

Common Variants in the TPH1 and TPH2 Regions Are Not Associated With Persistent ADHD in a Combined Sample of 1,636 Adult Cases and 1,923 Controls From Four European Populations

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The tryptophan hydroxylase 1 and 2 (*TPH1* and *TPH2*) genes encode the rate-limiting enzymes in the serotonin biosynthesis. Genetic variants in both genes have been implicated in several psychiatric disorders. For attention-deficit/hyperactivity disorder (ADHD) in children, the results are conflicting, and little is known about their role in adult ADHD patients. We therefore first genotype-tagged all common variants within both genes in a Norwegian sample of 451 patients with a diagnosis of adult ADHD and 584 controls. Six of the single nucleotide polymorphisms (SNPs) were subsequently genotyped in three additional independent European Caucasian samples of adult ADHD cases

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and controls from the International Multicenter persistent ADHD Collaboration (IMpACT). None of the SNPs reached formal study-wide significance in the total meta-analysis sample of 1,636 cases and 1,923 controls, despite having a power of >80% to detect a variant conferring an OR = 1.25 at $P = 0.001$ level. Only the *TPH1* SNP rs17794760 showed nominal significance [OR = 0.84 (0.71–1.00), $P = 0.05$]. In conclusion, in the single largest ADHD genetic study of *TPH1* and *TPH2* variants presented to date ($n = 3,559$ individuals), we did not find consistent evidence for a substantial effect of common genetic variants on persistent ADHD. © 2010 Wiley-Liss, Inc.

Key words: TPH1; TPH2; ADHD; common variants; genetic analysis

INTRODUCTION

Serotonin (5-HT) is a neurotransmitter and a hormone with important regulatory functions both in the peripheral organs and central nervous system (CNS). Serotonin dysfunction has been implicated in different psychiatric disorders such as major depression, schizophrenia, autism, and attention-deficit/hyperactivity disorder (ADHD). Tryptophan hydroxylase 1 and 2 (TPH1 and TPH2), which catalyze the rate-limiting step of serotonin biosynthesis [Walther et al., 2003; McKinney et al., 2005], have therefore been extensively studied for their putative role in the susceptibility to such psychiatric disorders. The human *TPH1* and *TPH2* genes are located on chromosomes 11p15.3-p14 and 12q21.1, respectively, and have different expression patterns. *TPH1* is found in the pineal gland, pituitary gland, and peripheral organs, including enterochromaffin cells of the gut, while *TPH2* is mainly expressed in the CNS and peripheral serotonergic neurons [Cote et al., 2007; Gutknecht et al., 2009; Zill et al., 2009].

ADHD is a common neuropsychiatric disorder conferring substantial impairment which manifests itself during childhood, but most patients will have symptoms that persist into their adult life [Biederman and Faraone, 2005; Faraone et al., 2006]. Non-remitting ADHD could represent a severe and highly familial subtype of

ADHD [Larsson et al., 2004]. Although most candidate studies in ADHD have targeted dopaminergic and noradrenergic signaling in ADHD, recent studies also indicate a serotonergic dysfunction underlying this psychiatric condition [Oades, 2007]. Sheehan et al. [2005] were the first to report an association between common *TPH2* variants and ADHD, but later studies have yielded inconsistent results [Walitza et al., 2005; Brookes et al., 2006; Sheehan et al., 2007; Baehne et al., 2008; Manor et al., 2008]. Since the discovery of *TPH2*, there has been less interest in the role of *TPH1* in ADHD, but *TPH1*-knockout mice have shown that its expression might still be important for the developing CNS [Cote et al., 2007; Savelieva et al., 2008; Gutknecht et al., 2009]. Furthermore, *TPH1* is one of the strongest candidate genes in meta-analyses of previous studies on schizophrenia [Allen et al., 2008].

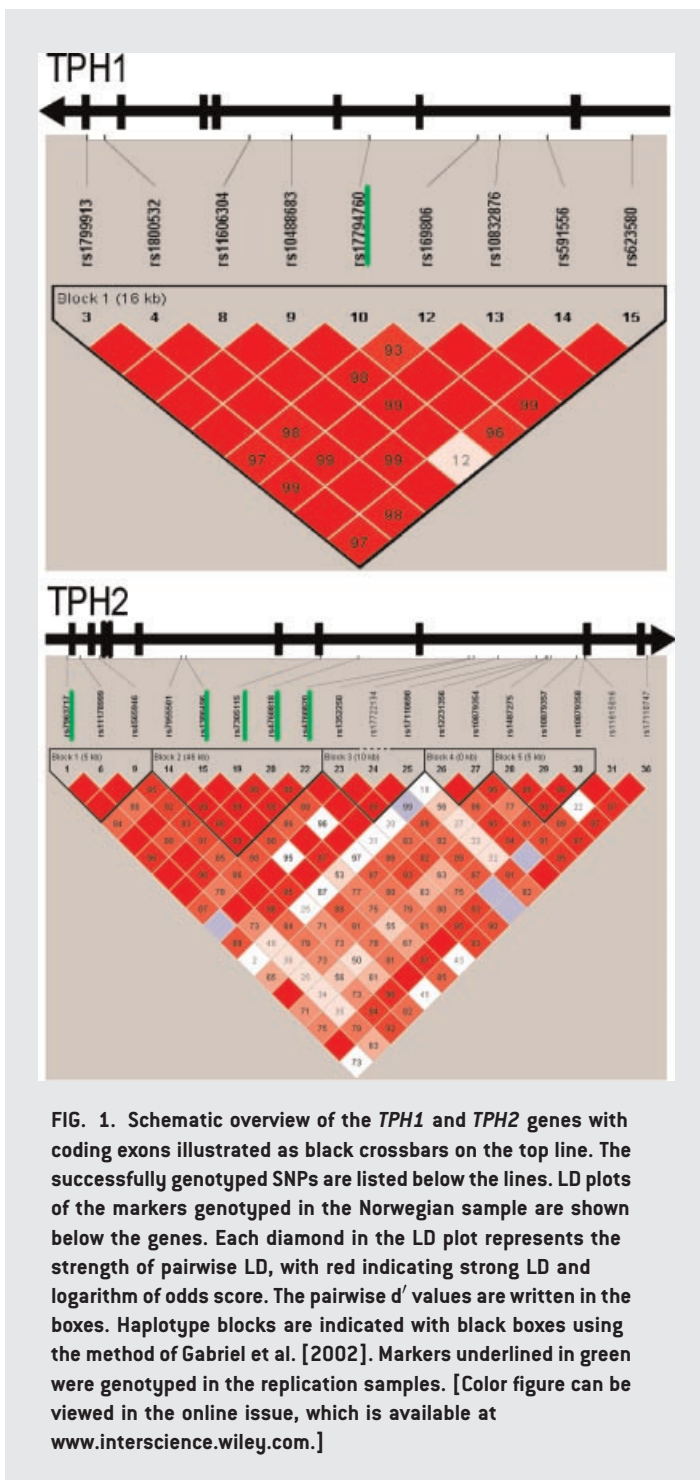
The recent success of whole genome association studies in other common disorders, such as type 2 diabetes, coronary artery disease, and obesity, has highlighted that large sample sizes are needed to sort out the true disease loci from spurious associations [Altshuler et al., 2008]. It is highly likely that the situation is similar for psychiatric disorders such as ADHD, where sample sizes have generally been small and testable phenotypes numerous. We therefore decided to increase power by applying a two-stage design with initial dense genetic tagging of all common single nucleotide polymorphisms (SNPs) within the *TPH1* and *TPH2* regions in a sample of 451 adult ADHD patients and 584 controls from Norway. These results were then followed by targeted replication attempts in three additional samples from Germany, The Netherlands, and Spain. The total sample of 1,636 cases and 1,923 controls is part of the International Multicenter persistent ADHD Collaboration (IMpACT).

MATERIALS AND METHODS

Patients and Controls

The total sample included 1,636 adult ADHD patients and 1,923 controls' all of Caucasian origin from Norway, Spain, Germany, and The Netherlands recruited into the IMpACT. Extensive description of the study samples can be found in Sanchez-Mora et al. [2009]. In short, all patients have been extensively examined by an experienced psychiatrist and diagnosed with ADHD according to the diagnostic criteria of Diagnostic and Statistical Manual for Mental Disorders-IV (DSM-IV), with onset of symptoms before the age of 7 years via retrospective diagnosis (which was confirmed by a family member, wherever possible), lifelong persistence of symptoms, and current diagnosis.

Measures of symptom severity in the Norwegian sample: severity of ADHD in adulthood was measured by the 18-item adult ADHD self-report rating scale (ASRS; mean = 45.9, SD = 12.1), where nine questions address the frequency of inattentive symptoms (mean = 23.8, SD = 6.6) and nine address the frequency of hyperactivity/impulsivity symptoms (mean = 22.1, SD = 6.8) [Kessler et al., 2005]. Childhood ADHD symptoms was measured by the 25-item Wender Utah Rating Scale (WURS; mean = 58.5, SD = 18.2) [Ward et al., 1993]. The distributions of the rating scales in the Norwegian patients are shown in Supplementary Figure 1.



Genotyping and Marker Selection

Information from CEU-HapMap-build 21-data was used in the Haploview-Tagger software [de Bakker et al., 2005] for selection of tagging SNPs. We applied the following criteria for marker selection: pairwise tagging only, r^2 threshold = 0.8, all SNPs in the genes and 1 kb surrounding region, with minor allele frequency (MAF) above 5%. Seven SNPs were found to be sufficient to tag all common variation within the *TPH1* gene. We also included a putative splice-site variant, rs1799913, located just upstream of exon 7, and the SNP

rs1800532 (known as 218A/C) based on previous claims of association with several psychiatric conditions (references). Applying the same criteria, 20 SNPs were initially selected to tag all common variation in a 96 kb region that includes the *TPH2* gene and the nearby flanking region. Two markers failed in the assays, but the final 18 successfully genotyped tagSNP set were still sufficient to tag all SNPs (MAF > 0.05) in the region with a mean maximal r^2 of 0.93 ($r^2 > 0.73$ for all non-genotyped variants) according to HapMap data (build 21).

Genotyping of the Norwegian samples: DNA was extracted either from whole blood, or from saliva using the Oragene™ DNA Self-Collection Kit from DNA Genotek (DNA Genotek Inc., Ontario, Canada). DNA from cases and controls were inter-mixed on 96-well plates with a minimum of two internal controls and two blank samples on each plate. Genotyping was performed using the MassARRAY iPLEX System (SEQUENOM, Inc., San Diego, CA) by CIGENE at the national technology platform, and supported by the Functional Genomics Programme (FUGE) in the Research Council of Norway. Total genotyping rate was >98% after removal of poorly performing markers and individuals. Concordance rate was 100% among 11 subjects with duplicate DNA spread over all assay plates.

Genotyping of the replication samples: samples from Spain and The Netherlands were genotyped together with an additional smaller Norwegian sample in one multiplex reaction using the MassARRAY iPLEX System at CIGENE in Norway. The German samples were genotyped using the same multiplex reaction at Würtzburg, Germany (primers are available upon request).

Statistical Analysis

For single marker analysis of dichotomous traits, we focused on an additive allelic model either with the chi-square test or by using logistic regression with gender as a cofactor as implemented in the PLINK software [Purcell et al., 2007]. For comparison, we also tested, but could not find, any evidence for a stronger association for either a dominant or recessive model in the Norwegian data set. For the meta-analysis, we considered only an additive allelic mode of inheritance and applied a random effect analysis as implemented by the “metan” command in Stata 8.2 (Stata Statistical Software, Stata Corp., College Station, TX), with the estimate of heterogeneity being taken from the Mantel–Haenszel model. Similar results were also obtained using the stratified Mantel–Haenszel test as implemented in the PLINK software. Haplotype analysis was performed in PLINK using a sliding window approach with three consecutive markers throughout the gene. The omnibus test is a test with $n-1$ degrees of freedom where n is the number of common haplotypes (1% frequency threshold). Haploview [Barrett et al., 2005] was used for linkage disequilibrium (LD) visualization. A possible association between SNPs and the quantitative traits, ASRS and WURS, was analyzed using linear regression (allelic model) in Norwegian cases only, with gender and current medication as cofactors. The traits were near normally distributed among cases, and results are therefore only presented without transformation (Supplementary Fig. 1). All the results are presented without correction for multiple testing.

TABLE I. Demographic Characteristics of All Cases and Controls Included in the Study

Country	Cases			
	Controls (% males)	Total (% males)	Combined subtype ^a (%)	Depression/anxiety (%)
Norway ^b	728 (44)	502 (53)	75	70
The Netherlands	490 (49)	246 (48)	81	68
Germany	393 (50)	589 (53)	66	53
Spain	312 (65)	299 (72)	65	50
Total	1,923	1,636		

^aPercentage of patients with combined subtype.

^bFifty-one cases and 144 controls of the Norwegian sample were part only in the replication sample.

RESULTS

Stage 1: Norwegian Sample

Demographic characteristics for the 451 ADHD patients and 584 controls from Norway are presented in Table I. The sample was successfully genotyped for nine SNPs covering all common variants in the *TPH1* region and 18 SNPs in the *TPH2* region. The pattern of LD was similar between the Norwegian sample and the HapMap CEU data set with relatively strong LD overall including a large LD block covering exons 5–8 for *TPH2* and one block of strong LD and limited haplotype diversity (only five common haplotypes, mean maximal $r^2 = 0.97$) for *TPH1* (Fig. 1).

Table II shows the results of the logistic regression analysis between *TPH1* markers and adult ADHD in 451 Norwegian individuals with persistent ADHD and 584 controls (adjusted for gender). Only rs17794760, located in intron 2, showed evidence of nominal association with ADHD (OR = 0.74, 95% CI: 0.59–0.94, $P = 0.01$). Haplotype analysis revealed that the suggested protective allele tagged a single haplotype that spans all nine markers (OR = 0.74, $P = 0.02$). We next considered the ASRS as a quantitative measure of severity of inattentive and hyperactive symptoms and found no further evidence of association with the tested markers.

Eighteen of the 20 selected *TPH2* SNPs were successfully genotyped in the entire Norwegian sample. Association results are depicted in Table III. In the single point analyses, the rare alleles of the two perfectly correlated markers rs1386496 and rs4760818

were slightly more common in cases than in controls. Haplotype analysis also showed additional evidence of association for a three-marker haplotype made up by rs7305115, rs4760818, and rs4760820 (omnibus $P = 0.02$; Table III). Results suggested one putative protective haplotype (15% in cases vs. 20% in controls, $P = 0.004$) and one susceptibility haplotype (14.2% in cases vs. 11.4% in controls, $P = 0.06$). In addition, quantitative analysis of ASRS symptom scores in only ADHD cases showed suggestive associations between the rare alleles of rs1386496 and rs4760818 and increased inattentive symptom score (using gender and current use of medication as cofactors in the analysis; Table IV).

Stage 2: Meta-Analysis in the IMPACT Sample

Based on the results from the Norwegian sample and previously reported studies, we chose one SNP in *TPH1* and five in *TPH2* for the replication study (markers underlined in green in Fig. 1) in an additional sample of 1,185 adult cases and 1,339 controls from The Netherlands, Germany, and Spain as well as an additional smaller sample from Norway (samples from IMPACT; Table I). The single *TPH1* SNP, rs17794760, showed nominal association with ADHD in the meta-analysis which could be ascribed to the Norwegian and Spanish samples (Fig. 2 and Supplementary Table I). However, this association did not survive correction for multiple testing. In addition, the meta-analysis did not support association between common *TPH2* variants or haplotypes and adult ADHD (Fig. 2 and Supplementary Table I). Marker allele frequencies (Supplementary

TABLE II. *TPH1* Single Point and Three-Marker Haplotype Associations in 451 ADHD Patients and 584 Controls From Norway

SNP	BP	MAF cases	MAF controls	OR (95% CI)	P	P_{gender}	Omnibus three-point P	Haplo P
rs1799913	18003831	0.402	0.383	1.08 (0.90–1.30)	0.39	0.37		
rs1800532	18004392	0.410	0.383	1.12 (0.94–1.35)	0.21	0.20	0.56	
rs11606304	18008824	0.084	0.080	1.05 (0.76–1.44)	0.77	0.78	0.05	
rs10488683	18010121	0.432	0.416	1.07 (0.89–1.27)	0.48	0.49	0.10	
rs17794760	18012496	0.157	0.200	0.74 (0.59–0.94)	0.012	0.014	0.06	0.02
rs169806	18015868	0.405	0.389	1.07 (0.89–1.28)	0.47	0.44	0.11	
rs10832876	18016505	0.271	0.244	1.15 (0.94–1.41)	0.17	0.18	0.17	
rs591556	18017976	0.161	0.166	0.96 (0.76–1.22)	0.74	0.79	0.20	
rs623580	18020553	0.319	0.356	0.85 (0.70–1.02)	0.09	0.09		

TABLE III. TPH2 Single Point and Three-Marker Haplotype Associations in 451 ADHD Patients and 584 Controls

SNP	Position	MAF cases	MAF controls	OR (95% CI)	P	P _{gender}	Omnibus three-point P	Haplo P
rs7963717	70617629	0.06	0.08	0.80 [0.57–1.13]	0.21	0.20		
rs11178999	70619837	0.21	0.23	0.87 [0.70–1.08]	0.20	0.18	0.56	
rs4565946	70623036	0.47	0.46	1.02 [0.86–1.22]	0.79	0.81	0.06	
rs7955501	70636293	0.41	0.38	1.12 [0.93–1.34]	0.23	0.23	0.06	
rs1386496	70637057	0.14	0.12	1.27 [0.98–1.64]	0.08	0.08	0.26	
rs7305115	70659129	0.43	0.40	1.11 [0.93–1.33]	0.24	0.24	0.19	
rs4760818	70665190	0.14	0.12	1.26 [0.97–1.64]	0.08	0.08	0.02	0.004
rs4760820	70683263	0.43	0.40	1.10 [0.92–1.31]	0.31	0.33	0.04	
rs1352250	70684051	0.42	0.40	1.09 [0.91–1.30]	0.33	0.33	0.08	
rs17722134	70687961	0.03	0.04	0.91 [0.56–1.48]	0.71	0.73	0.78	
rs17110690	70694264	0.26	0.25	1.04 [0.85–1.27]	0.69	0.65	0.96	
rs12231356	70695815	0.05	0.05	0.98 [0.66–1.46]	0.93	0.99	0.34	
rs10879354	70696049	0.39	0.35	1.19 [0.99–1.42]	0.06	0.06	0.30	
rs1487275	70696559	0.26	0.26	1.02 [0.83–1.25]	0.85	0.97	0.43	
rs10879357	70700830	0.37	0.35	1.08 [0.90–1.29]	0.44	0.47	0.70	
rs10879358	70702137	0.34	0.31	1.14 [0.95–1.38]	0.16	0.18	0.53	
rs11615016	70702261	0.08	0.09	0.88 [0.64–1.21]	0.42	0.43	0.56	
rs17110747	70712221	0.14	0.13	1.12 [0.87–1.45]	0.39	0.44		

Table I) and LD between markers (Supplementary Fig. 2) were relatively similar between populations.

Stratification for combined inattentive/hyperactive-impulsive (Supplementary Table II), or for primarily inattentive cases (data not shown), did not have any significant impact on the observed results for either *TPH1* or *TPH2*. Also, there were no significant differences in allele frequencies when stratification for

comorbid depression/anxiety (Supplementary Table III) or for gender was applied (data not shown).

DISCUSSION

Replicating genetic association findings in psychiatric disorders has been an important bottleneck in the identification of susceptibility

TABLE IV. Quantitative Analysis of TPH2 Allelic Association With Symptom Severity Score in Childhood (WURS) and at Present (ASRS) Among All Norwegian ADHD Patients Using Gender and Current Medication as Covariates

SNP	WURS (P)	ASRS		
		Hyperactivity (P)	Inattention (P)	Total (P)
rs7963717	0.05	0.90	0.47	0.84
rs11178999	0.02	0.86	0.64	0.77
rs4565946	0.78	0.20	0.87	0.39
rs7955501	0.83	0.29	0.59	0.36
rs1386496	0.29	0.27	0.05	0.10
rs7305115	1.00	0.35	0.47	0.34
rs4760818	0.29	0.27	0.06	0.11
rs4760820	0.42	0.29	0.45	0.30
rs1352250	0.86	0.35	0.59	0.39
rs17722134	0.40	0.93	0.33	0.57
rs17110690	0.43	0.80	0.33	0.74
rs12231356	0.72	0.49	0.85	0.66
rs10879354	0.25	0.66	0.92	0.77
rs1487275	0.66	0.74	0.55	0.60
rs10879357	0.93	0.96	0.26	0.53
rs10879358	0.79	0.88	0.31	0.64
rs11615016	0.13	0.27	0.52	0.32
rs17110747	0.29	0.68	0.45	0.51

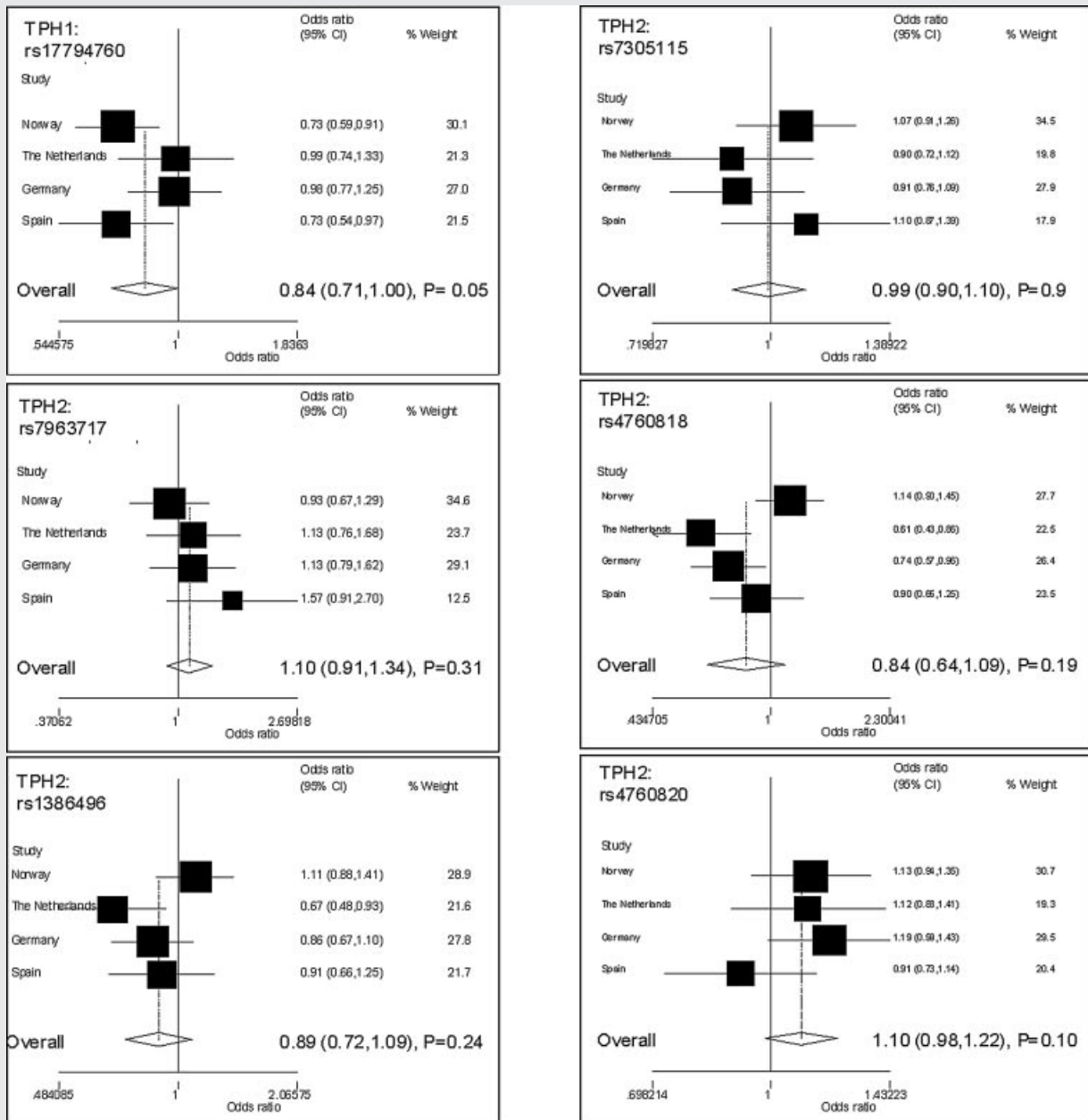


FIG. 2. Meta-analysis forest plots of the markers tested in all samples (1,636 cases and 1,923 controls). All results are presented using a random effect model.

genes for such disorders. In the present study, we therefore used a two-stage design: an initial explorative analysis with dense genetic tagging of common *TPH1* and *TPH2* SNPs in a clinical sample of 451 adult patients with persistent ADHD, and 584 controls from Norway was followed by attempts at replication in an additional sample of 2,524 individuals (1,185 cases and 1,339 controls) from IMPACT, a collaboration aimed to study the genetic predisposition to persistent ADHD.

Our two-stage study revealed no consistent evidence of association between persistent ADHD and common variants in the *TPH1* and *TPH2* regions. Previous *TPH2* studies mainly restricted to

childhood ADHD have also yielded inconsistent results: Sheehan et al. [2005] performed a family-based association study in 179 Irish families and found a strong over-transmission of the major allele for several markers in the region between introns 5 and 8 of the gene (most significant finding for rs1843809: OR = 2.36, $P = 0.0006$). However, the same group was later unable to replicate this finding in a smaller sample (107 families) of UK descent [Sheehan et al., 2007]. Another study by Walitza et al. [2005] found nominal association between ADHD and two SNPs within the promoter region of the gene in a sample of 103 families (225 affected family members) and later reported an association of these SNPs with

cognitive response control (but not ADHD per se) in both affected and unaffected individuals [Baehne et al., 2008]. Additionally, in the largest ADHD candidate gene study presented to date, Brookes et al. [2006] failed to find an association between ADHD and the promoter SNPs, but again found association for markers in introns 5–8 of *TPH2* in a sample of 776 combined type cases from the IMAGE study [Kuntsi et al., 2006]. However, it was the opposite allele compared to the initial study by Sheehan et al. [2005] that was overtransmitted to affected children ($OR = 1.40$, $P = 0.003$). Haplotype and LD analysis revealed that there were several markers which tagged a putative risk haplotype with a frequency of about 14% in the intron 5–8 region. Further support for this finding was found in a recent quantitative trait analysis in an Israeli sample partially overlapping with the IMAGE sample [Manor et al., 2008]. In this study, the same haplotype was found to be associated with combined type ADHD, worse performance on the continuance performance test, and improvement following methylphenidate treatment.

As all these studies had been performed in children with ADHD, the situation for persistent ADHD is even less clear. However, from the current study, it seems reasonable to conclude that the *TPH2* region does not contain common genetic variants with strong effects on persistent ADHD across populations, though we do not have the power required to exclude the existence of common variants of more modest effects ($OR: 1.05$ – 1.15) in, or near, the region. Furthermore, all of the above-mentioned studies including our own have focused only on the gene and core promoter region; so it remains possible that effects of putative, to our knowledge still unknown, more distant regulatory variants have been missed.

For *TPH1*, both the Norwegian and Spanish samples showed evidence of an association of rs17794760 with persistent ADHD, while there was no association in the German and Dutch sample ($OR = 0.84$, $P_{\text{random effect model}} = 0.05$, $P_{\text{fixed effect model}} = 0.006$). Two previous studies with subjects from Japan and China, respectively, did not find evidence for single marker association between the 218A>C SNP (rs1800532) and ADHD [Li et al., 2003, 2006]. Likewise, there was no evidence for association between ADHD and *TPH1* markers in two studies of samples from European populations [Brookes et al., 2006; Ribases et al., 2009]. However, none of the above-mentioned studies had full coverage of the common variation within the *TPH1* region and none involved any markers in strong LD with our top-hit marker rs17794760. Hence, it is not possible to exclude entirely a role of rs17794760 or another common risk variant tagged by this SNP within the *TPH1* region.

We chose to increase the power of our study by analyzing samples from several different populations. While this has proven very useful in many complex disorders [McPherson et al., 2007; Zeggini et al., 2008], it may come at the cost of introducing phenotypic heterogeneity into the study sample due to differences in the classification of ADHD or in recruitment strategies between different sites. In an attempt to limit this problem, we re-analyzed the data stratified on combined or inattentive cases, but the results remained similar in both sub-analyses. Importantly, our study design does not allow us to test for less-frequent variants which might explain more of the genetic variance of psychiatric disorders [McKinney et al., 2008; Goldstein 2009; Need et al., 2009].

In conclusion, we have performed the largest study of the role of *TPH1* and *TPH2* variants in ADHD to date, and our results do not support the idea that common variants within these genes have a substantial effect on persistent ADHD.

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