

Acute Shift in Glutamate Concentrations Following Experimentally Induced Panic with Cholecystokinin Tetrapeptide—A 3T-MRS Study in Healthy Subjects

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According to preclinical studies, glutamate has been implicated in the pathogenesis of anxiety. In order to elucidate the role of glutamate in anxiety and panic in humans, brain glutamate + glutamine (Glx) levels were measured during cholecystokinin-tetrapeptide (CCK-4)-induced panic using magnetic resonance spectroscopy (MRS). Eighteen healthy subjects underwent a CCK-4 challenge. MR spectra were obtained from the anterior cingulate cortex (ACC) using a single voxel point-resolved spectroscopy method and analyzed using LCModel. A combined fitting of Glx was performed. Panic was assessed using the Acute Panic Inventory (API) and Panic Symptom Scale (PSS) scores. Moreover, hypothalamic–pituitary–adrenal axis stimulation was monitored throughout the challenge. There was a significant panic response following CCK-4 as revealed by a marked increase in both the panic scores (API: $F(1,17) = 149.41$; $p < 0.0001$; PSS: $F(1,17) = 88.03$; $p < 0.0001$) and heart rate (HR: $F(1,17) = 72.79$; $p < 0.0001$). MRS measures showed a significant increase of brain Glx/creatine (Glx/Cr) levels peaking at 2–10 min after challenge ($F(1,17) = 15.94$; $p = 0.001$). There was also a significant increase in CCK-4-related cortisol release ($F(6,11) = 8.68$; $p = 0.002$). Finally, significant positive correlations were found between baseline Glx/Cr and both API_{max} ($r = 0.598$; $p = 0.009$) and maximum heart rate (HR_{max}) during challenge ($r = 0.519$; $p = 0.027$). Our results suggest that CCK-4-induced panic is accompanied by a significant glutamate increase in the bilateral ACC. The results add to the hypothesis of a disturbance of the inhibitory–excitatory equilibrium and suggest that apart from static alterations rapid and dynamic neurochemical changes might also be relevant for the neural control of panic attacks.

Neuropsychopharmacology (2013) **38**, 1648–1654; doi:10.1038/npp.2013.61; published online 17 April 2013

Keywords: panic attacks; anxiety; glutamate; CCK-4; magnetic resonance spectroscopy; anterior cingulate cortex

INTRODUCTION

With a lifetime prevalence of 3.5%, panic disorder (PD) represents one of the most frequent psychiatric disorders (Kessler *et al*, 1998; Roy-Byrne *et al*, 2000). The disorder is characterized by the occurrence of sudden, unexpected panic attacks, which are accompanied by several somatic symptoms such as palpitations, dyspnea, dizziness, headache, nausea, and others (White and Barlow, 2002). The etiology is complex and comprises both neurobiological and psychosocial aspects (White and Barlow, 2002; Gorman *et al*, 2000; Martin *et al*, 2010).

There is increasing evidence that an excitatory–inhibitory dysbalance might have an important role in the pathophysiology of PD. In this regard, several studies point towards a role of the gamma-amino-butyric acid (GABA) system, suggesting a decreased GABAergic tone as a key factor for the pathogenesis of panic and anxiety. Investigations using positron-emission tomography have shown that patients with PD have a decreased sensitivity of GABA_A receptors (Malizia *et al*, 1998; Hasler *et al*, 2008). Moreover, a decrease in cortical GABA concentrations has been demonstrated in studies using magnetic resonance spectroscopy (MRS) (Goddard *et al*, 2001). Alterations have also been found in concentrations of neuroactive steroids, which are known to act as allosteric modulators at the GABA_A receptor (Strohle *et al*, 2002). Finally, the well-known rapid and strong anxiolytic effects of benzodiazepines are mediated by the benzodiazepine-binding site at the GABA_A-receptor (Ballenger *et al*, 1988; Zwanzger and Rupprecht, 2005; Domschke and Zwanzger, 2008).

Glutamate represents the most important excitatory neurotransmitter and deploys its excitatory action via

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Received 9 November 2012; revised 5 February 2013; accepted 8 February 2013; accepted article preview online 5 March 2013

binding at the *N*-methyl-D-aspartate (NMDA) and the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor, which are predominantly located in the cortical and limbic structures (Bergink *et al*, 2004; Bliss and Collingridge, 1993). According to preclinical studies, glutamatergic signal transduction in the lateral amygdala has a significant role in fear-conditioning and extinction (Rogan *et al*, 1997). In humans, so far only few studies have looked at the possible role of glutamate in anxiety disorders. With regard to pharmacological intervention, the following studies point towards anxiolytic effects of glutamate antagonists: In patients with major depression and bipolar disorder, beneficial effects have been observed after treatment with NMDA antagonists (Berman *et al*, 2000). Moreover, positive results have been reported in experimentally induced anxiety using beta-carboline after treatment with the glutamate antagonist riluzole (Stutzmann *et al*, 1989).

Experimental panic induction has been established as a valid and reliable paradigm for the assessment of neurobiological correlates in panic attacks, anxiety, and acute stress. Among the available panicogenic agents, the neuropeptide cholecystokinin-tetrapeptide (CCK-4) has been shown to induce panic attacks both in patients with PD and in healthy volunteers (Zwanzger *et al*, 2012). Moreover, panic symptoms elicited by CCK-4 are attenuated after pharmacological treatment: Several studies have shown that treatment with antidepressants, which currently represent first-line treatment for PD, lead to a reduction of panic experimentally induced by CCK-4 (Bradwejn and Koszycki, 1994; Shlik *et al*, 1997). Similarly, anxiolytics such as the benzodiazepine alprazolam ameliorate CCK-4-induced panic attacks in healthy volunteers (Zwanzger *et al*, 2003). Such effects have also been observed in larger samples using XBD173, a ligand of the translocator (TPSO) protein (Rupprecht *et al*, 2009), which recently has been identified as a possible new treatment target for anxiety (Rupprecht *et al*, 2010; Nothdurfter *et al*, 2012). As shown by neuroimaging studies, experimentally induced panic attacks are accompanied by a significant activation of neuroanatomical key areas responsible for the processing of fear and anxiety, such as the amygdala, hippocampus, insula, and the anterior cingulate cortex (ACC) (Schunck *et al*, 2006; Eser *et al*, 2009). Finally, the ACC has been identified as a major target for benzodiazepines in CCK-4-induced panic (Leicht *et al*, 2013).

In order to further elucidate the role of an excitatory-inhibitory dysbalance in the pathogenesis of panic and anxiety, brain glutamate + glutamine (Glx) levels in the ACC were assessed during CCK-4-induced panic attacks by means of 3 Tesla proton magnetic resonance spectroscopy (3T-MRS). For correlation with subjects' individual stress response, hypothalamic-pituitary-adrenal (HPA) axis activity was assessed by monitoring cortisol levels along with MRS measurements during the entire procedure.

MATERIALS AND METHODS

Subjects

Eighteen healthy right-handed male subjects (mean age 26.9 ± 4.5 years) were investigated. Subjects were recruited

via advertising. Any history of mental disease was excluded before the study using the structured Mini International Neuropsychiatric Interview (Sheehan *et al*, 1997). Any somatic disease was ruled out by means of routine laboratory testing and physical examination. All subjects had to be free of any medication. Any drug intake was ruled out by urine toxicology screening. The protocol was approved by the Ethics Committee of the Medical Faculty at the University of Muenster, Muenster, Germany. Written informed consent was obtained from all the subjects after the procedure had been fully explained.

CCK-4 Challenge Procedure

Subjects were instructed to fast 10 h before the CCK-4 challenge. On the day of the CCK-4 challenge, an intravenous catheter was inserted into a forearm vein 60 min before MRS measure after subjects had been laid down in the scanner. After baseline (-5 min) MRS scan, $50 \mu\text{g}$ CCK-4 (Merck, Switzerland) were administered intravenously in a bolus injection.

Panic symptoms were assessed using the Acute Panic Inventory (API) (Dillon *et al*, 1987) and a DSM-IV-derived Panic Symptom Scale (PSS) (Bradwejn and Koszycki, 1994) at baseline shortly before CCK-4 injection (0 min) and 5 min after CCK-4 administration. Heart rate (HR) was recorded at baseline and peak levels after CCK-4 administration. For a categorical analysis of the panic response, a 'panic attack' was defined as a API total score ≥ 20 and an increment of ≥ 14 points above the pre-injection score.

Blood samples for determination of plasma cortisol and adrenocorticotrophic hormone (ACTH) were drawn by a technical assistant in the scanner room shortly before CCK-4 injection (-1 min) and continuously throughout the challenge procedure at 5, 10, 15, 20, 30, and 60 min. The samples were placed on ice and stored at -80°C after immediate plasma separation. Cortisol was quantified using a commercial enzyme immunoassay (ELISA) (Cortisol-ELISA, IBL International GmbH, Hamburg, Germany). The lower detection limit was 8.28 nmol/l . Intra- and inter-assay coefficients of variation were 2.4 and 6.4%, respectively.

Magnetic Resonance Spectroscopy (^1H -MRS)

The magnetic resonance imaging protocol included T1-weighted 3D-spoiled gradient echo acquisition of the whole brain and T2 and proton density-weighted fast spin echo sequences in transaxial and coronal orientation. For the MRS measurements, a single voxel point-resolved spectroscopy method (TE = 32 ms, repetition time = 2134 ms; voxel size = $35 \times 15 \times 15 \text{ mm}^3 = 7.875 \text{ cm}^3$, number of scans = 128) at 3 T (Gyrosan Intera Philips, Best, NL, USA) was used. Time resolution of the MRS series was 5 min. Six subsequent MRS spectra were acquired in total: 5 min before challenge (baseline, T0), with CCK-4 injection (T1), 5 (T2), 10 (T3), 15 (T4), and 20 (T5) min after injection. For statistical analysis, maximum values compared with baseline were used to test for potential influences of experimentally induced panic on neurochemical parameters. According to previous imaging studies showing CCK-4-induced panic to be accompanied by activation in the

ACC (Schunck *et al*, 2006; Eser *et al*, 2009), this region was chosen as the target area.

Voxel position was assigned according to Creutzfeldt (1983) to include Brodmann area 24/32 (Economo L_A/FDL) as described previously (Pfleiderer *et al*, 2002; see Figure 1). To assure high intraindividual reproducibility, scans were referenced to readily identifiable anatomic landmarks within the brain; the base of the voxel was aligned perpendicular to the tip of the genu corporis callosi (Pfleiderer *et al*, 2003).

Postprocessing was standardized using the LCModel program package (Provencher, 1993), with zero filling, Fourier transformation and automated phase, baseline, and eddy current correction. After postprocessing of the spectra, metabolite concentrations were calculated using the frequency domain-fitting procedure provided by LCModel. Due to the overlapping resonances of glutamate, glutamine, and GABA, only a combined fitting of Glx was performed. For robustness of results, only metabolite information with the fitting error (% SD) of <20% SD was included in the final analysis. The contribution of altered GABA levels may still have influenced our data. However, GABA concentration, in general, is much lower than combined Glx (Sanacora *et al*, 1999). Also, Glx signal is mostly dominated by glutamate (Auer *et al*, 2000).

Statistics

To facilitate the comparison of Glx levels at baseline and after CCK-4 injection, Glx values were normalized to creatine (Cr) and Glx/Cr values. Maximal deviation from baseline levels before CCK-4 administration in percentage was calculated (p (Glx_[max])).

To analyze brain metabolic changes, maximum concentrations of Glx/Cr after CCK-4 injection were compared with baseline using an analysis of variance (ANOVA) with repeated-measures design. Time (baseline and post-CCK-4 injection) was used as a within-subject factor. A forward linear regression analysis was used to evaluate the impact of glutamatergic baseline concentration on the extent of Glx/Cr increase. Subjective panic reaction (API, PSS) following CCK-4 challenge and maximum HR were analyzed in the

same manner. Stimulation of HPA axis after panic induction by CCK-4 was analyzed observing the time course of cortisol/ACTH concentrations as well as comparing baseline and peak hormone levels using an ANOVA with repeated measures design with time (minutes after challenge) as a within subject factor in both cases. To test for significant correlations between changes in Glx/Cr levels and behavioral data, Pearson correlations were performed. Results are expressed as mean \pm SD. $\alpha = 0.05$ was set as the nominal level of significance. The Statistical Package for Social Sciences SPSS (PASW 18) was used for statistical analyses (PASW 18, now IBM, Armonk, NY, USA).

RESULTS

Baseline and maximum levels after the CCK-4 challenge (post challenge) of behavioral data, HR, cortisol/ACTH and MRS measures are shown in Table 1.

Panic challenge with CCK-4 was followed by a rapid and significant increase in panic symptoms as revealed by significant changes in both the API (API_{max} vs API baseline; $F(1,17) = 149.41$; $p < 0.0001$) and the PSS score (PSS_{max} vs PSS baseline; $F(1,17) = 88.03$; $p < 0.0001$). Experimentally induced panic was furthermore accompanied by a significant increase in HR ($F(1,17) = 72.79$; $p < 0.0001$). In all, 15 out of 18 subjects fulfilled the criteria of a panic attack.

Moreover, MRS measurements indicated an increase of brain Glx/Cr concentrations. Mean Glx/Cr concentrations at the respective time points are illustrated in Figure 2. For statistical analysis, individual peak values throughout the whole challenge procedure were compared with baseline levels. Repeated-measures ANOVA revealed the significance of the change ($F(1,17) = 15.94$; $p = 0.001$). A forward linear regression analysis indicated that the glutamatergic baseline concentration seems to mainly determine the extent of percentage of Glx/Cr increase ($F(1,16) = 19.64$, $p < 0.0001$).

There was also a significant increase in CCK-4-related HPA axis stimulation mirrored by a significant effect on plasma cortisol levels 10–15 min after CCK-4 administration. Data for cortisol were missing in one subject. ANOVA for repeated measures revealed a significant effect ($F(6,10) = 8.68$; $p = 0.002$). There was also a trend towards

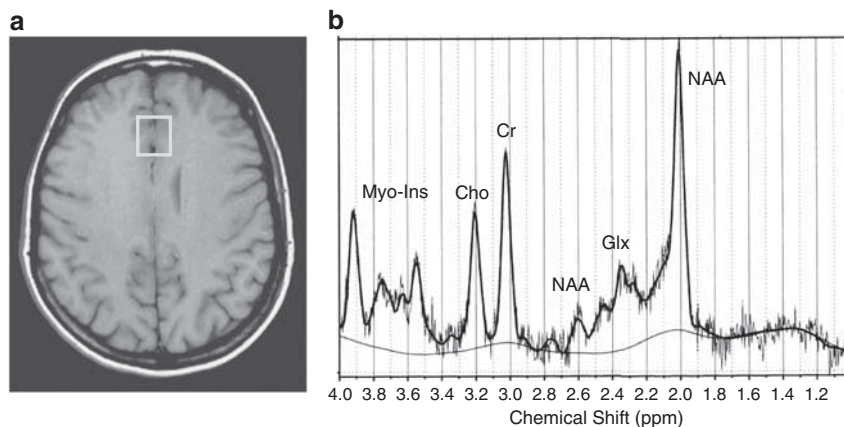
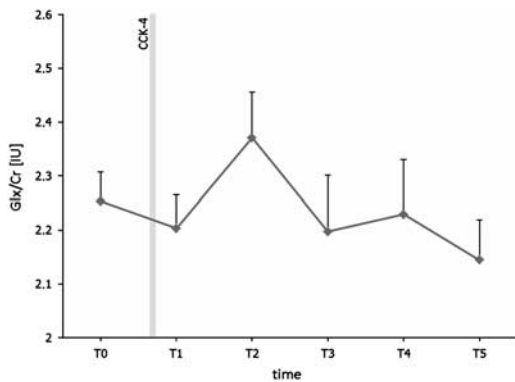
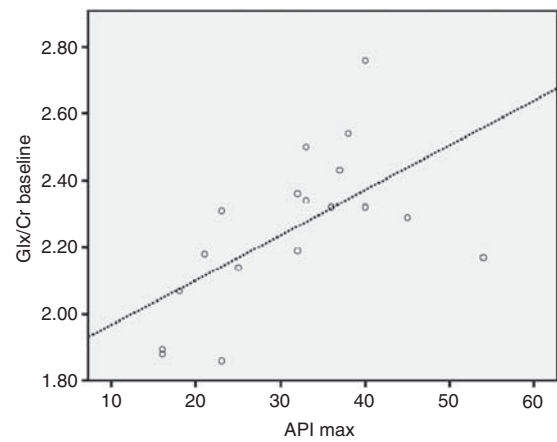


Figure 1 Glutamate + glutamine (Glx) in the anterior cingulate cortex (ACC) of healthy control subjects ($n = 18$) assessed by magnetic resonance spectroscopy (MRS). (a) Voxel localization; (b) Typical point-resolved spectroscopy spectrum acquired from human ACC at 3T at baseline. Peaks were assigned as follows: N-acetyl aspartate (NAA), total Cr, total choline (Cho), Glx, and Myo-Inositol (Myo-Ins).

Table 1 Baseline and Peak Values of Behavioral Data, Heart Rate, Cortisol/ACTH and MRS Measures Throughout the Entire Challenge Procedure

	Baseline (mean ± SD)	Peak values (mean ± SD)	F	P-value
Glx/Cr (IU)	2.25 ± 0.24	2.62 ± 0.27 ^a	15.94	0.001
API	4.28 ± 5.14	31.22 ± 10.54	149.41	<0.001
PSS	3.00 ± 3.80	26.28 ± 10.93	88.03	<0.001
HR (bpm)	65.94 ± 10.32	116.94 ± 22.76	72.79	<0.001
Cortisol (nmol/l)	121.78 ± 56.20	165.87 ± 52.73	29.41	<0.001
ACTH (pmol/l)	36.21 ± 54.99	56.43 ± 52.44	15.63	0.001

Abbreviations: API, Acute Panic Inventory; bpm, beats per minute; Glx/Cr, glutamate + glutamine/creatinine; HR, heart rate; PSS, Panic Symptom Scale. Repeated measures ANOVA, significance level at $p < 0.05$. ^aMean of individual maximum Glx/Cr levels (T1–T5).

**Figure 2** Time course of Glx/Cr (IU) levels (mean ± SEM) throughout the CCK-4 challenge. Levels provided represent the respective mean concentrations for each time point of MRS scans (T0–T5).**Figure 3** Significant correlation between baseline concentrations of glutamate + glutamine/creatinine (Glx/Cr) and maximum Acute Panic Inventory (API) panic score (API max); $r = 0.598$; $p = 0.009$.

significance for increases in ACTH plasma concentrations 5 min after CCK-4 challenge ($F(6,11) = 2.95$; $p = 0.057$). Correlation analyses were carried out in order to detect a possible relationship between glutamate metabolism and panic symptoms. Analyses showed a significant positive correlation between baseline Glx/Cr concentrations and API_{max} ($r = 0.598$; $p = 0.009$) (Figure 3). No effects were found for the correlation between maximum Glx/Cr concentrations and API_{max} . There was also a significant positive correlation between baseline Glx/Cr concentrations and HR_{max} ($r = 0.519$; $p = 0.027$). There was also a trend towards significance for a correlation between peak cortisol levels and percentage of Glx/Cr increase ($r = 0.445$; $p = 0.065$).

DISCUSSION

The aim of this study was to investigate brain Glx concentrations during experimental panic induction with CCK-4. The main finding was that CCK-4-induced panic was accompanied by a rapid and significant increase of Glx concentrations in the ACC. Moreover, panic was associated with a marked increase in the HPA axis stimulation as described previously (Kellner *et al*, 2000; Koszycki *et al*, 1998; Zwanzger *et al*, 2003).

Overall, with regard to behavioral effects observed after CCK-4 administration, the present results are in accordance

with the extensive body of literature on the panicogenic effects of CCK-4 (see Zwanzger *et al*, 2012).

Our results are in line with available data on glutamate metabolism in anxiety disorders. In patients with social anxiety disorder, glutamate levels were found to be significantly elevated in the ACC compared with controls (Phan *et al*, 2005). Also in other disorders with dysbalanced impulsivity such as in borderline personality disorder, an increase in ACC glutamate levels has been described (Hoerst *et al*, 2010). In line with our results, glutamate concentrations in the frontal cortex of healthy subjects might depend on anxiety levels as revealed by a study of Grachev and Apkarian (2000). The authors showed that subjects with high levels of anxiety display an increased overall chemical activity in the frontal cortex by >30% compared with subjects with low levels of anxiety. However, no significant correlation between maximum Glx and panic scores has been found in our study. Instead, a significant positive correlation between baseline Glx and panic response to CCK-4 was detected. It might be discussed whether in view of higher baseline levels in some subjects a possible ceiling effect may have masked a potential correlation.

From a preclinical point of view, the idea of increased glutamate in anxiety would be in line with the observation that excessive glutamate release within the limbic system is associated with fear-related learning (Walker and Davis,

2002). Intriguingly, decreased ACC glutamate concentrations in healthy subjects have been found to be associated with high levels of sensation-seeking behavior (Gallinat *et al*, 2007), which usually goes along with high risk taking and low levels of anxiety-related behavior according to preclinical studies (Blanchard *et al*, 2009).

The fact that Glx concentrations show a fast and immediate increase following experimental panic induction suggests that apart from static alterations in glutamatergic neurotransmission outlined above rapid and dynamic neurochemical changes might also be important for the neural control of acute anxiety and panic attacks. In similarly designed studies using the CCK-4 paradigm, an immediate shift of GABA_A-receptor modulating neuroactive steroids after experimentally induced panic was observed in patients with PD (Strohle *et al*, 2003) and healthy volunteers (Eser *et al*, 2005), with the latter study suggesting that the significant increase of 3 α -5 α -tetrahydrodeoxycorticosterone (3 α ,5 α -THDOC) as a positive allosteric modulator of the GABA_A-receptor might contribute to the termination of the anxiety response to CCK-4 (Eser *et al*, 2005). According to Salt and Eaton (1996), GABAergic and glutamatergic neurotransmission seems to be modulated by presynaptic metabotropic glutamate (mGlu) autoreceptors. Moreover, it has also been suggested that neurosteroids, synthesized in cortical glutamatergic neurons, may exert GABAergic activity through autocrine (targeting postsynaptic receptors at the same neuron) and/or paracrine (targeting receptors at distal neurons) mechanisms (Rupprecht *et al*, 2010). Finally, GABA released from GABAergic interneurons targets pre-, post-, and extrasynaptic receptors at glutamatergic principal output neurons (for a review, see Rupprecht *et al*, 2010). Thus, our results point towards a possible functional interaction of glutamatergic neurotransmission and neuroactive steroid regulation in the experimental panic model.

However, other anxiety-provoking paradigms yielded different results. In a threat-of-shock paradigm in healthy subjects, no changes in Glx levels were discerned (Hasler *et al*, 2010). Instead, a marked and significant decrease in prefrontal GABA concentrations was observed (Hasler *et al*, 2010). However, differences in the anxiety provocation paradigm might account for diverging findings: although the study of Hasler *et al* (2010) investigated the aspect of anticipatory anxiety, our study investigated acute panic using a well-established pharmacological challenge procedure.

In our study, CCK-4-induced panic attacks were furthermore accompanied by a marked and significant HPA axis stimulation, which is in line with several previous reports (Kellner *et al*, 2000; Koszycki *et al*, 1998; Zwanzger *et al*, 2003). Although data on the relationship of glutamate and HPA axis activity in humans are scarce, some reports show that glutamate is increased in stress-related situations. In a study investigating healthy volunteers, Glx was increased in the visual cortex following vigorous physical exercise (Maddock *et al*, 2011). Also, preclinical studies suggest a close relationship between glutamate metabolism and stress regulation: Corticotropin-releasing hormone neurons are co-localized with vesicular glutamate transporters (Herman *et al*, 2000). Glutamate microinjections into the paraventricular nucleus are followed by an increase of cortisol

(Brann and Mahesh, 1997). Moreover, glutamate-induced release of corticotropin-releasing hormone and cortisol has been shown to be mediated by NMDA receptors (Brann and Mahesh, 1997). Although the correlation analysis between maximum cortisol levels and panic scores only yielded borderline significance, these results still point to a close relationship between the glutamatergic and the HPA system and thus support findings in human studies.

Our results could also have implications for treatment of anxiety. There is ample evidence for anxiolytic properties of medication targeting the glutamate system. In both the animal and human studies, anxiolytic effects have been observed after treatment with modulators of ionotropic NMDA and mGlu receptors (Riaza Bermudo-Soriano *et al*, 2011; Bergink *et al*, 2004). It has been shown that modulators of the NMDA receptor are capable of blocking extinction learning (Kaplan and Moore, 2011). Moreover, the NMDA receptor agonist D-cycloserine has been shown to enhance effects of cognitive behavioral therapy in anxiety disorders in a couple of clinical studies based on its capacity to influence memory consolidation (Otto *et al*, 2010; Ressler *et al*, 2004). With regard to mGlu receptors, anxiolytic properties have been proposed for an allosteric modulator of the mGluR2/3 receptor subtype (Krystal *et al*, 2010). Moreover, patients with generalized anxiety disorder reported a significant decrease in anxiety sensitivity and worry after treatment with riluzole, which decreases glutamate release (Mathew *et al*, 2005; Pittenger *et al*, 2008). Finally, anxiolytic effects of anticonvulsants have, in part, also been attributed to their effect on the glutamatergic system (Riaza Bermudo-Soriano *et al*, 2011).

However, the present findings have to be considered in the light of several limitations and must therefore be interpreted with caution. The sample size is relatively small and includes only males. Thus, our study does not allow for extrapolation to a female sample. Moreover, no patients were included in the study. With regard to MRS, only a combined fitting of Glx was performed. Nevertheless, as mentioned in the Methods section, the Glx signal is assumed to be mostly dominated by glutamate (Auer *et al*, 2000). Additionally, no placebo injection was administered. Therefore, metabolic changes due to stress and anticipatory anxiety cannot be fully excluded. Finally, the present study did not control for the genetic background, eg, variation in the NMDA 2B receptor gene (GRIN2B), which has been shown to influence ACC Glx concentration (Arnold *et al*, 2009). Also, smoking or smoking cessation impacting anterior cingulate proton spectroscopy glutamate levels were not considered as potentially confounding factors (Mashhoon *et al*, 2011).

Taken together, our results show that CCK-4-induced panic is accompanied by a marked and significant glutamate increase in the ACC. The results are in accordance with the idea of a glutamatergic dysbalance and add to the hypothesis of a disturbance of inhibitory/excitatory mechanisms in anxiety and anxiety disorders. However, as interpretation of our data is certainly limited due to the small sample size and the lack of a patient group, more studies in larger samples of both the healthy subjects and patients, including a molecular genetic approach, are needed to further elucidate the possible role of the

glutamatergic system in the neurobiology of panic and anxiety.

ACKNOWLEDGEMENTS

This study was supported by 'Innovative Medizinische Forschung' (IMF) of the Medical Faculty, University of Muenster, Muenster, Germany (IMF ZW 210703). Furthermore, parts of this work were supported by the Deutsche Forschungsgemeinschaft (DFG) SFB TRR 58 subproject C1 to PZ and C2 to KD. Parts of this work were subject of the doctoral thesis of EG.

DISCLOSURE

All affiliations mentioned below have no relevance to the work covered in the manuscript: PZ has received speaker fees from Pfizer, Servier, Lilly, Astra Zeneca, and Bristol-Myers Squibb, he is on the advisory board of Pfizer, is a consultant for Ironwood Pharmaceuticals, and has received funding from Astra Zeneca. VA is member of advisory boards and/or gave presentations for the following companies: Astra Zeneca, Janssen-Organon, Lilly, Lundbeck, Servier, Pfizer, Otsuka, and Wyeth. He chaired the committee for the 'Wyeth Research Award Depression and Anxiety'. KD has received speaker fees from Pfizer, Lilly, and Bristol-Myers Squibb, She was a consultant for Johnson and Johnson and has received funding by Astra Zeneca. All the other authors have no conflicts of interest to declare, financial or otherwise.

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