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ATTENTIONAL BIASES TO THREAT AND SEROTONIN TRANSPORTER GENE PROMOTER (5-HTTLPR) POLYMORPHISMS: EVIDENCE FROM A PROBE DISCRIMINATION TASK WITH ENDOGENOUS CUES

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Abstract

Recent studies have investigated the association between serotonin transporter gene promoter (*5-HTTLPR*) functional polymorphisms and attentional biases to threat, a cognitive mechanism that probably contributes to the development and maintenance of anxiety. The present study genotyped a sample of N = 141 healthy volunteers for an insertion/deletion polymorphism and the rs25531 single-nucleotide polymorphism in *5-HTTLPR*. In order to investigate attentional biases to threat, we used a probe discrimination task in which the gaze direction of centrally presented fearful or neutral faces endogenously cued attention. The results indicated no significant differences in attentional biases to threat between *5-HTTLPR* genotype groups. However, we found that carriers of two low-expressing alleles (i.e., *S* or *LG*) of *5-HTTLPR* displayed a significant slowing of responses across trials with fearful compared to neutral faces. This effect may indicate that fearful faces triggered increased emotional arousal in these genotypes, which may have interfered with the processing of gaze direction and spatial cuing. These results suggest that using fearful faces as endogenous spatial cues may be problematic in genotypes associated with facilitated emotional arousal to these stimuli, and underscore the hypothesis that *5-HTTLPR* specifically influences automatic rather than consciously-controlled processes of attention.

Keywords

• Serotonin transporter gene • 5-HTTLPR • Attentional biases • Anxiety • Probe discrimination task.

1. Introduction

Psychological theories [1-3] have explained the development and maintenance of anxiety disorders through the interaction of several psychological factors, among which selective attention to threat is central [4,5]. These biases in the initial stimulus registration stage of cognitive processing [1,6] are reflected in the rapid and automatic allocation of attention to threat-relevant stimuli when attentional resources are limited. Threat cues include negative facial expressions (e.g., fearful or angry faces) or negative words (e.g., "disease", "failure") [7].

Attentional biases to threat have been extensively investigated using the probe *detection* task, which was described in a seminal study [8]. This experimental task generally involves the short presentation of a pair of words or pictures, which includes a threatening and a neutral stimulus, in two opposite locations on the screen. One of these cues is then replaced by a probe (e.g., a capital letter), which is detected with greater speed and accuracy in trials in which it replaces the cue toward which attention was allocated. Many studies have also used a related probe *discrimination* task, in which participants have to identify which of two alternative probes (e.g., the letters "T" and "L") is presented following two facial expression cues. An attentional bias to threat has been identified and replicated in patients with anxiety disorders [9-11], who respond significantly faster to probes replacing emotional (e.g., fearful) expressions that to probes replacing neutral expressions. Moreover, research showed that successful psychotherapy eliminated the attentional biases to threat [4,12], symptom amelioration due to psychotherapy correlated with the reduction in attentional biases to threat [13], and the re-emergence of these attentional biases predicted the return of anxiety at follow-up among patients treated for anxiety disorders [14].

Based on the relatively high heritability of anxiety disorders [15,16], diathesis-stress theories [1,17] suggested that attentional biases to threat and other cognitive factors that contribute to the maintenance of anxiety are secondary to genetic vulnerabilities. Molecular genetic studies have thus started to explore the associations of functional polymorphisms in genes related to neurotransmitters that are thought to play a role in anxiety, and cognitive features of anxiety [18]. In light of the therapeutic efficacy of selective serotonin reuptake inhibitors in anxiety disorders [19], and the impact of psychopharmacological manipulations of serotonin availability (e.g., tryptophan depletion) on anxiety symptoms [20], many genetic association studies have focused on the serotonin transporter gene (*5-HTT*). Several functional polymorphisms have been described in the promoter region

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(*5-HTTLPR*) of this gene, including an insertion/deletion (ins/del) polymorphism [21] that is functionally connected to a single-nucleotide polymorphism (SNP rs25531) [22,23]. The short (*S*) allele of the ins/del *5-HTTLPR* polymorphism, as well as the long allele in which an adenine was substituted by a guanine at the rs25531 locus (*LG*) are associated with decreased levels of *5-HTT* expression [22] and reduced availability of the serotonin transporter in the brain [24,25], in comparison to the *LA* allele.

Recent studies have begun to investigate the association of *5-HTTLPR* polymorphisms and attentional biases to threat. In the first study on this line [26], a small sample ($N = 27$) of psychiatric inpatients were genotyped for the ins/del *5-HTTLPR* polymorphism and tested using a probe detection task in which cues were anxious/neutral words. Carriers of the *S* allele displayed shorter detection latencies for probes that replaced the anxious word, which indicated a facilitated attentional engagement with the processing of anxious words. In this study [26], the *5-HTTLPR* genotype accounted for 26% of the variance in the attentional bias for anxious stimuli. A subsequent study [27] genotyped a larger sample of healthy volunteers for both the ins/del and the rs25531 *5-HTTLPR* polymorphisms. Using a probe discrimination task, this study also found that carriers of the low-expressing *S* and *LG* alleles had shorter latencies to discriminate probes that replaced angry faces [27]. There was even a gene dosage effect, such that the attentional bias to angry faces was the largest in carriers of two such low-expressing alleles (generally labeled *S'S'* for the sake of simplicity), followed by carriers of only one low-expressing allele (labeled *S'L'*) and homozygotes for the high-expressing *LA* allele (labeled *L'L'*) [27]. In contrast with these initial results, several other studies found that carriers of the low-expressing *5-HTTLPR* alleles displayed no attentional bias to threat in probe discrimination tasks with words [28,29] or images [30]. However, it was found that the performance of *LA* homozygotes in probe discrimination tasks was different from that of low-expressing allele carriers. This genotype was associated with shorter probe discrimination latencies in trials in which the probe replaced the neutral stimulus (i.e., in the location opposite of where the threat cue had been presented). Therefore, these authors suggested that *LA* homozygotes display an attentional avoidance of threat cues and that carriers of low-expressing alleles lack this potentially protective bias [28,30]. A recent meta-analysis [31], which included ten genetic association studies, supported an attentional bias to threat with medium effect size in *S'S'* genotypes, but not in *S'L' or L'L'* genotypes. Not surprisingly, this meta-analysis [31] emphasized the need for additional studies on the association between *5-HTTLPR* polymorphisms and attentional biases to threat.

The present study investigated the association between the ins/del and the rs25531 *5-HTTLPR* polymorphisms, and attentional biases to threat in a probe discrimination task in which the cues were fearful and neutral faces. The sample included randomly selected healthy volunteers. In line with previous studies, we hypothesized that *S'S'* genotype groups would display attentional biases to threat.

2. Methods

2.1 Participants

$N = 141$ participants (120 women) volunteered for this study. They were all Caucasians of Romanian descent, and came from the same well-circumscribed geographical area. Age ranged from 19 to 24 (*Mean* = 21.2 years). None of the participants reported neuropsychiatric conditions. Prior to study participation, written informed consent was obtained from all the volunteers. All the participants were compensated for their time. The study followed the recommendations of AMA's Declaration of Helsinki and it was approved by the Babeş-Bolyai University Research Council.

2.2 Genotyping

DNA was extracted from leukocytes (EDTA-anticoagulated blood) using Genomic DNA Extraction Kit (Fermentas, Vilnius, Lithuania) and kept at -20°C. Both the ins/del and the rs25531 genotyping were performed using published protocols [32,33]. Briefly, the polymerase chain reaction (PCR) assay conditions were optimized as follows: each reaction was carried out in a 25 µl volume [50 ng of genomic template, 12.5 µl PCR mastermix (2x)]; the forward primer (5'-GGCGTTGCCGCTCTGAATGC-3') and reverse primer (5'-GAGGGACTGAGCTGGACAACCAC-3'), from Generi-Biotech (Hradec Kralove, Czech Republic), were used to amplify a region encompassing *5-HTTLPR*. These primers yield amplicons of 529 (for *L* allele) or 486 bp (for *S* allele). Thermal cycling consisted of 3 minutes of initial denaturation at 94°C followed by 31 cycles of 94°C (40 s), 57°C (40 s) and 72°C (40 s), each with a final extension step of 4 min at 72°C. The *LG* and *LA* alleles were subsequently studied by enzymatic digestion of 10 µl of PCR products that were digested by *HpaII* (an isoschizomer of *MspI*) type FastDigest (Fermentas) in a 30 µl reaction assay at 37°C for 5 minutes. The restriction enzyme *MspI* recognizes and cuts a 5'-C/CGG-3' sequence resulting in the following fragments: 340 bp, 127 bp and 62 bp for the *LA* allele; 174, 166, 127 and 62 bp for the *LG* allele; 297 bp, 127 bp and 62 bp for the *SA* allele; and 166, 131, 127 and 62 bp for the *SG* allele. Finally, 10 µl of remaining PCR product and 15 µl of restriction enzyme assay solution were loaded onto a 2.5% agarose gel, run for 2h at 160 V in 0.5xTBE running buffer and visualized by ethidium bromide for size estimation.

2.3 Probe discrimination task

We used a probe discrimination task that was previously described [34,35]. This task involved presenting 384 unique trials in a random order, in which the cues were fearful or neutral facial expressions [36], displayed in the center of the screen, subtending a vertical visual angle of 7°; and the probe was the upper case letter "T" or "L", which subtended a 3° visual angle and were presented 5° from the midpoint of the screen on a 19-inch coloured monitor (for examples of trials see [34,35]). The sequence of events in a trial was: central fixation cross (675 ms); fearful/neutral face with eyes looking straight ahead (900 ms), followed by the same fearful/neutral face with the eyes either remaining in the same position (in central gaze trials), or moving to the right or the left side (in gaze congruent/incongruent trials), simulating the orientation of the gaze in that direction; then, the probe letter in the left/right side of the screen, displayed

until the participant pressed one of the two response buttons. In gaze-congruent trials, the target appeared in the location previously cued by the gaze direction; in the gaze-incongruent trials, the target appeared opposite to the cued location. The stimulus onset asynchrony (SOA) (i.e., time between the onset of cue and target) was 300 or 700 ms, counterbalanced between types of trials.

The participants were instructed to keep their eyes on the central fixation cross, and press a key when they discriminated the probe, as accurately and quickly as possible after the appearance of one of the two target letters. To minimize the interference of the left or right position of the probe, we labelled the keys vertically. In order to reduce the effect of the dominant hand, we asked the participants to respond only with their dominant hand. The task involved 12 practice trials and two blocks of testing with a rest period between them. We measured the latency (in milliseconds, ms) for each trial.

2.4 Statistical analyses

We checked whether the distribution of genotypes in our sample was in the Hardy-Weinberg equilibrium [37]. Then, repeated-measure ANOVA was used to test for the effects of the genotype on performance in the probe discrimination task. Considering that our sample was not balanced for sex, we report both the analyses on the whole sample and those on the women sub-sample. Performance (i.e., latencies) in the central gaze trials were grouped in the analyses by SOA (300 ms and 700 ms) and facial expression (neutral and fearful). The left- and right-sided gaze trials were grouped by the location of the target letters (obtaining two conditions: congruent and incongruent), and by facial expression (neutral and fearful). All the statistical analyses were run in SPSS.

3. Results

3.1 Genotypes

The frequency of alleles was 0.44 for the *S* allele, 0.06 for the L_G allele, and 0.5 for the *LA* allele. The genotypes were categorized as follows: *N* = 35 *S'S'* (i.e., *SS*, *SLG* and *LGLG*); *N* = 70 *S'L'* (i.e., *SLA* and *LGLA*); and *N* = 36 *L'L'* (i.e., *LALA*). These genotypes were in Hardy-Weinberg equilibrium ($\chi^2 = 0.17$, not significant).

3.2 Central gaze trials

Errors and outlying latencies less than 100 ms or greater than 1500 ms were removed. We entered the latencies from central gaze trials into an ANOVA with emotional expression (fearful vs. neutral) and SOA (300 vs. 700) as within-subject factors, and genotype (*S'S'* vs. *S'L'* vs. *L'L'*) as between-subject factor. There was a main effect of emotional expression (F[1, 139] = 6.244, $p = 0.014$, partial $\eta 2 = 0.044$), with slower responses in trials with fearful expressions (mean difference = 11.95 ms). There were also marginally significant interactions of SOA and emotional expression (F[1, 139] = 3.421, $p = 0.067$, partial $\eta 2 = 0.024$), and emotional expression and genotype (F[3, 137] = 2.358, $p = 0.074$, partial $\eta 2 = 0.049$). Follow-up pairwise comparisons indicated a significant slowing of responses in central gaze trials with fearful expressions compared with neutral expressions in the *SS'* group (mean difference = 8.601 ms; Scheffe post-hoc $p = 0.0004$) (Figure 1A), but not *SL'* and *LL'*.

These analyses were repeated on the women sub-sample (N = 120), in which *5-HTTLPR* genotypes were also in Hardy-Weinberg equilibrium ($\chi^2 = 0.3$, not significant). We replicated the significant interaction of SOA and emotional expression (F[1, 118] = 33.313, $p = 0.000$, partial $\eta 2 = 0.222$). The main effect of SOA (F[1, 118] = 3.456, $p = 0.066$, partial $\eta 2 = 0.029$) and the interaction of SOA × emotional expression × genotype (F[2, 117] = 1.883, $p = 0.157$, partial $\eta 2 = 0.031$) only approached the statistical significance threshold.

3.3 Congruent versus incongruent trials

Latencies from gaze congruent and gaze incongruent trials were entered into an ANOVA with emotional expression (fearful vs. neutral), SOA (300 vs. 700) and congruency (gaze incongruent vs. congruent) as within-subject factors, and genotype (*S'S'* vs. *S'L'* vs. *L'L'*) as between-subject factor. There were significant main effects of emotional expression (F[1, 139] = 197.614, $p = 0.000$, partial $\eta 2 = 0.589$), and congruency (F[1, 139] = 51.762, $p = 0.000$, partial $\eta 2 = 0.273$), as well as a significant interaction between emotional expression and congruency (F[1, 139] = 88.225, $p = 0.000$, partial $\eta 2 = 0.39$). In comparison to neutral expressions, fearful expressions were associated with faster responses in gaze congruent trials (mean differences = 8.027 ms) and slower responses in gaze incongruent trials (mean differences = 3.212 ms). There was also a significant interaction between emotional expressions and genotype (F[2, 138] = 3.087, $p = 0.049$, partial $\eta 2 = 0.043$), with a statistical tendency

Table 1. Response latencies (means ± standard error of the means, in milliseconds), collapsed across stimulus onset asynchrony (SOA), and divided by 5-HTTLPR genotypes

Genotypes	Central gaze trials		Gaze congruent trials		Gaze incongruent trials	
	Neutral face	Fearful face	Neutral face	Fearful face	Neutral face	Fearful face
SS	551.739 ± 13.307	543.085 ± 13.557	543.017± 12.73	533.029 ± 12.365	559.807 ± 14.774	555.826 ± 14.239
SLG	538.157 ± 27.681	532.62 ± 31.771	521.422 ± 31.23	519.328 ± 27.176	536.41 ± 28.901	543.17 ± 28.872
LGLG	570.44 ± 20.63	553.585 ± 17.145	532.82 ± 6.44	548.435 ± 24.605	559.145 ± 14.325	557.475 ± 17.205
SLA	570.942 ± 12.732	571.488 ± 12.306	561.312 ± 12.666	553.601 ± 12.073	572.783 ± 13.296	578.691 ± 12.506
LGLA	552.432 ± 30.173	548.73 ± 28.19	540.282 ± 29.927	531.59 ± 24.225	561.302 ± 33.997	572.827 ± 27.955
LALA	564.412 ± 16.128	550.198 ± 17.808	552.398 ± 14.915	543.089 ± 14.945	569.379 ± 16.545	571.484 ± 15.428

in the *S'S'* group to respond slower in trials with fearful compared to neutral expressions (mean difference = 4.911 ms) (Figure 1B). The expected three-way interaction between expression, congruency and genotype was not significant ($p = 0.247$) (Figure 2).

In addition, we calculated mean differences between neutral and fearful trials (neutral - fearful difference) by congruency. The genotype had no significant effect on speeding on congruent trials due to fearful versus neutral faces ($p = 0.907$), or slowing on incongruent trials due to fearful versus neutral faces ($p = 0.209$). For the latter bias, follow-up pairwise comparisons indicated a marginally significant difference between the genotypes, with a tendency of increased slowing in *S'S'* compared to *S'L'* (mean difference = 9.053 ms) and *L'L'* (mean difference = 5.259 ms) (Figure 3).

By repeating the analyses on raw response latencies from the women sub-sample, we replicated the significant effect of congruency ($F[1, 118] = 52.015$, $p = 0.000$, partial $\eta 2 = 0.308$) and the significant interaction of emotional expression × congruency ($F[1, 118] = 77.236$, $p = 0.000$, partial $\eta 2 = 0.398$). The interaction of emotional expression × genotype was marginally significant ($F[2, 117] = 2.826$, $p = 0.063$, partial $\eta 2 = 0.046$). The genotype had no significant effect on bias scores in the women sub-sample.

4. Discussion

The probe discrimination task that we used in this study allowed us to investigate whether fearful faces guided attention toward their direction of gaze more effectively than neutral faces. This effect was confirmed by faster responses in congruent trials with fearful compared to neutral faces (i.e., increased attentional engagement with threat), and slower responses in incongruent trials with fearful compared to neutral faces (i.e., reduced attentional disengagement from threat). However, we found no evidence of an influence of *5-HTTLPR* on this effect. The statistical interaction of genotype × expression

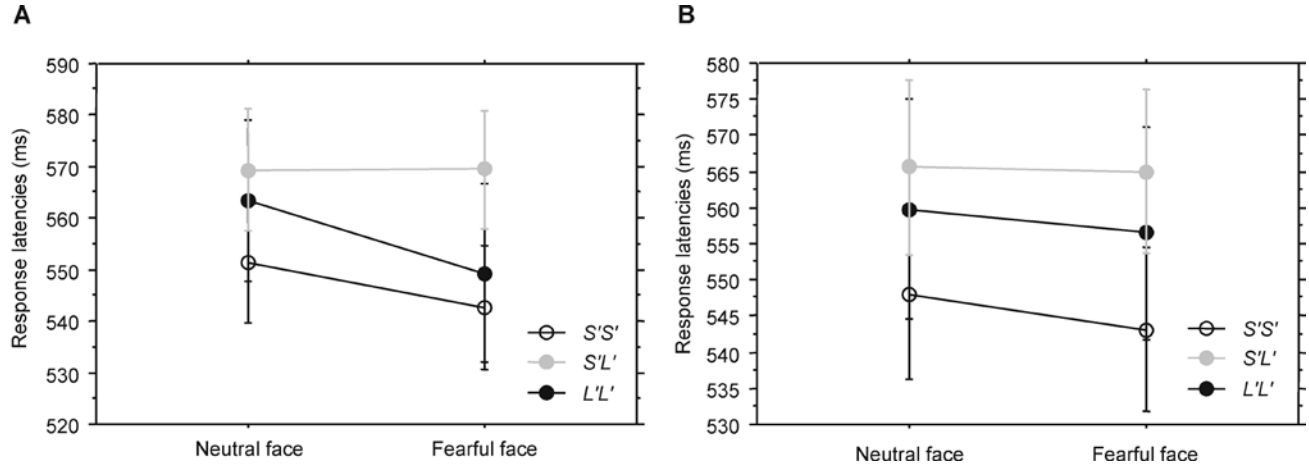

Figure 1. (A) Neutral *vs.* fearful face comparison of response latencies in central gaze trials, by 5-HTTLPR genotype. (B) Neutral *vs.* fearful face comparison of response latencies in congruent and incongruent trials (collapsed together), by 5-HTTLPR genotype.

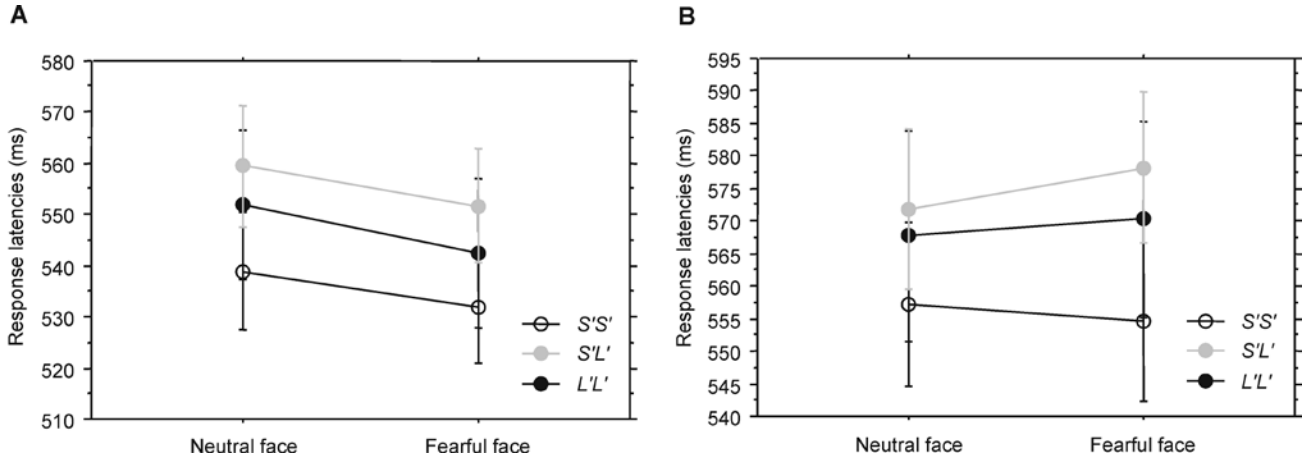

Figure 2. (A) Neutral *vs.* fearful face comparison of response latencies in congruent gaze trials, by 5-HTTLPR genotype. (B) Neutral *vs.* fearful face comparison of response latencies in incongruent gaze trials, by 5-HTTLPR genotype.

× congruency was not significant, which indicated that attention to the gaze direction of fearful faces is not differentially modulated in genotype groups. However, we found that fearful faces slowed responses both in central gaze, and gaze congruent and incongruent trials. This slowing due to fearful faces was present in the *S'S'* group, but not the *S'L'* or the *L'L'* group.

The non-significant association of *5-HTTLPR* with biases in the attentional engagement with threat or disengagement from threat is in line with a recent study [29]. Moreover, the absence of attentional biases to threat in the *S'S'* group is similar to the observations reported in two other studies [28,30]. However, these latter studies found an influence of *5-HTTLPR* on attentional biases to threat due to a significant speeding of the attentional disengagement from threat in *LA* homozygotes. As indicated in Figure 3, we did not observe this attentional bias in our sample of *LA* homozygotes.

The negative effects reported in the present study may diverge from some of the previous findings due to at least three reasons. First, the probe discrimination task used in this study was different from the tasks used in other studies. In a seminal study that described the spatial cuing task (from which the spatial discrimination task has derived), Posner [38] identified two types of cues that can direct attention to a spatial location: endogenous cues (e.g., a central arrow pointing to the right or left), which need to be processed semantically in order to guide attention; and exogenous cues (e.g., images or words that appear on the left or right side of the screen), which directly draw attention to the potential locations of the target. The spatial discrimination task used in the present study modeled the endogenous cuing condition. Instead of presenting pairs of emotional and neutral stimuli in each side of the screen, centrally presented faces were displayed, whose eyes gazed to the right or the left side of the screen. In order to test whether *5-HTTLPR* was associated with a selective processing of the eye gaze of threatening faces, this task included fearful and neutral faces that cued attention to either side of the screen. Our results supported the view that the gaze direction of fearful faces was more effective in directing attention to a spatial location. As in the other versions of the probe discrimination task, the probe appeared in either side of the screen in order to dissociate attentional engagement (i.e., when the probe appeared in the location indicated by the gaze) and attentional disengagement (i.e., when the probe appeared in the opposite location relative to the gaze). All the other studies that found a significant association of *5-HTTLPR* with attentional biases to threat, due to either increased engagement with threat in carriers of the low-expressing alleles [26,27], reduced disengagement from threat in carriers of the low-expressing alleles [39], or increased disengagement from threat in *LA* homozygotes [28,30] used probe discrimination tasks with exogenous cues. Exogenous cuing involves automatic processing and it is therefore possible that *5-HTTLPR* specifically biases automatic attentional processing of threat.

In the present probe discrimination task, we found that participants with the *S'S'* genotypes responded significantly slower across trials with fearful compared to neutral faces (i.e., both central gaze, and gaze congruent and incongruent trials collapsed together). We suggest that this effect reflects the higher emotional arousal triggered by centrally presented fearful faces in the *S'S'* group. This explanation is supported by convergent findings from previous studies, which showed that fearful or angry faces induce higher emotional arousal in carriers of the low-expressing alleles of *5-HTTLPR*, as reflected by amygdala activity [40,41]. It is possible that the higher emotional arousal triggered by fearful faces in the present study interfered with the semantic processing of the gaze direction in our *S'S'* group. This interference may have increased the difficulty of processing the endogenous cues in the probe discrimination task, and explain why *S'S'* participants responded slower on both gaze-congruent and gaze-incongruent trials with fearful compared to neutral faces.

A second explanation for the divergence between the present results and previous findings is related to the sample size. An a priori sample size estimation run in G-Power 2.0 [42] indicated a desirable sample size of $N \geq 159$ in order to detect a medium size effect (as found in the meta-analysis of [31]), with an alpha of 0.05 and power ≥ 0.08. The studies that reported a significant association of *5-HTTLPR* and attentional biases to threat [26-28,30,39] included samples between $N = 27$ [26] and $N = 144$ (Study 2 in [39]). Our sample of $N = 141$ is close, but still below the desirable sample size. Therefore, the divergent findings from the literature should be interpreted with caution, and future studies should strive to gather larger samples of participants in order to significantly advance this line of research. In addition, our sample was not balanced for sex.

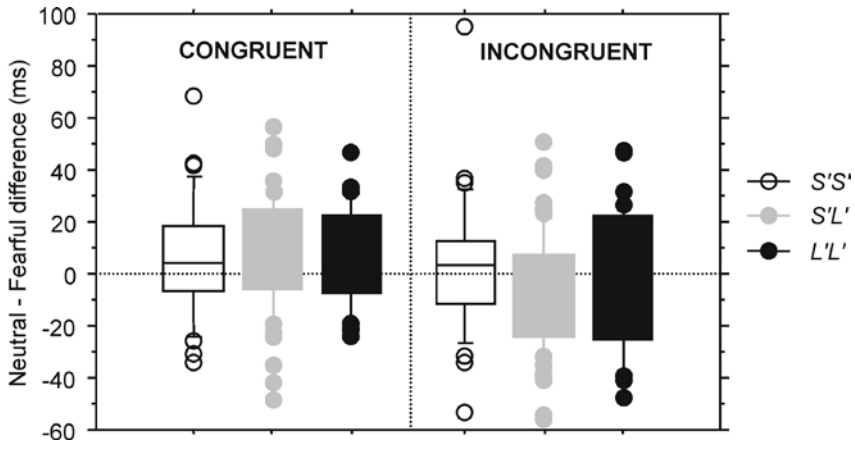

Figure 3. Attentional biases to threat scores in congruent (i.e., speeding on congruent trials due to fearful versus neutral faces) and incongruent trials (i.e., slowing on incongruent trials due to fearful versus neutral faces), by 5-HTTLPR genotype.

However, we replicated the null effects of the genotype in the women sub-sample: the effect of the emotional expression × congruency × genotype interaction on raw latencies was not significant, and the effect of the genotype on bias scores was also not significant. This suggests that sex did not confound our analyses, but future studies might include samples in which women and men and equally represented.

A third explanation for the differences between our findings and previous reports is related to genotyping both the ins/del and the rs25531 *5-HTTLPR* alleles. Some of the previous studies (e.g., [26,30]) have genotyped only the ins/del polymorphism and this may have resulted in the incorrect categorization of *LG* carriers as high-expressing allele homozygotes. We recently investigated the influence of genotyping rs25531 on resting respiratory sinus arrhythmia [43], a psychophysiological variable that has been associated with emotion dysregulation and anxiety susceptibility [44-47]. When the participants from that study were categorized based only on the ins/del *5-HTTLPR* alleles, our analyses indicated a significant influence of *5-HTTLPR* on respiratory sinus arrhythmia; however, when we re-categorized genotypes based on both the ins/del and the rs25531 polymorphisms, which indicated that 11.18% of *LG* carriers had been incorrectly categorized the first time, the association between *5-HTTLPR* and respiratory sinus arrhythmia was no longer significant [43]. This underscored the importance of genotyping both *5-HTTLPR* polymorphisms in order to reduce false positive effects. The present findings are based on a sample that was genotyped for both *5-HTTLPR* polymorphisms, which included ethnically homogenous participants recruited from a well-circumscribed geographical area.

In conclusion, this study found no evidence of an influence of *5-HTTLPR* on attentional biases to threat in a probe discrimination task in which the gaze direction of fearful/neutral faces endogenously cued attention. The *S'S'* group displayed a global slowing of responses in trials with fearful faces, and we explained this observation by an interference between the increased emotional arousal triggered by fearful faces and the processing of the gaze direction of these faces. This may have obscured our possibility to observe the potential effects of *5-HTTLPR* on biases in attentional engagement with threat or disengagement from threat. Therefore, we suggest that probe discrimination tasks with exogenous cues may be more appropriate in future genetic association studies on this topic, especially in light of the possibility that *5-HTTLPR* specifically biases automatic, rather than consciously controlled processes of attention.

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