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ORIGINAL INVESTIGATION

Effects of tryptophan depletion and tryptophan loading on the affective response to high-dose CO_2 challenge in healthy volunteers

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Abstract
Rationale It has been reported that in panic disorder (PD), tryptophan depletion enhances the vulnerability to experimentally induced panic, while the administration of serotonin precursors blunts the response to challenges.
Objectives Using a high-dose carbon dioxide (CO_2) challenge, we aimed to investigate the effects of acute tryptophan depletion (ATD) and acute tryptophan loading (ATL) on CO_2-induced panic response in healthy volunteers.
Methods Eighteen healthy volunteers participated in a randomized, double-blind placebo-controlled study. Each subject received ATD, ATL, and a balanced condition (BAL) in separate days, and a double-breath 35% CO_2 inhalation 4.5 h after treatment. Tryptophan (Trp) manipulations were obtained adding 0 g (ATD), 1.21 g (BAL), and 5.15 g (ATL) of l-tryptophan to a protein mixture lacking Trp. Assessments consisted of a visual analogue scale for affect (VAAS) and panic symptom list. A separate analysis on a sample of 55 subjects with a separate-group design has also been performed to study the relationship between plasma amino acid levels and subjective response to CO_2.
Results CO_2-induced subjective distress and breathlessness were significantly lower after ATD compared to BAL and ATL ($p<0.05$). In the separate-group analysis, ΔVAAS scores were positively correlated to the ratio Trp:ΣLNAA after treatment ($r=0.39$; $p<0.05$).
Conclusions The present results are in line with preclinical data indicating a role for the serotonergic system in promoting the aversive respiratory sensations to hypercapnic stimuli (Richerson, *Nat Rev Neurosci* 5(6):449–461, 2004). The differences observed in our study, compared to previous findings in PD patients, might depend on an altered serotonergic modulatory function in patients compared to healthy subjects.

Keywords Anxiety · Panic · Stress · 5-HT · Hypercapnia · Tryptophan · Carbon dioxide · CO_2 challenge · Fear · Respiration

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Introduction

In patients affected by panic disorder (PD), acute tryptophan depletion (ATD) enhances the response to a number of panicogenic agents. This effect of ATD has been shown in studies which used inhalation of 35% and 5% carbon dioxide (CO_2) (Miller et al. 2000; Schruers et al. 2000) and infusion of flumazenil (Bell et al. 2002; Davies et al. 2006) as challenges to induce panic, but no effect has been observed using infusion of cholecystokinin tetrapeptide (CCK-4) (Toru et al. 2006). Pre-treatment with 5-hydroxytryptophan (5-HTP), the immediate precursor of

serotonin (5-HT), has been shown to blunt the response to 35% CO_2 challenge, indicating a "protective" effect on experimentally induced panic (Schruers et al. 2002b). These findings seem to indicate that in PD patients, fear or anxiety provoked by some panicogenic challenges is negatively correlated to the availability of 5-HT precursors.

Studies in healthy volunteers yielded inconclusive findings: reports showed that ATD failed to modify the panicogenic effects of CCK-4 (Koszycki et al. 1996) as well as the effects of 5% CO_2 inhalation (Miller et al. 2000) or the Read rebreathing test (Struzik et al. 2002). Two studies have tested the effects of 35% CO_2 in tryptophan (Trp) depleted subjects, one showing an increase of CO_2-induced neurovegetative symptoms but not subjective anxiety (Klaassen et al. 1998), while the other did not find any significant difference between ATD condition and placebo with regard to CO_2-provoked subjective effects (Hood et al. 2006). Administration of 5-HTP in healthy volunteers was shown to reduce CCK-4-induced panic attacks and panic-related cognitive symptoms, specifically in females (Maron et al. 2004), but had no effects on the response to CO_2 (Schruers et al. 2002b).

The discrepancies observed between PD patients and healthy controls, in terms of the effects of ATD on the subjective response to challenges might be due to the relative low sensitivity exhibited by healthy subjects to panicogenic agents. We have previously showed in healthy volunteers that the inhalation of CO_2 dose-dependently induces panic-like symptoms, and that high doses of CO_2 (double-breath of 35% CO_2) in healthy subjects might be as effective as moderate doses of CO_2 are in PD patients (Griez et al. 2007; Schruers et al. 2004b). Here, we intended to perform a study in healthy subjects using a high-dose CO_2 challenge in order to test whether we can reproduce in a non-clinical population the same modulating effects of 5-HT manipulation observed in PD patients on experimental panic response; for this purpose, we investigated the effects of ATD on CO_2-induced panic response in healthy volunteers.

Additionally, since the Trp suppletion studies with panicogenic challenges conducted in healthy subjects gave inconclusive results (Maron et al. 2004; Schruers et al. 2002b), we also tested the effects of acute tryptophan loading (ATL) on subjective response to CO_2 challenge.

Material and methods

Subjects

Healthy volunteers were recruited among students or staff, through advertisements from within the Vijverdal Psychiatric Hospital Maastricht (Mondriaan Zorggroep) and throughout the Maastricht University locations. The Medical Ethics Committee of the Academic Hospital Maastricht and Maastricht University approved the study, and the subjects were paid for their participation in the experiment. After a complete description of the study, a written informed consent was obtained from the subjects. The volunteers underwent collection of medical history and physical examination. Inclusion criteria were age between 18 and 65 years and a good present and past physical and mental condition. The latter was established using a structured psychiatric interview (Mini International Neuropsychiatric Interview) performed by a physician. Exclusion criteria were current psychopharmacological or psychological treatment, recent alcohol intake, substance or caffeine-related disorders, excessive smoking (>10 cigarettes a day), respiratory or cardiovascular disease, hypertension (diastolic >100 mmHg; systolic >170 mmHg), personal or family history of cerebral aneurysm, pregnancy, and epilepsy. Individuals were also excluded if they reported common specific fears or if they had a history of panic attacks, or a history of PD in a first-degree relative.

Design

Subjects were randomized in double-blind placebo-controlled cross-over study design. The subjects received three different gelatin-based mixtures (GBM) on three different days in a randomized order, to induce respectively the ATD condition, a balanced condition (BAL) and the ATL condition. On each day, after GBM administration, they underwent a double-breath CO_2 challenge.

A separate analysis has been conducted on a larger sample of subjects in a randomized separate-group design, in order to investigate the relationship between the ratio Trp to the sum of large neutral amino acids in plasma (Trp:ΣLNAA) and the subjective response to CO_2. Subjects were randomly assigned to one of the three groups: ATD condition, BAL condition, and ATL condition. This sample also included subjects who were enrolled in the cross-over study for which only the first study day was taken into consideration in the analysis.

Procedure

The subjects arrived at the clinic after an overnight fast. Blood was drawn to measure plasma Trp and large neutral amino acids (LNAA) levels. GBM were administered in the morning at about 9:30. After drinking the GBM, the subjects remained on the ward and were allowed to read or watch a nature documentary on video. Subjects had ad libitum access to mineral water, but they were asked to refrain from eating and drinking any xanthine beverages. At 4.5 h after GBM administration, other blood samples were collected to monitor plasma Trp and LNAA levels. Ten minutes

later, the subjects underwent a double-breath inhalation of a gas mixture containing 35% CO_2 and 65% O_2.

Gelatin-based mixtures

The gelatin consists of a hydrolysate collagen-protein comprising the entire range of amino acids in the form of peptides, but completely lacking Trp. After administration, these peptides are decomposed into amino acids, and the mechanism of depletion is identical to that of the "classic" amino acid mixture (Sambeth et al. 2009). The GBM was kindly provided by PB Gelatins (Tessenderlo Group, Belgium) in form of powder. Amino acid composition of the GBM can be found in Table 1. The drink was prepared mixing 100 g of the powder with 200 ml water at 50–70°C. The drink was kept refrigerated at 4°C and then kept at room temperature for 30 min before administration. The three GBM were identical in composition, except that 1.15 g of l-tryptophan and 5.15 g of l-tryptophan was added to the mixtures for the BAL and ATL conditions, respectively. No l-tryptophan was added for the ATD condition. The three GBM had the same color and taste.

Carbon dioxide challenge

The 35% CO_2-inhalation procedure was performed in accordance to a standardized protocol developed at the Maastricht Academic Anxiety Center (Griez et al. 1987; Griez and Schruers 1998). A gas mixture containing 35% CO_2/65% O_2 was delivered through a nasal–oral exercise self-administration facemask, using a double vital capacity inhalation technique. Before the challenge, the inspired vital capacity of every subject was measured using an analogue respirometer (Wright respirometer Mark 20) connected to the self-administration mask. The same respirometer measured the gas volume delivered at each inhalation. The inspired vital capacity with a double breath of air was measured on each occasion, and a challenge was considered adequate if it was more than 80% of the baseline vital capacity. The subjects were then given the self-administration mask and asked to exhale as deeply as possible. They were asked to take a maximal inspiration through the mask and to make a complete expiration outside the mask, immediately followed by a second maximal inspiration. At the end of the second inhalation, the subjects were asked to hold their breath for 4 s to enhance the alveolar gas exchange, and finally make a complete expiration outside the mask again.

Table 1 Amino acid spectrum

Amino acid spectrum (typical weight% on ds protein)	
Alanine	8.4
Arginine	7.7
Aspartic acid/asparagine	4.5
Cysteine	0.0
Glutamic acid/glutamine	10.0
Glycine	23.3
Histidine	0.9
Hydroxylysine	1.5
Hydroxyproline	12.3
Isoleucine	1.2
Leucine	2.6
Lysine	3.3
Methionine	0.9
Phenylalanine	1.6
Proline	13.7
Serine	3.4
Threonine	1.9
Tryptophan	0.0
Tyrosine	0.6
Valine	2.2

Amino acid analysis

Samples for determination of plasma amino acid levels were taken at baseline (T0) and 4.5 h after GBM administration (T1). Blood (10 ml) was collected by venepuncture in sodium heparin tubes at each time point immediately after the rating of subjective assessments. After collection, the blood samples were immediately centrifuged at 4°C (10 min at 4,000 rpm). Subsequently, 100 μl of plasma was mixed with 8 mg of sulphasalicyl acid and frozen at −80°C until the amino acid analysis was performed (van Eijk et al. 1993). Plasma amino acids were determined using a fully automated high-performance liquid chromatography system after precolumn derivatization with *o*-phthaldialdehyde (OPA). OPA-AA derivates of the amino acids were quantified with fluorescence detection. The concentrations of plasma amino acids were expressed as micromoles per liter (μmol/l) (van Eijk et al. 1993). The ratio of total Trp:ΣLNAA (LNAA, i.e., tyrosine, phenylalanine, leucine, isoleucine, and valine) at baseline and 4.5 h after GBM were used as endpoints to monitor changes in Trp availability.

Assessments

Rating scales to assess panicogenic effects of CO_2 challenge were chosen with reference to the definition of panic attack in DSM-IV TR diagnostic criteria (APA 2000). We used a visual analogue scale for affect (VAAS) labeled "fear or discomfort", ranging from 0 (no fear/discomfort at all) to 100 (the worst imaginable fear/discomfort). The participants were instructed to indicate

the amount of the subjective disturbance, in case of feeling either fear or discomfort following an established procedure (Colasanti et al. 2008).

Panic symptoms were evaluated using the Panic Symptom List (PSL-IV) (Schruers et al. 2000). This consists of a questionnaire listing 13 items, each representing one of the DSM-IV TR symptoms (i.e., palpitations; sweating; trembling; sensations of shortness of breath or smothering; feeling of choking; chest discomfort; nausea or abdominal distress; feeling dizzy, lightheaded, or faint; derealization or depersonalization; fear of losing control; fear of dying; paresthesias; chills or hot flushes). The participants were asked to rate the intensity of each symptom from 0 (absent) to 4 (very intense). The total scores thus ranged from 0 to 52.

VAAS and PSL-IV were administered at baseline (T0; pre-GBM administration), 1.5 h (T1), 3 h (T2), 4.5 h post-GBM administration (T3), and after CO_2 challenge (Post-CO_2). Post-CO_2 scores indicated the worst moment experienced by the subjects after inhaling the gas mixture.

Mood states were measured with the shortened 32-item validated version of the Dutch translation of the Profile of Mood States Scale (POMS) (Wald and Mellenbergh 1990), which consists of five mood scales (depression, tension/anxiety, vigor, anger/hostility, and fatigue). The POMS was administered at T0, T1, T2, and T3. Subjects were asked to rate the scale according to how they felt at that moment.

Data analysis

All the data are presented as mean ± standard deviation (SD). Percentage changes in Trp: ΣLNAA ratio (Δ% Trp:ΣLNAA ratio) after GBM (T3) compared to baseline (T0) were calculated by the formula T3-T0/T0×100. CO_2-induced changes in VAAS and PSL scores were expressed as Δ scores (obtained by the formula POST-CO_2 scores− T3 scores). In the cross-over sample, all data were analyzed with analysis of variance (ANOVA) for repeated measures with time and treatment condition as within-subjects factors. The effects of time X treatment condition interaction were studied to investigate the influence of GBM condition (ATD, BAL, ATL) on the subjective response to CO_2 measured with VAAS and PSL scores. The analysis of VAAS scores has been repeated after controlling for gender, and the interaction time X treatment condition X gender has been studied using ANOVA per repeated measures.

To investigate the influence of GBM conditions on POMS scores, the effects of time per se and time X treatment condition interaction were studied with ANOVA per repeated measures.

If indicated by a significant ANOVA condition effect, time effect, or time X condition interaction effect, a subsequent evaluation on difference between individual conditions and between individual time points was done by within-subject repeated contrasts. The level of significance was set at 0.05. For the separate-group analysis the principal statistical analysis consisted of Spearman's non-parametric correlation between ΔVAAS scores and Trp:ΣLNAA levels. A partial correlation analysis between these two variables was repeated after controlling for gender. Moreover, baseline differences between treatments/groups were analyzed by one-way ANOVA for continuous variables (age, weight, Trp:ΣLNAA ratio, VAS, and PSL scores) and chi-square for non-parametric variables (gender distribution).

Results

Eighteen healthy volunteers (10 male) completed the cross-over study (mean age 25±5.5 years).

One subject was excluded because she reported nausea and vomiting after the administration of GBM.

Amino acid levels

Plasma amino acid levels are presented in Fig. 1. Total Trp:ΣLNAA ratio at T0 did not significantly differ between treatment conditions (F=0.88, p=0.42). A significant time X condition interaction was found with Δ% Trp:ΣLNAA ratio (from T0 to T3) being different between conditions (F=106.6; p<0.0001). ATD resulted in a decrease of 61.35±15.6% in Trp:ΣLNAA ratio compared to baseline, while ATL resulted in an increase of 361.53±154.05%. A 17.76±19% post-GBM increase was found in the BAL condition. Within-subject contrasts evidenced significant differences between changes in Trp:ΣLNAA ratio between ATD and BAL, between ATL and BAL, and between ATD and ATL (F=106.6; p<0.0001). In three cases of ATD condition, we observed a decrease in Trp:ΣLNAA ratio <50%, and in one case of BAL condition, an increase in Trp:ΣLNAA ratio >50% was observed. For all the other subjects, we found Trp:ΣLNAA changes >50% in ATD condition, <50% in balanced condition, >100% in the ATL condition. The analyses reported in the following sections have also been performed after exclusion of those three subjects, obtaining the same results as with the complete sample.

Subjective measures

VAAS scores (fear/discomfort) between conditions at different time points, are presented in Fig. 2. There were no significant differences in VAAS scores at T0, T1, T2, and T3 between conditions. No effect of GBM administration per se was observed on VAAS scores, as no significant difference was found between any of pre-CO_2 challenge

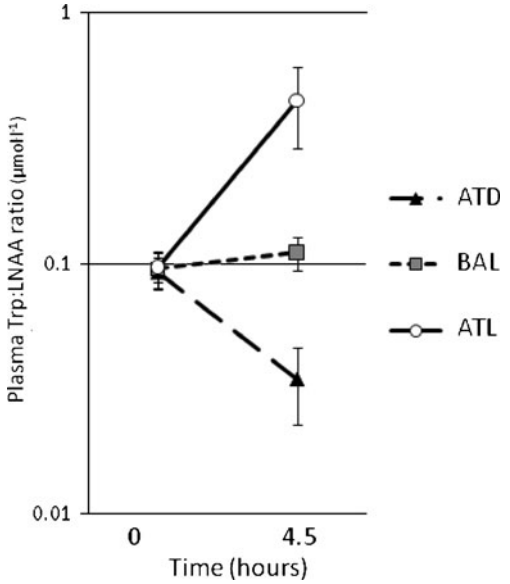

Fig. 1 Plasma Trp:ΣLNAA ratio at baseline and 4.5 h after treatment across conditions. Acute tryptophan depletion (*ATD*), balanced condition (*BAL*), acute tryptophan loading (*ATL*); time X condition interaction: $p<0.0001$; Trp:ΣLNAA ratio (μmol/l) is presented on logarithmic scale

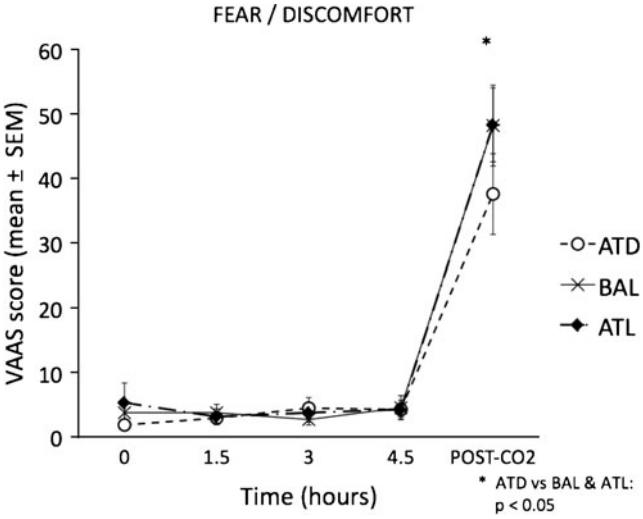

Fig. 2 VAAS scores for fear/discomfort at 0, 1.5, 3, 4.5 h after treatment, and after CO_2 inhalation, across treatment conditions. Acute tryptophan depletion (*ATD*), balanced condition (*BAL*), acute tryptophan loading (*ATL*); change in VAAS scores was significantly lower in ATD compared to BAL and ATL (time X condition interaction: $p<0.05$)

time points (T3, T2, T1) and T0 in any condition. CO_2 inhalation was followed by an increase in VAAS scores in all the conditions ($F=57.49$; $p<0.0001$). A significant time X condition interaction was found, indicating that ΔVAAS scores were significantly lower in ATD compared to BAL and ATL (33.89±26.18 vs 43.78±25.5 and 44.17±23.88, respectively; $F=5.79$ and $F=6.58$, $p<0.05$). No significant differences were found between BAL and ATL conditions. The findings remained identical after controlling for gender and no effects of time X gender X condition interaction have been found.

There were no significant differences in PSL scores between conditions at any time point. Total PSL scores did not change at T1, T2, and T3 compared to T0, but significantly increased after CO_2 inhalation, relative to T3 ($F=97.51$; $p<0.0001$). ΔPSL scores were similar between conditions (ATD, 11.11±6.35; BAL, 11.06±4.87; TL, 11.22±4.32; NS). Analyzing individual PSL items separately, the only significant effect of treatment conditions on ΔPSL scores was evident for the item "sensation of shortness of breath", indicating that ΔPSL scores after CO_2 were lower in ATD condition than in BAL and ATL conditions ($F=4.11$; $p<0.05$) (Fig. 3).

No order effect was found and ΔVAAS and ΔPSL scores were not affected by the order of administration of GBM conditions.

There was a significant effect of time on POMS-vigor ($p<0.0001$) and POMS-tension ($p<0.05$) scores, and within-subjects contrasts indicated that POMS-vigor scores were significantly higher at T0 relative to T1 ($p<0.01$), and significantly lower at T2 compared to T3 ($p<0.01$). POMS-tension scores were higher at T0 compared to T1 ($p<0.05$). There was no significant effect of the time X treatment condition interaction on POMS scores.

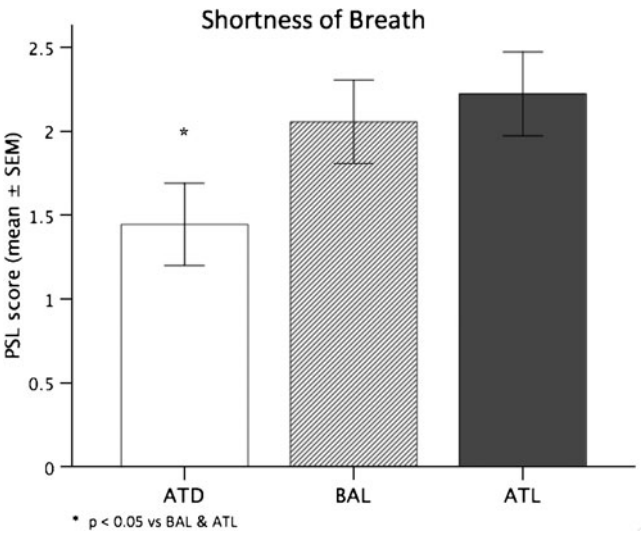

Fig. 3 Change in PSL scores for shortness of breath after CO_2 inhalation relative to T3, across treatment conditions. Acute tryptophan depletion (*ATD*), balanced condition (*BAL*), acute tryptophan loading (*ATL*); change in PSL scores was significantly lower in ATD compared to BAL and ATL (time X condition interaction: $p<0.05$)

Separate-group study

Fifty-five volunteers were enrolled in the separate-group study. Treatment groups consisted in 19 subjects (8 male; age: 24.84±5.33 years) in ATD condition, 19 subjects (10 male; age: 23.32±4.46 years) in BAL condition, and 17 subjects (10 male; 24.65±5.32) in ATL condition. Age, gender distribution, and weight did not significantly differ between groups. Trp:ΣLNAA ratio at T0 was similar between conditions and baseline VAAS scores and PSL scores did not significantly differ between groups either. The relationship between Trp:ΣLNAA ratio and ΔVAAS scores is presented in Figs. 4 and 5. ΔVAAS scores were positively correlated to Δ% Trp:ΣLNAA ratio and Trp:ΣLNAA ratio at T3 [Spearman's rho=0.395 ($p<0.005$) and 0.381 ($p<0.005$), respectively], indicating that higher VAAS scores were associated with larger increases in the ratio Trp:ΣLNAA.

No correlation was found between total PSL scores and Δ% Trp:ΣLNAA ratio or Trp:ΣLNAA ratio at T3.

Discussion

To investigate whether the amount of available 5-HT precursors influence the vulnerability to a panicogenic challenge, we tested the subjective response to a 35% CO_2 inhalation after acute Trp depletion, Trp loading, and placebo in healthy volunteers. We found a mild, but

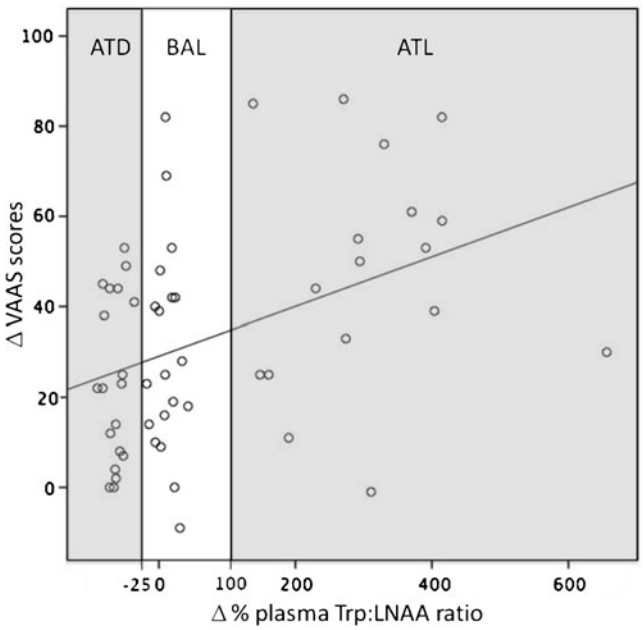

Fig. 4 Relationship between% change in Trp:ΣLNAA ratio after treatment and change in VAAS scores after CO_2 inhalation; change in VAAS scores after CO_2 inhalation was positively correlated to% change in Trp:ΣLNAA ratio [Spearman's rho=0.395, $p<0.005$]

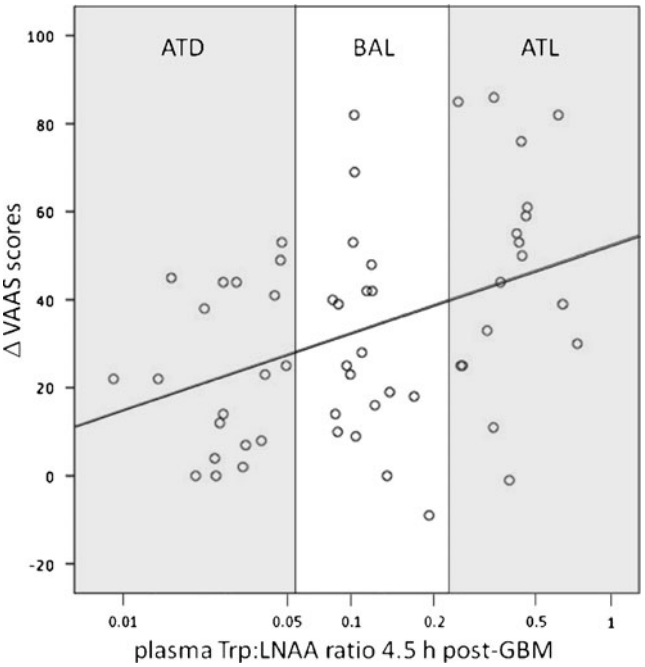

Fig. 5 Relationship between Trp:ΣLNAA ratio at T3 (4.5 h post-treatment) and change in VAAS scores after CO_2 inhalation; change in VAAS scores after CO_2 inhalation was positively correlated to Trp:ΣLNAA ratio at T3 [Spearman's rho=0.381, $p<0.005$]; Trp:ΣLNAA ratio (μmol/l) is presented on logarithmic scale

significant effect of treatment condition on the subjective scores of fear/discomfort, indicating that ATD is associated to a reduced response to a CO_2 inhalation relative to placebo and ATL. The same effect was also evident for scores on the panic symptom "shortness of breath". The analysis of the other separate individual symptoms and the total composite score of panic symptoms induced by CO_2 did not show any significant difference between conditions. In a separate analysis, we also investigated the relationship between Trp availability (Trp:ΣLNAA ratio) and subjective response to CO_2, as measured by VAAS scores, in a separate-group design. We found a relatively weak, but significant positive correlation between the Trp:ΣLNAA ratio and CO_2-induced changes in VAAS scores, indicating that lower availability of 5-HT precursors is associated to a blunted affective response to CO_2.

These findings are in contrast with previous results found in studies by our laboratory (Klaassen et al. 1998; Schruers and Griez 2004a; Schruers et al. 2000; Schruers et al. 2002a, b) and in other CO_2 challenge studies in healthy volunteers (Hood et al. 2006) and in PD patients (Miller et al. 2000). First, we will discuss potential explanations for the divergent findings observed in this study compared to other studies with inhaled CO_2 and other panicogenic agents in both healthy volunteers and PD patients. Furthermore, we will try to interpret our findings in the context of the complex relationship between panic, 5-HT, and CO_2, also in light of the recent data, which suggest that

5-HT neurons are sensitive to acute changes in CO_2 concentration. Methodological issues and limitations relative to the technique used to manipulate 5-HT precursors will be finally addressed.

Tryptophan depletion/suppletion studies in healthy volunteers and PD patients

A large body of evidence suggests that manipulation of 5-HT precursors availability has an influence in modulating panic and anxiety (see review by Maron et al. 2008). However, some of the findings from experimental studies, both in healthy volunteers and PD patients, are divisive and indicate that the role of 5-HT might not be unique.

Hood et al. (2006) and Klaassen et al. (1998) tested the effects of ATD on the response to single-breath 35% CO_2 challenge in healthy subjects. In a study on 14 healthy volunteers, no significant differences were found in subjective measures between ATD and balanced conditions, although ATD resulted in an increased CO_2-induced elevation of cortisol compared to the balanced condition (Hood et al. 2006). Klaassen et al. study (1998), including 15 volunteers, found a significant increase in CO_2-induced neurovegetative symptoms after ATD. In that study sample, mean net increases in total PSL scores and VAAS scores after CO_2 were <7 and <7, respectively (Klaassen et al. 1998). Subjective CO_2-induced anxiety was, therefore, very mild, hence indicating that single-breath 35% CO_2 in healthy subjects did not evoke "real" panic. In the present study, using double-breath CO_2, we found a more than 30% higher increase in PSL scores and more than 500% higher increase in VAAS scores after CO_2 compared to Klaassen et al. (1998) study. Average VAAS and PSL scores in the present double-breath 35% CO_2 study are comparable to those normally found in PD patients after single-breath 35% CO_2 (Verburg et al. 1998). Other challenges in healthy volunteers found no significant effect of ATD on responses to anxiogenic challenges. This has been showed using a 5% CO_2 challenge (Miller et al. 2000) and the hypercapnic Read rebreathing technique (Struzik et al. 2002), a simulated public speaking challenge (Monteiro-dos-Santos et al. 2000), and CCK-4 (Koszycki et al. 1996). In contrast, a study by (Goddard et al. 1995) showed increased nervousness in response to yohimbine challenge after ATD compared to yohimbine alone.

To enhance 5-HT availability in healthy subjects, (Maron et al. 2004) administered 5-HTP (direct precursor of 5-HT) and used CCK-4 as panicogenic challenge in 32 healthy volunteers. They observed a significant reduction compared to placebo in CCK-4-induced panic attacks and cognitive symptoms only in females, whereas in males only a decrease in somatic symptoms was observed. In Schruers et al. (2002b) study, 5-HTP did not alter the response to 35% CO_2 compared to placebo. Taken together, these data and our results indicate that the effects of Trp depletion in healthy volunteers largely depend on the type of challenge and its relative potency, and might be additionally confounded by gender effects.

Studies in PD patients demonstrated that ATD increased the response to single-breath 35% CO_2 (Schruers et al. 2000) and 5% CO_2 anxiety (Miller et al. 2000), and administration of 5-HTP blunted the response to single-breath 35% CO_2 (Schruers et al. 2002b)

ATD also increased the response to flumazenil infusion in PD patients successfully treated with selective serotonin reuptake inhibitors (SSRI) (Bell et al. 2002; Davies et al. 2006) or CBT (Bell et al. 2009). In contrast, subjective response to CCK-4 is not influenced by ATD (Toru et al. 2006) in SSRI-treated PD patients. Also, ATD did not have significant effects on the subjective response to anxiogenic challenges in OCD (Barr et al. 1994; Kulz et al. 2007) and in GAD SSRI-treated patients (Hood et al. 2010). It is interesting to note that in the latest study of Hood and colleagues (2010), using 7.5% CO_2 challenge, some subjective measures of anxiety ("something bad is going to happen", "anxious", "secure"), seemed to indicate an anxiolytic effect of ATD rather than anxiogenic; however, pairways comparison was not significantly different between conditions.

As a further confirmation of the role of 5-HT in modulating experimental panic, serotonergic drugs that are effective in treating panic disorder, like SSRI and tryciclics, also reduce the fear that patients with PD experience when they inhale CO_2 (Bertani et al. 2001; Bertani et al. 1997; Perna et al. 2004; Perna et al. 2002; Perna et al. 1997; Pols et al. 1996).

In summary, a number of studies in PD indicate that availability of 5-HT precursors is inversely related to vulnerability to CO_2 challenges, which is at odds with the present results. However, overall findings in anxiety disorder patients suggest that the effects of Trp manipulation specifically depend on the diagnosis and the type of anxiogenic challenge.

Panic, 5-HT, and CO_2: a complex relationship

Accumulating evidence from clinical and experimental research and genetic studies suggest a substantial role for the 5-HT system on the neurobiology of PD (see Maron and Shlik 2006). The relationship between 5-HT and panic is complex, as exemplified by the notion that SSRI are effective in reducing panic, but they may exacerbate anxiety during the initial phase of treatment (Sinclair et al. 2009). It has been hypothesized that panic is associated to either 5-HT excess (Iversen 1984) and 5-HT deficit (Deakin and Graeff 1991). Deakin & Graeff proposed that the 5-HT

system plays a dual role in the modulation of anxiety by inhibiting panic responses, but contributing to anticipatory or generalized anxiety. Our findings presented here are not in line with this theory, as in our experimental design increased 5-HT availability did not suppress CO_2-induced panic responses in healthy volunteers. However, recent studies suggest that other factors should be taken into account in understanding the relationship between 5-HT, CO_2 and anxiety: a subset of 5-HT neurons, located in the chemosensitive zone (ventrolateral medulla and raphe) and associated with large arteries, are intrinsically chemosensitive in vitro (Severson et al. 2003) and are stimulated by hypercapnia in vivo in unanaesthetized animals (Veasey et al. 1995, 1997). Furthermore, experiments using in vivo microdialysis showed that increasing inhaled CO_2 causes an increase in 5-HT release (Kanamaru and Homma 2007). Interestingly, mice selectively lacking 5-HT neurons display a blunted respiratory response to CO_2, indicating that 5-HT neurons are required for normal central chemoreception (Hodges and Richerson 2008). Richerson (2004) proposed that medullary 5-HT neurons control ventilatory responses to CO_2 and project to areas like forebrain and limbic system that are involved in affective regulation. Taking into account the panicogenic properties of CO_2, we have previously speculated that these neurons could be part of an adaptive protective mechanism alerting the organism against the risk of impending asphyxia (Griez et al. 2007). Taken all these preclinical findings together, it appears that acute hypercapnia stimulates serotonergic neurons and 5-HT release and that conversely, disruption of the 5-HT system blunts the neuronal response to CO_2.

Our data are in line with the above preclinical evidence; we have effectively reduced availability of 5-HT precursors and we have used CO_2 as a panicogenic challenge. In agreement with Hodges and Richerson (2008) data, we found that depletion of 5-HT precursors blunted the subjective response to CO_2, particularly the respiratory sensations, and Trp availability was positively correlated with the intensity of the subjective responses.

Methodological limitations

Some methodological considerations regard the validity of Trp manipulations to alter 5-HT availability. Trp depletion and Trp loading are relatively easy procedures to rapidly and reversibly change, decrease and increase respectively, the levels of 5-HT precursors (Hood et al. 2005). The first step in 5-HT biosynthesis is the conversion of Trp to 5-HTP by Trp hydroxylase. In the brain, this enzyme is only 50% saturated and the rate at which 5-HT is synthesized is limited only by substrate (Trp) availability. The LNAA transport system at the blood–brain barrier has a high affinity for all the LNAAs, including Trp. Therefore, the ratio Trp:ΣLNAA in plasma is generally used to predict the availability of Trp to the brain. A large body of literature provides evidence that manipulations of the levels of Trp in plasma results in a substantial and parallel alteration of 5-HT synthesis in the brain and availability of 5-HT and its metabolite in humans and animals (Biggio et al. 1974; Carpenter et al. 1998; Gessa et al. 1974; Leathwood 1987; Lieben et al. 2004; Williams et al. 1999). However, an altered release of 5-HT efflux (thought to reflect synaptic release, i.e., 5-HT neuronal activity) was only reported after chronic Trp depletion (Fadda et al. 2000) or after ATD in combination with 5-HT reuptake inhibition (Bel and Artigas 1996), but not after ATD alone (van der Plasse et al. 2007). In vitro studies suggest that an increase in Trp availability determines dose-dependent changes in 5-HT release under conditions of increased serotonergic neuronal activity but not on basal output of 5-HT (Sharp et al. 1992; Wolf and Kuhn 1986).

We assume that the alterations in the Trp:ΣLNAA ratio of the present study were followed by changes in brain 5-HT availability and synthesis in the same direction. However, at present, we cannot assure that Trp depletion and Trp loading actually influenced 5-HT release. Nevertheless, the possibility exists that the manipulations in the present study did affect 5-HT neuronal release, based on the abovementioned notions that acute hypercapnia stimulates serotonergic neurons and induces 5-HT release, and that Trp manipulations seem to be effective in altering 5-HT release only in stimulated neurons.

A number of other methodological issues need to be addressed. To manipulate brain Trp availability, the studies in healthy volunteers of both Klaassen et al. (1998) and Hood et al. (2006) included the use of the classic amino acid mixture (Young et al. 1985), the former including an addition of carbohydrates and fat. In these studies, ATD appeared to be as effective as it was in ours and resulted in a significant decrease of plasma Trp levels. However, the magnitude of the depletion seems difficult to compare with that in our study due to some methodological differences: in the study of Klaassen et al. (1998), no baseline Trp plasma levels were collected, and in the study of Hood et al. (2006), free Trp plasma levels instead of total TRP levels were used for calculation of the Trp:ΣLNAA ratio. It is still debated whether total Trp or free Trp levels in plasma are the most reliable indirect measures of brain Trp availability (Pardridge 1998). Therefore, methodological differences might account for the divergent findings in our study compared to previous studies in healthy volunteers.

Conclusions

It is recognized that 5-HT system plays a complex role in the regulation of the panic responses to CO_2, as different

serotonergic mechanisms can coexist, either inhibiting panic (Deakin and Graeff 1991) or promoting the aversive respiratory sensations to hypercapnic stimuli. The differences observed in our study in healthy volunteers, compared to previous findings in PD patients, might depend on the different relative contribution of these mechanisms in different populations.

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References

APA (2000) DSM-IV-TR diagnostic and statistic manual of mental disorders, fourth edition, Text Revision, 4th edn. American Psychiatric Association, Washington

Barr LC, Goodman WK, McDougle CJ, Delgado PL, Heninger GR, Charney DS et al (1994) Tryptophan depletion in patients with obsessive-compulsive disorder who respond to serotonin reuptake inhibitors. Arch Gen Psychiatr 51(4):309–317

Bel N, Artigas F (1996) Reduction of serotonergic function in rat brain by tryptophan depletion: effects in control and fluvoxamine-treated rats. J Neurochem 67(2):669–676

Bell C, Forshall S, Adrover M, Nash J, Hood S, Argyropoulos S et al (2002) Does 5-HT restrain panic? A tryptophan depletion study in panic disorder patients recovered on paroxetine. J Psychopharmacol 16(1):5–14

Bell C, Hood S, Potokar J, Nash J, Adrover M, Frampton C et al. (2009) Rapid tryptophan depletion following cognitive behavioural therapy for panic disorder. Psychopharmacology (Berl) (in press)

Bertani A, Perna G, Arancio C, Caldirola D, Bellodi L (1997) Pharmacologic effect of imipramine, paroxetine, and sertraline on 35% carbon dioxide hypersensitivity in panic patients: a double-blind, random, placebo-controlled study. J Clin Psychopharmacol 17(2):97–101

Bertani A, Caldirola D, Bussi R, Bellodi L, Perna G (2001) The 35% CO2 hyperreactivity and clinical symptomatology in patients with panic disorder after 1 week of treatment with citalopram: an open study. J Clin Psychopharmacol 21(3):262–267

Biggio G, Fadda F, Fanni P, Tagliamonte A, Gessa GL (1974) Rapid depletion of serum tryptophan, brain tryptophan, serotonin and 5-hydroxyindoleacetic acid by a tryptophan-free diet. Life Sci 14 (7):1321–1329

Carpenter LL, Anderson GM, Pelton GH, Gudin JA, Kirwin PD, Price LH et al (1998) Tryptophan depletion during continuous CSF sampling in healthy human subjects. Neuropsychopharmacology 19(1):26–35

Colasanti A, Salamon E, Schruers K, van Diest R, van Duinen M, Griez EJ (2008) Carbon dioxide-induced emotion and respiratory symptoms in healthy volunteers. Neuropsychopharmacology 33:3103–3110

Davies SJ, Hood SD, Argyropoulos SV, Morris K, Bell C, Witchel HJ et al (2006) Depleting serotonin enhances both cardiovascular and psychological stress reactivity in recovered patients with anxiety disorders. J Clin Psychopharmacol 26(4):414–418

Deakin J, Graeff F (1991) 5-HT and mechanisms of defence. J Psychopharmacol 5:305–315

Fadda F, Cocco S, Rossetti ZL, Melis G, Stancampiano R (2000) A tryptophan-free diet markedly reduces frontocortical 5-HT release, but fails to modify ethanol preference in alcohol-preferring (sP) and non-preferring (sNP) rats. Behav Brain Res 108(2):127–132

Gessa GL, Biggio G, Fadda F, Corsini GU, Tagliamonte A (1974) Effect of the oral administration of tryptophan-free amino acid mixtures on serum tryptophan, brain tryptophan and serotonin metabolism. J Neurochem 22(5):869–870

Goddard AW, Charney DS, Germine M, Woods SW, Heninger GR, Krystal JH et al (1995) Effects of tryptophan depletion on responses to yohimbine in healthy human subjects. Biol Psychiatry 38(2):74–85

Griez E, Schruers K (1998) Experimental pathophysiology of panic. J Psychosom Res 45(6):493–503

Griez E, Lousberg H, van den Hout MA, van der Molen MG (1987) CO2 vulnerability in panic disorder. Psychiatry Res 20:87–95

Griez EJ, Colasanti A, van Diest R, Salamon E, Schruers K (2007) Carbon dioxide inhalation induces dose-dependent and age-related negative affectivity. PLoS ONE 2(10):e987

Hodges MR, Richerson GB (2008) Contributions of 5-HT neurons to respiratory control: neuromodulatory and trophic effects. Respir Physiol Neurobiol 164(1–2):222–232

Hood SD, Bell CJ, Nutt DJ (2005) Acute tryptophan depletion. Part I: rationale and methodology. Aust N Z J Psychiatr 39(7):558–564

Hood SD, Hince DA, Robinson H, Cirillo M, Christmas D, Kaye JM (2006) Serotonin regulation of the human stress response. Psychoneuroendocrinology 31(9):1087–1097

Hood SD, Hince DA, Davies SJ, Argyropoulos S, Robinson H, Potokar J et al (2010) Effects of acute tryptophan depletion in serotonin reuptake inhibitor-remitted patients with generalized anxiety disorder. Psychopharmacology (Berl) 208(2):223–232

Iversen SD (1984) 5-HT and anxiety. Neuropharmacology 23 (12B):1553–1560

Kanamaru M, Homma I (2007) Compensatory airway dilation and additive ventilatory augmentation mediated by dorsomedial medullary 5-hydroxytryptamine 2 receptor activity and hypercapnia. Am J Physiol Regul Integr Comp Physiol 293(2):R854–R860

Klaassen T, Klumperbeek J, Deutz NE, van Praag HM, Griez E (1998) Effects of tryptophan depletion on anxiety and on panic provoked by carbon dioxide challenge. Psychiatr Res 77(3):167–174

Koszycki D, Zacharko RM, Le Melledo JM, Young SN, Bradwejn J (1996) Effect of acute tryptophan depletion on behavioral, cardiovascular, and hormonal sensitivity to cholecystokinin-tetrapeptide challenge in healthy volunteers. Biol Psychiatr 40 (7):648–655

Kulz AK, Meinzer S, Kopasz M, Voderholzer U (2007) Effects of tryptophan depletion on cognitive functioning, obsessive-compulsive symptoms and mood in obsessive-compulsive disorder: preliminary results. Neuropsychobiology 56(2–3):127–131

Leathwood PD (1987) Tryptophan availability and serotonin synthesis. Proc Nutr Soc 46(1):143–156

Lieben CK, van Oorsouw K, Deutz NE, Blokland A (2004) Acute tryptophan depletion induced by a gelatin-based mixture impairs object memory but not affective behavior and spatial learning in the rat. Behav Brain Res 151(1–2):53–64

Maron E, Shlik J (2006) Serotonin function in panic disorder: important, but why? Neuropsychopharmacology 31(1):1–11

Maron E, Toru I, Vasar V, Shlik J (2004) The effect of 5-hydroxytryptophan on cholecystokinin-4-induced panic attacks in healthy volunteers. J Psychopharmacol 18(2):194–199

Maron E, Shlik J, Nutt DJ (2008) Tryptophan research in panic disorder. International Journal of Tryptophan Research 1:3–12

Miller HE, Deakin JF, Anderson IM (2000) Effect of acute tryptophan depletion on CO2-induced anxiety in patients with panic disorder and normal volunteers. Br J Psychiatry 176:182–188

Monteiro-dos-Santos PC, Graeff FG, dos-Santos JE, Ribeiro RP, Guimaraes FS, Zuardi AW (2000) Effects of tryptophan depletion on anxiety induced by simulated public speaking. Braz J Med Biol Res 33(5):581–587

Pardridge WM (1998) Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. Neurochem Res 23 (5):635–644

Perna G, Bertani A, Gabriele A, Politi E, Bellodi L (1997) Modification of 35% carbon dioxide hypersensitivity across one week of treatment with clomipramine and fluvoxamine: a double-blind, randomized, placebo-controlled study. J Clin Psychopharmacol 17(3):173–178

Perna G, Bertani A, Caldirola D, Gabriele A, Cocchi S, Bellodi L (2002) Antipanic drug modulation of 35% CO2 hyperreactivity and short-term treatment outcome. J Clin Psychopharmacol 22 (3):300–308

Perna G, Bertani A, Caldirola D, Di Pasquale D, Migliarese G, Bellodi L (2004) Modulation of hyperreactivity to 35% CO2 after one week of treatment with paroxetine and reboxetine: a double-blind, randomized study. J Clin Psychopharmacol 24(3):277–282

Pols HJ, Hauzer RC, Meijer JA, Verburg K, Griez EJ (1996) Fluvoxamine attenuates panic induced by 35% CO2 challenge. J Clin Psychiatry 57(11):539–542

Richerson GB (2004) Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. Nat Rev Neurosci 5(6):449–461

Sambeth A, Riedel WJ, Tillie DE, Blokland A, Postma A, Schmitt JA (2009) Memory impairments in humans after acute tryptophan depletion using a novel gelatin-based protein drink. J Psychopharmacol 23(1):56–64

Schruers K, Griez E (2004) The effects of tianeptine or paroxetine on 35% CO2 provoked panic in panic disorder. J Psychopharmacol 18(4):553–558

Schruers K, Klaassen T, Pols H, Overbeek T, Deutz NE, Griez E (2000) Effects of tryptophan depletion on carbon dioxide provoked panic in panic disorder patients. Psychiatr Res 93 (3):179–187

Schruers K, van Diest R, Nicolson N, Griez E (2002a) L-5-hydroxytryptophan induced increase in salivary cortisol in panic disorder patients and healthy volunteers. Psychopharmacology (Berl) 161(4):365–369

Schruers K, van Diest R, Overbeek T, Griez E (2002b) Acute L-5-hydroxytryptophan administration inhibits carbon dioxide-induced panic in panic disorder patients. Psychiatr Res 113 (3):237–243

Schruers KRJ, van der Mortel H, Overbeek T, Griez E (2004) Symptom profiles of natural and laboratory panic attacks. Acta Neuropsychiatrica 16(2):101–106

Severson CA, Wang W, Pieribone VA, Dohle CI, Richerson GB (2003) Midbrain serotonergic neurons are central pH chemoreceptors. Nat Neurosci 6(11):1139–1140

Sharp T, Bramwell SR, Grahame-Smith DG (1992) Effect of acute administration of L-tryptophan on the release of 5-HT in rat hippocampus in relation to serotoninergic neuronal activity: an in vivo microdialysis study. Life Sci 50(17):1215–1223

Sinclair LI, Christmas DM, Hood SD, Potokar JP, Robertson A, Isaac A et al (2009) Antidepressant-induced jitteriness/anxiety syndrome: systematic review. Br J Psychiatr 194(6):483–490

Struzik L, Duffin J, Vermani M, Hegadoren K, Katzman MA (2002) Effects of tryptophan depletion on central and peripheral chemoreflexes in man. Respir Physiol Neurobiol 133(3):183–195

Toru I, Shlik J, Maron E, Vasar V, Nutt DJ (2006) Tryptophan depletion does not modify response to CCK-4 challenge in patients with panic disorder after treatment with citalopram. Psychopharmacology (Berl) 186(1):107–112

van der Plasse G, Meerkerk DT, Lieben CK, Blokland A, Feenstra MG (2007) Lack of evidence for reduced prefrontal cortical serotonin and dopamine efflux after acute tryptophan depletion. Psychopharmacology (Berl) 195(3):377–385

van Eijk HM, Rooyakkers DR, Deutz NE (1993) Rapid routine determination of amino acids in plasma by high-performance liquid chromatography with a 2-3 microns Spherisorb ODS II column. J Chromatogr 620(1):143–148

Veasey SC, Fornal CA, Metzler CW, Jacobs BL (1995) Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. J Neurosci 15(7 Pt 2):5346–5359

Veasey SC, Fornal CA, Metzler CW, Jacobs BL (1997) Single-unit responses of serotonergic dorsal raphe neurons to specific motor challenges in freely moving cats. Neuroscience 79(1):161–169

Verburg K, Pols H, de Leeuw M, Griez E (1998) Reliability of the 35% carbon dioxide panic provocation challenge. Psychiatr Res 78(3):207–214

Wald FD, Mellenbergh GJ (1990) De verkorte versie van de Nederlandse vertaling van de Profile of Mood States (POMS). Ned Tijdschr Psychol 45:86–90

Williams WA, Shoaf SE, Hommer D, Rawlings R, Linnoila M (1999) Effects of acute tryptophan depletion on plasma and cerebrospinal fluid tryptophan and 5-hydroxyindoleacetic acid in normal volunteers. J Neurochem 72(4):1641–1647

Wolf WA, Kuhn DM (1986) Uptake and release of tryptophan and serotonin: an HPLC method to study the flux of endogenous 5-hydroxyindoles through synaptosomes. J Neurochem 46(1):61–67

Young SN, Smith SE, Pihl RO, Ervin FR (1985) Tryptophan depletion causes a rapid lowering of mood in normal males. Psychopharmacology (Berl) 87(2):173–177