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SHORT COMMUNICATION

Elevated cannabinoid 1 receptor mRNA is linked to eating disorder related behavior and attitudes in females with eating disorders

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EDI-2;
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Summary
Objective: The endocannabinoid system is involved in the regulation of appetite, food intake and energy balance.
Methods: To study possible differences in CB_1 and CB_2 mRNA expression in eating disorders, 20 patients with anorexia nervosa (AN), 23 with bulimia nervosa (BN) and 26 healthy women were enrolled into the trial (Homocysteine and Eating Disorders, HEaD).
Results: We found significantly higher levels of CB_1 receptor mRNA in the blood of patients with AN (ΔCT: −3.9 (1.0); KW: 11.31; $P = 0.003$) and BN (ΔCT: −3.7 (1.7)) when compared to controls (ΔCT: −4.6 (0.6); Dunn's test AN vs. Controls: $P < 0.05$; BN vs. Controls: $P < 0.001$) measured by quantitative real-time PCR. No differences were found regarding the expression of CB_2 receptor mRNA. Higher CB_1 receptor expression was associated with lower scores in several eating disorder inventory-2 (EDI-2) subscales including perfectionism, impulse regulation and drive for thinness.
Conclusion: Our finding of elevated CB_1-receptor expression in AN and BN adds further evidence to the hypothesis of impaired endocannabinoid signaling in eating disorders.

1. Introduction

It has been proposed that disturbances in the neural circuitries regulating appetite, food intake and energy balance, including leptin and the endocannabinoid system, play a crucial role in the pathophysiology of eating disorders such as anorexia nervosa (AN) and bulimia nervosa (BN) (Monteleone et al., 2004; Holtkamp et al., 2006).

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Recently, elevated blood levels of anandamide have been reported in AN (Monteleone et al., 2005), as well as an association between a functional polymorphism of the cannabinoid receptor 1 (CB_1) gene (Cnr1) and restrictive AN (Siegfried et al., 2004).

The aim of the present study was to evaluate the hypothesis, that differences in peripheral mRNA levels of CB_1 and CB_2 exist between females with AN, BN and healthy women and if so, that these alterations are associated with changes in the epigenetic control of the genes' promoters.

2. Methods

2.1. Patients and design

This study was part of an observational study on homocysteine and eating disorders (HEaD) (Frieling et al., 2005). It was approved by the Local Ethics Committee (University of Münster, Germany). All patients met DSM-IV criteria for AN or BN. Comorbid psychiatric disorders were assessed using the German version of the structured clinical interview for DSM-IV diagnoses (SCID). Written informed consent was obtained from all patients after the procedures had been fully explained to them. All patients were inpatients in a psychosomatic hospital (Korso Hospital, Bad Oeynhausen, Germany). In the present analysis we included 43 patients, among them 20 women with AN (restrictive type: 6; purging type: 14) and 23 women with BN (purging type: 19; non-purging type: 4). Eating disorder symptomatology was assessed only in patients with the German version of the eating disorder inventory 2 (EDI-2), a self-rating instrument consisting of 91 items and 11 subscales (Thiel et al., 1997). Measurements were controlled against 26 age matched healthy women; absence of psychiatric disorders was assessed using the SCID interview by a senior psychiatrist (TH). Further characteristics of the study population are summarized in Table 1.

2.2. Laboratory analyses

Fasting blood samples were drawn in the morning of the first day after admission to the hospital. Total RNA was extracted from whole frozen EDTA–blood using Qiazol and the Rneasy Mini Kit (both: Qiagen, Hilden, Germany). After reverse transcription (iScript cDNA Synthesis System; BioRad, Hercules, CA), we performed quantitative real time PCR using iQ SYBR Green Super Mix buffer (BioRad). All reactions were run on an iCycler (BioRad) with a three-step standard protocol with an annealing temperature of 61 °C. β Actine was used to calculate ΔCT values. Following primer pairs were used:

CB_1mRNA-F: GCCTGGCGGTGGCAGACCTCC,
CB_1mRNA-R: GCAGCACGGCGATCACAATGG,
CB_2mRNA-F: CATGGAGGAATGCTGGGTGAC,
CB_2mRNA-R: GAGGAAGGCGATGAACAGGAG,
Beta Actine-F: CTGGAACGGTGAAGGTGACA,
Beta Actine-R: AAGGGACTTCCTGTAACAATGCA.

All experiments were repeated two times.

Analysis of promoter DNA methylation (CB1 only) was performed applying a methylation sensitive digestion assay as previously described (Bleich et al., 2006) followed by real-time PCR with following primers (Annealing temperature: 55 °C):

CB_1meth-F: TCCAAGAGTAGGGGTCATGTG,
CB_1meth-R: CAGGGCCAAGAAGACTGAAC.

All experiments were repeated two times. CT measurements were transformed using the formula $\% = [1-(2^{CT-HpaII}/2^{CT-MspI})] \times 100$ to calculate the percentage of methylation.

2.3. Statistical analysis

Comparisons between groups were made using either the Mann–Whitney-*U*-test or the Kruskal–Wallis test (KW) as most variables did not show equal variances. *P* values were adjusted for multiple comparisons (Dunn's test). Stepwise backward linear regression analysis was used to test for possible associations between different variables. A multivariate regression was entertained to test for possible associations between cannabinoid receptor mRNA levels and EDI-2 subscale scores, using a general linear model (dependent variables: EDI-subscales; independent: CB_1 mRNA, CB_2 mRNA). The results are presented as means (S.D.). A *P*-value of less than 0.05 (two-tailed) was considered to indicate statistical significance. Data were analyzed employing SPSS™ for Windows 16.01 (SPSS Inc., Chicago, IL) and Graph Pad Prism 5 (Graph Pad Inc., San Diego, CA).

3. Results

We found significantly higher levels of CB_1 mRNA in the blood of patients with AN (ΔCT: −3.9 (1.0); KW: 11.31; $P = 0.003$) and BN (ΔCT: −3.7 (1.7)) when compared to controls (ΔCT: −4.6 (0.6); Dunn's test AN vs. Controls: $P < 0.05$; BN vs.

Table 1 Characteristics of study sample.

	Anorexia ($n = 20$)		Bulimia ($n = 23$)		Control ($n = 26$)		*P*-value*
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Age (yrs)	26.4	10.6	25.9	7.9	21.3	2.2	0.518
BMI (kg/m^2)	15.9	2.0	22.5	2.6	20.9	1.6	0.001
Age of onset	17.2	5.5	16.9	6.5			0.879
Duration of illness (yrs)	9.7	10.8	9.0	5.9			0.793

* *P* values of the Kruskal–Wallis-test (age, BMI) or Mann–Whitney-*U* test (age of onset, duration of illness), respectively. Further statistical details are summarized in *Results*.

Controls: $P < 0.001$). No significant difference was found between AN and BN (Fig. 1). We found no significant differences between the groups regarding the expression of CB_2 mRNA (AN: ΔCT: −0.37 (1.6); BN: ΔCT −0.34 (3.1); Controls: ΔCT −0.72 (1.6); KW: 1.24; $P = 0.539$).

We found no significant differences in the promoter DNA methylation of the CB_1-gene (%methylated DNA: AN: 65.5 (26); BN: 70.2 (28.8); Controls: 71.8 (22.4); KW: 1.09; $P = 0.52$). As no differences in the expression of CB2 were observed, we did not analyze the methylation of the gene promoter.

In a stepwise backward linear regression model, using CB_1 or CB_2 mRNA as dependent and diagnosis, age, BMI and promoter methylation as independent variables ($R^2 = 0.148$, $P = 0.005$) only the diagnosis had a significant impact on the CB_1 mRNA expression ($\beta = -0.331$; $P = 0.005$), while the Cnr1 gene promoter DNA methylation showed a trend ($\beta = 0.21$; $P = 0.069$). None of these variables had an impact on CB_2 mRNA levels (data not shown). We divided the study sample according to the presence of binging behavior and purging behavior. We found neither a difference in CB_1 or CB_2 mRNA levels when comparing binging with no binging nor purging with no purging.

Using a general linear model to test for a possible association between CB_1 mRNA levels and EDI-2 (subscale) score, we found that higher expression of CB_1 occurred in patients with lower sum scores ($R^2 = 0.230$; $F = 11.594$; $P = 0.002$) and lower scores for drive for thinness ($R^2 = 0.178$; $F = 8.192$; $P = 0.007$), bulimia ($R^2 = 0.217$; $F = 10.688$; $P = 0.002$), body dissatisfaction ($R^2 = 0.106$; $F = 4.189$; $P = 0.047$), ineffectiveness ($R^2 = 0.118$; $F = 4.479$; $P = 0.041$), perfectionism ($R^2 = 0.202$; $F = 8.147$; $P = 0.007$), disturbed impulse regulation ($R^2 = 0.211$; $F = 8.838$; $P = 0.005$), and social insecurity ($R^2 = 0.098$; $F = 4.177$; $P = 0.048$; Pillai-Spur: 0.451; $F = 2.236$; $P = 0.040$; Fig. 2). No association between EDI-2 (subscale) scores and CB_2 mRNA levels was observed.

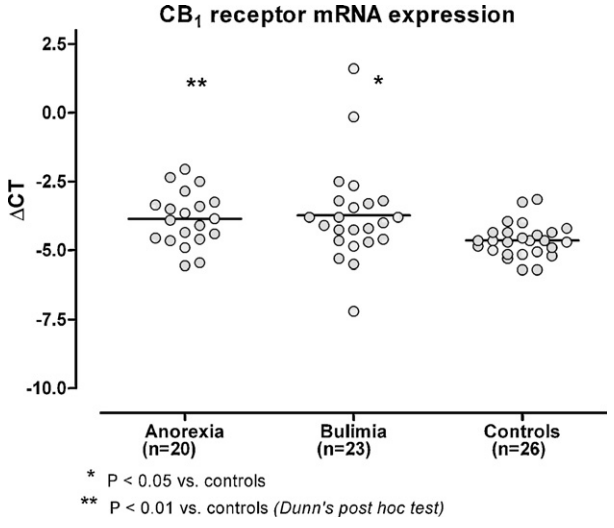

Figure 1 CB_1 receptor mRNA in anorexia, bulimia, and healthy controls. Significantly elevated mRNA expression of cannabinoid 1 receptor in peripheral blood of females suffering from anorexia or bulimia nervosa when compared to healthy controls. *P* values given are taken from the post hoc analysis (Dunn's) after Kruskal—Wallis test. Further details are summarized in *Results*.

4. Discussion

Recent studies have revealed disturbances in endocannabinoid signaling in eating disorders. Especially in AN elevated levels of the endocannabinoid anandamide have been found (Monteleone et al., 2005). In this study, in a small subgroup of patients ($n = 5$ for each: AN, BN, Binge eating disorders and controls), also the expression of the cannabinoid receptors 1 and 2 (CB_1/CB_2) was measured. The authors report no differences between the groups. Our findings are contrary to this result, probably due to the larger sample size, as we have found elevated levels of CB_1 mRNA in the blood of females with AN and BN. Our finding of no differences in CB_2 mRNA expression between groups is in line with the previous findings by Monteleone et al.

As we found no difference in the CB_1 mRNA between AN and BN and our sample size did not allow for sufficiently powered sub-group analyses, we decided to include all patients as a whole into the further analyses, as it has recently been shown, that ED diagnoses are often instable and changing throughout the life span (Milos et al., 2005). However, further studies are necessary with an increased sample size to facilitate sub-group specific analyses (especially comparing restricting vs. non-restricting types of AN).

Furthermore, we found an association between CB_1 mRNA expression and the scores in several EDI-2 sub-scales and in the sum score. Higher sum scores, representing more severe forms of the disorders, were associated with lower CB_1 expression. These findings are in line with recent reports about down-regulated CB_1 expression in an animal model of ADHD, that was especially associated with increased impulsivity (Adriani and Laviola, 2004). This finding is also important as it links the peripheral expression of cannabinoid receptors in the blood to central nervous processes making it more likely, that the observed disturbances reflect a disturbed endocannabinoid regulation in the central nervous system.

In our primary hypothesis, we assumed CB_1 mRNA to be downregulated, due to elevated endocannabinoids. In the light of our findings it seems more likely that the observed up-regulation of CB_1 mRNA may compensate for otherwise impaired endocannabinoid signaling that could be due to (hypothetically) reduced receptor sensitivity. CB1 receptor desensitation as a consequence of elevated endocannabinoids has recently been observed in diverticular disease (Guagnini et al., 2006). This explanation is further supported by the finding of a possible association of a functional polymorphism of the CB_1 receptor gene (Cnr1) with restricting type AN (Siegfried et al., 2004). Disorder—specific alterations downstream of the CB_1 receptor may also be responsible for the observed association between severity and CB_1 expression, e.g. HPA-axis disturbances (Di Marzo and Matias, 2005) or alterations in the involved G-proteins (Monteleone et al., 2003). Recent findings in T-lymphocytes may provide another explanation for the unexpected up-regulation of CB_1mRNA in our sample: When stimulated with anandamide, T-lymphocytes increase the expression of CB_1 mRNA and protein via a CB_2 dependent signal transduction (Borner et al., 2007). Therefore, the observed up-regulation of CB_1 mRNA in our sample of individuals suffering from eating disorders may be a physiological consequence of elevated endocannabinoid levels in these disorders. However, the

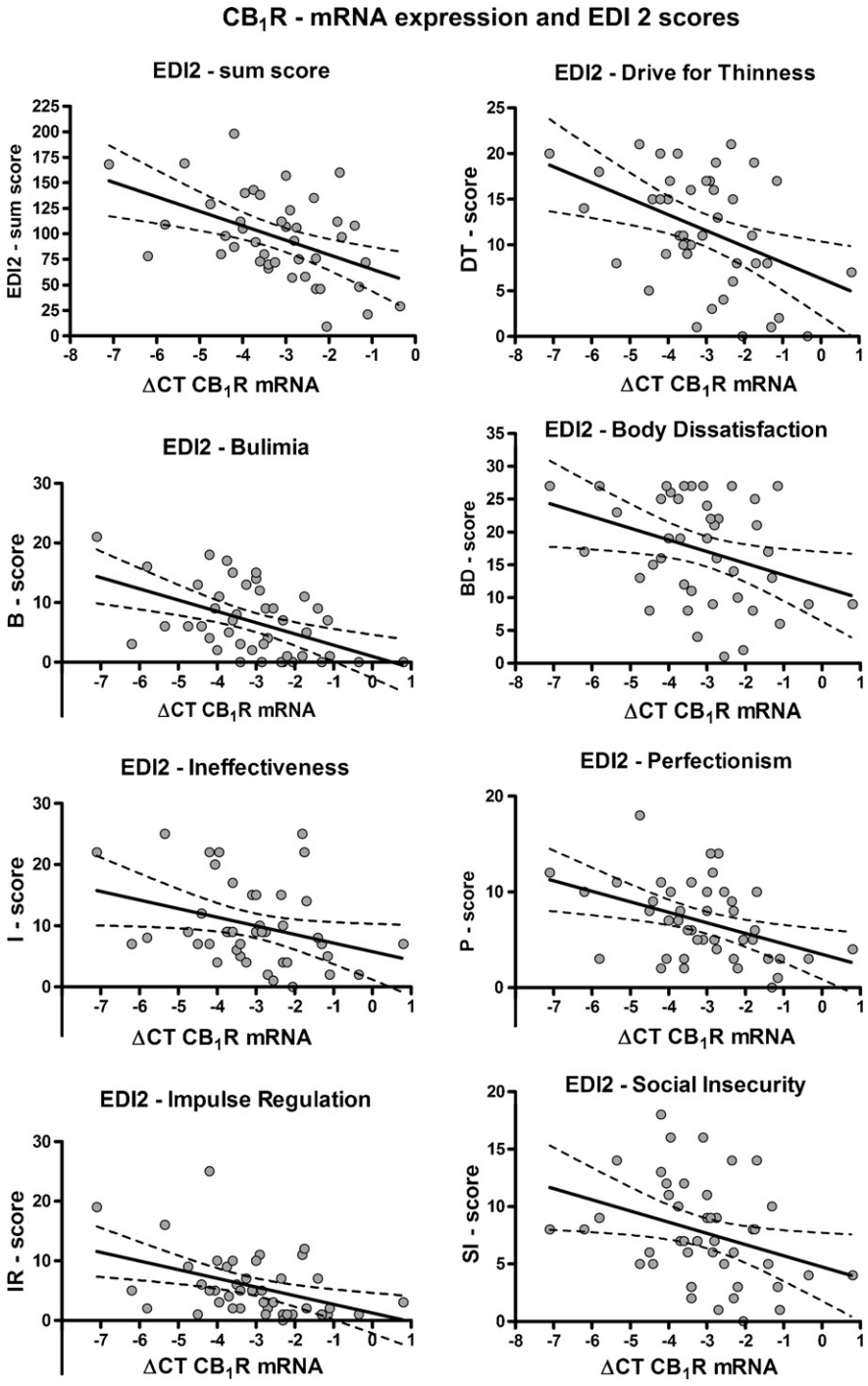

Figure 2 Association of CB_1 receptor mRNA with EDI-2 subscales. Association between CB_1 receptor mRNA with different subscales and the sum score of the eating disorder inventory 2. Further statistical details of the association are summarized in *Results*.

interplay between endocannabinoids in their receptors is complex and may be differentially regulated in different tissue (Matias and Di Marzo, 2007). Therefore, further studies are needed to elucidate this issue.

To investigate whether our finding of up-regulated CB_1 receptor mRNA in eating disorders may be explained by epigenetic changes in the Cnr1 gene, we analyzed the amount of DNA methylation of its promoter. We found no difference in promoter specific DNA methylation of the Cnr1 gene between AN, BN and controls. Nevertheless, the amount of DNA methylation seems to have a small impact on CB_1 mRNA expression as revealed by the almost significant association in the linear regression. These findings are in line with recent reports on the regulation of the Cnr1-gene, showing only limited relevance of DNA methylation (Zhang et al., 2004).

The present findings add further evidence to the hypothesis of impaired endocannabinoid signaling in eating disorders. Further studies are warranted, especially using a longitudinal

design to separate the acute effects of starvation from long-standing effects that may underlie the pathology of eating disorders.

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Conflict of interest

The authors declare that there exists no possible conflict of interest.

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