Article

Relation of Shyness in Grade School Children to the Genotype for the Long Form of the Serotonin Transporter Promoter Region Polymorphism

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Objective: Studies have shown that genetic factors are significant in predisposing individuals to shyness and social phobia. Toward further elucidating the genetic structure of shyness, the authors examined four functional polymorphisms that make biological sense for contributing to the development of this phenotype: serotonin transporter promoter region 44 base pair insertion/deletion (5-HTTLPR), dopamine D_4 receptor exon III repeat (DRD4), catechol *O*-methyltransferase (COMT), and monoamine oxidase A promoter region repeat (MAO_A).

Method: The authors assessed shyness after recruitment of a nonclinical sample (N=118, unscreened second-grade children) using a composite scale derived from questionnaires administered to the children, parents, and teachers. DNA from buccal smears successfully obtained from 98 children was genotyped by polymerase chain reaction methods for the 5-HTTLPR, DRD4, COMT, and MAO_A polymorphisms.

Results: Significant correlations were observed for parents', teachers', and children's ratings of shyness, and Cronbach's alpha reliability was high for all three scales. A significant association was observed between the long 5-HTTLPR polymorphism and shyness, both by the functional classification of Lesch as well as by consideration of all three genotypes. No significant association was observed for the DRD4, COMT, or MAO_A polymorphisms.

Conclusions: This study provisionally identifies a common genetic polymorphism, 5-HTTLPR, that modestly (effect size=7%) contributed to greater shyness scores in a nonclinical group of second-grade students. These first findings may be relevant to previous reports that have shown an association between the 5-HTTLPR long form and obsessive-compulsive disorder and autism.

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"Shyness," as used in developmental psychology, refers to a pattern of behaviors and emotional states that includes inhibition of approach behaviors and discomfort on exposure to unfamiliar people and situations, a constricted social life, and physiological markers such as high heart rate and cortisol levels (1). The term may encompass a broad range of severity, from normal traits such as mild social awkwardness to clinical disorders such as totally inhibiting social phobia. Shyness is often pervasive, in that it is manifest at school, at home (with strangers), and in other social situations. Various authors have suggested that its temperamental antecedents can be seen as early as the first 6 months of life (2) and that it is an important risk factor for clinical anxiety disorders in childhood and later (3).

Numerous studies have examined genetic factors involved in shyness, and socially related fears and heritability have been shown to be significant (4). However, to our knowledge, association of a specific gene to shyness has been shown in only one study of a phenotype—behavioral inhibition (5). In that study, four candidate genes first flagged in mouse models of emotionality were examined in a selected group of behaviorally inhibited children, and a modest association with glutamic acid decarboxylase was observed.

We assessed shyness after recruitment of unscreened second-grade children using a composite questionnaire administered to the children, parents, and teachers. Children were initially genotyped for the serotonin transporter promoter region 44 base pair deletion/insertion polymorphism (5-HTTLPR), which has been demonstrated to moderate transcriptional efficiency and was originally associated (reviewed in reference 6) with anxiety-related personality traits (7). Other studies have shown an association between the 5-HTTLPR polymorphism and obsessive-compulsive disorder (OCD) (8–13) and autism (14–16).

Three additional polymorphisms of considerable interest in human behavioral genetics—the dopamine D_4 receptor (DRD4) (17), catechol *O*-methyltransferase (COMT) (18), and the MAO_A promoter region (MAO_A) (19)—were also examined for association with shyness in our study sample.

Method

Subjects

Children (age=7.29 years, SD=0.45, range=7–8) were recruited from second-grade classes in schools in the Beer Sheva municipal area and represent a population-based sample. The interviews were carried out by a psychiatrist (M.G.). Parents and teachers completed a short questionnaire for each child participating in the study. Sixty-seven percent of the children were boys, and 33% were girls. Altogether, 118 children were recruited; DNA, genotyping, and questionnaires were completed for 98 children. After complete description of the study to the parents of the children, written informed consent was obtained. The study was approved by Ben Gurion University's institutional review board and the Israel Ministry of Health.

Assessment

We assessed shyness in second-grade children with three converging approaches: self-reports with questions from the Schedule for Affective Disorders and Schizophrenia for School-Age Children—Present and Lifetime Version (20), parental reports with questions from the Martin Temperament Assessment Battery (21) and the Achenbach Behavior Checklist (22), and teacher reports with questions from the teacher versions of the latter instruments. Relevant questions relating to shyness were taken from each of these questionnaires. The teachers' questionnaire consisted of eight questions, the parents' questionnaire eight questions, and the children's questionnaire six questions. The questionnaires used a Likert-type format.

Questions for Teachers

The teachers' questions were the following:

1. The child is bashful with adults (s)he doesn't know.
2. The child avoids new games or activities and prefers to watch others from the side.
3. The child "gets his/her act together" quickly when learning conditions change.
4. The child adapts to new activities and situations without reservations.
5. The child stands and performs in front of the class (sings, recites) without reservations, even for the first time.
6. When presented with a new learning task, the child reacts immediately and with interest.
7. The child prefers familiar games to new games.
8. The child is bashful when (s)he meets a child (s)he doesn't know.

Questions for Parents

The parents' questions were the following:

1. My child is shy with adults (s)he doesn't know.
2. My child does not feel at ease appearing or performing at home in front of unfamiliar guests.
3. When my child meets new children, (s)he is bashful.
4. In the playground, at a party, or during a visit, my child approaches children (s)he does not know and joins in their games.
5. When the family goes on a trip, my child quickly feels at ease in unfamiliar places.
6. My child makes friends easily and connects with unfamiliar adults visiting our home.
7. The first time (s)he stayed alone (without his/her parents) in a new place, my child felt very unsettled.
8. My child feels free to laugh and smile in the presence of people (s)he has just met for the first time.

Questions for Children

The children's questions were the following:

1. Many children are shy. Some children do not feel at ease with people outside their families. Have you ever been like those children?
2. Have you felt uneasy or tense with your teacher?
3. Have you felt uneasy or tense with other children at school?
4. Some children feel very shy with people they don't know and feel unable to say anything. Has this happened to you?
5. Is it hard for you to speak to children you don't know?
6. Some children do not like to answer questions in class or speak in front of the class even if they know the answer. Are you like those children?

Genotyping

DNA was obtained from a buccal smear (brush) from each child. The DNA was extracted by using a MasterPure kit (Epicentre, Madison, Wis.). The 5-HTTLPR (23), DRD4 (24), COMT (25), and MAO_A (26) polymorphisms were genotyped in our laboratory, as previously described.

Statistics

All statistical analyses were carried out by using SPSS for Windows (SPSS, Chicago). Associations with shyness scores were examined by using analysis of variance (ANOVA). In the univariate ANOVA analyses, the effect size of the genotype for shyness is given as eta squared. Sample size and power were estimated by using the program PS (W.D. Dupont and W.D. Plummer, Jr.), which is downloadable from Vanderbilt Medical Center (http://www.mc.vanderbilt.edu/prevmed/ps/index.htm).

Results

The children were rated on shyness by themselves, their parents, and their teachers. Significant correlations were observed between parents' and teachers' ratings and by self-ratings of shyness (child and teacher: r=0.38, N=116, p<0.001; child and parent: r=0.72, N=118, p<0.001; parent and teacher: r=0.35, N=116, p<0.001). Cronbach's alpha reliability, which measures internal consistency of test items, was also high for all three rating scales as well as for the combined scale (eight parent items: alpha=0.89, N=118; eight teacher items: alpha=0.88, N=116; six child self-rating items: alpha=0.70, N=118; 22 total items: alpha=0.90, N=116). For the genetic analysis, we used the combined rating scale, arrived at by summing the parent, teacher, and child questionnaire ratings.

Shyness scores were grouped by all three (long/long homozygotes, short/long heterozygotes, and short/short homozygotes) genotypes and also by a genotype classification (long/long versus short/short and short/long) first suggested by Lesch et al. (7), which reflects the effect of the polymorphism on transcription (Table 1). A significant association was observed between the long 5-HTTLPR polymorphism and shyness, both by the functional classification of Lesch et al. (ANOVA: F=7.47, df=1, 98, p=0.007, N=98; eta squared [a measure of effect size]=0.072, observed power=0.77) as well as by consideration of all three genotypes (ANOVA: F=4.00, df=2, 98, p<0.03, N=98; eta squared=0.078; observed power=0.70). Note that after

TABLE 1. Relation of Shyness Scale Scores to Genotype for a Serotonin Transporter Promoter Region (5-HTTLPR) for 98 Second-Grade Children

5-HTTLPR Genotype Classification and Alleles	N	Shyness Score[a] Mean	SD	Analysis F	df	p
Full genotype				4.00	2, 98	<0.03
Long/long (l/l)	32	73.00	20.90			
Long/short (l/s)	48	62.75	19.23			
Short/short (s/s)	18	58.76	16.23			
Total	98	65.36	19.89			
Lesch (7) scheme				7.47	1, 98	0.007
Long	32	73.00	20.91			
Short (l/s and s/s)	66	61.66	18.43			
Total	98	65.36	19.89			

[a] Composite of scores for answers by the child to six relevant questions from the Schedule for Affective Disorders and Schizophrenia for School-Age Children—Present and Lifetime Version (20), answers by the parents to eight questions from the Martin Temperament Assessment Battery (21) and the Achenbach Behavior Checklist (22), and answers by the teachers to eight questions from the teacher versions of the Martin and Achenbach instruments.

Bonferroni correction a significant difference at the p<0.05 level was observed between the long/long versus the short/short genotypes. The Bonferroni correction provided by ANOVA performed by SPSS is applicable only with more than two independent variables (in this instance, the long/long, long/short, and short/short genotypes). Of interest, the boys were the main contributors to the association (Lesch scheme [ANOVA], only boys: F=12.53, df=1, 64, p=0.001, N=65; only girls: F=0.04, df=1, 32, p=0.85, N=33). Genotype frequencies were distributed according to the Hardy-Weinberg equilibrium.

Another way of estimating the effect size of the 5-HTTLPR genotype on shyness scores is from the correlation coefficient derived from a linear regression analysis of our data. For example, in our data set, Pearson's correlation coefficient was 0.269 (p=0.004, one-tailed) for this analysis, which was obtained by regressing composite shyness scores on the Lesch genotype classification scheme. Such a correlation is similar to an odds ratio of ~1.2–1.9, which, by Cohen's definition (27), is a small effect.

Since population admixture and stratification may confound interpretation of association studies, we examined the distribution of the 5-HTTLPR genotype in a large sample that represents the two principal ethnic groups examined in the present study. These subjects were previously recruited in our studies of normal personality (6). No significant difference in genotype frequency (long/long, long/short, or short/short) for the 5-HTTLPR genotype was observed between the Ashkenazi (N=575) and non-Ashkenazi (N=383) Jewish populations (χ^2=0.01, df=2, p=1.00). Genotype frequencies were in Hardy-Weinberg equilibrium.

We also examined three additional polymorphisms for possible association with composite shyness scores: the DRD4, COMT, and MAO_A (Table 2). No association was observed for any of these genes with shyness (DRD4: F=0.76, df=1, 99, p=0.39, observed power=0.14; COMT: F=0.89, df=2, 96, p=0.41, observed power=0.20; MAO_A: F=0.75, df=1, 84, p=0.40, observed power=0.14). We included in Table 2 the estimated sample size for a significant effect for each of these genes at alpha=0.05, based on observed differences in mean values and standard deviations in the current sample.

TABLE 2. Relation of Shyness Scores to Genotypes for Dopamine D_4 Receptor (DRD4), Catechol *O*-Methyltransferase (COMT), and Monoamine Oxidase A (MAO_A) Polymorphisms for 98 Second-Grade Children[a]

Polymorphism and Genotype	N	Score Mean	SD	Analysis F	df	p	Estimated Sample Size for Alpha=0.05
DRD4				0.76	1, 99	0.39	
Non-seven-repeat	76	66.88	19.26				800
Seven-repeat	23	62.70	23.02				
COMT				0.89	2, 96	0.41	
Val/Val	26	63.56	20.99				6,000
Val/Met	43	68.37	17.89				
Met/Met	27	62.44	21.70				
MAO_A				0.75	1, 84	0.40	
Three-repeat	31	61.12	17.07				500
Four-repeat	53	65.02	21.51				

[a] The classification of D1RD4 into binary genotypes (seven-repeat versus non-seven-repeat) has been widely used in behavioral genetic studies of both normal personality (28, 29) and attention deficit hyperactivity disorder (ADHD) (30, 31). Binary classification preserves genetic information for individuals displaying less common repeats. The functional significance of the long and short classification is unclear, although a recent study (32) showed a dose-response effect of DRD4 number and score on a continuous performance test in children with ADHD. The classification of the MAO_A polymorphism into short and long alleles (2, 3 [3-repeat] versus 4, 5 [4-repeat]) is based on a functional characterization of this polymorphism in a luciferase assay that demonstrated that the longer alleles (3a, 4, and 5) were more active than allele 3 (33).

A Bonferroni correction for multiple testing (four genes tested) showed that the association with the 5-HTTLPR polymorphism was still significant (4×0.007=0.028).

Discussion

The current study shows an association between shyness measured in a group of second-grade schoolchildren by using a composite rating scale (of self-reports, parent reports, and teacher reports) and the long allele of the 5-HTTLPR polymorphism. Shyness, a phenotype akin to behavioral inhibition (34)—a well-described childhood temperament—has been suggested to be an antecedent of later anxiety disorders in adolescents and adults, especially social phobia (35). Although there is considerable evidence that genetic factors significantly contribute to the etiology of social phobia (4), the current study is one of a very few investigations (5, 36) that has examined the role of a specific and common gene variant in conferring risk for this temperament construct. Stein et al. (36) examined 17 multiplex families comprising 76 probands (from ages 18 to 65) who were diagnosed with social phobia and failed to observe linkage to 5-HTTLPR. However, as the authors noted, their study had power to observe only a major

gene effect. In contrast to these two previous studies, we examined a group of unscreened schoolchildren in the second grade. Although using subjects who exhibit extremes of a phenotype in genetic studies is generally considered the most efficient strategy, a recent genome-wide linkage study on human stature (height) (37) suggested otherwise. Indeed, the authors reached the conclusion that selective ascertainment of families may provide only modest gains in efficiency.

A longitudinal study from Australia that measured a broad range of temperament and behavioral problem variables found an association between the long 5-HTTLPR allele and higher anxiety at 13–14 and 15–16 years (38), which is consistent with the current findings of an association between the same allele and shyness. At earlier developmental stages, no association was observed between temperament traits and the transporter gene. In previous studies (39–42), we examined the role of the 5-HTTLPR polymorphism in human temperament from 2 weeks to 1 year. However, in contrast to the present findings and the Australian longitudinal study (38), it was the short allele (by an interaction with the DRD4 repeat variant) that enhanced avoidant-like behaviors during this early developmental period. It should be considered, however, that despite the apparent face validity of neonatal and infant temperament traits (distress to limitations, negative emotionality) to measures of adult behavioral traits (social and general anxiety), infant temperament is likely to be only weakly correlated with adult traits. Extrapolating gene effects on behavior from infancy to adult personality constructs, therefore, may lead to ambiguous conclusions. It is also worth noting that the original Lesch et al. finding (7) showed an association between the short 5-HTTLPR alleles that was shown to reduce gene transcription (and presumably make more serotonin available in the synapse) and that adult neuroticism is opposite from what might be expected from the action of selective serotonin reuptake inhibitors (SSRIs).

The currently reported association of the long 5-HTTLPR allele with shyness may be relevant to recent findings in adult anxiety disorder, OCD, and this gene (8–13). Both a family-based (12) and an association (11) study showed associations between the long 5-HTTLPR polymorphism and OCD. OCD is often comorbid (43) with social phobia and other anxiety disorders, suggesting the possibility that such disorders share some, but not all, common genetic determinants—for example, the 5-HTTLPR polymorphism. Such a notion is strengthened by the effectiveness of SSRIs across the range of anxiety disorders (44). Of interest, clinical response to fluvoxamine in OCD patients is related to the 5-HTTLPR genotype (45, 46), and carriers of the long allele are more responsive to treatment.

Some studies have also shown an association between the long 5-HTTLPR allele and autism (14–16). It is worth noting that Cook et al. (47) found an association between autism and the *short* HTTLPR allele. Tordjman et al. (16) suggested that this promoter region polymorphism by itself does not convey risk for autism but instead influences the behavioral phenotypic expression of autism. A core phenotype in autism is the repetitive behaviors that also characterize OCD. Most intriguing is the epidemiological study of Smalley et al. (48), showing that the frequency of social phobia—20.2%—in autistic families is approximately 10 times more common than that found among the relatives of comparison probands (2.4%).

Although we observed no differences between 5-HTTLPR genotype frequencies when grouped by the two main ethnic groups examined in this study, population stratification cannot be excluded as a contributing cause to the observed association. Future studies should include the use of more robust family-based designs, such as the transmission disequilibrium test (49, 50). Additionally, more sophisticated case-control designs (51) and the use of so-called genomic control subjects (52) offer alternative strategies for handling population admixture in association studies.

We also examined three additional polymorphisms (DRD4, COMT, and MAO_A) that have been studied in many other human behavioral genetic investigations and were therefore likely candidates for contributing to shyness. No association was observed between any of these polymorphisms and shyness in the current study. However, our failure to detect an association between these genes and shyness may reflect the difficulty in observing genes of small effect size in a sample of 98 children.

A word of caution is called for regarding association studies in general and the present report in particular. Ioannidis et al. (53) noted that "validation of statistical hypotheses in genetic epidemiology is a task of unprecedented scale" because of the increasing number of candidate genes generated by the mapping of the human genome that are being intensively examined in association studies of human disease. Meta-analysis offers a robust solution to making sense of numerous investigations.

Similarly, the provisional association reported here between shyness and the long 5-HTTLPR allele needs to be examined using both case-control and family-based designs in numerous independent study groups before the validity of this observation is either strengthened or weakened. Nevertheless, the role of shyness as a risk factor for subsequent psychopathology and the importance of elucidating its genetic architecture suggest the importance of the current study as a catalyst that will undoubtedly generate additional investigations of this and other genes in contributing to this intriguing phenotype.

Shyness in the children we examined may be considered an intermediate phenotype (a so-called endophenotype [54]) that is more proximally related to the genetic substrate than is the higher-order construct of a disorder (e.g., social phobia) (5). To the extent that shyness is an intermediate phenotype, genetic influence on it may be stronger and less complex, making shyness in children an

excellent target for genetic dissection that may shed light on adult anxiety disorders.

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