Small animal PET imaging of the type 1 cannabinoid receptor in a rodent model for anorexia nervosa

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Abstract

Purpose Several lines of evidence strongly implicate a dysfunctional endocannabinoid system (ECS) in eating disorders. Using [^{18}F]MK-9470 and small animal positron emission tomography (PET), we investigated for the first time cerebral changes in type 1 cannabinoid (CB1) receptor binding in vivo in the activity-based rat model of anorexia (ABA), in comparison to distinct motor- and food-related control conditions and in relation to gender and behavioural variables.

Methods In total, experiments were conducted on 80 Wistar rats (23 male and 57 female). Male rats were assigned to the cross-sectional conditions: ABA ($n=12$) and CONTROL ($n=11$), whereas female rats were divided between two settings: (1) a cross-sectional design using ABA ($n=13$), CONTROL ($n=9$), and two extra control conditions for each of the variables manipulated in ABA, i.e. DIET ($n=8$) and WHEEL ($n=9$), and (2) a longitudinal one using ABA ($n=10$) and CONTROL ($n=8$) studied at baseline, during the model and upon recovery. The ABA group was subjected to food

restriction in the presence of a running wheel, the DIET group to food restriction without wheel, the WHEEL group to a normal diet with wheel and CONTROL animals had a normal diet and no running wheel. Parametric CB1 receptor images of each group were spatially normalized to Paxinos space and analysed voxel-wise.

Results In the ABA model, absolute [^{18}F]MK-9470 binding was significantly increased in all cortical and subcortical brain areas as compared to control conditions (male +67 %; female >51 %, all $p_{\text{cluster}} < 6.3 \times 10^{-6}$) that normalized towards baseline values after weight gain. Additionally, relative [^{18}F]MK-9470 binding was increased in the hippocampus, inferior colliculus and entorhinal cortex of female ABA (+4.6 %; $p_{\text{cluster}} < 1.3 \times 10^{-6}$), whereas no regional differences were observed in male subjects. Again, relative [^{18}F]MK-9470 binding values normalized upon weight gain.

Conclusion These data point to a widespread transient disturbance of the endocannabinoid transmission, specifically for CB1 receptors in the ABA model. Our data also suggest (1) gender effects on regional CB1 receptor binding in the hippocampus and (2) add further proof to the validity of the ABA model to mimic aspects of human disease.

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Introduction

Anorexia nervosa (AN) is a devastating psychiatric disorder characterized by abnormal patterns of feeding behaviour, severe weight loss, fear of gaining weight and distorted body perception [1]. Despite increasing knowledge of neurotransmitter system pathophysiology, few effective treatments exist

for patients with AN and full recovery is seen in only 40–50 % of patients [2].

Several lines of evidence point to an important role of the endocannabinoid system (ECS) in the control of food intake and in eating disorders. The brain ECS consists of a family of naturally occurring lipids, the endocannabinoids [of which anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized substances], of degradation proteins and of cannabinoid receptors [3]. The type 1 cannabinoid (CB1) receptor mediates the majority of cannabinoid effects on the central nervous system and is found in nearly all brain areas, especially those involved in controlling food intake and reward processing [4, 5]. CB1 receptors are abundantly expressed presynaptically to directly or indirectly modulate major neurotransmitters such as glutamate, gamma-aminobutyric acid (GABA) and dopamine [6, 7].

In AN patients, peripheral endocannabinoid levels are increased and negatively correlated to leptin [8], while after prolonged starvation in mice, brain levels of 2-AG have shown to decrease [9]. CB1 receptor knockout mice eat less after food deprivation and are leaner [10]. Also, different alleles of the CB1 receptor gene have been associated with restrictive and binge/purging subtypes of AN [11]. Polymorphisms of the gene coding for the endocannabinoid degrading enzyme fatty acid amide hydrolase (FAAH) have been associated both with anorexia and bulimia nervosa [12]. Furthermore, cannabis and cannabinoid agonists with minimal psychoactive side effect profile have been used as eating stimulants in acquired immunodeficiency syndrome (AIDS) or cancer patients [13]. Also, in animals, an orexigenic effect of CB1 receptor agonists is present, even in satiated condition [14]. However, in one clinical trial in primary AN, patients received high doses of cannabis' psychoactive component Δ^9 -tetrahydrocannabinol (Δ^9 -THC), but no improvement in food intake and body weight was observed [15], still necessitating the need for improved understanding of the ECS system in AN for future therapy guidance. In female AN patients, we recently found a profound cortical and subcortical increase of CB1 receptor availability in vivo [16].

So far, brain studies of EC changes in experimental models of AN have focused on the hypothalamus, hippocampus and brainstem, but have not assessed other regions [9, 17]. Also unknown is whether brain CB1 receptor levels are changed in anorectic conditions in vivo, as only in vitro, electrophysiological, behavioural and post-mortem data have been reported so far. In vivo imaging of CB1 receptors in rat brain has become feasible due to the development of CB1-selective positron emission tomography (PET) radioligands, such as [^{18}F]MK-9470 [18].

A well-characterized and frequently used rat model of AN [19, 20] relies on the use of a limited feeding period per day in combination with free access to a running wheel. After 7–10 days, rats display hyperactivity and spontaneously restrict

the amount of food intake, leading to severe emaciation and often even death, to some extent modelling the behavioural aspects of AN [21]. The neuronal metabolic correlates of this model have previously been described using [^{18}F]fluorodeoxyglucose small animal PET, indicating changes in both motor and limbic circuitry [22, 23].

Our primary objective was to explore CB1 receptor changes in vivo using [^{18}F]MK-9470 and small animal PET in the activity-based rat model of AN (ABA). As a second objective, food intake, weight loss and motor activity, all shown to be altered in experimental AN [23], were investigated in the same animals and correlated to the regional CB1 receptor availability. To account for possible influence of gender or sex hormones, both female and male rats were studied cross-sectionally in separate designs. Finally, the reversibility of (possible) in vivo changes in CB1 receptor binding was longitudinally evaluated in female ABA rats until spontaneous weight recovery.

Materials and methods

Animals and model generation

All animal studies were reviewed and approved by the local Animal Ethics Committee and experiments were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

In total, experiments were conducted on 23 male and 57 female Wistar rats (on average 6 weeks old; body weight range at the start of the experiment 200–250 g and 108–200 g, respectively). All rats were housed in groups of two with food and water available ad libitum until the start of the experiments. A 12-h light portion of the light/dark cycle beginning at 6.00 a.m. was imposed.

Activity-based anorexia was induced in custom-made cages ($0.36 \times 0.36 \times 0.36$ m) with a running wheel (diameter 0.35 m; Campden Instruments, Loughborough, UK; Cat. No. 80350 and 80850; 1 wheel revolution equals 1.1 m). An electromagnetic rotary encoder (TWK-Elektronik GmbH, Düsseldorf, Germany; Cat. No. RBM22-06-64RK2P01) was mounted on the shaft of the running wheel for continuous registration of the position of the running wheel with a digital I/O card (National Instruments, Austin, TX, USA; Cat. No. DAQPad-6507) and LabVIEW 7.0 (National Instruments).

Study design

CB1 receptor binding was assessed in vivo in male Wistar rats using the following cross-sectional conditions: (1) an experimental group introduced in the ABA model, thus in cages with a fully functional running wheel ($n=12$), and (2) a control group housed in similar cages but without food

restriction and with the running wheel blocked (CONTROL, $n=11$). In addition, 39 of 57 female Wistar rats were cross-sectionally divided between the following experimental settings: (1) ABA ($n=13$) and (2) CONTROL ($n=9$), as described above, and (3) DIET ($n=8$) and (4) WHEEL ($n=9$) to create a control condition for each of the two variables manipulated in the ABA model. The DIET group was subjected to food restriction with a blocked running wheel and the WHEEL group to a normal diet with a functional running wheel. Finally, the remaining female rats ($n=18$) were used for assessment of reversibility in a separate longitudinal experiment and were randomized to ABA ($n=10$) and CONTROL ($n=8$). Binding of [^{18}F]MK-9470 to the CB1 receptor was assessed in these animals at baseline, at a chronic time point during the model (day 10) and after spontaneous weight recovery upon renewed unrestricted eating. A schematic representation of all experimental designs is shown in Fig. 1.

All groups had access to 50 g of food starting at 8.30 a.m. The remainder of the food was removed and weighed at 10.00 a.m. for the groups on a restricted diet and at 8.30 a.m. the next day for the groups on a normal diet. Weight restoration was performed by feeding all rats *ad libitum* again (50 g/24 h). Water was always available. Body weight was measured daily at 10.00 a.m. Room temperature was kept constant at $\pm 20^\circ\text{C}$. Daily food intake, relative weight loss, relative weight gain and running activity (number of wheel rotations) were monitored as covariates. For the female rats, vaginal cytological samples were systematically taken during 3 consecutive days, comprising the day of scanning. Early (pro-oestrus and oestrus) and late (dioestrus I and dioestrus II) phases of the ovarian cycle were determined by the type and number of different cells in the sample with light microscopy, as previously published [24].

Radiotracer preparation and imaging

CB1 receptor imaging was performed in all rats ($n=80$) using the radioligand [^{18}F]MK-9470 ((*N*-[2-(3-cyano-phenyl)-3-(4-(2-[^{18}F]fluoroethoxy)phenyl)-1-methylpropyl]-2-(5-methyl-2-pyridyloxy)-2-methylpropanamide), which is characterized by high specificity and high affinity for the CB1 receptor (rat $\text{IC}_{50}=0.9\text{ nM}$) [25]. The precursor for tracer synthesis was obtained from Merck Research Laboratories (MRL, West Point, PA, USA) and labelling was performed using [^{18}F]fluoroethyl bromide, as described previously [18]. The final product was obtained after high-performance liquid chromatography (HPLC) purification and had a radiochemical purity $>95\%$. Specific activity was on average 201 GBq/ μmol (specific activity range, 53–606 GBq/ μmol). The tracer was administered in a sterile solution of 5 mM sodium acetate buffer pH 5.5 containing 6 % of ethanol.

Image acquisition and reconstruction

Prior to small animal PET imaging, male rats were anaesthetized with 60 mg/kg sodium pentobarbital *i.p.* (Nembutal®, Ceva Santé Animale, Brussels, Belgium), while 2 % isoflurane in 2 l oxygen/min (Isoflurane USP®, Abbott Laboratories Ltd., Maidenhead, UK) was used for female subjects due to logistic reasons. Previous work showed that both anaesthetics induced similar regional changes in the CB1 receptor binding of [^{18}F]MK-9470 *in vivo* as compared to non-anaesthetized animals [26]. Tail veins were catheterized for injection of $21.4 \pm 2.0\text{ MBq}$ of [^{18}F]MK-9470 (specific activity range 100–760 GBq/ μmol ; total volume of approximately 500 μl). After overnight fasting, [^{18}F]MK-9470 measurements were obtained during a 20-min interval, starting 1 h post-injection [25].

Small animal PET imaging was performed using a FOCUS 220 tomograph (Siemens/Concorde Microsystems, Knoxville, TN, USA), which has a transaxial resolution of 1.35 mm full-width at half-maximum. Data were acquired in a $128 \times 128 \times 95$ matrix with a pixel width of 0.475 mm and a slice thickness of 0.796 mm. Sinograms were reconstructed using filtered backprojection (FBP) to avoid possible positive bias due to iterative methods [27]. No corrections were made for attenuation or scatter, due to the much smaller size of the rodent head and the lower thickness and density of the skull as compared to primates [28].

Image processing and data analysis

Parametric images based on (modified) standardized uptake values ((m)SUV) ($= \text{activity concentration (MBq/ml)} \times ((\text{body mass (g)} + x)/2)/\text{injected dose (MBq)}$) were generated as a measure of absolute [^{18}F]MK-9470 binding. In the mSUV formula, body weight was extra normalized to the average body weight (x) of an adult Wistar rat, as determined on the strain's standard growth curve (male: $x=400\text{ g}$, female: $x=250\text{ g}$) [29], to account for the systematic weight difference between anorectic and control rats. This index was previously validated to give reliable estimates of total volume of distribution as determined by full kinetic modelling in humans [30] and healthy rats [25]. Prior to study set-up, we also validated its use specifically for ABA. Full kinetic modelling in a small subset of ABA and control rats ($n=2/\text{group}$) showed a positive correlation of mSUV with total volume of distribution ($y=90x-57$; $R^2=0.9$). Also, ABA and control rats showed comparable [^{18}F]MK-9470 percentage fraction in arterial samples ($62.2 \pm 3.4\%$ vs $58.4 \pm 9.5\%$ and $12.8 \pm 7.4\%$ vs $7.1 \pm 0.5\%$ for ABA and control at 3 and 40 min post-injection, respectively). Non-parametric analysis did not detect significant differences in the AUC of the metabolite-corrected plasma input functions (Supplementary Fig. 1).

Fig. 1 Cross-sectional (a) and longitudinal (b) experimental designs. *N* number

We investigated both absolute (mSUV) and relative [^{18}F]MK-9470 binding. Relative [^{18}F]MK-9470 binding was expressed as mSUV normalized on whole-brain mSUV. The within subject test-retest variability of our imaging procedure is consistent in magnitude with human data, i.e. $< 5\%$ [31].

To obtain maximal use of information without a priori knowledge, PET data were analysed voxel-wise using SPM2 (Statistical Parametric Mapping, Wellcome Department of Cognitive Neurology, London, UK). For spatial normalization, individual PET data were standardized to custom-made rat brain templates in Paxinos stereotactic space [32]. This methodology allows reporting results in coordinates directly corresponding to the Paxinos coordinate system for the rat brain. Also, on this template, a predefined volume of interest (VOI) set of regions was defined for larger regional quantification purposes.

For SPM analysis, data were smoothed with an isotropic Gaussian kernel of 1.2 mm and analysed using a categorical subject design depending on gender (male: ABA vs CONTROL, and female: ABA vs CONTROL, WHEEL and DIET) and a condition $\times$ subjects interaction design for assessment of reversibility (conditions: baseline vs chronic vs recovery $\times$ subjects: ABA vs CONTROL). For analysis of absolute [^{18}F]MK-9470 binding, no proportional scaling was used and a relative (grey matter) analysis threshold of 80 % was used to exclude extracerebral activity. For relative [^{18}F]MK-9470 binding, proportional scaling was used with the same relative analysis threshold. In order to minimize false-positive findings, T-maps were interrogated at a peak voxel level of

$p=0.001$ (uncorrected) and extent threshold $k_E > 200$ voxels. Only significant clusters with $p < 0.05$ (corrected for multiple comparisons) were retained, as described previously [33].

In addition, a within-group voxel-based correlation analysis between [^{18}F]MK-9470 uptake and the following covariates was performed in ABA: (1) body weight at the time of small animal PET scanning relative to starting weight, defined as body weight loss; (2) body weight at the time of recovery relative to chronic anorectic weight, defined as body weight gain; (3) average daily food intake; and (4) the total number of wheel rotations.

General statistics

Parametric unpaired Student's t tests, analysis of variance (ANOVA) and correlation analysis for behavioural and VOI data were carried out using STATISTICA v8.0 (StatSoft, Tulsa, OK, USA). Tukey's HSD test was used for post hoc comparison. Significance was defined at the 95 % probability level. Data are mean $\pm$ SD.

Results

Subjects and behavioural data

Body weight relative to starting weight or anorectic weight, food intake and wheel activity of all conditions over time are summarized in Figs. 2 and 3.

Fig. 2 Behavioural data in male and female ABA. Comparison of relative body weight (%), food intake (g) and number of wheel rotations (/24 h) between male (ABA-CONTROL) and female (ABA-DIET-WHEEL-CONTROL) rats during the behavioural testing period. Mean

curves ± 1 SD are given for each condition. CB1 receptor small animal PET scanning took place on day 9 and on day 10 for male and female rats, respectively, due to logistic reasons

In male rats, body weight was significantly lower in the ABA group (175 ± 22 g, mean $\pm$ SD) compared to the CONTROL group (287 ± 28 g) on the day of PET scanning (day 9 relative to the start of the model; $p < 0.00001$). Likewise, the

ABA group had a significantly lower average daily food intake (9 ± 2 g) during the testing period compared to the CONTROL group (30 ± 3 g) ($p < 0.00001$). The ABA rats completed $21,559 \pm 7,147$ wheel revolutions during this

Fig. 3 Behavioural data in female ABA over time. Comparison of relative body weight (%), relative food intake (g) and number of wheel rotations (/24 h) between female ABA and CONTROL during model development (*left*) and upon renewed unrestricted eating (*right*). Mean

curves ± 1 SD are given for each condition. Body weight is expressed relative to baseline weight on the *left* and relative to anorectic weight on the *right*

testing period, which corresponds to a total distance of 23.7 ± 7.9 km.

At the time small animal PET scanning was performed on female Wistar rats in the cross-sectional design (day 10 relative to the start of the model), body weights of all four conditions were significantly different from each other ($p < 0.00001$). The average body weight for the ABA group was 130 ± 12 g, for the DIET group 149 ± 13 g, for the WHEEL

group 188 ± 8 g and for the CONTROL group 204 ± 8 g. All groups also differed from each other in their average daily food intake, except for the ABA and DIET groups, which were the two food-restricted groups ($p < 0.00001$). The ABA group ate on average 8 ± 2 g, the DIET group 10 ± 1 g, the WHEEL group 22 ± 2 g and the CONTROL group 24 ± 2 g per day. In this design, two groups had a running wheel available; the number of wheel revolutions for ABA rats was $39,086 \pm$

12,055 (43 ± 13.2 km), which was significantly different from the $18,295 \pm 10,830$ rotations (20.1 ± 11.9 km) ran by the WHEEL rats ($p < 0.00001$).

In the experimental set-up used for assessment of reversibility, female Wistar rats assigned to the ABA and CONTROL groups did not differ significantly in body weight at the time of baseline [^{18}F]MK-9470 small animal PET (126 ± 14 g and 125 ± 17 g, respectively). At the chronic time point, average body weight and food intake of ABA in comparison to CONTROL was 108 ± 13 g vs 142 ± 30 g and 6 ± 3 g vs 20 ± 5 g, respectively. ABA rats ran during the 10 days in the model $27,838 \pm 7,910$ wheel rotations, which represents 30.6 ± 8.7 km. Upon renewed unrestricted eating, ABA rats increased their weight by on average 17 %, compared to the weight at the chronic scan, and CONTROLS by 4 %, a difference which was significant from day 2 ($p < 0.05$). Food intake in the CONTROL group decreased over time in comparison to the ABA rats, the first eating 18 ± 3 g and the latter 20 ± 3 g.

On the day of chronic scanning in the ABA and DIET groups, 1 rat was in the early ovarian cycle phase compared to 19 and 7 rats in the late phase, respectively. In the WHEEL group, two rats were in the early phase and six in the late phase, while in the CONTROL group six were in the early phase and four in the late phase. Upon recovery, four ABA rats were found in the early ovarian cycle phase and three in the late phase, while five CONTROL rats were also in the latter one. Rats in the transition between early and late phases were not considered.

Categorical and interaction comparisons of small animal [^{18}F]MK-9470 PET

Absolute [^{18}F]MK-9470 binding was significantly increased in the whole brain of male ABA rats compared to CONTROL

rats (average difference 67 %, $p_{\text{cluster}} = 6.3 \times 10^{-6}$). Absolute binding of [^{18}F]MK-9470 to the CB1 receptor was also significantly increased in all cortical and subcortical areas in the female ABA group compared to WHEEL and CONTROL groups. The increases amounted to +51 % ($p_{\text{cluster}} = 7.6 \times 10^{-8}$) and +53 % ($p_{\text{cluster}} = 8 \times 10^{-8}$), respectively. The increase in absolute [^{18}F]MK-9470 binding in ABA compared to DIET (+20 %) was not significant, nor were the other group comparisons. In the experimental set-up used for assessment of reversibility, absolute [^{18}F]MK-9470 binding significantly increased between baseline and chronic phase (+36 %, $p_{\text{cluster}} < 2.5 \times 10^{-5}$) in all cortical and subcortical regions of ABA rats as compared to CONTROL, while absolute [^{18}F]MK-9470 binding subsequently decreased again in these regions of ABA upon recovery. No significant differences were noted for recovery versus baseline between both groups. Figure 4 shows the average images of absolute [^{18}F]MK-9470 binding in all experimental conditions, overlaid on a T2-weighted MR template. As can be seen from this figure, the pattern of [^{18}F]MK-9470 uptake observed in the rat control brain is similar to that previously reported ex vivo [34], i.e. a fairly uniform and high uptake in the cortex, cerebellum and caudate-putamen. Figure 5 shows absolute [^{18}F]MK-9470 binding in a regional VOI bar plot during anorectic state where the significant increases in all regions were confirmed ($p < 0.0001$).

When looking at regional changes by relative scaling to the global mean tracer binding, no differences in relative [^{18}F]MK-9470 binding were found in male rats. However, in female rats, a regional increase was detected in the ABA group compared to the CONTROL (+4.6 %, $p_{\text{cluster}} < 1.3 \times 10^{-6}$; Fig. 6a) and WHEEL (+2.8 %, $p_{\text{cluster}} < 3.1 \times 10^{-5}$; Fig. 6b) groups using the voxel-based and VOI-based approach. These increases encompassed the hippocampus, the visual and entorhinal cortex, cerebellum and inferior colliculus. The hippocampal increase was also found in the

Fig. 4 Mean [^{18}F]MK-9470 in ABA. **a** Coronal, transverse and sagittal brain slices of absolute [^{18}F]MK-9470 binding in the rat brain of male ABA and CONTROL animals (left) and of female ABA, DIET, WHEEL and CONTROL animals (middle). As reference, a T2-weighted MR image oriented in Paxinos space is shown on the right side. **b** Transverse

brain slices of absolute [^{18}F]MK-9470 binding in the rat brain of female ABA and CONTROL animals at baseline, at a chronic time point during the model and after spontaneous weight recovery. Mean images for the whole group are shown, with the intensity shown at a fixed mSUV scale

◀ **Fig. 5** Comparison of absolute [^{18}F]MK-9470 binding in ABA and controls. Bar diagrams showing absolute [^{18}F]MK-9470 binding expressed in mSUV per region of interest in male ABA and CONTROL animals (a) and in female ABA, DIET, WHEEL and CONTROL animals (b). Error bars indicate 1 SD

comparison of the DIET group with the CONTROL group (+3.4 %, $p_{\text{cluster}} < 6.2 \times 10^{-4}$; Fig. 6c). Furthermore, in the experimental set-up used for assessment of reversibility, the comparison of chronic to baseline confirmed the previous increase in relative [^{18}F]MK-9470 binding in the hippocampus, inferior colliculus and visual cortex (>3.4 %, $p_{\text{cluster}} < 0.04$), but also reported

increased binding in the caudate-putamen and somatosensory and auditory cortices of ABA rats in comparison to CONTROL. After recovery, no significant differences in relative [^{18}F]MK-9470 binding were detected for both groups in comparison to baseline. Detailed cluster peak locations and p values of categorical and interaction SPM comparisons on relative [^{18}F]MK-9470 binding are given in Table 1.

Regarding the comparison of male and female rats, absolute and relative [^{18}F]MK-9470 binding did not differ between male and female ABA rats ($p > 0.9$) or between male and female CONTROL rats ($p > 0.4$) (Fig. 7). The per cent differences between ABA and CONTROL found in both experimental settings were also not significantly different between male and

Fig. 6 Comparison of relative [^{18}F]MK-9470 binding in ABA and controls. Coronal brain sections showing overlays on the regions where a statistically significant increase (red) in relative [^{18}F]MK-9470 binding was observed between female ABA group compared to CONTROL (a) and WHEEL (b) groups and between female DIET group compared to CONTROL group (c) (figure given at $p_{\text{height}} \leq 0.001$ uncorrected). Significance is shown with a T statistic colour scale, which corresponds to the level of significance at the voxel level. The distance between the sections is 1.00 mm with the position relative to bregma (positive values for sections anterior to bregma) on top of the sections in the left corner. Images are oriented in neurological convention

Table 1 Peak locations for the clusters in the categorical and interaction comparisons of relative [^{18}F]MK-9470 binding (at $p_{\text{height}} \leq 0.001$ uncorrected, $k_E > 200$)

	Cluster level		Voxel level		Intensity difference ^a (%)	Structure			Name
	<i>p</i> _{corr}	<i>k</i> _E	T	<i>p</i> _{uncorr}		x	y	z	
Categorical relative [¹⁸ F]MK-9470 analysis									
Female: ABA>CONTROL	<0.001	4,587	7.45	<0.05	+4.6	0.8	−9.2	−2.6	Right hippocampus, right entorhinal cortex and inferior colliculus
				FWE		1.4	−5.2	−2.6	
			7.10	<0.05		2.2	−2.8	−3.6	
	<0.001	1,275	7.07	<0.05	+4.6	4.8	−8.4	−4.6	
				FWE		4.8	−5.8	−4.0	
			6.01	<0.05		5.0	−4.4	−8.0	
Female: ABA>WHEEL	<0.001	21,626	6.60	<0.001	+2.8	5.2	−4.4	−8.2	Right hippocampus, left visual cortex and left cerebellum
				FWE		−1.2	−8.4	−1.4	
			5.41	<0.001		−1.8	−10.6	−2.6	
Female: DIET>CONTROL	<0.001	2,557	5.51	<0.001	+3.4	1.8	−2.6	−3.8	Bilateral hippocampus and superior colliculus
				FWE		−0.2	−2.4	−4.0	
			4.99	<0.001		1.2	−5.8	−3.4	
	<0.001	2,593	5.32	<0.001	+3.4	0.6	−9.2	−2.4	Right inferior colliculus and left cerebellum
				FWE		−1.2	−11.4	−2.0	
			5.07	<0.001					
Interaction (condition x subject) relative [¹⁸ F]MK-9470 analysis									
(Chronic baseline) _{ABA} >(Chronic baseline) _{CONTROL}	0.03	819	4.63	<0.001	+3.6	−5.6	−2.6	−4.4	Left primary somatosensory cortex and left caudate-putamen
				FWE		−4.2	−1.4	−5.4	
			3.70	<0.001		6.0	−2.8	−5.2	
	0.017	967	4.48	<0.001	+3.6	5.0	−4.4	−4.6	Right hippocampus and right somatosensory, auditory and visual cortices
				FWE		5.2	−5.0	−2.8	
			3.92	<0.001					
	0.036	768	4.45	<0.001	+3.6	−2.8	−8.6	−3.8	Left inferior colliculus and left visual cortex
				FWE		−3.2	−7.4	−2.4	
			4.17	<0.001		−4.4	−8.0	−2.2	
		3.88	<0.001						

p_{corr} at cluster level: the chance (p) of finding a cluster with this or a greater size (k_E), corrected for search volume, k_E cluster extent, T measure of the statistical significance, p_{uncorr} at voxel level: the chance (p) of finding (under the null hypothesis) a voxel with this or a greater height (T statistic), uncorrected for search volume, x lateral distance in millimetres from the midline (negative values to the right side), y anteroposterior location relative to bregma (negative values: posterior to bregma), z dorsoventral position (based upon the Paxinos stereotactic atlas)

^a Intensity difference at the voxel level of ABA rats in comparison to controls

female rats ($p > 0.2$). For both ABA and CONTROL groups, body weight on scan day was significantly lower in the female rats compared to male rats ($p < 0.00001$). Also, in female rats, absolute and relative [^{18}F]MK-9470 binding did not differ between rats in early and late phases of the ovarian cycle for WHEEL and CONTROL groups (p value > 0.2).

Correlation analysis of [^{18}F]MK-9470 PET and behavioural data in ABA

As the above-mentioned differences in behavioural outcomes between ABA and CONTROL were highly significant, combining both groups for correlation analysis of these measurements would only reflect group differences. We therefore only

performed correlation analysis between [^{18}F]MK-9470 PET and behavioural data within the ABA group.

In the ABA group, average food intake was positively correlated with body weight on the day of scanning in both genders (Pearson's $r = 0.62$ and $r = 0.57$, respectively). Average food intake was also negatively correlated with relative body weight loss, but only in male rats ($r = -0.72$). Moreover, a negative correlation was found between number of wheel rotations and body weight on the day of scanning in male rats ($r = -0.70$), while a positive correlation of the number of wheel rotations with relative body weight loss was found in female subjects ($r = 0.79$). In the WHEEL group, the number of wheel rotations correlated positively with average food intake ($r = 0.82$).

Fig. 7 Comparison of absolute [^{18}F]MK-9470 binding between male and female ABA. Bar diagrams showing absolute [^{18}F]MK-9470 binding expressed in mSUV per region of interest in the ABA and CONTROL groups from both genders. Error bars indicate 1 SD

For both male and female ABA rats, no significant correlations were found between absolute or relative [^{18}F]MK-9470 binding and average food intake, total running activity, weight loss or weight gain.

Discussion

The aetiology of human AN remains largely unknown, but a complex interplay between genetic, neurobiological and environmental factors appears to play a role. These involve known neurochemical transmission disturbances in the serotonin and dopamine systems, but also a central role of the ECS as modulatory system may be present [35], either directly or through interaction with other neurotransmitter systems involved in eating behaviour.

In this study, we have for the first time characterized CB1 receptor alterations in vivo in the activity-based rodent model of AN using [^{18}F]MK-9470 and small animal PET. Compared to controls, ABA rats showed an in vivo increase in CB1 receptor availability in all cortical and subcortical areas that normalized again towards baseline values after weight gain. This finding of a widespread transient increase is not surprising considering its role in feeding modulation, in hedonics of food intake and generally in reward [36]. Stimulation of endocannabinoid signalling has an orexigenic effect, but the ECS is also physiologically involved in energy homeostasis of food intake [37]. Previously, compiled preclinical evidence has led to the likeliness of a hypoactive ECS in anorectic conditions based on several indirect arguments. CB1 receptor

inactivation via blockade or genetic deletion reduces food intake by suppressing the hedonic aspect [10, 17, 38–41]. CB1 receptor knockout mice also display a lean phenotype and are resistant to diet-induced obesity [42]. In addition, Hanus et al. reported decreased levels of 2-AG in the entire mouse brain, including hypothalamic and hippocampal regions, upon 12 days of food restriction in mice [9]. Frideri et al. stated that in the context of prolonged starvation, as seen in AN, decreased levels of 2-AG may aid survival by downregulating the orexigenic effect of EC signalling and reducing appetite and motivation to eat [43]. Although no direct data on the CB1 receptor status have been reported so far in experimental AN, it is conceivable from our observations that a widespread increase in CB1 receptor availability is in accordance with the above-mentioned impaired endocannabinoid tone, if one assumes that receptor upregulation occurs as a compensatory action due to a chronically hypoactive ECS, as often seen for other receptor systems [44]. Albeit that a decreased endocannabinoid tone could also drive a decrease in CB1 receptor expression [45] or that the change in CB1 receptor expression occurs independently from changes in the endocannabinoid content [46], a compensatory upregulation is plausible from the large intracellular CB1 receptor reserve previously reported [47]. Other contributing mechanisms such as changes in receptor affinity, in receptor trafficking and in conformational state cannot be excluded, as they cannot be distinguished with PET. Future studies simultaneously measuring brain endocannabinoid levels and CB1 receptor binding using in vivo microdialysis and small animal PET in the

ABA model are needed to further clarify the above issue. It remains also to be elucidated whether changes in expression and functionality of CB1 receptors are present in ABA. Nevertheless, the transiency of our finding suggests it to be secondary to ABA and not causal. Of special interest, we have observed a similar finding of a widespread increase in female anorectic patients using the same radioligand and PET [16], suggesting validity of our animal model to mimic human CB1 receptor alterations in AN.

In female ABA, especially hippocampal regions presented a regional increase in CB1 receptor availability. Our rank order of this increase among the different female conditions (i.e. ABA > DIET > WHEEL and CONTROL) is comparable to the ones of a recent *ex vivo* study looking at cell proliferation in this model [48]. Barbarich-Marsteller and co-workers observed a profound reduction in cell proliferation of the dorsal hippocampus in ABA rats using BrdU⁺ and Ki-67⁺ immunohistochemistry that was correlated to body weight and food intake, although the authors stated that various factors relevant to ABA, including food restriction, exercise and sleep deprivation, have an influence on neurogenesis. Of these various factors, especially running activity has been linked to the ECS [49]. Voluntary exercise in rats has been reported to influence hippocampal cell proliferation through modulating the levels of AEA and the density and functionality of CB1 receptors [49]. Female rats exhibiting ABA also showed elevated levels of GABA_A receptor subunits $\alpha 4$ and δ in the hippocampus [50], of which their transmission is known to be modulated via the ECS, but this effect did not show any rank order. We hereby confirmed the prominent role of the hippocampus in another species of female ABA rats than initially reported (Wistar vs Sprague-Dawley rats) [48] and observed no hippocampal effect in the male population. To further strengthen the influence of running activity on CB1 receptor changes in ABA, we also observed a regional increase in CB1 receptor binding in the inferior colliculus that is part of the midbrain tectum and controls motor responses [51].

With respect to the observed gender difference, it is conceivable that cannabinoid effects on neuronal processes are in part influenced by oestrogen and progesterone levels, especially in the hippocampus [52, 53]. However, we were unable to observe any difference in global CB1 receptor binding between male and female rats of the ABA and CONTROL groups. Also, female rats in the early phase of the menstrual cycle did not differ from those in the late one, at least not under the control conditions. In future studies, cycle phase synchronization and monitoring of cycle-dependent parameters, such as adrenocorticotrophic hormone (ACTH) and cortisol, could totally exclude potential interaction of this variable. Given the involvement of ECS in both eating and motor aspects of AN, multiple control groups should at least be included in these studies. We showed that the association of both seems necessary for significant changes in global and regional CB1 receptor binding.

From a methodological point of view, confounding effects of weight differences on [¹⁸F]MK-9470 quantification are unlikely. It is known that large differences in body weight between AN and control subjects may represent differences in [¹⁸F]MK-9470 distribution volume, e.g. in fatty tissues. However, we have explicitly validated the use of mSUV prior to study set-up. Full kinetic modelling as the gold quantification standard in a small subset of ABA and control rats showed a positive correlation of mSUV with total tracer distribution volume (V_T) ($y=90x-57$; $R^2=0.9$). Also, ABA and control rats showed comparable [¹⁸F]MK-9470 percentage fractions in arterial samples ($62.2\pm 3.4\%$ vs $58.4\pm 9.5\%$ and $12.8\pm 7.4\%$ vs $7.1\pm 0.5\%$ for ABA and control at 3 and 40 min post-injection, respectively), and non-parametric analysis did not detect significant differences in the AUC of the metabolite-corrected plasma input functions. Furthermore, the mSUV parameter is normalized by animal weight; consequently the systemically lower weight of ABA rats would imply an underestimation of CB1 receptor availability and does therefore not explain the global increase observed in this manuscript. In addition, we did not observe any difference in [¹⁸F]MK-9470 uptake between male and female rats within each condition, even though their body weight differed significantly.

Conclusion

In vivo cerebral mapping of a well-known rodent model for AN using [¹⁸F]MK-9470 small animal PET points to a widespread transient disturbance of the endocannabinoid transmission, specific for CB1 receptors and especially affecting the hippocampus in female subjects, that normalized upon recovery. Our findings also demonstrate molecular validity of the ABA model to mimic CB1 receptor alterations in human AN.

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Conflicts of interest None.

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