ORIGINAL ARTICLE

Attention-Deficit/Hyperactivity Disorder Symptoms in Offspring of Mothers With Impaired Serotonin Production

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Context: Exposure to adverse events during prenatal and postnatal development, as well as serotonin deficiency, have been implicated in disturbances of mood and impulsivity, but the underlying mechanisms are unknown.

Objective: To investigate the long-term effects of an impaired serotonin synthesis on the developing human brain, we studied the effects of nonsynonymous mutations affecting tryptophan hydroxylase (TPH) enzymes responsible for serotonin production in maternal reproductive tissues (*TPH1*) and the brain (*TPH2*).

Design: Family-based case-control and functional studies of candidate genes.

Setting: Adult outpatients with attention-deficit/hyperactivity disorder (ADHD), their family members, and random control subjects were recruited across Norway.

Participants: Nine pedigrees with *TPH1* and *TPH2* mutation carriers were identified among 459 patients with ADHD and 187 controls. The *TPH* genes were then sequenced in 97 additional family members, and information about psychiatric diagnoses and symptoms was obtained from 606 controls, the 459 patients, and their relatives.

Main Outcome Measures: The effects of maternal vs paternal *TPH1* mutations compared in all families.

Results: Nine different *TPH1* and *TPH2* mutations were found by sequencing in 646 individuals (1.0% and 0.2% allele frequency, respectively). In vitro studies showed that 8 TPH mutants had significantly impaired enzyme function. Family analysis of 38 *TPH1* mutation carriers and 41 of their offspring revealed that offspring of mothers carrying *TPH1* mutations reported 1.5- to 2.5-times-higher ADHD scores and related symptoms during childhood and as adults than did controls ($P < 10^{-6}$) or offspring of fathers with the corresponding *TPH1* mutations ($P < .001$).

Conclusions: Impaired maternal serotonin production may have long-term consequences for brain development and increase the risk of ADHD-related symptoms and behavior in offspring. Replication studies are required to form conclusions about the clinical implications of mutations affecting serotonin biosynthesis.

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SEROTONIN (5-HYDROXYTRYPTAMINE) is a hormone and transmitter that performs a broad range of physiological functions in the human body. In addition to its transmitter function in the mature nervous system, serotonin has an important role during development, being involved in neurogenesis, neural migration, differentiation, and synaptogenesis.[1] Mouse studies indicate that embryonic serotonin in early gestation is of maternal origin and that serotonin deficiency leads to growth retardation of the brain and other organs in the offspring.[2,3] The early arrival of serotonin in embryonic brain structures may regulate ingrowth and development of other monoaminergic neurons, in particular dopamine terminals.[4] Serotonin depletion in pregnant rats leads to decreased brain dopamine levels and increased locomotor activity in their adult offspring.[5]

Vertebrate genomes encode 2 different enzymes that are specifically involved in the biosynthesis of serotonin, that is, tryptophan hydroxylases 1 and 2 (TPH1 and TPH2), with slightly different struc-

ture and regulatory properties.[6-8] Tryptophan hydroxylase 1 is found in the pineal gland and peripheral organs, including female reproductive tissues, whereas TPH2 is responsible for nearly all serotonin production in the brain.[9,10] However, it has also been reported that significant amounts of TPH1 are expressed in various regions of the human brain, including the striatum and hippocampus.[11] Selective elimination of *Tph1* in mice is associated with an increased rate of malformations in their offspring, possibly mediated by an insufficient serotonin production in maternal reproductive tissues.[2,3]

A dysfunctional serotonin neurotransmission has been implicated in human psychiatric disorders such as bipolar disorder, depression, anxiety, autism, schizophrenia, and attention-deficit/hyperactivity disorder (ADHD).[12-16] Genetic variants within *TPH1* (NCBI Entrez Gene 7166), *TPH2* (NCBI Entrez Gene 121278), or a combination of these genes[17] has been reported to be associated with all of these conditions in different clinical samples. However, although disturbances of dopamine signaling have received more attention, conclusive evidence of abnormalities within the serotonin or dopamine pathways in patients with ADHD is still lacking. The problems of identifying strong susceptibility genes by using common genetic variants in association studies may be due to inadequate statistical power, population stratification, or failure to address the appropriate clinical phenotypes and/or target genes. Recent whole genome association studies suggest that common single-nucleotide polymorphisms are unlikely to explain most of the familiar aggregation of common psychiatric disorders. Attention has therefore shifted toward a search for rare variants of potentially stronger effects.[18] In addition to genetic factors, environmental agents also influence human phenotypes, including adult behavior, by their action in critical periods of embryonic development.[19]

Although human data also suggest a connection between disturbed serotonin function and birth defects,[20] the effect of an impaired serotonin synthesis on pregnancy outcome is unknown. To evaluate a possible causative role of serotonin deficiency in ADHD-related symptoms and behavior, we studied a large cohort of adult ADHD patients and multigenerational family members. By sequencing the *TPH1* and *TPH2* genes, we could show that children born to mothers with coding variants affecting TPH1 enzyme function have a high symptom load of inattention, hyperactivity, impulsivity, and other emotional problems.

METHODS

SUBJECTS

The patients in this study were adults (aged ≥18 years) with a clinical diagnosis of ADHD corresponding to *DSM-IV* criteria, recruited from all parts of Norway. Most of them were recruited from a national registry of adult ADHD patients in Norway from 1997 through 2005. To include patients diagnosed as having ADHD after 2005, additional patients were recruited directly from clinicians, using national guidelines based on the former registry assessment.

Controls were recruited from a random sample of the Norwegian population aged 18 to 40 years by using the National Public Registry. All of the sequenced controls were part of this sample. The total sample of 606 controls included 180 controls recruited from additional sources (students and friends of patients). The recruitment strategy and inclusion criteria of the patient and control samples have been described previously.[21,22]

Family members of patients were invited by the patients to participate in the family study. The families included in the present study were families of probands with *TPH1* mutations in which at least 3 family members agreed to participate. Seven families with a total of 86 members were included in the *TPH1* analyses.

An informed consent based on detailed written information about the project was obtained from all patients, controls, and family members. The study was approved by the Norwegian Regional Medical Research Ethics Committee West institutional review board 3.

TPH1 AND *TPH2* SEQUENCING

We extracted DNA from blood or saliva samples received from all study participants after informed consent. All coding exons and intron-exon regions of *TPH1* were successfully sequenced in 187 population-derived controls and 457 adult ADHD patients. Similarly, *TPH2* was successfully sequenced in 459 patients and 179 controls using a DNA analyzer (3730 series; Applied Biosystems, Foster City, California). An additional 9 patient samples and 4 control samples were excluded from analysis because sequence information was missing for more than 1 exon. Observers were blinded to patient groupings.

EXPRESSION, PURIFICATION, AND ANALYSIS OF HUMAN TPH1 VARIANTS

Human embryonic kidney cells (HEK293) were transfected with the pcDNA5/FRT-TPH1 and pcDNA5/FRT-TPH2 expression vector (wild-type [WT] and mutant forms) using lipofectamine (Gibco, Rockville, Maryland) as described by the manufacturer, and the TPH1 and TPH2 variants were expressed as previously described.[8] The TPH1 messenger RNA (mRNA) levels in the HEK293 were measured as described in the online-only supplemental "eMethods" section (http://www.archgenpsychiatry.com).

Expression of maltose-binding protein TPH1 and TPH2 fusion proteins in *Escherichia coli* was performed as described previously for TPH2,[23] except that for the TPH1 proteins, TOP10 competent cells (Invitrogen, Paisley, England) were used. The cell lysate was fractioned into soluble and insoluble parts by centrifugation at 10000*g* for 30 minutes. An equal volume of lysate buffer was added to the pelleted fraction and homogenized. Specific expression levels of fusion proteins in the soluble and pelleted fractions were determined by analyzing equal amounts (30 µg) by separation on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels that were stained with Coomassie blue or immunostained with anti-PH8 antibody.

Wild-type and mutant TPH1 and TPH2 were expressed and purified by a rapid batch method described previously for TPH2.[24] Fusion proteins were eluted with 10mM maltose in 20mM HEPES (pH, 7.4), 400mM sodium chloride, 10% glycerol, and complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Eluted fusion proteins were concentrated to approximately 1 mg/mL and analyzed after separation on 12% SDS-PAGE gel.

Table 1. Overview of *TPH1* and *TPH2* Coding Mutations Found by Sequencing the Entire Coding Sequence in 459 Adult ADHD Patients and 187 Population-Based Controls

Gene	Position	No. (%) Cases (n=459)	No. (%) Controls (n=187)	Exon	Amino Acid Change	Reference
TPH1	c.160A>C	1 (0.2)	0	2	p.Lys54Gln	Novel
	c.424C>T	1 (0.2)	0	4	p.Arg142Cys	Novel
	c.433C>T	1 (0.2)	0	4	p.Arg145X	Novel
	c.529G>A	2 (0.4)	1 (0.5)	5	p.Val177Ile	Ramaekers et al[26]
	c.820C>A	1 (0.2)	1 (0.5)	7	p.Leu274Ile	Novel
	c.898G>A	1 (0.2)	2 (1.1)	7	p.Ala300Thr	Novel
	c.1229T>A	1 (0.2)	0	10	p.Ile410Asn	Novel
	All	**8** (1.8)	**4** (2.1)			
TPH2	c.907C>T	1 (0.2)	0	7	p.Arg303Trp	McKinney et al[16]
	c.1417G>A	2 (0.2)	0	11	p.Asp473Asn	Novel
	All	**3** (0.7)	0			

Abbreviations; ADHD, attention-deficit/hyperactivity disorder; *TPH*, tryptophan hydroxylase.

Coomassie blue–stained maltose-binding protein TPH1 and TPH2 fusion protein band intensities were integrated using a molecular imaging system (VersaDoc MP 4000; Bio-Rad Laboratories, Hercules, California), whereas immunostained protein bands were integrated using a multicolor DNA analysis system (Fluorimager; Amersham Biosciences, Sunnyvale, California). Protein bands from both methods were analyzed using commercially available software (Quantity One; Bio-Rad Laboratories). The activity and stability of TPH were assayed as described previously.[24] Phosphorylation was performed as described previously.[24] Typically, we used a 1:10 ratio of cyclic adenosine monophosphate–dependent protein kinase C-subunit to TPH for phosphorylation. Phosphorylation stoichiometry was determined by measuring phosphorus P 32 incorporation at various time points using a scintillation counter (TriCarb 2900TR; Packard Bioscience, Meriden, Connecticut) and by visualization using a phosphoimager (Bio-Rad Laboratories) after separation on 12% SDS-PAGE at various time points. Quantification of band intensity was performed using the Quantity One software (Bio-Rad Laboratories).

MOLECULAR MODELING/ PREDICTION ANALYSIS

The crystal structure of double-truncated TPH1 (PDB database, 1MLW; http://www.pdb.org/pdb/home/home.do) and the full-length model of TPH1 (PDB database, 1IN9; http://www.pdb.org/pdb/home/home.do) were used to analyze enzyme mutants for differences in atomic contacts, van der Waals overlaps, hydrogen bonding, and salt bridges using the WhatIf protein structure analysis software[25] (Vriend group at the Centre for Molecular and Biomolecular Informatics, Nijmegen, the Netherlands).

STATISTICAL METHODS

Descriptive statistics for clinical data were performed with SPSS statistical software (version 15.01; SPSS Inc, Chicago, Illinois). We used χ^2 and Fisher exact tests for analyses of categorical variables, *t* test for independent samples, and 1-way analysis of variance (ANOVA) for continuous variables. Associations between parental genotype and ADHD symptom severity with and without covariates were analyzed using multiple linear regression analyses.

RESULTS

MUTATION SCREENING OF *TPH1* AND *TPH2*

To determine the frequencies of functional variants in *TPH1* and *TPH2*, their complete coding regions were first sequenced in a random sample of 187 Norwegian adults (**Table 1**). To examine whether *TPH1* and *TPH2* mutations were associated with ADHD, we obtained clinical information and sequenced these genes in 459 adult ADHD patients. In addition to previously reported common single-nucleotide polymorphisms, we found 5 novel (and 1 previously reported[26,27]) missense mutations and 1 novel nonsense mutation in *TPH1* (Table 1 and the eFigure). The total *TPH1* mutation carrier frequency was similar in ADHD cases and controls (1.8% vs 2.1%). In *TPH2*, we found 1 previously reported[16,24] and 1 novel missense mutation (2 individuals) in patients, compared with none in controls.

FUNCTIONAL CHARACTERIZATION OF MUTANT TPH1 PROTEINS

To determine the structural effects of the *TPH1* mutations, a full-length model of the human enzyme was prepared using a combination of the crystal structure of truncated TPH1[28] and homology and ab initio modeling (**Figure 1**). The model shows that the missense variants are distributed over all 3 enzyme domains. To examine the functional properties of the TPH1 protein variants, they were produced in *E coli*, and we analyzed their enzymatic activity, solubility, and stability as purified enzymes or in freshly prepared bacterial extracts. All variants were also transiently produced in HEK293 cells, and we measured their mRNA and protein abundance, protein solubility, and specific activity in the soluble fraction.

To determine whether the mutations affected protein expression levels, we calculated the amounts of TPH1 protein produced relative to mRNA levels. Some variability of protein expression was observed for the TPH1 mutants, but none of them were significantly different from the WT

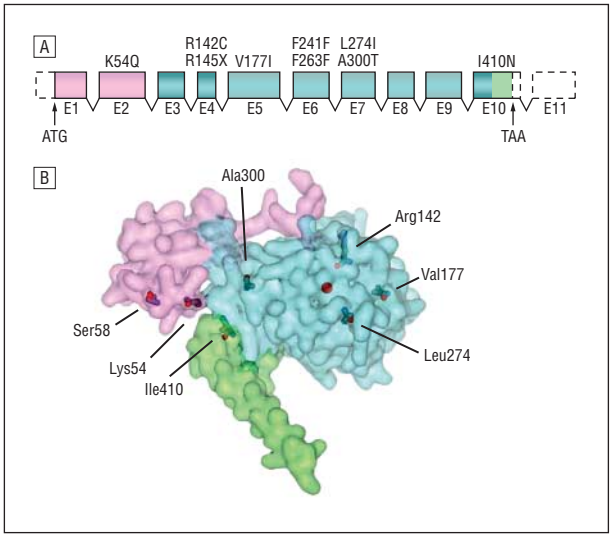

Figure 1. Positions of mutations in human tryptophan hydroxylase 1. A, Genomic structure and location of variants in *TPH1* gene. Exons encoding the regulatory domain are shown in pink (p.Met1-Thr104); the catalytic domain, in blue (p.Val105-Asn402); and the oligomerization domain in green (p.Pro403-Ile444). Modified from Haavik et al.[27] B, Molecular model of a full-length subunit of wild-type TPH1 illustrating the position of missense mutations and phosphorylation sites. The large red ball represents the active site iron atom. Arbitrarily determined domain boundaries are as shown in part A.

(**Figure 2**A). Except for TPH1 p.R145X, which lacks the catalytic domain and is completely inactive, all TPH1 variants had some residual catalytic activity when expressed in HEK293 cells (Figure 2B) or *E coli* (Figure 2F). In the bacterial homogenates, the protein solubility was intact for all TPH1 variants (Figure 2C). The specific activities of pure p.R142C, p.L274I, p.A300T, and p.I410N were 10% to 58% of WT TPH1, whereas the activities of p.K54Q and p.V177I were comparable to that of the WT (Figure 2F). Further kinetic studies of the TPH1 missense variants showed that their Michaelis-Menten constant (K_M) values for tryptophan or tetrahydrobiopterin were unaltered, but their specific activities (V_{max}) values were reduced, corresponding to the reduced enzyme activities shown in Figure 2F. Although we did not observe significantly changed mRNA or protein expression by any of the TPH1 variants, we cannot exclude additional effects of the mutations at the transcriptional or translational level.

Most disease-related mutations of aromatic amino acid hydroxylases are missense variants with reduced enzyme stability in vivo and in vitro.[24,27] Thus, the stability of the human TPH1 variants was also studied. Except for p.V177I, the purified enzyme variants had increased inactivation rates at 37°C, with apparent half-lives for p.A300T and p.I410N of only 10% to 15% of the WT (Figure 2D). The increased rate of inactivation could also explain why p.R142C has high activity in freshly prepared bacterial extracts but low specific activity after purification (Figure 2F).

The activity of TPH1 and TPH2 is regulated by phosphorylation and binding to 14-3-3 proteins.[25,29] To determine whether the missense variants of TPH1 had altered phosphorylation stoichiometry, the purified enzymes were phosphorylated with the catalytic subunit of cyclic adenosine monophosphate–dependent protein kinase. As shown in Figure 2E, all TPH1 variants were phosphorylated to approximately 1.5 mol phosphate per mole enzyme subunit, except for p.R145X, which incorporated only 0.7 mol phosphate/monomer. This indicates that the latter enzyme variant is lacking a phosphorylation site (eg, Ser260) in its C-terminal domain.[29]

To determine the possible effect of the novel TPH2 variant, p.D473N, we performed the same experiments as for the TPH1 variants. This variant had abundance and solubility similar to those of WT TPH2 but reduced specific activity (45%) (data not shown). Because we did not have access to family data, we did not pursue further studies on this mutation.

CLINICAL FEATURES OF *TPH1* AND *TPH2* MUTATION CARRIERS

As shown in Table 1, the frequency of *TPH1* mutations was similar in the population-derived sample and the ADHD clinical sample. We found only 3 patients with *TPH2* mutations and none in the control samples. By performing additional sequencing in 49 male and 48 female family members of persons with *TPH1* or *TPH2* mutations, we identified a total of 38 adults and 4 children (17 female and 25 male) who were heterozygous for *TPH1* missense or nonsense mutations and 4 adults who had *TPH2* mutations (eTable). According to our previous findings,[16,24] the inactivating *TPH2* mutation p.R303W appears to segregate with ADHD; however, family data were not available for the D473N mutation.

No obvious association of *TPH1* mutations and a diagnosis of ADHD was apparent in the patients or controls. Persons with 1 malfunctioning *TPH1* or *TPH2* allele appeared to have normal gross development and life span; 4 mutation carriers were healthy at ages 70 to 85 years, which is consistent with the mild phenotypes of *Tph1* and *Tph2* knockout mice.[9]

To obtain more information about the effects of inactivating *TPH1* or *TPH2* mutations, all study members reported symptoms and psychiatric diagnoses related to ADHD, depression, and substance abuse.[21,22] The adults reported childhood ADHD symptoms by using the 25-item Wender Utah Rating Scale (WURS; range, 0-100),[30] present ADHD symptoms on the 18-item adult ADHD Self-Report Rating Scale (ASRS; range, 0-72),[31] and bipolar spectrum symptoms on the Mood Disorder Questionnaire (MDQ; range, 0-13).[32,33] On average, individuals with coding variants in *TPH1* (n=38) had moderately higher scores on childhood and adult ADHD symptoms ($P=.03$ and $P=.07$, respectively, t test) and bipolar symptoms ($P=.22$) than did persons with the WT enzymes (eTable). Stratification by sex showed a more pronounced effect among men than women. However, the observed differences disappeared when the analyses were repeated after exclusion of all ADHD probands, indicating that the presence of 1 malfunctioning *TPH1* allele does not confer a strong risk of psychiatric symptoms.

EFFECTS OF MATERNAL *TPH1* MUTATIONS

Because TPH1 is expressed in peripheral organs, including female reproductive tissues,[34] and the maternal but not the paternal *Tph1* genotype affects the offspring in

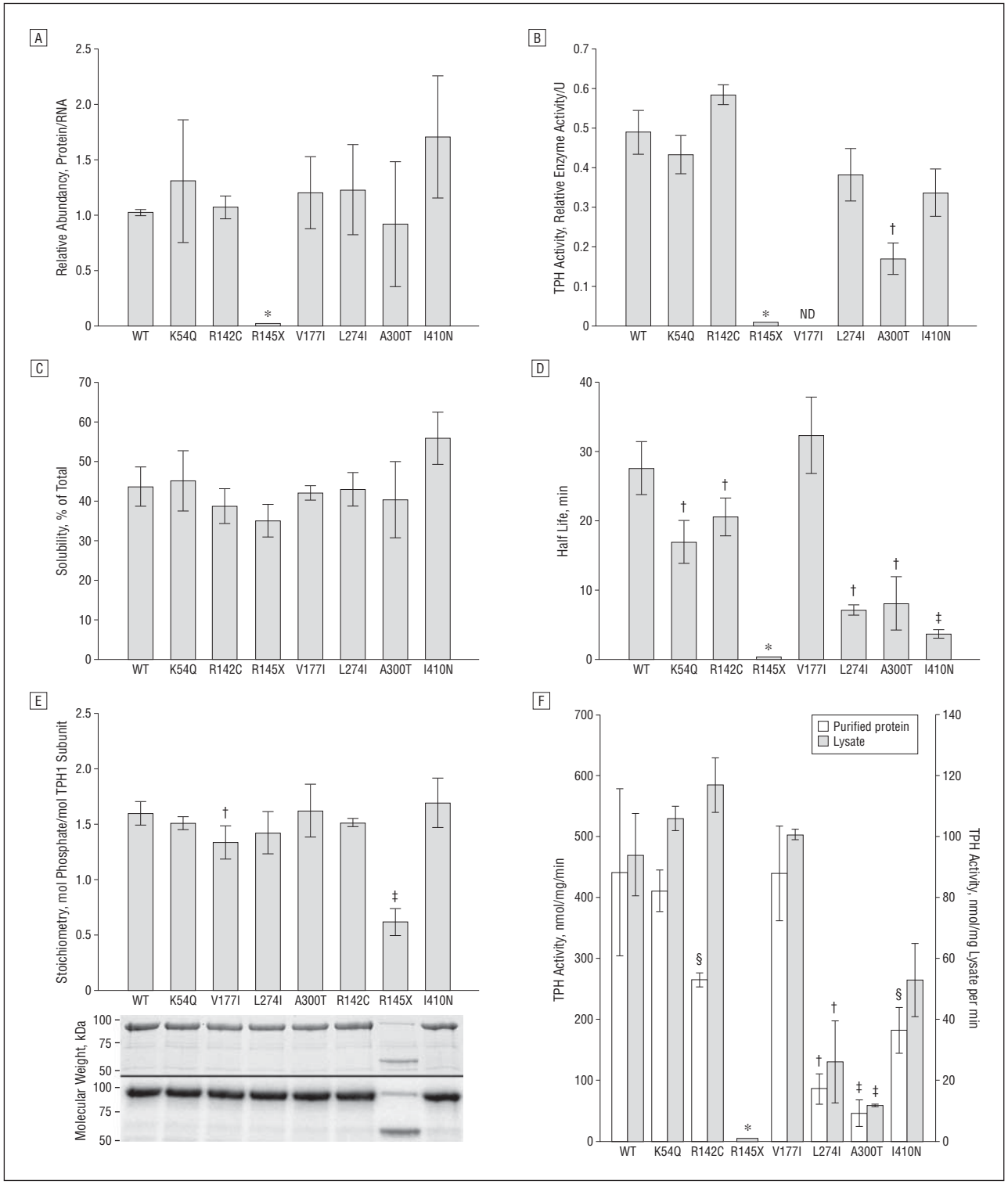

Figure 2. Biochemical properties of the wild-type (WT) and mutant tryptophan hydroxylase 1 (TPH1). A, Relative protein abundancy in transfected human embryonic kidney (HEK293) cells based on immunostaining and RNA levels. B, TPH1 activity in transfected HEK293 cell lysates. Levels of TPH1 protein were determined by immunostaining, and TPH activity of the soluble fractions of cell lysates was measured as described.[8] The activity of A300T was significantly lower than that of the WT ($P<.05$, *t* test). C, Relative solubility of TPH1 mutants. *S*oluble and insoluble fractions of *Escherichia coli* lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. D, Thermal stability of TPH1 WT and mutants at 37°C. The stability of p.K54Q, p.R142C, p.L274I, p.A300T, and p.I410N were significantly lower than that of the WT ($P<.05$, *t* test). E, Phosphorylation of TPH1 WT and coding variants by cyclic adenosine monophosphate–dependent kinase. Upper panel, phosphorylation stoichiometry of purified maltose-binding protein (MBP)–TPH1 fusion proteins ($n\geq3$). Middle panel, purified MBP-TPH1 fusion proteins separated by SDS-PAGE and stained with Coomassie blue. Lower panel, phosphoimage of phosphorylated MBP-TPH1 fusion proteins separated by SDS-PAGE. The positions of molecular weight standards are indicated (left). F, Activity of MBP-TPH1 WT and mutants as purified proteins and in *E coli* lysates adjusted for enzyme amount after immunostaining of TPH1. Bars (whiskers) represent mean (SEM); ND, not determined. *Not detectable. †$P<.05$, *t* test. ‡$P<.005$, *t* test. §$P=.06$.

Table 2. Linear Regression Analyses of Symptom Scores for Offspring of Mothers vs Fathers With *TPH1* Mutations

Variable	No. (%)		Linear Regression			
	Mothers With *TPH1* Mutations (n=20)	Fathers With *TPH1* Mutations (n=21)	Unadjusted β Value	Unadjusted *P* Value	Adjusted β Value[a]	Adjusted *P* Value[a]
Demographic and clinical data						
Age, mean (SD), y	28.9 (8.8)	33.3 (17.9)		.32		
Female sex	8 (40.0)	12 (57.1)		.27		
Mother smoking in pregnancy	13 (65.0)	6 (28.6)		.01		
Formal ADHD diagnosis	8 (40.0)	3 (14.3)		.17		
TPH1 mutation	13 (65.0)	11 (52.4)		.41		
Symptom score, mean (SD)						
WURS, total sum[b]	35.4 (26.7)	17.7 (21.9)	17.7	.04	16.6	.12
ASRS, total sum[c]	32.6 (13.8)	19.8 (11.1)	12.8	.004	11.8	.04
ASRS, inattentive score	17.8 (7.0)	11.9 (6.4)	5.96	.01	5.42	.07
ASRS, hyperactive/impulsive score	14.7 (7.9)	7.9 (5.2)	6.85	.005	6.41	.03
MDQ, sum[d]	7.2 (5.8)	2.9 (4.3)	4.23	.03	2.54	.26

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; ASRS, ADHD Self-Report Rating Scale; MDQ, Mood Disorder Questionnaire; *TPH1*, tryptophan hydroxylase 1; WURS, Wender Utah Rating Scale.
[a] Adjusted for age, sex, and maternal smoking in pregnancy.
[b] Score range, 0 to 100.
[c] Score range, 0 to 72.
[d] Score range, 0 to 13.

mice,[2] we hypothesized that an impaired maternal serotonin synthesis could affect brain development and behavior of human offspring. To correct for a possible recruitment bias resulting from an overrepresentation of persons with ADHD or related symptoms in relatives of *TPH1* mutation carriers, we performed a pairwise comparison of the effects of maternal vs paternal *TPH1* mutations for all mutations and families.

When comparing offspring of mothers with *TPH1* mutations (n=20) with offspring of fathers with the corresponding *TPH1* mutations (n=21) and a population-based control sample (assumed to represent the population distribution of *TPH1* variants, n=606), we found a strong effect of parental *TPH1* genotype. On average, the group with maternal *TPH1* mutations reported 1.5- to 2.5-times-higher symptom levels of ADHD and bipolar disorder than did children of fathers with the *TPH1* mutations or the population-derived control group (**Table 2** and **Figure 3**). Using ANOVA, the 3 groups showed significant differences in current symptoms of ADHD (ASRS score, $F=10.1$ [$P=5\times10^{-5}$]), childhood ADHD symptoms (WURS score, $F=15.2$ [$P=3.7\times10^{-7}$]), and symptoms of bipolar disorder (MDQ score, $F=14.6$ [$P=6.2\times10^{-7}$]). Data from an additional pairwise comparison of the 3 groups are shown in Figure 3.

The data on parental effect of the *TPH1* mutation derived from a total of 7 families, which varied in size (range, 3-31 family members), age (range, 5-81 years and 2-4 generations), and type of *TPH1* mutation (2 families with the V177I mutation and 1 each with the K54Q, A300T, R142C, L274I, and R145X mutations). Of a total of 86 included family members, 38 were *TPH1* mutation carriers, and 41 had a known parent with a *TPH1* mutation, including 20 with a maternal *TPH1* mutation and 21 with a paternal *TPH1* mutation. In addition, 13 individuals were predicted to have a mother or a father with a *TPH1* mutation because they were mutation carriers themselves, but they were not included in the analyses because information from their parents was not available. Thirty-five of the 41 individuals with a known parental origin of the mutation had 1 or more siblings included in the study, 18 in the maternal and 17 in the paternal offspring groups. Age, sex distribution, and proportions of individuals with a *TPH1* mutation and with a known diagnosis of ADHD were not statistically different in the maternal and paternal samples (Table 2), and sex-specific subanalyses of parental *TPH1* mutation effect did not alter the results (data not shown). We could not find any signs of biased patient recruitment; that is, the same proportion of fathers and mothers (12 vs 10) carrying the mutations were recruited. Furthermore, a similar number of offspring (20 vs 21) were recruited from both groups. The proportion of family members consenting to participate was also similar for the 2 groups.

Scatterplots of ASRS scores within each family in **Figure 4** illustrate that the effects of a maternal *TPH1* mutation were observed across all families and mutations. Furthermore, the effect of a maternal *TPH1* mutation remained essentially unchanged when parent probands with *TPH1* mutations (4 mothers and 3 fathers) were excluded from the analyses (ASRS score, $F=4.8$ [$P=.008$]; WURS score, $F=6.2$ [$P=.002$]; MDQ score, $F=10.9\times10^{-3}$ [$P=2\times10^{-5}$]). Experience with illegal drug use, which also may be considered an expression of impulsive behavior, was significantly different between individuals with the maternal vs paternal *TPH1* mutations (47.4% vs 11.8%, respectively, who had ever tried illegal drugs [$P=.03$, t test]) (Figure 3D).

To further investigate the effect of parental *TPH1* genotype and potential confounders on past or present symptoms in offspring, we performed a detailed comparison of demographic and clinical characteristics between individuals with mothers vs fathers with *TPH1* mutations. As shown in Table 2, the age and sex distribution was

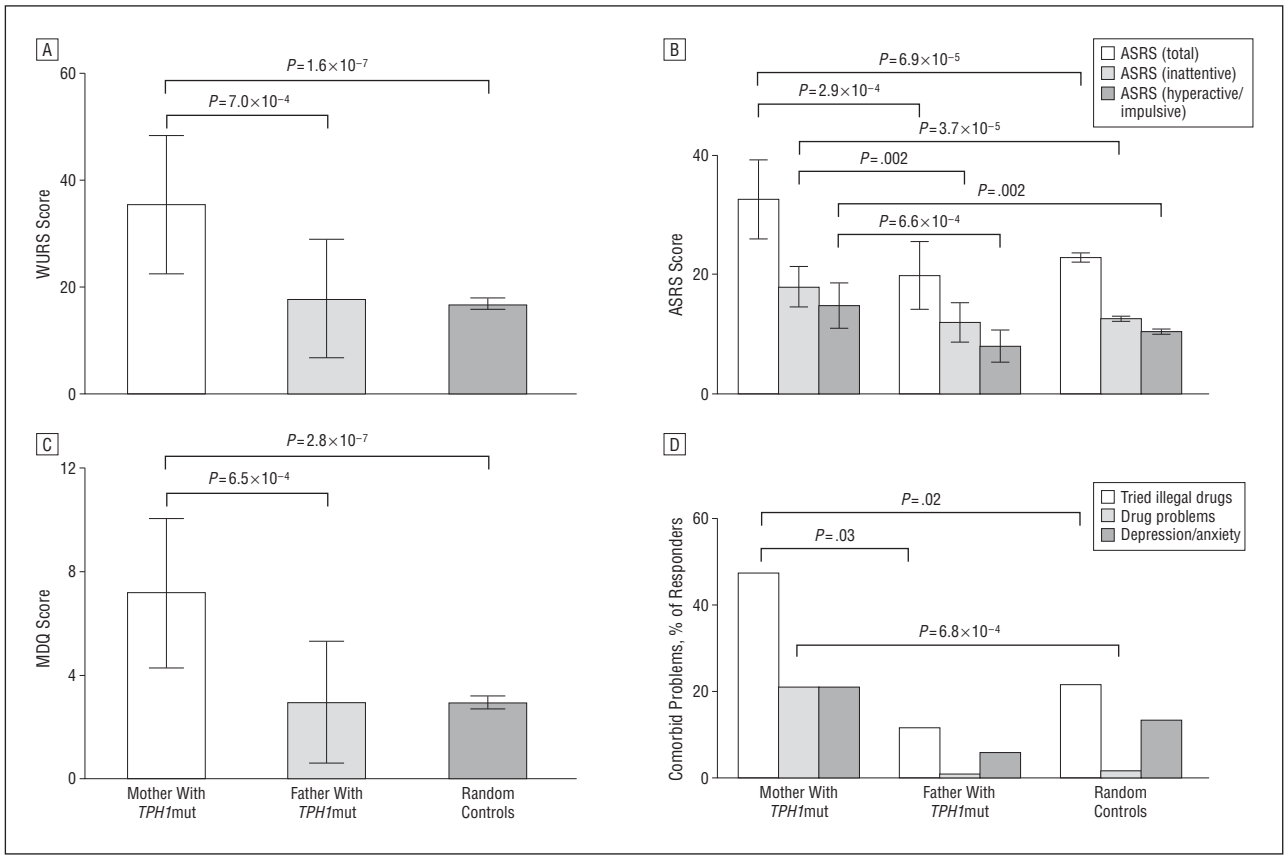

Figure 3. Effect of maternal mutations in the tryptophan hydroxylase 1 gene (*TPH1*mut) on symptoms of attention-deficit/hyperactivity disorder (ADHD) and related problems in offspring. Symptoms were measured in children of mothers with *TPH1*mut (12 male and 8 female offspring; mean [SD] age, 28.9 [8.8] years), a comparison group whose fathers carried the corresponding mutations (8 male and 13 female offspring; mean [SD] age, 33.3 [17.9] years), and a random population sample (n=606; mean [SD] age, 29.6 [8.1] years). A, Childhood ADHD symptoms (Wender Utah Rating Scale [WURS] sum). B, Current ADHD symptoms (ADHD Self-Report Rating Scale [ASRS] sum for the total scale and the inattentive and hyperactive/impulsive subscales). C, Bipolar symptoms (Mood Disorder Questionnaire [MDQ] sum). D, Proportions of individuals reporting having ever tried illegal drugs, having life-time drug problems, or having life-time occurrence of depression or anxiety. For parts A through C, data were compared using analysis of variance mean values of raw scores (bars) with 95% confidence intervals (whiskers) and *P* values from Tukey post hoc tests for multiple comparisons. For part D, data were compared using the Fisher exact test, with proportions in percentages and uncorrected *P* values.

similar in offspring of mothers and fathers with *TPH1* mutations, but maternal smoking during pregnancy was reported significantly more frequently in offspring of mothers with *TPH1* mutations. Likewise, there was a nonsignificant trend that offspring of mothers with *TPH1* mutations more frequently carried the *TPH1* mutation themselves and had previously been formally diagnosed as having ADHD. According to multiple linear regression analyses, the effect of parental *TPH1* genotype (β value) on the ASRS and WURS scores was almost unaltered after adjusting for age, sex, and maternal smoking during pregnancy. However, the differences in MDQ scores between the 2 groups were less apparent after this adjustment.

COMMENT

Herein we report the first sequencing of the human *TPH1* and *TPH2* genes in large nonclinical and adult ADHD samples, with special emphasis on the role of TPH1 protein variants. Because TPH1 is responsible for more than 90% of all serotonin production in peripheral tissues[3,7,9,35] and can influence embryonic development,[3] loss of TPH1 function could have a strong effect on human health. The total allele frequency of coding variants in *TPH1* and *TPH2* in Norway is approximately 1% and 0.2%, respectively. By extensive functional characterization of these variants, we have identified several loss-of-function mutations that are caused by decreased stability or by protein truncation (nonsense mutation). The in vitro activity and stability of coding variants of aromatic amino acid hydroxylases are generally well correlated with their in vivo enzymatic activity,[27] indicating that individuals with heterozygous loss-of-function *TPH1* mutations have an impaired serotonin biosynthetic capacity in peripheral tissues.

Although more than 99% of blood serotonin is stored in platelets, the free plasma serotonin is considered the active fraction. Mouse studies have shown that plasma serotonin levels in the offspring are determined by maternal *Tph1* genotype and maternal plasma serotonin levels but not by whole-blood serotonin levels.[2] Ideally, the plasma levels of serotonin in all *TPH1* mutation carriers should be measured directly to estimate its effect on the developing embryos. However, because it is difficult to obtain plasma completely free of contami-

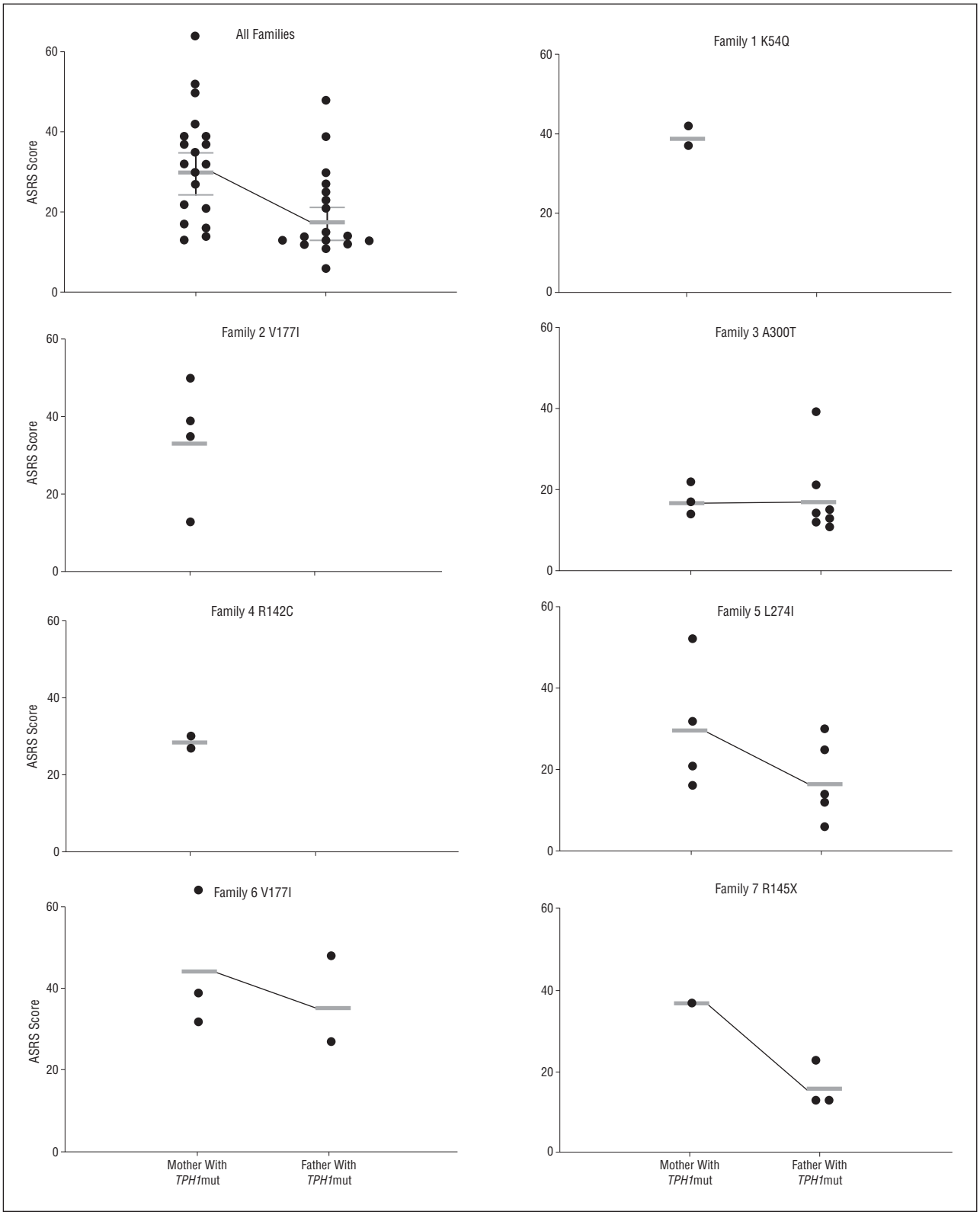

Figure 4. ADHD [attention deficit/hyperactivity disorder] Self-Report Rating Scale (ASRS) score within each family. The scatterplot was stratified by parental tryptophan hydroxylase 1 gene (*TPH1*) carrier status. Families are named by the type of *TPH1* mutation (*TPH1*mut). One dot represents 1 person. Only individuals with completed ASRS scores (>15 years) are represented (total, N=36; maternal, n=19; paternal, n=17). Mean values (gray horizontal bars) and 95% confidence intervals (whiskers; for all families only) are shown.

nating platelet serotonin and because serotonin levels fluctuate depending on dietary factors such as tryptophan intake, we considered that such measurements would not necessarily reflect the relevant maternal serotonin levels, for instance, during a critical gestational period. The serotonin transporter promoter polymor-

phism 5-HTTLPR is another functional variant within the serotonergic system that has been reported to have a strong influence on brain morphologic features and function.[36] However, the measured steady-state levels of serotonin or transporter function are not affected by this polymorphism,[37] illustrating the problems of predicting the genotype-phenotype relationships and biological markers in complex disorders.

The *TPH1* mutations in mothers seem to predispose to symptoms and behavior related to ADHD and affective disorder in their offspring, as demonstrated across different diagnoses and rating scales. As shown by the large variance in symptom load in different individuals with maternal *TPH1* mutations (Figure 4), the clinical outcome probably depends on a sum of many different genetic or environmental factors in addition to variations in maternal serotonin levels.

Studies in humans and animals have indicated that female sex hormones may modify the expression of several serotonergic genes, increasing TPH1 and TPH2 mRNA and protein levels.[38,39] Although the differences observed between offspring sexes in the present study were not significant, we cannot exclude a potential protective effect of female sex hormones in attenuating the impact of the TPH mutations by enhancing the expression of TPH. Clinical observations and experimental data indicate that many different factors, such as maternal stress experience, inadequate nutrition,[19] or endocrine factors such as levels of thyroid hormones[40] or glucocorticoids[41] early in pregnancy also may contribute to neurodevelopmental disorders in the offspring through their influence on placental function and embryonic development.

We propose that the effect of serotonin deficiency depends on the maternal *TPH1* status, independent of the patient's own genotype. This mechanism of genetic transmission could contribute to the total observed heredity of psychiatric disorders but would be difficult to detect in conventional molecular genetic studies. Although our findings are in accordance with previous animal studies and accumulated clinical evidence, we note that the number of mutation carriers still is small and that statistical evidence of some of the results is only indicative of an effect. Thus, replication studies in other large samples and possibly also reanalyses of previous family-based studies are required to verify this finding.

Although TPH1 is mainly expressed in peripheral tissues, *TPH1* variants have frequently been reported to be associated with psychiatric disorders, *TPH1* is a strong candidate gene for schizophrenia,[15] and both serotonin and melatonin have been suggested to be involved in the pathogenesis of autism spectrum disorders[42] and Tourette syndrome.[43] An association between low endogenous serotonin function and high alcohol preference has been reported[44] and is consistent with the observed effect of *TPH1* maternal genotype on reported illicit drug use in our study (Figure 3D). Thus, the effects of maternal TPH1 dysfunction are not limited to ADHD symptoms but may be linked to more general phenotypic traits, such as dysregulated impulsivity/inhibition, that are found in many psychiatric disorders and may be mediated by different transmitter systems.

We have not examined the functional role of the intronic markers used in previous association studies of *TPH1*, but our data support the idea that *TPH1* plays a role in shaping human behavior and the possibility that some of the previously used single-nucleotide polymorphisms may have a functional role or could tag other nearby functional variants. In addition to a direct trophic role of maternal serotonin on embryonic development, the effect of maternal *TPH1* genotype could be mediated by altered maternal nurturing behavior, as demonstrated in mice with disrupted serotonin neuronal differentiation.[45]

Mouse studies have also indicated that maternal *Tph1* could act in concert with embryonic *Tph2* during brain development,[3] and a combination of *TPH1* and *TPH2* risk alleles have been reported to increase the risk of bipolar disorder through genetic interaction.[17] Although such an interaction in theory is possible, the very low allele frequency of *TPH1* and *TPH2* coding mutations (<1% for each gene) makes this combination very rare and would not influence the results of our study.

An important challenge when studying the effects of rare functional mutations is the need to sequence large population cohorts. Compared with conventional genetic association studies of frequent genetic markers, our sample of 38 *TPH1* mutation carriers and 41 offspring of *TPH1* mutation carriers is rather small. Based on the *TPH1* mutation frequency estimate approximately equal to 1%, we would have to sequence a random population of 2000 individuals to obtain this sample size. However, because we had access to large multigenerational pedigrees, we were able to identify mutation carriers more efficiently and to systematically compare the effects of parental mutation carrier status within all families and for each mutation (Figure 4).

Our results may also have public health implications. Whether it is caused by genetic vulnerability, chronic inflammation, malnutrition, or other processes,[19] maternal serotonin deficiency during pregnancy might predispose to neuropsychiatric disorders and cardiovascular illnesses.[3] Recently, potent inhibitors of TPH1 and TPH2 activity have undergone clinical testing for use against functional gastrointestinal disorders,[46,47] problems that are most frequently reported in young female patients.[48] Tryptophan hydroxylase inhibitors can block serotonin production in the periphery and in the brain.[46,47] Our findings indicate that even 50% inhibition of TPH1 during pregnancy could have lasting behavioral effects on the offspring. However, further replication studies, preferably in larger samples, will be required to corroborate this relationship.

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Correction

Error in Byline and Author Affiliations. In the article "Support for the Vascular Depression Hypothesis in Late-Life Depression: Results of a 2-Site, Prospective, Antidepressant Treatment Trial" by Sheline et al, published in the March issue of the *Archives* (2010;67[3]:277-285), there were errors in the byline and Author Affiliations. In the byline on page 277, the name Kathleen Welsh-Boehmer, PhD, should appear as Kathleen Welsh-Bohmer, PhD. On page 284, in the Author Affiliations, Dr Welsh-Boemer should appear as Dr Welsh-Bohmer.