Influence of the Serotonin Transporter Promoter Gene and Shyness on Children’s Cerebral Responses to Facial Expressions

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Background: Childhood shyness can predate social anxiety disorder and may be associated with biased discrimination of facial expressions of emotions.

Objective: To determine whether childhood shyness, or the serotonin transporter promoter polymorphism genotype, can predict participants’ visual event-related potentials in response to expressions of children of similar ages.

Design: Study group drawn from an inception cohort of 149 subjects characterized 1 year before the present study by their degree of shyness.

Setting: Third- and fourth-grade schoolchildren.

Participants: Forty-nine of the inception cohort children, randomly selected.

Main Outcome Measures: Latencies and amplitudes of the N400 waveform in response to happy, neutral, and angry expressions.

Results: Shyness predicted significantly smaller N400 amplitudes in response to anger (at Pz: P ≤ 0.04) and to a neutral expression (at Pz: P ≤ 0.047). Shyness was significantly different across the 3 genotypes, the SS genotype being associated with higher shyness levels (analysis of variance: F(2,42)=4.47, P=0.02; Tukey honestly significant difference, SS vs LL, P≤.01). An analysis of covariance showed that neither the type of expression nor the genotype per se influenced the N400 amplitudes, but a significant expression × genotype interaction was found (F(4,72)=3.37, P=0.01), sustained by the difference in amplitude of the SS and S carrier subjects compared with the LL subjects when exposed to the anger expression (Tukey honestly significant difference, P≤.02).

Conclusion: Children who manifest higher levels of shyness or have 1 or 2 copies of the short allele of the serotonin transporter promoter gene appear to have a different pattern of processing affective stimuli of interpersonal hostility.

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In adult patients with social phobia, compared with control subjects during simulated public speaking, a decreased cortical (secondary visual, parietal, retrosplenial, temporal, and insular cortex) activity occurs typically in association with increased amygdala activation (regional cerebral blood flow).16 Likewise, exaggerated amygdala activation has been reported in controlled studies of adults with social phobia exposed to angry or contemptuous and neutral facial expressions, which are usually categorized as mildly hostile or ambiguous.10

There is also initial, but consistent, evidence of biased processing and discrimination of the facial expressions of emotions in adults and children at heightened risk for social phobia. A study of visual attention showed that adults with social phobia avoid salient facial features when they watch facial expressions,11 while children with high indexes of social anxiety or shyness have biased recognition of emotions, particularly the neutral and the angry expressions.12,13

The brain responses to basic elements of social communication in children at risk of developing social anxiety or avoidant personality disorder can thus help clarify the developmental pathways to social phobia.

A temperamental disposition toward the avoidance of novel and uncertain situations together with a set of behaviors that indicate shyness and discomfort in social interactions are comprehensively named childhood shyness, or behavioral inhibition (BI).14 Children with high indexes of shyness-BI are at a heightened risk of developing anxiety disorders, in particular social phobia,14 and subjects who fall within the BI–social phobia developmental continuum show specific patterns of neurophysiologic responses to pictures of facial expressions. Adults who had been categorized as behaviorally inhibited at the age of 2 years exhibited a higher amygdala activation in response to unknown vs familiar faces16 compared with adults who were uninhibited as children, consistent with the notion that novel or ambiguous environmental stimuli of potential biological relevance activate the amygdala.17 Turning to genes that can influence the neurobiological bases of the processing of emotions, 2 common alleles, the short (S) and the long (L), in a variable repeat sequence of the serotonin transporter (5-HTT) promoter polymorphism (5-HTTLPR) on human chromosome 17q11 have been differently associated with greater amygdala activity in response to angry or fearful faces18 in healthy adults. The L and S alleles influence 5-HTT transcription activity,19 with the S allele conveying reduced transcription, lower transporter levels, and diminished serotonin uptake. The presence of 1 or 2 copies of the S variant influences predisposition to anxiety, avoidant behaviors, and interpersonal negative emotionality according to several, but not all, genetic association studies of adults,20 while a recent study of childhood shyness-BI21 found an association in the opposite direction (ie, with the LL genotype).

The cerebral visual event-related potentials (ERPs) are scalp potentials that occur within a few hundred milliseconds after the presentation of a visual stimulus. The ERP waveforms contain components22-24 that span a continuum between the earlier, “exogenous” potentials (reflex responses controlled by the physical properties of an external eliciting event) and the later, “endogenous” potentials (manifestations of information processing determined more by the nature of interaction between the subject and the event). Meta-analytic evaluations of ERP studies in twins show substantial heritability of some late (the P300 and N400 waveforms) ERP components.25

Children from the age of 10 years show the same ERP late components26-28 found in adults when they look at pictures of facial affects.29 These include a characteristic negative waveform that occurs at about 400 milliseconds (N400) believed to reflect specific cognitive processing of human facial information30,31 in the absence of verbal-semantic information.32 More broadly, the N400 has been claimed to reflect a temporal correlate of a corticoamygdala pathway of emotional processing.33,34 In keeping with positron emission tomographic and functional magnetic resonance imaging studies that showed activation of primary and secondary visual areas and of the somatosensory cortex during the act of processing face expressions,3 ERP studies suggest that expression identification is associated with a centroparietal effect, indexing attention and motivation processes of adaptive significance.35,36

The present study analyzes the N400 visual ERP responses of children to different emotional facial expressions. We hypothesized that individual differences in shyness-BI and genetic variability at the 5-HTTLPR could predict a pattern of diminished cortical activation in response to hostile compared with neutral or prosocial expressions, which could be put into a relationship with the finding of biased recognition of expressions of emotions in shy children,12,13 and with the patterns of central nervous system activation found by brain-imaging studies of social phobia in tasks of perceptually induced anxiety.1

### METHODS

One year before this study (time 0), an inception cohort of 149 schoolchildren were characterized by their degree of shyness and ability to discriminate facial expressions of emotions.37 Resources were available to study a fraction of the original sample through interviews and electrophysiologic evaluation; 70 of the 149 children were therefore randomly selected and invited to participate in the present study. After a complete description of the study, 55 of the 70 invited children and their parents agreed to participate, and parents signed a declaration of informed consent. The procedures of this study were accepted by the ethical committee of the participating institutions. Six of the 55 participating children were not included in the final sample for different reasons, such as minor physical illnesses on the day of the ERP recording, or unavailability of an adult person to accompany the child to the laboratory. This left 49 white children of Italian ancestry with normal or corrected-to-normal visual acuity to take part in the study. Post hoc comparisons showed no differences in demographic and psychometric characteristics between 49 participating vs 100 nonparticipating children (Table 1). Although there were 49 participants, results are shown for numbers of participants that vary between 34 and 49 because of removal of subjects who had artifacts like eyeblinks in some recordings, or who had an unsuccessful DNA collection (which occurred in 4 children). At time 0, the children’s degree of shyness-BI was evaluated by a questionnaire that was filled in by appropriately trained teachers, and by direct observation of the number of spontaneous comments made in the presence of an unfamiliar adult, based on previous descriptions of children with BI.12,14 The questionnaire included a set of items seeking to identify temperamental dis-
position to BI and symptoms of possible social anxiety disorder proper, and included the Italian translations of the Stevenson-Hinde and Glover Shyness to the Unfamiliar,\textsuperscript{13} Cloninger and coworkers' Harm Avoidance Scale,\textsuperscript{36} and the Liebowitz Social Anxiety Scale adapted for children; SES, socioeconomic status calculated on the basis of the Hollingshead scale (lower range, score of 1-3; middle, score of 4-6; upper, score of 7-9); SU, Stevenson-Hinde and Glover Shyness to the Unfamiliar scale.

**BEHAVIORAL MEASURES**

A principal component analysis of the items collected by questionnaire at time 0 provided 3 factors with the following eigenvalues (and percentages of explained variance): 11.99 (59.9%), 2.1 (9.8%), and 1.6 (6%). Among the items belonging in the first factor, the following 7 had maximal factorial loading (range, 0.80-0.89), maximal reciprocal correlations (r = 0.58-0.86, all p ≤ .001), and maximal internal consistency (Cronbach α = 0.89): (1) the child fears or avoids being at the center of attention; (2) the child fears or avoids raising his or her hand and answering questions in front of the class; (3) the child fears or avoids unfamiliar people; (4) the child speaks easily with unfamiliar adults (negative loading); (5) the child tends to avoid new visitors and strangers; (6) the child plays readily with new children (negative loading); and (7) when the child meets new children, it takes him or her a long time to start talking. These items were selected to build a concise index of BI-shyness.

**DNA EXTRACTION AND GENOTYPING**

Genomic DNA was extracted from mouthwash samples collected in 4% sucrose by means of a reagent for isolation of genomic DNA (DNAzol Genomic DNA Isolation Reagent; Molecular Research Center Inc, Cincinnati, Ohio). The polymorphism in the transcriptional control region upstream from the 5-HTT coding sequence (5-HTTLP) was analyzed by polymerase chain reaction according to the method reported by Lesch et al\textsuperscript{39} and Heils et al.\textsuperscript{41} Two fragments were generated: a short variant (S) of 484 base pairs and a long variant (L) of 528 base pairs. All amplification reactions were performed on a thermocycler (Mastercycler; Eppendorf, Milan, Italy). The amplified products were analyzed on 2% agarose gels.

For 4 of the 49 children, the DNA extraction was unsuccessful, and therefore genetic data were available for only 45 subjects.

**PROTOCOL**

**Rationale for Stimulus Selection**

We chose to use standardized faces of other children of similar age (models aged 8-9 years), instead of adults, for 2 reasons: first, schoolchildren spend most of their time among other children, complete, 20 minutes; range, 17-22 minutes), the number of the spontaneous comments made by every participant was counted. Second, after the ERP recording, all mothers and children were interviewed individually by trained clinical psychologists with the Italian version of the Schedule for Affective Disorders and Schizophrenia for School-age Children (K-SADS)\textsuperscript{38} interview to collect the children's lifetime DSM-IV symptoms of social phobia, simple phobia, depression, enuresis, generalized anxiety disorder, separation anxiety disorder, panic disorder, attention-deficit/hyperactivity disorder, obsessive-compulsive disorder, conduct disorder, oppositional disorder, and tic disorder. The presence of symptoms of DSM-IV childhood disorders was established on the basis of blind review sessions chaired by a senior child psychiatrist (Dr Marino) of all the information obtained by both the mother and the child's K-SADS interview protocols. All children were included in the study, regardless of the presence or absence of a full psychiatric diagnosis. Third, mothers filled in the Child Behavior Checklist 4-18\textsuperscript{39} and answered questions on parental occupations to calculate the socioeconomic status on the basis of the Hollingshead scale.\textsuperscript{40}

**Table 1. Demographic and Psychometric Features of Participants vs Nonparticipants in the ERP Experiment, Based on Evaluations Made 1 Year Before the ERP Recordings (Time 0)**

<table>
<thead>
<tr>
<th></th>
<th>Participants (n = 49)</th>
<th>Nonparticipants (n = 106)</th>
<th>t Value/χ²</th>
<th>df</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, y</td>
<td>8.84 ± 0.72</td>
<td>8.67 ± 0.60</td>
<td>1.52</td>
<td>147</td>
<td>.13*</td>
</tr>
<tr>
<td>LSAS score, mean ± SD</td>
<td>7.96 ± 5.23</td>
<td>7.71 ± 5.07</td>
<td>0.28</td>
<td>147</td>
<td>.78*</td>
</tr>
<tr>
<td>SU score, mean ± SD</td>
<td>11.33 ± 5.39</td>
<td>11.77 ± 5.27</td>
<td>-0.48</td>
<td>147</td>
<td>.63*</td>
</tr>
<tr>
<td>HA score, mean ± SD</td>
<td>3.67 ± 4.01</td>
<td>4.35 ± 3.97</td>
<td>-0.97</td>
<td>147</td>
<td>.33*</td>
</tr>
<tr>
<td>No. of spontaneous comments at time 0, mean ± SD</td>
<td>7.42 ± 1.76</td>
<td>7.51 ± 1.75</td>
<td>-0.29</td>
<td>147</td>
<td>.77*</td>
</tr>
<tr>
<td>Sex, No. (%); male</td>
<td>26 (53)</td>
<td>53 (53)</td>
<td>0.02</td>
<td>1</td>
<td>.86†</td>
</tr>
<tr>
<td>SES, No.</td>
<td>Lower 6</td>
<td>14</td>
<td>0.15</td>
<td>2</td>
<td>.93†</td>
</tr>
<tr>
<td></td>
<td>Middle 24</td>
<td>46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Upper 19</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ERP, event-related potential; HA, Harm Avoidance Scale of Cloninger Junior Temperament and Character Inventory; Parent Version; LSAS, Liebowitz Social Anxiety Scale adapted for children; SES, socioeconomic status calculated on the basis of the Hollingshead scale (lower range, score of 1-3; middle, score of 4-6; upper, score of 7-9); SU, Stevenson-Hinde and Glover Shyness to the Unfamiliar scale.

*By t test.
†By χ² test.
The joy expression is processed by brain networks at least partially independent from those involved in processing hostile expressions and evokes neurofunctional responses that can be in part predicted by the extroverted, but not by the introverted, temperamental dimensions. The neutral expression was included because it is usually categorized as mildly hostile or ambiguous and was significantly more often misclassified by children with higher indexes of social anxiety at time 0.

The angry expression was included because it is significantly more often misclassified by children with higher indexes of social anxiety and it has been found to elicit heightened subcortical amygdala activity in adults with social phobia and in adults with 1 or 2 copies of the 5-HTT allele.

Trials

On each trial the children were first presented with a child’s face (total time on screen, 1300 milliseconds), which they were instructed to watch carefully until a blue circle appeared superimposed around the center of the picture. As soon as a blue circle appeared (700 milliseconds after the appearance of the stimulus), they had to click a mouse. Thus, the ERPs relevant to this study were all generated before the motor task, which was merely set up to stimulate children’s participation and attention. The monitor screen remained dark between trials for periods that varied randomly from 1200 to 1600 milliseconds. The stimuli were presented to all the children in a sequence that alternated male and female pictures and that avoided close repetition of the same expression. To simulate a real video game and reinforce participation, a screen picture with increasing score appeared about every 6 pictures. Each stimulus was presented 20 times to ensure sufficient ERP acquisition (total, 120 presentations in a complete session).

Every child was exposed to a preexperiment trial of 6 pictures not belonging to the same set used for the experiment to make sure she or he understood the procedure well.

Each child received a gift of a value equivalent to €30.

**ERP ACQUISITION AND ANALYSIS**

As in other studies of ERP and facial expressions of children and adults,26,27,29,37 electroencephalographic activity was recorded at sites Fz, C3, Cz, C4, and Pz of the 10-20 system with the use of silver–silver chloride electrodes referred to linked mastoids with an amplifier (Neuroscan SynAmp; Neuroscan Labs, Sterling, Va), with head preamplified gain 150 and acquisition software (SCAN, version 4.2, Neuroscan Labs). The ground electrode was attached to the forehead. Electro-oculographic activity was recorded from electrodes placed above and below the right eye.

Electrode impedance was maintained below 5 kΩ. The electroencephalogram and electro-oculogram were amplified (gain 500), analogically bandpass filtered (1-30 Hz), digitized, and acquired at a 1000-Hz sampling rate. Electroencephalogram and electro-oculogram epochs between –50 and 1300 milliseconds from the stimulus onset were obtained by means of different trigger codes for each image, allowing for later off-line artifact rejection, sorting, and digital averaging with the Neuroscan EDIT software (Neuroscan Labs). All epochs from all electrodes were rejected if affected by artifacts (greater than +65 μV or less than –65 μV between –50 and 700 milliseconds). The ERP averages were constructed from artifact-free epochs for each trigger code and for each electrode. Amplitudes were measured according to the distance between peaks and troughs for each identified waveform.

**RESULTS**

**RELATIONSHIP OF THE SHYNESS-BI INDEX TO OTHER BEHAVIORAL MEASURES**

Table 2 summarizes the cross-correlations of the behavioral indexes used in the study.

The shyness-BI index significantly predicted the number of spontaneous comments made by children (mean ± SD, 3.39 ± 4.87; range, 0-17) while the electrodes were being placed on their skulls, and the number of lifetime symptoms of social phobia (mean ± SD, 2.26 ± 2.72; range, 0-8) collected by the K-SADS interview, but no other symptoms...
of mental disorders assessed with the K-SADS (the prediction closest to significance pertained to separation anxiety, with \( P = .18 \)). Moreover, among the 9 narrow-band dimensions of problem behaviors measured by the Child Behavior Checklist, the Withdrawn scale (possible scores, 0-16; mean ± SD, 3.02 ± 2.81; range, 0-11) was the only one to correlate significantly with the shyness-BI index.

**ERP ANALYSES**

**Analyses of General Effects**

Analyses of the waveforms generated by the facial expressions (Figure 1 and Table 3) showed an enhanced late negativity occurring after stimulus presentation at a mean of 256 milliseconds for the Fz, 368 milliseconds for the。

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Figure 1. Grand averages of waveforms generated by facial expressions at the reference electrodes, with an enlargement of the waveforms at the Pz electrode. EOG indicates electro-oculogram.
To ascertain the degree of variability of measures across trials, we performed a repeated-measures ANOVA with 2 factors: (1) type of expression (on 3 levels) and (2) time (on 2 levels: first block, encompassing the first 50% of repetitions of each expression; and second block, with the remaining repetitions) for both amplitudes and latencies at all electrodes. The results showed the absence of significant effects of time or time expression factors for the N400 waveforms (Table 3; values and statistics are given for Pz; for other electrodes, data are available from authors on request).

We also found no effect of sex or sex expression on the N400 characteristics (Table 3), and therefore subjects of both sexes were pooled in all subsequent analyses.

**Effects of Shyness-BI on ERP Waveforms**

To test the hypothesis that shyness-BI predicts N400 characteristics when children are presented with other children's expressions of emotions, 2 multiple linear regression procedures were performed separately for N400 amplitudes and latencies, with the N400 amplitudes (or latencies) for each expression (joy, neutral, anger) at the different electrodes (Fz, Cz, Pz, C3, C4) as dependent variables, and the degree of shyness-BI as the independent variable.
Table 4. Influence of 5-HTTLPR on Shyness-BI Index, N400 Waveforms, and Misclassifications of Expressions at Time 0*

<table>
<thead>
<tr>
<th>5-HTTLPR Genotype</th>
<th>SS (n = 9)</th>
<th>LS (n = 23)</th>
<th>LL (n = 13)</th>
<th>S Carriers (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, y†</td>
<td>8.44 ± 0.52</td>
<td>8.65 ± 0.64</td>
<td>9.2 ± 0.59</td>
<td>8.54 ± 0.56‡</td>
</tr>
<tr>
<td>Sex, No. (%), male†</td>
<td>5 (67)</td>
<td>11 (48)</td>
<td>6 (46)</td>
<td>17 (55)</td>
</tr>
<tr>
<td>Education, No.†</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Grade 3</td>
<td>5</td>
<td>15</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Grade 4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>SES, No. (%):‡</td>
<td>Lower</td>
<td>2 (22)</td>
<td>2 (9)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Middle</td>
<td>3 (33)</td>
<td>12 (52)</td>
<td>7 (54)</td>
<td>15 (47)</td>
</tr>
<tr>
<td>Upper</td>
<td>4 (44)</td>
<td>9 (39)</td>
<td>5 (38)</td>
<td>13 (41)</td>
</tr>
<tr>
<td>Shyness-BI index, mean ± SD</td>
<td>15.44 ± 8.72§</td>
<td>10.04 ± 5.10</td>
<td>7.69 ± 5.19</td>
<td>11.74 ± 6.88</td>
</tr>
<tr>
<td>N400 amplitude, µV ‡</td>
<td>19.76 ± 9.95</td>
<td>–19.83 ± 6.07</td>
<td>–17.5 ± 4.35</td>
<td>–19.81 ± 7.18</td>
</tr>
<tr>
<td>Joy</td>
<td>n = 8</td>
<td>20</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>Neutral</td>
<td>n = 8</td>
<td>20</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>Anger</td>
<td>n = 8</td>
<td>20</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>No. of misclassified expressions at time 0, mean ± SD</td>
<td>3.00 ± 1.22**</td>
<td>2.91 ± 1.47</td>
<td>1.92 ± 1.12</td>
<td>2.9 ± 1.43††</td>
</tr>
</tbody>
</table>

Abbreviations: 5-HTTLPR, serotonin transporter promoter polymorphism; SES, socioeconomic status calculated on the basis of the Hollingshead scale (lower range, score of 1-3; middle, score of 4-6; upper, score of 7-9).

*Time 0 refers to a trial of facial expression discrimination task taken by children 1 year before the event-related potential recordings. DNA analysis was successfully completed in 45 children. The "S carriers" column represents the SS and LS genotypes combined.

†For age, by analysis of variance (ANOVA), F1,45=10.1, P<.003. Sex was equally distributed across genotypes (χ²=1.11, P=.51) and in LL vs S carrier subjects (χ²=0.55, P=.46). Education by grade was equally distributed across genotypes (χ²=2.41, P=.129) and in LL vs S carrier subjects (χ²=3.24, P=.07). The SES was equally distributed across genotypes (χ²=1.71, P=.79) and in LL vs S carrier subjects (χ²=0.68, P=.71).

‡For LL vs S carrier subjects, t43=−2.20, P=.05.

§Association of 5-HTTLPR to shyness-BI index: ANOVA, F2,42=4.47, P=.02; independent samples, 2-tailed t test of LL vs S carrier subjects, t42=−2.155, P=.05.

|| Variability of N400 amplitude measures across trials (habituation effects) by 5-HTTLPR: repeated-measures analysis of covariance (ANCOVA) (3 5-HTTLPR genotypes): time: F1,44=1.52, P=.20; time×expression, F2,44=1.38, P=.26; time×expression×genotype: F2,44=0.79, P=.53, repeated-measures ANCOVA (LL subjects and S carriers); time×expression×genotype: F2,44=0.53, P=.59.

¶ANCOVA on N400 amplitudes at Pz by expression (3 levels), with genotype as independent variable and shyness-BI index and age as covariates: type of expression: F2,44=1.25, P=.29; mean square (MS) effect=18.3, MS error=14.59; genotype: F2,44=0.11, P=.929; MS effect=8.49, MS error=76.64; expression×genotype interaction: F2,44=3.57, P=.05, MS effect=52.0, MS error=14.58; MS error=0.18, observed power=0.88. Post hoc comparison: SS vs LL subjects on anger, Tukey HSD, P=.03.

#ANCOVA on N400 amplitudes at Pz by expression (3 levels), with LL or S carrier as independent variable and shyness-BI index and age as covariates: type of expression: F2,44=0.17, P=.84; LL or S carrier: F2,44=0.11, P=.84. Expression by LL or S carrier interaction: F2,44=5.70, P=.005. Post hoc comparison: S carrier vs LL subjects on anger, Tukey HSD, P=.05.

**Linear regression on number of misclassified expressions at time 0 by 5-HTTLPR genotype (0, 1, or 2 alleles): F1,44=4.08, P=.05, β=0.29, adjusted R²=0.065.

†† t Test for number of misclassifications of LL vs S carrier subjects: t43=−2.20, P=.03.

Children’s shyness-BI predicted the N400 amplitude for anger and the neutral expression (Table 3) at Pz, Cz, and C4, always in the direction of a reduction of amplitude, but not the amplitude for the joy expression on any of the electrodes. Similarly, no significant prediction for any of the expressions was provided by regression applied to the N400 latency for any of the electrodes.

Genotypes and Allelic Frequencies

The allelic frequencies were 49 (54%) for the L allele and 41 (46%) for the S allele (Table 4); both were in Hardy-Weinberg equilibrium, without sex-related differences in distribution (respectively, χ²=1.09, P=.37; and χ²=0.43, P=.51). All ANOVAs dealing with the 5-HTTLPR genotype were conducted with the 3 genotypes separately and with the classification10 that combines the LS and SS genotypes into “S carriers.”

While socioeconomic status, grade, and sex were equally distributed across genotypes and in LL vs S carrier subjects, children with the SS genotype and S carriers were slightly, but significantly, younger (Table 4).

Genetic Analyses

A polynomial univariate ANOVA, where the factor was the genotype at the 5-HTTLPR gene and the dependent variable was the shyness-BI index, showed that shyness was significantly different across the genotypes, with the SS genotype being associated with higher shyness-BI index (Table 4); similarly, S carriers vs LL subjects showed a trend toward significantly higher shyness-BI index (Table 4).
These results for the anger expression could not be explained by habituation effects on the different expressions that could have occurred between the first and the second block of repetitions, as shown by repeated-measures analysis of covariance on amplitudes with 3 factors: (1) time (on 2 levels: first and second blocks of repetitions), (2) expression, and (3) genotype, with shyness-BI index and age as covariates (Table 4).

Because the results of the ERP analyses suggested a role for both shyness-BI index and the 5-HTTLPR genotype in determining different patterns of information processing of hostile (angry) and/or neutral facial expressions, we tested post hoc whether the 5-HTTLPR genotype could predict a biased discrimination of expressions in the trial completed 1 year before the ERP recordings. A linear regression was then performed on the total number of misclassifications made at time 0 by the 45 children whose genotyping was available, with the 5-HTTLPR genotype (0, 1, or 2 S alleles) as the independent variable. The number of misclassifications was predicted significantly, in keeping with the previous findings. Similarly, an independent samples, 2-tailed t test of the number of misclassifications between S carriers and LL subjects showed a trend toward significantly worse performance for the former (Table 4).

To evaluate whether the 5-HTTLPR genotype had greater power to predict the phenotype of shyness-BI index, or the endophenotype of N400 amplitude at Pz in response to anger, we specified a linear trend, with weights of –1, 0, and 1 for the 0, 1, and 2 S alleles, and a quadratic trend, with weights of –1, 2, and –1 for the 0, 1, and 2 S alleles, respectively, and then entered these coded vectors simultaneously in the regression equations to predict shyness-BI index and the N400 amplitude (Table 5).

### Table 5. Regressions of 5-HTTLPR Genotype on N400 Amplitude Elicited by the Anger Expression at Pz and on the Shyness-BI Index

<table>
<thead>
<tr>
<th></th>
<th>Anger N400 Amplitude at Pz Model: F_{1,42} = 5.55, P = .008</th>
<th>Shyness-BI Index Model: F_{1,42} = 4.57, P = .02</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β</strong></td>
<td><strong>t</strong></td>
<td><strong>P Value</strong></td>
</tr>
<tr>
<td>Linear trend</td>
<td>3.30</td>
<td>.002*</td>
</tr>
<tr>
<td>Quadratic trend</td>
<td>–0.16</td>
<td>–0.34</td>
</tr>
</tbody>
</table>

Abbreviations: BI, behavioral inhibition; 5-HTTLPR, serotonin transporter promoter polymorphism.

*R^2 = .24; adjusted R^2 = .19.
†R^2 = .18; adjusted R^2 = .14.

In light of the foregoing relationships, and after checking for the homogeneity of covariance matrices (Bartlett χ^2 not significant on all dependent variables and the covariate; box M = 19.98, χ^2 = 16.15, P = .70), we performed analysis of covariance where the independent variables were the genotypes at the 5-HTTLPR, and the dependent variables were the N400 amplitudes evoked by facial expressions at the Pz electrode, the repeated-measures factor was the expression (joy, neutral, anger), and the covariates were the shyness-BI index and age. Neither the type of expression nor genotype per se influenced the N400 amplitudes, but a significant expression × genotype interaction was found (Table 4). The post hoc comparison showed that the significance was sustained by smaller amplitude elicited by anger in the SS subjects compared with the LL subjects (Figure 2, Figure 3, and Table 4). The analysis of covariance, contrasting LL subjects with S carriers, provided very similar results, ie, the absence of genotype or expression effects, but a significant expression × genotype interaction effect, sustained by smaller N400 elicited by anger in S carriers (Table 4).
Shyness-BI index and the presence of 1 or 2 copies of the 5-HTTLPR S allele predicted smaller ERP N400 amplitudes in response to overtly hostile and neutral facial expressions at centrotemporal regions, which are interconnected with emotion processing networks encompassing the amygdala and the prefrontal cortex. As such, the N400 reflects the neural processes involved in recognition of facial expressions, and predicts partially impaired reading in response to some facial affects, consistent with the findings of biased discrimination of the hostile and ambiguous expressions at time 0 by the same subjects. Smaller ERP waveforms in response to angry faces have been reported in a controlled study of posttraumatic stress disorder and have been interpreted as a relative reduction of cortical activity, co-occurring with heightened subcortical activity, in response to hostile social stimuli. Indeed, a relatively decreased activity in the cortices that are involved in emotional evaluative processes (including the secondary visual and parieto-occipital areas) has been demonstrated to co-occur with exaggerated activation of the amygdala in adults with social phobia during simulated social interaction investigated by functional brain imaging techniques. A decreased cortical—as opposed to subcortical—perfusion is contrary to the pattern of activation in normal controls in conditions of simulated social interaction. Likewise, adults with social phobia show heightened subcortical, and specifically amygdala, activation in response to neutral and angry facial expressions, and carriers of 1 or 2 copies of the S allele have an increased amygdala response to anger. The increased subcortical, and decreased cortical, activation in response to emotional stimuli of social relevance has been interpreted as the excessive involvement of a phylogenetically “older” system of recognition of dangerous, novel, or ambiguous stimuli. Heightened amygdala activation in response to novel faces in adults who had been categorized as shy in childhood may be seen as broadly consistent with these data.

Consistent with the finding that the neurofunctional responses to happiness expressions can be partly predicted by the extraverted, temporal dimensions, childhood shyness-BI and/or the 5-HTTLPR genotype did not predict the N400 potentials evoked in response to a prosocial (joy) expression. The greater $R^2$ found by regressing the 5-HTTLPR genotype on the endophenotype of N400 than on the shyness-BI phenotype is consistent with the suggestions that the effect of functional genetic polymorphisms is more robustly assessed by assays of brain physiology than with behavioral phenotypes. However, while the association between the SS 5-HTTLPR genotype or S carrier status and shyness-BI is consistent with most studies of adult human emotionality, a study found the LL genotype associated with childhood shyness-BI.

Although these results need replication, they show that genetic variation at 5-HTTLPR contributes to shape the N400 waveform, a possible index of complex neuronal activity that occurs when expressions of emotions are being processed. A pattern of decreased cortical activation in response to some specific social cues (including peers’ nonverbal signs of refusal or neutrality) may then constitute a heritable basis for biased discrimination of some forms of socially relevant information, which in turn may hamper social interactions and ultimately reinforce a child’s disposition to shyness-BI.

Shy children have been shown to provide relatively distinct physiologic responses in a variety of contexts. These data suggest that a biased pattern of processing emotional information of social relevance can be recognized and characterized psychophysically early in life in children with heightened indexes of shyness-BI or 1 or 2 copies of the 5-HTTLPR S allele.

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