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Association testing of panic disorder candidate genes using CCK-4 challenge in healthy volunteers

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ABSTRACT

Despite continuing efforts to determine genetic vulnerability to panic disorder (PD), the studies of candidate genes in this disorder have produced inconsistent or negative, results. Laboratory panic induction may have a potential in testing genetic substrate of PD. In this study we aimed to explore the effects of several genetic polymorphisms previously implicated in PD on the susceptibility to cholecystokinin-tetrapeptide (CCK-4) challenge in healthy subjects. The study sample consisted of 110 healthy volunteers (47 males and 63 females, mean age 22.2 ± 5.2) who participated in CCK-4 challenge test. Nine gene-candidates, including 5-HTTLPR, MAO-A VNTR, TPH2 rs1386494, 5-HTR1A −1019C–G, 5-HTR2A 102T–C, CCKR1 246G–A, CCKR2 −215C–A, DRD1 −94G–A and COMT Val158Met, were selected for genotyping based on previous positive findings from genetic association studies in PD. After CCK-4 challenge, 39 (35.5%) subjects experienced a panic attack, while 71 subjects were defined as non-panickers. We detected significant differences for both genotypic and allelic frequencies of 1386494A/G polymorphism in TPH2 gene between panic and non-panic groups with the frequencies of G/G genotype and G allele significantly higher in panickers. None of the other candidate loci were significantly associated with CCK-4-induced panic attacks in healthy subjects. In line with our previous association study in patients with PD, we detected a possible association between TPH2 rs1386494 polymorphism and susceptibility to panic attacks. Other polymorphisms previously associated with PD were unrelated to CCK-4-induced panic attacks, probably due to the differences between complex nature of PD and laboratory panic model.

Panic disorder (PD) is a common and potentially disabling condition characterized by unexpected panic attacks (PA), fear of their recurrence or harmful consequences and frequently developing agoraphobia [1]. The National Comorbidity Survey replication has shown lifetime prevalence of PD with or without agoraphobia at 4.8% and isolated PA at 22.7% with all panic syndromes associated with serious impairment [11]. The data from twin and family studies suggest an involvement of genetic factors in the development of PD with a heritability estimate near 40% [8]; however, the genetic substrates of the panicogenesis are not known. The association studies with candidate genes have so far produced mostly negative or inconsistent results with only few gene variants in key neurotransmitter systems showing suggestive link to PD phenotypes [14]. The favored among them are serotonin (5-HT)-related gene variants, particularly the 5-HT transporter-linked polymorphism (5-HTTLPR) [15], MAO-A gene uVNTR polymorphism [4,15], 5-HT receptor (5-HTR) 1A [16,23] and 5-HTR2A [9,16] as well as tryptophan hydroxylase gene isomer 2 (TPH2) [19]. Encouraging but not always consistent findings were also observed with cholecystokinin-related genes, particularly a CT repeat polymorphism of the CCK2 receptor (CCK2R) gene [7,10]. Our SNP-array study [16] showed no significant associations between various other CCK-related polymorphisms and pure PD phenotype, although CCK2R polymorphism −215C–A showed an association in the PD group with comorbid mood disorders. We also detected an association of CCK1R 246G-A polymorphism with the phenotype of comorbid, but not pure PD. In addition, a dopamine type 1 receptor (DRD1) polymorphism and related haplotype was significantly associated with the pure PD phenotype [16]. Finally, a functional

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variant Val158Met (472G/A-Val/Met) in the coding sequence of the catechol-*O*-methyltransferase (COMT) gene, has attracted a number of studies in PD, demonstrating its possible involvement in PD [5,24].

Although the genetic research on PD has grown tremendously in the last decade, the yield of these studies has been limited for a number of reasons, including a clinical heterogeneity of PD, small sample sizes and subsequent lack of power as well as possible ethnic differences. Furthermore, the findings have demonstrated that genetic influence may be differently related to specific PD phenotypes, such as with or without agoraphobia, pure or comorbid, and can be gender-dependent. The laboratory panic challenge models used in the investigations of panic phenomenology may help in testing the premises of genetic predisposition to panic. In this study we aimed to explore the effects of several genetic polymorphisms previously implicated in PD or PA on the susceptibility to cholecystokinin-tetrapeptide (CCK-4) challenge in healthy subjects. CCK-4 test is a well-established laboratory model of PAs [2,6] that are observed in up to 100% of PD patients and in up to 50% of healthy subjects, depending on the dose. We hypothesized that validation of previous findings in a panic challenge could clarify the impact of these candidate genes on the panicogenesis.

The study sample consisted of 110 healthy volunteers (47 males and 63 females, mean age 22.2 ± 5.2) recruited by flyer advertisement. The inclusion criteria were: age between 18 and 50 years, no personal or family psychiatric history, and good physical health as determined by medical history and physical examination. The subjects were questioned using the Mini International Neuropsychiatric Interview (M.I.N.I. 5.0.0). None of the volunteers had a positive urine test for benzodiazepines, cocaine, amphetamines, hallucinogens, opioids or cannabis at the time of screening visit. All female participants had a negative urine pregnancy test. All participants were required to abstain from alcohol or any medications for at least 2 weeks before the study. None of the subjects participated in our previous CCK-4 challenge studies The Human Studies Ethics Committee of the University of Tartu approved the study protocol and the informed consent form.

The CCK-4 challenge in a dose of 50 μg in 2.5 ml of normal saline solution was conducted as a bolus push after 30 min rest. Behavioural response to CCK-4 injection was rated on a Visual Analogue Scale (VAS; 100 mm line from most relaxed to most anxious) and on the DSM criteria-based panic symptom scale (PSS) [2], which assesses presence and intensity of 18 panic symptoms on a scale from 0 (not present) to 4 (extremely severe). Baseline anxiety was rated 5 min before the CCK-4 injection. Within 5 min after CCK-4 injection the participants marked on VAS the level of anxiety they experienced on the peak of the CCK-4-induced symptoms. The variables derived from the PSS were the sum intensity score, defined as the sum of all individual item ratings, and occurrence of PA, defined as presence of four or more symptoms with sudden onset and at least moderate severity plus "fear/apprehension" item with a score 3 or 4 [20]. Another variable used for categorization of panic response was the change in VAS-rated anxiety from pre- to post-challenge; net increase equal or more than 50 mm was defined as strong and less then 50 mm as mild anxiety response. The subjects who met the definition of PA and demonstrated strong anxiety net increase were defined as panickers; the subjects who did not meet these criteria were defined as non-panickers.

Nine gene-candidates were selected for this study based on the previous positive findings from genetic association studies in PD (Table 1). DNA was extracted from 5 ml of venous blood using a standard phenol–chloroform extraction. Genotyping of genetic polymorphisms in 5-HTTLPR, MAO-A VNTR, and TPH2 rs1386494 loci was performed as described previously [12,19,25]. Following oligonucleotides were used to get amplicons that contain polymorphisms in 5-HTR1A 1019CG (rs6295), 5-HTR2A 102TC (rs6313), CCKR1 246GA, CCKR2 215CA (rs1799721), DRD1 94GA (rs5326) and COMT Val158Met (rs4680) loci: rs6295F: GGA AGA AGA CCG AGT GTG TCA T; rs6295R: GGC TGG ACT GTT AGA TGA TAA CG; rs6313F: AGC AGA AAC TAT AAC CTG TT; rs6313R: CAA GTG ACA TCA GGA AAT AG; CCK1RF: GGA GCA GGG AGG GAG TGA TTT; CCK1RF: GTA GCC AGG CTG TCT GTA CT; rs1799721F: GGG GGT GGG GGC GGG TGA TA; rs1799721R: CCG CCT GGG TTC CCG TCC CT; rs5326F: GCT CTG ACA CCC CTC AAG TT; rs5326R: GCA GCA AGG GAG TCA GAA GA; rs4680F: CGG ATG GTG GAT TTC GCT CGC; rs4680R: CGC TCC AAC CAC AAG GGT GAC. Amplification was followed by digestion with restriction endonucleases and agarose gel electrophoresis. Following restriction endonucleases were used from Fermentas: Bse GI, MspI, MboII, SmuI, FSpB1 and Bsh 1236 I for loci rs6295, rs6313, CCK1R 246 GA, rs1799721, rs5326 and rs4680, respectively. For all genotypes tested, 25 samples were genotyped twice and no discrepancies were found.

Table 1
Associations between selected candidate genetic polymorphisms and panic disorder

Polymorphism	Main findings
5-HTTLPR	Association with LL genotype and L allele in Estonian sample (158 cases/215 controls; $p = 0.01$–0.02) [15]
MAO-A VNTR	Association with longer alleles in different female samples ($p = 0.001$–0.02), but not males [4,15]
TPH2 rs1386494	Association only in females with pure phenotype (58 cases/212 controls; $p = 0.01$–0.02) [19]
5-HTR1A −1019C–G	Association with some phenotypes ($p = 0.03$–0.05) [16,23]
5-HTR2A 102T–C	Association only with pure phenotype in Japanese ($n = 63$; $p = 0.016$) and Estonian ($n = 42$; $p = 0.01$) samples [9,16]
CCKR1 246G–A	Association with comorbid, but not pure phenotype [16]
CCKR2 −215C–A	Suggestive association in PD group with comorbid mood disorders in Estonian sample ($n = 127$; $p = 0.05$) [16]
DRD1 −94G–A	Association only with pure phenotype ($n = 42$; $p = 0.02$), supported by haplotype analysis ($p = 0.03$) [16]
COMT Val158Met	Association in different samples (most $p < 0.01$) [5,14,24]

The genotype and allele frequencies between the panic and non-panic groups were compared by chi-square tests using the software package STATISTICA 5.1. Odds ratio (OR) values and 95% confidence intervals (CI) were calculated using STATA 6.0. The results were considered nominally significant at the level of $p < 0.05$ without correction for multiple comparison.

After CCK-4 challenge, 39 subjects experienced a panic attack, while 71 subjects were defined as non-panickers. The descriptive data on panic and anxiety variables by groups are presented in Table 2. After genotyping the two CCK receptor gene polymorphisms, CCK2R −215C–A and CCK1R 246G–A, were excluded from association analyzes due to absence of subjects with A alleles in both SNPs in our sample. The distributions of genotypes

Table 2
Characteristics of panickers and non-panickers

	Panickers	Non-panickers	Statistic
Sex, male/female	16/23	31/40	$p = 0.79$
Age	21.4 ± 4.1	22.6 ± 5.6	$p = 0.23$
VAS baseline	9.0 ± 8.7	9.4 ± 9.7	$p = 0.84$
VAS peak	82.0 ± 10.4	54.9 ± 20.1	$p < 0.001$
PSS total	27.0 ± 8.8	15.9 ± 6.0	$p < 0.001$
PSS somatic	21.3 ± 6.8	13.0 ± 5.5	$p < 0.001$
PSS cognitive	5.7 ± 3.1	2.9 ± 1.7	$p < 0.001$
Number of panic symptoms	8.2 ± 2.5	4.6 ± 2.2	$p < 0.001$

PSS, panic symptom scale; VAS, visual analogue scale.