Association between Serotonin-related Genetic Polymorphisms and CCK-4-induced Panic Attacks with or without 5-hydroxytryptophan Pretreatment in Healthy Volunteers

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Summary
Genetic regulation of the function of serotonin (5-HT) may be important for the neurobiology of panic disorder. In order to evaluate the influence of 5-HT-related gene variants on the vulnerability to panic attacks, we genotyped 32 healthy volunteers who participated in the study of the effect of 5-hydroxytryptophan on panic attacks induced with cholecystokinin tetrapeptide (CCK-4). The polymorphisms of interest included those of 5-HT transporter (S-HTTLPR) and monoamine oxidase A (MAO-A promoter region) genes. The results showed significant associations between certain genotypes and panic rate in females but not in male volunteers. Specifically, there was a significantly lower rate of CCK-4-induced panic attacks in female subjects who had MAO-A lower alleles or S-HTTLPR short allele gene variants. These data suggest that functional genetic polymorphisms of the 5-HT system may influence the vulnerability to panic attacks and add to the growing evidence of inhibitory function of 5-HT in the neuronal circuitry of panic.

Key words: serotonin, genetic polymorphism, cholecystokinin, panic attacks.

Introduction
There is strong evidence that the function of brain serotonin (5-hydroxytryptamin, 5-HT) has a crucial role in the neurobiology of panic attacks and panic disorder (PD). Treatment studies have shown that medications preferentially increasing the availability of brain 5-HT, such as selective 5-HT re-uptake inhibitors (SSRIs) and monoamine oxidase A enzyme (MAO-A) inhibitors, are highly effective in PD (Kent et al. 1996; Tyrer and Shawcross 1988). Support for the 5-HTergic dysregulation in PD also comes from a number of studies demonstrating anxiogenic and heightened neuroendocrine responses to direct 5-HT agonists, such as m-chlorophenylpiperazine (m-CPP) and D-fenfluramine (Kahn et al. 1988; Targum and Marshall 1988) as well as reduced binding properties of brain 5-HT transporter (S-HTT) and 5-HT1A receptors in patients with PD (Maron et al. 2003; Neumeister et al. 2004). Experimental studies so far have shown that tryptophan depletion increased panic response to carbon dioxide (CO2) challenge in healthy subjects and in patients with PD (Klaassen et al. 1998; Schuerer et al. 2000). On the other hand, acute increase of brain 5-HT availability by administration of 5-HT precursor 5-hydroxytryptophan (5-HTP) restrained panicogenic effects of CO2 in patients with PD but not in healthy volunteers (Schuerer et al. 2002).

Cholecystokinin-tetrapeptide (CCK-4) is an agonist of the central subtype of CCK receptors with well-established panicogenic properties in both PD patients and healthy subjects (Bradwejn et al. 1991; Shlik et al. 1997). In an earlier study tryptophan depletion did not affect the panicogenic response of CCK-4 in healthy males, although it did augment CCK-4-mediated neuroendocrine activation (Kozyczki et al. 1996). In a recent study we investigated the effects of acute pre-treatment with 5-HTP on CCK-4 panic responses in healthy subjects. We found that 5-HTP administration reduced the rate of CCK-4-induced panic attacks and intern-
ity of cognitive panic symptoms in females and reduced somatic panic symptoms in males (Maron et al. 2004).

The availability of 5-HT in the brain depends on various factors including genetic regulation. The genes of particular interest are those with a principal role in the control of 5-HT neurotransmission, such as 5-HTT and MAO-A. Several studies have indicated that allelic variation in these genes may contribute to the expression of anxiety symptoms or disorders. For instance, short (S) allele in the 5-HTT linked promoter region (5-HTTLPR) was related to a decreased 5-HTT expression and 5-HTT uptake in lymphoblasts as well as in the brain (Heinz et al. 2000; Lesch et al. 1996). It has been demonstrated that the S variant of 5-HTTLPR is associated with anxiety-related traits in healthy subjects (Lesch et al. 1996), but not with the diagnosis of PD (Deckert et al. 1997; Hamilton et al. 1999). However, a higher frequency of the long allele (L) genotype was observed in females with PD (Hamilton et al. 1999). A recently identified polymorphism upstream of the MAO-A gene has been shown to affect the activity and expression levels of MAO-A enzyme. MAO-A promoter region variants containing longer alleles displayed higher enzymatic activity compared to shorter alleles (Deckert et al. 1999; Sabol et al. 1998). Clinically, a significant excess of functionally more active MAO-A promoter alleles was observed in female, but not male patients with PD (Deckert et al. 1999). However, another study did not find associations between PD and MAO-A promoter polymorphism (Hamilton et al. 2000) and there was no evidence of a major interaction between MAO-A and 5-HTT promoter polymorphisms in patients with PD (Sand et al. 2000). Several studies have attempted to find associations between 5-HT-related gene variants and clinical features or treatment response. For instance, Moreno et al. (2002) have shown that remitted depressive patients with the S allele of 5-HTTLPR had significantly less depressive symptoms in response to tryptophan depletion compared to LL homozygotes, whereas the short variant of 5-HTTLPR had a negative influence on the therapeutic effect of SSRIs in depressive patients and in females with PD (see Catalano 2001 for review).

Based on these data we hypothesized that susceptibility to panic attacks in healthy subjects in our study of the effects of 5-HTP on panic challenge with CCK-4 was influenced by functional polymorphisms in 5-HT-related genes. Specifically, we predicted that genotypes leading to an increased 5-HT availability would be associated with lower panic rate after CCK-4 challenge. To test this hypothesis we genotyped the above-mentioned polymorphisms in 5-HTT and MAO-A genes and examined their associations with CCK-4-induced panic rate. In addition, we examined the effect of these polymorphisms with respect to sex and 5-HTP pre-treatment.

Experimental procedures

• Subjects and study design
The study procedures and characteristics of the subjects have been described in detail elsewhere (Maron et al. 2004). In short, 32 healthy volunteers participated in the investigation of the effect of acute pre-treatment with 5-HTP on the CCK-4-induced panic attacks. All subjects gave written informed consent after explanations about procedures, substances and genetic analyses. The Human Studies Ethics Committee of the University of Tartu approved the study protocol and informed consent form. The inclusion criteria were age between 18 and 40 years, no personal or family psychiatric history, negative urine drug screen and good physical health. Pregnancy was excluded in all females on a urine pregnancy test and females were studied within the first week of their menstrual cycle phase. Equal number of males and females were randomized to receive either 200 mg of 5-HTP or identical placebo capsules in a double-blind parallel-group design. CCK-4 was injected 90 minutes after administration of 5-HTP or placebo in a dose of 50 mg in 2.5 ml of normal saline solution as a bolus push. Within 5 minutes after CCK-4 injection the participants were asked to rate peak intensity of their symptoms on 18-items Panic Symptom Scale (Bradwejn et al. 1991) on a scale from 0 (not present) to 4 (extremely severe). The presence of at least four symptoms with a score of 2 or more plus “anxiety/apprehension/fear” item with a score of 3 or more was a priori criterion for the occurrence of panic attack.

• DNA analysis
DNA was extracted from 5 ml of venous blood using a standard phenol-chloroform extraction. For genotyping of deletion/insertion polymorphism in the promoter of 5-HTT gene, the region was amplified using primers HTT-LPR-A (5'-GGC GTT GCC GCT CTC AAT GC) and HTT-LPR-B (5'-GAG GGA CTG AGC TGG ACA ACC AC); PCR products were resolved by electrophoresis in a 2% agarose gel. MAO-A promoter region polymorphisms were identified using PCR with primers MAOaP1/2 (GAA CGG ACC CTC CAT TGC GA) and MAOaP1/3 (ACA GCC TGA CCG TGG AGA AG), followed by electrophoresis in polyacrylamide gel and staining with ethidium bromide.

• Data analysis
The data were analysed using the software package STATISTICA 5.1 (StatSoft Inc, Tulsa, OK, USA). The proportions were compared by Pearson Chi-square tests. The results were considered significant at the level of p<0.05.
Results

• Distribution and grouping of genotypes
  The distribution of genotypes in the total sample for 5-HTTLPR was as follows: LL 31.9% (n=10), LS 56.6% (n=18) and SS 11.5% (n=4); and for MAOA-A: 1-1 34.9% (n=11), 3-1 22% (n=7) 3- (3) 38% (n=12) and 4-3 6% (n=2). There was no significant difference in the distribution of the genotypes frequencies between males and females. Distribution of the genotypes in females was similar in the 5-HTP and placebo groups. In male subjects distributions of 5- HTTLPR but not of MAOA-A genotypes significantly differed between 5-HTP and placebo groups (χ²=7.37, df=2, p=0.02).

For the further analysis genotypes were organised into two groups similar to previous studies (Lesch et al. 1996; Deckert et al. 1999). The 5-HTTLPR alleles were combined in one group with all 5 allele carriers (SI and 5L genotypes) and another group with LL-homozigous only. The MAOA-A alleles were divided into a group with all carriers of shorter alleles (1-1) and 1-3 genotypes) and a group of carriers of longer alleles (all subjects with 3-3 and 3-4 genotypes).

• Effect of 5-HTP on CCK-4-induced panic rate
  Three subjects (18.8%; 1 female, 2 males) from the 5-HTP group and seven (43.8%; 6 females, 1 male) from the placebo group experienced a panic attack after CCK-4 challenge. The between-group difference in panic rate was not statistically significant (χ²=2.3, df=1, p=0.13); however, in females panic rate was significantly lower after 5-HTP than after placebo pre-treatment (11.1% vs. 66.7%; χ²=5.8, df=1, p=0.016).

Table 1

<table>
<thead>
<tr>
<th>Total</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Panic</td>
<td>No panic</td>
</tr>
<tr>
<td>5-HTP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LL</td>
<td>5 (2)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>SS</td>
<td>17 (7)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Subtotal</td>
<td>χ²=2.14, df=1, p=0.14</td>
<td>χ²=4.01, df=1, p=0.042</td>
</tr>
<tr>
<td>MAOA-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-1</td>
<td>1 (0)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>3-3</td>
<td>11 (9)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Subtotal</td>
<td>χ²=0.73, df=1, p=0.39</td>
<td>χ²=0.52, df=1, p=0.47</td>
</tr>
</tbody>
</table>

• Associations between 5-HT-related genotypes and CCK-4-induced panic
  The associations between genotype groups and rate of panic attacks are presented in Table 1. In the total sample the presence of MAOA-A polymorphisms with longer alleles was associated with a lower panic rate. There were no associations between panic rate and genotypes of 5-HTTLPR. The following analysis by sex showed lack of significant associations between MAOA-A or 5-HTTLPR polymorphisms and panic rate in males. In females panic rate was significantly lower in the group with longer alleles of MAOA-A polymorphism as well as in the group with S alleles of 5-HTTLPR. Additionally, panic rate was significantly lower in females with longer alleles of MAOA-A polymorphism who received placebo, but did not differ in the 5-HTP pre-treatment group (χ²=5.14, df=1, p=0.02 and χ²=1.41, df=1, p=0.24, respectively). On the contrary, panic rate in females was significantly lower in the group with 5-HTTLPR S alleles after 5-HTP but not placebo pre-treatment (χ²=3.94, df=1, p=0.047 and χ²=2.25, df=1, p=0.13 respectively). In the total sample and individually in males there were no associations between candidate polymorphisms and rate of panic response separately in 5-HTP and placebo groups (data not shown).

Discussion

The main finding of this study is a lower frequency of CCK-4-induced panic attacks in healthy subjects with high activity MAOA-A gene allele variants. In addition, CCK-4-induced panic rate was significantly lower in females with high activity MAOA-A alleles or with short allele of the 5-HTTLPR compared to females with low activity MAOA-A alleles or with LL-genotype of the 5-HTTLPR. Thus, CCK-4 induced panic rate was affected by genetic polymorphisms with identified functional activity and therefore apparently related to the regulation of 5-HT neurotransmission.

Interpretation of these findings is confounded by the use of 5-HTP pre-treatment in half of the study subjects, which in turn reduced panic rate in female subjects. Conceivably, the effects of 5- HTP and functional 5-HT-related gene polymorphisms may have additive antipanic properties. Indeed there was a synergistic overlap in the observed association of 5-HT gene polymorphism leading to lower activity of 5-HT reuptake with the reduction of panic attacks and the antipanic effect of 5-HTP in females. Moreover, a strong association with S allele in females was present under 5-HTP but not placebo pre-treatment suggesting the antipanic direction of the interaction between the effects of 5-HTP and low-activity 5-HTTLPR variant. On the other hand, the association between high activity MAOA-A polymorphism and reduced panic attacks in females contradicts the hypothesis of an inhibitory effect of increased 5-HT availability on panicogenesis. It is of interest that pre-treatment with 5-HTP decreased the strength of association between highly active MAOA-A polymorphisms and reduced occurrence of panic attacks in females, although the
association remained significant in the placebo group. Thus, the effect of 5-HTP may have compensated the influence of high activity MAO-A polymorphisms on the panic rate. A possible explanation of these contradictory results is related to the different roles of 5-HT in the modulation of panic responses on the level of 5-HT-ergic neuronal cell bodies in the midbrain raphe region and on the level of the 5-HT-ergic neuronal terminals in other brain regions. Perhaps an increased availability of 5-HT in the midbrain raphe and a decreased 5-HT tone in the 5-HT-ergic projections to other brain regions may both have antipanic effects. Moreover, in addition to the 5-HT-related mechanisms, the increased activity of MAO-A enzyme could have antipanic effects due to increased inactivation of noradrenaline resulting in a lower noradrenergic tone in the panic-related brain structures.

So far little is known about the role of genetic regulation of 5-HT function in PD. Previous studies have failed to find associations between PD and different 5-HTT-related gene polymorphisms, such as 5-HTTRIB 861 G>C (Fehr et al. 2000a), 5-HTTRIB Cys233Ser (Fehr et al. 2000b), Fnu4I1 polymorphism of the MAO-A (Tadic et al. 2003) or 5-HTTLPR (Decket et al. 1997; Hamilton et al. 1999). However, Hamilton et al. (1999) have reported more frequent occurrence of 5-HTT LL genotype in female PD probands compared to female controls. This finding is in line with our results showing that females with LL genotype are more sensitive to CCK-4 panic compared to S allele genotype. Furthermore, our recent association study in PD patients revealed significantly higher frequencies of 5-HTTLPR long allele as well as LL genotype in patients than in healthy subjects (Maron et al. unpublished data). These preliminary results suggest that 5-HTTLPR long allele variant associated with higher functional activity of 5-HTT may have a role in the predisposition to PD. Another previous finding is a significant association between PD and high activity MAO-A gene polymorphism in female patients (Decket et al. 1999). This relationship differs from our finding of a protective effect of this polymorphism in CCK-4 challenge that may indicate a distinct genetic background of panic attacks in patients with PD and experimental panic attacks in healthy subjects. Another explanation may be related to the complexity of the PD phenotype that in addition to panic attacks include anticipatory and phobic anxiety, whereas MAO-A gene may have a different influence on various components of PD (Decket et al. 1999).

In healthy volunteers the short allele of 5-HTTLPR has been associated with anxiety-related personality traits (Lesch et al. 1996) and with greater activation of the amygdala in response to fearful face stimuli (Hariri et al. 2002). Thus, lower activity of 5-HTT may be related to anxiety proneness that is opposite to the antipanic direction of this genotype in our results. This discrepancy may be explained by the dual role of the 5-HT system in neuronal circuits of anxiety and panic attacks according to the theory of Deakin and Graeff (1991). The pharmacological interventions that affect the 5-HT system also demonstrate different effects on anxiety, fear and panic. The administration of the nonselective 5-HT1 and 5-HT2 receptor agonist m-CPP led to increased anxiety and hostility in patients with anxiety disorders, although its true panicogenic effect was not clearly established (Germaine et al. 1992; Charnley et al. 1987). Additionally, the 5-HT uptake blocker and 5-HT releasing agent D-fenfluramine tended to increase generalised or anticipatory anxiety rather than induce the panic attacks (Graeff et al. 1996). Both the partial 5-HT1A agonist buspirone and the 5-HT2A receptor antagonist ritanserin may have beneficial effects in the treatment of generalised anxiety, but their efficacy in PD was not supported (Bell and Nutt 1998; Den Boer and Westenberg 1990; Bressa et al. 1967). Furthermore, the results from animal studies support the evidence that distinct 5-HT-ergic pathways regulate neural mechanisms underlying anxiety and panic (Sena et al. 2003). Altogether these data are in agreement with the hypothesis of Deakin and Graeff (1991) proposing that 5-HT system oppositely modulates different forms of anxiety with facilitation of generalised or anticipatory anxiety and inhibiting of panic responses.

Considering the sex differences, our study did not reveal antipanic properties of 5-HTT in CCK-4 challenge in healthy males. Furthermore, neither MAO-A nor 5-HTTLPR gene variants affected the frequency of panic attacks in male subjects. A likely reason of this failure to demonstrate antipanic effects of 5-HTP and/or 5-HT-ergic genes is the small number of panic attacks induced by CCK-4 in males in this study. However, sex-related differences in the genetic background of 5-HT as well as in the susceptibility to panic attacks should be taken into account (Decket et al. 1999; Hamilton et al. 1999).

The major limitations of the current study in respect to the genetic findings are concurrent use of 5-HTP and the small sample size, which may have led to false positive results. The study was primarily designed to test putative antipanic properties of 5-HTP, however the detection of genotype influences in the secondary analysis underlines the importance of incorporating factors of genetic variability in this type of study. Further investigations in larger samples are needed for the validation of current findings and more reliable conclusions. The role of 5-HT-related genetic variants requires more extensive
investigation in healthy subjects and in patients with PD and other anxiety disorders. Use of panic challenge with CCK-4 or another appropriate laboratory agent could help to uncover vulnerability to panic and relate it to a genetic disposition to panic attacks and PD.

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References


BRIEF REPORT


