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Norepinephrine transporter and catecholamine-O-methyltransferase gene variants and attention-deficit/hyperactivity disorder symptoms in adults

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Summary. Attention deficit/hyperactivity disorder (ADHD) is a complex, highly heritable psychiatric condition. Neuropsychological and pharmacological studies suggest a dysregulation of central noradrenergic neurotransmission in addition to dopaminergic and serotonergic mechanisms. Only a few studies have focused on the association of noradrenergic susceptibility genes with ADHD.

In this study, we investigated the association of several ADHD symptom scores (German short form of the Wender Utah Rating Scale, WURS-k; ADHD self report, ADHD-SB, and the German validated version of the WRAADDS, WRI) with haplotypes of the catechol-O-methyltransferase (*COMT*) and the norepinephrine transporter (*SLC6A2*) genes. Subjects were genotyped for three *SLC6A2* (rs5569, rs998424, rs2242447) and two *COMT* single nucleotide polymorphisms (rs4680, rs4818). In addition, psychosocial adversity in childhood was assessed in order to evaluate putative gene-environment interactions.

We did not find main effects of the *COMT* and *SLC6A2* NET1 gene haplotypes on any ADHD symptom severity score. Childhood psychosocial adversity was strongly associated with number of ADHD symptoms. No gene-environment interaction was found. A specific combination of two *COMT* and *SLC6A2* gene haplotypes, containing the low functioning COMT variant was nominally associated with low ADHD scores in all scales. Results do not support the hypothesis that common variants in the *SLC6A2* and *COMT* genes in particular are associated with ADHD, but might give some evidence for interactive effects between these gene variants on ADHD severity.

Keywords: ADHD; adult; *COMT*; *SLC6A2*; genetic association

Introduction

Attention deficit/hyperactivity disorder (ADHD) is a complex psychiatric trait that arises due to interactions of environmental and biological influences with a strong underlying genetic aetiology (Rowland et al. 2002; Biederman and Faraone 2005). Family, twin, and adoption studies reported heritability estimates ranging from 60% to 80% and provide compelling evidence that genes play a strong role in mediating susceptibility to ADHD (Waldman and Gizer 2006).

Convergent findings in multiple samples which have been confirmed by meta- or pooled analyses (Faraone et al. 2005; Thapar et al. 2005a) suggest that several common genetic variants increase the risk for ADHD. These include the 7-repeat allele of a 48 bp VNTR in the dopaminergic D4 receptor (*DRD4*) gene (Faraone et al. 2001), the 10 repeat allele of a 480 bp VNTR in the dopamine transporter gene (*DAT1*) (Faraone et al. 2005), a microsatellite marker in the dopamine D5 receptor (*DRD5*) gene (Lowe et al. 2004), and a 44 bp insertion/deletion polymorphism in the promoter region of the serotonin transporter gene (*SLC6A4*) (Faraone et al. 2005; Retz et al. 2007).

In addition to dopaminergic and serotonergic mechanisms, neuropsychological and pharmacological studies also suggest a dysregulation of central noradrenergic neurotransmission, which is associated with certain aspects of higher cortical function including attention and arousal (Biederman and Spencer 2000). Treatment with drugs that enhance noradrenergic function in the frontal cortex shows efficacy in the treatment of ADHD (Arnsten et al. 1996; Wilens 2006). These findings have stimulated genetic association studies on variants in genes regulating noradren-

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ergic neurotransmission. Several studies suggested an association of polymorphisms of the adrenergic alpha1c receptor (*ADRA1C*) gene, the adrenergic alpha2a receptor (*ADRA2A*) gene and the adrenergic alpha2c receptor (*ADRA2C*) gene with ADHD (Comings et al. 1999; Barr et al. 2001; Xu et al. 2001; Roman et al. 2003; Park et al. 2005; Stevenson et al. 2005; Deupree et al. 2006; Schmitz et al. 2006; Wang et al. 2006). These findings, however, were not always replicated and have not yet been confirmed by meta-analyses (Faraone et al. 2005).

The norepinephrine transporter (*SLC6A2* alias NET) plays an important role in the regulation of catecholaminergic neurotransmission. In the frontal cortex, which is a brain region strongly involved in ADHD (Spencer et al. 2002) but shows low dopamine transporter density, not only norepinephrine but also dopamine re-uptake depends primarily on the NET (Moron et al. 2002). The norepinephrine transporter is a 617-amino acid protein and the coding gene (*NET1*, *SLC6A2*) is located on chromosome 16q12.2, consisting of 14 exons (Bruss et al. 1993). Two studies reported nominal association of ADHD with two single nucleotide polymorphisms (SNPs) (rs998424 and rs3785157) in the *SLC6A2* gene, and in one study an additional association with a third SNP (rs2242447) was found (Bobb et al. 2005; Xu et al. 2005). Brookes et al. (2006) could not corroborate these findings in a pooled sample of 776 individuals with ADHD combined-type, but they found evidence for association of ADHD with two other SNPs (rs3785143 and rs11568324). Other studies, however, reported negative findings for the reported variants (Barr et al. 2002; McEvoy et al. 2002; DeLuca et al. 2004). A recent study described an association with a new functional polymorphism influencing promoter function of the *SLC6A2* gene (A-3081T) (Kim et al. 2006).

The catechol-O-methyltransferase (COMT) is another important enzyme involved in the regulation of norepinephrine, epinephrine and dopamine in the human brain. Although COMT is expressed widely throughout the brain, its enzymatic activity appears to be particularly important in the prefrontal cortex, where it inactivates dopamine (Matsumoto et al. 2003). COMT occurs in two distinct isoforms: as a soluble protein in the cytoplasm (S-COMT; 221 aa) and as a longer membrane-bound isoform (MB-COMT 271 aa) (Tenhunen et al. 1994). The MB-COMT is predominantly expressed in brain neurons, while the S-COMT is predominantly expressed in blood cells and tissues like liver and kidney (Tenhunen et al. 1994). The COMT protein is encoded by a single gene with six exons that has been mapped to chromosome 22q11.21 (Grossman et al. 1992). In exon 4, a functional polymorphism has been described (rs4680, Lachman et al. 1996), which codes for a substitution of valine (val) to methionine (met) (G > A). The met variant produces a COMT enzyme with lower thermostability, resulting in decreased enzyme activity. Studies on the association with this functional variant in the *COMT* gene and ADHD resulted in diverging findings; some studies reported evidence for association, others did not. Two recent meta-analyses could not confirm a role for *COMT* val158met in the aetiology of ADHD (Faraone et al. 2005; Cheuk and Wong 2006).

Most of the reported genetic association studies have been performed in samples of children and adolescents with ADHD. As into adulthood persistent ADHD, however, might represent a stronger genetically determined trait than childhood ADHD (Thapar et al. 2005a) and no studies on *SLC6A2* and *COMT* variants have been performed in samples with adult ADHD to date, we aimed to investigate the association between variants in these two genes, as they both play a role in regulating noradrenergic as well as dopaminergic neurotransmission. In addition, we assessed gene-gene interaction and the role of psychosocial environmental risk factors, which have previously been shown to increase ADHD symptoms in adults with ADHD (Retz et al. 2007).

Methods

Subjects and instruments

A sample of 184 unrelated males (mean age 34.1 years, SD 11.7 years), consecutively referred for psychiatric examination to the Institute of Forensic Psychiatry of the Saarland University, entered the study after providing written informed consent and explanation of the aims of the study. Non-Caucasians and non-German speaking subjects were not included to avoid stratification artefacts. The local Ethical Committee approved the study.

To assure uniformity of data collection, all subjects underwent the same semi-structured psychiatric interview by well-trained psychiatrists and a neurological examination. Psychiatric diagnoses were assessed according to ICD-10 criteria by a standardized assessment procedure, using modified, standardized checklists (Hiller et al. 1990). Subjects with a diagnosis of current substance dependence, acute schizophrenia, major depression/bipolar disorder, or any other severe axis-I diagnosis according to DSM-IV as well as subjects with the diagnosis of mental retardation (IQ < 70) were excluded from the study. Cluster B personality disorders were present in 27.2%, personality disorders of Clusters A and C in 7.6% of the sample.

Regarding childhood adverse psychosocial risk factors, all participants of the study were rated by an independent investigator, who was blinded to the diagnostic status and genotype of the subjects, with regards to the following criteria towards childhood (defined as 0–10 years) environment: social status, family structure, emotional family climate, social integration, school education. The items were graded from 0 to 2 points, with higher ratings indicating worse environment conditions. A mean score of adverse childhood environment was calculated for each subject, giving a relative total childhood adverse environment index (CAEI) of 0 (optimal childhood environment) to 2 (most adverse childhood environment), which has been described elsewhere (Reif et al. 2007).

Table 1. *PCR conditions and primer pairs used in amplification of target DNA*

Gene	SNP	Primer sequences (5′ > 3′)	Ta	Cy	Size, bp	RE	Genotype
COMT	rs4818	cac ctg tgc tca cct ctc ct ggg ttt tca gtg aac gtg gt	60	36	348	*BclI*, 50°C	CC = 348 bp; GG = 189 + 159 bp
	rs4680	cac ctg tgc tca cct ctc ct ggg ttt tca gtg aac gtg gt	60	36	348	none	direct sequencing (G > A)
SLC6A2	rs5569	tcc agg gag acc cta att cc ttg act tta ttg aaa tgc gg	58	35	251	none	direct sequencing (G > A)
	rs998424	tcc agg gag acc cta att cc ttg act tta ttg aaa tgc gg	58	35	251	*MnlI*, 37°C	AA = 124 + 42 + 32 + 18 + 12 + 6 + 5 + 2bp GG = 156 + 42 + 18 + 12 + 6 + 5 + 2 bp
	rs2242447	tgg gtc tcc ttc aag tct gg gaa aaa cac ccc ctt cct tc	59	36	628	*RsaI*, 37°C	TT = 271 + 228 + 129 bp CC = 499 + 129 bp

Fragment length (*Size*) observed in base pair (*bp*), annealing temperature (*Ta*) in °Celsius and number of PCR cycles (*Cy*) together with the restriction enzyme used (*RE*) are shown. When specified, the amplicon was direct sequenced with a multicolour fluorescence-based 16-capillary electrophoresis ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the nucleotide sequence analyzed with the Staden processing software package.

ADHD symptoms were assessed according to their relative ADHD-scores following two self-report scales (ADHD-SB and WURS-k), the Wender-Reimherr diagnostic interview (WRI), which is the German validated version of the Wender Reimherr Adult Attention Deficit Disorder Scale (WRAADDS; Wender 1995) and validated background data. The WURS-k is the German validated short form of the Wender-Utah Rating Scale designed for the retrospective assessment of childhood ADHD symptoms. This self-report consists of 25 items rated on a 0–4 Likert scale (Retz-Junginger et al. 2002, 2003). The ADHS-SB includes 18 psychopathological items of ADHD according to DSM-IV criteria, which are rated on a 0–3 Likert scale and has been validated for the use in German speaking populations (Rösler et al. 2004). The WRI consists of 28 items rated from 0 (not present) to 2 (severe), which contribute to 7 psychopathological domains: inattentiveness, hyperactivity, mood lability, irritability and hot temper, impaired stress tolerance, disorganization, and impulsivity. Complete data sets regarding ADHS-SB and WRI were available from 171 and 147 subjects, respectively. These groups did not differ from the entire sample concerning age (ADHS-SB $p = 0.716$; WRI $p = 0.908$), childhood CAEI score (ADHS-SB $p = 0.927$; WRI $p = 0.540$), personality disorders (ADHS-SB $p = 0.378$; WRI $p = 0.227$) and substance use disorders (ADHS-SB $p = 0.459$; WRI $p = 0.080$).

Genotyping

DNA isolation for genotyping was performed from 10 ml EDTA-stabilized blood with a commercial Invisorb Blood Giga Kit (Invitek, Berlin, Germany) according to the manufacturer's protocol as previously reported (Kissling et al. 2007). The quantity of extracted DNA was adjusted using a PicoGreen fluorometric assay (Molecular Probes Inc.). A 25-µl polymerase chain reaction (PCR) was performed, which contained 10 ng of genomic DNA in a premixed ready-to-use master-solution, consisting of *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers (Promega, Germany). Primers (MWG Biotech, Germany) were designed using the Primer3 programme (Rozen and Skaletsky 2000). After PCR, restriction enzyme digestion (RFLP) and/or direct sequencing techniques were used under the conditions as specified in Table 1. Twenty five percent of the total sample was randomly selected, and re-sequenced for all SNPs confirming the genotyping results.

Statistics

Descriptive statistics were performed by χ^2- and *t*-tests as appropriate. Correlations of two continuous variables were assessed by the Spearman correlation coefficient. Variants in *SLC6A2* and *COMT* were assessed as haplotypes, as respective variants did show moderate to high linkage disequilibrium (LD measured by D′, assessed by FastEH; Zhao and Sham 2002). Haplotypes of two *SLC6A2* and two *COMT* variants, respectively were individually assigned by PHASE (Stephens et al. 2001; Stephens and Donnelly 2003). Haplotypes were coded as either present (= 1) or not present (= 0), subsuming both, homozygote and heterozygote haplotypes, under "present". Association of haplotypes with the dimensional ADHD measures was assessed either by linear regression or non-parametric ANOVA, adjusted for age, history of substance abuse and adverse childhood environmental effects. As WURS-k values were obtained in all individuals and were normally distributed, the WURS-k score was the primary outcome of interest, for which haplotype by haplotype and haplotype by environment interaction was assessed by stepwise linear regression with backward elimination of interaction effects not showing any influence on the dependent variable. Only the final model showing significant effects of a haplotype by haplotype interaction on WURS-k scores was also assessed for the two other outcome measures of interest, the ADHD-SB score and the ADHD score obtained by the WRI. As both measures were not normally distributed, analyses were performed by non-parametric ANCOVA (SAS-macro npar, http://www.ams.med.uni-goettingen.de/Projekte/makros/index.html). As the sample size was relatively low, results were not adjusted for multiple testing.

Results

Two of the three *SLC6A2* variants were in complete linkage disequilibirum (LD; $D' = 1.0$; rs5569 and rs998424). Therefore, only rs998424 was assessed further. LD of rs998424 and rs2242447 was $D' = -0.68$ (SD 0.08). Haplotypes were individually assigned with a probability of > 0.85 in the full sample, and with a probability of 1.0 in the sample of $N = 143$, in whom all questionnaires were obtained. At least one haplotype containing A of rs998424 and T of rs2242447 was present in 101 individuals (*SLC6A2* AT; 55%), G of rs998424 and C of rs2242447 in 91 individuals (*SLC6A2* GC; 51%), and G of rs998424 and T of rs2242447 in 112 individuals (*SLC6A2* GT; 61%).

Both variants in the *COMT* gene did show high LD ($D' = 0.90$; SD = 0.03). Haplotypes were individually assigned with probability of > 0.98 in the full sample. The most frequent haplotypes constructed from rs4818 and rs4680 were CA in 137 (*COMT* CA, 74%), CG in 30 (*COMT* CG, 16%) and GG in individuals 124 (*COMT* GG, 74%).

Table 2. *Interaction of SLC6A2 and COMT haplotypes with regard to self-reported ADHD symptoms in childhood*

Independent variable	Type III sums of squares	Df	*F*-value	*p*-value
Age	1901.4	1	10.8	0.001
SLC6A2-GT	51.0	1	0.3	0.591
COMT-GG	268.8	1	1.5	0.218
SLC6A2-GT** *COMT*-GG	991.2	1	5.6	0.019
History substance abuse	130.4	1	0.7	0.390
Adverse childhood environment	6536.3	1	37.2	<0.0001

Dependent variable: WURS-k score.
Model DF = 6, error DF = 177; model F-value 11.1, $p < 0.0001$; $R^2 = 0.27$.

History of personality disorder was not associated with any of the 6 haplotypes assessed, nor with any of the three ADHD outcome measures (WURS-k score; ADHD-SB, WRI) ($p_{all} > 0.05$), therefore it was not controlled for in further analyses. History of substance abuse was less frequent in individuals carrying *COMT* CG, therefore, it was controlled for in further analyses.

Results of the final linear regression model obtained for the dependent variable WURS-k are shown in Table 2. Higher WURS-k scores were obtained in younger individuals ($\rho = -0.32$) who did show more childhood environmental risk factors ($\rho = 0.46$). An interaction effect of haplotypes *SLC6A2* GT and *COMT* GG was observed, however, independent main effects of both haplotypes were not observed. In addition, no interaction of any haplotype with the CAEI score was detected (models not shown). The interaction effect of the two haplotypes reflects lower self reported childhood ADHD symptoms in individuals not carrying any of both, the *SLC6A2* GT nor the *COMT* GG haplotype (Table 3). Similar effects were observed for the other two ADHD measures assessing current ADHD symptoms, the ADHD-self report score (obtained in 171 individuals; interaction effect *SLC6A2* GT* *COMT* GG $T = 4.6$, 1 DF, $p = 0.033$) and the Wender-Reimherr-Interview Score (obtained in 147 individuals; interaction effect NET-GT*COMT-GG $T = 4.1$, 1 DF, $p = 0.042$; Table 3). Again, age and CAEI did show strong effects on both scores as well ($p_{all} < 0.005$).

Table 3. *ADHD measures by SLC6A2*-GT and *COMT*-GG

	N, mean (SD)		
	WURS-k ($N = 184$)	ADHD-SB ($N = 171$)	Wender-Reimherr-interview ($N = 147$)
SLC6A2-GT not present	24	23	19
COMT-GG not present	23.0 (16.4)	8.8 (8.6)	12.1 (9.9)
SLC6A2-GT present	36	32	25
COMT-GG not present	26.8 (14.5)	14.2 (12.1)	18.2 (12.8)
SLC6A2-GT not present	48	46	40
COMT-GG present	28.9 (15.4)	13.3 (10.8)	17.5 (11.6)
SLC6A2-GT present	76	70	63
COMT-GG present	27.4 (15.3)	13.5 (11.7)	18.3 (12.8)

Discussion

In frontal cortical brain areas, norepinephrine transporters and *COMT* play a crucial role in the modulation and degradation of dopamine and other catecholamines. Therefore, genetic determined variations of these molecules are under discussion regarding dysfunction of frontal cortex in several psychiatric disorders including ADHD. Up to date, results of only few association studies concerning the *SLC6A2* and *COMT* gene have been reported.

In this study we did not find convincing evidence for a particular association of the assessed *SLC6A2* and *COMT* gene haplotypes with ADHD symptom scores. The negative findings with respect to *SLC6A2*, however, do not exclude this gene as a candidate for ADHD, as a functional and therefore the most promising variant in this gene thus far, described by Kim et al. (2006) is not at all in LD with the SNPs examined in the study. Moreover, the results of this study might give some support for an interaction between *SLC6A2* and *COMT* regarding severity of ADHD symptoms. Reduction of both, childhood and present ADHD symptoms, were detected when the *SLC6A2* GT haplotype and the *COMT* GG haplotype were absent, whereas all other *SLC6A2* GT/*COMT* GG haplotype combinations were associated with higher mean ADHD scores. Concerning the relevance of the val158met polymorphism for COMT activity, this finding supports the notion, that the low enzymatic activity variant of the *COMT* gene (not containing the G-allele coding for valine, but the A-allele, coding for methionine) might provide some protective influence against ADHD symptomatology, but only in carriers of special *SLC6A2* gene variants. Since there is no information about the functional relevance of the *SLC6A2* polymorphisms investigated in this study, further interpretation of this gene-gene interaction is yet not possible. Our findings, therefore, are in contrast to results of a study by Reuter et al. (2006), who have reported higher ADHD scores in adult carriers of the *COMT* A/A genotype.

Although ADHD has been conceptualized as a polygenetic disorder determined by the interaction of several genes with minor effects as well as environmental influences, there are only few studies, which have investigated epistatic gene effects so far. Carrasco et al. (2006) for example reported that interaction of the *DRD4* and the *DAT1* genes increases the risk for ADHD, whereas Qian et al. (2007) did not find interactive effects of several dop-

minergic genes and the *COMT* gene. However, further investigations of gene-gene interactions in ADHD might provide new insights in the aetiology of this disorder.

According to earlier reports, ADHD symptoms were strongly associated with age and influenced by psychosocial adversity during childhood in our study (Biederman et al. 1995; Retz et al. 2007). We did not find an interaction between psychosocial childhood adversity and genetic variants of the *SLC6A2* and *COMT* gene with respect to the extent of ADHD symptoms. However, we could confirm the effect of psychosocial childhood adversity on the development and persistence of ADHD. Thapar et al. (2005b) have reported an interactive effect of the *COMT* gene and low birth weight on early-onset conduct disorder in children with ADHD, suggesting that adverse effects of environmental factors are more susceptible in ADHD subjects carrying the high enzymatic activity G/G (val/val) *COMT* genotype. These results, however, were not replicated in another sample of ADHD children (Sengupta et al. 2006). Concerning the *SLC6A2* gene, there are no studies reported in literature up to date, which have focused on putative gene-environment interactions on psychiatric phenotypes.

As the subjects assessed in this study were not recruited from general population but from a forensic sample, which is a population enriched with ADHD cases (Rösler et al. 2004), some caution should be used in generalizing the results. Another limitation of the study is the retrospective assessment of childhood ADHD symptoms and psychosocial adversity. Although each subject was interviewed carefully for psychosocial risk factors and history of ADHD by the use of standardized instruments, recall bias and invalid diagnoses due to missing information of parents or spouses cannot fully be excluded. Further it has to be considered that the sample size was small in this study and no adjustment for multiple testing was made. Therefore, the assessment of the interacting risk alleles needs replication in a larger sample with higher power.

In conclusion, in our study no main effects on the assessed ADHD symptom scores of common variants in the *SLC6A2* and *COMT* genes were found. The nominal effect of the interaction of two particular haplotypes in these genes on ADHD symptom severity, however, provides evidence for some possible contribution of noradrenergic mechanisms on ADHD psychopathology. Interaction analyses such as the present one may help to explain inconsistencies in analyses of individual polymorphisms and genes between studies by controlling for the effects of other polymorphisms. Concerning that the majority of association studies in ADHD with noradrenergic genes have generated negative results, more emphasis should be put on gene-gene and gene-environment interactions in further studies.

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