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A Follow-Up Case–Control Association Study of Tractable (Druggable) Genes in Recurrent Major Depression

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The High-Throughput Disease-specific target Identification Program (HiTDIP) aimed to study case–control association samples for 18 common diseases. Here we present the results of a follow-up case–control association study of HiTDIP in major depressive disorder (MDD). The HiTDIP in MDD was conducted in a sample of 974 cases of recurrent MDD of white German origin collected at the Max-Planck Institute (MP-GSK) and 968 ethnically matched controls screened for lifetime absence of depression. Six genes were identified as of interest for a follow-up, based on the strength of the association and based on the interest as potential candidate target for developing new treatment for depression: Solute Carrier Family 4 Member 10 (*SLC4A10*), Dipeptidyl Peptidase IV (*DPP4*), Dopamine Receptor D3 (*DRD3*), Zinc Finger Protein 80 (*ZNF80*), Nitric Oxide Synthase 2A (*NOS2A*) and Peroxisome Proliferator-Activated Receptor-Gamma, Coactivator 1, Alpha (*PPARGC1A*). Within the current study, we attempted to follow-up these findings in a sample from the UK, the Depression Case Control (DeCC) sample consisting of 1,196 cases and 842 screened controls, phenotyped using exactly the same methods as the MP-GSK sample. Performing Cochran–Mantel–Haenzel statistics to test for genotypic and/or allelic differences between the DeCC and MP-GSK samples, we found no significant differences, thus being able to combine the two samples for association testing. In the combined sample of 2,170 MDD cases and 1,810 controls, there were positive findings in the Nitric Oxide Synthase 2A (*NOS2A*) gene both using single SNP analysis and haplotype analysis.

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BACKGROUND

Major depressive disorder (MDD) is a major clinical problem with a median lifetime prevalence of 16.1% (range 4.4–18%) [Wittchen, 2000; Waraich et al., 2004]. MDD is a recurrent disorder and 50–85% of patients who experience an episode will eventually have another [Keller et al., 1986; Mueller et al., 1999]. Although approximately 50–70% [Bauer et al., 2002] of patients respond to treatment with antidepressants, up to 75% have a recurrence within 10 years and a very high proportion of sufferers remain undiagnosed and untreated. The importance of genetic factors is well established for major affective disorders although the mode of inheritance is uncertain. Together, the high heritability of the disease and the need for new treatments provides the foundation for a genetic study which would facilitate the identification of new therapeutic targets for MDD.

The ''High-Throughput Disease-specific target Identification Program (HiTDIP)'' [Roses et al., 2005] consists of a set of case–control studies of common disorders where DNA samples were genotyped for approximately 6000 single nucleotide polymorphisms (SNPs) in a panel of 1,800 ''druggable'' candidate genes [for additional details see Roses et al., 2005]. The HiTDIP in MDD has been made public in 2008 through a patent application by S. Chissoe, the ''Genes associated with unipolar depression'' United States Patent Application 20080108076 Kind Code: A1 (http://www.freepatentsonline.com/y2008/0108076.html). The purpose of the study was to identify genes coding for tractable targets that are associated with MDD, to develop screening methods to identify compounds that act upon such targets, and to develop such compounds as medicines to treat MDD. A ''tractable'' or ''druggable target'' is a biological molecule that is known to be responsive to manipulation by small molecule chemical compunds, for example, 7-transmembrane receptors, ion channels, nuclear receptors, kinases, proteases and integrins. Here we report the summary results of the most significant original HiTDIP analysis (P.A. 20080108076) and detailed results from a follow-up case–control study of HiTDIP in MDD, combining the original HitDIP MDD sample and a sample collected in UK (Table I).

TABLE I. Summary of Demographic Variables

Sample	Gender (female/male)	Mean age at interview (years)
Cases		
DeCC	69%/31%	47.9
MP-GSK	67%/33%	51.6
Controls		
DeCC	60%/40%	47.2
MP-GSK	67%/33%	51.5

All patients were suffering from recurrent major depression, that is at least two separate episodes of unipolar depression.

METHODS

Subjects

A total of 974 Caucasian individuals diagnosed with recurrent MDD (67.04% women) and 968 Caucasian age- and gender-matched non-affected controls (66.84% women) were recruited in Southern Germany (MP-GSK). The cases were selected from three centers: Max-Planck Institute of Psychiatry in Munich (307 cases), Klinikum Ingoldstadt (320 cases) and BKH Augsburg (347 cases). The controls were all collected by the Max-Planck Institute of Psychiatry [Muglia et al., 2010]. All subjects gave informed consent for the use of their DNA in the study. All patients were evaluated by experienced research assistants (who had received training at WHO Training and Research Centres) using the semi-structured Schedules for Clinical Assessment in Neuropsychiatry (SCAN) instrument [Wing, 1990]. Each participant completed a questionnaire regarding his/her demographics, family and individual history and ethnicity. Patients were included in the study if they were diagnosed with recurrent MDD (i.e., at least two separate episodes of unipolar depression) of moderate or severe intensity according to DSM-IV or ICD-10, as resulted by analysis of the SCAN2.1 interview using the computerized algorithm CATEGO [Celik, 2003]. Patients were excluded from the study if they had: experienced mood incongruent psychotic symptoms, a lifetime history of intravenous drug use or diagnosis of drug dependency, depression secondary to alcohol or substance abuse or depression as clear consequence of medical illnesses or use of medications. Patients with diagnosis of schizophrenia, schizoaffective disorders and other axis I disorders other than anxiety disorders were excluded from the study. Patients with co-morbid anxiety disorders with the exception of obsessive compulsive and post-traumatic stress disorders, were included considering that anxiety and MDD very often co-occur and are also likely to share some of the genetic factors [Hettma, 2008]. Controls were selected randomly from a Munich-based community sample and recruited at the Max-Planck Institute of Psychiatry. They were screened for the presence of anxiety and mood disorders using the Composite International Diagnostic Screener [Wittchen et al., 1999a]. Only individuals without mood and anxiety disorders were collected as controls. The mean age at interview was 51.6 ($\pm$13.7) years for the cases, and 51.5 ($\pm$13.9) years for the controls.

The Medical Research Council (MRC) funded Depression Case control (DeCC) sample was collected from three sites, London, Birmingham, and Cardiff. The DeCC sample consists of 1,196 cases aged 18 years or more (69% women) who have suffered from recurrent depression (defined as having had 2 or more episodes of unipolar depression of at least moderate severity, separated by at least 2 months of remission, as defined by DSM-IV and/or ICD-10). Cases were excluded if they: had psychotic symptoms that were clearly mood incongruent or were prominent at a time when there was no evidence of mood disturbance; had been an intravenous drug user with a lifetime diagnosis of dependency; had depression that only ever occurred in relation to or as a result of alcohol or substance abuse or dependence; or had depression that was only secondary to medical illness or medication. The mean age at interview was 47.87 years (SEM 0.29, range 19–85 years). Older subjects were only included if their onset of depression was before

age 65. The controls consist of 60% women and have a mean age 47.24 years (SEM 0.31, range 20–69 years). They comprise 842 subjects contacted via the MRC general practice research framework and screened using the Sham et al. [2000] composite index (G) of depressive and anxiety symptoms and the remainder are healthy volunteers who are staff or students of King's College London. Potential control subjects were interviewed face to face or by telephone using a modified version of the Past History Schedule [McGuffin et al., 1986] and were included if there was no evidence of past or present clinically significant psychiatric disorder.

Diagnostic instruments. All depressed cases were given a face to face semi-structured diagnostic interview, the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) [Wing, 1990] by trained interviewers to establish the diagnosis of recurrent depression with symptoms rated for the worst and second worst episodes of depression. The clinical methodology exactly followed our Depression Network (DeNt) linkage study collection (further details are provided by Farmer et al. [2004]). The computerized version of the SCAN2.1 is built on top of the IShell system, which is a computer aided personal interviewing tool produced by the World Health Organisation which provides diagnoses according to DSM-IV and ICD-10 operational definitions [Celik, 2003].

The Munich case–control collection (HiTDIP) was closely modeled on the DeCC study that was begun slightly earlier in UK and all clinical interviewers of the two studies were trained by AEF and participated in a calibration and reliability meeting. The two case–control collections should also represent reasonably homogenous and ethnically related North European populations.

Genotyping

Genomic DNA of the HiTDIP sample (Germany) was isolated from whole blood using a standard salting-out procedure. Samples were arrayed and normalized in water to a standard concentration of 5 ng/μl. Twenty-nanogram aliquots of the DNA samples were arrayed into 96-well PCR plates. For purposes of quality control, 3.4% of the samples were duplicated on the plates and two negative template control wells received water. The samples were dried and the plates were stored at −20°C until use. Genotyping was performed by a modification of the single base chain extension (SGCE) assay previously described [Taylor et al., 2001]. Assays were designed by Glaxo Smith Kline R&D (Verona, Italy) in-house primer design program and then grouped into multiplexes of 50 reactions for PCR and SBCE. Following genotyping, the data were scored using a modification of Spotfire Decision Site Version 7.0. Genotypes passed quality control if: (a) duplicate comparisons were concordant, (b) negative template controls did not generate genotypes, and (c) more than 80% of the samples had valid genotypes. Genotypes for assays passing quality control tests were exported to an analysis database.

Genomic DNA of the DeCC sample (UK) was extracted from bloods and cheek swabs collected as described previously [Freeman et al., 1997, 2003]. Genotyping of the DeCC sample was performed using SNPlex™ Genotyping System (PE Applied Biosystems, Foster City, CA) as described previously [Schosser et al., 2010]. One SNP, rs1394015 in *ZNF80* could not be typed using SNPlex™ and was genotyped using a Taqman® SNP genotyping platform (PE Applied Biosystems). Genotyping was performed blind to all phenotypic information, including affection status, and 2,038 samples were successfully genotyped. Analysis of the raw data were performed using GeneMapper Software v3.7 and Microsoft Office Excel 2003. The following quality control criteria were applied: SNPs were omitted from analysis if poor genotype clustering prevented GeneMapper from making calls. Individual genotypes were omitted if their peak heights were <20% of the average for that genotypic group across the entire sample to avoid false heterozygosity assignment due to background noise in poor quality samples. As low call rates may indicate inaccurate genotyping, markers were omitted if the call rate after the previous exclusions was less than 80%.

Statistical Analyses

The association analysis of the primary sample was conducted using a Fisher's exact test to contrast single-point genotype or allele frequencies between cases and controls. SNPs that showed an Hardy–Weinberg Equilibrium $P < 0.001$ in controls were removed. To rank the most significant genes accounting for the different number of SNPs tested in each gene, a gene-based permutation test accounting for the number of SNPs tested for each gene was performed using 5,000 permutations.

A Cochran–Mantel–Haenzel-test was performed to test for genotypic and/or allelic differences between the two samples before combining them and analyzing them together.

To test for deviation from Hardy–Weinberg Equilibrium (HWE), the computer program FINETTI (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to perform exact statistics, and cases and controls were considered separately. To test for genotypic association with each SNP, a standard Chi-square (χ^2) statistic was calculated using SPSS version 15.0. The computer program FINETTI (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to calculate the Cochran–Armitage trend statistic to test for allelic association. UNPHASED version 3.0.10 program [Dudbridge, 2003] was applied using two- and three-marker slide windows to analyze for haplotypic association. Individuals with incomplete genotyping data were excluded from haplotype analyses. Individual haplotypes were also tested for association by grouping the frequencies of all other haplotypes together. Haploview 4.0 program [Barrett et al., 2005] was used to perform linkage disequilibrium (LD) analysis of all SNPs in our sample. The measure of LD, denoted as D' and r^2, was calculated from the haplotype frequency using the Expectation–Maximization (EM) algorithm. To calculate the power of our case–control sample, we used the PS program [Dupont and Plummer, 1990]. All *P*-values reported in this study were two-tailed. Multiple testing corrections were performed by application of the false discovery rate [FDR, Benjamini et al., 2001] to both single-marker and haplotype analyses. In the case of single-marker analyses, it was assumed that 29 independent tests were performed when testing 29 SNPs. Haplotype analyses were corrected for the number of sliding windows used.

RESULTS

The original HiTDIP in MDD study analyzed 6,500 SNPs, of these 6,391 SNPs resided within a gene while for 109 SNPs no

gene could be identified (http://www.freepatentsonline.com/y2008/0108076.html). A total of 1,827 genes (1,759 autosomal and 68 X-linked) were analyzed. The mean number of SNPs per gene was 3.6 and the number of SNPs per gene was comprised between 1 and 52. The gene associations were ranked based on the permutaion *P*-value to account for different number of SNPs tested for each gene. The results of the 49 genes that showed the most significant permutation p value are summarized in Supplement 1. Among those, six genes (*SLC4A10*, *DPP4*, *DRD3*, *ZNF80*, *NOS2A*, and *PPARGC1A*) were selected because compounds capable of modulating the protein were available, and drug development programs to rapidly lead to study in human subjects or patients could be initiated. In HiTDIP, several SNPs within these genes were associated with MDD before correction for multiple testing: 2 out of 16 SNPs located in the Solute Carrier Family 4 Member 10 (*SLC4A10*) gene, 1 out of 14 SNPs in Dipeptidyl Peptidase IV (*DPP4*), 7 out of 20 SNPs in Dopamine Receptor D3 (*DRD3*) and between *DRD3* and Zinc Finger Protein 80 (*ZNF80*), 3 out of 10 SNPs in Nitric Oxide Synthase 2A (*NOS2A*) and 7 out of 26 SNPs in Peroxisome Proliferator-Activated Receptor-Gamma, Coactivator 1, Alpha (*PPARGC1A*). Within the current study, 29 of the SNPs genotyped in the HiTDIP study (MP-GSK) were genotyped in a large depression case–control sample from UK (DeCC) that used an exactly similar clinical design. One SNP, rs3794764, had an overall call rate of 64%, and was therefore excluded from further analyses. All the other markers had call rates above 90%.

We performed Cochran–Mantel–Haenszel statistics to test for genotypic and/or allelic differences between the DeCC and MP-GSK samples (see Table II), in order to confirm for each SNP that there is no difference between the two samples, and assuming no population stratification in our analysis. Applying the FDR method to correct for multiple testing, none of the

TABLE II. Results of Cochran–Mantel–Haenszel Test Comparing the DeCC (UK) and MP-GSK (Germany) Sample, and Results of Single-Marker Analyses Are Shown

					N		Total (genotypic)		Total (allelic)		Cochran–Mantel–Haenszel			
SNP ID	Gene	Alleles	MAF_{cases}	$MAF_{controls}$	Cases	Controls	χ^2	*P*	χ^2	*P*	Genotypic M^2	Genotypic *P*	Allelic χ^2	Allelic *P*
rs6432706	*SLC4A10*	C/T!	0.294	0.303	2,099	1,733	2.021	0.364	0.83	0.362	1.751	0.417	0.454	0.5
rs2176135	*SLC4A10*	A/G!	0.227	0.243	2,054	1,741	5.371	0.068	2.59	0.108	5.388	0.068	2.532	0.112
rs6707646	*SLC4A10*	C/T!	0.478	0.460	2,128	1,754	2.593	0.274	2.52	0.112	1.652	0.438	1.586	0.208
rs4637136	*SLC4A10*	A/G!	0.416	0.407	2,107	1,718	2.223	0.329	0.74	0.388	1.45	0.484	0.227	0.634
rs1558958	*DPP4*	C!/G	0.193	0.211	2,071	1,731	4.124	0.127	3.87	0.049	4.468	0.107	4.039	**0.044**
rs2287509	*DPP4*	G!/T	0.419	0.399	2,139	1,764	3.353	0.187	3	0.083	2.375	0.305	2.044	0.153
rs7565794	*DPP4*	C!/T	0.324	0.323	2,128	1,740	0.104	0.949	0.01	0.919	0.198	0.906	0.119	0.73
rs2300755	*DPP4*	C/T!	0.373	0.380	2,142	1,774	0.514	0.773	0.38	0.538	0.154	0.926	0.072	0.789
rs2111850	*DPP4*	C!/T	0.322	0.330	1,797	4,290	0.526	0.769	0.52	0.471	0.18	0.914	0.148	0.701
rs10930040	*DPP4*	A/G!	0.338	0.333	2,101	1,757	1.506	0.471	0.21	0.647	1.594	0.451	0.363	0.547
rs3788979	*DPP4*	A!/G	0.137	0.134	2,122	1,761	0.116	0.944	0.12	0.734	0.201	0.904	0.171	0.679
rs6741949	*DPP4*	C!/G	0.436	0.421	2,113	1,729	2.155	0.34	1.63	0.202	1.445	0.486	0.966	0.326
rs6733162	*DPP4*	C/G!	0.400	0.393	2,093	1,708	0.763	0.683	0.32	0.574	0.954	0.621	2.95	0.086
rs741529	*DPP4*	A!/G	0.117	0.117	2,064	1,731	0.5	0.779	0.01	0.94	0.392	0.822	0.003	0.958
rs2216447	*DPP4*	C!/T	0.472	0.420	1,998	1,625	2.965	0.227	2.45	0.118	4.762	0.093	4.279	**0.039**
rs1861975	*DPP4*	A/C!	0.342	0.333	2,134	1,802	0.859	0.651	0.73	0.392	0.317	0.853	0.196	0.658
rs2399496	*DRD3*	A!/T	0.503	0.490	2,091	1,763	1.676	0.433	1.29	0.257	1.658	0.437	1.366	0.243
rs167771	*DRD3*	A/G!	0.173	0.167	2,122	1,755	5.325	0.07	0.58	0.445	4.951	0.084	0.605	0.437
rs167770	*DRD3*	A/G!	0.273	0.269	2,140	1,763	4.327	0.115	0.12	0.727	4.656	0.098	0.062	0.803
rs10934256	*DRD3*	A!/C	0.186	0.191	2,148	1,779	1.491	0.474	0.28	0.599	1.78	0.411	0.489	0.484
rs6280	*DRD3*	A/C!	0.322	0.314	2,140	1,800	3.635	0.162	0.58	0.448	3.806	0.149	0.379	0.538
rs1394015	*ZNF80*	C!/G	0.363	0.370	2,304	2,177	1.919	0.383	0.39	0.53	1.917	0.384	0.37	0.543
rs2297518	*NOS2A*	A!/G	0.206	0.190	2,052	1,753	3.721	0.156	3.24	0.072	3.579	0.167	2.843	0.092
rs3794764	*NOS2A*	A!/G	0.239	0.210	1,676	1,533	7.658	**0.022**	7.3	**0.007**	7.562	**0.023**	0.121	0.728
rs8072199	*NOS2A*	C/T!	0.447	0.441	2,135	1,757	0.537	0.765	0.33	0.564	0.56	0.7558	0.31	0.578
rs2779248	*NOS2A*	C!/T	0.405	0.369	2,154	1,799	10.189	**0.006**	10.11	**0.001**	9.996	**0.007**	9.834	**0.002**
rs768695	*PPARGC1A*	A!/G	0.511	0.487	2,154	1,805	8.035	**0.018**	4.53	**0.033**	7.196	**0.027**	4.197	**0.04**
rs3755863	*PPARGC1A*	A!/G	0.405	0.400	2,151	1,783	3.126	0.209	0.23	0.634	2.551	0.279	0.086	0.777
rs17576121		C!/T	0.308	0.325	2,143	1,769	3.075	0.215	2.79	0.095	2.573	0.276	2.246	0.134

All *P*-values are prior to correction for multiple testing (for corrected *P*-values see Results Section). The minor allele of each SNP is labeled with '!' [N, number of cases/controls for this SNP; MAF, minor allele frequency; SLC4A10, Solute Carrier Family 4 Member 10; DPP4, Dipeptidyl Peptidase IV; DRD3, Dopamine Receptor D3; ZNF80, Zinc Finger Protein 80; NOS2A, Nitric Oxide Synthase 2A; PPARGC1A, Peroxisome Proliferator-Activated Receptor-Gamma Coactivator 1 Alpha].

genotypic and/or allelic differences between the two samples (see Table II) remained significant. We therefore combined them and analyzed them together, resulting in a total of 2,170 cases and 1,810 controls.

As for marker rs2216447, cases were significantly out of Hardy–Weinberg equilibrium ($P = 0.0099$), but not the controls ($P = 0.192$). For two additional SNPs (rs4637136 and rs167771), controls show deviation from HWE ($P = 0.045$ and $P = 0.043$) before multiple testing correction, all other SNPs genotyped were in HWE.

We found significant genotypic and allelic differences between cases and controls for two SNPs in *NOS2A* and one SNP in *PPARGC1A*: rs3794764 (genotypic $P = 0.022$ [FDR$p = 0.128$]; allelic $P = 0.007$ [FDR$p = 0.068$]) and rs2770248 (genotypic $P = 0.006$ [FDR$p = 0.068$]; allelic $P = 0.001$ [FDR$p = 0.029$]) within the *NOS2A* gene and rs768695 (genotypic $P = 0.018$ [FDR$p = 0.128$]; allelic $P = 0.033$ [FDR$p = 0.159$]) within the *PPARGC1A* gene. With the exception of the significant allelic association of rs2770248 within the *NOS2A* gene, none of the significant *P*-values resisted multiple testing correction (see FDRp in [] brackets). None of the other investigated SNPs showed significant case–control differences in the combined UK and German sample. The genotypic and allelic case–control comparisons for each SNP are summarized in Table II.

Haplotype analyses applying 2- and 3-SNPs sliding windows using UNPHASED program (see Table III) showed significant global association between *NOS2A* rs2297518–rs8072199–rs2779248 haplotype ($P = 0.017$, FDR$p = 0.026$) and MDD: the A-T-C haplotype was overrepresented in cases compared to controls (13% vs. 11%). The same holds true for the corresponding two-marker combinations ($P = 0.038$, FDR$p = 0.038$ and $P = 0.016$, FDR$p = 0.026$).

There was no global significance found with the three-marker haplotype in *PPARGC1A* ($P = 0.099$), however with one of the possible two-marker haplotypes (rs768695–rs3755863, $P = 0.024$, FDR$p = 0.024$): the A–G haplotype was overrepresented in cases compared to controls (11% vs. 9%).

After multiple testing correction, none of the haplotypes in *DRD3* or *SLC4A10* were significantly associated with MDD.

Within the *DPP4* gene, we found two highly significant three-marker haplotypes (rs2300755–rs2111850–rs10930040, $P = 1.289 \times 10^{-7}$, and rs2111850–rs10930040–3788979, $P = 4.872 \times 10^{-5}$) and a highly significant two-marker haplotype (rs2111850–rs10930040, $P = 3.964 \times 10^{-6}$), all with the same alleles. However, although the significance resisted multiple testing correction, these extremely small *P*-values appeared to be driven by very rare haplotypes (frequencies of controls 0% for both haplotypes, frequencies of cases 0.01% and 0.02%). All SNPs of these highly significant haplotypes were in strong LD (see Table IV). Two additional three-marker haplotypes showed global significance, rs6733162–rs741529–rs2216447 ($P = 0.004$, FDR$p = 0.0057$) and rs741529–rs2216447–rs1861975 ($P = 0.041$, FDR$p = 0.037$), as well as a two-marker haplotype (rs2216447–rs1861975, $P = 0.039$, 0.039, FDR$p = 0.037$), however the latter again driven by a rare haplotype (frequency of controls 0% and of cases 0.02%). Linkage disequilibrium (LD) between the analyzed loci of the investigated genes is shown in Table IV.

DISCUSSION

The HiTDIP study in unipolar depression predated genome-wide association studies (GWAS) and tested genes that encode for potential tractable targets to identify genes that are associated with the occurrence of unipolar depression and to provide methods for screening to identify compounds with potential therapeutic effects. Therefore, this approach had the potential of identifying new therapeutic targets allowing to rapidly move from genes association to the testing of novel pharmacological interventions. From the most significant genes identified in the original HiTDIP analysis, six genes (*NOS2A*, *DRD3*, *ZNF80*, *SLC4A10*, *DPP4*, and *PPARGC1A*) for which molecules already available that could be progressed into drug development programs in human subjects or patients were selected for attempting replication in additional MDD sample from UK (DeCC) that used an exactly similar clinical design.

We found significant single marker and haplotypic association with SNPs in the *NOS2A* gene. One SNP (rs2779248) showed significant allelic association after multiple testing correction, however, it has to be pointed out that this SNP showed both genotypic and allelic differences between the DeCC (alleles cases: C = 40.1%, T = 59.9%; alleles controls: C = 37.9%, T = 62.1%/genotypes cases: CC = 16.0%, CT = 48.3%, TT = 35.8%; genotypes controls: CC = 14.2%, CT = 47.4%, TT = 38.5%) and MP-GSK samples (alleles cases: C = 40.9%, T = 59.1%; alleles controls: C = 36.1%, T = 63.9%/genotypes cases: CC = 17.7%, CT = 46.3%, TT = 35.9%; genotypes controls: CC = 13.4%, CT = 45.4%, TT = 41.2%), although theses differences did not withstand multiple testing correction. Therefore, this finding has to be interpreted with caution. The haplotype comprising the three *NOS2A* SNPs showed uncorrected global significance, which resisted multiple testing correction. The same holds true for the two-marker haplotypes; of note, the two-marker haplotype without rs2779248 was significant after multiple testing correction. The frequencies of alleles and genotypes for rs2297518 and rs8072199 did not show genotypic and/or allelic differences between the two samples. Nitric oxide (NO) is an important signaling molecule [Schulman, 1997] and affects neurodevelopmental processes in the CNS [Black et al., 1999]. It has not only been implicated in several physiological functions (e.g., noradrenalin and dopamine release, memory and learning), but also in the pathophysiology of MDD, bipolar disorder (BPD) and schizophrenia (SZ) [Herken et al., 2001; Papageorgious et al., 2001; Savas et al., 2002]. NO is also involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis [Bernstein et al., 1998] and mood disorders are frequently associated with HPA axis dysregulation [Daban et al., 2005; Pariante and Lightman, 2008]. The NOS-2A isoform of this enzyme plays a central role in CNS demyelination and glial-induced neuronal death [Brown and Bal-Price, 2003].

We found single marker association with one SNP in the *PPARGC1A* gene, rs768695, however not withstanding multiple testing correction. The two-marker haplotype including this SNP was significant after multiple testing correction, although this SNP showed genotypic and allelic differences between the DeCC and MP-GSK sample. The *PPARGC1A* gene maps to chromosome 4p15.1, a region that has been linked to BPD and MDD, as well

TABLE III. UNPHASED Version 3.0.10 Program [Dudbridge, 2003] Was Applied Using Two- (a) and Three-Marker Slide Windows (b) to Analyze for Haplotypic Association

Gene	SNP	Haplotypes										
a												
NOS2A	rs2297518	A										
	rs8072199	T	T									
	rs2779248		C									
	Global *P*	**0.038**	**0.016**									
	Individual *P*	**0.008**	**0.007**									
	Freq. cases/controls	0.15/0.12	0.19/0.17									
DPP4	rs1558958	C										
	rs2287509	T	T									
	rs7565794		T	T								
	rs2300755			T	C							
	rs2111850				T	T						
	rs10930040					G	A					
	rs3788979						G	G				
	rs6741949							G	G			
	rs6733162								C	G		
	rs741529									G	G	
	rs2216447										C	T
	rs1861975											A
	Global *P*	0.053	0.101	0.777	0.795	**3.964e−006**	0.983	0.193	0.325	0.653	0.058	**0.039**
	Individual *P*	**0.026**	**0.048**	0.436	0.486	**3.184e−008**	0.853	0.095	0.075	0.519	**0.007**	**0.012**
	Freq. cases/controls	0.19/0.21	0.26/0.28	0.05/0.06	0.63/0.62	0.02/0.00	0.66/0.67	0.43/0.45	0.31/0.34	0.29/0.28	0.36/0.33	0.60/0.63
DRD3 (ZNF80)	rs2399496	T										
	rs167771	A	G									
	rs167770		G	G								
	rs10934256			C	C							
	rs6280				C	C						
	rs1394015					G						
	Global *P*	0.421	0.847	0.619	0.368	0.672						
	Individual *P*	0.102	0.385	0.339	0.1342	0.219						
	Freq. cases/controls	0.41/0.43	0.11/0.11	0.09/0.08	0.14/0.11	0.22/0.21						
SLC4A10	rs6432706	C										
	rs2176135	A	A									
	rs6707646		T	C								
	rs4637136			A								
	Global *P*	**0.038**	0.222	0.308								
	Individual *P*	**0.02**	0.12	0.179								
	Freq. cases/controls	0.48/0.46	0.48/0.46	0.53/0.54								
PPARGC1A	rs768695	A										
	rs3755863	G	G									
	rs17576121		C									
	Global *P*	**0.024**	0.33									
	Individual *P*	**0.015**	0.075									
	Freq. cases/controls	0.11/0.09	0.20/0.22									
b												
NOS2A	rs2297518	A										
	rs8072199	T										
	rs2779248	C										
	Global *P*	**0.017**										
	Individual *P*	**0.02**										

(*Continued*)

TABLE III. (*Continued*)

Gene	SNP	Haplotypes									
	Freq. cases/controls	0.13/0.11									
DPP4	rs1558958	C									
	rs2287509	T	T								
	rs7565794	T	T	T							
	rs2300755		C	T	C						
	rs2111850			T	T	T					
	rs10930040				G	G	G				
	rs3788979					G	C	G			
	rs6741949							G	C		
	rs6733162							C	G	G	
	rs741529								C	C	A
	rs2216447										
	rs1861975										
	Global *P*	0.095	0.246	0.928	**1.289e−007**	**4.872e−005**	**0.034**	0.482	0.644	**0.004**	**0.041**
	Individual *P*	**0.02**	0.092	0.701	**1.352e−015**	**3.18e−007**	**0.0015**	0.074	0.126	**0.001**	**0.039**
	Freq. cases/controls	0.19/0.21	0.21/0.22	0.05/0.05	0.01/0.00	0.02/0.00	0.02/0.01	0.27/0.29	0.31/0.33	0.35/0.31	0.06/0.04
DRD3 (ZNF80)	rs2399496	T									
	rs167771	A	G								
	rs167770	G	G	G							
	rs10934256		C	C	C						
	rs6280			C	C						
	rs1394015				G						
	Global *P*	0.665	0.937	0.498	0.641						
	Individual *P*	0.127	0.415	0.218	0.063						
	Freq. cases/controls	0.02/0.02	0.09/0.08	0.09/0.08	0.12/0.10						
SLC4A10	rs6432706	C									
	rs2176135	A	G								
	rs6707646	T	C								
	rs4637136		A								
	Global *P*	**0.052**	0.38								
	Individual *P*	0.068	0.148								
	Freq. cases/controls	0.48/0.45	0.23/0.24								
PPARGC1A	rs768695	A									
	rs3755863	G									
	rs17576121	T									
	Global *P*	0.099									
	Individual *P*	**0.018**									
	Freq. cases/controls	0.09/0.07									

Global and individual *P*-values of the haplotypes are shown (significant haplotypes in bold), prior to correction for multiple testing (for corrected *P*-values see Results Section). SLC4A10, Solute Carrier Family 4 Member 10; DPP4, Dipeptidyl Peptidase IV; DRD3, Dopamine Receptor D3; ZNF80, Zinc Finger Protein 80; NOS2A, Nitric Oxide Synthase 2A; PPARGC1A, Peroxisome Proliferator-Activated Receptor-Gamma Coactivator 1 Alpha.

TABLE IV. Pair-Wise Comparisons (Linkage Disequilibrium, LD) of the SNPs Investigated in the NOS2A Gene, DPP4 Gene, DRD3/ZNF80 Gene, SLC4A10 Gene, and PPARGC1A Gene, Performed Using Haploview 4.0 Program [Barrett et al., 2005]

NOS2A	rs2297518	rs3794764	rs8072199	rs2779248
rs2297518		0.799	0.44	0.783
rs3794764	0.045		0.941	0.125
rs8072199	0.06	0.207		0.037
rs2779248	0.238	0.007	0.001	

DPP4	rs1558958	rs2287509	rs7565794	rs2300755	rs2111850	rs10930040	rs3788979	rs6741949b	rs6733162	rs741529	rs2216447	rs1861975
rs1558958		0.99	0.983	0.99	0.976	0.909	0.952	0.747	0.762	0.598	0.261	0.399
rs2287509	0.173		0.985	0.993	0.993	0.946	0.987	0.689	0.088	0.465	0.316	0.337
rs7565794	0.118	0.321		0.979	0.98	0.979	0.964	0.895	0.477	0.515	0.412	0.469
rs2300755	0.151	0.412	0.761		0.993	0.957	0.992	0.643	0.369	0.523	0.353	0.362
rs2111850	0.117	0.33	0.953	0.791		0.996	0.998	0.912	0.482	0.504	0.419	0.47
rs10930040	0.107	0.312	0.906	0.767	0.949		0.998	0.849	0.463	0.505	0.407	0.417
rs3788979	0.036	0.106	0.302	0.257	0.321	0.309		0.9	0.369	0.783	0.139	0.119
rs6741949	0.106	0.439	0.287	0.187	0.3	0.273	0.095		0.129	0.469	0.374	0.43
rs6733162	0.097	0.004	0.165	0.125	0.17	0.163	0.032	0.008		0.962	0.807	0.954
rs741529	0.012	0.02	0.074	0.06	0.069	0.068	0.013	0.022	0.187		0.725	0.941
rs2216447	0.01	0.083	0.047	0.044	0.049	0.047	0.002	0.106	0.246	0.04		0.852
rs1861975	0.021	0.084	0.054	0.04	0.054	0.044	0.001	0.126	0.305	0.06	0.636	

DRD3	rs2399496	rs167771	rs167770	rs10934256	rs6280	rs1394015
rs2399496		0.022	0.225	0.766	0.316	0.336
rs167771	0.0		0.535	0.091	0.874	0.525
rs167770	0.019	0.156		0.995	0.983	0.106
rs10934256	0.138	0.0	0.613		0.98	0.074
rs6280	0.047	0.334	0.771	0.474		0.139
rs1394015	0.064	0.032	0.002	0.002	0.005	

SLCA10	rs6432706	rs2176135	rs6707646	rs4637136
rs6432706		0.973	0.977	0.975
rs2176135	0.123		0.974	0.966
rs6707646	0.362	0.257		0.998
rs4637136	0.286	0.203	0.795	

PPARGC1A	rs768695	rs3755863	rs17576121
rs768695		0.987	0.212
rs3755863	0.659		0.154
rs17576121	0.021	0.007	

D' values are shown in the upper right triangle, r^2 values are shown in the lower triangle.

as to SZ [Blackwood et al., 1996; Detera-Wadleigh et al., 1999; Visscher et al., 1999; Als et al., 2004; Itokawa et al., 2004; Le Hellard et al., 2007]. A recent association analysis of this region [Christoforou et al., 2007] identified significant associations of BPD and SZ within the chromosome 4p15–p16 linkage region, including the *PPARGC1A* gene region.

No single marker but haplotypic association was found for SNPs in the *DPP4* gene. We found two highly significant three-marker haplotypes and one highly significant two-marker haplotype, all with the same alleles. It has to be pointed out that although the significance resisted multiple testing correction, these extremely small *P*-values appeared to be driven by very rare haplotypes. Two further three-marker haplotypes showed global significance after multiple testing correction, as well as a two-marker haplotype, however the latter again driven by a rare haplotype. Although these highly significant haplotypic associations were all driven by very rare haplotypes, further investigations are warranted in the light of the emerging copy number variations (CNV) data from other neuropsychiatric phenotypes. Due to its enzymatic activity on numerous substrates including peptide hormones involved in glucose homeostasis (such as Glucagon-like peptide 1 and PACAP) and neuropeptides (such as substance P and neuropeptide Y), DPP4 offers multiple biological rationales for its association with depression [Hildebrandt et al., 2000; Lambeir et al., 2003]. Of particular interest is the observation of glucose metabolism abnormalities in depression, such as the high frequency of insulin resistance [Winokur et al., 1988; Tashiro et al., 1997] and the high comorbidity between type 2 diabetes and depression [Katon, 2008], which suggest a potential role for DPP4 in depression through its effect on incretin hormones. In addition, DPP4 is known to be able to modulate neurotransmission by degradation of neuromodulatory peptides. Preclinical evidences suggests the above as the mechanism by which inactivation of DPP4 brings about anxiolytic-like profile and reduced stress-like responses in rodents, a phenomenon presently not targeted by DPP4 inhibitors [Karl et al., 2003; Frerker et al., 2009]. Previous reports have shown that psychiatric disorders such as depression are associated with decreased levels and activity of DPP4 [Maes et al., 1991; Elgün et al., 1999]. So far, no association of the *DPP4* gene with mood disorder has been reported, however strong associations between MDD and various physical diseases mediated via increased BMI have been shown [Farmer et al., 2008].

We found no evidence for single marker association with any of the SNPs in the *SLC4A10* gene, a gene that is highly expressed in the cerebral cortex and hippocampus, and the only significant two-marker haplotype did not withstand multiple testing correction. Similarly, there was no evidence of association with any of the SNPs in the *DRD3* and *ZNF80* genes.

We have good power to detect associations with markers where there is an odds ratio of 1.3 or higher and a minor allele frequency of 0.2 or higher (92.8% power at an alpha level of 0.05). However, if the odds ratio is as low as 1.2 or the minor allele frequency is as low as 0.1, we have only 42.6% power to detect association at an alpha level of 0.05. Another limitation of the current study is that controls were not matched for age. Finally, since the selection of genes from the primary HiTDIP study was based on the availability of molecules that could be progressed into drug development programs in human subjects or patients, this bias in selection of the genes together with the poor coverage of SNPs per gene are the key limits of this study.

In conclusion, the HiTDIP study of major depression suggested six candidate genes as depression susceptibility loci. In a follow-up case–control study of HiTDIP in MDD, we combined the HiTDIP sample with an independent sample collected in UK. In the combined analyses of both samples, we found single marker and haplotypic association of SNPs in the *NOS2A* gene, but not in *SLC4A10*, *DPP4*, DRD3, *ZNF80*, or *PPARGC1A*.

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