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ORIGINAL INVESTIGATION

Impaired recognition of fear facial expressions in 5-HTTLPR S-polymorphism carriers following tryptophan depletion

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Abstract
Rationale Genotype at the 5′ promoter region (5-HTTLPR) of the serotonin transporter has been implicated in moderating the effects of acute tryptophan depletion on neurocognitive functioning. Acute tryptophan depletion has been associated with the processing of fear-relevant cues, such as emotional expressions, but the effect of genotype at the 5-HTTLPR has not been assessed.
Objective The present study investigated the effects of acute tryptophan depletion on the recognition of standardized facial expressions of emotions in healthy volunteers classified as ll homozygotes or s carriers.
Materials and methods A double-blind between-groups design was used with volunteers randomly selected to ingest capsules containing an amino acid mixture specifically lacking tryptophan, or placebo capsules containing lactose. 5 h after capsule ingestion, subjects were required to identify anger, disgust, fear, happiness, sadness, and surprise expressions that progressed from neutral to each full emotional expression in 5% steps.
Results Tryptophan depletion significantly impaired the recognition of fearful facial expressions in s carriers but not ll homozygotes. This impairment was specific to fear expressions. No significant differences in the recognition of other expressions were found. Free tryptophan levels were correlated with fear recognition in s carriers but not ll homozygotes.
Conclusions The effects of acute tryptophan depletion on the processing of emotional expressions varies as a function of genotype at the 5-HTTLPR. Depletion impairs the recognition of fear in s carriers but not ll homozygotes. This finding reinforces the importance of considering genotype when assessing the behavioral effects of pharmacologic modulation.

Keywords Tryptophan depletion · Fear · Serotonin · 5-HTTLPR · Amygdala

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Introduction

Serotonergic systems play an important role in the neural processes underlying fear and anxiety. The administration of pharmacological compounds that reduce serotonin availability or uptake has been seen to reduce both conditioned fear responding and the ability to process fear facial expressions (Harmer et al. 2003b; Hellewell et al. 1999; Hensman et al. 1991; Silva et al. 2001). Extensive data support the role of the amygdala in fear-relevant

conditioning and in the identification of fear expressions (Adolphs 2002; Blair 2003; LeDoux 2003; Phillips et al. 2004). These effects are believed to represent the modulation of serotonin function in the amygdala (Graeff et al. 1997; Harmer et al. 2003a,b). Serotonin functioning in the amygdala is itself influenced by an individual's genotype at the 5′ promoter region (5-HTTLPR) of the serotonin transporter gene (Hariri et al. 2002). Individuals carrying the short (s) allele of this gene show increased sensitivity to serotonergic manipulation relative to long (l) allele homozygotes (Neumeister et al. 2002; Roiser et al. 2005, 2006). The present study examined the interaction between 5-HTTLPR genotype and serotonergic modulation on fear expression processing.

A wealth of research has implicated serotonin in fear and anxiety-relevant processes. The modulation of serotonin levels affects anxiety symptoms after anxiogenic challenges (Richell et al. 2005; Schruers et al. 2000) and sensitivity to fear-relevant social cues (Harmer et al. 2003a,b, 2004; Munafo et al. 2006). Acute tryptophan depletion (ATD), a standard method of reducing brain synthesis of serotonin, is associated with decrements in the recognition of the fear facial expression (Harmer et al. 2003b). Acute administration of tryptophan, by contrast, increases the subjects' ability to recognize the fear expression (Attenburrow et al. 2003). Similar effects of fear recognition were seen after the administration of citalopram, an SSRI, which also increases serotonin levels (Harmer et al. 2003a).

The behavioral effects of tryptophan depletion may reflect modulation of activity in the amygdala. In particular, recognition of the fear expression relative to other expressions is frequently associated with amygdala functioning. Lesion studies in humans consistently find that amygdala damage is associated with impaired recognition of emotional facial expressions, and that this impairment is most marked for the fear expression (Adolphs et al. 1999; Adolphs 2002). Although non-specific amygdala activation in response to multiple emotional expressions was reported (Winston et al. 2003; Fitzgerald et al. 2006), other studies have found that the amygdala is most activated by fear expressions relative to other expressions (Whalen et al. 1998; Morris et al. 1996; Phillips et al. 2004).

The effects of serotonin vary as a function of genotype at the 5-HTTLPR. Carriers of the short (s) 5-HTTLPR allele show decreases in transcriptional activity of the serotonin transporter relative to the ll homozygotes, which leads to reduced serotonin uptake. S carriers have heightened risk of anxiety and depression (Wurtman 2005) and at baseline they show increased amygdala activity relative to ll homozygotes in response to fear expressions (Hariri et al. 2002, 2005). S carriers also appear to be more sensitive to manipulations of serotonin levels (Bell et al. 2005). S carriers are less sensitive to reinforcement contingency after acute tryptophan depletion than are ll homozygotes (Roiser et al. 2006), and female ss homozygotes show enhanced risk of depression during tryptophan depletion (Neumeister et al. 2002).

These data suggest that the effects of serotonin depletion on fear recognition will vary as a function of 5-HTTLPR genotype. S carriers, who are generally more responsive to tryptophan depletion, may show a greater decrement in fear recognition than ll homozygotes. In support of this notion, it was found that individual differences in threat sensitivity, which is associated with the s allele, interact with the effect of tryptophan depletion in predicting amygdala responses to fear facial expressions (Cools et al. 2005).

Study design

In this study, we used a tryptophan depletion paradigm in healthy volunteers who completed a standard facial expression recognition task. Subjects were genotyped as ll homozygotes or s carriers (ss homozygotes or sl heterozygotes). Previous study results have indicated that reducing serotonin impairs fear expression processing and that s carriers show decreases in functional responsivity of the serotonin system (Smith et al. 2004; Roiser et al. 2006) and heightened sensitivity to the effects of tryptophan depletion. We thus predicted that depletion would be associated with more impaired fear expression processing in s carriers relative to ll homozygotes.

Materials and methods

Subjects

Twenty-six healthy volunteers (12 males, 14 females, *M* age=27.77 years, SD=7.88) took part in this investigation. Subjects underwent screening at the National Institutes of Health using the DSM-IV criteria via a standardized psychiatric interview, a medical history and physical exam performed by a clinician, and blood and urine screening tests. No participants in whom current or past major affective disorder, anxiety disorder, psychotic disorder, substance dependence, anorexia nervosa or bulimia was indicated were included in the study. Participants with any first-degree family member with past or current depression were also excluded. Subjects were currently free of psychotropic medications. Urine toxicity screens also excluded subjects in which recent drug use was indicated. All subjects gave informed written consent and were paid for their participation.

In a double-blinded between-subjects design, subjects were randomly assigned to receive either amino acid caplets lacking tryptophan (ATD) or placebo caplets containing

lactose. Seven females were assigned to the ATD condition and seven to the placebo condition. Subjects' IQs, as measured by the Wechsler Abbreviated Scale of Intelligence (Wechsler 1999) were evenly matched across conditions.

Amino acid mixtures

The composition of the 70 capsules ingested by ATD subjects was: L-isoleucine (4.2 g), L-leucine (6.6 g), L-lysine (4.8 g), L-methionine (1.5 g), L-phenylalanine (6.6 g), L-threonine (3.0 g), and L-valine (4.8 g). All amino acids were provided in powder form. The placebo consisted of 70 capsules containing a total of 31.5 g of lactose. These mixtures were selected based on prior experience with this mixture within our institute (Neumeister et al. 2004, 2005). All capsules were taken with water (Wolfe et al. 1995).

Total tryptophan determination

Serum was collected in pre-chilled EDTA tubes, and immediately after collection was centrifuged for 15 min at 300 rpm and 4°C. They were subsequently stored at −70°C. Plasma tryptophan concentrations were determined by reverse-phase high performance liquid chromatography (HPLC) in conjunction with fluorescence endpoint detection. For total tryptophan, plasma proteins were removed by precipitation with 3% trichloroacetic acid followed by centrifugation. For the estimation of free tryptophan, protein bound TRP was separated from free by filtration through 10 K cutoff microfilters via a centrifugation process. LNAAs were analyzed via gradient HPLC with utilization of pre-column derivatization and fluorescence endpoint detection.

DNA acquisition

DNA for each subject was prepared from 16 ml of peripheral blood and was extracted by a collaborator blinded to the subjects' identities. Approximately 300 μg were obtained for each subject. All 5-HTTLPR analyses were accomplished via amplification of the region followed by size separation of the alleles using HPLC. Positive (known genotype) and negative control (no DNA) standards were run with each assay, a method demonstrated to have an error rate of <1%.

Procedure

All subjects were instructed to follow a low-protein diet (10–15 g protein) for the 24 h preceding testing. Then, they were instructed to fast from the midnight before testing until the completion of testing (approximately 7 h after amino acid administration). All subjects arrived for testing at 8:30 a.m. and serum was drawn to obtain baseline plasma tryptophan levels. Additional serum was drawn for 5-HTTLPR genetic analysis. 5 h after the subjects finished swallowing the capsules, serum was drawn again to assess changes in free tryptophan and total tryptophan:large neutral amino acids (LNAA) ratios. All subjects then completed the facial expression task, approximately 5 h after ingestion of the last capsule. This task was preceded by a passive avoidance learning task described in Finger et al. (2006). Mood states were assessed by visual analog scales and verbal report.

The morphed facial expression task

The facial expression recognition task featured six basic emotions (anger, disgust, fear, happiness, sadness, and surprise) taken from the Pictures of Facial Affect series (Ekman and Friesen 1976). These expressions are well-validated and are consistently recognized across a variety of cultures. The task used in this study was described previously (Blair et al. 2001; Blair and Curran 1999). In the task, subjects watched a computer screen on which initially neutral faces change over 20 stages into one of the six basic emotions. Subjects were asked to indicate the emotion they perceived to be expressed as soon as they identified it, and were also asked to provide a final judgment on the identity of each expression. In this way, both data on accuracy (final judgments) and latency (number of stages required before accurate judgment) were collected. After a practice phase consisting of one example of each of the six types of expressions, the subjects saw the test faces in randomized order (36 test stimuli in all).

Results

Biochemical results

A 2 (drug group: ATD vs placebo) by 2 (time point: pre-capsules vs 5 h post-capsules) ANOVA was conducted on serum free tryptophan levels. This revealed a main effect of drug group (ATD or placebo) ($F(1,24)=6.75$, $P<0.05$), and a significant drug group by time point interaction ($F(1,24)=24.09$, $P<0.001$). Examination of this interaction with follow up t tests revealed no significant differences in the baseline free tryptophan levels between the drug groups ($t(24)<1$, n.s.) but a significant reduction in free tryptophan levels in the ATD group relative to the placebo group after capsule ingestion ($t(24)=6.39$, $P<0.001$) (M[free ATD in μg/ml]=0.225, SEM=0.04; M[free placebo]=0.693], SEM=0.06). This represented a reduction of 80% in free tryptophan levels in the ATD group compared to a 38% reduction in the placebo group (both Ps<0.05). Similarly, there were no

significant differences between-groups at baseline in the LNAA ratio ($t(24)<1$, n.s.), while at 5 h post-capsule ingestion, there was a significantly greater reduction of the LNAA ratio in the ATD group compared to the placebo group ($t(24)=2.10$, $P<0.05$).

5-HTTLPR results

There were no significant differences in age or IQ scores between the ll vs s carrier groups. Two (genotype: ll vs s carriers) by two (drug group: ATD or placebo) by two (pre/post) ANOVAs with repeated measures on the third variable were conducted on tryptophan levels. The results demonstrated no significant effect of genotype on baseline or 5 h post-capsule free tryptophan levels or LNAA ratios and no significant interactions involving genotype.

Morphed facial expression task results

Identification accuracy

The subjects' accuracy was assessed by comparing the subjects' final responses for each expression using the unbiased hit rate analysis (Wagner 1993; Marsh and Ambady 2006). The procedure calculates the conventional percentage accuracy hit rate multiplied by one minus the rate of false alarms, then normalizes the score using an arcsine transformation. Then, the expected value due to chance guessing is calculated, analogous to calculating expected values for a chi-square analysis. Thus, all accuracy scores used in the analysis represented that which would be expected above the accuracy that would be expected due to guessing.

Given that tryptophan depletion may affect s carriers differently from ll homozygotes, performance differences among groups were assessed using the multivariate analysis of variance (MANOVA). The between-group factors were amino acid condition (ATD, placebo) and polymorphism (s carriers, ll homozygotes), and the dependent variables were accuracy levels for the six facial expressions. Significant interactions were followed up with pair-wise comparisons to assess the direction of the effects.

For all facial expressions, no significant main effect was found for amino acid condition or genotype (Ps>0.05). A

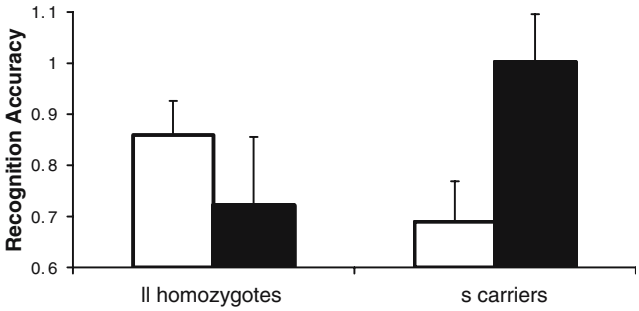

Fig. 1 Correct identification of fear facial expressions. *Dark bars*: After ingestion of placebo capsules; *light bars*: after ingestion of tryptophan-free amino acid capsules. Values represent the means± 1 SEM

significant interaction between amino acid condition and genotype was found for fear expression only [$F(1,22)=4.408$, $P=0.047$, partial $\eta^2=0.167$], suggesting that the effects of tryptophan depletion on the recognition of fear are modulated by genotype (Fig. 1). Applying a stepwise Bonferroni correction (Rice 1989), no significant interactions were found for any of the five remaining expressions (Ps>0.30, partial η^2s<0.05) (Table 1). Separate analysis of the two groups of individuals indicated that tryptophan depletion impaired fear recognition in s carriers [$t(13)=2.552$, $P=0.024$, $r^2=0.33$] but did not affect fear recognition in ll homozygotes [$t(9)=0.738$, $P=0.480$, $r^2=.06$]. A contrast analysis ($\lambda=3, -1, -1, -1$) indicated that s carriers in the placebo condition obtained fear recognition accuracy scores higher than subjects in the remaining three groups [$t(22)=2.10$, $P=0.048$].

In addition, correlations were calculated between free tryptophan levels and LNAA ratios after tryptophan depletion and accuracy of recognizing the fear expression. Free tryptophan levels were positively associated with the accuracy of identifying fear in s carriers ($r(13)=0.542$, $P=0.037$) but not ll homozygotes ($r(11)=-0.218$, n.s.). A marginally significant relationship was found between LNAA ratios and fear recognition in s carriers ($r(13)=0.452$, $P=0.091$) but not ll homozygotes ($r(11)=0.219$, n.s.).

Correct identification latency

Latency was assessed using the same technique as for accuracy scores, except that the data comprised the total

Table 1 Different scores represent the difference in accuracy scores for placebo relative to acute tryptophan depletion (ATD); negative scores indicate impairment in ATD relative to placebo

	Anger	Disgust	Fear	Happiness	Sadness	Surprise
S carriers (*N*s: *P*=7/TD=8)	−0.23(.09)	0.00(.08)	−0.31(.07)	0.00(.01)	−0.08(.07)	−0.16(.09)
LL homozygotes (*N*s: *P*=7/TD=4)	0.00(.08)	−0.03(.05)	0.14(.09)	−0.04(.04)	0.15(.10)	0.10(.08)

SEMs are indicated in parentheses.

number of stages each subject required to identify each kind of emotion, corrected again for false alarm rates using the unbiased hit rate analysis. For latency data, no significant main effects or interactions were found for correct recognition of any of the six facial expressions (all *P*s>0.10).

Gender

Previous studies have found gender to modulate the effects of tryptophan depletion on facial expression recognition (Harmer et al. 2003b). This study was not powered to enable the simultaneous examination of the effects of pharmacological challenge condition, genotype, and gender. However, MANOVAS testing the effects of gender and ATD on the recognition of emotional expressions did not find any significant main effects or interactions involving gender for emotion recognition accuracy or latency for any of the six expressions (all *P*s>0.10).[1]

Subjective mood state

No significant changes in mood were associated with tryptophan depletion, genotype, or gender. Inclusion of VAS score (indicating mood after tryptophan depletion) as a covariate did not affect the interaction between genotype and tryptophan depletion on facial expression recognition accuracy.

Analysis of TPH2 variants

We also examined whether tryptophan depletion may differently affect the GT and GG variants of TPH2. However, analysis of these variants did not reveal any significant main effects or interactions on either expression recognition generally or the recognition of any specific expression (*P*s>0.05).

Discussion

We report the effects of acute tryptophan depletion and 5-HTTLPR long and short variants on the recognition of facial expressions of emotion. These factors were found to significantly interact such that s carriers showed deficits in fear expression recognition after tryptophan depletion, whereas ll homozygotes did not. These results were specific to the recognition of fear expressions. No significant effects were found for the recognition of the other five emotional expressions. This study demonstrates the influential role of genotype on fear-relevant emotional processing during pharmacologic challenges.

The relevance of serotonergic mechanisms to fear and anxiety-relevant processes is well-known (Harmer et al. 2003a,b; Hellewell et al. 1999; Hensman et al. 1991; Silva et al. 2001). In humans, serotonin synthesis can be reliably manipulated via ATD, entailing the administration of amino acid mixtures selectively lacking in tryptophan. However, individuals differ in their susceptibility to the effects of serotonergic manipulation. For example, ATD produces mood symptoms in subjects with a history of depression, but not in individuals without such a history (Munafo et al. 2006; Neumeister et al. 2004). Individuals who carry short alleles of the 5-HTTLPR gene also show more sensitivity to ATD as measured by their behavior on neurocognitive tasks and mood measures (Caspi et al. 2003). In general, however, it should be noted that the effects of ATD on mood may only be found in the context of an environmental stressor or personal or family history of mood disorder (Bell et al. 2005; Caspi et al. 2003; Davies et al. 2006; Neumeister et al. 2002).

Consistent with these findings, we found that s carriers but not ll homozygotes show decrements in fear recognition in response to tryptophan depletion. Relative to the s allele, the l allele of the 5-HTTLPR is associated with increased transporter production in vitro (Heils et al. 1997). This may be related to findings that individuals who carry the s allele show enhanced susceptibility to serotonergic depletion (Bell et al. 2005; Roiser et al. 2006). However, the present data do not permit a complete understanding of the mechanisms underlying the interaction among 5-HT availability, genotype, and emotional expression recognition. A full understanding of these interactions may not be reached until a better understanding of the mechanisms by which these variables affect behavior at a molecular and anatomical level is reached. Such research may help to resolve contradictions in the current literature, such as findings that TD increases anxious mood (Davies et al. 2006; Miller et al. 2000) while decreasing in some individuals sensitivity to anxiety-relevant stimuli such as fear facial expressions. Moreover, as the mechanism by which 5-HTTLPR genotype influences phenotype may be developmental, an improved understanding of the developmental effects of genotype on behavioral correlates of 5-HT availability will also be important (Parsey et al. 2006).

Of particular relevance to the present study, Hariri et al. (2002, 2005) have demonstrated that fearful faces elicit increased amygdala activity in s carriers relative to ll homozygotes. This may explain why s carriers in the placebo condition of the present study show enhanced fear

[1] Data were collected from an additional 10 subjects for whom no genotype information was available. When the MANOVAs were conducted including these additional 10 subjects (*N*=36), we continued to find no evidence for a main effect of either ATD or gender on recognition of any of the six emotional expressions (all *P*s>0.10).

recognition abilities. Accurate recognition of the fear facial expression is frequently associated with the functioning of the amygdala (Adolphs 2002; Calder et al. 2001; Fine et al. 2001). The present findings suggest impairments in fear expression recognition in s carriers after ATD. This suggests the possibility that ATD may impair amygdala functioning in s carriers. Planned future imaging studies will test this hypothesis.

In this context it is interesting to note that the s allele of the 5-HTTLPR was suggested as a susceptibility factor for affective disorders because it enhances responsivity of the amygdala to fear-relevant environmental cues (Hariri et al. 2005). It is speculated that serotonergic modulation of the amygdala plays a role in normalizing certain anomalous cognitive processes (Harmer et al. 2006). However, enhanced fear responding is not necessarily detrimental. Improved processing of socioemotional cues such as fear expressions is likely to be advantageous under some conditions, and may be associated with beneficial personality traits such as empathy (Blair et al. 1997; Marsh and Ambady 2006). That the population frequency of the s allele is high—ranging from 40% to over 70% in some populations (Heils et al. 1997; Sakado et al. 2003)—further supports the notion that possession of the s allele may not be so risky as to have been selected out of the population. Rather, as for many genes, the extent to which this allele proves risky vs beneficial may depend on environmental demands.

One prior study examining the effects of ATD on facial expression recognition found no main effect of ATD, but found an interaction between gender and ATD on the recognition of fear expressions (Harmer et al. 2003b). Unlike that study, we found no interactions between gender and ATD or 5-HTTLPR. Our findings are consistent with the findings of other studies that have found no effect of gender on facial expression recognition after serotonergic manipulation (Roiser et al. 2006; Attenburrow et al. 2003; Harmer et al. 2003a,b). They are also consistent with the findings of two prior studies assessing 5-HTTLPR genotype and ATD, neither of which found gender effects (Roiser et al. 2006; Finger et al. 2006). Like these studies, we found behavioral effects resulting instead from the interaction of 5-HTTLPR genotype and ATD. It is also important to consider the size of the current study when considering our effects. Although our sample size is comparable to other studies that have considered the effects of ATD on healthy volunteers as a function of 5-HTTLPR genotype (e.g., Neumeister et al. 2002), future studies involving larger samples will be important to confirm the effects found in smaller studies. Larger samples will also permit assessment of study results according to the triallelic classification of 5-HTTLPR (Parsey et al. 2006), which the current study was not sufficiently powered to explore. However, that eight of our ll homozygous subjects were "high" expressers should be considered when interpreting our results.

It is also important to consider the between-subjects design used in this study. We selected this design to minimize practice effects and enhance comparability to prior studies of the effects of serotonergic manipulation on facial expression recognition (e.g., Harmer et al. 2003a,b). The random assignment to study conditions that we used is designed to prevent group differences at baseline. However, it should be noted that failure of randomization could result in spurious apparent effects of the study manipulation.

Considering the relative reduction in biochemical differences across groups is also important in interpreting our results. A lower dosage of amino acids was used in this study relative to Harmer et al. (2003b) and unlike that study, we did not provide tryptophan supplementation to the comparison group. The comparison group used by Harmer et al. (2003b) showed an increase in total tryptophan levels, whereas our control group showed a decrease. This resulted in a smaller difference in total tryptophan levels between ATD and placebo groups after pharmacological challenge in our study. This small difference may have resulted from our use of lactose capsules as the placebo and the ingestion of low-tryptophan meals by all subjects. This combination may have influenced the T/LNAA ratio in the control group. The relative reduction in group differences at the biochemical level is an important consideration in the interpretation of our results. It is possible that increasing the difference in total tryptophan levels between conditions through the use of a tryptophan-containing balanced amino acid placebo would result in a main effect of ATD on the recognition of fear expressions. However, on the basis of our current results, we would still predict a stronger effect of ATD on s carriers than ll homozygotes.

Conclusions

This study sought to investigate the relationship between 5-HTTLPR polymorphism and the processing of emotional facial cues after ATD. After prior findings, it was predicted that ATD would reduce sensitivity to fear expressions and that this effect would be greater in s carriers than in ll homozygotes. In accordance with this prediction, ATD reduced fear sensitivity in s carriers but not in ll homozygotes. In addition, free tryptophan levels were associated with enhanced fear expression recognition. The finding that individuals vary in their susceptibility to emotional processing changes after tryptophan depletion reinforces the importance of considering genotype when assessing the behavioral effects of pharmacologic modulation.

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