errors), probably amplified by insufficient correction for multiple testing. Testing this, for example, with funnel plots, is not appropriate for the approach followed here as our literature search strategy did not include negative candidate gene studies. Second, given that MDD is a rather heterogeneous disorder, and that it was diagnosed with different instruments across studies, previous and current study samples may have differed phenotypically (see Hek *et al*.[27] for a discussion of this point). A third explanation is that associations between genes and etiologically complex diseases may depend on genetic (gene $\times$ gene interactions or epistasis) and environmental (gene $\times$ environment interactions) contexts, which may differ in samples from different populations.[28] Thus, previous and current samples may have been different genetically or in their contextual factors.

Given the modest support for the replicated candidate genes (one *P*-value <0.01 and three *P*-values <0.05) it is possible that even the four replications of our analysis are false-positive results. With the number of SNPs ($n = 65$) been tested and under the null hypothesis of no true associations of any of the candidate SNPs in previous studies, one would expect three significant findings. Thus, it is possible that our two significant findings in *C5orf20* and *NPY* are false-positive results. In addition, under a similar null hypothesis for the 55 candidate genes, two significant findings were expected, implying that the single gene-wide significant result of *TNF* might also be a false-positive result. This is supported by the lack of overall significance for the *TNF* SNP ($P = 0.33$). And finally, the significant finding for *SLC6A2*, the only gene showing a larger number of significant SNPs than expected, might also be a chance finding.

Table 3 Gene-wide results for the 57 candidate genes identified by the systematic literature search

Gene	*Genotyped*	*Imputed*	*Total*	*Minimal* P *(analytical)*	*Minimal* P *(permuted)*	*Gene-wide* P	*Overall* P	*rs-id*	*SNPs at* P < *0.05*	P-*value SNPs*
TNF	2	18	20	**0.00029**	**0.00094**	**0.0034**	0.34	rs769178	1	0.32
DISC1	105	386	491	**0.0025**	**0.0015**	0.29	0.95	rs7533169	18	0.56
SLC6A2	25	75	100	**0.0061**	**0.0058**	0.19	1.00	rs5564	19	**0.039**
DRD2	12	68	80	**0.0079**	**0.0077**	0.15	1.00	rs7131056	3	0.32
HTR3B	6	33	39	**0.0081**	**0.0077**	0.11	1.00	rs7945926	3	0.21
CHRM2	43	157	200	**0.0097**	**0.0081**	0.32	1.00	rs6944132	3	0.54
MTHFR	6	37	43	**0.0098**	**0.0080**	0.11	1.00	rs3737967	4	0.18
PDE10A	107	392	499	**0.013**	**0.014**	0.74	1.00	rs7741623	8	0.80
APOE	0	5	5	**0.015**	**0.012**	0.057	1.00	rs10119	1	0.11
TACR1	31	141	172	**0.016**	**0.019**	0.50	1.00	rs1012686	24	0.10
TPH2	21	134	155	**0.017**	**0.016**	0.29	1.00	rs17722134	6	0.29
PDE11A	66	576	642	**0.018**	**0.016**	0.63	1.00	rs6433706	11	0.57
DRD3	13	41	54	**0.018**	**0.021**	0.23	1.00	rs1800827	3	0.27
PDE5A	7	108	115	**0.019**	**0.020**	0.20	1.00	rs13104219	21	0.08
NR3C1	14	60	74	**0.024**	**0.025**	0.37	1.00	rs4607376	12	0.11
MAOA	5	0	5	**0.026**	**0.028**	0.078	1.00	rs5906883	1	0.11
ESR1	50	272	322	**0.027**	**0.025**	0.84	1.00	rs17081749	1	0.93
ACE	3	28	31	**0.028**	**0.031**	0.21	1.00	rs4344	8	0.08
NPY	6	38	44	**0.030**	**0.031**	0.29	1.00	rs1073317	6	0.15
PDE9A	25	133	158	**0.033**	**0.031**	0.69	1.00	rs13046735	1	0.75
HTR2A	35	100	135	**0.037**	**0.037**	0.72	1.00	rs2770292	3	0.56
C5orf20	10	35	45	**0.038**	**0.038**	0.36	1.00	rs12520799	5	0.13
TPH1	6	24	30	**0.040**	**0.038**	0.25	1.00	rs10741734	2	0.19
PLA2G4A	26	92	118	**0.040**	**0.042**	0.68	1.00	rs7555140	1	0.68
BCR	14	110	124	**0.041**	**0.045**	0.69	1.00	rs11090231	1	0.67
ESR2	43	155	198	**0.042**	**0.040**	0.80	1.00	rs1255949	2	0.68
P2RX7	21	87	108	**0.047**	**0.049**	0.62	1.00	rs11065464	1	0.57
AGTR1	8	44	52	0.054	**0.049**	0.58	1.00	rs12721331	0	0.77
PDE6C	18	52	70	0.055	0.056	0.71	1.00	rs10882288	0	0.84
CYP2C9	8	47	55	0.057	0.056	0.48	1.00	rs1799853	0	0.72
CCKAR	4	17	21	0.060	0.058	0.33	1.00	rs11726159	0	0.64
NGFR	4	16	20	0.065	0.064	0.55	1.00	rs741073	0	0.73
TFCP2	4	59	63	0.066	0.064	0.53	1.00	rs2029686	0	0.72
CRHR2	12	35	47	0.083	0.085	0.70	1.00	rs2267717	0	0.77
GPR50	3	0	3	0.090	0.092	0.24	1.00	rs5969987	0	0.57
HTR2C	10	0	10	0.092	0.091	0.39	1.00	rs6318	0	0.62
CRHBP	3	9	12	0.093	0.092	0.43	1.00	rs7721799	0	0.63
PAM	7	92	99	0.096	0.090	0.81	1.00	rs1650500	0	0.79
SLC6A4	6	26	32	0.11	0.11	0.72	1.00	rs12150465	0	0.72
M6PR	3	21	24	0.11	0.10	0.54	1.00	rs1805729	0	0.65
BDNF	7	29	36	0.11	0.11	0.60	1.00	rs11030104	0	0.68
GABRA3	10	0	10	0.11	0.10	0.50	1.00	rs994423	0	0.63
WFS1	3	55	58	0.12	0.13	0.51	1.00	rs4689393	0	0.63
COMT	5	31	36	0.13	0.13	0.78	1.00	rs2871047	0	0.72
CCL2	0	9	9	0.14	0.14	0.55	1.00	rs2857655	0	0.62
DRD1	5	16	21	0.17	0.17	0.79	1.00	rs5326	0	0.69
PDE2A	16	62	78	0.20	0.19	0.97	1.00	rs11604811	0	0.81
CRHR1	4	42	46	0.22	0.22	0.86	1.00	rs242939	0	0.69
AR	1	0	1	0.23	0.23	0.23	1.00	rs2207040	0	0.52
GMIP	0	10	10	0.23	0.23	0.83	1.00	rs12003	0	0.66
DRD4	0	6	6	0.29	0.30	0.74	1.00	rs3758653	0	0.59
ACSL4	2	0	2	0.31	0.30	0.50	1.00	rs7887981	0	0.55
HP	1	5	6	0.32	0.32	0.77	1.00	rs5475	0	0.59
GNB3	3	10	13	0.39	0.39	0.96	1.00	rs3759348	0	0.23
HTR1A	0	5	5	0.54	0.55	0.79	1.00	rs10042486	0	0.55
AVPR1B	0	0	0	NA	NA	NA	NA	NA	NA	NA
CHRFAM7A	0	0	0	NA	NA	NA	NA	NA	NA	NA

Abbreviations: NA, not applicable; SNP, single-nucleotide polymorphism; rs-id, reference SNP identification number.
Analytical *P*-values were determined using SNPTEST for the autosomes and logistic regression in PLINK for the X chromosome. Permutations ($N = 10\,000$) were performed to determine uncorrected SNP *P*-values, gene-wide corrected *P*-values, overall corrected *P*-values and *P*-values for number of SNPs with $P < 0.05$. All *P*-values were calculated assuming an additive model. *P*-values of < 0.05 are shown in bold.

On the other hand, the above calculations may be too conservative when assuming that at least some of the previously found associations of candidate genes with MDD were true findings. In that case, our approach of replicating candidate genes is more or less hypothesis driven, thus not requiring the same multiple testing penalty as the genome-wide approach.[4] Nonetheless, the few replications in our study are rather sobering and to uncover whether the replicated SNPs and genes are truly associated to MDD, confirmation in independent samples is crucial. As such, meta-analytical results from the Psychiatric GWAS Consortium are also eagerly awaited.[29]

From the most recent meta-analysis of genetic studies on MDD, Lopez-Leon *et al*.[9] concluded that statistically significant evidence exists for six MDD susceptibility genes, that is, *APOE*, *DRD4*, *GNB3*, *MTHFR*, *SLC6A3* and *SLC6A4*. Our study offers little support for these genes. Given our sample size, we had 80% power to detect ORs of >1.15 for allele frequencies >5% under an additive disease model. All of the above genes meet these criteria. The association of *GNB3* and *MTHFR* with MDD was directly tested but could not be replicated in the GAIN MDD sample, although the effect of *MTHFR* showed a trend in the expected direction ($P=0.074$; OR TT versus CC = 1.14). In addition, *APOE*, *DRD4* and *SLC6A4* were not associated with MDD in our study, but the previously identified genetic variants were length polymorphisms instead of SNPs. Hence, these could not directly be tested and as it is unsure whether these variants are tagged by the SNPs in the corresponding genes, we cannot refute the associations. *SLC6A3* was not identified in our literature search as a candidate gene for MDD, because individual studies did not report significant effects for this gene[30,31] and it only reached significance in the pooled meta-analysis. We also tested for significance in our sample *post hoc* and did not find any association (59 SNPs, most significant SNP: rs27072, $P=0.096$).

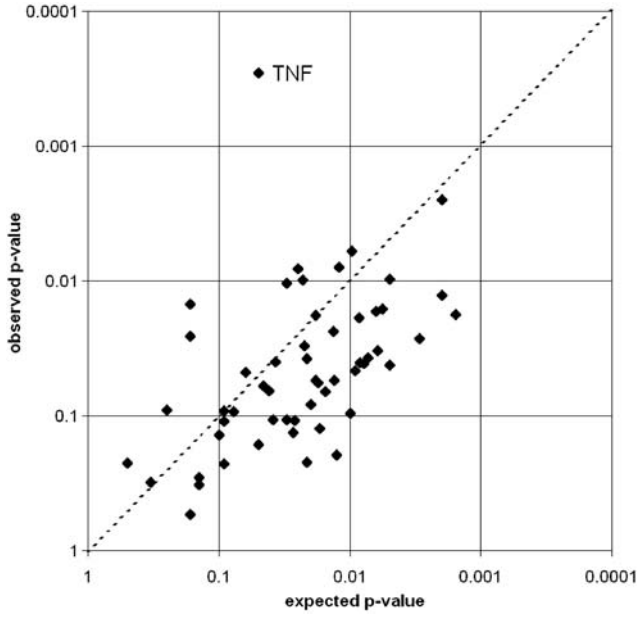

Figure 3 Observed minimal *P*-value versus the expected minimal *P*-value based on the number of SNPs in the gene.

In the context of the non-replication of many of the selected candidate genes as discussed earlier, two limitations of our study need to be noted. First, we did not have direct or indirect information on one-third of the candidate SNPs, as these were not present on the genotyping chip and could not be imputed using the HapMap CEU data. Second, we were unable to test candidate length polymorphisms previously associated with MDD. Among these was the 44-bp insertion/deletion polymorphism (or 5-HTTLPR) in the promoter region of the serotonin transporter gene *SLC6A4* (*SERT*). Length polymorphisms are often difficult to tag with single SNPs because LD information between SNPs and length polymorphisms is either unavailable or LD with SNPs is insufficiently strong.[32] However, a recent study by Wray *et al*.[33] identified a two-SNP haplotype proxy for 5-HTTLPR.

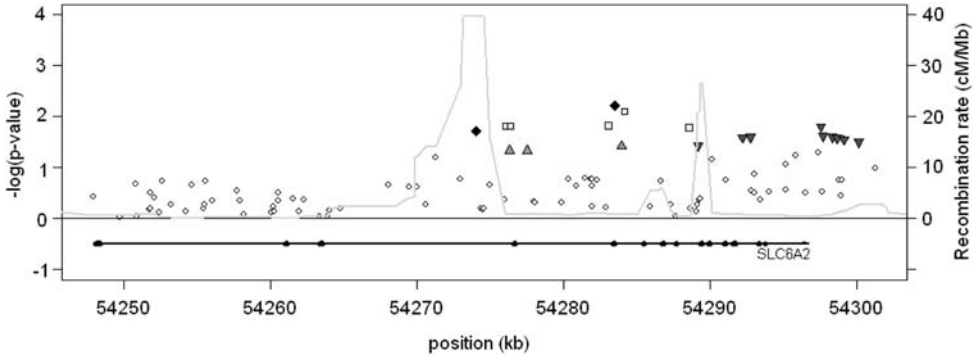

Figure 4 Association of SLC6A2 (NET) with MDD. Along the *x* axis the location of all 100 SNPs within 5 kb of SLC6A2 is shown and the $-\log^{10}$(*P*-value) is on the *y* axis. A total of 19 SNPs have a *P*-value of <0.05, which is significantly more than expected ($P=0.039$). Of these, 17 lie in three haplotype blocks (squares–upward triangles (light grey) and downward triangles (dark grey)) (r^2 values HapMap CEU: squares–upward triangles: 0.020; squares–downward triangles: 0.661; and upward triangles–downward triangles: 0.013). The other two (black diamonds) SNPs are not in LD with the haplotype blocks or with each other. The light grey line shows the recombination rate in this area (axis on the right).

In conclusion, the GWAS data of the GAIN MDD study failed to replicate all but four of the previously reported candidate gene associations with MDD. However, given the number of candidate SNPs and genes that were tested, even these significant may well be false-positives, implying that we found no replication at all. This might point to publication bias and false-positive findings in previous studies, and also to heterogeneity of the MDD phenotype as well as variations in contextual genetic or environmental factors.

Conflict of interest

The authors declare no conflict of interest.

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