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Cross-Disorder Analysis of Bipolar Risk Genes: Further Evidence of *DGKH* as a Risk Gene for Bipolar Disorder, but also Unipolar Depression and Adult ADHD

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Recently, several genome-wide association studies (GWAS) on bipolar disorder (BPD) suggested novel risk genes. However, only few of them were followed up and further, the specificity of these genes is even more elusive. To address these issues, we genotyped SNPs in *ANK3*, *CACNA1C*, *CMTM8*, *DGKH*, *EGFR*, and *NPAS3*, which were significantly associated with BPD in previous GWAS, in a sample of 380 BPD patients. Replicated SNPs were then followed up in patients suffering from unipolar depression (UPD; $n = 387$) or adult attention-deficit/hyperactivity disorder (aADHD; $n = 535$). While we could not confirm an association of *ANK3*, *CACNA1C*, and *EGFR* with BPD, 10 SNPs in *DGKH*, *CMTM8*, and *NPAS3* were nominally associated with disease, with two *DGKH* markers surviving correction for multiple testing. When these were followed up in UPD and aADHD, seven *DGKH* SNPs were also associated with UPD, while one SNP each in *NPAS3* and *CMTM8* and four in *DGKH* were linked to aADHD. Furthermore, a *DGKH* haplotype consisting of rs994856/rs9525580/rs9525584 GAT was associated with all disorders tested, while the complementary AGC haplotype was protective. The corresponding haploblock spans a 27-kb region covering exons coding for amino acids 65–243, and thus might include functional variants yet to be identified. We demonstrate an association of *DGKH* with BPD, UPD, and aADHD by applying a two-stage design. These disorders share the feature of mood instability, so that this phenotype might be associated with genetic variation in *DGKH*.

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INTRODUCTION

Bipolar disorder (BPD) is a severe psychiatric disorder and affects up to 4% of the adult population worldwide (Bauer and Pfennig, 2005; Merikangas *et al*, 2007). Approximately 20% of the patients die of suicide (Kilbane *et al*, 2009). Additionally, co-morbid disorders are frequent and include adult attention-deficit/hyperactivity disorder (aADHD), anxiety disorders, and substance abuse (Kessler *et al*, 2006; Merikangas *et al*, 2007). Taken together, this results in severe psychosocial adversity and leads to serious economic burden. The etiology of BPD remains largely unknown but it is evident that genetic factors have an important role (Kieseppa *et al*, 2004; McGuffin *et al*, 2003).

Consistently replicated risk genes for BPD are still lacking. However, a variety of research tools were applied to detect susceptibility genes for BPD. This includes linkage studies, candidate gene association studies, and finally genome-wide association studies (GWAS). More than 40 linkage scans for BPD have been published to date.

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Different meta-analysis found the strongest evidence for susceptibility loci on 13q and 22q (Segurado *et al*, 2003). In a combined analysis, 6q21–q25 and 8q24 showed genome-wide significance (McQueen *et al*, 2005). The underlying genes, however, have not yet been identified. With regard to candidate gene studies, several genes were shown to be associated with BPD, but none of them has been established as a specific BPD susceptibility gene. Among the best-replicated genes are *DISC1*, *DAOA/G72*, *BDNF*, *TPH2*, *NRG1*, *ARNTL/CLOCK*, and *FAT* (Barnett and Smoller, 2009). Another approach to identify genetic factors predisposing to diseases is the search for gross structural variations (Zhang *et al*, 2009).

As there are no common loci of large effect, but several genes with small effect sizes increasing the risk toward BPD, GWAS might be more fruitful. Using this rationale, several novel risk genes have recently been published. By examining an US and German population, Baum *et al* (2008a) reported genome-wide significance of rs10120253 in intron 1 of *DGKH* (diacylglycerol kinase eta). *DGKH* also is a promising functional candidate gene as its gene product is involved in the phosphatidyl inositol pathway, which is assumed to have an important role in lithium action. The UK Wellcome Trust Case–Control Consortium (WTCCC; WTCCC, 2007) demonstrated genome-wide significance for a marker next to *PALB2*, *NDUFAB1*, and *DCTN5*, and Sklar *et al* (2008) reported significant findings for *MYO5B*, *TSPAN8*, and *EGFR*. Finally, also including meta-analytic treatment of the WTCCC and Sklar data sets found strong evidence for *CACNA1C* (α-1 subunit of a voltage-dependent calcium channel) and *ANK3* (ankyrin 3) (Ferreira *et al*, 2008). Taken together, these GWAS have provided risk genes that have been replicated in some cases (*DGKH* (Baum *et al*, 2008b; Ollila *et al*, 2009); *SORCS2* and *DFNB31* (Ollila *et al*, 2009); *CACNA1C* (Green *et al*, 2009); *ANK3* (Lee *et al*, 2010; Schulze *et al*, 2009; Scott *et al*, 2009; Smith *et al*, 2009); *TSPAN8* (Scholz *et al*, 2010)), while other replication attempts were negative. Several promising hits, however, were never attempted to replicate, and the potential impact of these candidate genes on other disorders displaying by mood disturbance has not yet been assessed. We have, therefore, picked the most promising risk genes and attempted their replication in an independent BPD sample. Confirmed risk genes thereafter were tested in samples consisting of patients suffering from unipolar depression (UPD) or aADHD, which is also characterized by severe mood dysregulation (Jacob *et al*, 2007).

MATERIALS AND METHODS

Samples

A detailed description of the BPD sample is available elsewhere (Lundorf *et al*, 2005; Reif *et al*, 2006b; Scholz *et al*, 2010). This sample consisted of 214 unrelated bipolar patients (mean age 51.7 ± 14.1 years, 65% female), from the German Lower Franconia area for whom an ICD-10 diagnosis was established by means of an extensive, semi-structured interview analogous to the AMDP interview (AMDP, 2000) carried out by two experienced psychiatrists at the University of Würzburg. Furthermore, the OPCRIT system was used in these patients (McGuffin *et al*, 1991). A further 166 unrelated bipolar patients (mean age 43.0 ± 11.5 years, 49% female) were ascertained according to ICD-10 diagnostic criteria for research (DCR) by means of a semi-structured interview (SCAN ver. 2.1.; World Health Organization, 1998) at the Center for Psychiatric Research, Århus University Hospital, giving a total number of 380 patients suffering from BPD. In all, 387 unrelated patients of German origin suffered from UPD and were enrolled also in the lower Franconia region ($n = 120$, mean age 54.1 ± 16.3 years, 54% female) as well as at the Department of Psychiatry, University of Münster ($n = 267$, mean age 49.7 ± 15.4 years, 57% female) as described (Baune *et al*, 2008). The diagnosis for UPD was ascertained by trained psychiatrists according to the ICD-10 DCR on the basis of semi-structured interviews. Co-morbidity data for UPD and BPD, respectively, with aADHD could not be obtained as these patients were ascertained during acute disease episodes where it is not possible to reliably establish a diagnosis of aADHD.

The aADHD study sample has been described previously (Franke *et al*, 2010a; Jacob *et al*, 2007; Gross-Lesch *et al*, in preparation) and consisted of 535 unrelated patients (mean age 33.7 ± 10.2 years, 46% female), recruited at the University of Würzburg in the Lower Franconian region, who completed a structured interview from which diagnoses of DSM-IV aADHD were determined by two experienced psychiatrists. In all, 60% of the aADHD patients suffered from combined type ADHD, 30 of inattentive type ADHD, and 10% of hyperactive-impulsive type ADHD. In all, 57% of patients suffered from co-morbid lifetime depression, 32% of lifetime anxiety disorders, and 43% of lifetime substance abuse disorders (mainly alcohol abuse). BPD has been an exclusion criterion for these patients. None of the patients showed significant neurologic co-morbidity, mental retardation, or other somatic disorders, suggesting organic psychosis. Patients with substance-induced disorders were excluded as well.

The control sample consisted of 630 healthy subjects and was composed of blood donors, staff members, and volunteers all originating from the Lower Franconia region. A total of 284 control subjects (mean age 35 ± 13 years, 47% female), consisting of healthy blood donors originating from Würzburg, were enrolled. The sample was not screened for psychiatric disorders; however, all subjects were free of medication, and the study was explained to them, so that the likelihood of severe psychiatric disorders in the control sample was low. An additional 356 subjects (mean age 33.7 ± 10.2 years, 51% female) were recruited and screened for the absence of psychiatric disorders by conducting the Structured Clinical Interview for DSM-IV (SCID-I). All case as well as control subjects were of self-reported German, or Danish, respectively, ethnicity. Only subjects who gave written informed consent were enrolled in the study, which complied with the Declaration of Helsinki and was approved by the Ethics Committees of the Universities of Würzburg, Münster, and Århus.

SNP Selection and Genotyping

Genes of interest were compiled from published GWAS on BPD: *NPAS3*, *ARNT2*, *CACNA1C*, *ANK3* (Ferreira *et al*,

2008), *NXN, SLC39A3, SORCS2, DGKH* (Baum *et al*, 2008a; Ollila *et al*, 2009), *NALCN, SLC19A3, SLC29A3, DFNB31, CMTM8* (Ollila *et al*, 2009; WTCCC, 2007), and *EGFR* (Sklar *et al*, 2008). Genes were selected due to strength of association signals, biological rationale, and involved pathways; significant SNPs were selected from previous studies. The study focused on *CACNA1C*, *ANK3*, *DGKH*, *CMTM8*, and *EGFR*, as in our hands those were the most promising candidate genes. For the other genes, only one or two top SNPs were tested as pilot investigations; nevertheless, they were also included in this analysis to fully account for multiple testing. Taken together, 14 genes that contained associated SNPs were tagged with 99 SNPs. In a first step, we tested for an association of these SNPs with BPD (single marker data, Supplementary Table 1; haplotype analysis, Supplementary Table 2). Second, we genotyped the 23 SNPs that were found to be nominally associated with BPD (Table 1) in the UPD (single marker data, Supplementary Table 3; haplotype analysis, Supplementary Table 4) and aADHD (single marker data, Supplementary Table 5; haplotype analysis, Supplementary Table 6) samples to assess whether these SNP associations were specific for BPD.

SNP genotyping was performed using Sequenom's MassArray system (Sequenom, San Diego, CA) according to the instructions supplied by the manufacturer. All PCR reactions were done using the iPlex chemistry following the manufacturer's standard operation procedure. All primer sequences are given in Supplementary Table 7.

Statistical Analysis

Statistical analysis of genotype data was performed with PLINK V1.07. (Purcell *et al*, 2007) and HaploView V4.1 (Barrett *et al*, 2005). Only polymorphic SNPs with a call rate (CR) $\geqslant$75% were included in the study; of those, genotype frequencies were ascertained for overall Hardy–Weinberg equilibrium (HWE; χ^2 HWE p-value $\geqslant$0.01). In the BPD sample, 87 of 99 typed SNPs passed these inclusion criteria, 12 SNPs accordingly were excluded from further analysis (rs41274688, rs35776153, rs999940, and 35065420 were monomorphic; rs4955274, rs11914777, rs17172438, and 7984523 departed from HWE; rs4411993, rs7683874, rs10234806, and 10994336 had a CR $<$75%). Of the 23 SNPs genotyped in UPD patients, three SNPs (rs4979416 and rs12496256 due to low CR and rs17455703 due to HWE departure) in the Würzburg subsample and one SNP (rs11773818, low CR) in the Münster subsample did not fulfill the inclusion criteria. In the aADHD sample, all 23 typed markers complied with the quality criteria.

Single marker associations were calculated by comparison of allele counts in 1-degree-of-freedom χ^2 tests; results were adjusted for multiple testing using the conservative Bonferroni correction. For multi-marker association tests, haplotype blocks were defined according to the four-gamete rule (Wang *et al*, 2002); inferred haplotype counts in groups were compared with logistic regression. For each haplotype, this multi-marker association test was permuted 10 000 times to generate an empirical probability distribution; this

Table 1 Nominally Significant Findings for Bipolar Disorder (Combined BPD Sample), Along With the Corresponding Results for Unipolar Depression (Combined UPD Sample) and Adult Attention-Deficit Hyperactivity Disorder (aADHD)

SNP	Alleles		$N = 630$	Complete BPD sample ($N = 380$)			Complete UPD sample ($N = 387$)			Adult ADHD sample ($N = 535$)		
	Minor	Major	Controls % d	Cases % d	Nominal p-value	Bonferroni p-value	Cases % d	Nominal p-value	Bonferroni p-value	Cases % d	Nominal p-value	Bonferroni p-value
CMTM8; chromosome 3												
rs12496256	G	A	0.336	0.282	**0.013**	1	0.303	0.181	1	0.313	0.250	1
rs6803740	G	A	0.165	0.210	**0.014**	1	0.183	0.289	1	0.198	**0.041**	0.941
DGKH; chromosome 13												
rs1170191	A	G	0.159	0.197	**0.026**	1	0.213	**0.003**	0.059	0.170	0.455	1
rs1170169	G	C	0.303	0.405	$\mathbf{1.5 \times 10^{-4}}$	**0.013**	0.402	$\mathbf{2.8 \times 10^{-4}}$	**0.006**	0.354	**0.046**	1
rs2148004	G	A	0.317	0.280	0.056	1	0.256	**0.005**	0.114	0.289	0.152	1
rs994856	G	A	0.401	0.455	**0.017**	1	0.454	**0.024**	0.556	0.454	**0.012**	0.277
rs9525580	A	G	0.232	0.312	$\mathbf{8.1 \times 10^{-5}}$	**0.007**	0.284	**0.012**	0.267	0.293	**0.001**	**0.023**
rs9525584	C	T	0.481	0.423	**0.013**	1	0.391	$\mathbf{1.2 \times 10^{-4}}$	**0.003**	0.444	0.083	1
rs1170101	G	A	0.277	0.349	$\mathbf{7.1 \times 10^{-4}}$	0.063	0.353	$\mathbf{4.0 \times 10^{-4}}$	**0.009**	0.304	0.152	1
rs347405	C	G	0.449	0.493	0.056	1	0.468	0.423	1	0.492	**0.043**	0.987
NPAS3; chromosome 14												
rs17455703	G	A	0.296	0.253	**0.041**	1	0.252	0.070	1	0.250	**0.016**	0.359
SLC39A3; chromosome 19												
rs4806874	G	A	0.314	0.269	**0.033**	1	0.293	0.327	1	0.309	0.788	1

Results are shown along with minor/major alleles (converted to the coding strand for the genes), the associated disease, allele frequencies for cases and controls, nominal p-values, as well as Bonferroni-corrected p-values.
Bold values denote $P < 0.05$.

was used to estimate p-values that control the family-wise error rate (FWER). With our study population, nominal association tests have a power of 55 and 50% to detect SNPs and haplotypes, respectively, conveying an odds ratio (OR) of 1.5 (corresponding to a relative risk of 1.48) to develop BPD assuming a co-dominant model and an MAF of 0.05 (Menashe *et al*, 2008). Using the same parameters, the power for SNP and haplotype associations was 66 and 64% for aADHD, while for UPD, the power is 59 and 56%, respectively.

Furthermore, meta-analytic treatment of rs9315885 and rs1170191 was performed by including the studies by Baum *et al* (2008a), Ollila *et al* (2009), and Squassina *et al* (2009) (as these SNPs were not genotyped in the study by Tesli *et al* (2009), this study could not be included in the meta-analysis): ORs were calculated as a measure for effect size; thereafter, the Q-statistic was applied to assess heterogeneity. Inconsistency across studies was quantified with I^2 metric (I^2 = Q–df/Q). In the absence of heterogeneity, ORs were combined using fixed-effects models; if significant heterogeneity was detected, joint ORs were derived from random-effects models. Calculations were performed using R version 2.10 along with the package metafor version 0.5–7.

RESULTS

Single Marker Analysis

In order to replicate and assess the specificity of 14 selected genes from published GWAS on BPD, we analyzed 88 tag SNPs in the genes *ANK3*, *ARNT2*, *CACNA1C*, *CMTM8*, *DFNB31*, *DGKH*, *EGFR*, *NALCN*, *NPAS3*, *NXN*, *SLC19A3*, *SLC29A3*, *SLC39A3*, and *SORCS2* in our BPD sample. After correction for multiple testing, two SNPs (rs1170169 and rs9525580) in *DGKH* remained significantly associated with BPD; at the nominal level, eight further significant findings were detected in the combined BPD sample, while another 13 SNPs were associated in only one of the BPD subsamples (Table 1; Supplementary Table 1). In order to examine the specificity of these associations for BPD, we further analyzed all 23 nominally associated markers (from the genes *CMTM8*, *EGFR*, *DFNB31*, *DGKH*, *NPAS3*, and *SLC39A3*) in UPD (Table 1; Supplementary Table 3) and aADHD (Table 1; Supplementary Table 5). SNPs from the other eight genes showed no significant association with BPD and were thus not analyzed further.

Cross-disorder genotyping revealed a total of 12 SNPs in four genes (*CMTM8*, *DGKH*, *NPAS3*, and *SLC39A3*) that were associated with at least one of the three examined phenotypes at the nominal level (Table 1). Ten association p-values were nominally significant in the combined BPD sample (the remaining other two had a borderline significant $p = 0.056$), seven in the combined UPD sample, and six in the aADHD sample, with seven SNPs being associated with two and two SNPs with all three disorders. Only those SNPs that were associated with BPD in the combined sample replicated in either UPD or aADHD (however, including rs2148004 and rs347405 with $p = 0.056$), but not those that were only associated in one of the subsamples (compare Supplementary Table 1 with Table 1). Most of the replicated SNPs mapped to *DGKH* (BPD: six SNPs; UPD: seven SNPs; aADHD: four SNPs), which was the only gene in our study that contained SNPs, which were significant following Bonferroni correction (Table 1). Noteworthy, all associations found with UPD mapped to *DGKH*. In *CMTM8*, only one SNP (rs6803740) featured an overlapping association between BPD and aADHD. The same disorders also overlapped in their association regarding the *NPAS3* SNP rs7455703. The *SLC39A3* association of rs4806874 was found to be exclusive for BPD (Table 1).

Haplotype Analysis

Haplotype analysis was then performed with all cross-disorder genotyped SNPs (BPD, significant findings: Table 2, complete data are given in Supplementary Table 2; UPD significant findings: Table 2, complete data are given in Supplementary Table 4; aADHD significant findings: Table 2, complete data are given in Supplementary Table 6). The strongest association found in all analyzed disorders was in *DGKH* block 2 (rs994856–rs9525580–rs9525584; Figure 1) haplotype GAT, which is exclusively composed of each single marker's risk alleles; this was consistent in all three examined phenotypes. Accordingly, GAT frequency was increased in all case groups as compared with controls; although this was nominally significant in all three disorders, the FWER was below 5% only in BPD and aADHD, but slightly above this threshold in UPD (permutation $p = 0.056$). The GAT haplotype can, therefore, be assumed to predispose to at least two, but possibly to any of the three disorders (see Table 2). In terms of frequency, GAT follows its 'complementary' haplotype AGC, which is composed of those alleles that have a higher MAF in controls. The expected protective effect conveyed by AGC, however, was only significant in UPD following FWER correction, and nominally also in BPD (Table 2). A similar phenomenon was seen in *DGKH* block 1 (rs1170191–1170169–rs2148004) haplotype GCG, whose frequency was lower in all case groups as compared with controls, but the presumed protective effect was nominally significant only in BPD and UPD. The haplotype GGA in turn was enriched in all cases; following FWER, this was significant in BPD, whereas nominally it was also associated with aADHD and UPD, respectively ($p = 0.051$; Table 2). Two further risk haplotypes were exclusively found to be associated with UPD (Table 2).

Haplotype associations in genes other than *DGKH* were found to be restricted to specific psychiatric disorders and did not withstand correction for multiple testing. In *CMTM8*, block 1 (rs6550109–rs12496256; Figure 2) haplotype TG was significantly protective, while block 3 (rs4276227–rs6803740) haplotype CG was associated with risk for BPD (Table 2). The *CMTM8* block 2 (rs4955272–rs7644602–rs7632109) haplotype GGG was the only significant haplotype association in aADHD and presumed to be protective (Table 2). *NPAS3* rs8015959–rs17455703 had two alleles associated with BPD, the protective CG and the risk haplotype CA (Table 2).

Meta-Analysis

To compare our findings with previous studies, we have subjected the significant SNPs rs9315885 and rs1170191

Table 2 Association of Haplotypes With the Complete BPD, UPD, and aADHD Samples Containing At Least One Significant Haplotype

CMTM8											
Chromosome 3; block 1			**Complete BPD sample ($N=380$)**			**Complete UPD sample ($N=387$)**			**Adult ADHD sample ($N=535$)**		
rs6550109	**rs12496256**		**Case/control frequencies**	**Nominal *p*-value**	**Permutation *p*-value**	**Case/control frequencies**	**Nominal *p*-value**	**Permutation *p*-value**	**Case/control frequencies**	**Nominal *p*-value**	**Permutation *p*-value**
T	**G**		**0.274/0.336**	**0.004**	**0.183**	**0.298/0.336**	**0.124**	**0.942**	**0.311/0.336**	**0.185**	**0.959**
T	A		0.133/0.110	0.158	1	0.134/0.110	0.166	0.990	0.113/0.110	0.799	1
C	A		0.593/0.554	0.151	1	0.569/0.554	0.579	1	0.576/0.554	0.347	0.999
Chromosome 3; block 2											
rs4955272	**rs7644602**	**rs7632109**									
G	G	A	0.183/0.218	0.054	0.949	0.211/0.218	0.709	1	0.244/0.218	0.151	0.925
G	**G**	**G**	**0.239/0.23**	**0.667**	**1**	**0.199/0.23**	**0.134**	**0.973**	**0.185/0.23**	**0.016**	**0.251**
A	T	G	0.299/0.287	0.573	1	0.285/0.287	0.916	1	0.290/0.287	0.923	1
G	T	G	0.279/0.265	0.537	1	0.305/0.265	0.068	0.674	0.281/0.265	0.313	0.997
Chromosome 3; block 3											
rs4276227	**rs6803740**										
C	**G**		**0.210/0.165**	**0.013**	**0.519**	**0.186/0.165**	**0.225**	**0.952**	**0.194/0.165**	**0.070**	**0.691**
T	A		0.306/0.325	0.393	1	0.335/0.325	0.627	1	0.328/0.325	0.870	1
C	A		0.484/0.510	0.270	1	0.479/0.510	0.170	0.853	0.478/0.510	0.086	0.768
DGKH											
Chromosome 13; block 1											
rs1170191	**rs1170169**	**rs2148004**									
G	**C**	**G**	**0.269/0.318**	**0.021**	**0.677**	**0.257/0.318**	**0.005**	**0.197**	**0.286/0.318**	**0.107**	**0.835**
A	G	A	0.189/0.161	0.112	0.998	0.211/0.161	0.006	0.140	0.168/0.161	0.706	1
G	**G**	**A**	**0.218/0.159**	**5.4×10^{-4}**	**0.030**	**0.188/0.159**	**0.051**	**0.563**	**0.192/0.159**	**0.009**	**0.151**
G	**C**	**A**	**0.324/0.362**	**0.051**	**0.941**	**0.343/0.362**	**0.288**	**0.990**	**0.354/0.362**	**0.579**	**1**
Chromosome 13; block 2											
rs994856	**rs9525580**	**rs9525584**									
A	**G**	**C**	**0.417/0.48**	**0.009**	**0.406**	**0.39/0.48**	**1.6×10^{-4}**	**0.021**	**0.445/0.48**	**0.120**	**0.873**
G	**A**	**T**	**0.312/0.228**	**2.9×10^{-5}**	**0.001**	**0.288/0.228**	**0.004**	**0.056**	**0.296/0.228**	**3.0×10^{-4}**	**0.004**
G	G	T	0.145/0.17	0.153	1	0.166/0.17	0.810	1	0.159/0.17	0.479	1
A	**G**	**T**	**0.127/0.122**	**0.737**	**1**	**0.155/0.122**	**0.039**	**0.867**	**0.101/0.122**	**0.132**	**0.899**
NPAS3											
Chromosome 14; block 1											
rs8015959	**rs17455703**										
C	**G**		**0.253/0.296**	**0.039**	**0.883**	—	—	—	—	—	—
T	A		0.024/0.026	0.823	1	—	—	—	—	—	—
C	**A**		**0.723/0.678**	**0.038**	**0.877**	—	—	—	—	—	—

Bold values denote $p<0.05$.

from the studies by Baum *et al* (2008a), Ollila *et al* (2009), and Squassina *et al* (2009) to a formal meta-analysis (Figure 3; Supplementary Table 8). While rs9315885, which was significant in the three other studies but not our data set, proved to be highly significantly associated in the meta-analysis (Figure 3a), this was not the case for rs1170191, as the effect direction was reversed in our as compared with other studies (Figure 3b).

DISCUSSION

The present study had two major aims: (1) to replicate risk genes for BPD obtained through GWAS and (2) to assess the specificity of associated risk genes by testing all nominally associated SNPs in UPD and aADHD as well. We chose to specifically test risk variants in *CACNA1C*, *ANK3*, *DGKH*, *CMTM8*, and *EGFR*, while for several other genes only a few SNPs were tested, which we report here as well to fully account for multiple testing. In the following discussion, we will elaborate on a gene-by-gene wise manner, yet genes where only a few SNPs were tested and not found to be associated will not be commented further upon (*ARNT2*, *DFNB31*, *NALCN*, *NXN*, *SLC19A3*, *SLC29A3*, *SLC39A3*, and *SORCS2*). An important caveat that has to be considered in the interpretation of our data is the use of a single control group, which has been compared against all three diagnostic groups. Significant deviation of our control group from the population's allele distribution, therefore, would bias our association data. We have thus compared the MAFs of our

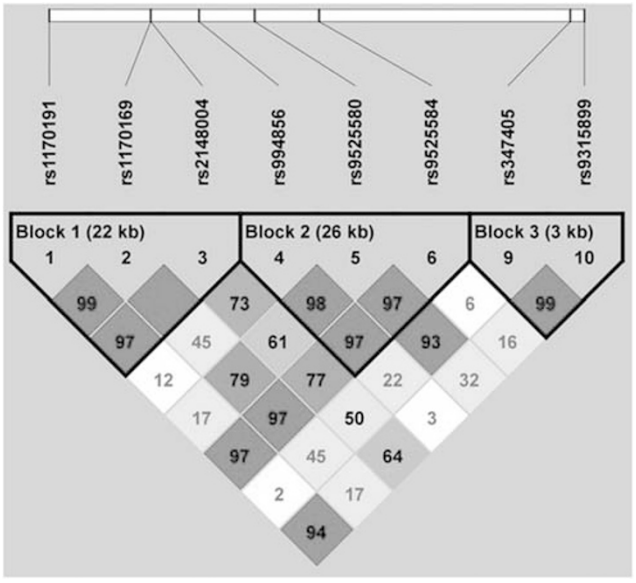

Figure 1 LD plot of *DGKH*, according to the four-gamete rule.

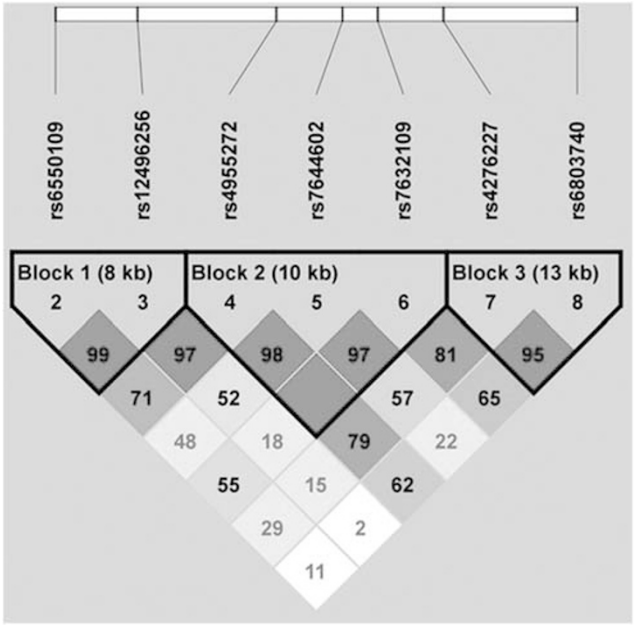

Figure 2 LD plot of *CMTM8*, according to the four-gamete rule.

control sample to a set of German population based controls, which have been ascertained via the HNR/KORA studies and genotyped on an Illumina microarray ($n = 795$; M Mattheisen and S Cichon, personal communication). From the 86 SNPs entering our analysis, 52 could be retrieved from the microarray. Only one of these 52 SNPs however significantly differed from our control sample (nominal p-value $p = 0.017$, rs1370717; all other p-values were > 0.15, data not shown), which argues against the assumption of a systematic bias due to the use of an unusual control group.

ANK3 and *CACNA1C*

In our panel of candidate genes, we have also included the top SNPs of the most replicated BPD risk genes so far: *ANK3* (Ferreira *et al*, 2008; Lee *et al*, 2010; Schulze *et al*, 2009; Scott *et al*, 2009; Smith *et al*, 2009), which was also associated with schizophrenia (Athanasiu *et al*, 2010) and *CACNA1C* (Ferreira *et al*, 2008; Keers *et al*, 2009; Sklar *et al*, 2008; WTCCC, 2007), which was as well demonstrated to be associated with schizophrenia (Green *et al*, 2009; Moskvina *et al*, 2009; Nyegaard *et al*, 2010), UPD (Green *et al*, 2009), and its endophenotypes (Casamassima *et al*, 2010). *CACNA1C* was shown to exert effects on verbal fluency and functional (Erk *et al*, 2010; Krug *et al*, 2010; Wessa *et al*, 2010) and structural (Franke *et al*, 2010b; Kempton *et al*, 2009) neuroimaging. We have aimed to replicate these genes in our bipolar sample, yet there was no significant association of either *ANK3* or *CACNAC1C* so that we abstained from testing them further. Several reasons for this lack of replication have to be considered: (1) lack of power owing to the sample size of $n = 380$ bipolar patients, as compared with the huge number of patients tested in current GWAS; (2) missed common variants, as we did not tag the whole gene but rather focused on previously associated SNPs however, including *CACNA1C* rs1006737, which was tested in the genomic imaging studies outlined above; (3) missed rare variants causing an association of common variants in the discovery samples (Dickson *et al*, 2010), thus escaping replication attempts due to differing LD substructures in the examined population; and (4) genetic heterogeneity of BPD, resulting in an association of risk genes in some, but not all populations (which might be very likely as associations were hitherto restricted to US American, UK, and Irish populations in the case of *CACNA1C*).

CMTM8 and *EGFR*

These two genes are considered together, as *CMTM8* (CKLF-like MARVEL transmembrane domain containing 8) appears to be a negative regulator of EGF-induced signaling (Jin *et al*, 2005, 2007), which is mediated by the EGF receptor EGFR (previously termed ErbB). Thus, a common pathway of EGFR and CMTM8 seems reasonable. While the first evidence for an involvement of *EGFR* in BPD came from the GWAS by Sklar *et al* (2008), *CMTM8* was identified in the WTCCC data set (WTCCC, 2007) yet not replicated later (Ollila *et al*, 2009). While there is almost no information on *CMTM8*, there is a vast body of literature on EGFR. This receptor kinase signals through PI3K/Akt as well as RAS/RAF/MEK/ERK (Wong and Guillaud, 2004), leading to downstream mechanisms including cell proliferation and survival. Accordingly, EGFR was shown to regulate neural stem cell proliferation (Cesetti *et al*, 2009; Grimm *et al*, 2009; Suh *et al*, 2009) and migration (Kim *et al*, 2009). Most interestingly, NO exerts its effect on neural stem cell proliferation by preventing EGFR-induced Akt phosphorylation (Torroglosa *et al*, 2007). Thus, EGFR and it regulators are excellent candidate molecules for neuropsychiatric disorders. Although other ErbB isoforms have gained much interest due to their interaction with neuregulin-1 (Birchmeier, 2009), there are almost no studies on EGFR/ErbB1 and its pathway. In the present study, we

Sample rs9315885	Cases Events	Cases Total	Controls Events	Controls Total	Odds Ratio M-H, Fixed, 95% CI
Weber et al.	214	690	384	1234	1.00 [0.81, 1.22]
Baum et al. (German)	462	1644	606	1752	0.81 [0.70, 0.94]
Baum et al. (NIMH)	242	922	360	1124	0.76 [0.62, 0.92]
Squassina et al.	158	394	282	600	0.75 [0.58, 0.98]
Total (95% CI)		**3650**		**4710**	0.82 [0.75, 0.91]
Total events	1076		1632		

Heterogeneity: $Chi^2 = 4.675$; df = 3 (p = 0.197)
Test for overall effect: Z = -4.03 (p= 0.0001)
Cochran-Mantel-Haenzel-Test: CMH =16.07; df = 4 (p = 0.0001)

Sample rs1170191	Cases Events	Cases Total	Controls Events	Controls Total	Odds Ratio M-H, Random, 95% CI
Weber et al.	144	730	196	1240	1.31 [1.03, 1.66]
Baum et al. (German)	233	1544	350	1752	0.71 [0.59, 0.85]
Baum et al. (NIMH)	122	922	202	1124	0.70 [0.55, 0.89]
Squassina et al.	83	393	144	600	0.85 [0.62, 1.15]
Total (95% CI)		**3589**		**4716**	0.83 [0.74, 0.94]
Total events	582		892		

Heterogeneity: $Tau^2 = 0.077$; $Chi^2 = 18.83$; df = 3(P = 0.0003); $I^2 = 84.07\%$
Test for overall effect: Z = -1.008 (P=0.314)

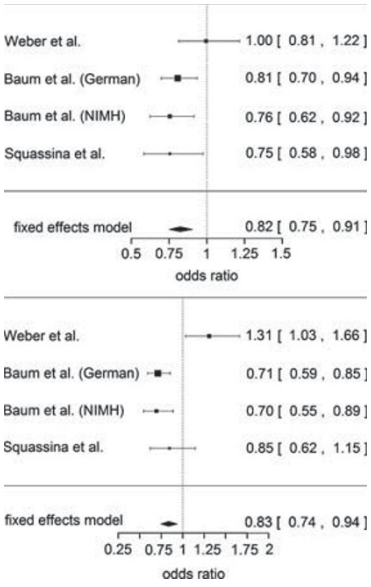

Figure 3 Forest plots displaying meta-analyses of minor *vs* major allele of rs9315885 (a) and rs1170191 (b).

could however not find support for an involvement of *EGFR* variation in BPD, in contrast to Sklar *et al* (2008). Again, this might be due to population-specific associations, yet the independent finding on *CMTM8* in the WTCCC GWAS underscores the notion that GWAS, when combined, can indentify novel pathways and thereby provide a starting point for more mechanistic studies. As we could replicate *CMTM8* as a bipolar—and, with borderline significance, aADHD—risk gene, we consider further studies worthwhile. Interestingly, when looking up *CMTM8* in our pooled GWAS on aADHD (Lesch *et al*, 2008), rs9833771 which is just 18 kb away from our most significant *CMTM8* finding rs6803740 was associated with aADHD at $p = 0.0002$, adding further support for our notion that it is involved in aADHD.

NPAS3

Several lines of evidence link the transcription factor neuronal PAS domain protein 3 (NPAS3) to psychiatric disorders (Pickard *et al*, 2006). Kamnasaran *et al* (2003) reported on a family in which a disruption of *NPAS3* segregates with schizophrenia and was also associated with learning disability (Pickard *et al*, 2005). This finding was picked up soon thereafter in animal studies, demonstrating that *Npas3* deletion mutant mice display schizophrenia-like behavioral abnormalities (Erbel-Sieler *et al*, 2004). Most interestingly, this was paralleled by a marked reduction of hippocampal adult neurogenesis (Pieper *et al*, 2005), which is suggested to have a role in schizophrenia (Reif *et al*, 2006a). Later, Pickard *et al* (2009) could show that common genetic variation in the *NPAS3* gene is associated with both schizophrenia and BPD. Furthermore, coding non-synonymous variants were identified and demonstrated to be associated with schizophrenia (Macintyre *et al*, 2010), which might well underlie the association of common, intronic variants (Dickson *et al*, 2010). Additionally, *NPAS3* was identified in two GWAS to be associated with iloperidone response (Lavedan *et al*, 2009) and, interestingly, BPD (Ferreira *et al*, 2008). Thus, both hypothesis-free and hypothesis-driven genetic data as well as animal models argue for a role of *NPAS3* in psychoses. Indeed, one (rs17455703) of the two *NPAS3* SNPs tested in the present study again was associated with BPD. A nominally significant association for the same SNP with aADHD was also found, arguing that the connection between *NPAS3* and psychiatric disorders crosses diagnostic boundaries. In line with this, three out of 282 *NPAS3* SNPs tested in our aADHD GWAS (Lesch *et al*, 2008) were associated with disease, also following correction for multiple testing on a gene-based level (rs4503707, rs10483437, rs12100538). Further studies have to reveal whether *NPAS3* is involved in cognitive functioning or rather emotional regulation, as both domains are affected in all three disorders (schizophrenia, BPD, and aADHD).

DGKH

The most prominent finding of our study however related to *DGKH*, suggested to be associated with BPD in the GWAS by Baum *et al* (2008a, b). However, replication failed in studies on BPD and lithium response (Manchia *et al*, 2009; Tesli *et al*, 2009), while two other studies were ambiguous (Ollila *et al*, 2009; Squassina *et al*, 2009). We attempted to replicate previously associated SNPs (rs9315885 and rs1170191), and by calculating a meta-analysis a role for rs9315885 was confirmed (Figure 3a), which however was not due to a signal in our sample. Our most significant SNPs were tagging SNPs, which have not been previously reported. While our data thus are again arguing for a role of *DGKH* in BPD, they cannot be considered a replication in a strict statistical sense and thus follow-up studies have to further test the top SNPs described here. Nevertheless, a recent report demonstrated increased expression of DGKH in BPD (Moya *et al*, 2010), lending further support to the notion for an involvement of this molecule in BPD. The role of DGKH is to metabolize diacylglycerol (DAG), which is produced upon cleavage of PIP2 into IP3 and DAG by phospholipase C. DAG, in turn, activates protein kinase C

(PKC), which phosphorylates a variety of proteins including Disheveled, an inhibitor of GSK3β. Thus, although the precise role of DGKH is not known yet, it clearly is involved in crucial pathways for psychiatric disorders and especially the mechanism of action of lithium. Intriguingly, *DGKH* knockdown in HeLa cells impaired the MEK/ERK pathway activated by EGF, while overexpression of the gene activated the pathway (Yasuda *et al*, 2009). Thus, DGKH is also linked to EGFR/CMTM8 mentioned above.

In our study, 6 out of 21 tested SNPs in *DGKH* were associated with BPD, and two SNPs withstood correction for multiple testing. Re-analysis of our aADHD GWAS (Lesch *et al*, 2008) revealed that 8 out of 52 SNPs were nominally associated with disease, one of which also survived correction on the gene level (rs10492444; nominal $p = 0.0004$, corrected $p = 0.0212$) and was located 505 bp away from rs9525584 being at the 3′ end of the risk haploblock delineated below. Furthermore, two frequent haplotypes were significantly associated with disease, especially rs994856/rs9525580/rs9525584 GAT. Apart from rs347405, all associated SNPs were also nominally associated with UPD (with three SNPs surviving correction for multiple testing) and again the haploblock rs994856/rs9525580/rs9525584 was associated. In aADHD, four of the eight SNPs replicated, one of which withstanding correction and again, rs994856/rs9525580/rs9525584 GAT was associated with disease. When looking at absolute haplotype frequencies, it becomes apparent that the GAT haplotype is always more frequent in cases (controls, 23%; BPD, 31%; UPD, 29%; aADHD, 30%), while the AGC haplotype is always less frequent in cases (controls, 48%; BPD, 42%; UPD, 39%; aADHD, 45%). While our data as well as HapMap CEU suggest that AGT are the major alleles, the sub-Saharan HapMap subset suggest that the AGC alleles are evolutionary older, that is the AGC haplotype can be considered ancient. The GAT haplotype appears to be evolutionary younger and thus it seems to convey the risk variant. The haploblock spans a genomic region of 27 kb covering exons two to six of the gene, that is amino acids 65–243. Three non-synonymous variants (no frequency data available) are deposited in databases, rs59790803 causing a G > A transition resulting in a possibly damaging Asp > Asn exchange (Polyphen-2 (Adzhubei *et al*, 2010) score: 0.736, sensitivity: 0.81, specificity: 0.90) in exon 6 at position 107; rs9566925, causing a stop mutation at position 90; and rs1344286, causing a A > C transition, resulting in a Thr > Pro exchange at position 65. These regions contain a pleckstrin homology (PH) domain (AA 65–158) and a phorbol-ester/DAG-type zinc finger (AA 175–225). While the latter is the DAG sensor, the PH binds phosphatidylinositols and proteins such as PKC. This domain is, therefore, involved in intracellular targeting and enables DGKH to interact with other signal transduction pathways. Both variants, therefore, might well alter the function of the protein by either impairing catalysis or changing protein–protein interactions, thereby disturbing intraneuronal second- and third-messenger pathways.

Outlook and Conclusions

By applying a two-stage design, we here demonstrated an association of *DGKH* with BPD, UPD, and aADHD. These disorders share the feature of mood instability with varying amplitude and frequency. Thus, genetic variation at the *DGKH* locus might be associated with this psychopathological phenotype. This is yet another example that a common genetic variant is associated with more than one psychiatric phenotype, which was also the case with other risk genes picked up by GWAS, for example, *CACNA1C* and *ANK3*. The integration of such findings might pinpoint distinct molecular pathways whose identification might enhance psychiatric diagnostics and research on neurobiological underpinnings.

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DISCLOSURE

AR and KD have received funding by Astra Zeneca. KD is on the speakers' board of Pfizer, Lilly, and Bristol-Myers Squibb. These affiliations have no relevance to the work covered in the manuscript. None of the other authors reported any biomedical financial interests or potential conflicts of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)