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Serotonergic modulation
of CCK-4- induced panic

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ABBREVIATIONS

A	Adenine
ACTH	Adrenocorticotropic hormone
A.M.	Before noon
APA	the American Psychiatric Association
API	*Acute Panic Inventory*
Asp	Aspartate
BDI	*Beck Depression Inventory*
BP	Blood pressure
BP_{ND}	Non-displaceable binding potential
c	Cytosine
C	Carbon
CCK	Cholecystokinin
CCK-4	Cholecystokinin tetrapeptide
CCK-5	Cholecystokinin pentapeptide, pentagastrin
CCK1R, CCKAR	Cholecystokinin 1 (alimentary subtype) receptor
CCK-2R, CCKBR	Cholecystokinin 2 (brain subtype) receptor
CGI	*Clinical Global Impression* (scale)
CGI-I	*Clinical Global Impression of Improvement* (scale)
CI	Confidence intervals
CIT	Citalopram
COMT	Catechol-*O*-methyltransferase
CO_2	Carbon dioxide
CV	Coefficients of variation
DA	Dopamine
df	Degrees of freedom
DNA	Deoxyribonucleic acid
D1R	Dopamine 1 receptor
DSM-IV	*Diagnostic and Statistical Manual of Mental Disorders*, 4-th Edition
ESC	Escitalopram
EST-Q	*Emotional State Questionnaire*
FDR	False discovery rate
FWHM	Full width at half maximum
G	Guanine
GABA	Gamma-aminobutyric acid
h	Hour
HAS	*Hamilton Anxiety Rating Scale*
HIV	Human immundeficency virus
HPLC	High-performance liquid chromatography
HR	Heart rate

HV	Healthy volunteer
ICD-10	*International Classification of Diseases*, 10-th Revision
L	Long (allele of 5-HTT gene)
LNAA	Large neutral aminoacids
MADAM	N,N-dimethyl-2-(2-amino-4-methylphenyl thio)-benzylamine
MADRS	*Montgomery-Åsberg Depression Rating Scale*
MAO-A	Monoamine oxidase A
MBq	Mega becquerel
m-CPP	Meta-chloro-phenylpiperazine
MDMA	3,4-Methylenedioxymethamphetamine
Met	Methionine
mg	Milligram
min	Minute
M.I.N.I. 5.0.0	*Mini International Neuropsychiatric Interview*, Version 5.0.0
ml	Millilitre
MR	Magnetic resonance
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
n	Number
N	Nitrogen
NA	Noradrenalin
ng	Nanogram
NH2	Amido group, amidogen
OR	Odds ratio
p	*p*-value
PA	Panic attack
PD	Panic disorder
PDSS	*Panic Disorder Severity Scale*
PET	Positron emission tomography
Phe	Phenylalanine
P.M.	After noon
PSS	*Panic Symptom Scale*
R	Pearson product-moment correlation coefficient
rmANOVA	Repeated measures analysis of variance
s	Second
S	Short (allele of 5-HTT gene)
SD	Standard deviation
SNP	Single nucleotide polymorphism
SPECT	Single photon emission computed tomography
SPM2	Statistical parametric mapping version 2
SPM8	Statistical parametric mapping version 8
SSRI	Selective serotonin re-uptake inhibitor
t	Thymine
T	Tesla

TD	Tryptophan depletion
TRP	Tryptophan
TPH2	Tryptophan hydroxylase gene isomer 2
UHPLC	Ultra high performance liquid chromatography-mass spectrometry
UV	Ultra violet
VNTR	Variable number tandem repeat
Val	Valine
VAS	*Visual Analogue Scale*
VAS-A	*Visual Analogue Scale* of Anxiety
VAS-D	*Visual Analogue Scale* of Distress
VAS-S	*Visual Analogue Scale* of Similarity (to natural panic attacks)
VOI	Volumes of interest
μg	Microgram
χ^2	Chi-square
5-HT	Serotonin (5-hydroxytryptamine)
5-HTP	5-hydroxytryptophan, oxitriptan
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin transporter-linked polymorphism
5-HT1AR	Serotonin 1A receptor
5-HT3R	Serotonin 3 receptor
[^{11}C]MADAM	[^{11}C]N,N-dimethyl-2-(2-amino-4-methylphenyl thio)-benzylamine

INTRODUCTION

A panic attack (PA) is a period of intense fear or discomfort, developing abruptly and peaking within 10 minutes, and requiring at least four of the following thirteen symptoms: palpitations or accelerated heart rate; sweating, trembling or shaking; sensations of shortness of breath or smothering; feeling of choking, chest pain or discomfort; nausea or abdominal distress; feeling dizzy, unsteady, lightheaded, or faint; derealisation or depersonalisation; fear of losing control or going crazy; fear of dying; paresthesias; chills or hot flashes ((DSM-IV; APA, 1994). Panic attacks are common, occurring in 22.7% of population (Kessler *et al.*, 2006). Recurrent PA are the hallmark of panic disorder (PD). Panic disorder is a major anxiety disorder characterized by recurrent unexpected PA and persisting fear of their recurrence or harmful consequences, and is often complicated by agoraphobia (DSM-IV, ICD-10) (WHO, 1992; APA, 1994). Epidemiological studies have found a high prevalence (one year prevalence of 2.8%, lifetime prevalence of 4.7%) and early onset of PD, with female preponderance, and serious impairment associated with all panic syndromes (Kessler *et al.*, 2006).

Cholecystokinin (CCK) is a peptide neurotransmitter that has been implicated in the aetiology of PA and PD (Bradwejn & Koszycki, 1994a; Bradwejn & Koszycki, 2001). Panic induction with cholecystokinin-tetrapeptide (CCK-4) has been established as a valid experimental model of human PA (Bradwein *et al.*, 1991; Eser *et al.*, 2007; Kellner, 2012) used to investigate the mechanisms of PA and anxiolytic treatments under controlled conditions. CCK-4, acting as an agonist of the central subtype of CCK receptors, induces PA in patients with PD and, to a lesser extent, in healthy volunteers (HV). CCK-induced panic symptoms resemble spontaneous PA of PD patients (Bradwejn *et al.*, 1991; Abelson & Nesse, 1994) suggesting the suitability of such experimental challenge to clinical investigation of panic phenomena. Despite the nearly 20 years of research using the CCK-4 challenge paradigm, the mechanisms underlying the panic induction by CCK-4 are not yet fully understood.

In the context of the interactions between CCK and other neuronal systems relevant to the mechanisms of PA, one of the most intriguing neurotransmitters is serotonin (5-hydroxytryptamin, 5-HT). Serotonin, potentially having a restraining effect on panic symptoms (Deakin & Graeff, 1991), has been suggested to have a crucial role in the neurobiology of PD (Bell & Nutt, 1998; Maron & Shlik, 2005) and serotonergic drugs are considered to be the treatment of choice for PD (Kasper & Resinger, 2001; Pollack *et al.*, 2003). On the other hand, 5-HT agonists, such as meta-chloro-phenylpiperazine (m-CPP) and fenfluramine, acutely induce panic and/or anxiety symptoms in PD patients (Charney *et al.*, 1987; Targum & Marshall, 1989; Kahn *et al.*, 1991). Several studies have focused on the modifying role of 5-HT and its precursor tryptophan (TRP) on CCK-4-induced symptoms in patients with PD (Bradwejn & Koszycki, 1994b; Shlik *et al.*, 1997; van Megen *et al.*, 1997) and in HV

(Koszycki *et al.*, 1996; Depot *et al.*, 1999; Maron *et al.*, 2004c). These studies have shown that effective antipanic treatment with a serotonergic drug, assumingly increasing availability of synaptic 5-HT, lessens CCK-4-induced PA in patients with PD. In HV, the effects of 5-HT and TRP on CCK-4-induced symptoms have not been so uniform.

HV and patients with PD seem to differ both, in their sensitivity to CCK-4 challenge and in their responsiveness to a serotonergic modulation of CCK-4-induced panic response. Studies suggest that these dissimilarities may at least partly be explained by the differences in the brain 5-HT receptor and transporter systems (Maron *et al.*, 2004a; Neumeister *et al.*, 2004; Nash *et al.*, 2008) as well as in the genes regulating 5-HT system (Hamilton *et al.*, 1999; Maron *et al.*, 2004b; Maron *et al.*, 2005a; Perna *et al.*, 2005).

Altogether, the accumulated data suggested that the functional states of and the interaction patterns between CCK and 5-HT systems are distinct in patients with PD as compared to HV warranting further research in this area.

REVIEW OF THE LITERATURE

Serotonin – cholecystokinin interplay

Preclinical data

Experimental preclinical studies provide ample evidence of interactions between the 5-HT and CCK systems. For example, it was observed that central injections of CCK-4 stimulate the metabolism of 5-HT in the rat brain (Itoh *et al.*, 1988). In the guinea pig, intraperitoneal administration of butylocarbonyl (BOC)-CCK-4 amplified the rise in extracellular 5-HT normally observed in the elevated plus-maze model of anxiety, and produced anxiogenic effects. Pretreatment with L 365,260, a CCKB receptor antagonist, opposed both effects. When administered alone, L 365,260 showed anxiolytic properties, decreased basal 5-HT levels, and prevented the rise in 5-HT induced by exposure to the elevated plus-maze test (Rex *et al.*, 1994).

On the other hand, increasing brain 5-HT was shown to lead to an increased release of brain CCK (Raiteri *et al.*, 1993; Rosen *et al.*, 1995). These data are supported by preclinical data on SSRI administration, showing initial increases in CCK transmission and the number of CCK receptors (Rosen *et al.*, 1995; Koks *et al.*, 1999). These effects however, normalize upon chronic treatment as demonstrated by Harro and colleagues (Harro *et al.*, 1997), who found no changes in the density of CCK receptors or in the content of CCK-related peptides after long-term treatment with various antidepressants, including 5-HT reuptake inhibitors.

The modulation of endogenous 5-HT3 receptor (5-HT3R) activity also has been shown to influence the CCK system. Animal studies have demonstrated that 5-HT and 1-phenylbiguanide, a 5-HT3R agonist, enhanced the depolarisation-evoked release of CCK from synaptosomes of the rat cerebral cortex and the nucleus accumbens. This effect was not observed under basal conditions or after pretreatment with 5-HT3R antagonists such as MDL 72222, ICS 205–930, and ondansetron. In contrast, the 5-HT1/5-HT2 receptor blockade by methiothepin did not antagonize CCK release by 5-HT, indicating that the effect is most likely mediated by 5-HT3 receptors located on CCK-releasing nerve terminals (Paudice & Raiteri, 1991; Raiteri *et al.*, 1993). The importance of 5-HT3 receptors in the regulation of anxiety was further confirmed by Vasar and colleagues (Vasar *et al.*, 1993) who showed that intraperitoneal pretreatment of rats with ondansetron completely reversed the antiexploratory effect of subcutaneously injected caerulein, a nonselective agonist of CCKA/CCKB receptors.

Clinical data

Manipulation of synaptic 5-HT level
Two approaches for manipulating synaptic 5-HT level have been used in human studies. One is the acute tryptophan depletion (TD), which causes a substantial transient decrease in the brain 5-HT levels (Young *et al.*, 1985; Bell *et al.*, 2001). The opposite effect could be achieved with the use of 5-HT precursor 5-hydroxytryptophan (5-HTP) or a drug acting to increase the brain concentrations of 5-HT (den Boer & Westenberg, 1990; van Vliet *et al.*, 1996). Both methods are evidently more informative under conditions of experimental challenge with panicogenic agents, such as inhaled carbon dioxide (CO_2) or CCK-4.

Previous studies have demonstrated that while TD by itself is not panicogenic in unmedicated PD patients (Goddard *et al.*, 1994), it increased ventilation in PD patients (Kent *et al*., 1996) and revealed susceptibility to PA when combined with challenge agents, such as the alpha-2-adrenergic antagonist yohimbine (Goddard *et al*., 1995) or inhaled CO_2 (Miller *et al.*, 2000; Schruers *et al.*, 2000). Furthermore, TD reversed the antipanic effect of treatment with an SSRI, paroxetine in PD patients when challenged with flumazenil, a benzodiazepine receptor antagonist (Bell *et al.*, 2002). Conversely, the administration of 5-HTP inhibited the panic symptoms induced by CO_2 inhalation in patients with PD (Schruers et al., 2002). There is also an earlier observation that PD patients felt relief from the effect of 5-HTP (den Boer & Westenberg, 1990), and as noted earlier, 5-HT-ergic drugs possess antipanic efficacy presumably due to increasing the level of synaptic 5-HT (Kasper & Resinger, 2001; Pollack *et al.*, 2003). These findings suggest that a decrease in 5-HT neurotransmission predisposes to PA, and that the antipanic effect of SSRIs depends upon the availability of 5-HT in the brain.

The respective findings in HV have been less robust than in patients with PD. For instance, (Schruers *et al*., 2002) did not find any significant influence of acute administration of 5-HTP on CO_2-induced panic in HV, although they found significant reduction in panic response by 5-HTP in patients with PD. Similarly, the augmenting effects of TD on CO_2-induced panic have been more pronounced in patients with PD than in HV (Klaassen *et al.*, 1998; Schruers *et al.*, 2000).

Several studies have focused on the modifying role of 5-HT and TRP on CCK-4-induced symptoms in patients with PD (Bradwejn & Koszycki, 1994b; Shlik *et al.*, 1997; van Megen *et al.*, 1997) and in HV (Koszycki *et al.*, 1996; Depot *et al.*, 1999; Maron *et al.*, 2004c). Initially it was shown that effective antipanic treatment with nonselective monoamine reuptake inhibitor imipramine (Bradwejn & Koszycki, 1994b) or a SSRI (Shlik *et al.*, 1997; van Megen *et al.*, 1997) lessens CCK-4-induced PA in PD patients. It has been assumed that these treatments, in accordance with the theory of Deakin and Graeff (1991), lessen CCK-4-induced PA by increasing availability of synaptic 5-HT. In HV, the modulating effect of 5-HT or TRP on CCK-4-induced symptoms does not

appear so uniform. Koszycki and colleagues (1996) demonstrated that TD augmented CCK-4-induced release of adrenocorticotropic hormone (ACTH), cortisol and prolactin in healthy males, without influencing the panicogenic effect of CCK-4, suggesting that 5-HT system has a role in neuroendocrine, but not psychological effects of CCK-4. Maron and colleagues (Maron *et al.*, 2004c) demonstrated that acute administration of 5-HTP significantly lowered the panic rate and intensity of cognitive panic symptoms in female HV, and intensity of somatic symptoms in healthy males, indicating gender differences in the 5-HT-ergic influence on CCK-4-induced panic. However, no whole group antipanic effect was found in this study. Recently Kellner and colleagues (2009) found that escitalopram (ESC) had no inhibitory effect on panic symptoms elicited by CCK-4 in healthy men.

Apart from the studies modulating CCK-4-induced PA by increasing or decreasing synaptic availability of 5-HT, a few studies undertook to manipulate specific 5-HT receptors, in particular the 5-HT3 receptor. After acute administration, ondansetron, the antagonist of 5-HT3 receptor, attenuated CCK-4-induced PA in HV (Depot *et al.*, 1999), but did not prevent the panicogenic effects of CCK-4 analogue pentagastrin in patients with PD (McCann *et al.*, 1997), demonstrating a difference in modulatory potential of this 5-HT3 receptor antagonist on CCK induced panic between the HV and PD patients. In the same study (Depot *et al.*, 1999) it was found that, although after acute administration ondansetron attenuated CCK-4-induced PA in HV significantly more than placebo, after chronic administration (28 days), while ondansetron and placebo groups differed by some hormonal parameters, they did not significantly differ in any of the assessed behavioral or cardiovascular parameters.

Thus, the interaction patterns between 5-HT and CCK neuronal systems as well as antipanic potential of 5-HT-ergic agents seem to be different in HV vs. PD patients, in males vs. females as well as in case of acute vs. chronic administration.

Brain imaging of 5-HT receptor
The brain imaging studies in PD have so far shown both structural and functional abnormalities in several brain regions (Graeff & Del-Ben, 2008). Additionally, neurochemical alterations have been found in neuroimaging studies of gamma-aminobutyric acid (GABA) (Kuikka *et al.*, 1995; Malizia *et al.*, 1998; Hasler *et al.*, 2008), 5-HT (Maron *et al.*, 2004a; Neumeister *et al.*, 2004; Nash *et al.*, 2008), neurokinin (Fujimura *et al.*, 2009) and dopamine (Maron *et al.*, 2010b) systems. Different 5-HTT and 5-HT1A (both assumingly crucial in 5-HT turnover) receptor binding properties in several brain regions have been visualized in multiple brain imaging studies suggesting both state and trait dependent differences in 5-HT-ergic system of PD patients and HV as well as a possibility of different treatment response to a 5-HT-ergic agent. In a study using single photon emission computed tomography (SPECT), Maron and

colleagues (Maron *et al*., 2004a) found that patients with current PD had significantly lower 5-HTT binding in the midbrain raphe, the temporal lobes, and the thalamus as compared with HV. In contrast, patients with PD in remission had normal 5-HTT binding properties in the midbrain and the temporal regions, but still a significantly lower thalamic 5-HTT binding. Neumeister and colleagues (Neumeister *et al*., 2004), using PET, have demonstrated a marked reduction of 5-HT1A receptor binding in the anterior and posterior cingulate cortices, and in the midbrain raphe in patients with PD compared to HV. In another PET study, Nash and colleagues **(**Nash *et al*., 2008) have detected reduced 5-HT1A receptor binding in the raphe region and in the amygdala, as well as in the orbitofrontal and temporal cortices in untreated PD patients, and still reduced density of 5-HT1A receptors in the raphe and in the hippocampus after recovery with an SSRI paroxetine.

Also gender-dependent differences in 5-HT-ergic function have been found, e.g. lower brain 5-HT metabolism in females compared to males was discovered in a PET brain imaging study by Nishizawa and colleagues (Nishizawa *et al*., 1997).

5-HT and CCK system genes
The data from twin and family studies suggest an involvement of genetic factors in the development of PD with a heritability estimate near 40% (Hettema *et al*., 2001). However, the respective genetic substrates are not known and the association studies with candidate genes have so far produced mostly negative or inconsistent results with only few gene variants in the key neurotransmitter systems showing suggestive link to PD phenotypes. The favoured among them have been 5-HT-related gene variants, particularly the 5-HTT-linked polymorphism (5-HTTLPR) (Maron *et al.*, 2005a), MAO-A gene VNTR polymorphism (Deckert *et al.*, 1999; Maron *et al.*, 2005a), 5-HT receptor 1A (5-HTR1A) (Rothe *et al.*, 2004; Maron *et al.*, 2005a) and 5-HT receptor 2A (5-HTR2A) (Inada *et al.*, 2003; Maron *et al.*, 2005a) as well as tryptophan hydroxylase gene isomer 2 (TPH2) (Maron *et al.*, 2007).

In respect to CCK genes, some promising, although not always consistent associations with PD have been observed, particularly for a CT repeat polymorphism of the CCK2 receptor (CCK2R) gene (Kennedy *et al.*, 1999; Hamilton *et al.*, 2001). Maron and colleagues (Maron *et al.*, 2005b) in their SNP-array study, have shown no significant associations between several other CCK-related polymorphisms and pure PD phenotype, although CCK2R polymorphism −215C–A showed an association in the PD group with comorbid mood disorders. They also detected an association of CCK1R 246G-A polymorphism with the phenotype of comorbid, but not pure PD.

The laboratory panic challenges have been now applied to test the premises of genetic predisposition to panic. Several studies addressed the influence of 5-HT related gene variants on experimentally induced panic responses as well as on treatment effects of 5-HT-ergic antipanic drugs. Most of these studies have

focused on the allelic variants of 5-HTTLPR. Schmidt and colleagues (Schmidt *et al*., 2000) have reported that subjects homozygous for the long variant of 5-HTTLPR were at greater risk for behavioural hyperreactivity to 35% CO_2 challenge than those with short-allele genotypes. On the contrary, in PD patients CO_2 reactivity was not influenced by 5-HTTLPR genotype (Perna *et al*., 2004). There are also preliminary data suggesting that 5-HTTLPR variance might influence the treatment response to a 5-HT-ergic agent in a gender-dependent manner both in PD patients and in HV. Perna and colleagues showed that the effect of paroxetine on spontaneous PA was significantly lower in female PD patients with the S/S genotype in comparison to subjects with L/L or S/L genotypes, but not in men (Perna *et al*., 2005). Also, a gender dependent effect of 5-HTTLPR allelic variance on 5-HTP pretreatment modulation of CCK-4-induced panic was noted (Maron *et al*., 2004b) where females, but not males, with S alleles of the 5-HTTLPR showed a significantly lower panic rate to CCK-4. In a recent study of Kellner and colleagues (Kellner *et al*., 2009), the effect of 5-HTTLPR on treatment response was also demonstrated in healthy men. Although they did not detect the expected difference in panic response to CCK-4 between L/L and S/S genotypes after placebo-pretreatment, CCK-4-induced panic after pretreatment with ESC was significantly more pronounced in healthy males with 5-HTTLPR S/S genotype.

AIMS OF THE STUDIES

The general objective of the present work was to study the modulating role of the 5-HT system on CCK-4-induced panic response. Apart from the effects of direct pharmacological manipulation of synaptic 5-HT levels, some factors of hypothetical importance, such as gene polymorphisms and binding properties of 5-HT transporter system were investigated.

The specific aims and hypotheses of conducted studies were as follows:

1. To establish the effect of TD on CCK-4-induced symptoms in patients with PD who responded to treatment with an SSRI citalopram.

Our hypothesis was that in treatment-responders the antipanic action of citalopram will be reversed by TD, as indicated by a higher rate and greater intensity of CCK-4-induced panic attacks under TD condition as compared to the control condition (Study 1).

2. To explore the effects of genetic polymorphisms of nine candidate genes (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) previously associated with PD on the susceptibility to CCK-4 challenge in healthy subjects.

We hypothesized that the listed genetic polymorphisms will be associated with higher intensity of CCK-4-induced panic response in healthy subjects (Study 2).

3. To assess brain 5-HTT non-displaceable binding potential (BP_{ND}) in male and female patients with PD and matched healthy controls using PET with a tracer [^{11}C]MADAM.

Our hypothesis was that brain 5-HTT BP_{ND} will differ between PD patients and HV reflecting the differences in 5-HT system between these two groups (Study 3).

4. To assess the effect of 6-week treatment with an SSRI escitalopram on CCK-4-induced symptoms in HV, who previously responded to CCK-4 challenge with a panic attack.

Our hypothesis was that in CCK-4 sensitive HV the effect of CCK-4 challenge will be attenuated, as indicated by a lower rate and intensity of CCK-4-induced panic attacks, after 6-week treatment with escitalopram as compared to the placebo (Study 4).

MATERIALS AND METHODS

All studies were conducted at the Department of Psychiatry of the University of Tartu (Tartu, Estonia). PET and MRI scans were performed in Turku PET Centre (Turku, Finland).

Ethical considerations

All study protocols and informed consent forms were approved by the Human Studies Ethics Committee of the University of Tartu (Tartu, Estonia), and for Study 3 also by the Human Studies Ethics Committee of the University of Turku (Turku, Finland). All participants provided written informed consent.

Subjects

Study 1 The inclusion criteria for the treatment phase were current DSM-IV diagnosis of PD (APA, 1994) and absence of current psychiatric or somatic comorbidity. Treatment response criteria were panic-free status for at least 2 weeks and a rating of "much" or "very much improved" on the Clinical Global Impression Improvement (CGI-I) scale (Guy *et al*., 1976) by the 10th week of treatment maintained throughout the experimental phase of the study. Treatment responders were included in the experimental part of the study while continuing citalopram treatment. In total, 18 responders (6 males, 12 females, mean age 34.5±9.3) were included in this study. Among them, 72% had concurrent agoraphobia and 44% had a history of major depression. For other key clinical characteristics see Table 1 (Paper I).

    Study 2 The study sample consisted of 110 HV (47 males and 63 females; mean age 22.2±5.2) recruited by a flyer advertisement. The inclusion criteria were: age between 18 and 50 years, no personal or family psychiatric history, and healthy physical status as determined by medical history and physical examination. None had a positive urine test for psychoactive drugs at the time of a screening. 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 had participated in previous CCK-4 challenge studies.

    Study 3 Eleven medication-free outpatients with PD (5 males, 6 females; mean age 31.1±8.6) and 24 HV (12 males, 12 females; mean age 36.9±6.9) were included. All were Caucasians, right-handed and in good physical health as confirmed by medical history, physical examination, routine blood tests and magnetic resonance imaging (MRI) of the brain at 1.5 T. The patients were recruited at the Psychiatry Clinic of the Tartu University Hospital. Three patients met criteria for current mild depressive episode considered secondary

to PD, and one patient had comorbid social anxiety disorder. Healthy subjects, matched to the patients by age and sex, were recruited by newspaper advertisements in Tartu, Estonia and in Turku, Finland. Only those HV without personal or family (defined as first-degree relatives) history of psychiatric disorders were included in this study. All participants abstained from alcohol and benzodiazepines for at least 2 weeks prior to PET scans as confirmed by questioning and medical records. None of them were heavy smokers or had current or lifetime diagnosis of alcohol dependence or abuse. None of the subjects had taken any antidepressant or other medication known to affect 5HTT binding for at least 4 months before the study.

Study 4 The study consisted of two phases with separate protocols. From the total of 82 subjects (29 men, 53 women; mean age 22.3 ± 5.0), who underwent a challenge with intravenous CCK-4 (50 μg), 37 (45.1%) experienced a pre-defined PA and were asked to participate in the second phase of the study, 27 of them agreed. In the course of the study phase II, 4 volunteers dropped out: one male and 2 females due to side-effects of study drug; one female due to an un-expected pregnancy. Treatment non-compliance was detected in four subjects, whose data were omitted from further analysis. To maintain the comparability of groups the data of one female in Dp group (order of treatments – Drug, followed by placebo) with uncommonly high baseline VAS anxiety score prior to the first challenge was also excluded. Thus, the data of 18 subjects (10 males, 8 females, mean age 22.5 ± 5.8) were included in final analysis: 11 subjects in the Dp group (45% male; mean age 22.9 ± 6.1), and 7 in the pD group (order of treatments – placebo, followed by Drug) (71% male; mean age 22.4 ± 6.1).

Assessments

The diagnostic assessments in all studies were done by experienced psychiatrists using the Estonian translation of a structured diagnostic instrument, the Mini International Neuropsychiatric Interview - M.I.N.I. 5.0.0 (Sheehan *et al*., 1998).

In all the challenge studies (Studies 1, 2, 4) the subjective effects of CCK-4 challenge were assessed with a set of Visual Analogue Scales (VAS), based on the scales of Bond and Lader (Bond & Lader, 1974) consisting of 100-mm lines for the dimensions of anxiety (VAS-A), feeling of overall discomfort (VAS-D) and the similarity of the experience to spontaneous PA (VAS-S) (the latter 2 only in Study 1). CCK-4-induced symptoms were rated on the Panic Symptom Scale (PSS; (Bradwejn *et al*., 1991)) assessing the intensity of 18 symptoms derived from the DSM-IV criteria for a PA from 0 (not present) to 4 (extremely severe). Measures derived from the PSS were the number of symptoms scored at least 1, sum intensity score, defined as the sum of all individual item ratings, and subscale scores for somatic and cognitive symptoms.

The arterial blood pressure (BP) and heart rate (HR) were measured with an automatic sphygmomanometer (Dinamap Pro 100, Criticon, Tampa, FL, USA). Maximum changes in the studied parameters were calculated by subtracting baseline scores from the highest value obtained within 120 s after injection.

Study 1 The treatment response was assessed with the CGI (Guy *et al*., 1976) and Panic Disorder Severity Scale – PDSS; (Shear *et al*., 1997). For the assessment of mood and generalized anxiety symptoms, the Montgomery–Åsberg Depression Rating Scale (MADRS; (Montgomery & Asberg, 1979)), Beck Depression Inventory (BDI; (Beck *et al*., 1961)) and Hamilton Anxiety Rating Scale (HAS; (Hamilton, 1969)) were used. The a priori criteria for the occurrence of a PA were: (a) sudden onset of at least four panic symptoms; (b) anxiety/apprehension/fear PSS item scored at least 2; (c) at least moderately severe (>35%) anxiety or fear on VAS-A; and (d) at least moderate (>35%) similarity of experience to spontaneous PA on VAS-S.

Study 3 On the day of PET scan, the patients were assessed with the PDSS; (score range 0–28) (Shear *et al*., 1997) and with the Hamilton Anxiety Rating Scale (HAS; score range 0–42; (Hamilton, 1969)).

Studies 2&4 The baseline anxiety and depression were evaluated by Hamilton Anxiety Rating Scale (HAS; (Hamilton, 1969)) two subscales of the self-rated Emotional State Questionnaire (EST-Q; (Aluoja *et al*., 1999)) and VAS-A. The *a priori* criteria for the occurrence of a PA were: (a) sudden onset of at least 4 PSS panic symptoms, with at least moderate intensity (PSS score 2); (b) PSS item anxiety/apprehension/fear scored at least 3 (severe). In Study 2 additionally a net increase equal or greater than 50 mm on VAS-A was requested.

Substances

The CCK-4 (Trp-Met-Asp-Phe-NH2) (Clinalfa, Merck Biosciences AG, Switzerland) (Studies 1, 2, 4) was diluted in sterile physiological solution before the challenge, and 25 µg (Study 1) or 50 µg (Studies 2, 4) of CCK-4 was used for the challenge as a bolus injection via an intravenous cannula.

Study 1 Citalopram (CIT) was used as Cipramil® (H. Lundbeck A/S, Denmark) in 20mg tablets. Amino acids were obtained from SHS International Ltd (UK) and prepared as described by Young and colleagues (Young *et al*., 1985).

Study 3 The precursor N-desmethyl-MADAM and MADAM were obtained from PharmaSynth AS, Tartu, Estonia. [^{11}C]Methane was produced at the Accelerator Laboratory of Åbo Akademi with a 103-cm isochronous Efremov cyclotron using the 14N(p,a)11C reaction. Highly specific radioactivity [^{11}C]methyl iodide was prepared from [11C]methane. The preparation of [^{11}C]MADAM from [^{11}C]methyl triflate was performed according to a published procedure with minor modifications (Halldin *et al*., 2005).

Study 4 Escitalopram was used as Cipralex® (H. Lundbeck A/S, Denmark) in 5 mg (1st week) and 10 mg (the rest of 6 weeks) tablets. Placebo tablets, identical in size and colour as well as a standard ESC used for the assessment of ESC plasma concentrations were the courtesy of H. Lundbeck A/S, Denmark.

Treatments

Study 1 The patients were treated with a flexible dose of CIT (mean final dose of CIT was 18.9±4.4 mg/day; range 10–30 mg/day) for 10 weeks. The dose of CIT was 10 mg/day for the first week and then optimized according to response and tolerability. Alprazolam (0.25–0.5 mg/day) was used in three patients for the first 2–4 weeks due to an initial increase in anxiety.

Study 4 Subjects enrolled in the second phase of the study were randomized to two groups. One group (group Dp; D – Drug, p – placebo) received ESC for 6 weeks (5 mg/day for one week and then 10 mg/day) and, after a 1-week washout period, placebo for another 6 weeks. The other group (group pD) received placebo first and then, after a 1-week washout, was switched to ESC. Each treatment period was concluded by a challenge with CCK-4 (50 µg).

Procedures

Study 1 TD/CCK-4 challenges were carried out in a double-blind, placebo-controlled, balanced crossover design, with each patient taking part in two similar test days. Test days were about 1 week apart (mean 9 days; range 5–20 days), the first day approximately during the 11th week (between days 74 and 105) and the second day approximately during the 12th week (between days 81 and 112) of the study. Patients had a tryptophan-free amino-acid drink on the depletion day and a drink containing tryptophan on the control day. The order of the drinks was randomly allocated. The TD test was carried out according to a standard protocol (Young *et al*., 1985). Patients followed a 24-h low tryptophan diet on the day before the test and fasted starting at midnight on both test days.

On the test day, patients arrived at the research unit at 8:00 A.M. During the procedure, they stayed in the same room lying on a bed or sitting and were allowed to drink only water. At 8:20 initial assessments were made, baseline values of BP, HR and VAS-A were registered and blood samples for baseline levels of TRP and LNAA were obtained. At 8:30, subjects consumed the amino acid or control drink. BP, HR and VAS-A were registered in 2-h intervals. At 1:00 P.M., the intravenous cannula was inserted into an antecubital vein and saline infusion was started. After pre-challenge assessments, at 1:20, the blood sample for the TRP and LNAA assay was drawn through the cannula. At 1:30, after registration of BP, HR and VAS-A, a bolus injection of CCK-4 (25 µg) was given through the cannula. BP, HR and VAS-A were registered in 30-s

intervals for 2 min and at 5 and 15 min after CCK-4 injection. After CCK-4-induced symptoms had abated, patients' peak symptoms were rated with the PSS, VAS-A, VAS-D and VAS-S. After completion of the last measurements, the cannula was removed and the patient allowed to leave when comfortable and given an option of phone contact in the next 24 h.

Study 3 The radioligand used in this study for imaging of 5-HTT binding was [N-methyl-^{11}C]N,N-dimethyl-2-(20-amino-40methylphenyl-thio)ben-zylamine ([^{11}C]MADAM) (Lundberg *et al*., 2005). PET experiments were conducted using a brain-dedicated, high-resolution PET scanner HRRT (Siemens Medical Solutions, Knoxville, TN) as previously described (Hirvonen *et al*., 2008). Briefly, after a transmission scan was performed with a caesium-137 point source, emission data were collected after radioligand injection for 75 minutes using 17 frames (3x1, 4x3, and 10x6 min) in list mode. Images were reconstructed into $1.2x1.2x1.2$ mm^3 volumes using speed-optimized OP-OSEM-3D (Ordinary Poisson-OSEM in full 3D) reconstruction. The head was fixed using an individually moulded thermoplastic mask. The antecubital vein was cannulated and 400–500 MBq [^{11}C]MADAM was injected as a rapid bolus flushed with saline. Injected mass and specific radioactivity for HV and patients with PD were 0.59±0.40 vs. 0.72±0.54 mg, and 284.6±117.1 vs. 270.6± 149.0 MBq/ nmol, respectively, with no statistically significant differences between the groups in any variable. Radiochemical purity was >98%.

Studies 2&4 Upon arrival at the research unit at about 10 A.M. on challenge days the subjects completed the EST-Q and HAS. An intravenous cannula was inserted into an antecubital vein and saline infusion started to keep the cannula open. The subjects stayed in the same room resting. At 11 A.M., after the registration of baseline values of BP, HR and VAS-A, a bolus injection of 50 μg of CCK-4 was given through the cannula (during ca 3 seconds). The subjects were asked to describe any symptoms they experienced after the injection. The BP, HR and VAS-A were registered every 30 seconds for 2 minutes, then at 5 and 15 minutes. After the CCK-4-induced symptoms had abated, the subjects were assessed on PSS and rated VAS-A for the peak symptoms. After completion of the last measurements the cannula was removed and the subject was allowed to leave when comfortable with an option of phone contact over the next 24 hours.

For genotyping, the blood samples were obtained (Studies 2&4; for further details see chapter "Laboratory analysis").

To check for compliance with study medication, samples were collected from every subject at four time points (one sample on each challenge day and one on randomly chosen visit during each treatment period) (Study 4).

Laboratory analyses

Study 1 Plasma free- and total tryptophan (TRP) were measured by isocratic high-performance liquid chromatography (HPLC) with UV end-point detection. The inter- and intra-assay coefficients of variation (CV) were 9% and 5.2% (n=9) and 13.5% and 6.9% (n=5) for the total and free TRP respectively. Large chain amino acids (LNAA) were measured by gradient-elution HPLC with utilization of fluorescence end-point detection. The amino acid residues were derivatised pre-column to enhance the detection signal. Validation analysis showed that the CV over the range of the standard amino acid standard curves was <14% for all acids of interest.

Study 2 Nine candidate genes were selected for this study based on the previous positive findings from genetic association studies in PD (Table 1, Paper II). DNA was extracted from 5ml 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 (Lesch *et al.*, 1996; Sabol *et al.*, 1998; Maron *et al.*, 2007). For oligonucleotides used to get amplicons that contain respective poly-morphisms in 5-HTR1A 1019CG (rs6295), 5-HTR2A 102TC (rs6313), CCKR1 246GA, CCKR2 215CA (rs1799721), DRD1 94GA (rs5326) and COMT Val158Met (rs4680) loci refer to Paper II. Amplification was followed by digestion with restriction endonucleases and agarose gel electrophoresis. Restriction endonucleases were used from Fermentas (see paper II). For all genotypes tested, 25 samples were genotyped twice and no discrepancies were found.

Study 4 Plasma ESC concentrations were assessed using the ultra high performance liquid chromatography-mass spectrometry (UHPLC) system (Agilent Series 1290 Infinity LC, Santa-Clara, CA, USA), in two samples collected during the treatment period with an active substance, and in one randomly chosen sample collected during the placebo treatment period, after the study had ended. For genotyping, the blood samples were obtained, DNA extracted and 5-HTTLPR genotype determined as described by Maron et al (Maron *et al.*, 2004b).

Statistical analysis

Study 1 The data were analysed using the software package STATISTICA 5.1 (StatSoft, Tulsa, OK, USA). The Wilcoxon matched-pairs test and Mann–Whitney U test were used to analyze the nonparametric data. The proportions were compared by Pearson Chi-square tests. One-way analysis of variance (ANOVA) was used to compare the cardiovascular responses to CCK-4 injection between TD and placebo groups. The data were presented as mean values and standard deviation (SD). The sample size of 18 subjects allowed

detection of a 50% change in panic rate with 90% power at a significance level of 5%.

Study 2 The genotype and allele frequencies between the panic and nonpanic 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 comparisons.

Study 3 Image analysis and modelling of [^{11}C]MADAM kinetics [^{11}C]MADAM PET images were corrected for frame misalignment and co-registered with T1-weighted MR images as previously described using SPM2 (Hirvonen *et al.*, 2008). Volumes of interest (VOIs) were manually delineated on co-registered T1-weighted MR images using Imadeus software (version 1.2, Forima Inc., Turku, Finland). The following VOIs were drawn: dorsal, ventral and subgenual anterior cingulate cortex; dorso- and ventrolateral and medial prefrontal cortex; lateral and medial orbitofrontal cortex; angular and supra-marginal gyri; superior, middle and inferior temporal gyri; anterior and posterior insular cortex; hippocampus and amygdala; dorsal caudate, dorsal putamen, ventral striatum, and thalamus; dorsal raphe nuclei; grey matter of cerebellum. Regional time-activity curves were derived within these VOIs from the dynamic PET images. Specific binding of [^{11}C]MADAM was estimated as BP_{ND}, which is the ratio at equilibrium of specific binding to non-displaceable uptake (Innis *et al.*, 2007). BP_{ND} was estimated using the simplified reference tissue model with the cerebellum as reference region (Lammertsma & Hume, 1996; Lundberg *et al.*, 2005). Prior to modelling, time-activity curves were weighted according to frame duration and counts in frame (Hirvonen *et al.*, 2003).

Voxel-based analysis To confirm the results from the regional analysis, we conducted voxel-based analysis on parametric BP_{ND} maps using SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8) (Friston KJ *et al.*, 1995). Parametric BP_{ND} maps were first calculated using the basis function imple-mentation of the simplified reference tissue model (Gunn *et al.*, 1997). These parametric maps were then spatially normalized using transformation para-meters that had been estimated from summed PET images and an in-house ligand-specific template for [^{11}C]MADAM. Spatially normalized parametric BP_{ND} maps were then smoothed with a 12-mm (FWHM) Gaussian kernel. Group differences were estimated with a voxel-wise independent samples t-test. Statistical significance in the exploratory analysis was inferred as t<2.4 at voxel level and corrected p<0.05 at cluster level.

Statistical analysis Statistical analysis was carried out using SPSS 13.0 for Windows (Release 13.0.1, copyright SPSS Inc., 1989–2004). BP_{ND} data was modelled by means of repeated measures analysis of variance (rmANOVA), with group status and sex as between-subject predictors, brain region as a within subject predictor, and age as a covariate. The interaction term group х sex х region was used to assess region-specific and sex-dependent group effects,

while the interaction term group x sex assessed sex-dependent group effects across brain regions, effectively avoiding the problem of multiple comparisons. Finally, group differences were assessed in each brain region using post hoc t-tests. The associations between BP_{ND} and symptom ratings were assessed using Pearson product-moment correlation coefficients (R). The correlation analysis was exploratory and its results not considered primary due to the small sample size for each gender. To control for positive bias due to violation of the sphericity assumption associated with interaction terms including within-subject factors with three or more levels, degrees of freedom in the averaged tests of significance were adjusted using the Greenhouse–Geisser method. Observed p-values were corrected for multiple comparisons using the false discovery rate (FDR) method, which keeps the overall proportion of false positives below 0.05 (Benjamini & Hochberg, 1995).

Study 4 To assure comparability of the groups we used Fisher exact test, t-tests and Wilcoxon rank-sum tests as appropriate. The effect of treatment was estimated using random intercept models. At first, the results of the second and third challenges were compared. Thereafter the data of the first challenge were taken into account by subtracting these from the readings of response variables of second and third challenges. In both models, the time effect (time measured as provocation serial number), drug effect and the carry-over effect as interaction of both were estimated. Additionally, main effect of genetic variation of 5-HTTLPR polymorphism with three levels (SS, SL, LL) and the interaction between the genetic variation and treatment were estimated. Similar analysis was applied to gender effect. For PA intensity, HAS, and EST-Q anxiety scores the generalized mixed models were used; for the rest of the variables, linear mixed models were used. Baseline diastolic BP and baseline VAS anxiety score were transformed to have a normal distribution. For the effect sizes, 95% confidence intervals were calculated. Statistical software R version 2.9.0 with lme4 package was used.

RESULTS AND DISCUSSION

Study 1. The effect of tryptophan depletion on response to CCK-4 challenge in patients with panic disorder after treatment with citalopram.

Plasma tryptophan levels. There were no significant differences in baseline measures of plasma tryptophan between TD and control days. Five hours after ingestion of the TRP-depleted mixture, there were drops of 73% and 69% in mean values of total and free plasma TRP, respectively. After the control drink, mean plasma levels of free and total plasma TRP increased 369% and 138%, respectively. The mean TRP/LNAA ratio decreased 92% on the TD day and 46% on the control day (Table 2, Paper I). These data indicated that TD effectively decreased the availability of TRP in the brain.

Pre-challenge effects. TD did not cause any significant changes in psychological and cardiovascular measures, the pre-challenge baseline variables were similar on the two test days (Table 3, Paper I).

Post-challenge effects. CCK-4 challenge caused a PA in about 1/3 of the patients on both TD and control days (n=5, 27.8% and n=6, 33.3%, respectively; $\chi 2=0.13$, df=1, p=0.72). Neither the PSS sum intensity score nor cognitive or somatic subscores were significantly affected by TD (Figure1). The mean maximum ratings of VAS-A and VAS-D did not differ significantly between the two test days. The changes in BP and HR values were also similar on the two test days (Table 4, Paper I). A secondary analysis with a breakdown by gender did not reveal any significant differences in panic rate between TD and control days.

Further examination of the results, applying more stringent criteria for treatment response (panic-free status for at least 2 weeks plus at least 50% decrease on the PDSS maintained throughout the experimental phase; n=14) and in a subset of patients in full remission (panic-free status plus PDSS score ≤3, with no score >1 on any PDSS item; n=8), no differences were revealed between the TD and control conditions in any of the psychological or cardiovascular measures.
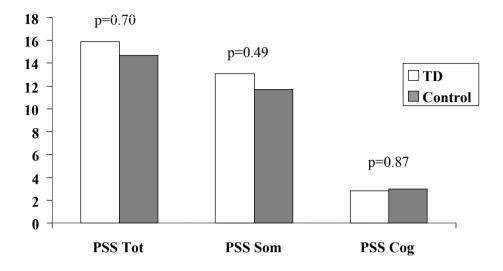
Figure 1. Intensity of panic symptoms on PSS (mean scores)
Abbreviations: *TD* Tryptophan depletion, *PSS* Panic Symptom Scale, *Tot* Total score, *Som* Subscore of somatic symptoms, *Cog* Subscore of cognitive symptoms

Discussion. The results of this study showed that an acute lowering of brain 5-HT availability by TD did not affect the behavioural or cardiovascular response to CCK-4 challenge in PD patients who responded to a 10-week treatment with an SSRI CIT. A nearly 30% panic rate, similar to what was previously observed with CCK-4 challenge after an SSRI treatment in patients with PD (Shlik *et al.*, 1997; van Megen *et al.*, 1997) implies that the sensitivity to CCK-4 challenge was effectively decreased by treatment with CIT in this study. The lack of the effect of TD in our study was at odds with previous findings of Bell and colleagues (2002), demonstrating that TD reversed the protective effect of treatment with another SSRI, paroxetine, in panic challenge with flumazenil. Thus, the reduced sensitivity to CCK-4 after SSRI treatment may be related to mechanisms other than 5-HT availability in the brain and the effects of CCK-4 and flumazenil may be modulated differently by 5-HT.

In addition to studies showing the reduction of the CCK-4 sensitivity as a result of treatment with serotonergic antidepressants (Bradwejn & Koszycki, 1994b; Shlik *et al.*, 1997; van Megen *et al.*, 1997), assuming increasing the level of synaptic level 5-HT, it has been observed that in HV, an acute pre-treatment with 5-HT precursor 5-hydroxytryptophan (5-HTP) significantly reduced manifestations of CCK-4-induced panic, particularly in females (Maron *et al.*, 2004c). Considering the hypothesis of a panic-restraining nature of 5-HT (Deakin & Graeff, 1991), it seemed plausible that a reduction in the 5-HT levels

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would lead to an increased CCK-4 sensitivity. These interactions, however, may be more complex.

Koszycki and colleagues (1996) have shown that, without influencing the panicogenic effect of CCK-4, TD augmented CCK-4-induced release of adrenocorticotropic hormone, cortisol and prolactin in healthy males. These findings suggest that 5-HT systems may have a role in the neuroendocrine, but not the psychological, actions of CCK-4. Subsequently, Depot and colleagues (1999) reported that acute administration of ondansetron, a selective 5-HT3-receptor antagonist, significantly decreased CCK-4-induced panic in healthy males and reduced cortisol, growth hormone and prolactin response. Thus, at least in regard to 5-HT3 receptors, a reduction rather than an increase in 5-HT effects was associated with the alleviation of CCK-4-induced panic. Furthermore, these effects dissipated upon chronic treatment with ondansetron, indicating that adaptive changes that abolish the initial effect are likely to occur.

One explanation for our findings is that the SSRIs may alter the CCK transmission system, perhaps through desensitisation of the postulated CCK-2R receptor hypersensitivity (Bradwejn & Koszycki, 1994a), where variation in CCK-2R sensitivity may also explain the difference in panicogenic properties of CCK-4 between PD patients and HV (Bradwejn *et al.*, 1991). Increasing brain 5-HT leads to an increased release of brain CCK (Raiteri *et al.*, 1993; Rosen *et al.*, 1995), which might over time down-regulate CCK receptors. Thus, one theory is that while SSRIs initially increase CCK as a result of making more 5-HT available, perhaps explaining the early worsening seen with these drugs in some patients, the later desensitization of CCK-2R is independent of 5-HT availability in the synaptic cleft. Such an explanation is compatible with the preclinical data on SSRI administration, which shows an initial increase in CCK transmission and the number of CCK receptors (Rosen *et al.*, 1995; Koks *et al.*, 1999). These effects apparently normalize upon chronic treatment, as demonstrated by Harro and colleagues (1997), who found no changes in the density of CCK receptors or in the content of CCK-related peptides after long-term treatment with various antidepressants, including 5-HT reuptake inhibitors. If the sensitivity of CCK-2R, and not the synaptic availability of 5-HT, is a critical factor determining the reactivity to a CCK-4 challenge, then effects of acute TD on CCK-4 sensitivity would not be as strong as the effects seen with other challenge agents in both HV (Goddard *et al.*, 1995; Klaassen *et al.*, 1998; Miller *et al.*, 2000) and, even more robustly, in PD patients (Kent *et al.*, 1996; Miller *et al.*, 2000; Schruers *et al.*, 2000) and those in remission (Bell *et al.*, 2002). This hypothesis also fits with the above-mentioned study by Koszycki and colleagues (1996) showing that TD did not influence the panicogenic effect of CCK-4 in HV.

Among the other limitations of our study, it should be noted that the mean TD-induced decrease in free plasma TRP remained just below 70%, whereas, a more robust depletion may have been needed to elicit behavioural effects. Also,

this study had statistical power to detect only a large, 50% difference in panic rate; a bigger sample size would have been required to find a smaller effect.

Study 2. Association testing of panic disorder candidate genes using CCK-4 challenge in healthy volunteers.
After CCK-4 challenge, 39 (35.5%) subjects of 110 experienced a PA, while 71 subjects were defined as non-panickers. The descriptive data on panic and anxiety variables by groups are presented in Table 2 (Paper II). Two CCK receptor gene polymorphisms, CCK2R −215C–A and CCK1R 246G–A, were excluded from association analyzes after genotyping due to absence of subjects with A alleles in both SNPs in our sample. The distributions of genotypes for all polymorphisms included in the analyses did not deviate from Hardy–Weinberg equilibrium (p=0.11–1.00). The genotype frequencies of 5-HTTLPR, MAO-A, 5-HT1A, 5-HT2A, DRD1 and COMT polymorphisms are shown in Table 3 (Paper II) and the allelic and genotypic distributions of TPH2 gene are presented in Table 4 (Paper II).

There were no differences in the distribution of 5-HTTLPR genotypes and allele frequencies between panic and non-panic groups. There were no associations between panic rate and genotypes of 5-HTTLPR separately in females. Of note, the subjects with LL genotype demonstrated significantly higher level of baseline anxiety measured by VAS immediately before CCK-4 injection than the carriers of S alleles (LL: 12.1±10.7, LS: 7.7±7.6, SS: 5.4±7.2; F(2.11)=4.2; p=0.02). None of other measurements of anxiety or panic responses were associated with 5-HTTLPR polymorphism.

The comparisons of MAO-A promoter region variants between the groups were made according to functional classification of Sabol et al (1998). There were no significant differences in genotype or allele frequencies of MAO-A promoter region polymorphism between the panic and non-panic groups. Separate analysis of this polymorphism in females did not show any significant difference in genotype frequencies of MAO-A promoter region polymorphisms between groups (p=0.44). VNTR polymorphism of MAO-A did also not associate with any other anxiety or panic variables.

There were significant differences for both genotypic and allelic frequencies of rs1386494 A/G SNP of TPH2 gene between panic and non-panic groups with the frequencies of G/G genotype (p=0.03) and G allele (p=0.009) significantly higher in panickers (Table 1). Further analysis showed significant difference in genotypic and allelic frequencies of this SNP between panic and non-panic groups in females (p=0.038 and p=0.048, respectively), but not in males (p=0.26 and p=0.09, respectively). No associations between any other variables of anxiety or panic symptoms and rs1386494 A/G SNP were observed.

Table 1. Distribution of genotypes and allele frequencies of TPH2 1386494 A/G polymorphism

	GENOTYPE FREQUENCIES (%)			ALLELE FREQUENCIES (%)	
	GG	**AG**	**AA**	**G**	**A**
Non-panic	74.7	23.9	1.4	86.6	13.4
Panic	94.9	5.1	0	97.4	2.6

Finally, no significant differences were found between panickers and non-panickers in genotypic or allele distributions for any other studied candidate gene polymorphisms. Subsequent analysis showed that the subjects with CC genotype of 5-HT1A −1018 polymorphism demonstrated higher cognitive symptoms on the PSS (4.8±2.9) then carriers of CG (3.4±2.6) or GG (3.5±1.8) genotypes (F(2.11)=3.5; p=0.03). The subjects with TT genotype of 5-HT2A 102 polymorphism demonstrated higher increase on the VAS after CCK-4 injection (78.5±15.6) then carriers of CT (61.0±22.9) or CC (65.0±20.3) genotypes (F(2.11)=3.1; p=0.049).

Discussion. The results of this study revealed that the TPH2 rs1386494, but none of other polymorphisms previously implicated in PD, is possibly associated with CCK-4-induced panic in HV.

This finding is intriguing in light of predominant expression of TPH2 in the brain stem, the major locus of the 5-HT-producing neurons (Zill *et al*., 2004b) and the proposed role of 5-HT in the pathogenesis of PD (Maron & Shlik, 2005). An increased level of 5-HT synaptic concentration may be a necessary factor of antipanic effect of SSRIs and particularly for their potency to reduce the occurrence of PA (Nutt, 1998). However, acute lowering of brain 5-HT availability with TD did not modify the reduced sensitivity to CCK-4 challenge in PD patients effectively treated with CIT (Tõru *et al*., 2006). Previously, Maron and colleagues have demonstrated that acute administration of 5-HT precursor, 5-HTP, significantly reduced the rate of CCK-4-induced PA in healthy females (Maron *et al*., 2004c). Subsequently a positive association between rs1386494 polymorphism of TPH2 gene in a subgroup of female PD patients without affective comorbidity was observed (Maron *et al*., 2007). Particularly, the frequencies of the both G/G genotype and G allele were significantly lower in females with pure PD as compared to healthy females, whereas this association was not seen in the total female group or in the subgroup of PD females with major depression. On the contrary, Zill and colleagues (2004a) recently demonstrated the positive association between rs1386494 SNP and major depression with greater prevalence of the G allele in patient groups. In the present study, the HV categorized as panickers had significantly higher frequencies of G/G genotype and G allele than those with less panic response to CCK-4 challenge. Importantly, the association between

rs1386494 polymorphism and CCK-4-induced panic was also seen in females similarly to our previous finding in PD female patients. However there was lack of associations between this SNP and panic or anxiety severity on the used scales, probably due to differences between categorical and dimensional assessments of panic responses. Notably, the association of rs1386494 polymorphism with PA rates remained significant when panickers were compared to the subjects with very weak response to CCK-4 challenge (n=21) who did not meet PA criteria and had anxiety net increase less than 50mm on the VAS scale (p=0.03 for genotypes and p=0.005 for alleles).

Nevertheless the divergence in allelic distributions and associations with PD phenotype in patient group and CCK-4-induced PA in HV indicate that further investigations on the functional effects of rs1386494 polymorphism are needed.

Lim and colleagues (2006) have examined the presence of functional or *cis*-acting polymorphisms in the TPH2 gene that may affect mRNA expression. Their analysis of allele-specific expression of TPH2 mRNA in sections of human pons revealed several SNPs for which heterozygosity was highly correlated with allelic expression imbalance and overall expression of TPH2 mRNA. Although identified SNPs are closely linked to rs1386494 poly-morphism, the last variant itself was not associated with allelic mRNA expression of TPH2. Further, Chen and colleagues (2008) reported that the polymorphisms within the 5' regulatory region play an important role in the regulation of TPH2 gene expression. Particularly they found that 90A/G (rs11178998) polymorphism affects, in most cases up-regulates, gene expression at both transcriptional and post-transcriptional levels, whereas the regulation of gene expression by this SNP is apparently cell-specific.

The findings of the present study did not confirm our earlier preliminary evidence of the associations between 5-HTTLPR and panic responses to CCK-4 challenge test in smaller sample of HV (Maron *et al*., 2004b). Particularly, we have found that healthy females with the LL genotype were more sensitive to CCK-4-induced PA than those carrying the S allele. Pertinently, only our group has also demonstrated a positive association between longer variations of 5-HTTLPR and PD phenotype in the patients, most of them with affective or anxiety comorbidity (Maron *et al*., 2005a). Earlier Schmidt and colleagues (2000) have reported that subjects homozygous for the L-variant were at a greater risk for behavioural hyper-reactivity to 35% CO_2 challenge than those with S-allele genotypes. On the contrary, in patients with PD, CO_2 reactivity was not influenced by 5-HTTLPR genetic variants (Perna *et al*., 2004). Our current larger study did not confirm the major role of 5-HTTLPR in panicogenesis, at least in a laboratory panic with CCK-4. However, there was some evidence of a link of this polymorphism to anxiety manifestations, as the subjects with LL genotype had higher anticipatory anxiety preceding CCK-4 challenge than those with shorter alleles. Notably, the levels of anticipatory anxiety did not significantly differ between panickers and non-panickers at the baseline and did not predict the occurrence of PA.

Other candidate polymorphisms, including MAO-A VNTR, 5-HT1A −1018, DRD1 −94 and COMT Val158Met, were also unrelated to occurrence of PA in the present study. Furthermore, we were not able to examine the involvement of CCK-related polymorphisms, CCK2R −215C–A and CCK1R 246G–A, due to absence of rare alleles in our sample. The lack of association between 5-HTR2A 102T−C polymorphism and CCK-4-induced PA in HV may not be a sufficient argument against the involvement of this variant in PD, but may suggest that the detected association in PD patients is related to comorbid agoraphobia (Inada *et al.*, 2003; Maron *et al.*, 2005a). In our study, the subjects with 102TT genotype demonstrated higher net increase of anxiety in response to CCK-4 challenge than those with C alleles. This finding at least partly confirms the role of 102T–C polymorphism in anxiety.

Various factors need to be taken into account while interpreting the current findings and their discrepancies with previous data. The genetic substrates of different manifestations of PD, such as PA, anticipatory anxiety or agoraphobia may be heterogeneous and affected by environmental factors. The findings from previous association studies in PD may have been confounded by a complex nature of PD and high prevalence of other psychiatric or somatic morbidity. Indeed, most of the polymorphisms explored in this study were previously investigated in samples with high prevalence of comorbid disorders, including mood and anxiety disorders. Plausibly, more complex clinical phenotypes may have stronger genetic substrates than pure panic condition. Although our study was conducted in a reasonably large sample of HV, we cannot exclude that sample size was not sufficient to detect more subtle associations. The candidate polymorphisms in this study were chosen mainly based on our group's positive findings in PD patients. For this reason, and in order to validate previous positive associations, we did not correct for multiple testing; however, a conservative estimation of significance with correction for multiple comparisons would have eliminated the major finding in the TPH2 gene. Notably, there are numerous recent investigations on other genetic polymorphisms potentially associated with PD (Maron *et al*., 2008a), which could be candidates for further challenge studies.

In summary, our findings lend tentative support to the possible importance of TPH2 rs1386494 SNP in the susceptibility to PA.

Study 3. Gender differences in brain serotonin transporter availability in panic disorder.
The overall rmANOVA showed a highly significant group × sex interaction (F=15.93, p<0.001), as well as a significant group × sex × region interaction (F=5.51, p=0.015). These effects suggested region-specific and sex-dependent group differences in 5-HTT BP_{ND}.

Next, the sex-dependent group differences were modelled in each brain region separately. Consistent with the overall analysis, statistically significant group × sex interactions specifically in the dorsal anterior cingulate cortex

(F=12.20, p=0.002), medial (F=6.59, p=0.016) and dorsolateral (F=10.18, p=0.003) prefrontal cortex, anterior (F=7.26, p=0.011) and posterior (F=10.35, p=0.003) insula, medial (F=13.39, p=0.001) and lateral (F=7.54, p=0.010) orbitofrontal cortex, middle (F=7.75, p=0.009) and superior (F=5.89, p=0.021) temporal gyri, angular gyrus (F=10.44, p=0.003), hippocampus (F=4.34, p=0.046) and the raphe nuclei (F=7.11, p=0.012) were observed.

Upon establishing sex-dependent group differences in 5-HTT BP_{ND} in several brain regions relevant to the pathophysiology of PD, group differences in these specific brain areas were estimated in both sexes separately. Consistent with region-specific group × sex interactions, male patients with PD demonstrated significantly and considerably elevated 5-HTT BP_{ND} compared with male control subjects in several brain regions, except for the hippocampus, which had significantly reduced 5-HTT BP_{ND} (Table 2). No statistically significant differences between patients with PD and HV were observed among females.

Table 2. Regional 5-HTT binding potential values in PD patients and healthy controls

ROS	SERT BP (mean ± SD) in males				SERT BP (mean ± SD) in females			
	Patients ($n=5$)	Controls ($n=12$)	Difference (%)	t-test p-value	Patients ($n=6$)	Controls ($n=12$)	Difference (%)	t-test p-value
ACD	0.57 ± 0.08	0.45 ± 0.06	+29	**0.001***	0.43 ± 0.08	0.46 ± 0.08	−7	0.42
ACS	0.64 ± 0.11	0.52 ± 0.08	+24	**0.02***	0.48 ± 0.11	0.49 ± 0.12	−5	0.68
ACV	0.53 ± 0.12	0.45 ± 0.12	+16	0.27	0.39 ± 0.11	0.41 ± 0.07	−5	0.67
AMY	0.90 ± 0.11	1.04 ± 0.18	−14	0.12	0.93 ± 0.16	0.93 ± 0.15	0	0.97
ANQ	0.30 ± 0.11	0.20 ± 0.03	+51	**0.01***	0.22 ± 0.06	0.24 ± 0.05	−8	0.46
DCA	1.10 ± 0.14	0.98 ± 0.14	+12	0.13	0.97 ± 0.15	1.06 ± 0.15	−8	0.27
DLP	0.41 ± 0.10	0.32 ± 0.06	+29	**0.03**	0.30 ± 0.07	0.37 ± 0.08	−18	0.11
DPU	1.22 ± 0.11	1.09 ± 0.12	+12	0.06	1.22 ± 0.11	1.20 ± 0.13	+1	0.79
GTM	0.35 ± 0.08	0.25 ± 0.05	+41	**0.004***	0.25 ± 0.04	0.27 ± 0.08	−9	0.48
GTS	0.36 ± 0.07	0.25 ± 0.06	+46	**0.004***	0.26 ± 0.09	0.25 ± 0.08	+4	0.80
HIP	0.39 ± 0.09	0.60 ± 0.21	−35	**0.048**	0.49 ± 0.10	0.47 ± 0.09	+6	0.59
INSA	0.54 ± 0.09	0.42 ± 0.08	+30	**0.01***	0.42 ± 0.06	0.45 ± 0.10	−6	0.52
INSP	0.77 ± 0.17	0.56 ± 0.08	+38	**0.003***	0.63 ± 0.12	0.61 ± 0.10	+3	0.78
MFC	0.44 ± 0.09	0.29 ± 0.06	+53	**0.0007***	0.34 ± 0.12	0.34 ± 0.09	−1	0.95
ORBL	0.25 ± 0.04	0.16 ± 0.05	+55	**0.005***	0.19 ± 0.09	0.21 ± 0.07	−7	0.71
ORBM	0.45 ± 0.13	0.28 ± 0.04	+65	**0.0005***	0.30 ± 0.12	0.36 ± 0.09	−16	0.25
RAPHE	4.07 ± 2.82	1.98 ± 0.61	+106	**0.02***	2.80 ± 1.35	2.82 ± 0.73	−1	0.96
THA	1.40 ± 0.13	1.34 ± 0.11	+5	0.32	1.36 ± 0.18	1.30 ± 0.38	+4	0.73
VLP	0.34 ± 0.06	0.25 ± 0.05	+35	**0.01***	0.25 ± 0.07	0.37 ± 0.30	−34	0.32
VST	1.45 ± 0.19	1.36 ± 0.21	+7	0.40	1.29 ± 0.14	1.26 ± 0.30	+3	0.79

ACD: dorsal anterior cingulate cortex, ACS: subgenual anterior cingulate cortex, ACV: ventral anterior cingulate cortex, AMY: amygdale, ANQ: angular gyrus, DCA: dorsal caudatus, DLP: dorsolateral prefrontal cortex, DPU: dorsal putamen, GTM: medial temporal gyrus, GTS: superior temporal gyrus, HIP: hippocampus, INSA: anterior insular cortex, INSP: posterior insular cortex, MFC: medial frontal cortex, ORBL: lateral orbitofrontal cortex, ORBM: medial orbitofrontal cortex, RAPHE: nucleus raphe, THA: thalamus, VLP: ventrolateral prefrontal cortex, VST: ventral striatum.
*p-values: adjusted p-values which remained significant after FDR correction.

Voxel-based analysis of BP_{ND} maps confirmed the findings from the regional analysis by showing a widespread increase in BP_{ND} in males with PD compared with male controls (Figure 2, Paper III). Consistent with the regional analysis, the largest effect size was found in the left orbitofrontal cortex (data not shown). No significant correlations were observed between 5-HTT BP_{ND} and

clinical scale scores obtained before PET scans neither in total sample nor in female PD patients separately.

In male patients, HAS scores were positively correlated with 5-HTT BP_{ND} in the dorsal anterior cingulate cortex (R=0.90, p=0.038), dorsal caudate (R=0.94, p=0.020), anterior insula (R=0.98, p=0.003), medial frontal cortex (R=0.94, p=0.019), and the lateral (R=0.98, p=0.003) and medial orbitofrontal cortex (R=0.93, p=0.024). Similarly, PDSS scores were positively associated with 5-HTT BP_{ND} in the ventral anterior cingulate cortex (R=0.96, p=0.010), dorsal caudate (R=0.94, p=0.019) and lateral orbitofrontal cortex (R=0.91, p=0.031), whereas a negative association was seen in the hippocampus (R= –0.97, p=0.006). Notably, the positive correlations were driven by one male subject with the highest symptom scores and also highest 5-HTT BP_{ND}.

Discussion. The results provided evidence of global changes in 5-HTT density in the brain, including the structures implicated in neuronal network of PD in males with PD as compared with HV. Specifically, male patients showed a higher 5-HTT BP_{ND} in the brainstem raphe, temporal gyri, anterior cingulate, insular, orbitofrontal, prefrontal and frontal cortices, but lower 5-HTT availability in the hippocampus. Thus, a reduced synaptic concentration of 5-HT in the raphe and several cortical regions, as a result of higher 5-HT re-uptake, but an elevated 5-HT neurotransmission in the hippocampus, as a result of lower 5-HT re-uptake, may together contribute to the manifestation of PD in males.

Such pattern is consistent with the concept of Deakin and Graeff (1991), which linked a deficit of 5-HT neurotransmission to panicogenesis and proposed a dual role of 5-HT in modulation of different types of anxiety. Our findings also suggest that 5-HT-ergic pathways to the hippocampus have distinctive impact on the panicogenesis independent from the cortical pathways. Similar regional differences were previously observed for benzodiazepine/ GABA-A receptor binding, which was reduced in multiple areas of the frontal, temporal, and parietal cortices (Malizia *et al*., 1998; Hasler *et al*., 2008), but increased in the hippocampus/parahippocampal region in patients with PD (Hasler *et al*., 2008). Pertinently, a recent PET study in male patients with PD demonstrated a global reduction of 5-HT1A receptor binding in overlapping brain regions (Nash *et al*., 2008), suggesting that reduced 5-HT neurotrans-mission via these receptors is one of the pathways contributing to the develop-ment of PD. This study also demonstrated that males with PD in remission after treatment with paroxetine showed normalized density of 5-HT1A postsynaptic receptors, but remaining reduction in the density of 5-HT1A receptors in the raphe and in the hippocampus, suggesting a trait nature of these alterations. According to the Deakin–Graeff hypothesis, the median raphe nucleus – hippocampal 5-HT pathway promotes resistance to chronic, unavoidable stress via 5-HT1A receptors, whereas failure of this mechanism may lead to depres-sion. Moreover, the neuronal network between the amygdala, hippocampus and

cortical structures may be involved in cognitive attributions of fear stimuli and the enhancement of memory function facilitating adaptive responses (Goddard & Charney, 1997). Thus, the dysfunction of the 5-HT system in the hippo-campus in males with PD may reflect an impaired tolerance of or a maladaptive responsiveness to stressors. The exact role of the hippocampal 5-HT system and its specificity to panicogenesis require further clarification.

Notably, the present study did not replicate the findings of our earlier SPECT study, which showed a reduced 5-HTT binding in the brainstem, the temporal lobes and the thalamus of females with PD. As compared with the former SPECT investigation, the present PET study had certain advantages, including higher resolution, regional volume co-registration and correction by MRI, and estimation of 5-HTT binding potential for more brain regions. Furthermore, the previously used tracer, [123I]nor-b-CIT, has high affinity to both 5-HTT and dopamine transporter, which may have biased the findings for brain 5-HTT availability. Importantly, females with PD in the current sample had more severe panic and anxiety symptoms on PDSS and HAS than female patients in previous sample, which may also account for discrepant results. As previously suggested (Maron & Shlik, 2005), the reduced 5-HTT availability in females with PD may reflect compensatory processes in the 5-HT system leading to mitigation of panic symptoms. If 5-HTT binding potential in PD is state-dependent, then such compensation in 5-HTT functioning is perhaps less obvious in more severe cases. However, the small sample size is another likely reason of the failure to demonstrate significant changes of 5-HTT availability in female patients in the present study.

The results of the present study may help to delineate the functional status of the 5-HT system in PD and other neuropsychiatric conditions. Indeed, our findings of increased 5-HTT BP_{ND} in males with PD are clearly distinguishable from available PET studies of 5-HT in various other neuropsychiatric conditions. Notably, all other 5-HTT PET studies have used tracers different from ours, mostly [11C]DASB and [11C]McN5652, and most of them were not designed to examine the brain 5-HTT density separately in male and female subjects. So far, a decreased regional 5-HTT BP_{ND} was demonstrated in patients with variety of diagnoses, including obsessive-compulsive disorder (Matsumoto *et al.*, 2010), unipolar and bipolar depression (Hammoud *et al.*, 2010; Oquendo *et al.*, 2007; Parsey *et al., 2006*), high-functioning autism (Nakamura *et al.*, 2010), impulsive aggression (Frankle *et al.*, 2005a) and methamphetamine abuse (Sekine *et al.*, 2006). 5-HTT density was found unaffected or normal in recovered male patients with depression (Bhagwagar *et al.*, 2007), former male users of MDMA 'ecstasy' (Selvaraj *et al.*, 2009) and in patients, mostly males, with type II alcoholism (Martinez *et al.*, 2009) or schizophrenia (Frankle *et al.*, 2005b). On the contrary, the recent PET study by Cannon and colleagues (2007) found that patients with unipolar and bipolar depression had similarly elevated 5-HTT density in the thalamus, insula and striatum, but showed distinct abnormalities in the brainstem. However, the effect of gender was not assessed

in this study, which included more females than males. Additionally, while no difference in 5-HTT BP_{ND} was detected between patients with acute unipolar depression and HV, the subgroup of patients with highly negativistic dysfunctional attitudes had significantly higher 5-HTT density in several brain regions, including prefrontal cortex, anterior cingulate, thalamus, bilateral caudate and bilateral putamen (Meyer *et al.*, 2004). The effect of gender in this study was not reported. Conclusively, PET studies have shown that only bipolar and probably some subtypes of unipolar depression might be characterized by higher brain 5-HTT availability; however, these data lack consistence and are not supported for males distinctively.

Several other aspects should be taken into account when interpreting our data. Although some structural MRI studies had detected a higher incidence of anatomical abnormalities in the brain of PD patients, particularly decreased volume in the temporal lobe, amygdala, parahippocampal gyrus and putamen (Vythilingam *et al.*, 2000; Massana *et al.*, 2003; Uchida *et al.*, 2003; Yoo *et al.*, 2005), but increased grey matter volume in the midbrain and left insula (Protopopescu *et al.*, 2006; Uchida *et al.*, 2008) as well as greater cingulate white matter connectivity (Han *et al.*, 2008), no significant regional volume differences were detected between PD patients and HV in the present study, indicating that increased 5-HTT BP_{ND} in our male patients could not be explained by structural changes. Importantly, control subjects underwent PET scans during the same seasonal intervals as PD patients to minimize possible seasonal effects on 5-HTT binding (Praschak-Rieder *et al.*, 2008). Additionally patients and controls did not significantly differ in respect to body mass index, smoking and allelic or genotypic frequencies of 5-HTT-linked promoter region polymorphism, which also minimize possible bias. Finally, although most of the studied brain regions withstood FDR correction, larger samples are still required to replicate our findings.

In summary, we obtained evidence of gender-dependent differences in the availability of brain 5-HTT in patients with PD. Male but not female PD patients demonstrated widespread alterations in the density of brain 5-HTT. These findings advance and challenge current understanding of the involvement of 5-HTT in panicogenesis. Other neuroimaging studies have demonstrated lower brain 5-HTT availability in depressed (Staley *et al.*, 2006) and healthy (Jovanovic *et al.*, 2008) females, as well as lower brain 5-HT synthesis rate (Nishizawa *et al.*, 1997), but higher 5-HT1A receptor BP (Jovanovic *et al.*, 2008) in females than in males. However, no sex differences in brain 5-HTT availability were detected in a large sample of HV (Praschak-Rieder *et al.*, 2008). Thus, further investigations are needed to clarify the impact of gender-distinct functions of the 5-HT system on the expressions of various mood and anxiety disorders, including PD.
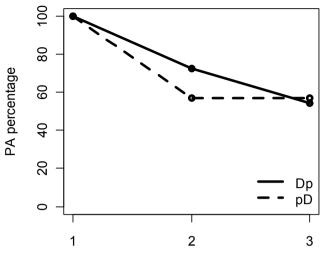
Study 4. The effect of 6-week treatment with escitalopram on CCK-4 challenge: a placebo-controlled study in CCK-4-sensitive healthy volunteers.
Pre-challenge data. The groups (Dp vs pD) did not significantly differ in their gender (p=0.367) or mean age (p=0.490). The distribution of 5-HTTLPR genotypes was similar between the groups: 42.9% in pD group and 36.4% in Dp group with LL genotype; 28.6% in pD and 45.5% in Dp with LS genotype; and 28.6% in pD and 18.2% in Dp with SS genotype (p=0.846). The groups were also similar in all other pre-challenge variables. No significant effect of active treatment or placebo on any of the pre-challenge variables was observed, and no significant differences between the groups in any of the pre-challenge baseline variables (psychological or cardiovascular) were detected between different challenge days.

Post-challenge effects - panic attacks. After the first treatment period, 8 of 11 (72.7% (95% CI 39.0...94.0%)) treated with ESC and 4 of 7 (57.1% (95% CI 18.4...90.1%)) treated with placebo had a PA during the second challenge. On the 3rd challenge performed after the second treatment period, 4 of 7 (57.1% (95% CI 18.4...90.1%)) treated with ESC and 6 of 11 (54.5% (95% CI 23.4...83.3%)) treated with placebo experienced a PA (Figure 2).

Pooling the data of all subjects independent of treatment, a remarkable reduction in panic rates was observed in the course of the first treatment period: from the 18 subjects who all had a PA on the 1st challenge, 12 (66.7% (95% CI 41.0...86.7%)) had PA on the 2nd challenge and 10 (55.6% (95% CI 30.6...78.5%)) on the 3rd challenge. The difference between the 2nd and 3rd challenge was not significant (χ^2=0.005, df=1, p=0.943). Comparing the frequencies of PA in the ESC-treated subjects on the 2nd and 3rd challenge no significant differences were found between the challenges (p=0.742). The same appeared to be valid for the groups of placebo pre-treated subjects (p>0.9).

Pooling the data of both treatment types (second and third challenge), 10 subjects of 18 (55.6%) had a PA during challenge after treatment with placebo, (4 of 7 (57.1%) in the pD group and 6 of 11 (54.5%) in the Dp group). During the challenge after treatment with ESC, 12 of 18 subjects (66.7%) had PA (4 of 7 (57.1%) in the pD group and 8 of 11 (72.7%) in the Dp group). The carry-over effect of treatment, the time effect as well as the drug effect all were not significant. As there was no carry-over or time effects, the proportions of PA were compared among drug receivers (12 of 18) and placebo receivers (10 of 18), and the difference was not significant (χ^2=0.1169, df=1, p=0.732).

Figure 2. Proportion of subjects responding to CCK-4 challenge with a panic attack. Abbreviations: *PA*- Panic attack, *Dp* – group that received ESC prior to second provocation and placebo prior to third provocation; *pD* – group that received placebo prior to second and ESC prior to third provocation.

Other variables. Also for the rest of the variables the carry-over effect, the time effect and the drug effect were not significant. After Holm-Bonferroni correction the difference of second and third provocation from the first did not appear significant for any of the variables assessed.

Effect of 5-HTTLPR polymorphism. The effect of 5-HTTLPR polymorphism was assessed comparing SS vs. SL vs. LL as well as SS+SL vs. LL and LL+LS vs. SS. Neither main effect of 5-HTTLPR polymorphism nor the effect of 5-HTTLPR polymorphism and treatment interaction were significantly associated with any of the outcome variables.

Gender effect. A secondary analysis with breakdown by gender did not reveal any significant differences in panic rates by gender after correction for multiple testing. No significant gender or gender-treatment interaction effects were detected for any other variables after correction for multiple testing.

Discussion. In the present study we observed a significant reduction in the rate of CCK-4-induced PA after 6-week treatment with either ESC or placebo in CCK-4-sensitive HV. After the first treatment period, the overall pre-treatment CCK-4-induced panic rate of 100% was reduced to 72.7% (8 of 11) with ESC and 57.1% (4 of 7) with placebo.

These findings partly concur with studies in patients with PD, showing that 5-HT-ergic antipanic antidepressants lessen CCK-4-induced PA (Bradwejn &

Koszycki, 1994b; Shlik *et al.*, 1997; van Megen *et al.*, 1997; Tõru *et al.*, 2006). However, in contrast to the findings in patients with PD, where the drug outperformed placebo (van Megen *et al*., 1997), no significant difference in response to CCK-4 challenge was observed between the ESC and placebo conditions. The same was valid for the second treatment period. While the reduction in CCK-4-induced panic rate in PD patients treated with a 5-HT-agent has been attributed to an increased availability of synaptic 5-HT, the similarity between the reduction in CCK-4-induced panic rate after both ESC and placebo treatments in our study questions the importance of synaptic availability of 5-HT in CCK-4-induced panic proneness in HV.

Similarly to our study, Kellner and colleagues (2009) found no inhibitory effect of ESC upon CCK-4-induced panic symptoms in HV. Plausibly, level of synaptic 5-HT may be affected differently in PD patients than in HV, e.g. SSRI may change the level of 5-HT in PD patients, but not, or much less so, in HV.

Differences in 5-HTT and 5-HT1A receptor densities between PD patients and HV may explain different responses to ESC. So, Maron and colleagues (2004a) showed in a SPECT study that patients with current PD had significantly lower 5-HTT binding in the midbrain raphe, in the temporal lobes and in the thalamus, while the patients in remission had significantly lower 5-HTT binding only in the thalamus. Neumeister and colleagues (2004), using PET, demonstrated a marked reduction of 5-HT1A receptor binding in the anterior and posterior cingulate cortices, and in the midbrain raphe in patients with PD compared to HV. Nash and colleagues (2008) have demonstrated in another PET study a reduced 5-HT1A receptor binding in the raphe region and in the amygdala as well as in the orbitofrontal and temporal cortices in untreated PD patients, with reduced density in the raphe and in the hippocampus persisting after recovery with an SSRI paroxetine. Altogether these data suggest state-, trait- and regional differences in brain 5-HT function between HV and patients with PD that may result in a different response to a 5-HT-ergic treatment.

However SSRIs do seem to increase 5-HT synaptic levels in HV as demonstrated in the studies by Attenburrow and colleagues (2001), Bhagwagar and colleagues (2002), and Kellner and colleagues (2009), showing significant increases of plasma cortisol and prolactin after active treatment, indicating the central effects of the drug. Also Argyropoulos and colleagues (2008) have found major effects of most SSRIs on 5HT-sensitive sleep measures in HV.

Notably, not all the patients receiving treatment with a 5-HT-ergic agent improve, e.g. according to Otto and colleagues (2001), ca 45% of PD patients treated with an SSRI in controlled trials failed to achieve panic-free status. This may further imply that increased level of synaptic 5-HT may not be sufficient for achieving panic-free status, at least not in all patients. Also the data of Schruers and Griez (2004), who found that 6-week treatment with tianeptine, an antidepressant that in contrast to SSRIs is believed to increase 5-HT reuptake, had an antipanic effect in 35% CO_2 panic challenge in PD patients similarly to the effect seen with the SSRI paroxetine, support the notion that the hypothesis

of a direct causal relationship between increased level of synaptic 5-HT and protection from PA may be oversimplified, or at least depends on the type of challenge used (Bell *et al.*, 2002).

The CCK receptor function hypothesis for PD proposed by Bradwejn and Koszycki (1994b) and further outlined by Tõru and colleagues (2006) also deserves consideration in the present context. It suggests that SSRIs alter CCK neurotransmission in PD patients through desensitizing the postulated CCK-2 receptor (CCK-2R) hypersensitivity (Bradwejn & Koszycki, 1994a). It is theorized that an increase in synaptic 5-HT causes an increased release of CCK (Raiteri *et al.*, 1993; Rosen *et al.*, 1995) resulting over time in a downregulation of CCK receptors, and thus, in a decreased sensitivity to CCK-4 challenge. Assuming that CCK-2R sensitivity in HV is already relatively low, the increased 5-HT levels are not likely to decrease CCK-2R sensitivity or cause only a small decrease that we could not detect.

The reduction in panic rates on repeated challenges seen in our study warrants a discussion on its possible causes. While panic rates decreased with both treatments after the first treatment episode, active treatment did not differ from placebo and numerically the reduction in panic rates was even more pronounced after placebo. This suggests that the observed reduction resulted from the placebo effect. After the second treatment episode, the reduction in panic rates seen with placebo was again numerically, but not significantly, bigger than with ESC, implying that ESC did not have any specific effect. In the first of the two earlier CCK-4 challenge studies with SSRIs that used placebo-controlled design, Van Megen and colleagues (van Megen *et al.*, 1997) found a significant decrease in panic rate after treatment with fluvoxamine (from 76% to 29%), but not after placebo (from 67% to 56%), supporting the idea of superiority of 5-HT-ergic drug over placebo. On the other hand, while comparing the treatment groups with respect to decline in panic rate from day 1 to day 56, they did not find significant group effect, suggesting that the difference between placebo and active treatment may have been not robust. In a more recent study, Kellner and his group (2009) showed no significant effect of treatment (ESC vs. placebo) on pre-CCK-4 challenge or CCK-4-induced increases in panic symptom scores in healthy males. Moreover, in the subjects with 5-HTTLPR s/s genotype, the increase in panic scores after CCK-4 was significantly higher after ESC, suggesting a stronger effect of placebo. A substantial placebo effect on CCK-4-induced panic was also observed in several other studies assessing the treatment effect of non-SSRI drugs (Depot *et al.*, 1999; Zwanzger *et al.*, 2003; Kronenberg *et al.*, 2005). Thus, the accumulating data suggest at least some role for placebo effect in modulating the panicogenic effects of CCK-4.

The aspects of novelty or anticipation may also be pertinent to the decreasing rate of CCK-4-induced PA on repeated challenges. The novelty may result in anxious interpretation of the emerging symptoms that could amplify overall intensity of response according to the cognitive model of clinical PA (Wells, 1997). In some subjects, after eliminating the novelty component, the potential of CCK-4 to induce PA may become lower. Such habituation might fit

with the present data where only the subjects who responded to the initial CCK-4 challenge with PA were selected for further study. It is plausible, that cognitive factors e.g. higher anticipation rather than the hypothesized higher sensitivity of CCK-2 receptors were crucial in determining panic reaction to the first challenge in some subjects. As by the second challenge the novelty had disappeared, the potential of CCK-4 to induce panic might have fallen below the critical level in some subjects explaining at least partly the reduction of panic rates from the 1st to 2nd challenge. Some, but not all previous studies have also implied a role for anticipation and habituation in response to panic challenges (Bradwejn *et al.*, 1992; 1994; Aluoja *et al.*, 1997; Zwanzger *et al.*, 2001; Radu *et al.*, 2003; Eser *et al.*, 2007; 2008; Hinkelmann *et al.*, 2010; Tõru *et al.*, 2010). Thus, anticipation or other psychological factors may be involved in determining the response to a laboratory challenge.

In the present study, apart from reduction in panic rates, no significant effects of either treatment were detected on any other panic or anxiety-related characteristics after corrections for multiple testing. Also in the study of Kellner and colleagues (2009) no significant differences in treatment effect on any CCK-4-induced panic indices were observed Thus, the findings in HV are in contrast to the findings in patients with PD, where in addition to changes in panic rates, treatment induced changes in several other characteristics of panic response have been observed, although not consistently (Bradwejn & Koszycki, 1994b; Shlik *et al.*, 1997; van Megen *et al.*, 1997). These differences may be explained by more robust panic responses to CCK-4 and higher sensitivity to antipanic treatment in the patients leading to more pronounced changes in panic responses as a result of treatment. On the other hand, corrections for multiple comparisons seemed to be omitted in the earlier studies, leaving the option open for overstating significance of the differences.

Effect of gender
Although PD is more frequent in females than in males (Kessler *et al.*, 2006) the CCK-4-induced panic does not seem to be robustly gender-dependent. In our recent study with more than 100 HV we found that panic rate was similar in males and females (Tõru *et al.*, 2010). Nevertheless, as gender dependent regional differences in brain 5-HTT binding properties (Maron *et al.*, 2011) as well as in some genes involved in regulation of serotonergic activity (Maron *et al.*, 2010a) have been reported, one might assume gender effects on 5-HT-CCK interaction patterns. However, the findings of the present study argue against a major role of gender in the treatment response to an SSRI or placebo.

Effects of 5-HTTLPR
Although the major influence of 5-HTTLPR in PD remains unproven (Blaya *et al.*, 2007; Maron *et al.*, 2010a), there are data suggesting that 5-HTTLPR variance might have at least some role (Hamilton *et al.*, 1999; Maron *et al.*, 2005a). There are also some data demonstrating that 5-HTTLPR genotype may influence

vulnerability to experimentally induced panic (Schmidt *et al.*, 2000). On the contrary, in PD patients CO_2 reactivity was not influenced by 5-HTTLPR genotype (Perna *et al.*, 2004). There are also preliminary data suggesting that 5-HTTLPR variance gender-dependently influences the treatment response to a 5-HT-ergic agent both in PD patients and in HV (Maron *et al.*, 2004b; Perna *et al.*, 2005; Kellner *et al.*, 2009). Our present data did not reveal any significant effects of 5-HTTLPR variants on treatment response. It cannot be excluded however, that our negative result is a consequence of insufficient sample size resulting in very small genotype subgroups. Also, we did not take into account the third functional variant of the l allele (Neumeister *et al.*, 2006).

Several methodological issues of the present study should be noted. First, there is a possibility that the drug treatment was insufficient. Although the ESC dose of 10 mg/day is commonly used and effective in clinical practice (Stahl *et al.*, 2003), it positions on the lower end of dosages used (Townsend & Conrad, 2007). Further, the mean ESC plasma levels in our sample (15.24 in Dp group and 23.9 in pD group), although comparable to mean values seen in clinical settings (Baumann *et al.*, 2004; Reis *et al.*, 2009), fell into the lower part of recommended therapeutic range (Baumann *et al.*, 2004). Also, the ESC plasma concentrations in our sample varied remarkably between the individuals and the different assessments of the same individual (expanding from 1.1 ng/ml to 41.35 ng/ml), thus concentration of ESC could have been below the effective level in some cases. Further, the 6-week duration of treatment may have been insufficient to cause changes in the 5-HT system necessary for the reduction of panic response to CCK-4. Finally, the possible confounding effect of the menstrual cycle phase on response to CCK-4 (Le Melledo *et al.*, 1999) could not be taken into account in present study due to protocol demands for challenge times.

In conclusion, the present study demonstrates that in CCK-4-sensitive HV 6-week treatment with an SSRI ESC or placebo both significantly reduce the rate of CCK-4-induced PA. However, differently from what was found in patients with PD, the effect of medication was not different from placebo. Accumulating data suggest that the phenomena of placebo effect and habituation may have a bigger role in the context of pharmacological modulation of CCK-4-induced panic than assumed after initial studies. The exact nature of mechanisms underlying the reduction in CCK-4 sensitivity seen in our sample e.g. increased 5-HT availability, placebo effect of both treatments, desensitization of receptors, dissipation of novelty, remains to be clarified. Our data call for caution in applying CCK-4 challenge in HV to screen antipanic properties of drugs, particularly in respect to the effects of chronic treatment, and question the validity of pre-selected CCK-4-sensitive HV as a proxy for patients with PD in this type of studies.

GENERAL DISCUSSION

The presented studies were conducted in order to investigate the modulatory role of 5-HT on CCK-4-induced PA in respect to the pathophysiology of PD. Studies 1 and 4 focused on the direct influence of 5-HT availability on CCK-4-induced panic response. Studies 2 and 3 investigated the relationship of panic propensity to biogenetic markers, such as the variations in 5-HT-ergic genes and the brain 5-HTT binding, which could explain the differences between HV and patients with PD in their susceptibility to PA and response to treatment.

Two opposing hypotheses have been put forth to explain panic phenomena by 5-HT-ergic dysfunction: 5-HT excess or overactivity (Iversen, 1984; Kahn *et al.*, 1988a; Kahn *et al.*, 1988b) and 5-HT deficit or underactivity (Deakin & Graeff, 1991; Bell & Nutt, 1998). While the 5-HT excess theory suggests that patients with PD either have an increased level of 5-HT release or a hyper-sensitivity in postsynaptic 5-HT receptors, the 5-HT deficit theory proposes that, in particular brain regions, such as the dorsal periaqueductal gray (PAG), 5-HT has a restraining effect on panic behavior and a 5-HT deficit may facilitate panic. Although both theories are substantiated by some data, the accumulating evidence from clinical and experimental research preferably supports the inhibitory influence of 5-HT on panic (Maron & Shlik, 2005).

A number of studies have addressed the antipanic effect of 5-HT in the context of experimental panic challenge with CCK-4. Several investigations in patients with PD have shown that the treatment with 5-HT-ergic antidepressants reduced CCK-4 sensitivity (Bradwejn & Koszycki, 1994b; Shlik *et al.*, 1997; van Megen *et al.*, 1997), assumingly by increasing the level of synaptic 5-HT. In HV, an acute pre-treatment with 5-HT precursor 5-HTP significantly reduced manifestations of CCK-4-induced panic, particularly in females (Maron *et al.*, 2004c). Our present results (Tõru *et al.*, 2006); Tõru *et al.*, submitted), concurring with some other studies (Koszycki *et al.*, 1996; Depot *et al.*, 1999; Kellner *et al.*, 2009) challenge this straightforward hypothesis and suggest that the interactions between 5-HT and CCK may be more complex. Specifically, we found (Tõru *et al.*, 2006) that an acute decline in the central availability of 5-HT did not reverse the protective effect of SSRI treatment on CCK-4-induced panic in HV. Although a nearly 30% panic rate, similar to what was previously observed with CCK-4 challenge after SSRI treatment in patients with PD (Shlik *et al.*, 1997; van Megen *et al.*, 1997), suggested that the sensitivity to CCK-4 challenge was effectively decreased by the treatment with CIT in this study, the lack of the effect of TD seems to contradict the notion of crucial importance of synaptic 5-HT level in determining the panic response to CCK-4 challenge, inferred from the earlier studies. Specifically, although TD by itself was not panicogenic in unmedicated PD patients (Goddard *et al.*, 1994), it increased ventilation in PD patients (Kent *et al.*, 1996) and revealed susceptibility to PA when combined with such challenge agents as adrenergic stimulant yohimbine (Goddard *et al.*, 1995) or inhaled carbon dioxide (Miller *et al.*, 2000; Schruers

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et al., 2000). Furthermore, TD reversed the antipanic effect of treatment with an SSRI paroxetine in PD patients when using a challenge with flumazenil, a benzodiazepine receptor antagonist (Bell *et al*., 2002). On the other hand, Koszycki and colleagues (1996) demonstrated that without influencing the panicogenic effect of CCK-4, TD augmented CCK-4-induced release of ACTH, cortisol and prolactin in healthy males, suggesting that 5-HT systems may have a role in the neuroendocrine, but not in the psychological actions of CCK-4.

Altogether these findings seem to support the notion that the increase in synaptic 5-HT conveys protection against panic. However, while some of these studies suggest, that the antipanic effect of SSRI-s may crucially depend upon the availability of 5-HT in the brain, and that the decrease in 5-HT neuro-transmission has a potential to increase the vulnerability to PA, others indicate, that the effect of reduction in brain 5-HT on panic propensity is not very robust, and may affect only part of panic symptomatology, if at all. Furthermore, a study by Depot and colleagues (Depot *et al*., 1999) suggested that CCK-5-HT interactions may be complex. They reported that an acute administration of a selective 5-HT3-receptor antagonist ondansetron significantly decreased CCK-4-induced panic in healthy males and reduced cortisol, growth hormone and prolactin response. Thus, at least in regard to 5-HT3 receptors, a reduction rather than increase in 5-HT effects was associated with the alleviation of CCK-4-induced panic. Furthermore, these effects dissipated upon chronic treatment with ondansetron, indicating that adaptive changes that abolish the initial effect are likely to occur.

In contrast to the findings in patients with PD, treatment with an antipanic SSRI ESC in CCK-4-sensitive HV did not cause a reduction of CCK-4-induced PA beyond the effect of placebo (Tõru *et al*., submitted). While the reduction in CCK-4-induced panic rate in PD patients treated with a 5-HT-ergic agent has been attributed to an increased availability of synaptic 5-HT, the similarity between the reduction in CCK-4-induced panic rate after ESC and placebo treatments in our study questions the importance of synaptic availability of 5-HT in CCK-4-induced panic proneness in HV. Similarly to our study, Kellner and colleagues (2009) found no inhibitory effect of ESC upon CCK-4-induced panic symptoms in HV. Plausibly, the level of synaptic 5-HT may be affected differently in PD patients than in HV, e.g. SSRI may change the level of 5-HT in PD patients, but not, or much less so, in HV. The difference in response to 5-HT-ergic modulation of CCK-induced panic between HV and PD patients was also observed in the studies with a 5-HT3 receptor antagonist ondansetron, which acutely attenuated CCK-4-induced PA in HV (Depot *et al*., 1999), but did not prevent the panicogenic effects of CCK-4 analogue pentagastrin in patients with PD (McCann *et al*., 1997).

The brain imaging studies of 5-HTT and 5-HT1A have been used to test the 5-HT functional differences between patients with PD and HV. The initial SPECT study by Maron and colleagues (Maron *et al*., 2004a) showed that when compared to HV, the patients with current PD had significantly lower 5-HTT

binding in the midbrain raphe, in the temporal lobes and in the thalamus, while the patients in remission had significantly lower 5-HTT binding only in the thalamus. Neumeister and colleagues (2004), using PET, have demonstrated a marked reduction of 5-HT1A receptor binding in the anterior and posterior cingulate cortices, and in the midbrain raphe in patients with PD compared to HV. Nash and colleagues (2008) have demonstrated, in another PET study, a reduced 5-HT1A receptor binding in the raphe region and in the amygdala, as well as in the orbitofrontal and temporal cortices in untreated PD patients, and still reduced density of 5-HT1A receptors in the raphe and in the hippocampus after recovery achieved with an SSRI paroxetine. Although the preliminary findings of reduced 5-HTT binding in PD (Maron *et al.*, 2004a) were not replicated in a recent PET study (Maron *et al.*, 2011), a higher 5-HTT binding potential in males with PD than in male controls was detected in the majority of the studied brain regions. However, no significant differences were found between the female PD patients and female controls. Altogether, these data suggest gender, state and trait dependent regional differences in the brain 5-HT turnover between the HV and patients with PD that may result in distinctive response to 5-HT-ergic treatment.

Another line of research has addressed the genetic aspects of 5-HT system in HV and PD patients. In line with the findings in patients with PD (Maron *et al.*, 2007), we detected an association between TPH2 rs1386494 polymorphism and susceptibility to CCK-4-induced PA in HV (Maron *et al.*, 2008b). This finding indicates possible importance of this polymorphism, respective gene, and 5-HT in the mechanisms of panic. However, none of the other 5-HT-related polymorphisms previously associated with PD (5-HTTLPR, MAO-A VNTR, 5-HTR1A 1019CG (rs6295), 5-HTR2A 102TC (rs6313)) was linked to CCK-4-induced PA with the exception of 5-HTR2A 102T-C, where T alleles were associated with a higher anxiety response to CCK-4 challenge. The TPH gene finding remains inconclusive, as it may not withstand a more conservative estimation of significance with correction for multiple comparisons. Moreover, the allelic distributions of this variation and associations with PD phenotype in patients and CCK-4-induced PA in HV were reversed, indicating the need for replication and explanation of such discrepancy. Notably, the findings of this study did not confirm our preliminary evidence of the associations between 5-HTTLPR and panic response to CCK-4 challenge in smaller sample of HV (Maron *et al.*, 2004b). Overall, the data concerning genetic factors modulating susceptibility to PA and PD at present stage need to be interpreted with caution, as most of the findings are negative or inconclusive, and only the COMT Val158Met (rs4680) polymorphism has been implicated in several studies and confirmed in a recent meta-analysis.

Aside of possible differences in 5-HT turnover between HV and PD patients, the SSRIs increase 5-HT synaptic levels in HV as demonstrated in the studies by Attenburrow and colleagues (2001), Bhagwagar and colleagues (2002), and Kellner and colleagues (2009), showing significant effect on plasma cortisol

and prolactin after active treatment, indicating the central effects of the drug. Also Argyropoulos and colleagues (2008) have found major effects of most SSRIs on 5HT-sensitive sleep measures in HV. Thus, the lack of antipanic efficacy of SSRI-s in HV seen in our study (Tõru *et al*., submitted) as well as in the study of Kellner and colleagues (2009) cannot be ascribed to the lack of central effects of the drug. Notably, the clinical studies show that not all PD patients respond to SSRIs (Otto *et al*., (2001), implying that increased level of synaptic 5-HT may not always be sufficient for achieving panic-free status. Also the findings of Schruers and Griez (2004) that 6-week treatment with tianeptine, an antidepressant that in contrast to SSRIs is believed to increase 5-HT reuptake, had an antipanic effect in 35% CO_2 panic challenge in PD patients similarly to the effect seen with the SSRI paroxetine, support the notion that the hypothesis of a direct causal relationship between increased synaptic 5-HT and protection from PA may be oversimplified, or at least depends on the type of challenge used (Bell *et al*., 2002).

Altogether, the data presented so far suggest that the reduced sensitivity to CCK-4 after SSRI treatment may be related to mechanisms other than 5-HT availability in the brain. One possible explanation is that SSRIs alter CCK neurotransmission, perhaps through the desensitization of postulated CCK-2R receptor hypersensitivity (Bradwejn & Koszycki, 1994b), where variation in CCK-2R sensitivity may also explain the difference in panicogenic properties of CCK-4 between PD patients and HV (Bradwejn *et al*., 1991). Increasing brain 5-HT leads to an increased release of brain CCK (Raiteri *et al.*, 1993; Rosen *et al.*, 1995) that might over time down-regulate CCK receptors. Thus, SSRIs may initially increase the effects of CCK, perhaps explaining the early worsening in anxiety seen with these drugs in some patients, but the later desensitization of CCK-2R occurs independently of 5-HT availability in the synaptic cleft. Such explanation is compatible with the preclinical data on SSRI administration showing an initial increase in CCK transmission and the number of CCK receptors (Rosen *et al.*, 1995; Koks *et al.*, 1999). These effects apparently normalize upon chronic treatment, as demonstrated by Harro and colleagues (1997), who found no changes in the density of CCK receptors or in the content of CCK-related peptides after long-term treatment with various antidepressants, including SSRIs. If the sensitivity of CCK-2R, and not the synaptic availability of 5-HT, is a critical factor determining the reactivity to a CCK-4 challenge, then the effects of acute TD on CCK-4 sensitivity would not be as strong as the effects seen with other challenge agents in both, HV (Goddard *et al.*, 1995; Klaassen *et al.*, 1998; Miller *et al.*, 2000) and, even more robustly, PD patients (Kent *et al.*, 1996; Miller *et al.*, 2000; Schruers *et al.*, 2000), including those in remission (Bell *et al*., 2002). This hypothesis also concurs with the findings of Koszycki and colleagues (1996) showing lack of a robust effect of reduced level of synaptic 5-HT on CCK-4-induced panic. It is also compatible with the results of Kellner and colleagues (2009) and our study in HV (Tõru *et al*, submitted). Namely, assuming that CCK-2R sensitivity in HV is already relatively low, the

SSRI-induced higher 5-HT levels are not likely to decrease CCK-2R sensitivity or cause only small decrease that remained undetected.

Another explanation of the observed reduction in CCK-4-induced panic rates after treatment with an SSRI is the placebo effect. In the first of the two earlier CCK-4 challenge studies with SSRIs that used placebo-controlled design, Van Megen and colleagues (1997) found a significant decrease in CCK-4-induced panic rate after treatment with fluvoxamine, but not after placebo. However, on comparison of treatment groups over the course of treatment, they did not find a significant group effect, suggesting that the difference between placebo and active treatment may have not been robust. In a more recent study, Kellner and colleagues (2009) showed no significant effect of treatment with ESC vs. placebo on pre-CCK-4 challenge or CCK-4-induced increases in panic symptoms in healthy males. Moreover, in the subjects with 5-HTTLPR S/S genotype, the increase in panic intensity after CCK-4 was significantly higher after ESC, suggesting a stronger effect of placebo. A substantial placebo effect on CCK-4-induced panic was also observed in several other studies assessing the treatment effect of non-SSRI drugs (Depot *et al.*, 1999; Zwanzger *et al.*, 2003; Kronenberg *et al.*, 2005). Thus, the accumulating data suggest at least some role for placebo effect in modulating the panicogenic effects of CCK-4.

The aspects of novelty or anticipation may also be pertinent to the decreasing rate of CCK-4-induced PA on repeated challenges. The novelty may result in anxious interpretation of the emerging symptoms that could amplify overall intensity of response according to the cognitive model of clinical PA (Wells, 1997). In some subjects, after eliminating the novelty component, the potential of CCK-4 to induce PA may become lower. Such habituation might fit with the data of our study (Tõru *et al.,* submitted), where only the subjects who responded to the initial CCK-4 challenge with PA were included. It is plausible that cognitive factors, e.g. higher anticipation, were crucial in determining panic reaction to the first challenge in some subjects. As by the second challenge the novelty was lacking, the potential of CCK-4 to induce panic might have fallen below the critical level explaining the reduction of panic rates from the 1st to 2nd challenge. Some (pro refs), but not all (con refs) previous studies have also implied a role for anticipation and habituation in response to panic challenges (Bradwejn *et al.*, 1992; Bradwejn *et al.*, 1994; Aluoja *et al.*, 1997; Zwanzger *et al.*, 2001; Radu *et al.*, 2003; Eser *et al.*, 2007; Eser *et al.*, 2008; Hinkelmann *et al.*, 2010; Tõru *et al.*, 2010). Thus, anticipation or related psychological factors may be involved in determining the response to a laboratory challenge.

To conclude, it would apparently be an oversimplification to consider 5-HT system as the single or primary modulatory factor in determining the CCK-4-induced panic response as well as to assume a direct modulatory effect of 5-HT on CCK-4 induced panic. The molecular and receptor mechanisms behind the 5-HT effects on panic manifestations observed in the context of CCK-4 challenge warrant further studies. The specific effects of different subsets of 5-HT system and their respective regulating mechanisms, e.g. receptor subtypes,

regional differences in receptor binding properties and gene effects, require clarification. Importantly, both CCK and 5-HT systems affect and are influenced by many other neurotransmitters in the brain circuitry involved in processing and expression of panic and anxiety (Coplan & Lydiard, 1998). To capture an integrated view on panic mechanisms, the complex character of the 5-HT system itself as well as the interaction patterns between different neurotransmitter systems and psychological modulators have to be taken into account. A better understanding of 5-HT - CCK interaction patterns and underlying mechanisms should lead toward more comprehensive view on the mechanisms of PA and contribute to the improved clinical management of PD.

CONCLUSIONS

1. Acute decline in the central availability of 5-HT by means of TD did not reverse the protective effect of SSRI treatment on CCK-4-induced panic. This outcome differs from the findings with another panicogen, flumazenil, suggesting a distinctive modulatory role of 5-HT in response to various challenge agents.

2. On a panel of candidate genes, only rs1386494 A/G polymorphism in TPH2 gene was associated with CCK-4-induced panic attacks in HV with the frequencies of G/G genotype and G allele significantly higher in panickers. Other polymorphisms previously associated with PD were unrelated to CCK-4-induced PA, probably due to the differences between complex nature of PD and laboratory panic model.

3. 5-HTT BP_{ND} was significantly higher in 13 of 20 studied brain regions, including several cortical and raphe areas, but lower in the hippocampus in males with PD as compared with healthy males. No significant differences in 5-HTT BP_{ND} were observed between female patients and controls. These results suggest gender-dependent regional differences in the brain 5-HTT availability and converge with previous PET findings of reduced 5-HT1A receptor binding in similar brain areas in PD. Distinctive functioning of the 5-HT system in males and females may underlie certain gender differences in expressions of PD.

4. The treatment with SSRI ESC did not reduce the CCK-4-induced PA in CCK-4-sensitive HV beyond the effect of placebo in contrast to the findings in patients with PD. These results suggest that placebo effect and habituation may have a bigger role in the context of pharmacological modulation of CCK-4-induced panic than assumed previously. The exact nature of the mechanisms underlying the reduction in CCK-4 sensitivity (e.g. increased 5-HT availability, placebo effect of both treatments, desensitization of receptors, dissipation of novelty) remains to be clarified. Our data call for caution in applying CCK-4 challenge in HV to screen antipanic properties of drugs, particularly in respect to the effects of chronic treatment, and question the validity of pre-selected CCK-4-sensitive HV as a proxy for patients with PD in this type of studies.

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SUMMARY IN ESTONIAN

CCK-4 poolt indutseeritud
paanikavastuse serotonergiline modulatsioon

Sissejuhatus

Paanikahäire, üks põhilistest ärevushäiretest, on sage (esinemissagedus aasta jooksul 2.8% ning elu jooksul 4.7%) ning tõsine probleem nii seda põdevale haigele kui ühiskonnale (Kessler *et al*., 2006). Paanikahäiret iseloomustavad korduvad ootamatud paanikahood ning hirm nende taastekkimise või nendest tingitud võimalike kahjulike tagajärgede ees, mis sageli viib agorafoobia lisandumiseni (DSM-IV, RHK-10) (WHO, 1992; APA, 1994). Paanikahäire ja paanikahoogude uurimisel on edukalt rakendatud erinevaid eksperimentaalseid mudeleid, teiste hulgas paanikahoogude esilekutsumist koletsüstokiniini tetrapeptiidi CCK-4-ga (Eser *et al.*, 2007; Kellner, 2012). CCK-4 on endogeense neuropeptiidi sünteetiline analoog, mis, sõltuvalt annusest, tingib paanikahäirega haigetel spontaanselt esinevate paanikahoogudega sarnaste paanikahoogude tekke nii paanikahäirega haigetel kui tervetel vabatahtlikel, kusjuures viimased on vähem tundlikud (Bradwejn *et al*., 1991).

Paanikafenomeni paremaks mõistmiseks on uuritud ka CCK ning teiste neurotransmitterite interaktsioone. Mitmed uuringud on keskendunud serotoniini ning selle eelkäija trüptofaani moduleerivale mõjule CCK-4-poolt esile kutsutud sümptomitele nii paanikahäirega haigetel (Bradwejn & Koszycki, 1994b; Shlik *et al.*, 1997; van Megen *et al.*, 1997) kui tervetel vabatahtlikel (Koszycki *et al.*, 1996; Depot *et al.*, 1999; Maron *et al.*, 2004c). Nimetatud uuringud on näidanud, et tõhus paanikavastane ravi serotonergilise ravimiga, mis eeldatavasti tõstab sünaptilise 5-HT taset, vähendab paanikahäirega haigetel CCK-4 poolt indutseeritud paanikahoogude hulka ning intensiivsust. Tervete vabatahtlike puhul on tulemused 5-HT ning trüptofaani moduleeriva mõju kohta CCK-4 poolt indutseeritud paanikasümptomitele olnud vastuolulisemad.

Varasemad uuringud osutasid erinevustele nii paanikahäirega haigete ning tervete vabatahtlike tundlikkuses CCK-4-mõjule kui ka selle tundlikkuse serotonergilises moduleerimises. Leitud erinevused tundusid olevat, vähemalt osaliselt, seletatavad erinevustega paanikahäirega haigete ning tervete vabatahtlike aju serotonergilistes retseptor- ning transportersüsteemides (Maron *et al.*, 2004a; Neumeister *et al.*, 2004; Nash *et al.*, 2008) ning serotoniinisüsteemi tööd reguleerivate geenide aktiivsuses (Hamilton *et al.*, 1999; Maron *et al.*, 2004b; Maron *et al.*, 2005a; Perna *et al.*, 2005).

Uurimistöö põhieesmärgid

Käesoleva uurmistöö peaeesmärgiks oli täiendavalt hinnata serotoniinisüsteemi moduleerivat rolli CCK-4 poolt indutseeritud paanikavastusele paanikahäirega haigetel ja tervetel vabatahtlikel. Lisaks aju serotoniintaseme otsese farmakoloogilise manipuleerimise mõjule uuriti ka mõnede hüpoteetiliselt oluliste taustafaktorite (mõnede serotonergiliste geenide variatsioonide ning aju serotoniini transporteri süsteemi) rolli.

Töösse kaasatud uuringute spetsiifilised eesmärgid olid järgmised:

1. Uurida trüptofaani akuutse depletsiooni mõju CCK-4 poolt tingitud paanikasümptomitele paanikahäirega haigetel, kes olid eelnevalt positiivselt reageerinud ravile SSTI tsitalopraamiga.

2. Hinnata üheksa varasemalt paanikahäirega seostatud kandidaatgeeni variatsioonide (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) seoseid CCK-4 poolt tervetel vabatahtlikel esile kutsutud paanikavastusega.

3. Hinnata võrdlevalt serotoniini transporteri sidumisvõimet (a non-displaceable brain 5-HTT binding potential (BP_{ND})) aju erinevates piirkondades paanikahäirega mees- ja naishaigetel ning soo ja vanuse mõttes sobitatud tervetel vabatahtlikel kasutades positronemissioon-tomograafiat ning radioaktiivset markerit [11C]MADAM.

4. Hinnata SSTI estsitalopraami 6-nädalase ravikuuri mõju CCK-4 poolt indutseeritud paanikasümptomitele CCK-4-le „tundlikel" tervetel vabatahtlikel (olid eelnevalt reageerinud CCK-4 manustamisele paanikahooga).

Uuringute metoodika

Kõik uuringud teostati Tartu Ülikooli Psühhiaatriakliinikus, PET ja MRT uuringud teostati Turu Ülikooli PET Keskuses. Kõigi uuringute protokollid olid heaks kiidetud vastavate institutsioonide eetikakomiteede poolt, kõik uuritavad allkirjastasid informeeritud nõusoleku vormi.

Esimene uuring hindas trüptofaani depletsiooni (TD) poolt tingitud aju serotoniinitaseme ajutise languse mõju CCK-4 poolt tingitud paanikavastusele paanikahäirega haigetel, kes olid eelnevalt reageerinud 10 nädalasele ravile SSTI tsitalopraamiga. Uuringu eksperimentaalsesse faasi kaasati 18 paanikahäirega haiget (6 meest, 12 naist, keskmine vanus 34.5±9.3), kelle seisund uuringu alguses oli vastanud käesoleva paanikahäire diagnostilistele tunnustele, kellel 10.-nädalaks ei olnud tsitalopraamikuuri (keskmine annus 18.9±4.4 mg/p; vahemik 10–30 mg/p) mõjul esinenud vähemalt 2 eelnenud nädala vältel paanikahoogu ning kelle seisund oli raviarsti hinnangul ravi käigus palju või väga palju paranenud; eksperimentaalse perioodi kestel ravi jätkus. TD/CCK-4 provokatsioonid teostati topeltpimedal, platseebokontrollitud, tasakaalustatud ristuvate gruppide meetodil, mille kohaselt iga haige osales ca nädalase vahega

kahel sarnase korraldusega provokatsioonil. Depletsioonipäeval said haiged provokatsioonile eelnevalt trüptofaanivaba ning platseebopäeval trüptofaani sisaldava aminohappeid sisaldava joogi. Jookide järjekord määrati juhuslikult, joogid segati, järgides standardset protokolli (Young *et al.*, 1985). 5 tundi pärast aminohapete manustamist võeti vereanalüüs trüptofaani sisalduse määramiseks ning teostati CCK-4 provokatsioon – veenisisese süstena manustati ca 3 sekundi jooksul 25 μg füsioloogilises lahuses lahustatud CCK-4. Vastavalt protokollile hinnati CCK-4 mõju mitmetele käitumuslikele ning kardiovaskulaarsetele parameetritele.

Teine uuring hindas võimalikke seoseid 9 varasemalt paanikahäirega seostatud geenipolümorfismi (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) ning CCK-4 poolt tervetel vabatahtlikel esile kutsutud paanikavastuse vahel. Uuringus osales 110 ajalehekuulutuse peale pöördunud tervet vabatahtlikku (47 meest, 63 naist, keskmine vanus 22.2±5.2). Kaasamiseks pidi uuritav olema vanuses 18–50 aastat, nõutav oli psüühikahäirete puudumine käesolevalt ning ka anamneesis (seda nii uuritaval kui lähisugulastel), hea kehaline tervis ning eelneva CCK-4 provokatsioonikogemuse puudumine. Uuringupäeval manustati uuritavatele eelnevalt paigaldatud veenikanüüli kaudu kiire süstena füsioloogilises lahuses lahustatud CCK-4 (50 μg). Süste eelselt ning järgselt mõõdeti regulaarselt protokollis määratud käitumuslikke ning kardiovaskulaarseid karakteristikuid. Genotüpiseerimiseks võeti vereanalüüs, millest DNA eraldati kasutades standardset fenool-kloroform ekstraktsioonimeetodit. Polümorfismide määramine toimus vastavalt varem kirjeldatud protokollidele (Lesch *et al.*, 1996; Sabol *et al.*, 1998; Maron *et al.*, 2007)**.** Hinnati võimalike seoste olemasolu määratud geenivariatsioonide ning CCK-4 poolt indutseeritud paanikahoogude, aga ka muude mõõdetud provokatsioonivastuse karakteristikute vahel.

Kolmas uuring hindas võrdlevalt serotoniin transporteri sidumisvõimet (a non-displaceable brain 5-HTT binding potential (BP_{ND})) aju erinevates piirkondades paanikahäirega mees- ja naishaigetel ning soo ja vanuse mõttes sobitatud tervetel vabatahtlikel kasutades positron- emissioontomograafiat ning radioaktiivset markerit [^{11}C]MADAM. Uuringus osales 11 ravita (vähemalt 4 kuud) paanikahäirega haiget (5 meest, 6 naist; keskmine vanus 31.1±8.6) ning 24 tervet vabatahtlikku (12 meest, 12 naist; keskmine vanus 36.9±6.9). Kõik uuritavad olid kehaliselt terved. Haiged värvati SA TÜK Psühhiaatriakliiniku haigete hulgast, terved vabatahtlikud (nõutav oli psüühikahäirete puudumine anamneesis nii uuritaval kui lähisugulastel) kuulutuse vahendusel (Tartust ning Turust). [11C]MADAM valmistati vastavalt varem avaldatud protokollile (Halldin *et al.*, 2005). PET uuringud toimusid Turu Ülikooli PET keskuses kasutades kõrge lahutusvõimega spetsiaalset PET kaamerat HRRT (Siemens Medical Solutions, Knoxville, TN). PET uuring ning järgnev kujutiste analüüs toimus varemkirjeldatud protokolli kohaselt (Hirvonen *et al.*, 2008).

Neljas uuring hindas 6-nädalase estsitalopraami kuuri mõju CCK-4 provokatsioonivastusele eelnevalt CCK-4 mõjul paanikahoo saanud tervetel vabatahtlikel. Uuring koosnes 2-st osast. 82 uuritavat (29 meest, 53 naist; keskmine vanus 22.3±5.0), osales uuringu esimeses osas (manustati 50µg CCK-4). 37 (45.1%) uuritavale, kellel avaldus provokatsiooni käigus paanikahoog, tehti ettepanek osaleda uuringu teises osas, 27 neist nõustus. Uuringu teine osa koosnes topeltpimeda disainiga 6-nädalasest raviperioodist estsitalopraami (10 mg/p) või platseeboga, sellele järgnenud nädalasest väljauhtumisperioodist ning järgmisest 6 nädalasest raviperioodist ümbervahetatud ravimiga. Kummagi ravifaasi lõpus leidis aset CCK-4 provokatsioon (50µg CCK-4). Uuringu ravifaasis langes 4 vabatahtlikku välja (3 kõrvaltoimete, 1 raseduse tõttu). 4 uuritavat lülitati edasisest analüüsist välja puuduliku ravisoostumuse ning üks uuritav statistilis-metodoloogiliste kaalutluste tõttu, lõppanalüüsi lülitati 18 uuritava (10 meest, 8 naist, keskmine vanus 22.5±5.8) andmed. Plasma estsitalopraami kontsentratsiooni määramiseks võeti vereanalüüs kummagi raviperioodi kahes ajapunktis, kontsentratsiooni määramiseks kasutati vedelikkromatograafilist – massspektromeetrilist süsteemi (Agilent Series 1290 Infinity LC, Santa-Clara, CA, USA), uuritavate genotüpiseerimine toimus vastavalt varemkirjeldatud protokollile (Maron *et al*., 2004b).

Peamised tulemused

Esimene uuring. TD tingis kogu- ning vaba trüptofaani keskmise taseme languse vastavalt 73 ning 69%; pärast kontrolljooki tõusid vaba- ja kogutrüptofaani keskmised tasemed vastavalt 369 and 138%; keskmine TRP/LNAA suhe langes TD päeval 92% ning kontrollpäeval 46%. CCK-4 põhjustas mõlemal provokatsioonipäeval paanikahoo võrdselt ca 1/3-l haigetest (TD n=5, 27.8%, kontroll n=6, 33.3%; χ2=0.13, df=1, p=0.72); ka ühegi teise hinnatud näitaja osas depletsioonipäeva ning kontrollpäeva vahel erinevusi ei avaldunud.

Teine uuring. CCK-4 provokatsiooni käigus koges 110-st uuritavast paanikahoogu 39 (35.5%). Pärast genotüpiseerimist lülitati edasisest analüüsist välja kaks CCK retseptorite geenipolümorfismi – CCK2R −215C–A ning CCK1R 246G–A, kuna uuritavate hulgas ei esinenud kummagi SNP A alleeli kandjat. 5- HTTLPR genotüüpide ning alleelisageduste osas gruppide vahel olulisi erinevusi ei olnud. Võrreldes S alleeli kandjatega oli LL genotüübiga uuritavatel vahetult enne CCK provokatsiooni oluliselt kõrgem baasärevuse tase (VAS-iga mõõdetud) (*p* = 0.02). Ükski muu hinnatud ärevuse parameeter 5-HTTLPR polümorfismiga ei seostunud. MAO-A VNTR polümorfism ei korreleerunud paanikahoogude ega ühegi muu hinnatud ärevuse parameetriga. TPH2 geeni rs1386494 A/G polümorfismi osas esines gruppide vahel oluline erinevus – nimelt esines paanikahoo saanud uuritavatel G/G genotüüpi ning G alleeli oluliselt enam. Edasine analüüs näitas gruppidevahelist olulist erinevust naiste (vastavalt p=0.038 ning p=0.048), mitte aga meeste osas (vastavalt p=0.26 ning p=0.09). Ühegi muu mõõdetud parameetriga nimetatud polümorfism oluliselt

seotud ei olnud. Ülejäänud uuritavatest polümorfismidest ükski CCK-4 poolt indutseeritud paanikavastusega ei seostunud. Täiendav analüüs näitas, et 5-HT1A −1018 polümorfismi CC genotüübiga uuritavatel oli kõrgem skoor PSS kognitiivsete sümptomite alaskaalal kui CG või GG genotüübi kandjatel (F(2.11)=3.5; p=0.03) ning et 5-HT2A 102 polümorfismi TT genotüübi kandjatel tingis CCK-4 kõrgema ärevuse tõusu VAS skaalal kui CT või CC genotüübi kandjatel (F(2.11)=3.1; p=0.049).

Kolmas uuring. Analüüs näitas olulist interaktsiooni grupi ning uuritavate soo vahel (F=15.93, p<0.001) ning ka grupi, uuritavate soo ja ajupiirkonna vahel (F=5.51, p=0.015), mis osutas gruppide soost sõltuvatele regionaalsetele erinevustele serotoniini transporteri sidumisvõimes (5-HTT BP_{ND}). Erinevused avaldusid mitmes paanikahäire patofüsioloogia seisukohalt olulises ajupiirkonnas, nimelt dorsaalses eesmises tsingulaarkoores (F=12.20, p=0.002), mediaalses (F=6.59, p=0.016) ja dorsolateraalses (F=10.18, p=0.003) prefrontaalkoores, insula eesmises (F=7.26, p=0.011) ja tagumises (F=10.35, p=0.003) osas, mediaalses (F=13.39, p=0.001) ja lateraalses (F=7.54, p=0.010) orbitofrontaalkoores, keskmises (F=7.75, p=0.009) ja ülemises (F=5.89, p=0.021) temporaalkäärus, angulaarkäärus (F=10.44, p=0.003), hipokampuses (F=4.34, p=0.046) ning raphe tuumades (F=7.11, p=0.012). Edasine analüüs näitas, et PH-ga meestel oli 5-HTT BP_{ND} kõnealustes piirkondades võrreldes tervete kontrollisikutega oluliselt kõrgem, välja arvatud hipokampuses, kus 5-HTT BP_{ND} oli oluliselt madalam. Naiste puhul gruppide (PH vs TV) vahel olulist erinevust ei avaldunud. Kogu grupi nagu ka naissoost uuritavate osas 5-HTT BP_{ND} ning enne PET uuringut täidetud kliiniliste skaalade skooride vahel olulist seost ei avaldunud. Meeshaigetel korreleerusid 5-HTT BP_{ND} väärtused HAS skooridega positiivselt dorsaalses eesmises tsingulaarkoores (R=0.90, p=0.038), dorsaalses sabatuumas (R=0.94, p=0.020), eesmises insulaarkäärus (R=0.98, p=0.003), mediaalses frontaalkoores (R=0.94, p=0.019) ning lateraalses (R=0.98, p=0.003) ja mediaalses orbitofrontaalkoores (R=0.93, p=0.024) ning PDSS skooridega ventraalses eesmises tsingulaarkoores (R=0.96, p=0.010), dorsaalses sabatuumas (R=0.94, p=0.019) ning lateraalses orbitofrontaalkoores (R=0.91, p=0.031); negatiivne korrelatsioon avaldus hipokampuses (R= – 0.97, p=0.006).

Neljas uuring. Grupid (Dp (ravim-platseebo) vs pD (platseebo-ravim)) ei erinenud üksteisest ei soolise jaotuse, uuritavate keskmise vanuse, 5-HTTLPR genotüüpide jaotuse, ega ühegi muu hinnatud provokatsioonieelse karakteristiku (ei psühholoogilise ega kardivaskulaarse) poolest ühelgi provokatsioonipäeval. Kumbki, ei estsitalopraam ega platseebo, avaldanud olulist mõju ühelegi hinnatud provokatsioonieelsetest karakteristikutest.

Peale esimest ravikuuri, II provokatsioonil, sai paanikahoo 11 estsitalopraamiga ravitud uuritavast 8 (72.7% (95% CI 39.0...94.0%)) ning 7-st platseeboga ravitud uuritavast 4 (57.1% (95% CI 18.4...90.1%)). III provokatsioonil sai 7-st estsitalopraamiga ravitud uuritavast paanikahoo 4 (57.1% (95% CI 18.4...

90.1%)) ning 11-st platseeboga ravitud uuritavast 6 (54.5% (95% CI 23.4...
83.3%)).

Summeerides kõigi uuritavate andmed (sõltumata ravist), avaldus esimese
ravikuuri tulemusena oluline paanikahoogude sageduse langus: 18 uuritavast,
kes kõik olid saanud paanikahoo I provokatsioonil, said II provokatsioonil
paanikahoo 12 (66.7% (95% CI 41.0...86.7%)) ning III provokatsioonil 10
(55.6% (95% CI 30.6...78.5%)). II ja III provoktasiooni erinevus oli statistiliselt
ebaoluline (χ^2=0.005, df=1, p=0.943). Nii ravimõju edasikandumise, provo-
katsiooni järjekorra, kui ka ravimi efekt olid statistiliselt ebaolulised. Paanika-
hoogude esinemissagedus ravimisaajate (12 uuritavat 18-st) ning platseebo-
saajate (10 uuritavat 18-st) vahel oluliselt ei erinenud (χ^2=0.1169, df=1, p=
0.732). Ka ühegi muu hinnatud parameetri osas gruppide vahel erinevusi ei
olnud.

5-HTTLPR polümorfism (võrreldes genotüüpe SS vs. SL vs. LL aga ka
SS+SL vs. LL ning LL+LS vs. SS), ega ka nimetatud polümorfismi ning ravi
interaktsioon oluliselt ühegagi hinnatud parameetritest ei seostunud. Samuti ei
olnud ühelegi hinnatud parameetrile olulist mõju uuritavate sool ega ka soo ning
ravi interaktsioonil.

Järeldused

1. Trüptofaani depletsioonist tingitud aju serotoniinitaseme ajutine alandamine
ei kaota SSTI raviga paranenud paaanikahäirega haigetel CCK-4 provokat-
sioonil avalduvat tsitalopraami paanikavastast toimet. Saadud tulemus erineb
teist tüüpi panikogeeni – flumaseniiliga saadud tulemustest, osutades võimalike-
le erinevustele 5-HT süsteemi moduleerivas rollis erinevate panikogeenide
korral.

2. Trüptofaani hüdroksülaasi 2 isovormi polümorfism 1386494A/G seostus
positiivselt CCK-4 poolt tingitud paanikahoogudega tervetel vabatahtlikel,
kusjuures G/G genotüübi ja G alleeli esinemissagedus oli oluliselt kõrgem
uuritavatel, kes said provokatsiooni käigus paanikahoo. Käesolev tulemus, toe-
tades varasemat paanikahäirega haigetel läbiviidud uuringus saadud tulemust,
osutab TPH2 rs1386494 polümorfismi ning paanikavalmiduse vahelisele
võimalikule seosele. Ükski teine uuritud kandidaatgeenide variatsioonidest
tervetel vabatahtlikel CCK-4 poolt indutseeritud paanikahoogudega ei assot-
sieerunud, osutades muuhulgas ilmselt teatud erinevustele kompleksse loomu-
sega paanikahäire ning laboratoorse paanikamudeli vahel.

3. Serotoniin transporteri sidumisvõime (5-HTT BP_{ND}) oli paanikahäirega
meestel võrreldes tervete meestega oluliselt kõrgem 13-s uuritud 20-st ajupiir-
konnast, sealhulgas mitmes kortikaalses ning raphe piirkonnas; madalam aga
hipokampuse piirkonnas. Naiste puhul tervete ning paanikahäirega haigete
vahel aju serotoniin transporteri (5-HTT BP_{ND}) sidumisvõime osas piirkond-
likke erinevusi ei avaldunud. Saadud tulemused osutavad uuritavate soost
sõltuvatele regionaalsetele erinevustele serotoniini transporteri tiheduses.

Leitud funktsionaalsed erinevused 5-HT süsteemis meeste ja naiste vahel võivad osaliselt seletada mõningaid soolisi erinevusi paanikahäire avaldumises.

4. Erinevalt paanikahäirega haigetest, ei alandanud ravi SSTI–ga CCK-4-le tundlike tervete vabatahtlike puhul paanikahoogude sagedust enam kui platseebo. Meie tulemused osutavad, et CCK-4 toime farmakoloogilise modulatsiooni kontekstis võib platseebo- ja/või habituatsiooniefektil olla esialgselt oletatust olulisem tähendus. Käesolevas uuringus täheldatud CCK-4–tundlikkuse languse täpsed põhjused (aju 5-HT taseme tõus, retseptorite desensitisatsioon, platseeboefekt, uudsuse kadu) vajavad selgitamist edasistes uuringutes. Saadud tulemused süvendavad kahtlusi, mis on tekkinud CCK-4 provokatsiooni rakendatavuse osas uute paanikavastaste ravimite katsetamiseks tervetel vabatahtlikel ning muudavad küsitavaks eelnevalt selekteeritud CCK-4 suhtes tundlike tervete vabatahtlike kasutamise otstarbekuse sedalaadi uuringutes.

ACKNOWLEDGEMENTS

PUBLICATIONS

CURRICULUM VITAE

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Education

1989–1995   University of Tartu, Faculty of Medicine
1995–1997   University of Tartu, Faculty of Medicine; Internship
1997–2001   University of Tartu, Faculty of Medicine; residency in psychiatry
2001–2006   University of Tartu, Faculty of Medicine; doctoral studies

Professional employment

2001   Tartu University Hospital, Clinic of Psychiatry; psychiatrist
2008   Tartu University Hospital, Department of Psychiatry; senior
              assistant, research fellow

Scientific work

My research interest has been mainly focused on neurobiological, genetic and psychological factors modulating the cholecystokinin tetrapeptide-induced panic response.

Membership in professional organizations

Estonian Association of Psychiatrists; Estonian Association of Cognitive and Behavioral Therapy.

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2001   Tartu Ülikooli Kliinikumi Psühhiaatriakliinik; arst-õppejõud psühhiaatria alal
2008   Tartu Ülikooli Psühhiaatriakliinik; vanemassistent, teadur

Teadustöö

Uurimistöö on põhiliselt keskendunud koletsüstokiniini poolt indutseeritud paanikavastust moduleerivate neurobioloogiliste, geneetiliste ning psühholoogiliste faktorite uurimisele.

Kuuluvus erialaühendustesse

Eesti Psühhiaatrite Selts; Eesti Kognitiivse ja Käitumisteraapia Assotsiatsioon.

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83. **Helgi Kolk.** Dyspepsia and *Helicobacter pylori* infection: the diagnostic value of symptoms, treatment and follow-up of patients referred for upper gastrointestinal endoscopy by family physicians. Tartu, 2003.

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