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Serotonin Transporter Promoter Polymorphism, Peripheral Indexes of Serotonin Function, and Personality Measures in Families with Alcoholism

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Abstract

A functional polymorphism in the regulatory region of the serotonin transporter gene (5-HTTLPR) is considered to be a plausible candidate gene for anxiety-related personality traits and for alcoholism. Empirical support for the association between 5-HTTLPR and psychological traits has been somewhat inconsistent; however, observations of the functional dominance of the low-activity s-allele over the l-allele have been more consistent. When studying the influence of particular genes on psychological traits, it seems useful also to assess more biological intermediate traits that may mediate the effects of those genes on the traits of interest. The present study examined relationships between 5-HTTLPR genotype, whole blood serotonin (5-HT) level, and platelet 5-HT binding in 150 Caucasian subjects from 50 biological families. Individuals with the s-allele had lower average platelet 5-HT binding availability than those with the l/l genotype ($P < 0.025$). Whole blood 5-HT level was not associated with 5-HTTLPR genotype. In adult men, those with the s-allele had higher mean scores on the NEO-FFI personality trait of openness than did those with the l/l genotype ($P = 0.002$). The effect was not statistically significant in women ($P = 0.42$), although it was in the same direction. Our findings do not support an association of 5-HTTLPR genotype with alcoholism diagnosis, alcoholism subtype, or the personality trait of

neuroticism. The results of this pilot study suggest that further work should examine the mediation of the genetic effects on personality traits by biochemical measures and their moderation by gender.

Keywords: association study, 5-HT, 5-HTTLPR, binding availability, openness, neuroticism

Perhaps the most promising candidate gene in psychiatric genetics is a variant in the promoter region of the serotonin transporter, called 5-HTTLPR [Heils *et al.*, 1996, 1997; Lesch *et al.*, 1996]. The two common alleles, short (s) and long (l), have functional differences in lymphocytes [Lesch *et al.*, 1996], in blood platelets [Greenberg *et al.*, 1999], and in brain [Little *et al.*, 1998]. 5-HTTLPR is considered to be an important candidate to influence risk for psychiatric disorders [Stoltenberg and Burmeister, 2000], such as obsessive-compulsive disorder (OCD) [Hanna *et al.*, 1998; Bengel *et al.*, 1999], affective disorders [Collier *et al.*, 1996], autism [Cook *et al.*, 1997], and alcoholism [Sander *et al.*, 1997; Edenberg *et al.*, 1998; Hallikainen *et al.*, 1999; Hammoumi *et al.*, 1999; Ishiguro *et al.*, 1999].

Associations between 5-HTTLPR and normal variation in personality traits, especially those related to anxiety, have been widely studied [Lesch *et al.*, 1996; Mazzanti *et al.*, 1998; Gustavsson *et al.*, 1999; Greenberg *et al.*, 2000]. The s-allele appears to be associated with higher scores on anxiety-related measures in some populations [Greenberg *et al.*, 2000].

The present pilot study examines associations between 5-HTTLPR and indexes of 5-HT function (whole blood 5-HT [WB-5-HT] and platelet 5-HT transporter binding) in families. In addition, associations between 5-HTTLPR genotype and personality traits measured by the NEO-Five Factor Inventory (NEO-FFI) [Costa and McCrae, 1992] are examined in adults. Finally, we compare allele frequencies in adult males grouped by alcoholism diagnosis and by alcoholism subtype.

The Michigan-Michigan State Longitudinal Study (MMSLS) [Zucker, 1987; Fitzgerald *et al.*, 1995; Zucker *et al.*, 1996, 1997] is following a population-based sample of children of alcoholics, both biological parents, and an ecologically comparable but nonalcoholic group of control families at 3-year intervals (i.e., Waves), beginning when the target boys were ages 3 to 5 and the target girls were between ages 3 and 11 [Zucker *et al.*, 2000]. Alcoholism diagnosis was assessed using the Feighner diagnostic criteria [Feighner *et al.*, 1972] within a structured clinical interview format [Robins *et al.*, 1981].

Wave one involved 215 alcoholic and 96 nonalcoholic control families of primarily lower to lower-middle socioeconomic class and all of non-Hispanic Caucasian background. Inclusion criteria for the larger study included having a biological son between age 3 and 6 living in an intact family with both biological parents. Fetal alcohol syndrome in target children was exclusionary. Maternal alcoholism was neither inclusionary nor exclusionary for alcoholic families, although a subset of women married to antisocial alcoholic men (41%) met lifetime DSM-III-R [American Psychiatric Association, 1987] alcohol abuse/dependence diagnosis. Families were subtyped by paternal alcoholism and antisocial personality disorder diagnoses in earlier analytic work with the Longitudinal Study (antisocial alcoholism, nonantisocial alcoholism, nonalcoholic control) [Wong *et al.*, 1999; Poon *et al.*, 2000].

Subjects in the present study were 161 Caucasian biological family members from 50 families drawn from the MMSLS. These 50 families served as a pilot sample for initial examinations of biology and behavior relationships in association to alcoholism with reports from the children previously reported [Twitchell *et al.*, 1998, 2000, 2001]. The basic family structure includes a male target child (MTC) and his biological father and mother ($n = 30$ families); some also included a female target child (FTC; $n = 14$) and six were missing data from one or more members.

Medication screening, collection of demographic data, and blood sampling were performed by G.R.T. during an in-home session. Twenty-one-gauge needles were used in blood collection to ensure platelet integrity. Informed consent was obtained prior to data collection and the blood draw. At that time, the children ranged in age from 7 to 15 (45 boys, 11.05 ± 1.82 ; 17 girls, 10.43 ± 2.50). This protocol was approved by institutional review boards at the University of Michigan and Michigan State University. All subjects donated their blood samples and were paid for filling out questionnaires. Samples assayed for whole blood serotonin (WB-5-HT) were immediately transported to the laboratory and stored at -70°C until the time of batch assay.

Blood samples used for platelet 5-HTT binding availability assay were immediately transported on ice to the laboratory for processing. Assays were performed blind to demographic and diagnostic information.

WB-5-HT assays and genotyping methods were described previously [Hanna *et al.*, 1998]. WB-5-HT was analyzed by high-pressure liquid chromatography (HPLC) with fluorometric detection [Anderson *et al.*, 1981]. 5-hydroxytryptophan was used as an internal standard. Intra-assay and interassay coefficients of variation were 0.8% and 3.6%, respectively.

Oligonucleotide primers flanking the *HTT* polymorphic regulatory region (stpr5, 5'-GGCGTTGCC-GCTCTGAATGTC; stpr3, 5'-GAGGGACTGAGCTGGACAACCCAC) were used to generate a 484/528 bp fragment [Heils *et al.*, 1996, 1997]. A second primer (HTTp2A, 5'-TGAATGCCAGCACCTAACCC-3'; HTT-p2B, 5'-TTCTGGTGGCCACCTAGACGC-3') successfully amplified a 406/450 bp fragment from DNAs that failed to amplify with the first set. PCR was performed in a 10 μl volume containing approximately 50 ng of genomic template, 1 μM of each primer, 200 μM each dATP, dCTP, and dTTP, 100 μM each of dGTP, and 7'-deaza-dGTP, 0.6 units of *Taq* polymerase (AmpliTaq; Perkin Elmer), 1.5 mM MgCl_2 , 5% DMSO, 10 mM Tris-HCl, 50 mM KCl, and 0.001% gelatin. Samples were processed in a Perkin Elmer GeneAmp PCR System 9600 through 40 cycles of 30 sec at 95°C , 30 sec at 61°C , and 1 min at 72°C , followed by 10 min at 72°C . PCR products were visualized on 4% agarose (Perkin Elmer) gels containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide.

The methods used to assay [^{125}I]RTI-55 (also referred to as [^{125}I] β -CIT) binding have been previously described [Little *et al.*, 1993]. Briefly, the [^{125}I]RTI-55 assay (specific activity 2,200 Ci/mmol; New England Nuclear/ Dupont) utilized a 0.32 M sucrose and 10 μM Na_2HPO_4 buffer at pH 7.4. A saturating concentration of [^{125}I]RTI-55 (3 nM, approximately $8 \times \text{Kd}$ previously determined, 0.37 nM) was employed at equilibrium conditions (1.5 hr at 20°C). Incubations were performed with approximately 2 mg of pelleted platelets, followed by rapid filtration over Whatman GF/B glass fiber filters presoaked in 0.01% polyethylenimine. Nonspecific binding was defined with 30 μM (-)-cocaine. Specific binding averaged 90-95% of the total. Protein concentrations were determined using a commercial assay kit (BioRad, Claremont, CA).

Personality characteristics in the adults were assessed with the NEO-FFI [Costa and McCrae, 1992]. The NEO-FFI consists of 60 items (12 items per factor) and is designed to assess the five personality domains of neuroticism (negative affect), extraversion (sociability, assertiveness), openness (intellectual curiosity, unconventionality), agreeableness (altruism, sympathy), and conscientiousness (self-discipline, deliberation). Internal consistency coefficients for the NEO-FFI range from 0.68 to 0.86 for domain scales; validity is well established [Costa and McCrae, 1992].

For association analysis of WB-5-HT level and of [^{125}I]RTI-55 binding, three models were tested: one additive (s/s, s/l, l/l) and two dominance (sDom = s/s + 1/s vs. l/l, and lDom = s/s vs. l/s + 1/l) using the ASSOC module of

the Statistical Analysis for Genetic Epidemiology software package [SAGE, 1998].

General linear models (SPSS) were used to assess associations between 5-HTTLPR genotype and NEO-FFI scores. For fathers, we tested associations between 5-HTTLPR genotype, alcoholism diagnosis, and sub-type (antisocial alcoholic [AAL], nonantisocial alcoholic [NAAL], or nonalcoholic [control]) [Zucker *et al.*, 1996; Puttier *et al.*, 1998] with a case control design. Given the exploratory nature of these analyses, we were more willing to commit type I than type II errors; therefore, corrections for multiple testing were not performed.

Eleven subjects were excluded from all analyses because of medication use. Allele frequencies were calculated using only parental genotypes. The frequency of the 5-HTTLPR long allele l was 0.54 and the short allele s was 0.46 ($n = 86$). Genotypes were in Hardy-Weinberg equilibrium (chi-square = 0.24, 2 df, NS).

Overall mean WB-5-HT level was 188.08 ± 63.54 ng/ml, ($n = 150$). [125 I]RTI-55 binding was assayed for the final 54 samples collected. Mean [125 I]RTI-55 binding level was 147.96 ± 74.24 cpm/mg protein. Levels of WB-5-HT and [125 I]RTI-55 binding were not correlated in parents ($r = -0.01$; $P = 0.96$; $n = 33$) or in children ($r = -0.29$; $P = 0.20$; $n = 21$).

To control for age differences in WB-5-HT, we tested sex differences separately among men and women and among boys and girls. None were significant (all P values > 0.17); therefore, we did not control for age or sex in the SAGE analysis.

Model fit was assessed with the likelihood ratio statistic that is distributed as chi-square with degrees of freedom being 1 less than the number of genotype groups (e.g., 2 for additive and 1 for dominance models). For WB-5-HT, including marker data did not improve model fit, though the additive model fit best ($s/s = 208.43$, $l/s = 187.36$, $l/l = 175.55$; chi-square = 4.10; $P > 0.10$). Patterns of mean [125 I]RTI-55 binding levels were consistent with the dominance of the s-allele ($s/s = 138.50$, $l/s = 133.31$, $l/l = 190.15$); accordingly, including sDom marker data improved model fit (chi-square = 5.19; $P < 0.025$). Neither the lDom nor the additive marker data improved model fit (i.e., P values > 0.05).

Table I presents the mean raw NEO-FFI adult personality scores by genotype. One-way ANOVAs were used to test the effects of 5-HTTLPR on each of the personality measures. Only mean openness scores were significantly different for the genotype groups (S [s/s and l/s] = 38.34, L [l/l] = 34.54; $F = 7.04$; $P = 0.01$).

To examine the observed association further, we performed one-way ANOVAs separately for men and women. Among men, mean openness scores were different for the genotype groups ($S = 38.25$, $L = 32.50$; $F = 10.60$; $P = 0.002$); among women, they were not ($S = 38.43$, $L = 36.58$; $F = 0.68$; $P = 0.42$). The effect of 5-HTTLPR on openness appears to be moderated by gender.

Father's genotype was not related to his alcoholism sub-type (Fisher exact statistic = 2.24; $P = 0.73$; Table II). Similarly, when the genotype frequencies of those with any alcoholism diagnosis (AAL + NAAL) were compared to nonalcoholic controls, no relationship was observed (chi-square = 1.50, 4 df, $P > 0.75$).

Patterns in binding availability of the 5-HTT in blood platelets (indexed by [125 I]RTI-55 binding) were consistent with the dominance of the s-allele for lower function. Levels of 5-HTT binding in the L-group (l/l) were 42% higher than in the S-group (l/s and s/s). This closely replicates the difference observed in lymphocyte membrane preparations reported by Lesch *et al.* [1996].

Levels of WB-5-HT were neither associated with 5-HTTLPR genotype nor correlated with platelet 5-HTT binding availability. WB-5-HT content likely reflects a number of different processes in addition to 5-HT uptake, so it is not surprising that WB-5-HT content and [125 I]RTI-55 binding levels were not correlated. These results suggest that general statements about serotonergic dysfunction should be made cautiously.

In this study, we observed a difference in mean neuroticism scores of approximately 1/10 of a point ($L = 28.63$, $S = 28.74$; $n = 86$). This is in contrast to the findings of Lesch *et al.* [1996] and a large replication sample of mostly women (primarily sibling pairs) collected by the same group that found higher levels of neuroticism in individuals with the s-allele [Greenberg *et al.*, 2000]. Other studies have failed to replicate the association [Flory *et al.*, 1999; Jorm *et al.*, 2000].

Table I. Mean NEO-FFI Personality Traits Scores and Standard Deviations for Groups Defined by 5-HTTLPR Genotype*

Genotype	NEO-FFI scores by genotype				F	S - L	P	P ^a
	l/l (group L)	l/s	s/s	l/s & s/s (group S)				
Number per group	24	45	17	62				
Neuroticism	28.63 ±8.60	29.20 ±9.39	27.53 ±6.76	28.74 ±8.73	0.003	0.11	0.96	0.44
Extraversion	41.04 ±6.44	41.98 ±6.08	42.41 ±5.47	42.10 ±5.88	0.59	1.06	0.47	0.41
Openness	34.54 ±5.01	38.29 ±6.86	38.47 ±4.53	38.34 ±6.27	7.04	3.80	0.01	0.005
Agreeableness	45.13 ±4.79	44.71 ±6.47	46.71 ±6.88	45.26 ±6.59	0.008	0.13	0.93	0.90
Conscientiousness	46.92 ±8.14	43.69 ±8.15	48.06 ±6.27	44.89 ±7.88	1.13	-2.03	0.29	0.17

*Mean scores and standard deviations for the five major traits measured by the NEO-FFI stratified by genotype and group. F = one-way ANOVA statistics; S - L = mean score for S-genotype group minus mean score for L-genotype group; P = two-tailed significance.

^aTwo-tailed significance level after controlling for sex and father's alcoholism status in a general linear model.

Table II. Observed 5-HTTLPR Genotypes and Frequencies of s-Allele for Fathers Categorized by Alcoholism Subtype*

	s/s	l/l	l/s	N	F(s)
AAL	4	10	3	17	0.53
NAAL	4	6	4	16	0.50
Control	2	6	5	13	0.38
Total	10	22	12	44	0.48

*AAL = antisocial alcoholic; NAAL = nonantisocial alcoholic; Control = nonalcoholic.

Father's genotype is not associated with his alcoholism status (Fisher exact statistic = 2.24; $P = 0.73$).

It may be that differences between the sample populations can account for such inconsistencies. The sample analyzed in the present study, while not a treatment population, its diagnostic characteristics both for alcoholism and comorbid psychiatric disorders clearly establish it as a clinical one [Zucker *et al.*, 2000].

In this study, 5-HTTLPR genotype groups differed on the personality trait of openness. The association was statistically significant only among men; although among women, the pattern was in the same direction (i.e., higher mean scores for individuals with an s-allele). Because mean platelet 5-HTT binding availability was lower in individuals with an s-allele, these results suggest that the effect of 5-HTTLPR on openness may be partially mediated through 5-HTT binding availability and moderated by gender. However, in the present study, we could not fully support this mediational hypothesis because the correlation between [¹²⁵I]RTI-55 binding and openness was not significant ($n = 33$; $r = -0.18$; $P = 0.31$), but this may be due to low statistical power. Testing mediational hypotheses between genes, biochemical, or physiological measures and behavior may prove to be an important approach to understanding heredity-behavior relations.

In the study by Greenberg *et al.* [2000], the pattern of mean openness scores in their new sample is similar to the one we report, although the differences were not statistically significant. This pattern is not seen in their combined sample. The samples that Greenberg *et al.* [2000] added are those reported by Lesch *et al.* [1996], who appear to be younger, more ethnically diverse, better educated, and perhaps contain different distribution of sexual orientation than our study population. It is possible that such differences account for the observed discrepancies.

Higher openness scores are related to a history of depression, and this effect is over and above the association between neuroticism and depression [Wolfenstein and Trull, 1997]. It may be that the s-allele increases risk for depression through either neuroticism or openness. That the mean openness scores for those with s/s and l/s genotypes are different from those with the VI genotype in the present study provides some measure of confidence because it mirrors the pattern seen for [¹²⁵I]RTI-55 binding. The P value of the association of 5-HTTLPR genotype and openness when controlling for sex and father's alcoholism status ($P = 0.005$) survives Bonferroni correction for the 10

comparisons conducted for genotype and NEO-FFI scores. However, further confidence will require replication. Until then, these findings should be regarded as preliminary and interpreted with caution.

Father's alcoholism diagnosis or alcoholism subtype was not associated with 5-HTTLPR genotype. This is consistent with findings from the COGA group [Edenberg *et al.*, 1998]. However, other groups have reported associations between 5-HTTLPR and alcohol use disorders in other populations (e.g., dependence in French [Hammoumi *et al.*, 1999], anti-social alcoholism in Finnish [Hallikainen *et al.*, 1999], and early-onset alcoholism in Japanese [Ishiguro *et al.*, 1999]). Allelic variation at 5-HTTLPR has also been found associated with low response to alcohol [Schuckit *et al.*, 1999] and high ethanol tolerance [Turker *et al.*, 1998]. Taken together, these conflicting reports (and others) highlight the complexity inherent in the study of psychiatric genetics [Stoltenberg and Burmeister, 2000] and suggest that in different environments and populations the pattern of 5-HTTLPR effects may be different. In other words, genotype \times environment and/or genotype \times genotype interactions may exist for 5-HTTLPR effects on alcoholism.

In summary, this study replicated the dominance pattern of the s-allele for reduced 5-HTTLPR function as indexed by platelet [¹²⁵I]RTI-55 binding. Results did not support for the association of 5-HTTLPR genotype with neuroticism or alcoholism; however, increased mean openness scores were associated with the s-allele.

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