TITLE PAGE**Exercise training and high fat diet elicit endocannabinoid system modifications in the rat hypothalamus and hippocampus.**

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Short title: Endocannabinoid system in brain, diet and exercise effects

ABSTRACT

The purpose of the present study was to examine the effect of chronic exercise on the hypothalamus and hippocampus levels of the endocannabinoids (eCBs) anandamide (AEA), 2-arachidonoylglycerol (2-AG), and of two AEA congeners, and on the expression of genes coding for CB1, CB2 receptors (*Cnr1* and *Cnr2* respectively) and the enzymes responsible of eCB biosynthesis and degradation, in rats fed with a standard or high fat diet. Male Wistar rats (n=28) were placed on a 12-week high fat (HFD) or standard diet period, followed by 12 weeks of exercise training for half of each group. Tissue levels of eCBs and related lipids were measured by liquid chromatography mass spectrometry, and expression of genes coding for CB1, CB2 receptors and eCB metabolic enzymes were measured by quantitative real time Polymerase Chain Reaction (qPCR). HFD induced a significant increase in 2-AG ($p<0.01$) in hypothalamus. High fat diet paired with exercise training had no effect on AEA, 2-AG and AEA congener levels in the hypothalamus and hippocampus. *Cnr1* expression levels were significantly increased in the hippocampus in response to HFD, exercise and the combination of both ($p<0.05$). Our results indicate that eCB signaling in the CNS is mainly sensitive to diet and/or exercise.

Key words: Key words: 2-Arachidonoylglycerol, Anandamide, CB1 receptor, CB2 receptor, hippocampus, hypothalamus

INTRODUCTION

Strong evidence supports a role of the Endocannabinoid System (ECS) in the impaired regulation of food intake and energy metabolism that contribute to the development of obesity [15, 43]. The ECS is a complex endogenous signaling system comprising 7-transmembrane domain receptors (cannabinoid type 1 (CB1) and type 2 (CB2) receptors), their endogenous lipid-derived ligands (the endocannabinoids, eCBs) and enzymes for eCB biosynthesis and degradation. The two most studied eCBs are *N*-arachidonylethanolamine (AEA), also known as anandamide, and 2-arachidonoylglycerol (2-AG). eCBs are not stored in cells but are synthesized on demand from arachidonic acid containing phospholipid precursors through enzyme activation by multiple pathways in the cell membrane of most mammalian cells such as neurons, adipocytes and skeletal muscle cells, possibly in response to elevated levels of intracellular calcium [6], membrane depolarization and/or receptor stimulation [36].

In the central nervous system (CNS), the ECS interacts with the different systems involved in control of food intake and energy expenditure [15]. In rodents, AEA injection in hypothalamus elicits increased feeding via CB1 activation [27] by modulating probably the expression and the action of orexigenic and anorectic mediators, such as the neuropeptide melanin concentrating hormone or the corticotropin-releasing hormone [9, 28]. In addition to the regulation of food intake, the hypothalamic ECS is also involved in energy expenditure regulation by acting on thermogenesis [29].

The ECS plays also a role in hedonic aspect of food intake by modulating brain area activities of the reward system such as the nucleus accumbens, ventral tegmental area or hippocampus [31] [34]. Kirkham et al. 2002 [30] observed that the injection of 2-AG into the nucleus accumbens shell produced a short term stimulatory action on feeding behaviour in free-feeding rats. The ECS seems to facilitate the mesolimbic dopamine signaling that stimulates appetite, as Verty et al. [49] observed that a dopamine D1 receptor antagonist attenuates feeding induced by a CB1 agonist.

Previous studies in obese rodents suggest the presence of ECS dysregulation in the hypothalamus and hippocampus [14, 34]. Di Marzo et al. [14] found that genetically obese rats and mice with disrupted leptin signaling (Zucker *fa/fa* rats and *db/db* mice), as well as mice lacking leptin (*ob/ob* mice), have higher hypothalamic endocannabinoid levels compared with wild-type animals. Similar findings were also reported in the hippocampus of mice with diet-induced obesity [34]. These central ECS alterations, together with peripheral eCB disturbances in the adipose tissue and skeletal muscle may participate in excessive and/or ectopic fat accumulation and related metabolic disorders [12, 35].

In human obesity, ECS dysregulation is supported by the observation of changes in AEA and/or 2-AG levels in the plasma and adipose tissue [3, 18]. The ECS represents a primary target for the treatment of abdominal obesity and associated metabolic changes, whether its dysregulation is a consequence or a cause of obesity. It is noteworthy that before being withdrawn from the market due to psychiatric side effects such as anxiety and depression [8], CB1 antagonists were clearly shown to be effective at reducing body weight and waist circumference in obese subjects [38].

Exercise is a recognized treatment of obesity [37]. One bout of exercise triggers eCB signaling by elevating AEA plasma levels [45] [23, 40]. However, there is some evidence suggesting that a more long lasting healthy lifestyle approach may be effective to reverse ECS dysregulation [3, 17] and may represent a safe alternative to the pharmacological approach. A 1-year lifestyle

modification program including physical activity induced a significant decrease in fasting plasma AEA (-7.1%) and, most importantly, 2-AG (-62.3%) levels in viscerally obese men [17]. In subcutaneous and visceral adipose tissues, chronic exercise limits CB1 gene expression increase induced by a high fat diet in rodents [51]. Thus, chronic exercise may counteract ECS dysregulation in these tissues.

In the CNS, the ECS mediates exercise-induced hippocampus plasticity [25] and reward [20]. However, it remains unknown whether exercise has beneficial effects on the ECS in the CNS of obese animals, and more specifically the hypothalamus and hippocampus, involved in the control of energy balance [32, 48].

This study aimed therefore at identifying potential changes elicited by exercise training in the brain ECS of rats on a high fat diet, compared to rats on a standard diet. With this purpose we determined the hypothalamic and hippocampal levels of AEA, 2-AG, and two AEA congeners, *N*-oleylethanolamine (OEA) and *N*-palmitoyl-ethanolamine (PEA); the expression of genes encoding for eCB receptors (CB1, CB2) and enzymes involved in eCB anabolic (α / β -hydrolase 4, glycerophosphodiesterase 1, N-acylphosphatidylethanolamine-phospholipase D, protein tyrosine phosphatase N22 (respectively ABHD4, GDE-1, NAPE-PLD and PTNP-22) for AEA, OEA and PEA; diacylglycerol lipase α (DAGL- α) and diacylglycerol lipase β (DAGL- β), for 2-AG) and catabolic (fatty acid amide hydrolase (FAAH), for AEA, OEA and PEA; α / β -hydrolase 6 (ABHD6), α / β -hydrolase 12 (ABHD12) and monoacylglycerol lipase (MAGL), for 2-AG) pathways [16]. We also determined the expression of the transient receptor potential vanilloid type-1 (TRPV1) channel, which is activated by eCBs and AEA congeners, and is an ionotropic receptor for eCBs [52].

MATERIALS AND METHODS

Animals and general procedure

General procedures were already described previously [21]. Briefly, 28 male Wistar rats (3 weeks old) were housed in groups of two or three per cage. After one week of acclimatization, rats were randomly divided into 2 groups and fed ad libitum either with a standard diet (Energy equivalent: 2.90 Kcal.g⁻¹) or a high fat diet (Energy equivalent: 5.05 Kcal.g⁻¹) during 24 weeks. After 12 weeks, half of the rats were submitted to 12 weeks of exercise training (Ctl+training and HFD+training). The second half of the rats remained untrained for 12 weeks (Ctl and HFD groups for rats on standard and high fat diets respectively). Before and after the training period, Ctl+training and HFD+training performed a maximal aerobic velocity (MAV) test on the treadmill. Five days before sacrifice, all rats were subjected to an oral glucose tolerance test (OGTT). At the end of the exercise training period, blood samples were collected by cardiac puncture, after which all rats were euthanized and the hypothalamus and hippocampus removed.

All procedures described were approved by the Agricultural and Forest Ministry and the National Education Ministry (Veterinary service of health and animal protection) and were in accordance with the European Union Directive of 22 September 2010 (2010/63/UE).

High fat diet

Rats were fed with two different types of diet during the 24 weeks of the experimentation:

- A high fat diet (Purified Diet 231 HF, Safe, Augy, France) with an energy equivalent of 5.05 kcal.g⁻¹, and containing 26.9% of proteins, 39.7% of lipids and 10.1% of carbohydrates.
- A standard diet with an energy equivalent of 2.90 Kcal.g⁻¹. It contained 16% of proteins, 3 % of lipids, 60% of carbohydrate and 21% of other components (fiber, mineral, humidity).

Fatty acid compositions of the two diets are described in Table 1. Food and caloric intake by each rat and their weight gain were estimated 2 times per week during the experimentation.

Maximal aerobic velocity test

Animals of in the HFD+training and Ctl+training were familiarized with treadmill running (L810, Bioseb. France) during 10 min for 5 days at a velocity of 20 cm.s⁻¹ and a 0° slope. Electric shocks (intensity < 1.2 mA) were used to motivate the rat to run. After the familiarization period, Ctl+training and HFD+training groups performed a graded exercise test to voluntary exhaustion. The test started at 20 cm.s⁻¹ for 5 min, followed by speed increment of 3 cm.s⁻¹ every 3 minutes until the animal could no longer keep up with the treadmill speed. Exhaustion was reached when animal sat longer than 10 s on electric shock grid. MAV was defined

as the velocity of the last 3 min stage completed. The same protocol was repeated 1 week before rats sacrifice to determine the change in MAV with exercise training (two days before the OGTT).

Exercise training

The day after the baseline MAV test, Ctl+training and HFD+training groups started the 12-week exercise training period that consisted of treadmill running for 1hour/day, 5 days/week at an intensity set between 70 – 80 % of the MAV. The intensity was increased by 1 cm.s⁻¹ every week to take into the adaptations to exercise training. Animal exercised at the same hour of the day at the end of the room dark cycle (7:30 am). Control groups were in the same room during the training session and handled in the same way to induce a similar stress level. Three day before the sacrifice, exercise training was stopped to avoid the confounding fatigue or stress effect of acute exercise.

Oral Glucose Tolerance Test

Five days before sacrifice, the animals were fasted overnight. Basal blood glucose level, defined as T0, was determined using an automatic glucometer (Accu-Chek Performa; Roche Diagnostics) before oral administration (4 ml.kg⁻¹ of body weight) of a D-glucose solution (50%). Tail vein blood glucose was then measured at 30, 60, 90, 120 min after the administration. Total Area under the curve (AUC) was calculated using the trapezoidal method [39].

Samples collection

The day before the end of the experiment, rats were fasted in order to obtain the same nutritional state for each. Animals were anesthetized with pentobarbital sodium (60 mg.kg⁻¹ of body weight, ip) and blood samples were collected by cardiac puncture. Samples were drawn directly into pre-cooled 5-mL EDTA tubes. EDTA blood was immediately centrifuged (less than 5 min after sampling) and plasma was removed and frozen (- 80 °C) until analysis. Then rats were sacrificed by decapitation. The head was immediately surrounded with ice and the hypothalamus and hippocampus were quickly removed, weighed and immediately frozen in liquid nitrogen. Then they were stored at -80°C until analyses.

Plasma analyses

Fasting levels of glucose and insulin were determined in plasma. The content of glucose in plasma was measured using a commercially available colorimetric assay kit (Cayman Chemical Company, USA). Plasma insulin was determined using a commercially available rat insulin enzyme immunoassay kit (SPI-BIO, France).

Measurements of endocannabinoids

The extraction, purification, and quantification of EC from tissues have been performed as previously described [26]. Briefly, the tissues were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mmol.l⁻¹ pH 7.5 (2:1:1, vol/vol) containing internal standards ([²H]₈ AEA; [²H]₅ 2-AG, [²H]₅ PEA and [²H]₄ OEA, 5 pmol each). The lipid-containing organic phase was dried down, weighed, and pre-purified by open-bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1, 90:10 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA, 2-AG, PEA and OEA quantification by liquid chromatography – atmospheric pressure chemical ionization – mass spectrometry by using a Shimadzu high-performance liquid chromatography apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadruple mass spectrometry via a Shimadzu atmospheric pressure chemical ionization interface as previously described [26]. The amounts of analytes in tissues, quantified by isotope dilution with the above mentioned deuterated standards, were expressed as picomole per gram or milligram of wet tissue weight.

RNA purification and quantitative real-time PCR (qPCR)

Total RNA was isolated from native tissues by use of the TRI-Reagent (Sigma-Aldrich, Milan, Italy), reacted with DNase-I (1 U/ml; Sigma-Aldrich) for 15 min at room temperature, and followed by spectrophotometric quantification. Final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥ 1.7 . Isolated mRNA was reverse transcribed by use of SuperScript III Reverse Transcriptase [Life Technologies, Monza (MI), Italy]. The quantitative real-time PCR was carried out in CFX384 real-time PCR detection system [Bio-Rad, Segrate (MI), Italy] with specific primers [26]) by the use of SYBR Green master mix kit [Bio-Rad, Segrate (MI)] (See table 2 for primers sequence). Samples were amplified simultaneously in quadruplicate in one- assay run with a non-template control blank for each primer pair to control for contamination or primer-dimers formation, and the ct (cycle threshold) value for each experimental group was determined. The housekeeping gene (the hypoxanthine-guanine phosphoribosyltransferase, hprt) was used as an internal control to normalize the ct values, using the $2^{-\Delta Ct}$ formula; differences in mRNA content between groups were as expressed as $2^{-\Delta\Delta Ct}$.

Statistical analyses

Data are shown as means $\pm$ SE. Normal Gaussian distribution of the data was verified by the Shapiro-wilk test. Repeated measure analysis of variance (ANOVA) was used to evaluate the evolution of weight and accumulated caloric intake during the first 12 weeks of the experiment, the evolutions of weight and MAV during the exercise-training period and mean caloric intake of the next to last week of each period and the glycemia during the OGTT. Multiple comparisons were made with the Newman-Keul post-hoc test. A two-way ANOVA was used to evaluate the effects of diet, exercise training and the diet – exercise interaction on metabolic parameters (fasting glucose, insulin, AUC during OGTT), and on eCBs and congeners tissue levels. Multiple comparisons were made with the Bonferroni post-hoc test if significant main effects or interaction were observed with ANOVA.

Concerning gene expression data, Ctl group was compared with other groups by using Student's t-Test. Statistical significance was set at $p = 0.05$ level for all analysis. All calculations were made with Statistica 6.0 (Statsoft, Tulsa, USA).

RESULTS

Effect of diet and/or exercise on body mass, caloric intake, maximal aerobic velocity, basal glucose and insulin levels, and glucose tolerance

Figure 1 shows body weight gain over the diet and exercise training period. Over the first 12 weeks, body mass increased with time and this is all the more the case of the high fat diet (Table 3). From the 10th week, rats fed with the high fat diet were significantly heavier than rats fed with the control diet ($p < 0.01$; Figure 1). During the exercise period, rats fed with the high fat diet continued to gain more weight with time while exercise training slowed down the time-induced body gain, especially for the HFD+training group (table 3).

Accumulated caloric intake is described in Figure 2. Over the first and second period of the protocol, we observed that rats fed on the high fat diet accumulated more and more rapidly calorie than rats fed on the standard diet (table 3). From the 8th week, rats fed with the high fat diet accumulated significantly more calorie than rats fed with the control diet ($p < 0.01$; Figure 2). However, over the second period, the accumulated caloric intake was slowed down by exercise training (Table 3).

MAV was measured only in the exercise trained groups to avoid a familiarization in Ctl and HFD groups that could affect the results. Two-way ANOVA for MAV revealed significant effects of diet and time, but no significant interaction between both factors (Table 4). There were no significant differences for fasting plasma insulin levels between groups. Fasting plasma glucose concentration was increased by the high fat diet but this increase was strongly reduced by exercise training (Table 4). Between group comparison indicated that in the HFD group glycaemia was significantly higher than other groups (Table 4). Glucose AUC during the OGTT performed one week before the animal sacrifice was also significantly increased by the high fat diet (Table 4) but without significant protecting effect from exercise training. Regarding glucose kinetics during the OGTT (Figure 3), the ANOVA revealed a significant effect of diet ($F_{(1, 26)} = 85.23$, $p < 0.006$ and interactions between time and diet ($F_{(4, 104)} = 3.97$, $p < 0.008$) as well time, training and diet ($F_{(4, 96)} = 6.59$, $p < 0.0001$). The HFD group showed higher level of blood glucose than other groups at 30 min ($p < 0.02$). At 90 and 120 min, blood glucose levels for the HFD groups were higher than Ctl+training group ($p < 0.05$ for all).

Effect of diet and/or exercise on AEA, 2-AG, PEA, OEA levels and the expression of genes coding for eCB receptors and eCB metabolic enzymes in brain tissues.

Hypothalamus

Hypothalamic eCB and AEA congener levels are reported in Table 5. Diet had no significant effect on AEA, and AEA congeners, whereas 2-AG levels were increased by HFD ($p < 0.01$). Gene expression of the enzymes implicated in eCB synthesis or degradation were affected by the HFD (Fig. 4(A)). Expression of genes coding for ABDH4, NAPE-PLD (*Abdh4* and *Nape-pld* respectively), i.e. two enzymes potentially involved in AEA, OEA and PEA biosynthesis, were significantly decreased in the HFD group compared to control rats ($p < 0.05$), and so was the mRNA coding for FAAH (*Faah*), a major enzyme for AEA, OEA and PEA degradation ($p < 0.05$). Thus, the concurrent down-regulation of biosynthetic and inactivating enzymes expression may explain the lack of effect of the HFD on levels of AEA and its congeners. Concerning 2-AG, the expression of the mRNA coding for its biosynthetic enzymes (*Magl* and *Dagl- α* or *Dagl- β*) was unchanged with diet while the mRNA coding for its degradation enzymes was either decreased (*Abdh6* gene) or increased (*Abdh12* gene) in the HFD group ($p < 0.05$). Finally, the expression of mRNAs coding for CB1 and CB2 (*Cnr1* and *Cnr2*, respectively) was not altered in the HFD group, whereas *Trpv1* mRNA was significantly decreased (Fig. 4(A) $p < 0.05$).

Exercise had no significant effect on AEA, 2-AG or AEA congener levels (Table 5) nor on the mRNA expression of genes coding for eCB receptors (CB1, CB2). *Gde-1* and *Nape-pld* mRNA expression was significantly decreased with exercise in rats on the standard diet ($p < 0.05$ for both) but not in rats on HFD (Fig. 4(A)). Expression of mRNA for PTNPN-22 (*Ptnpn-22*), another enzyme potentially involved in AEA, OEA and PEA biosynthesis, was instead lowered by exercise training only in the rats fed with the HFD. Concerning 2-AG biosynthesis or degradation, only *Abdh6* mRNA levels were significantly decreased with exercise training in lean rats (Ctl+training group, $p < 0.05$).

Hippocampus

Hippocampal eCB and AEA congener levels are reported in Table 5 and results of qPCR analyses for the hippocampus are presented in Figure 4(B). The levels of eCBs and AEA congeners were not affected by diet or exercise, whereas *Cnr1* mRNA expression was significantly increased in HFD, HFD+training and Ctl+training groups compared to the Ctl group ($p < 0.05$ for all). *Trpv1* mRNA levels were also increased in the HFD+training group compared to the Ctl group ($p < 0.05$). Concerning gene expression of eCB biosynthetic or degrading enzymes, none of them was modified by the HFD. The mRNA expression of *Gde-1* was increased and that of *Nape-pld* was decreased for Ctl + training and HFD + training groups ($p < 0.05$ for both) and *Faah* mRNA levels were increased in these 2 groups compared to the respective ctl groups ($p < 0.05$ for both). The mRNA expression of the 2-AG biosynthetic enzyme DAGL- α was significantly increased in HFD + training group as was that of the 2-AG degrading enzymes ABDH6, ABDH12 and MAGL ($p < 0.05$ for all).

DISCUSSION

This study aimed at identifying changes in the hypothalamic and hippocampal tissue concentrations of 2-AG, AEA and two AEA congeners, OEA and PEA, together with corresponding alterations in the expression of genes encoding for eCB receptors (CB1, CB2) and enzymes involved in the anabolic (ABHD4, GDE-1, NAPE-PLD and PTNP-22, for AEA, OEA and PEA; DAGL- α and DAGL- β , for 2-AG) and catabolic (FAAH, for AEA, OEA and PEA; ABHD6, ABHD12 and MAGL, for 2-AG) pathways of these mediators, after 12 weeks of endurance training in Wistar rats fed with a standard diet or a HFD.

Previous studies in diet-induced obesity (DIO) mice or genetic models of obesity reported a dysregulation of the eCBs and particularly an increase of 2-AG in the hypothalamus [14] [4]. We confirm these results in all Wistar rats fed with the HFD in the present study. As the ECS has an important role in the regulation of food intake through hypothalamic pathways [30], this 2-AG increase might participate in the higher accumulated caloric intake observed in these rats. According to the gene expression results of biosynthetic and degrading enzymes, this 2-AG increase is difficult to explain for two main reasons. First, the HFD group presented at the same time a decrease and an increase in gene expression of two 2-AG degrading enzymes (*Abdh6* and *Abdh12* respectively) that are known to participate only for about 15% in 2-AG hydrolysis [5]. Secondly, these results on *Abdh6* and *Abdh12* expression were not observed in the exercise training group fed the HFD, where the same trends should have been observed to account for the similar changes in 2-AG levels. Thus, more than the mRNA expression of biosynthetic and / or degrading enzymes, enzymatic activities and eCB biosynthetic precursors might be involved in 2-AG level increase induced by HFD. Di Marzo et al. [14] indeed explain this 2-AG increase with defective leptin signaling. Leptin inhibits 2-AG biosynthesis by likely decreasing the formation of diacylglycerol (DAG) precursors [14]. Nevertheless, these results should be taken with caution as this leptin decrease is not systematically observed in Wistar rats fed with high fat diet [7], and other feeding-regulated hormones, such as grehlin and glucocorticoids are also involved in the regulation of hypothalamic eCB levels [43]. Furthermore, in the lateral hypothalamus and arcuate nucleus (two areas of the hypothalamus) of mice fed a HFD, the increased 2-AG levels have been recently shown to be accompanied by increased DAGL- α protein expression [10] [11].

Our results confirm at the mRNA level that the expression of hypothalamic CB1 is not affected by HFD in rats. Previous rat studies have shown a lack of alteration in CB1 density with HFD [22] models. Considering that CB1 agonist levels might regulate CB1 receptors density in the hypothalamus [44] this is consistent with the fact that only the levels of 2-AG, and not also AEA, were found here to be altered in this brain area. Our results however do not exclude transient changes in CB1 or *Cnr1* expression over the course of the study. Indeed, South et al. [44] observed a transient increase in mouse hypothalamic CB1 density after 3 weeks of HFD that was normalized at the end of the 20 weeks of HFD, suggesting temporal CB1 alterations during obesity induction in rodents.

Interestingly, *Trpv1* gene expression was decreased with the HFD. To our knowledge only Baboota et al. [2] has reported a down regulation in the expression of this gene in the mouse hypothalamus after a HFD period. The down-regulation of *Trpv1*, observed

now here also in rats, may play a role in the hyperglycemia observed in the HFD group. Indeed, Zsombok [52] suggested that TRPV1 activation in the paraventricular nucleus of the hypothalamus could lower blood glucose.

Unlike the hypothalamus, the HFD did not alter 2-AG levels in the hippocampus in our study, nor the levels of AEA and its congeners. However, the ECS in this brain area was previously shown to be sensitive to this kind of diet [34, 41], with increased AEA and 2-AG hippocampal concentrations in mice after a 12-week HFD [34], and Rivera et al. [41] reporting increased AEA, and other acylethanolamide (OEA, PEA), but not 2-AG, levels also in rats with 12-week HFD. While these previous studies reported an unbalanced ratio between the expression of eCB biosynthesis and degradation enzymes, we did not observe any significant change in the levels of the mRNAs coding for these enzymes, which may explain the absence of eCB level alterations. Differences in the diet composition between studies may partly explain these discrepancies. Different amounts of polyunsaturated fatty acid precursors in the diet are for example known to modulate the availability of eCB biosynthetic precursors [1]. Nevertheless, as shown in a previous study [34], where the mRNA coding for CB1 (*Cnr1*) and the CB1 protein were overexpressed, the hippocampal ECS may be altered following a HFD and contribute to the development of obesity, or related metabolic alterations. Accordingly, an increase in CB1 expression was shown to participate in the regulation of neuroplasticity involved in the hedonic aspect of eating [34]. Here we found that rats fed with HFD showed increased *Cnr1* mRNA expression as compared to rats fed with standard diet, which correlates with a higher accumulated caloric intake in HFD rats.

The up-regulation of hippocampal *Cnr1* expression was also found with exercise training combined with HFD. Chronic exercise did not appear to counteract ECS overactivation and, in fact, seems even to induce this effect independently of the diet. Indeed, *Cnr1* mRNA and the majority of the genes coding for biosynthetic and degrading eCB enzymes were increased in Ctl+training rats, suggesting an increase in eCB turnover and signaling. This result is not surprising as Hill et al. [25] have already observed an increase in AEA levels and in CB1 agonist binding density after 8 days of free access to a running wheel in the hippocampus of lean Wistar rats. They demonstrated that this ECS overactivation in this brain area was required for chronic exercise-induced neuroplasticity. However, exercise does not produce the reversal of CB1 overactivation induced by HFD in spite of the slowing down of calorie intake accumulation observed with exercise training. Studies of different hippocampal regions will be needed to determine whether exercise and HFD selectively affect eCB signaling in different subpopulations of neurons in this brain area. Altogether, these data suggest that exercise training induces hippocampal CB1 signaling, which may be potentially involved in enhanced neuroplasticity, thus possibly explaining the improved cognitive performance and mood observed typically with exercise [47].

In contrast to the hippocampus, the hypothalamic ECS seems to be less sensitive to exercise, as no change in CB1 and CB2 were observed in HFD and Ctl rats, whether they exercised or not. These results may differentiate chronic exercise from chronic stress, considering that repeated stress exposure leads to ECS alterations in the hypothalamus and other brain area involved in the stress response [42]. Wamsteeker et al. [50] showed a functional downregulation of CB1 receptor in the paraventricular nucleus of the

hypothalamus after repetitive immobilization stress in adolescent Sprague Dawley rats. Indeed, exercise differs from other stressors. It activates a number of systems related to the stress response but other mechanisms associated with chronic exercise exist to reduce the negative effect of this stressor [46], thus possibly avoiding endocannabinoid-signaling impairment.

Our study suffers from several limitations. First, gene expression quantification without data on receptor or enzyme protein levels or function limits the interpretation of our results. Secondly, the brain areas studied were limited to the hippocampus and hypothalamus. Other brain structures involved in the control of eating and voluntary exercise behaviors, such as the ventral tegmental area and striatum are highly sensitive to eCB signaling [13, 20, 44]. Measurements of ECS activities in these areas would have allowed to provide a global view of the different structures affected by exercise and food intake. Third, treadmill was chosen instead of running wheel as exercise protocol. Even though both these protocols are known to induce neural plasticity [33] the forced exercise could induce additional stress compared to the voluntary exercise [19] and thus affect the ECS differently. However, our results (i.e. *Cnr1* upregulation in the hippocampus) do not seem to be the mere consequence of chronic stress induced by forced exercise. In fact, Hill et al. [24] reported hippocampal CB1 downregulation following 21 days of chronic stress.

In summary, we have confirmed here that HFD's are accompanied by changes in the expression of genes encoding for eCB receptors and enzymes involved in the anabolic and catabolic pathways of the ECS in the hippocampus. These changes, and particularly the alteration of hippocampal CB1 receptor gene expression, may participate in weight gain and glucose metabolic perturbation observed with the HFD. While chronic exercise improves weight alterations and avoids hyperglycemia induced by HFD, the alterations in ECS gene expression occurring during the latter diet are not reversed, and chronic exercise may even result in several hippocampal ECS responses similar to those observed with HFD. These results highlight the need for additional investigations about the role of the ECS in the beneficial brain adaptations induced by chronic exercise.

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327 **ACKNOWLEDGMENTS**

328 The authors thank Gamain J., Barbez P., Max and Mahault G. for their advices and technical assistances.

329 The authors have no conflicts of interest that are directly relevant to the content of this article.

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TABLES

Table 1. Fatty acid compositions of the standard (Ctl) and the high fat diet (HFD).

	Ctl	HFD
Total Fat (g/kg)	27.50	395.47
Total Saturated Fat (g/kg)	6.34	140.36
C10:0	-	0.32
C12:0	0.03	0.32
C14:0	0.17	4.19
C15:0	0.03	0.32
C16:0	5.30	86.93
C17:0	0.03	1.29
C18:0	0.58	45.97
C20:0	0.10	0.94
C22:0	0.06	0.07
C24:0	0.06	-
Total Monounsaturated Fat (g/kg)	5.61	171.18
C16:1	0.19	8.14
C17:1	0.03	0.65
C18:1	5.09	158.96
C19:1	-	0.00
C20:1	0.30	2.80
C22:1	-	0.65
Total Polyunsaturated Fat (g/kg)	15.57	86.54
C18:2	13.70	78.14
C18:3	1.13	3.24
C18:4	-	2.58
C20:2	0.03	1.29
C20:3	-	0.65
C20:4	0.06	-
C20:5	0.14	-
C22:1	0.19	-
C22:4	-	0.32
C22:5	0.06	0.32
C22:6	0.22	-
C24:1	0.06	-
Total ω3 fatty acids (g/kg)	1.57	3.91
Total ω6 fatty acids (g/kg)	13.70	75.35

Table 2. Primers sequence used in qPCR analysis

Gene	FORWARD Sequence (5'→3')	REVERSE Sequence (5'→3')	Enter Number	Accession	Product length (bp)
<i>abdh12</i>	CAGGCGTGCGGTCGAAACCA	TCAAGCTGCAGTCGGCGTCC	NM_001024314.1		189
<i>abdh4</i>	TCTGGCGTCAAGCGGAGGGA	ACGCCACCCCCAAAGCCATG	NM_001108866.1		299
<i>abdh6</i>	AGCGTCTGCTCCCATCCCCA	TGGCTTGCCAGTGGCGTGAA	NM_001007680.1		255
<i>cnr-1</i>	CTGAGGGTTCCCTCCCGGCA	TGCTGGGACCAACGGGGAGT	NM_012784.4		285
<i>cnr-2</i>	GCGGCTAGACGTGAGGTTGGC	TCCTTCAGGACCAAGAGTCTCAGCCT	NM_020543.4		335
<i>dagla</i>	GGCCGCACCTTCGTCAAGCT	ATCCAGCACCGCATTGCGCT	NM_001005886.1		380
<i>daglβ</i>	AGACCCGGGTGCAATGCTGC	GCCCTGGTGTGTGGGTACG	NM_001107120.1		212
<i>faah</i>	GGCAGAGCCACAGGGGCTATCA	TGGGGCTACAGTGCACAGCG	NM_024132.3		349
<i>gde-1</i>	GCAGCCCCTTCAACGCCTGT	GATGGCCGCCAGCGTGTCT	NM_019580.4		172
<i>magl</i>	CGGAACAAGTCGGAGGTTGA	TGTCCTGACTCGGGGATGAT	NM_138502.2		220
<i>nape-pld</i>	AGGCTGGCCTACGAATCACGT	ATGGTACACGGGGGACGGCG	NM_199381.1		150
<i>ptpn-22</i>	TGGTCGTGGGAGAGCCGCTT	GGGCCACTTTTTGCGCCTGC	NM_001106460.1		263
<i>trpv1</i>	AGACATCAGCGCCCGGGACT	CCAGCTTCAGCGTGGGGTGG	NM_031982.1		151

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Table 3. F-statistic and its associated degrees of freedom and p-value of Analyses of Variance used to compare the effects of diet and / or exercise on body weight, food intake, maximal aerobic velocity (MAV), basal glucose and insulin levels, oral glucose tolerance test in control, high fat diet, control with chronic exercise and high fat diet with chronic exercise groups

	Main effects by ANOVA	F values	p
Weight (g)			
<i>During diet period</i>	Diet	$F_{(1, 26)} = 53.80$	0.00001
	Time	$F_{(11, 297)} = 1714.96$	0.00001
	Time x Diet	$F_{(11, 286)} = 30.56$	0.00001
<i>During exercise period</i>	Diet	$F_{(1, 26)} = 38.91$	0.00001
	Ex	$F_{(1, 26)} = 0.60$	NS
	Diet x Ex	$F_{(1, 24)} = 0.59$	NS
	Time	$F_{(12, 288)} = 392.85$	0.00001
	Time x Diet	$F_{(12, 312)} = 5.34$	0.00001
	Time x Ex	$F_{(12, 312)} = 16.72$	0.00001
	Time x Diet x Ex	$F_{(12, 288)} = 3.48$	0.0001
Accumulated Caloric Intake (Kcal)			
<i>During diet period</i>	Diet	$F_{(1, 26)} = 63.94$	0.00001
	Time	$F_{(11, 297)} = 9136.67$	0.00001
	Time x Diet	$F_{(11, 286)} = 71.46$	0.00001
<i>During exercise period</i>	Diet	$F_{(1, 26)} = 61.06$	0.00001
	Ex	$F_{(1, 26)} = 0.42$	NS
	Diet x Ex	$F_{(1, 24)} = 1.28$	NS
	Time	$F_{(10, 270)} = 12597.78$	0.00001
	Time x Diet	$F_{(10, 260)} = 28.08$	0.00001
	Time x Ex	$F_{(10, 260)} = 2.99$	0.01
	Time x Diet x Ex	$F_{(10, 260)} = 0.28$	NS

The main effects from ANOVA are as follows: Time, time effect; Diet, diet effect; Ex, exercise training effect; x, Interaction between variables

NS:

Table 4. Effect of diet and / or exercise on body weight, food intake, maximal aerobic velocity (MAV), basal glucose and insulin levels, oral glucose tolerance test (OGTT) in control (Ctl), high fat diet (HFD), control with chronic exercise (Ctl+training) and high fat diet with chronic exercise (HFD+training) groups.

		Ctl	HFD	Ctl+training	HFD+training	Main effects by ANOVA	F values	p
MAV (cm.s⁻¹)								
<i>During diet period</i>				45.9 ± 5.2	41.6 ± 3.9	Time	<i>F</i> _(1, 13) = 95.80	0.0001
<i>During exercise period</i>				61.9 ± 6.0	55.8 ± 5.0	Diet	<i>F</i> _(1, 12) = 5.41	0.05
						Time x Diet	<i>F</i> _(1, 12) = 0.36	NS
Plasma insulin (ng.ml⁻¹)		2.8 ± 0.9	2.9 ± 0.5	2.2 ± 0.8	2.5 ± 0.3	Diet	<i>F</i> _(1, 26) = 0.46	NS
						Ex	<i>F</i> _(1, 26) = 2.19	NS
						Diet x Ex	<i>F</i> _(1, 24) = 0.08	NS
Plasma glucose (mg.dL⁻¹)		83 ± 3	103 ± 12 [*]	86 ± 5	92 ± 5	Diet	<i>F</i> _(1, 26) = 21.09	0.0001
						Ex	<i>F</i> _(1, 26) = 2.91	NS
						Diet x Ex	<i>F</i> _(1, 24) = 5.70	0.05
OGTT (AUC)		247.3 ± 12.4	306.7 ± 20.4	247.7 ± 16.5	286.1 ± 9.8	Diet	<i>F</i> _(1, 26) = 71.07	0.0001
						Ex	<i>F</i> _(1, 26) = 3.02	NS
						Diet x Ex	<i>F</i> _(1, 24) = 3.26	NS

Data are means ± SD;
The main effects from ANOVA are as follows: Time, time effect; Diet, diet effect; Ex, exercise training effect; x, Interaction between variables
* Significantly different from all groups, p <0.05

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Table 5. Hypothalamic and hippocampal concentrations of endocannabinoids and anandamide congeners in control (Ctl), high fat diet (HFD), control with chronic exercise (Ctl+training) and high fat diet with chronic exercise (HFD+training) groups.

	Ctl	HFD	Ctl + training	HFD + training	Main effects by ANOVA	<i>F</i> values	<i>p</i>
Hypothalamus							
<i>AEA</i> (<i>pmol.g⁻¹</i>)	27.82 ± 22.13	37.66 ± 16.61	24.54 ± 9.82	36.16 ± 14.82	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 2.56 <i>F</i> _(1, 26) = 0.12 <i>F</i> _(1, 24) = 0.01	NS NS NS
<i>2-AG</i> (<i>pmol.mg⁻¹</i>)	3.71 ± 1.02	4.82 ± 1.06	3.17 ± 1.14	4.62 ± 0.69	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 9.24 <i>F</i> _(1, 26) = 0.78 <i>F</i> _(1, 24) = 0.16	0.01 NS NS
<i>PEA</i> (<i>pmol.mg⁻¹</i>)	1.98 ± 0.27	1.90 ± 0.26	1.91 ± 0.23	1.98 ± 0.38	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 0.01 <i>F</i> _(1, 26) = 0.00 <i>F</i> _(1, 24) = 0.40	NS NS NS
<i>OEA</i> (<i>pmol.mg⁻¹</i>)	0.56 ± 0.86	0.42 ± 0.09	0.53 ± 0.09	0.54 ± 0.11	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 2.64 <i>F</i> _(1, 26) = 1.84 <i>F</i> _(1, 24) = 3.66	NS NS NS
Hippocampus							
<i>AEA</i> (<i>pmol.g⁻¹</i>)	61.38 ± 24.61	64.20 ± 17.79	58.20 ± 16.44	59.78 ± 18.27	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 0.07 <i>F</i> _(1, 26) = 0.21 <i>F</i> _(1, 24) = 0.01	NS NS NS
<i>2-AG</i> (<i>pmol.mg⁻¹</i>)	1.68 ± 0.31	2.38 ± 1.52	2.03 ± 0.27	1.78 ± 0.40	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 0.39 <i>F</i> _(1, 26) = 0.12 <i>F</i> _(1, 24) = 1.75	NS NS NS
<i>PEA</i> (<i>pmol.mg⁻¹</i>)	1.00 ± 0.25	1.07 ± 0.52	0.83 ± 0.15	0.80 ± 0.21	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 0.03 <i>F</i> _(1, 26) = 2.90 <i>F</i> _(1, 24) = 0.15	NS NS NS
<i>OEA</i> (<i>pmol.mg⁻¹</i>)	0.33 ± 0.07	0.40 ± 0.15	0.36 ± 0.11	0.31 ± 0.06	Diet Ex Diet x Ex	<i>F</i> _(1, 26) = 0.05 <i>F</i> _(1, 26) = 0.37 <i>F</i> _(1, 24) = 1.79	NS NS NS

Data are means ± SD ;

The main effects from ANOVA are as follows : Diet, diet effect; Ex, exercise training effect ; x, interaction between variables.

NS : non significant

FIGURES

Figure 1. Evolution of body weight during the 24 weeks of the protocol in control (ctl), high fat diet (HFD), control with chronic exercise (ctl+training) and high fat diet with chronic exercise (HFD+training) groups. The dashed line corresponds to the introduction of exercise in ctl+training and HFD+training groups. * Significant difference between rats fed with high fat and standard diet during the first period of the protocol: $p < 0.01$.

Figure 2. Accumulated caloric intake during the 24 weeks of the protocol in control (ctl), high fat diet (HFD), control with chronic exercise (ctl+training) and high fat diet with chronic exercise (HFD+training) groups. The dashed line corresponds to the introduction of exercise in ctl+training and HFD+training groups. * Significant difference between rats fed with high fat and standard diet during the first period of the protocol: $p < 0.01$.

Figure 3. Blood glucose levels at 0, 30, 60, 90 and 120 minutes during the oral glucose tolerance test in control (ctl), high fat diet (HFD), control with chronic exercise (ctl+training) and high fat diet with chronic exercise (HFD+training) groups. * Significant difference between HFD group and all other groups: $p < 0.01$. † ctl+training significantly different from HFD and HFD+training groups: $p < 0.05$.

Figure 4. Expression level analysis of the genes related to endocannabinoid metabolism and function in control (ctl), high fat diet (HFD), control with chronic exercise (ctl+training) and high fat diet with chronic exercise (HFD+training) groups. mRNA expression levels of genes encoding for endocannabinoid receptors (*cnr1*, *cnr2*, *trpv1*) or anabolic (*abdh4*, *gde-1*, *nape-pld*, *ptpn22*, *dagla*, *daglb*) and catabolic (*faah*, *abdh6*, *abdh12*, *magl*) enzymes were measured in the hypothalamus (A) and the hippocampus (B). The results obtained by qPCR are reported using the $2^{-\Delta\Delta ct}$ formula using *hprt* as housekeeping gene. Each column represents the mean $\pm$ S.E.M. of at least four independent determinations performed each in quadruplicate. * Significantly different from ctl group: $p < 0.05$

Figure 1