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Serotonin transporter gene and schizophrenia: evidence for association/linkage disequilibrium in families with affected siblings

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The serotonergic (5-HT) system has been implicated in the etiopathogenesis of psychoses. Since the 5-HT transporter plays an important role in regulation of 5-HT transmission, its gene can be considered as a candidate for vulnerability to psychiatric disorders. Two polymorphic sites of the 5-HT transporter gene—5-HTTLPR, a VNTR in the 5′ regulatory region, and a VNTR in the second intron—were studied in a sample of 61 families with schizophrenia for transmission disequilibrium. Each family contained at least two siblings affected with schizophrenia or schizoaffective disorder (mainly schizophrenic). One hundred and thirty-nine affected offspring with parental information for genotyping, were available for analysis. No preferential transmission of either short or long alleles of the promoter polymorphism was observed. However, a transmission distortion was detected for alleles of the intronic VNTR polymorphism ($\chi^2_{TDT\ max}$ =14.33; *P* = 0.0002; corrected *P* value = 0.0003) resulting in more frequent than expected transmission of the 12 repeat allele. This finding adds additional evidence to the idea that the serotonergic system may be involved in development of psychoses. *Molecular Psychiatry* (2000) **5,** 91–95.

Disturbances in neurotransmission are considered to play a role in the development of psychiatric disorders. Several lines of evidence suggest that a master control neurotransmitter of complex neuronal communication may be serotonin (5-hydroxytryptamine, 5-HT), as reviewed by Lesch and Moessner.[1] While it is well established that the 5-HT system is involved in affective disorders,[1,2] its role in schizophrenia is currently being evaluated.

It has been shown that the 5-HT receptor agonist *m*-chlorophenylpiperazine (mCPP) can induce psychosis in schizophrenic patients,[3] while clozapine, the best known atypical antipsychotic drug,[4] displays an antagonist-activity at several 5-HT receptors.[5,6] Since the 5-HT transporter (5-HTT) may provide fine tuning for the 5-HT system,[1] alterations in the function of this protein could be involved in the development of schizophrenia. A reduced number of 5-HTT uptake sites has been detected in post-mortem brains of schizophrenics,[7,8] and Hernandez and Sokolov[9] reported an increase of 5-HTT mRNA in the cortex of schizophrenic individuals examined post-mortem.

Two polymorphic sites in the 5-HTT gene: VNTR-17, a 17-bp repeat element in the second intron[10] and 5-HTTLPR, a VNTR with a 22-bp repeat element in the promoter region,[11] have been discovered. While the function of the intronic VNTR-17 is not yet clear, it has been demonstrated by Lesch and colleagues[12] that 5-HTTLPR regulates expression of the 5-HT transporter. The short variant of the two polymorphic forms reduces the transcriptional activity of the 5-HTT gene promoter, resulting in decreased 5-HTT expression and uptake.

Several groups investigated a possible association of both polymorphisms with schizophrenia using case-control designs.[13–17] With the exception of Malhotra and colleagues[14] who demonstrated an association of intensity of hallucinations in neuroleptic-free schizophrenic patients with the 5-HTTLPR l/l genotype, all other studies failed to detect association with schizophrenia.

In the present study we investigated linkage and association of both 5-HT transporter polymorphisms with schizophrenia in a large sample of families multiply affected with schizophrenia applying the transmission disequilibrium test (TDT).

One hundred and thirty-three trios (offspring + parents) were obtained from 61 multiplex families and genotyped for both polymorphisms (Table 1). Out of 264 possible allele transmissions (for each marker one offspring escaped genotyping), 133 transmissions of VNTR-17 and 105 transmissions of 5-HTTLPR alleles were from heterozygous parents and therefore informative for TDT analysis.

The contingency table (Table 2) shows that allele 10, which represents ten repeats of VNTR-17 element, was transmitted 43 times (86 times not transmitted) while allele 12, representing twelve VNTR-17 repeats, was transmitted 90 times (47 times not transmitted). The rare allele with nine repeats was four times transmitted

Table 1 Composition of the family sample (for each family both parents available)

	Families	*Number of affected offspring*
	54 with pairs	108
	Four with triplets	12
	Two with quadruplets	8
	One with quintuplet	5
Total	61	133

Table 2 5-HTT gene VNTR alleles transmitted and not transmitted from heterozygous parents

		Not transmitted		
		9	**10**	**12**
Transmitted	**9**	0	0	4
	10	0	33	43
	12	4	86	94

$\chi^2_{\text{TDT max}} = 14.33$; $P = 0.0002$ ($P = 0.0003$).[a]
[a]*P* value corrected according to Morris *et al*.[43]

and four times not transmitted (both from 9/12 genotype). These differences in transmission were statistically significant ($\chi^2_{\text{TDT max}} = 14.33$, $P = 0.0003$) (Table 2).

On the other hand, neither of the alleles of the 5-HTTLPR polymorphism showed preferential transmission ($\chi^2_{\text{TDT}} = 0.24$, $P = 0.692$). The longer allele was transmitted 50 times and the shorter allele 55 times from heterozygous parents (Table 3).

We investigated allele transmission for two polymorphisms in the 5-HTT gene, 5-HTTLPR in the promoter region and VNTR-17 bp repeat in the second intron, in a large family sample with high genetic loading of schizophrenic disorders: each family consisted of more than one affected sibling. Besides multiple affected offspring, family history revealed additional affected family members for almost every family. Preferential transmission was detected for the VNTR-17 (allele 12), but not for 5-HTTLPR, applying TDT analysis. This suggests association/linkage of the 5-HTT gene with schizophrenia.

The intronic polymorphism has been mainly investigated in affective illness. Association of allele 9 with unipolar depression was shown by Ogilvie and colleagues[18] but could not be replicated by Stoeber *et al*.[19] Allele 12 has been shown to be associated with bipolar disorder by Collier *et al*[20] and this finding has been replicated by Rees *et al*[21] and Kunugi *et al*.[22]

A number of association studies have been reported, investigating the promoter polymorphism in affective

Table 3 Short (s) and long (l) 5-HTTLPR alleles transmitted and not transmitted from heterozygous parents

		Not transmitted	
		l	**s**
Transmitted	**l**	104	50
	s	55	55

$\chi^2_{\text{TDT}} = 0.24$; $P = 0.6256$.

disorders and in behavioral traits. Positive association for the short allele has been found in unipolar and bipolar disorders,[23,24] as well as in anxiety,[12] but could not be confirmed by other authors.[25–28] With the exception of Esterling *et al*,[28] who studied association/linkage of the 5-HTT gene with bipolar affective disorder by the transmission disequilibrium test, all of the above mentioned studies used a case-control design for investigation of potential disease association with affective disorders or schizophrenia. Since case-control studies are prone to false positive association, caused by population stratification rather than by disease-association, the preferred method for studying association should be a design with internal, family-based controls.[29] We applied the transmission disequilibrium test (TDT) which has the particular advantage of being applicable also to population samples where stratification may be a problem.[30] When the TDT is applied to a sample consisting of multiplex families, it is valid as a test for the null hypothesis of no linkage, but will have reasonable power only in the presence of linkage disequilibrium between marker and disease. Since there was no *a priori* evidence for linkage between the 5-HTT gene region and schizophrenia, we considered this as an appropriate null hypothesis to be tested.

The fact that the TDT is a valid test for linkage between marker and disease in structured populations,[30] does not diminish the possibility of type I error. Therefore, replication of positive results in independent samples will be mandatory.

Excess transmission of one particular allele could also result from segregation distortion, ie from preferential transmission in the meiotic process itself.[30] Since there are not enough unaffected siblings available in our sample, we cannot exclude this possibility.

Our sample has been used previously for testing eight candidate genes (the dopamine receptors D1–D5, the dopamine transporter, and the G-proteins Gs_α and G-olf_α), and association/linkage disequilibrium has been detected for G-olf_α.[31] Polymorphisms of the 5-HT transporter gene were investigated independently of these studies and correction for multiple testing may be too conservative. Nevertheless, the *P*-value remained statistically significant when Bonferroni correction for a number of 10 markers was applied ($P = 0.003$).

The negative result which we obtained in our sample

for the promoter polymorphism is in accordance with recent case-control studies in schizophrenia reported by Mendes de Oliveira *et al*,[15] Rao *et al*[16] and Stoeber *et al*.[17] Association of the 5HTTLPR l/l genotype with intensity of hallucinations in schizophrenic patients was found by Malhotra and colleagues.[14] Since our sample was not subtyped for psychotic symptoms in schizophrenia, we have not been able to attempt replication of this finding.

In contrast to the promoter polymorphism, the TDT for the intronic polymorphism was statistically highly significant ($P = 0.0003$) in our sample, displaying a preferential transmission of the 12 repeat allele. To our knowledge, this represents the first report of potential association/linkage disequilibrium of the intronic polymorphism and schizophrenia. No association was detected by Stoeber *et al*[17] in a case control study using 180 schizophrenic individuals and 233 blood donors as controls for the intronic polymorphism. In contrast to this study, our sample consisted entirely of familiar cases of schizophrenia, each family having at least two affected members. Consequently, our sample may be enriched for severe cases where different genetic risk factors may contribute to the development of the illness.

With the exception of the study of Ogilvie *et al*[18] who found association of the rare allele 9, the so far reported positive association studies of the intronic polymorphism with bipolar disorder were due to a higher frequency of allele 12 in the disease-sample.[20–22] This may point to the existence of a potential susceptibility gene shared by both disorders. Preliminary evidence, suggesting common susceptibility genes for the functional psychoses, has been provided by recent linkage studies. In our family sample with schizophrenia, as well as in independent samples of families with affective disorders, evidence for overlap of linked regions has been obtained for chromosome 18,[31–33] chromosome 10[34–37] and chromosome 22.[38]

Association/linkage disequilibrium of the intronic polymorphism with schizophrenia may have the following implications: (1) the 17-bp VNTR region in the second intron could be involved in development of the disease, in analogy to the VNTR at the insulin gene locus (IDDM2);[39] (2) the region may be in linkage disequilibrium with a near-by locus which confers susceptibility to the disease. No transmission disequilibrium between the promoter polymorphism and schizophrenia could be detected in our sample, even though linkage disequilibrium between the promoter- and the intronic-polymorphism[17,20] has been reported and also observed in our data. Whereas it may seem to be intuitive to expect significant TDT results for both markers in this situation, it was pointed out by Abel and Müller-Mysok[40] that the power of TDT can differ dramatically for marker loci with different allele frequencies, even if the two markers are in complete linkage disequilibrium. The latter is not the case for the two 5-HTT polymorphisms.

In conclusion, our study has provided evidence for association/linkage disequilibrium of the serotonin transporter gene with schizophrenia. Whether the serotonin transporter gene itself, or a gene close by may be involved in schizophrenia remains an open question and requires further studies.

Materials and methods

Subjects

All individuals were interviewed using the Schedule for Affective Disorder Schizophrenia—Lifetime Version (SADS-L) or the Structured Clinical Interview for DSM-III-R Disorders (SCID-II).[41] Case records were evaluated by Opcrit.[42] An independent psychiatrist reviewed the complete interview form, family history information and the medical record of each individual, without prior knowledge of the family relationship to the index patient, nor of the morbidity in the family. The sample consisted of 61 families with at least two affected siblings per family. Fifty-three families were from Germany (Haar, Mainz and Bonn) and eight from Israel. The sample contained 139 affected offspring comprising core diagnoses of schizophrenia or schizoaffective disorder, schizophrenic type according to the Research Diagnostic Criteria (RDC) (Table 1). The study was approved by the local ethics committees. All participants gave their informed consent after description and explanation of the study.

Genotyping

DNA was isolated from whole blood using a commercially available isolation kit (Qiagen, Hilden, Germany). PCR for the intronic polymorphism was performed in a total volume of 10 μl containing 2 mM $MgCl_2$, 0.5 μM primers (forward: 5′-GTCAGTATCA CAGGCTGCGAG-3′, reverse: 5′-TGTTCCTAGTCTTA CGCCAGT-3′), and 300 ng of genomic DNA. After an initial 3-min denaturation step at 95°C, 45 cycles were performed consisting of 30 s at 95°C, 30 s at 53°C, and 45 s at 72°C, followed by a final step of 5 min at 72°C. PCR products were separated on 3% MetaPhor (Biozym, Hess Oldendorf, Germany) agarose gel. PCR for the promoter polymorphism was carried out in a 15-μl reaction mixture containing 1.5 mM $MgCl_2$, 0.5 μM primers (forward: 5′-GGCGTTGCCGCTC TGAATGC-3′, reverse: 5′-GAGGGACTGAGCTGGA-CAACCAC-3′), and 150 ng of genomic DNA using a Taq polymerase from Qiagen, with the addition of Q-solution, as recommended by the manufacturer. After an initial 3-min denaturation step at 95°C, 40 cycles were performed consisting of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by a final step of 5 min at 72°C. PCR products were separated on 2% agarose (Biozym) gel.

Statistical analysis

The transmission disequilibrium test[29] and a generalization of this test to multi-allelic marker loci[43] were applied to test each of the two HTT loci for linkage in the presence of association/linkage disequilibrium.

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