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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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## Abstract

Recently, several genome-wide studies (GWA) 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 ADHD (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 to 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*.

**Key words:** association, bipolar disorder, depression, adult ADHD, NPAS3, CMTM8

## 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 to serious economic burden. The etiology of BPD remains largely unknown but it is evident that genetic factors play 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. 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 towards 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*. (Baum *et al*, 2008a) reported

genome wide significance of rs10120253 in intron 1 of *DGKH* (diacylglycerol kinase eta) (Baum *et al*, 2008a). *DGKH* also is a promising functional candidate gene as its gene product is involved in the phosphatidyl inositol pathway, which is assumed to play 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*. reported significant findings for *MYO5B*, *TSPAN8* and *EGFR* (Sklar *et al*, 2008). Finally, also including meta-analytic treatment of the WTCCC and Sklar datasets found strong evidence for *CACNA1C* (alpha-1 subunit of a voltage dependent calcium channel) and *ANK3* (ankyrin 3) (Ferreira *et al*, 2008). Taken together, these GWAS have provided risk genes which 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  $51.7 \pm 14.1$  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  $43.0 \pm 11.5$  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. 387 unrelated patients of German origin suffered from UPD and were enrolled also in the lower Franconia region ( $n=120$ , mean age  $54.1 \pm 16.3$  years, 54 % female) as well as at the Department of Psychiatry, University of Münster ( $n=267$ , mean age  $49.7 \pm 15.4$  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  $33.7 \pm 10.2$  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. 60% of the aADHD patients suffered from combined type ADHD, 30 of inattentive type ADHD, and 10% of hyperactive-impulsive type ADHD. 57% of patients suffered from co-morbid life-time depression, 32% of life-time anxiety disorders and 43% of life-time substance abuse disorders (mainly alcohol abuse). Bipolar disorder 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  $35 \pm 13$  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  $33.7 \pm 10.2$  years, 51% female) were recruited and screened for 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, Suppl. Table 1; haplotype analysis, Suppl. 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, Suppl. Table 3; haplotype analysis, Suppl. Table 4) and aADHD (single marker data, Suppl. Table 5; haplotype analysis, Suppl. 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)  $\geq 75\%$  were included in the study; of those, genotype frequencies were ascertained for overall Hardy-Weinberg equilibrium (HWE;  $\chi^2$  HWE p-value  $\geq 0.01$ ). 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 less than 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  $\chi^2$  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 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 of 1.5 (corresponding to a relative risk of 1.48) to develop bipolar disorder assuming a co-dominant model and a 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.*, 2010, this study could not be included in the meta-analysis): Odds ratios (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 ( $I^2=Q\text{-df}/Q$ ). In 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, Suppl. Tab. 3) and aADHD (Table 1, Suppl. Tab. 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 twelve 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 which 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 which were only

associated in one of the subsamples (compare Suppl. Table 1 with Table 1). Most of the replicated SNPs mapped to *DGKH* (BPD: 6 SNPs; UPD: 7 SNPs; aADHD: 4 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 is given in Suppl. Table 2; UPD significant findings: Table 2, complete data is given in Suppl. Tab. 4; aADHD significant findings: Table 2, complete data is given in Suppl. Tab. 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 to 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 (Tables 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 to 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 from the studies by Baum et al, 2008a, Ollila et al, 2009 and Squassina et al, 2009 to a formal meta-analysis (Figure 3, Supplemental Table 8). While rs9315885, which was significant in the three other studies but not our dataset, 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 to the 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 which 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 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 (Athanasios *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 *CACNA1C* so that we abstained from testing them further. Several reasons for this lack of replication have to be considered: a) lack of power owing to the sample size of n=380 bipolar patients, as compared to the huge number of patients tested in current GWA studies; b) 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; c) 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; d) 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; Jin *et al*, 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 and associates (Sklar *et al*, 2008), *CMTM8* was identified in the WTCCC dataset

(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 its 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 could however not find support for an involvement of *EGFR* variation in BPD, in contrast to Sklar and colleagues (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 GWA studies, when combined, can identify 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). In 2003, Kamnasaran reported on a family in which a



disruption of *NPAS3* segregates with schizophrenia (Kamnasaran *et al*, 2003) 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 and associates could show that common genetic variation in the *NPAS3* gene is associated with both schizophrenia and BPD (Pickard *et al*, 2009). 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 and colleagues (Baum *et al*, 2008a; Baum *et al*, 2008b). 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 GSK3 $\beta$ . 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, six 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 eight 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, i.e. 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, i.e. amino acids 65 to 243. Three non-synonymous variants (no frequency data available) are deposited in databases, rs59790803 causing a G>A transition resulting in an 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 protein kinase C. 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 either by 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 studies, e.g. *CACNA1C* and *ANK3*. The integration of such findings might pinpoint distinct molecular pathways whose identification might enhance psychiatric diagnostics and research on neurobiological underpinnings.

### **Disclosure/Conflict of Interest**

None of the authors reported any biomedical financial interests or potential conflicts of interest.

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## Tables

**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). 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.

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
<b>CMTM8</b> ; Chromosome 3												
rs12496256	G	A	0.336	0.282	<b>0.013</b>	1	0.303	0.181	1	0.313	0.250	1
rs6803740	G	A	0.165	0.210	<b>0.014</b>	1	0.183	0.289	1	0.198	<b>0.041</b>	0.941
<b>DGKH</b> ; Chromosome 13												
rs1170191	A	G	0.159	0.197	<b>0.026</b>	1	0.213	<b>0.003</b>	0.059	0.170	0.455	1
rs1170169	G	C	0.303	0.405	<b>1.5*10<sup>-4</sup></b>	<b>0.013</b>	0.402	<b>2.8*10<sup>-4</sup></b>	<b>0.006</b>	0.354	<b>0.046</b>	1
rs2148004	G	A	0.317	0.280	0.056	1	0.256	<b>0.005</b>	0.114	0.289	0.152	1
rs994856	G	A	0.401	0.455	<b>0.017</b>	1	0.454	<b>0.024</b>	0.556	0.454	<b>0.012</b>	0.277
rs9525580	A	G	0.232	0.312	<b>8.1*10<sup>-5</sup></b>	<b>0.007</b>	0.284	<b>0.012</b>	0.267	0.293	<b>0.001</b>	<b>0.023</b>
rs9525584	C	T	0.481	0.423	<b>0.013</b>	1	0.391	<b>1.2*10<sup>-4</sup></b>	<b>0.003</b>	0.444	0.083	1
rs1170101	G	A	0.277	0.349	<b>7.1*10<sup>-4</sup></b>	0.063	0.353	<b>4.0*10<sup>-4</sup></b>	<b>0.009</b>	0.304	0.152	1
rs347405	C	G	0.449	0.493	0.056	1	0.468	0.423	1	0.492	<b>0.043</b>	0.987
<b>NPAS3</b> ; Chromosome 14												
rs17455703	G	A	0.296	0.253	<b>0.041</b>	1	0.252	0.070	1	0.250	<b>0.016</b>	0.359
<b>SLC39A3</b> ; Chromosome 19												
rs4806874	G	A	0.314	0.269	<b>0.033</b>	1	0.293	0.327	1	0.309	0.788	1

**Table 2:** Association of haplotypes with the complete BPD, UPD and aADHD samples containing at least one significant haplotype. Bold, p<0.05

<b>CMTM8</b>			<b>Complete BPD Sample (N=380)</b>			<b>Complete UPD Sample (N=387)</b>			<b>Adult ADHD Sample (N=535)</b>		
Chromosome 3; Block 1			Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation
rs6550109	rs12496256		Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value
		T	G		<b>0.274/0.336</b>	<b>0.004</b>	<b>0.183</b>	<b>0.298/0.336</b>	<b>0.124</b>	<b>0.942</b>	<b>0.311/0.336</b>
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			Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation
rs4955272	rs7644602	rs7632109	Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value
			G	G	A	0.183/0.218	0.054	0.949	0.211/0.218	0.709	1
G	G	G	<b>0.239/0.23</b>	<b>0.667</b>	<b>1</b>	<b>0.199/0.23</b>	<b>0.134</b>	<b>0.973</b>	<b>0.185/0.23</b>	<b>0.016</b>	<b>0.251</b>
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			Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation
rs4276227	rs6803740		Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value
		C	G		<b>0.210/0.165</b>	<b>0.013</b>	<b>0.519</b>	<b>0.186/0.165</b>	<b>0.225</b>	<b>0.952</b>	<b>0.194/0.165</b>
T	A		0.306/0.325	0.393	1	0.335/0.325	0.627	1	0.328/0.325	0.870	1
			0.484/0.510	0.270	1						
C	A					0.479/0.510	0.170	0.853	0.478/0.510	0.086	0.768

**DGKH**

Chromosome 13; Block 1			Complete BPD Sample (N=380)			Complete UPD Sample (N=387)			Adult ADHD Sample (N=535)		
rs1170191	rs1170169	rs2148004	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation
			Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value
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 <sup>-4</sup>	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			Complete BPD Sample (N=380)			Complete UPD Sample (N=387)			Adult ADHD Sample (N=535)		
rs994856	rs9525580	rs9525584	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation
			Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value
A	G	C	0.417/0.48	0.009	0.406	0.39/0.48	1.6*10 <sup>-4</sup>	0.021	0.445/0.48	0.120	0.873
G	A	T	0.312/0.228	2.9*10 <sup>-5</sup>	0.001	0.288/0.228	0.004	0.056	0.296/0.228	3.0*10 <sup>-4</sup>	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
<b>NPAS3</b>											
Chromosome 14; Block 1			Complete BPD Sample (N=380)			Complete UPD Sample (N=387)			Adult ADHD Sample (N=535)		
rs8015959	rs17455703		Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation	Case/Control	Nominal	Permutation
			Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value	Frequencies	P-Value	P-Value
C	G		0.253/0.296	0.039	0.883	---	---	---	---	---	---
T	A		0.024/0.026	0.823	1	---	---	---	---	---	---
			0.723/0.678	0.038	0.877						
C	A					---	---	---	---	---	---

## Figures

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

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

**Figure 3.** Forest Plots displaying meta-analyses of minor vs. major allele of rs9315885 (A) and rs1170191 (B).

rs1170191

rs1170169

rs2148004

rs994856

rs9525580

rs9525584

rs347405

rs9315899

Block 1 (22 kb)

1

2

3

Block 2 (26 kb)

4

5

6

Block 3 (3 kb)

9

10

99

97

73

98

97

6

99

45

61

97

93

16

12

79

77

22

32

17

97

50

3

97

45

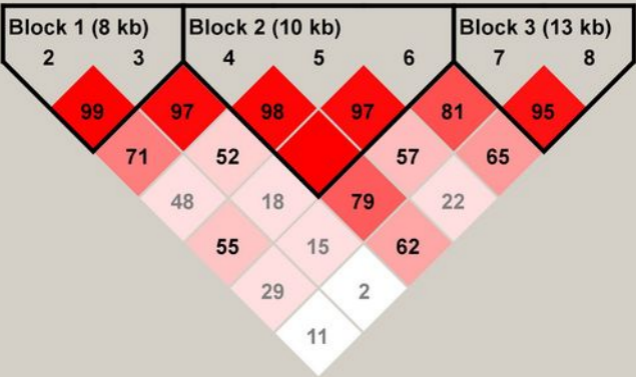
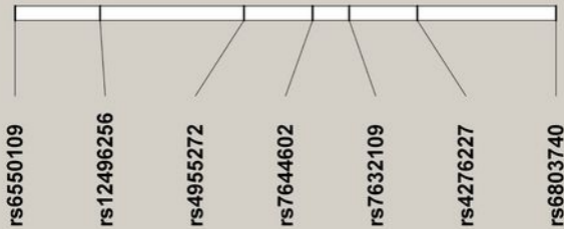
64

2

17

94





Sample rs9315885	Cases		Controls		Odds Ratio M-H, Fixed, 95% CI
	Events	Total	Events	Total	
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)**

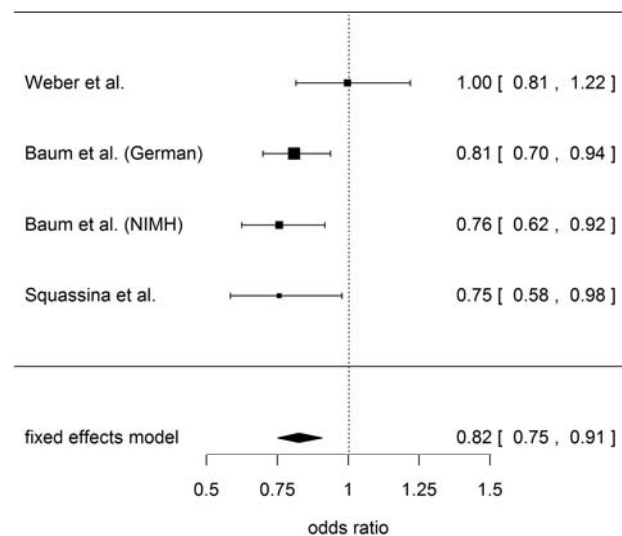
Total events                      **3650**                      **4710**                      0.82 [0.75, 0.91]

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-Haenszel-Test:  $CMH = 16.07$ ;  $df = 4$  ( $p = 0.0001$ )



Sample rs1170191	Cases		Controls		Odds Ratio M-H, Random, 95% CI
	Events	Total	Events	Total	
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)**

Total events                      **582**                      **892**                      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:  $Z = -1.008$  ( $P = 0.314$ )

