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***DIRAS2* is associated with adult ADHD, related traits, and co-morbid disorders**

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Abstract

Several linkage analyses implicated the chromosome 9q22 region in attention-deficit / hyperactivity disorder (ADHD), a neurodevelopmental disease with remarkable persistence into adulthood. This locus contains the brain-expressed GTP-binding RAS-like 2 gene (*DIRAS2*) thought to regulate neurogenesis. As *DIRAS2* is a positional and functional ADHD candidate gene, we conducted an association study in 600 patients suffering from adult ADHD and 420 controls. Replication samples consisted of 1035 adult ADHD patients and 1381 controls, as well as 166 families with a child affected from childhood ADHD. Given the high degree of co-morbidity with ADHD, we also investigated patients suffering from bipolar disorder (n=336) or personality disorders (n=622). Twelve SNPs covering the structural gene and the transcriptional control region of *DIRAS2* were analyzed. Four SNPs and two haplotype blocks showed evidence of association with ADHD, with nominal p-values ranging from p=0.006 to p=0.05. In the adult replication samples, we obtained a consistent effect of rs1412005 and of a risk haplotype containing the promoter region (p=0.026). Meta-analysis resulted in a significant common OR of 1.12 (p=0.04) for rs1412005 and confirmed association with the promoter risk haplotype (OR=1.45, p=0.0003). Subsequent analysis in nuclear families with childhood ADHD again showed an association of the promoter haplotype block (p=0.02). rs1412005 also increased risk towards bipolar disorder (p=0.026) and cluster B personality disorder (p=0.031). Additional SNPs showed association with personality scores (p=0.008-0.048). Converging lines of evidence implicate genetic variance in the promoter region of *DIRAS2* in the etiology of ADHD and co-morbid impulsive disorders.

Introduction

Attention deficit/hyperactivity disorder (ADHD) is a prevalent psychiatric condition in childhood associated with substantial impairment. In up to 30% of all affected cases, the disorder persists into adulthood (adult ADHD, aADHD; (Faraone *et al*, 2000), although the core symptoms become less prominent, as adaptive mechanisms interfere with the presentation of the disease and as co-morbid conditions modify or even dominate the clinical picture. The latter include substance use disorders, affective and personality disorders (Jacob *et al*, 2007). Furthermore, there is accumulating evidence for neurobiological overlap with bipolar disorder (BD), with a high percentage of adult ADHD patients also suffering from BD (Klassen *et al*, ; Wingo and Ghaemi, 2007) and even more from bipolar symptoms on the dimensional level (Halmoy *et al*). Conversely, approximately 10% of BD patients suffer from ADHD, suggesting a subgroup of patients where pathologies overlap and presumably risk genes are shared (Nierenberg *et al*, 2005; Tamam *et al*, 2008). Adult ADHD patients feature a distinct personality profile, with increased scores in the personality domain *Neuroticism* as measured with the NEO PI-R (Costa and McCrae, 1992), while *Conscientiousness*, *Extraversion*, and *Openness* are decreased (Jacob *et al*, 2007). Likewise, personality assessment using the TPQ (Cloninger *et al*, 1993) reveals that *Novelty Seeking* and *Harm Avoidance* are increased in aADHD patients (Jacob *et al*, 2007). Taken together, the clinical appearance of aADHD represents a complex clinical phenotype.

ADHD in children (cADHD) was repeatedly shown to feature a strong genetic component, and a meta-analysis of twenty studies provided a heritability index of 0.76 (Faraone *et al*, 2005). However, despite this considerable heritability, only a few risk genes with small effects have been identified. This might be due to the facts that cADHD a) is a heterogeneous clinical condition (with the risk of phenocopies), b) might feature substantial ethnic background effects, and c) is a genetically complex disorder with common variants conveying low risk (Franke *et al*, 2009) as well as individually rare variants with variable penetrance conveying high risk (Becker and Knapp, ; Lesch *et al*, ; Williams *et al*). Adult ADHD has come into the focus of genetic research more recently, but has also been shown to have a genetic basis (IMpACT Collaboration, submitted). Molecular genetic findings are even more sparse; this might partly be due to the fact that not only risk variants causing disease as such come into play, but also genes modifying the developmental trajectory of the disorder – i.e., persistence into adulthood (e.g., *SLC6A3*; (Franke *et al*) – may be of importance.

To overcome the problematic issues coming along with candidate gene studies, genome-wide approaches were increasingly used in the last years. These include genome-wide linkage analyses, eight of which have been published so far; seven of these were included in a meta-analysis (Zhou *et*

al, 2008). Furthermore, genome-wide association studies (GWAS) were employed to find new risk genes (Franke *et al*, 2009; Neale *et al*, ; Neale *et al*). We have previously reported on a pooled GWAS in aADHD (Lesch *et al*, 2008), where two single nucleotide polymorphisms (SNPs) in the top 500 localized to the intergenic region between *DIRAS2* and *SYK* on chromosome 9q22.2. The *DIRAS2/SYK* locus is also supported by two linkage scans, one of which was performed in individuals from the same catchment population as the aADHD GWAS (Romanos *et al*, 2008), while the other was conducted in the UK (Asherson *et al*, 2008). Further interest in this region comes from a pooled GWAS in personality disorder, where *SYK* was the number 11 top-locus for Cluster B personality disorder (in preparation). Thus, three independent and hypothesis-free studies provide converging evidence that this locus might contribute to the genetic risk for disorders of cognitive control featuring increased impulsivity. *DIRAS2* is strongly expressed in the brain, while *SYK* is preferentially expressed in peripheral tissues, making *DIRAS2* the prime candidate gene at this locus. Intriguingly, in a recent GWAS on cognitive performance of the aging brain, *DIRAS2* was associated ($p=8 \times 10^{-6}$) with performance in the Boston naming test arguing for a role of *DIRAS2* in cognitive processes (Seshadri *et al*, 2007).

These findings prompted us to conduct a study using large discovery and replication aADHD samples to assess the genetic relevance of *DIRAS2* for adult ADHD. In addition, we attempted generalization of the findings to cADHD and to related disorders of impulsivity. As the discovery samples also were assessed for personality factors, we were also able to test the influence of *DIRAS2* on impulsivity-related traits which might be considered an intermediate phenotype of ADHD.

Materials and Methods

Samples

Discovery sample: adult ADHD sample

The sample was previously described in greater detail (Franke *et al*, ; Jacob *et al*, 2007). Briefly, 600 patients were extensively examined using a semi-structured interview as well as by applying the SCID-I and SCID-II. Personality assessment was done using NEO PI-R and TPQ. Eligibility criteria for the study were aADHD according to the diagnostic criteria of DSM-IV, onset before the age of 7 years, life-long persistence, current diagnosis and age at recruitment between 18 and 65 years. Exclusion criteria were a current diagnosis of drug/alcohol abuse/dependence (prior to detoxification /withdrawal), or a lifetime diagnosis of BD, schizophrenia and mental retardation. A more detailed sample description including the used rating scales can be obtained from Supplementary Methods. The control group was recruited from the Lower Franconia area in Germany and consisted of 420

healthy volunteers. This sample of volunteers was also used as a control for the association analysis for personality disorder and BD.

Family-based childhood ADHD sample

Detailed information on the sample was reported previously (Renner *et al*, 2008). Briefly, total sample size was 166 families, of whom 109 were trios, 42 quartets and 15 were multi-sibling families. We recruited families with at least one child affected by ADHD, combined type according to DSM-IV as diagnosed by a structured interview with the parents (KIDDIE-SADS), parent and teacher questionnaires and direct clinical observation. The index child was aged 8 years or above. Exclusion criteria were IQ below 80, co-morbid autistic or somatic disorders, primary affective disorders including BD, Tourette's syndrome, psychotic or other severe primary psychiatric disorders.

IMpACT adult ADHD samples

The adult ADHD replication samples have been described in detail elsewhere (Franke *et al*, 2010). The total sample included 1035 adult ADHD patients and 1381 controls and originated from The Netherlands (285 patients and 508 controls), Norway (497 cases and 562 controls), Spain (253 patients and 311 controls), and was made available within the framework of the IMpACT consortium. All patients were evaluated by experienced psychiatrists and diagnosed with persistent ADHD according to DSM-IV (Diagnostic and Statistical Manual for Mental Disorders) criteria and also using standardized rating scales. Consensus eligibility criteria for this study across all study sites were a diagnosis of ADHD according to the diagnostic criteria of DSM-IV, onset before the age of 7 years by retrospective diagnosis (which was confirmed by a family member, wherever possible), life-long persistence and current diagnosis. Further information on the samples as well as used rating scales can be obtained from Supplementary Methods.

Personality disorder sample

This sample has been described in detail previously (Jacob *et al*, 2005). A total of 622 inpatients and outpatients of the Department of Psychiatry and Psychotherapy, University of Würzburg with personality disorders according to DSM-IV criteria participated in the study. Patients were drawn from the general population of hospital patients; every patient with a clinical diagnosis of personality disorder treated in the department from 2000 to 2004 was approached and asked to participate in the study. Inclusion criteria were personality disorder (PD) according to DSM-IV (antisocial, histrionic, borderline, narcissistic, avoidant, dependent, and obsessive-compulsive personality disorder) and age between 18 and 60 years. Exclusion criteria were medical conditions and lifetime diagnosis of schizophrenia or other psychotic disorders. The SCID-II was used to diagnose PDs, and PDs were

allocated to cluster B and C operationalized as follows: cluster B (77%) (dramatic-emotional) encompasses antisocial, borderline, histrionic, and narcissistic PDs. Cluster C (37%) (anxious-fearful) includes avoidant, dependent, and obsessive-compulsive PDs. Furthermore, co-morbid axis-I disorders were assessed using the SCID-I as well as extensive clinical interview. Thereby, a lifetime diagnosis of BD (type I and II) was established in 17 of the 622 personality disorder patients (2.7%). The assessment of the PDs, including all psychometric testing, was performed by a single experienced psychiatrist (C.P.J.).

Bipolar disorder sample

The present study investigated a sample described previously in detail (Scholz et al, 2010). This sample consisted of 170 unrelated bipolar patients (mean age 53 ± 14 years, 68 % 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 336 patients suffering from BPD. Co-morbidity data for BD 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. None of the subjects showed significant neurological co-morbidity, epilepsy, mental retardation, or other somatic disorders suggesting organic psychosis. Patients with substance-induced disorders were excluded.

In silico replication in the SHIP GWAS

Data from the Study of Health in Pomerania (SHIP) were used (Grabe et al, 2005). The target population was comprised of adult German residents (20-79 years) in a northeastern German population of 212,157 individuals. The net sample comprised 6,267 eligible subjects, of which 1,859 consecutively recruited subjects were eligible for the present analyses. The assessment of personality traits was performed with the NEO-FFI-30 (Korner et al, 2008). The sample was genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0. As most of the *DIRAS2* SNPs genotyped in the discovery sample (Table 1) were not captured, genotype data were derived from standard imputation procedures using the software IMPUTE v0.5.0 based on HapMap II.

All cases and controls of the above samples were of self reported German (or Danish, respectively) ancestry. The study was approved by the respective ethics committees of the participating centers and complied with the latest version of the Declaration of Helsinki. Written informed consent was provided by all participants.

Marker selection and genotyping

14 SNPs were selected, which tagged the promoter region, the 5' and 3' UTRs as well as the transcribed exon of the *DIRAS2* gene, spanning a chromosomal region of 33 kb. Marker selection was performed using the Tagger algorithm (parameter: pair-wise tagging only, r^2 threshold=0.8, minor allele frequency above 5%) as implemented in HaploView (www.broad.mit.edu/mpg/haploview) (Figure 1; the haplotype structure was comparable in each sample). SNPs were genotyped using the Sequenom MassArray® system (Sequenom, San Diego, CA) coupled to a Bruker Autoflex mass spectrometer (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions. PCR was performed using iPLEX chemistry as recommended in the MassArray® iPLEX standard operating procedure. Primer sequences can be obtained on request. rs12350590, located proximal of the *DIRAS2* gene, and rs9695432 in intron 1, were excluded from further analyses due to minor allele frequency (MAF) <0.01 in the discovery sample.

Statistical analysis

For all samples, we analyzed only SNPs passing quality check which was defined by MAF>0.01, call rate>90% and p value of χ^2 test for Hardy-Weinberg equilibrium>0.01 in controls or parents. Inconsistent genotypes in the family sample were revealed and removed by means of PedCheck (O'Connell and Weeks, 1998). Generalized linear models (GLM) implemented in the R function glm() were carried out to test for an association between SNP genotype as independent variable and a trait of interest as dependent variable (binary: ADHD, bipolar, cluster B and C personality disorder; quantitative: NEO-PI R (*Neuroticism, Extraversion, Openness, Agreeableness, Conscientiousness*) and TPQ (*Novelty Seeking, Harm Avoidance, Reward Dependence*)). Binomial logit link or Gaussian identity link was used for binary or quantitative traits, respectively. We also performed Fisher's exact test in order to assure the asymptotic test results of glm(). Family-based association analysis was carried out using pedigree disequilibrium test (sumPDT, (Martin *et al*, 2000)) to assess whether an allele is more frequently transmitted to affected offspring by taking relationships between the offspring into account.

We used the omnibus test implemented in famhap (10000 simulations; (Becker *et al*, 2004) to test the global hypothesis that association with ADHD may be contributed by at least one of the estimated haplotypes. The LD blocks were defined by pairwise $D'>0.65$ assessed using Haploview.

After the haplotype ACGCTT from block 2 (consisting of SNPs rs1331503, rs2297354, rs1331504, rs7848810, rs1412005, rs689687) was identified as the sole haplotype consistently increasing risk across all samples, a haplotype-specific association test using Unphased (Dudbridge, 2008) was carried out.

For the meta analysis, we assumed a common effect of the rs1412005 T allele and the ACGCTT haplotype on the risk for ADHD, as there was no effect heterogeneity across all four considered aADHD case-control samples (p value = 0.43 and 0.29, respectively, Woolf's test for heterogeneity, obtained with the R function `meta.MH()`). A joint analysis assessing the common OR was done by means of GLM described above, adjusted for fixed country effect. This approach was also applied to the total data set including all patients (adult ADHD, bipolar and personality disorders) and controls in order estimate the cross disorder effects of *DIRAS2* SNPs.

Unless otherwise specified, p values are two-sided p values unadjusted for multiple testing. The significance level was set to 0.05. Our study primarily aimed at detecting ADHD-associated SNP variants of *DIRAS2*, whereas analyses for other traits were exploratory. Therefore, we used the Bonferroni method to calculate p values adjusted for testing multiple SNPs and haplotypes for association with ADHD.

Power calculations were performed under a two-sided nominal significance level of 0.05 using QUANTO Version 1.0 (<http://hydra.usc.edu/gxe>). We assumed an effect size between 1.1 and 1.5 for the rs1412005 T allele and between 1.3 and 3.0 for the ACGCTT haplotype. By given disease prevalence of 5%, we computed the power to detect these variants in the replication sample consisting of 1035 aADHD patients and 1381 controls from IMpACT, under a frequency of 25% and 5% for the rs1412005 T allele and the ACGCTT haplotype, respectively. All assumptions nearly corresponded with nominal 95% confidence intervals and frequencies estimated from the discovery sample.

Results

Association of DIRAS2 with adult ADHD

Table 1 displays information about functional and physical location of each SNP in/near *DIRAS2*. In the discovery sample of 600 adult ADHD patients and 420 controls from Germany, four out of 12 investigated SNPs had $p < 0.05$ in the association test with aADHD (Table 2). An empirical test at the gene level for association of at least one of 12 SNPs yielded a $p = 0.0127$ (10,000 permutations). Upon haplotype testing, both LD blocks (Figure 1) showed evidence of association with nominally significant p values ($p = 0.006$ for block 1 and $p = 0.033$ for block 2).

Replication in 1035 aADHD patients and 1381 controls from IMpACT did not show any significant SNP association (all $p > 0.07$; Table 2). Also for each individual country, we found neither an association on the single marker ($p > 0.1$, Suppl Table 1) nor on the haploblock level ($p > 0.09$). However, consistency of the effect size estimates was observed indicating increased aADHD risk for carriers of the A allele of rs1331503, the T allele of rs1412005 and the haplotype ACGCTT from block 2 (consisting of SNPs rs1331503, rs2297354, rs1331504, rs7848810, rs1412005, and rs689687; Table 3) across the four samples. Association testing for this specific haplotype provided a significant result in the initial sample from Germany ($p = 0.0006$) which replicated in the additional independent combined samples from IMpACT ($p = 0.026$). To further explore the association of *DIRAS2* with aADHD, we performed five meta-analyses, for the best four SNPs of the discovery sample (rs7854469, rs16906711, rs1331503, rs1412005) and for the risk haplotype ACGCTT, in all 1635 aADHD cases and 1801 controls from Germany, The Netherlands, Spain and Norway taking population strata into account. A $p = 0.04$ was found for the rs1412005 T allele in the association test with aADHD (p value adjusted for five tests: 0.22; OR=1.12, 95%-CI: 1.00-1.25; Figure 2). The risk haplotype ACGCTT (9.0% in cases and 6.5% in controls) was associated with an increased risk for aADHD, and this effect was statistically significant even after adjusting for multiple testing ($p = 0.00028$, p value adjusted for five tests: 0.001, OR=1.45, 95% CI: 1.19-1.77; Figure 3). This haplotype contains the T allele of SNP rs1412005.

DIRAS2 and childhood ADHD

In order to extend our findings for *DIRAS2* to cADHD, we also analyzed 166 independent nuclear families with offspring affected by cADHD. The rs1412005 T allele was not significantly over-transmitted ($p = 0.65$), but the C allele of the neighboring SNP rs7848810 – also a promoter SNP – was ($p = 0.046$, transmission rate: 53%; Table 4). Haplotype analysis provided evidence of significant association of the haploblock 2 ($p = 0.0231$) with a p value for the specific haplotype ACGCTT slightly exceeding 0.05 ($p = 0.0692$, frequency among transmitted versus non-transmitted haplotypes: 11% versus 6.5%, OR=2.01, 95% CI: 0.80-5.00). rs7848810 was also associated with co-morbid dyslexia ($p = 0.015$).

Expanding the phenotype: association of DIRAS2 with personality disorders and bipolar disorder

To examine whether *DIRAS2* is specifically linked to ADHD or whether the association generalizes to disorders sharing the feature of impulsivity and emotional instability (independent of co-morbidity with ADHD), we performed association analyses in two further independent samples: patients suffering from personality disorders and BD. We found nominal evidence of association of rs1412005, the SNP implicated in aADHD (see above), with Cluster B personality disorder ($p = 0.031$) and BD ($p = 0.026$). The OR of the T allele for both disorders was estimated at around 1.26 (Table 5).

The T allele also tended towards increasing risk for cluster C personality disorder ($p=0.086$) with the same OR estimate of 1.26.

DIRAS2 relates to personality factors linked to aADHD

The Würzburg aADHD and personality disorder samples had also been tested for personality dimensions, as assessed by two questionnaires (NEO-PI R: *Neuroticism, Extraversion, Openness to Experience, Agreeableness, Conscientiousness*; and TPQ: *Novelty Seeking, Harm Avoidance, Reward Dependence*). Given the association of *DIRAS2* variants with personality disorders (see above), and considering that aADHD goes along with a distinct personality profile (Jacob *et al*, 2007), we additionally investigated whether personality scores may be also be affected by *DIRAS2* variants. Table 6 represents all nominally significant association results. In the aADHD sample, associations with $p<0.05$ were found for *Neuroticism, Extraversion, Agreeableness, Conscientiousness* and *Harm Avoidance* (p values between 0.002 and 0.033). The three SNPs rs1331503, rs1412005 and rs689687, which were associated with these personality traits, are located in haplotype block 2 and the transcriptional control region of the gene. In the PD sample, nominal evidence of association was found for *Extraversion, Agreeableness, Conscientiousness, Novelty Seeking* and *Harm Avoidance* (p values between 0.008 and 0.040; Table 6). In comparison to the aADHD sample, different SNPs showed association in this sample and almost all of them are located in the haplotype block 1. However, rs7854469 was associated both with aADHD (see above) *and* the ADHD-relevant personality trait *Extraversion* ($p=0.0075$, Table 6) in PD. Also the aADHD-associated SNP rs7848810 was associated with TPQ *Novelty Seeking* ($p=0.0396$, Table 6) and NEO PI-R *Conscientiousness* ($p=0.0123$, Table 6) in the PD sample.

Due to the pitfalls of multiple testing, inevitable in personality genetics, replication attempts are paramount. We have thus analyzed 1859 subjects from a general population study (SHIP) who have been examined for personality factors; apart from rs7848810, SNPs had to be imputed from data acquired on the Affymetrix Human SNP Array 6.0 platform. In doing so, rs16906711 was found to be associated with NEO PI-R *Neuroticism* ($p=0.046$, Suppl Table 2), and rs1331504 with NEO PI-R *Conscientiousness* ($p=0.002$, Suppl Table 2).

Discussion

Based on the results of four hypothesis-free approaches converging on the chromosomal locus 9q22.2 - two linkage scans for cADHD, a pooled GWAS of adult ADHD and a pooled GWAS of personality disorder - we examined the *DIRAS2* gene by genotyping 14 markers, selected to tag the

promoter region, the 5' and 3' UTRs and the only coding exon of the gene, in a total of seven independent patient samples. ADHD findings replicated across adult ADHD samples, with meta-analytic treatment implicating the ACGCTT haplotype (which spans the regulatory region of *DIRAS2*) and one of the SNPs contributing to this haplotype (rs1412005, T allele), and generalized to ADHD in childhood. However, after correction for multiple testing, only the association of the haplotype remained significant arguing that the association of *DIRAS2* with ADHD most likely is not due to one of the examined single markers but rather to a variant segregating with this risk haplotype. Furthermore, expanding the phenotype to impulsivity disorders and impulsivity-related traits pointed towards a role for *DIRAS2*. While two of the associated SNPs are located in the 3' UTR and potentially involved in expression regulation (rs7854469 and rs16906711), two others (rs7848810 and rs1412005) were located upstream of the transcription start site (Figure 1). Scanning of the transcriptional control region SNPs for putative overlapping transcription factor binding motifs revealed that the sequence carrying the major allele of rs7848810 contained predicted response elements for PBX1 and for the PBX-HOXA9 dimer. PBX1 is known for its developmental regulation of neuropeptide Y expression (Mayer *et al*, 2003), whereas PBX-HOX dimers generally play important roles in vertebrate development (Moens and Selleri, 2006). Minor allele carriers may therefore have an impaired developmental expression of *DIRAS2*. Meta-analytic treatment of the aggregated ADHD data provided further evidence especially for ACGCTT haplotype, which includes the rs1412005 T allele and spans the regulatory region of *DIRAS2*, to be associated with aADHD. We therefore hypothesize that alterations in the expressional control of *DIRAS2* underlie the ADHD association, although further studies have yet to corroborate our findings.

Evidently, association of *DIRAS2* is not specific to ADHD, but also occurs for other phenotypes such as bipolar and personality disorders. A cross disorder analysis for all disorders combined (aADHD, bipolar, and personality disorder) provided an OR effect for the rs1412005 T allele of 1.13 (95%CI: 1.02-1.26, $p=0.02$). Given the considerable co-morbidity of these disorders with aADHD, our findings may explain an (although presumably small) part of the genetic variance underlying these clinical observations. Clues to which neurobiological (endo-)phenotype is modulated by *DIRAS2* might be obtained from personality genetics, as nine *DIRAS2* SNPs were associated with personality domains in aADHD or PD patients. Specifically, aADHD patients carrying the rs1412005 T allele, showing meta-analytic evidence of increasing risk towards aADHD, exhibit on average 3.6 point lower *Conscientiousness* and 1.4 point higher *Harm Avoidance* scores than non-carriers (Table 6). Thus, *DIRAS2* seems to predispose to a characteristic personality profile going along with ADHD, which might best be characterized by reduced *Conscientiousness* and increased *Harm Avoidance*. However,

it has to be noted that associations were quite disparate between samples so that these data can only be considered preliminary ahead of further replication.

What are the molecular mechanisms underlying these association findings? As almost nothing is known about the function of *DIRAS2*, we can only speculate. The expression profile of *DIRAS2* is specific to the brain displaying marked expression in the prefrontal cortex, the anterior cingulate cortex and the amygdala. While the former two structures are involved in working memory, error detection and executive control – all of which are impaired in ADHD – the latter is also a key component in fear conditioning and anxiety; dampened amygdala response might well lead to increased risk taking behavior. While *DIRAS2*'s expression pattern endorses a potential pathophysiological role in ADHD and personality, the relevant molecular cascades are elusive. Di-Ras2, the protein encoded by *DIRAS2*, belongs to the small GTPase Ras family, whose members have role in cell morphogenesis and neurogenesis. Within the Ras family, Di-Ras2 seems to have unusual biochemical properties (Kontani *et al*, 2002). Unlike other Ras kinases, it fails to activate the MAP kinase, phosphoinositide 3-kinase or AKT pathways. To date, no effector mechanisms have been described, and due to its specific catalytic characteristics, it is even unclear whether Di-Ras2 is regulated by GTP binding at all. Highly selective expression in raphe serotonin neuron subpopulations (Wylie *et al*) points to a role in the regulation of the caudal serotonergic system; however, precise pathways again remain elusive. Despite it being a commonplace statement, in this case further studies are clearly needed to even speculate on the neurobiological and behavioral role of Di-ras2.

Several caveats have to be considered in the interpretation of our data. Given the genetic effect sizes (odds ratio) estimated in the discovery sample (1020 German individuals), our replication sample (2416 individuals from IMpACT) had sufficient power to detect the effects of the rs1412005 T allele (59-100%) and the ACGCTT haplotype (88-100%). Despite this fact, there still is a chance of false-positive findings. This is relevant as most associations on the single marker level would not withstand correction for multiple testing, which becomes an even more prominent issue when examining multi-axial dimensional data such as personality traits. However, since we achieved the essential goal of targeted replication in several independent populations at least for the risk haplotype, and in conjunction with previous evidence from linkage studies and GWAS, we are confident to report a true association. As all nearby (\pm one megabase) genes are not expressed in the brain, it appears unlikely that genetic variants outside of *DIRAS2*, but in linkage disequilibrium with the scrutinized polymorphisms are pathophysiologically relevant. We therefore suspect that genetic variation within *DIRAS2*, in linkage disequilibrium with the interrogated SNPs, are the “true” underlying risk alleles, a hypothesis which will have to be tested in further studies using re-sequencing approaches followed

by functional studies. Finally, as we did not obtain strong positive findings from all samples, small individual sample size, background, epistatic or founder effects or even gene-environment interaction may also play a role explaining that *DIRAS2* genetic variants contribute to ADHD and related phenotypes in some, but not all populations.

In conclusion, converging evidence from several hypothesis-free and hypothesis-driven approaches suggest that *DIRAS2* is a candidate gene for ADHD and related behavioral domains. Due to lack of data on the encoded protein, the underlying molecular mechanisms nevertheless are far from being understood, so that future functional studies will have to bridge the genotype-phenotype gap.

Conflict of interests: In the past 3 years, Jan K. Buitelaar has been a consultant to/member of advisory board of and/or speaker for Janssen Cilag BV, Eli Lilly, Bristol-Myer Squibb, Organon/Shering Plough, UCB, Shire, Medice and Servier. He is not an employee of any of these companies. He is not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents or royalties. Sandra Kooij has received research grants from Janssen BV en Shire, and is on the speaker's bureau of Janssen and Eli Lilly. All other authors declare no conflicts of interest.

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Tables and Figures

Figure 1. LD plot of *DIRAS2* aligned to the gene structure and the significant SNPs from all samples. Please note that the LD plot is not exactly matched to the SNPs, as precise scaling was not possible: rs9695432 is the last SNP of block 1, while rs1331503 is the first SNP of block 2. rs16906711 is associated with aADHD, Cluster C PD, Cluster B PD, and Harm Avoidance in PD; rs9695432 with aADHD and personality domains; rs1331503 with aADHD and personality domains; rs2297354 with aADHD and personality domains; rs7848810 with aADHD, Cluster B PD, BD, and personality domains; and rs1412005 with aADHD and personality domains.

Figure 2. Forest plot of the rs1412005 T allele for the aADHD case-control association study.

Figure 3. Forest plot of the ACGCTT haplotype for the aADHD case-control association study.

Table 1. General SNP information with minor allele frequency (MAF, %).

Table 2. SNP results of case-control based association analyses for aADHD.

Table 3. Association analysis of the ACGCTT haplotype for the aADHD case-control samples.

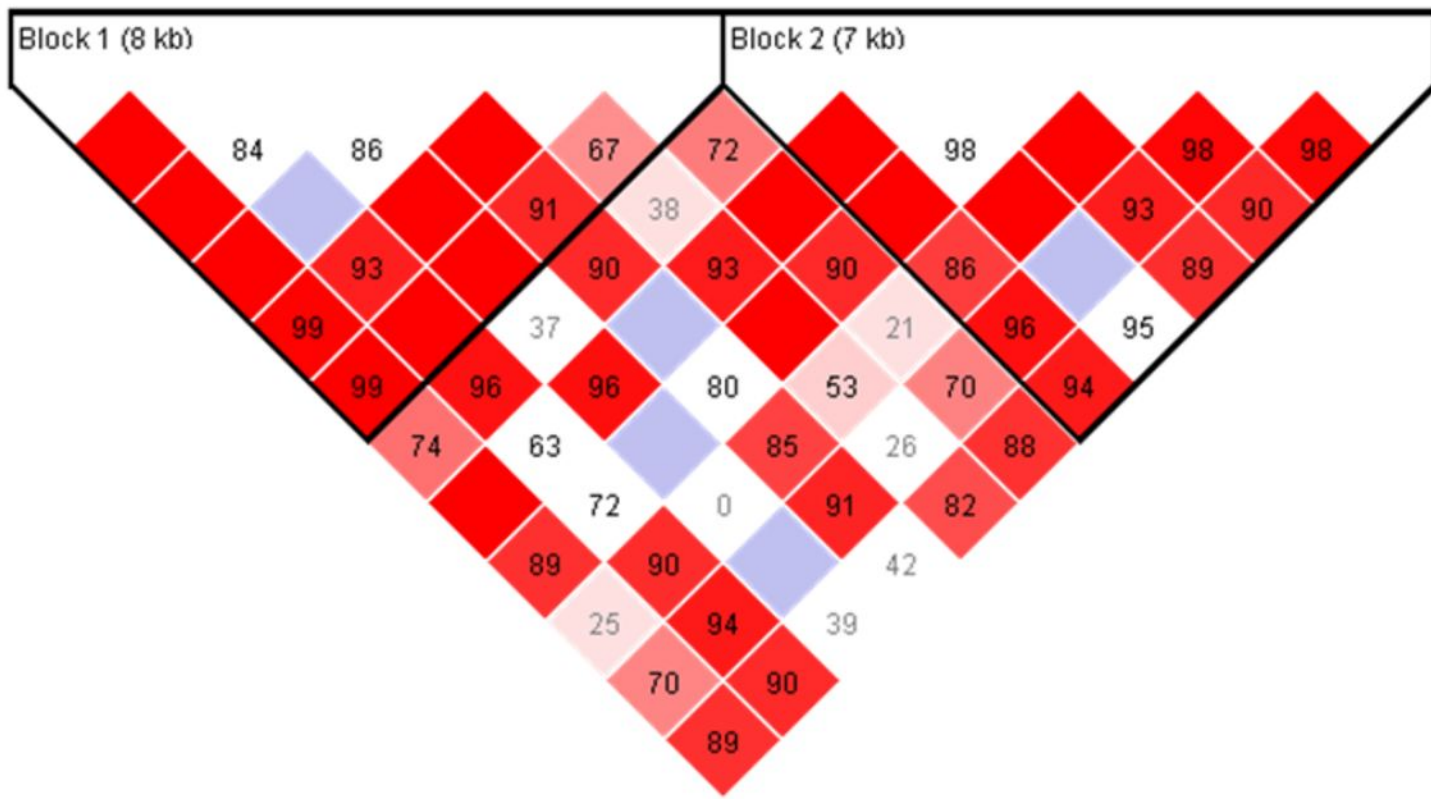
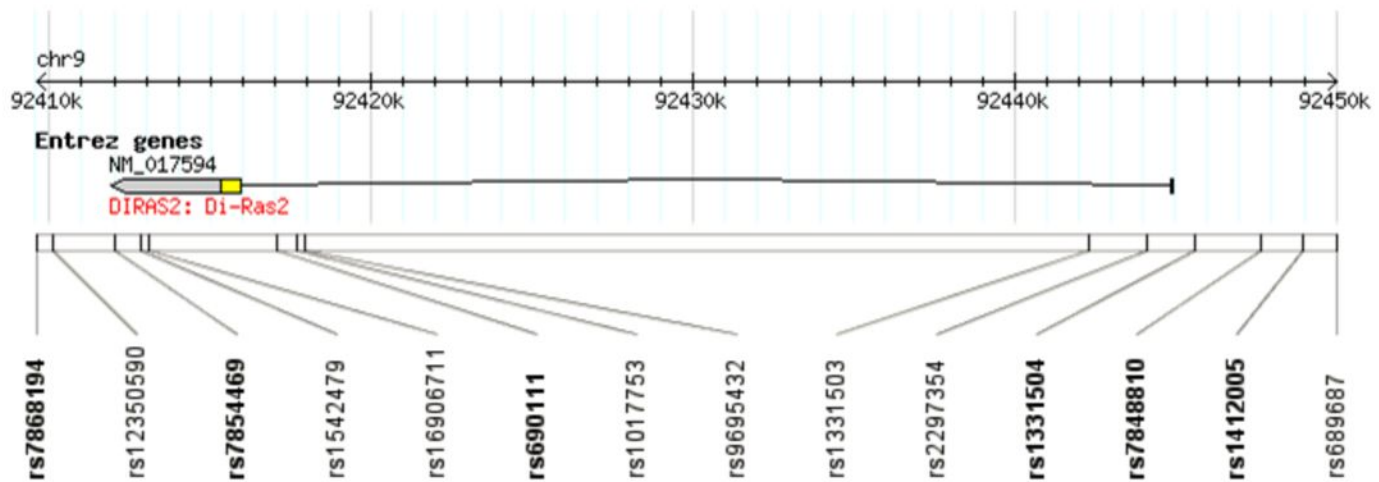
Table 4. Results of family-based association analysis for cADHD.

Table 5. Results of case-control association analysis for co-morbid disorders (BD and personality disorder).

Table 6. Nominally significant results of association analysis for quantitative personality traits.

Suppl Table 1. SNP results of case-control based association analyses for aADHD by population.

Suppl Table 2. Personality domains in the SHIP study.



The Effects of the rs1412005 T Allele

Sample

Germany

Netherlands

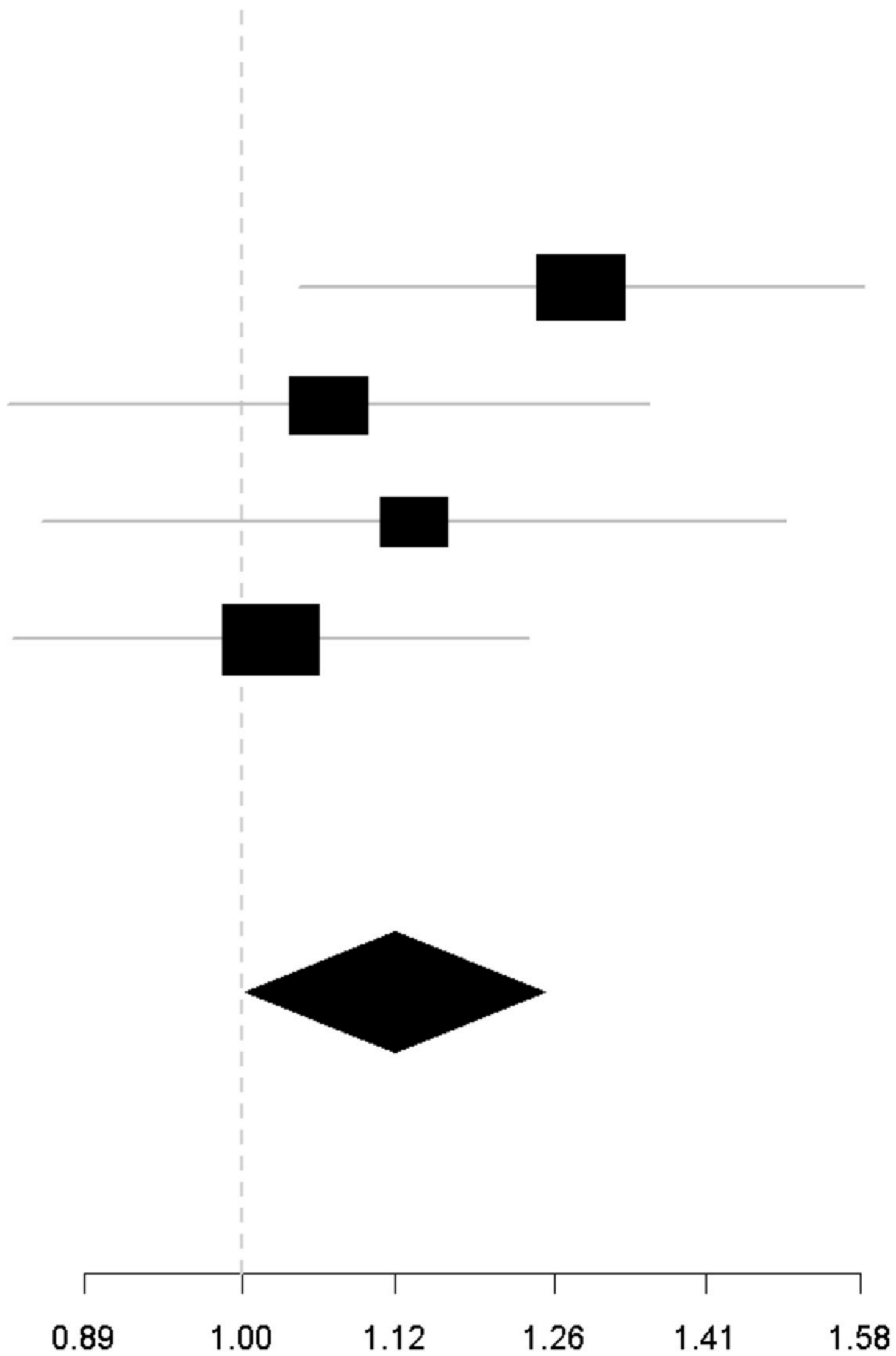
Spain

Norway

Summary

0.89 1.00 1.12 1.26 1.41 1.58

Log-additive Odds Ratio



The Effects of the ACGCTT Haplotype

Sample

Germany

Netherlands

Spain

Norway

Summary

1.00 1.26 1.58 2.00 2.51 3.16

Odds Ratio

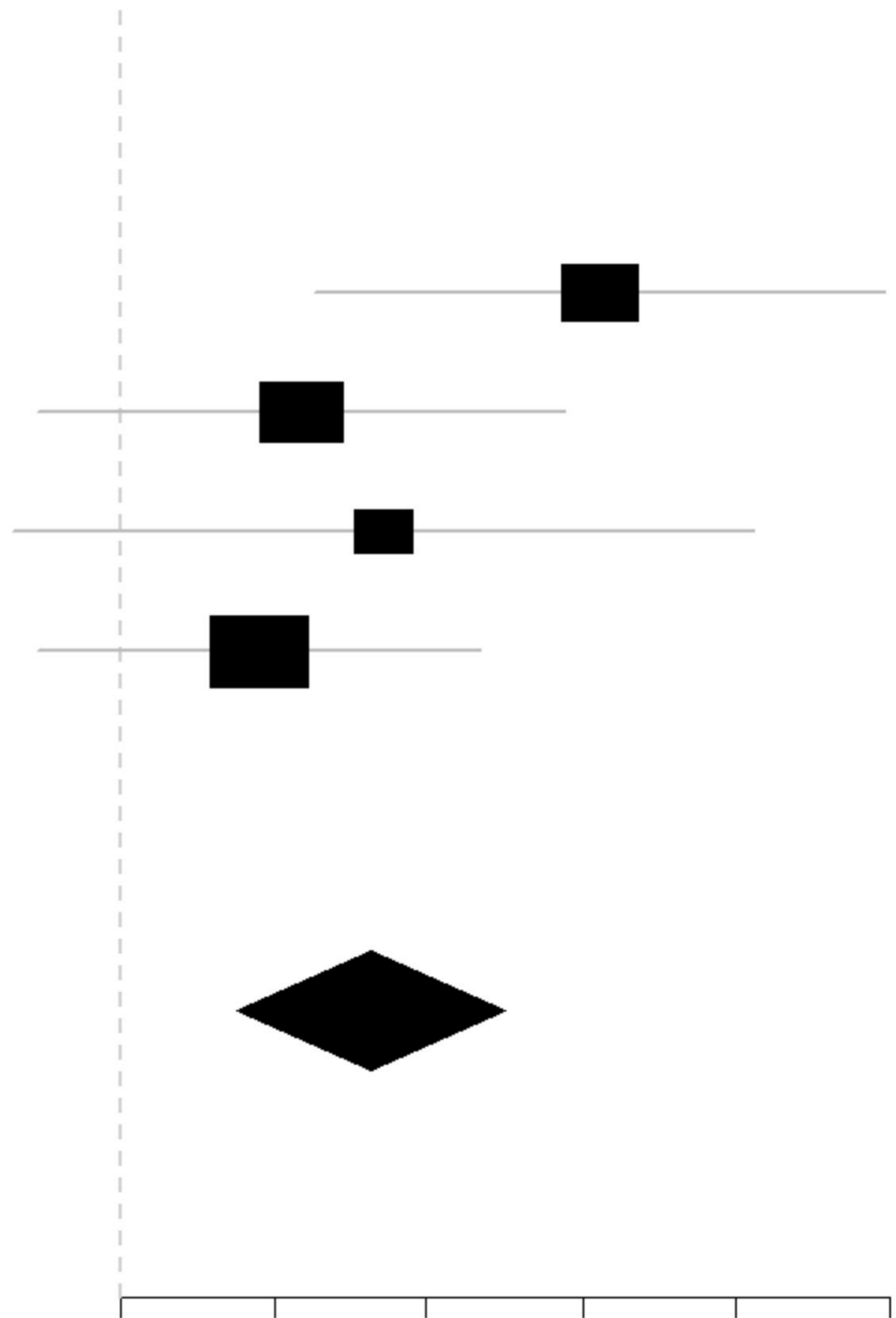


Table 1. General SNP information with minor allele frequency (%).

SNP NR	SNP ID	Physical position	Region	Major allele	Minor allele	German aADHD		German cADHD		Spanish aADHD		Dutch aADHD		Norwegian aADHD		Bipolar disorder	Personality disorder
						cases n=600	controls n=420	parents n=166	offspring n=244	cases n=253	controls n=311	cases n=285	controls n=508	cases n=497	controls n=562	cases n=336	cases n=622
1	rs7868194	92409604	3' region	A	G	43.2	47.0	45.4	45.2	46.7	50.3	48.4	47.9	45.9	46.9	43.4	47.4
2*	rs12350590	92410160	3' region	C	G	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	rs7854469	92412049	3' UTR	T	A	7.5	10.2	9.8	8.1	14.9	11.6	11.4	8.9	9.0	8.9	9.9	9.1
4	rs1542479	92412869	3' UTR	G	A	4.3	2.8	3.7	2.8	3.6	5.4	4.6	4.4	4.7	5.2	3.5	3.5
5	rs16906711	92413098	3' UTR	C	G	2.9	1.5	2.2	1.5	2.8	3.4	2.5	2.5	2.3	2.3	2.0	3.0
6	rs690111	92417110	Intron 1	G	T	26.1	24.1	29.3	29.9	24.7	21.8	26.3	26.6	25.8	26.5	27.3	24.3
7	rs1017753	92417721	Intron 1	C	T	47.7	50.1	49.5	49.8	49.8	45.6	47.8	48.7	49.0	48.2	47.4	49.7
8*	rs9695432	92417994	Intron 1	G	A	0.5	1.5	0.4	0.5	1.6	0.7	1.8	0.9	1.5	0.6	0.5	0.9
9	rs1331503	92442337	Intron 1	T	A	37.8	32.3	32.1	33.4	35.9	35.7	34.1	32.9	33.9	33.1	35.7	36.4
10	rs2297354	92444160	Intron 1	C	G	2.0	1.2	1.3	1.1	1.2	0.7	1.6	0.7	2.1	2.6	1.5	1.0
11	rs1331504	92445648	Intron 1	G	C	8.7	8.4	6.9	6.8	7.8	8.3	7.1	6.4	7.4	6.6	6.7	8.1
12	rs7848810	92447678	Promoter	C	A	28.2	31.5	30.3	28.0	33.1	29.7	28.5	28.3	22.2	24.3	28.0	28.4
13	rs1412005	92448981	Promoter	G	T	27.9	23.0	26.1	25.7	26.3	23.9	26.4	25.2	26.4	26.0	28.2	27.6
14	rs689687	92450021	Promoter	T	C	19.4	18.5	16.1	13.7	19.0	19.8	17.0	17.2	17.3	19.0	20.3	19.0

*SNPs were excluded from further analyses due to low MAF (<0.01)

Table 2. SNP results of case-control based association analyses for aADHD.

SNP NR	SNP ID	German discovery sample			IMpACT replication sample			Pooled data		
		P value	OR (allele)	95%-CI	P value	OR (allele)	95%-CI	P value	OR (allele)	95%-CI
1	rs7868194	0.0880	1.18 (A)	0.98 - 1.42	0.4395	1.05 (A)	0.93 - 1.18	0.1174	1.08 (A)	0.98 - 1.20
3	rs7854469	0.0394	1.40 (T)	1.02 - 1.93	0.0720	1.19 (A)	0.98 - 1.43	0.6023	1.04 (A)	0.89 - 1.23
4	rs1542479	0.0905	1.55 (A)	0.93 - 2.59	0.3378	1.15 (G)	0.87 - 1.51	0.9958	1.00 (A)	0.79 - 1.27
5	rs16906711	0.0453	1.97 (G)	1.01 - 3.81	0.7288	1.07 (C)	0.74 - 1.55	0.4271	1.14 (G)	0.83 - 1.55
6	rs690111	0.3392	1.11 (T)	0.90 - 1.37	0.8043	1.02 (T)	0.89 - 1.16	0.4684	1.04 (T)	0.93 - 1.17
7	rs1017753	0.2741	1.11 (C)	0.92 - 1.33	0.4749	1.04 (C)	0.93 - 1.17	0.2353	1.06 (C)	0.96 - 1.17
9	rs1331503	0.0125	1.27 (A)	1.05 - 1.54	0.5812	1.03 (A)	0.92 - 1.17	0.0678	1.10 (A)	0.99 - 1.22
10	rs2297354	0.2101	1.62 (G)	0.76 - 3.44	0.6599	1.11 (G)	0.70 - 1.77	0.2880	1.24 (G)	0.84 - 1.82
11	rs1331504	0.8241	1.04 (C)	0.75 - 1.44	0.5302	1.07 (C)	0.86 - 1.34	0.5195	1.06 (C)	0.88 - 1.28
12	rs7848810	0.1244	1.17 (C)	0.96 - 1.42	0.9898	1.00 (A)	0.88 - 1.14	0.3942	1.05 (C)	0.94 - 1.17
13	rs1412005	0.0182	1.29 (T)	1.04 - 1.58	0.3785	1.06 (T)	0.93 - 1.21	0.0437	1.12 (T)	1.00 - 1.25
14	rs689687	0.6039	1.07 (C)	0.84 - 1.36	0.3791	1.07 (T)	0.92 - 1.24	0.6344	1.03 (T)	0.91 - 1.17

Nominal p-values < 0.05 are in bold print

Table 3. Association analysis of the ACGCTT haplotype* for the studied aADHD case-control samples.

Sample	Frequency: N (%)		Association test		
	cases	controls	P value	OR	95%-CI
Germany	89 (8.94)	30 (4.57)	0.0006	2.05	1.34 - 3.14
Netherlands	46 (9.54)	66 (7.45)	0.1815	1.31	0.88 - 1.94
Spain	28 (6.48)	25 (4.46)	0.1815	1.48	0.85 - 2.58
Norway	76 (10.08)	78 (8.35)	0.2213	1.23	0.88 - 1.71

* Haplotype consists of SNPs rs1331503, rs2297354, rs1331504, rs7848810, rs1412005 and rs689687

Table 4. Results of family based association analysis for cADHD.

SNP NR	SNP ID	Over-transmission		PDT
		Allele	Rate (%)	P value
1	rs7868194	G	50.9	0.6507
3	rs7854469	T	50.8	0.2059
4	rs1542479	G	50.5	0.1779
5	rs16906711	C	50.2	0.3711
6	rs690111	G	50.9	0.4982
7	rs1017753	T	50.9	0.6911
9	rs1331503	A	52.2	0.4245
10	rs2297354	C	50.3	0.2253
11	rs1331504	G	50.1	0.8997
12	rs7848810	C	52.8	0.0463
13	rs1412005	T	51.4	0.6565
14	<i>rs689687</i>	<i>T</i>	<i>51.3</i>	<i>0.1456*</i>

Nominal p-values < 0.05 are in bold print

*call rate=77%

Table 5. Results of case-control association analysis for co-morbid bipolar disorder and personality disorders.

SNP NR	SNP ID	Bipolar			Cluster B			Cluster C		
		P value	OR (allele)	95%-CI	P value	OR (allele)	95%-CI	P value	OR (allele)	95%-CI
1	rs7868194	0.1794	1.15 (A)	0.94 - 1.42	0.9272	1.01 (G)	0.84 - 1.22	0.3893	1.11 (G)	0.88 - 1.39
3	rs7854469	0.8687	1.03 (T)	0.73 - 1.44	0.0868	1.35 (T)	0.96 - 1.91	0.5588	1.12 (A)	0.77 - 1.63
4	rs1542479	0.4349	1.26 (A)	0.70 - 2.26	0.5663	1.17 (A)	0.68 - 2.00	0.3782	1.34 (A)	0.70 - 2.56
5	rs16906711	0.4935	1.32 (G)	0.59 - 2.94	0.0454*	1.95 (G)	1.01 - 3.75	0.0220*	2.33 (G)	1.13 - 4.80
6	rs690111	0.1752	1.18 (T)	0.93 - 1.50	0.7622	1.03 (T)	0.83 - 1.29	0.8047	1.03 (G)	0.79 - 1.36
7	rs1017753	0.2906	1.12 (C)	0.91 - 1.38	0.9950	1.00 (C)	0.83 - 1.21	0.3930	1.10 (T)	0.88 - 1.39
9	rs1331503	0.1923	1.16 (A)	0.93 - 1.44	0.1416	1.16 (A)	0.95 - 1.42	0.1950	1.18 (A)	0.92 - 1.50
10	rs2297354	0.6345	1.24 (G)	0.51 - 3.02	0.5963	1.28 (C)	0.51 - 3.18	0.8929	1.07 (G)	0.38 - 2.99
11	rs1331504	0.2404	1.27 (G)	0.85 - 1.89	0.6503	1.08 (G)	0.77 - 1.53	0.5419	1.14 (G)	0.74 - 1.76
12	rs7848810	0.1645	1.17 (C)	0.94 - 1.47	0.1065	1.18 (C)	0.96 - 1.46	0.7176	1.05 (C)	0.82 - 1.34
13	rs1412005	0.0256	1.32 (T)	1.03 - 1.67	0.0305	1.27 (T)	1.02 - 1.59	0.0855	1.26 (T)	0.97 - 1.64
14	rs689687	0.3988	1.12 (C)	0.86 - 1.47	0.7428	1.04 (C)	0.81 - 1.34	0.8141	1.04 (C)	0.77 - 1.40

Nominal p-values < 0.05 are in bold print

**p*HWE=0.001

Table 6. Nominally significant results of association analysis for quantitative personality traits.

SNP NR	SNP ID	Personality trait	aADHD cases			PD patients		
			P value	Beta (allele)	Se	P value	Beta (allele)	Se
9	rs1331503	<i>Neuroticism</i>	0.0241	3.51 (A)	1.55	0.3113	1.72 (A)	1.70
14	rs689687	<i>Neuroticism</i>	0.0327	4.14 (C)	1.93	0.5747	1.15 (C)	2.05
3	rs7854469	<i>Extraversion</i>	0.4172	2.07 (A)	2.55	0.0075	6.32 (T)	2.36
9	rs1331503	<i>Extraversion</i>	0.0065	3.68 (T)	1.35	0.6695	0.59 (T)	1.38
13	rs1412005	<i>Extraversion</i>	0.0088	3.83 (G)	1.46	0.7600	0.44 (T)	1.42
3	rs7854469	<i>Agreeableness</i>	0.8963	0.26 (T)	1.96	0.0116	4.24 (A)	1.67
10	rs2297354	<i>Agreeableness</i>	0.2065	4.78 (C)	3.78	0.0288	10.03 (C)	4.57
14	rs689687	<i>Agreeableness</i>	0.0284	2.80 (C)	1.28	0.2645	1.28 (T)	1.15
6	rs690111	<i>Conscientiousness</i>	0.9152	0.17 (T)	1.60	0.0448	2.87 (T)	1.43
9	rs1331503	<i>Conscientiousness</i>	0.0173	3.24 (T)	1.36	0.1876	1.77 (T)	1.34
12	rs7848810	<i>Conscientiousness</i>	0.9051	0.18 (C)	1.52	0.0123	3.49 (A)	1.39
13	rs1412005	<i>Conscientiousness</i>	0.0135	3.61 (G)	1.46	0.1013	2.26 (G)	1.38
14	rs689687	<i>Conscientiousness</i>	0.0043	4.84 (T)	1.69	0.1166	2.50 (T)	1.59
3	rs7854469	<i>Novelty Seeking</i>	0.8075	0.16 (A)	0.65	0.0224	1.35 (T)	0.59
12	rs7848810	<i>Novelty Seeking</i>	0.3585	0.35 (A)	0.38	0.0396	0.73 (C)	0.36
1	rs7868194	<i>Harm Avoidance</i>	0.1634	0.62 (A)	0.45	0.0126	1.08 (G)	0.43
7	rs1017753	<i>Harm Avoidance</i>	0.0897	0.74 (C)	0.43	0.0257	0.95 (T)	0.43
9	rs1331503	<i>Harm Avoidance</i>	0.0023	1.30 (A)	0.42	0.7309	0.16 (A)	0.46
13	rs1412005	<i>Harm Avoidance</i>	0.0018	1.43 (T)	0.46	0.7284	0.17 (T)	0.48
14	rs689687	<i>Harm Avoidance</i>	0.0033	1.54 (C)	0.52	0.6540	0.25 (T)	0.55

SNPs with p-value < 0.05 in at least one analysis are in bold print. Se, standard error.