THE ROLE OF THE ENDOCANNABINOID SYSTEM IN THE REGULATION OF ENERGY BALANCE

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Research described in this thesis was funded by an unrestricted grant from Kruidvat (Hutchinson Whampoa Ltd) and University of Groningen

Printing of this thesis was kindly supported by the University of Groningen, Graduate school for drug exploration (GUIDE) and the University Medical Center Groningen

Colofon
Cover and lay-out by Legatron Electronic Publishing, Rotterdam, The Netherlands
Printed by Ipskamp drukkers, Enschede, The Netherlands

RIJKSUNIVERSITEIT GRONINGEN

The role of the endocannabinoid system in the regulation of energy balance

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 9 juni 2010
om 14.45 uur

door

Anna Hendrike Koolman
geboren op 1 november 1973
te Warffum

Promotores:	Prof. dr. F. Kuipers
	Prof. dr. G. van Dijk
	Prof. dr. P.J.J. Sauer
Beoordelingscommissie:	Prof. dr. R.F. Witkamp
	Prof. dr. A.J.W. Scheurink
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ISBN: 978-90-367-4360-0

Voor Robert, Giel en Jort

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CHAPTER 1

General introduction

Introduction

Obesity and obesity-related diseases have become major health concerns in industrialized countries.[1] Excessive caloric intake and, at the same time, a more sedentary life style are the main factors contributing to the current obesity epidemic. This combination has been referred to as a patho-environment, stressing a cause of this pathology outside the patient. The problem is, however, much more complex, and involves an interplay between genetic, epigenetic, psychological, pedagogical, socio-economical and nutritional factors. Since a large variation in body weight exists within populations despite exposure to the same nutrient availability and to the same lifestyle factors, it is of great relevance to understand intrinsic factors contributing to the development of obesity. Moreover, broad advertisements 'to eat less and exercise more' did not prevent the dramatic increase in prevalence of obesity and there is an urgent need for new strategies for treatment and prevention. Consequently, it is pivotal to understand mechanisms involved in maintenance of energy balance and to define possible mechanisms contributing to the current increase in prevalence of obesity. The endocannabinoid system (ECS), recently defined as an important modulator of energy homeostasis, constitutes an interesting research field and a promising target for pharmaceutical intervention.[2]

The Endocannabinoid system

The endocannabinoid system is a signalling system with multiple functions. It is a phylogenetically old system found in most species, indicating an essential role in vital functions.[3,4] The name "endocannabinoid" system is derived from "cannabis". Among others, cannabinoid receptors have binding affinity for Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of cannabis.[5-7]

Medicinal properties of cannabis are known since ancient history but only recently, in 1988, receptors for cannabis were identified and in the early nineties endogenous ligands for these cannabinoid (CB) receptors were characterized.[7-10] Since then, major progress has been made in understanding the role of the endocannabinoid system as an important modulator of energy homeostasis (Table 1).[11,12]

There are at least two types of cannabinoid receptors, *i.e.*, the CB_1 and CB_2-receptors, both G-protein coupled receptors.[5,6,13] CB_1-receptors are the most abundant G-protein coupled receptors in the central nervous system (CNS), but are also widely expressed

outside the CNS among others in liver and fat tissue.[14] CB_2-receptors are primarily found in cells of the immune system.[2,15] Several endogenous ligands for these cannabinoid receptors have been identified with arachidonoylethanolamide (anandamide) and 2-arachydonoylglycerol (2-AG) being the most extensively investigated ones.[12]

Table 1. History of identifying components of the endocannabinoid system.

	Key findings	First author
B.C.	Therapeutic use of cannabis in Eastern medicine	
19th century	Introduction to Western Medicine	reviewed by Abel 1975
1964	Identification of THC as major psychoactive component of cannabis	Gaoni and Mechoulam
1988	Identification of cannabinoid receptors	Devane
1990	Cloning of cannabinoid receptors	Matsuda
1992-1995	Isolation of endocannabinoids	Devane, Mechoulam, Sugiura
1994	development of rimonabant	Rinaldi-Carmona
1996	cloning of FAAH	Cravatt
2002	recognizing the role of MAGL in the hydrolysis of 2-AG	Dinh
2003-2004	cloning of enzymes responsible for synthesis of anandamide and 2-AG	Bisogno, Okamoto

The endocannabinoids anandamide and 2-AG are eicosanoids, a class of lipids derived from arachidonic acid. The synthetic pathway of anandamide involves two major steps (Figure 1); 1) synthesis of *N*-arachidonyl-phosphatidylethanolamine (NAPE) from arachidonic acid and phosphatidylethanolamine catalyzed by the enzyme *N*-acyltransferase and 2) synthesis of anandamide and phosphatidic acid from NAPE *via* activity of *N*-arachidonyl-phosphatidylethanolamine phospholipase (NAPE-PLD). There are two pathways suggested for synthesis of 2-AG (Figure 1). In both pathways 2-AG is derived from phosphatidylinositol. These pathways either involve diacylglycerol lipase (DAGL) or phospholipase C.[4]

In line with the fact that endocannabinoids are lipids, which makes intermediate storage in vesicles impossible, endocannabinoids are synthesized on demand. Synthesis of endocannabinoids is stimulated by an increase in intracellular Ca^{2+} and cAMP.[4,12]

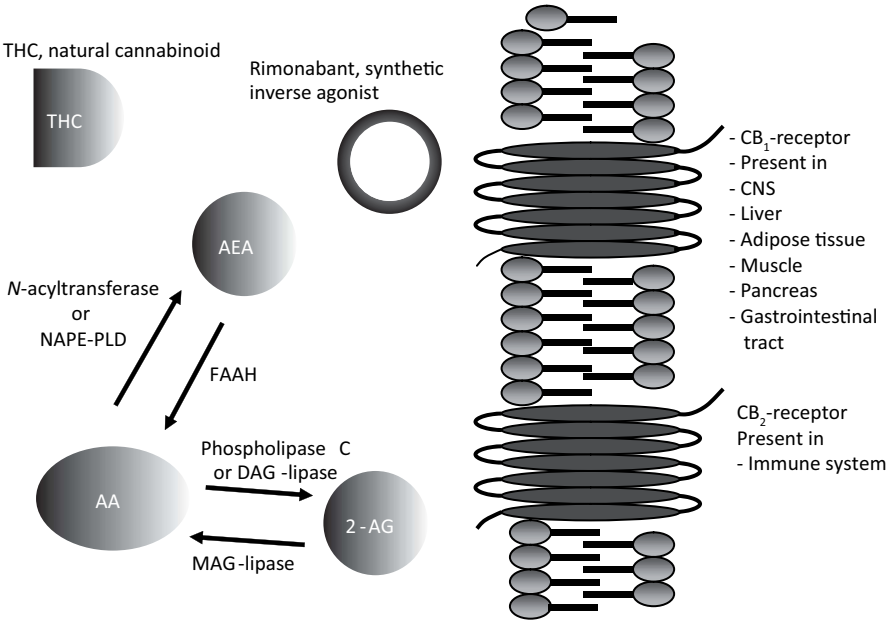

Figure 1. Schematic representation of the endocannabinoid system (ECS)*:* The ECS comprises cannabinoid (CB) receptors, endogenous ligands for these receptors called endocannabinoids and enzymes responsible for synthesis and degradation of endocannabinoids. CB receptors can bind THC, the psychoactive component of cannabis. There are at least two different CB receptors, the CB_1 and the CB_2-receptor. Anandamide (AEA) and arachidonoylglycerol (2-AG) are endocannabinoids. Both are derived from arachidonic acid (AA). *N*-arachidonyl-phosphatidylethanolamine phospholipase (NAPE-PLD) and *N*-acyltransferase, and diacyl-glycerol lipase (DAG-lipase) and phospholipase C are enzymes important in synthesis of respectively anandamide and 2-AG. Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAG-lipase) are enzymes important in degradation of respectively anandamide and 2-AG. Rimonabant is a synthetic inverse agonist-antagonist of the CB_1-receptor.

Endocannabinoids are thought to act either autocrine or paracrine. Because of their lipid characteristics they can move across the membrane of the producing cell and signal *via* CB receptors on the same cell. To reach CB receptors on (more distant) target cells, endocannabinoids need to bind specific transport proteins because of their hydrophobic properties.[4]

Since endocannabinoids act as intercellular messengers, the magnitude of effect of endocannabinoids is not only determined by binding affinity for CB receptors and rate of synthesis and release, but also by rate of removal from the extracellular space and rate of intracellular degradation.[11] Clearance of anandamide and 2-AG from the extracellular space is thought to be mediated by protein transporters.[7,11] Intracellularly, fatty acid amide hydrolase (FAAH) is responsible for the degradation of anandamide, whereas monoacylglycerol lipase is involved in the degradation of 2-AG.[11,16]

Thus, endocannabinoids are produced following an increase in intracellular Ca^{2+} or cAMP levels, and are inactivated when paracrine or autocrine cannabinoid activation is to be terminated *via* active transport mechanisms and intracellular degradation.[12]

Activation of cannabinoid receptors is coupled to several intracellular signalling pathways including 1) inhibition of adenylate cyclase; 2) stimulation of mitogen-activated protein kinases (MAPK); 3) inhibition of voltage-activated Ca^{2+} channels; 4) stimulation of inwardly rectifying K^{+} channels. It depends on the agonist which transduction pathway is activated.[17]

Functions of the endocannabinoid system

Endocannabinoid signalling has been found to be involved in control of a variety of processes.[17-19] In general, functions of endocannabinoid signalling concern repair of (local) homeostasis as stated by Di Marzo: "excessive neuronal activity, cell damage, exaggerated stimulation of inflammatory cytokine receptors and physiological perturbations of homeostasis (i.e., food deprivation) trigger endocannabinoid production, which, together with other homeostatic signalling systems, helps cells return to their steady state."[12]

Several characteristics of the endocannabinoid system perfectly suit a modulatory functions; 1) signal transduction *via* inhibition of voltage-activated Ca^{2+} channels; 2) stimulation of synthesis of endocannabinoids by increased intracellular Ca^{2+} and activation of other G-protein coupled receptors; and 3) presynaptic localization.[17] Predominantly presynaptic localization of CB_1-receptors in the nervous system allows retrograde signalling. In this way activation of CB_1-receptors results in modulation of release of several neurotransmitters including gamma-aminobutyric acid (GABA), dopamine, noradrenaline, glutamate and serotonin (5-HT). This process is called depolarization-induced suppression of inhibitory and excitatory neurotransmission.[17] In this manner, the endocannabinoid system is able to modulate a wide range of

physiological functions including neuroprotection; nociception; cardiovascular and respiratory effects (regulation of blood pressure, heart rate and bronchial functions); gastrointestinal functions; regulation of energy homeostasis; reproduction; cognitive functions; emotion; reward and addiction; modulation of movement and posture; modulation of immune and inflammatory responses; and bone formation.[7,17]

The endocannabinoid system as a modulator of energy balance

Research so far clearly indicates a crucial role for the endocannabinoid system in regulation of energy balance. Contribution of endocannabinoids to maintainance of energy balance involves regulation of both food intake (often referred to as "central effects") and food-intake independent mechanisms ("peripheral mechanisms").

Central regulation of energy balance by endocannabinoid signalling

Following the identification of cannabinoid receptors and endocannabinoids, it has been shown that these receptors and their endogenous ligands are present in areas in the central nervous system important for regulation of food intake. Administration of endocannabinoids, systemic as well as hypothalamically, stimulates food intake in animal species, consistent with earlier established effects of administration of cannabis or synthetic cannabinoids.[15,20-23]

Since regulation of feeding behaviour by endocannabinoids is acting in brain stem and in hypothalamic circuits regulating hunger/satiety as well as in the limbic forebrain circuitry (for control of consummatory and rewarding aspects of feeding behaviour), it is suggested that the endocannabinoid system is involved in both the homeostatic and hedonic aspects of feeding behaviour.[4,24] Endocannabinoid control of homeostatic aspects of feeding either involves a direct route *via* activation of CB_1-receptors in hypothalamic centres or involves activation of CB_1-receptors at peripheral afferent nerves that project to the brain stem and hypothalamus.[25]

In general, food intake is influenced by a complex network of orexigenic (appetite-promoting) and anorexigenic (appetite-inhibiting) pathways. Interconnections between endocannabinoids and these regulatory pathways are reviewed by Pacher *et al.*[18] Endocannabinoids have been found to interact with both orexigenic factors including NPY, orexins, ghrelin, and endogenous opioids and with anorexigenic factors such as α-melanocyte-stimulating hormone (α-MSH), corticotrophin-releasing hormone (CRH), and the product of the cocaine and amphetamine-related transcript (CART).[3,11,18,20,26,27]

Leptin, a key signal in the long-term regulation of food intake and energy balance, was also found to interact with endocannabinoid activity. Tonic inhibition of endocannabinoid levels by leptin was suggested based on observed elevated endocannabinoid levels in mouse models unable to produce leptin (ob/ob mice) and in animal models with dysfunctional leptin receptors (Zucker rats and db/db mice).[26,28,29] On the other hand, endocannabinoids have been suggested to increase leptin levels, since mice with a genetic deletion of the CB_1-receptor show decreased leptin levels and increased leptin sensitivity compared to wild type mice.[30]

Nutritional state contributes to observed orexigenic effects of CB_1-activation with maximal effects in pre-satiated animals.[22] Kirkham *et al.* directly measured brain endocannabinoid levels in response to fasting, feeding and satiation in rats. Fasting levels of anandamide and 2-AG were increased in the limbic forebrain and to a lesser extend in the hypothalamus while hypothalamic 2-AG levels declined during eating. No changes were detected in satiated rats. Endocannabinoid levels in the cerebellum, a control region not directly involved in regulation of feeding behaviour, were unaffected by nutritional state.[24,31] Orexigenic effects of (endogenous) cannabinoids are prevented by administration of exogenous CB_1-receptor antagonists (*e.g.*, Rimonabant), confirming that orexigenic effects of cannabinoids and endocannabinoids are mediated by CB_1-receptors.[30,32,33]

A role of CB_1-receptors in the endocannabinoid regulation of food intake was reinforced by studies in CB_1-receptor-deficient mice ($CB_1^{-/-}$). Di Marzo *et al.* showed that food intake in previously fasted $CB_1^{-/-}$ mice was reduced compared to their wild type littermates and that food intake of $CB_1^{-/-}$ was not affected by treatment with a CB_1-receptor antagonist whereas food intake of wild type mice was reduced to levels found in untreated $CB_1^{-/-}$ mice.[26]

Peripheral effects of endocannabinoid regulation of energy homeostasis

Studies on effects of cannabis in humans showed that treatment with cannabis led to a transient increase in food intake in combination with a more sustained increase in body weight. Conversely, treatment with CB_1-receptor antagonists in animal models and humans resulted in a sustained reduction in body weight despite merely a transient decrease in food intake, suggesting that endocannabinoids have food-intake independent effects on energy balance.[30,32-34]

By showing that $CB_1^{-/-}$ mice have decreased body weight compared to pairfed wild type littermates Cota *et al.* clearly pointed out that there are food intake-independent

mechanisms contributing to endocannabinoid regulation of energy balance (Figure 2).[20] Several mechanisms and target organs were suggested to underlie peripheral effects. Presence of CB_1-receptors and endocannabionoids has been described for most organs involved in energy homeostasis (*i.e.*, liver, gastrointestinal tract, muscle, adipose tissue, pancreas). Presumed peripheral effects of endocannabinoid signalling are summarized in Figure 3.

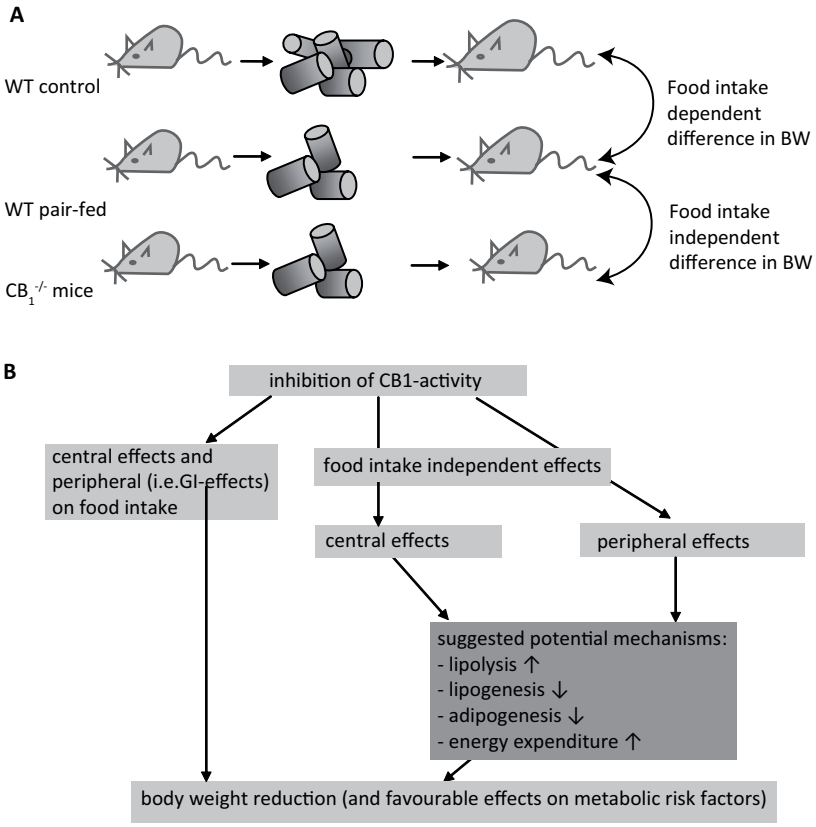

Figure 2. Food intake independent effects: A) CB_1-receptor deficient ($CB_1^{-/-}$) mice, as compared with pair-fed wild type (WT) mice show reduced body weight, indicating a food intake independent effect of CB_1-ablation on energy balance besides an effect on food intake. B) Inhibition of CB_1-signalling results in reduced body weight *via* central effects on intake, *via* peripheral effects on intake (gastrointestinal (GI) satiety signals) and *via* central and peripheral determined mechanisms independent of intake.

In summary, the presumed role of endocannabinoid signalling in energy homeostasis is to facilitate energy storage, as stated by Woods; "the net effect of endocannabinoids is anabolic, facilitating increased energy intake, decreased energy expenditure, and increased accumulation of body fat."[4,35] The effect on food intake upon manipulating endocannabinoid signalling appears to be transient, whereas intervening with endocannabinoid signalling has a more persistent effect on body weight and body composition and pair feeding experiments confirmed that both central and peripheral mechanisms are involved in the role of endocannabinoid signalling in the regulation of energy homeostasis.

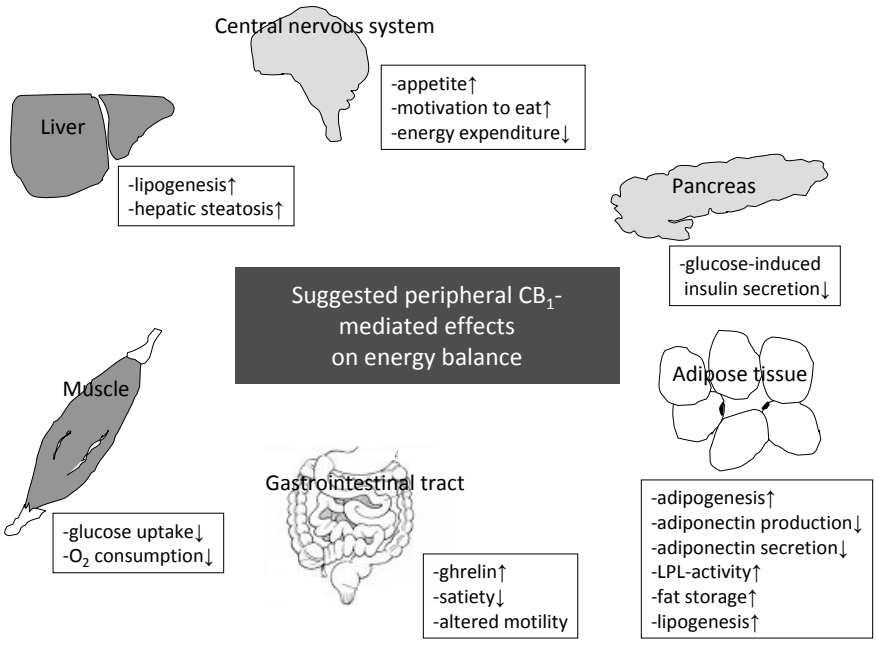

Figure 3. Metabolic effect of endocannabinoid receptor activation: So far, several mechanisms, summarized in this figure, have been suggested for peripheral mechanisms by which endocannabinoid activation affects energy balance.

The endocannabinoid system and implications for treatment of disturbance of energy balance

Since the endocannabinoid system has emerged as a crucial modulator in the regulation of energy homeostasis, it is recognized as a potential target for drug treatment in obesity and related disease, but also in (disease-associated) cachexia.[36]

The endocannabinoid system and obesity

There are several findings to indicate that increased endocannabinoid tone might contribute to the development of obesity. First of all, compared to lean controls, obese rodents are more sensitive to the anti-obesity effects of treatment with CB_1-receptor antagonists. Secondly, diet-induced obesity in animal models is accompanied by elevated levels of endocannabinoids and adipocytes of obese rats show a higher CB_1-receptor expression. Thirdly, $CB_1^{-/-}$ mice are resistant to diet-induced obesity. Moreover, in obese humans significantly higher levels of endocannabinoids were observed in blood and visceral fat. Finally, plasma 2-AG levels, but not anandamide levels, are positively correlated to amount of intra-abdominal fat in humans.[37-39] It has been suggested that obesity-related overactivity of the endocannabinoid system could be the consequence of a reduced degradation of endocannabinoids. Interesting in view of this is a reported association between obesity and a missense polymorphism in the *Faah* gene, since FAAH is responsible for the degradation of anandamide (explained in Figure 1).[40] However, this finding was not confirmed by others.[41] Alternative causes of obesity-related elevated endocannabinoid tone are obesity-associated insulin resistance and leptin resistance, since these hormones were found to suppress endocannabinoid tone.[12] Endocannabinoid activity could hence be increased progressively in a feed-forward manner further contributing to the problem, but, at the same time allows disruption of this vicious circle by pharmacologically blocking endocannabinoid signalling at the CB_1-receptor.[4]

With the identification of the endocannabinoid system as a target for weight loss medication, new pharmaceutical therapies for obesity have emerged. However, the first CB_1 antagonist (in fact an inverse agonist), rimonabant, was not designed for the treatment of obesity, but rather as an anti-psychotic agent. Although rimonabant was not successful in ameliorating symptoms when tested in schizophrenic patients, it was well-tolerated by the patients and it was found to reduce body weight. Since

then, large clinical trials have been conducted to test rimonabant as an anti-obesity drug. Besides succesfully reducing weight, weight loss-independent improvement of metabolic risk factors was observed with rimonabant-treatment. However, so far safety concerns have obstructed approval by the FDA. Registration of rimonabant in a number of European countries has been withdrawn after increased risks of depression and suicide were reported. Since these side-effects are related to central functions of the endocannabinoid system, it is essential to find pharmaceutical strategies that bypass these unwanted central effects. To be able to design drugs specifically targeted to evoke weight-reducing effects and favourable effects on metabolic risk factors without adverse central effects, it is essential to elucidate exactly how the endocannabinoid system is involved in the regulation of fuel metabolism and energy balance.

Interaction between the endocannabinoid and dietary long chain polyunsaturated fatty acids (LC-PUFA) intake

Dietary fatty acids can affect cellular functions by incorporation into membrane phospholipids. Since arachidonic acid (20:4n-6, AA) is a precursor of endocannabinoids, the presence of AA in membranes is expected to be a factor in control of endocannabinoid tone. This leads to the hypothesis that dietary fatty acid composition can influence endocannabinoid tone. It was also suggested that n-3 LC-PUFA might act as competitive inhibitors in the peripheral endocannabinoid system, thereby promoting energy metabolism and exerting anti-obesity effects.[42,43] Only a few studies were aimed at investigating how dietary fatty acids could affect endocannabinoid levels. First, an *in vitro* study by Matias *et al.* showed that in mouse adipocytes incubation with (22:6n-3, DHA) significantly decreased both the proportion of AA in membrane phospholipids and the cellular concentrations of anandamide and 2-AG, while incubation with AA significantly increased AA in membrane phospholipids and the 2-AG concentration, but not the concentration of anandamide.[44] Secondly, short-term maternal supplementation of both AA and DHA were found to increase anandamide levels in brain of newborn piglets.[45] However, Watanabe *et al.* found that n-3 LC-PUFA deficiency elevates 2-AG levels in the brain of mice and supplementation with a n-3 enriched diet reduces brain 2-AG levels in mice.[46] Finally, in a study in rats fed AA, higher levels of anandamide were found in brain, liver and small intestine but higher levels of 2-AG were found in small intestine. Rats fed fish oil showed lower levels of anandamide in small intestine and liver.[47] Since LC-PUFA supplementation is currently recommended

for its presumed favourable metabolic effects, the relation between dietary LC-PUFA levels and endocannabinoid tone and the possible interaction is of great interest and needs further investigation.[48]

Scope of this thesis

The aim of this thesis is to explore the role of the endocannabinoid system in the regulation of energy balance in mice with a focus on peripheral metabolic effects of endocannabinoid signalling and on the interaction between dietary fatty acid intake and effects of intervening with endocannabinoid signalling. Since increased prevalence of obesity and associated comorbidities is especially alarming in children, we did not study the endocannabinoid system in a model of established obesity, but we choose deliberately for adolescent and young adult mice.

In the experiments described in this thesis two models were used; 1) mice treated with rimonabant, a CB_1-receptor antagonist, compared to controls; and 2) mice with a targeted deletion of the CB_1-receptor, CB_1-receptor-deficient mice ($CB_1^{-/-}$), compared to wild type littermates.

Previously reported data on body weight gain in young $CB_1^{-/-}$ mice showed reduced body weight compared to wild type mice, however, since these pups had respectively $CB_1^{-/-}$ parents or WT parents, this reduction in body weight could be a consequence of maternal phenotype. Therefore we first studied phenotype of pups of heterozygous breeding to determine the contribution of genotype and maternal phenotype to the decrease in body weight gain in $CB_1^{-/-}$ pups compared to WT pups (**chapter 2**). The experiment described in chapter 2 was furthermore designed to determine phenotype differences between male and female pups. Based on these results we only used male mice in the following experiments.

Based on reported effects suggesting a potential role of endocannabinoid signalling in lipogenesis, adipogenesis and LPL-activity, we next explored possible mechanisms for CB_1-mediated regulation of lipid metabolism. Since endocannabinoids are derived from PUFA and since PUFA are known to influence lipid fluxes (*i.e.*, decrease hepatic lipogenesis), we hypothesized that dietary fatty acid intake would influence the effects of treatment with rimonabant. In **chapter 3** effects of treatment with rimonabant in mice on either a chow (standard low fat diet), or a high-fat (HF) diet or a high-fat fish-oil enriched (HF/FO) diet are described. In **chapter 4** potential mechanisms in the

resistance of $CB_1^{-/-}$ mice to diet-induced hepatic steatosis are evaluated in mice on chow, HF diet or HF/FO diet.

Data on hepatic lipogenesis described in chapter 3 and 4 were not able to explain peripheral effects of endocannabinoid activity, and therefore we shifted our focus on adipose tissue. Adipogenesis, adipocyte morphology, adipose tissue lipolysis, and adipose tissue lipogenesis were studied in $CB_1^{-/-}$ mice in relation to dietary fat intake and these data are described in **chapter 5**.

Since metabolic effects of endocannabinoid signalling are partly independent of food intake, an effect on energy expenditure might be expected. Data on energy expenditure, substrate utilization and circadian rhythm in nutrient partitioning in $CB_1^{-/-}$ mice compared to WT mice are presented in **chapter 6**.

Finally, in **chapter 7**, results of the studies in this thesis are summarized and discussed. Clinical implications are considered and future directions are suggested.

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CHAPTER 2

Postnatal regulation of weight gain by endocannabinoid signalling in mice

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Abstract

The endocannabinoid system (ECS) plays an important role in energy homeostasis. It was previously demonstrated that cannabinoid 1 receptor deficient ($CB_1^{-/-}$) mice have decreased body weight compared to wild type (WT) mice during lactation, which was attributed to reduced suckling behaviour of $CB_1^{-/-}$ mice. Since offspring in these experiments was obtained from homozygous breeding pairs, a potential maternal contribution to reduced body weight gain of $CB_1^{-/-}$ offspring *versus* WT mice could not be excluded. To rule out such an effect, we investigated offspring parameters during lactation of 41 heterozygous $CB_1^{+/-}$ breeding couples. At weaning on day 20 there was a significant decrease in body weight of $CB_1^{-/-}$ pups compared to WT pups in both sexes. In males reduction in body weight was based on reduction in both lean and fat mass, whereas in females only reduction in fat mass was responsible for reduced body weight in $CB_1^{-/-}$ pups compared to WT littermates. Intermediary body weights were found in heterozygous $CB_1^{+/-}$ pups.

Conclusions: These results confirm that the CB_1-receptor contributes to bodyweight gain during lactation in both male and female mice, potentially in a gene-dose effect relationship. Secondly, they rule out that these effects are mediated through differences in the maternal environment.

Introduction

Failure to thrive (FTT) is a common problem in paediatric practice. FTT is diagnosed in an infant or child whose growth is significantly less than that of his or her peers.[1] Organic FTT is growth failure based on an underlying medical condition. In the majority of patients, however, an underlying medical condition can not be found and these patients are categorized as non-organic FTT.[1] Originally, cases of non-organic FTT were attributed to psychosocial factors (i.e. emotional deprivation, failure to offer adequate calories, inadequate meal-time settings, and maternal depression).[2] Alternatively, non-organic FTT could originate from an imbalance in hypothalamic orexigenic and anorexigenic pathways in the newborn. These neural pathways are formed relatively late in fetal development, but are nonetheless necessary to thrive during the early postnatal stage.[3]

Recently, the endocannabinoid system (ECS) emerged as a potentially important system to regulate nutrient intake and handling of the newborn. First of all, it has been shown that anandamide and 2-arachidonoyl glycerol (2-AG), the two most studied endocannabinoids, are present in mammalian milk (bovine as well as human) at sufficient concentrations to stimulate suckling behaviour in the newborn by acting on CB_1-receptors.[4-6] Secondly, levels of 2-AG in the rat brain peak during the first day postpartum.[7] Fride *et al.* suggested that derangements in endocannabinoid signalling play a role in non-organic FTT based on data showing that CB_1-receptor deficient ($CB_1^{-/-}$) mice have decreased body weight compared to wild type (WT) mice during lactation, which they attributed to reduced suckling behaviour of $CB_1^{-/-}$ mice.[8] Since offspring in their experiments was obtained from homozygous breeding couples *i.e.*, a comparison of offspring from $CB_1^{+/+}$ x $CB_1^{+/+}$ and $CB_1^{-/-}$ x $CB_1^{-/-}$, a potential maternal contribution to reduced body weight gain of $CB_1^{-/-}$ offspring compared to WT mice could not be excluded. The ECS not only plays a role in the neonatal period but also in control of adult energy homeostasis. Since food intake during pregnancy and lactation should be optimal, it can be expected that endocannabinoid activity is maximized during pregnancy and lactation. Endocannabinoid deficiency in the mother may result in alterations in maternal care, maternal nutritional and neuro-endocrine status which may have secondary effects on prenatal and postnatal development. Therefore, targeted deletion of the CB_1-receptor in the mothers ($CB_1^{-/-}$ mothers) could very well have secondary consequences in the offspring, apart from the primary consequences resulting from their genetic defect. To evaluate postnatal development of mice lacking CB_1-receptors,

independent of the effects of maternal nutritional factors, we investigated growth in offspring of 41 heterozygous $CB_1^{+/-}$ breeding couples during lactation. We compared body weight, growth and body composition of male and female WT, heterozygous and $CB_1^{-/-}$ littermates.

Material and methods

Animals

CB_1-deficient mice ($CB_1^{-/-}$) with a C57BL/6J background and their WT-littermates were bred in our own facility. Initial breeding pairs were kindly provided by Prof. Dr. A. Zimmer, Laboratory of Molecular Neurobiology, Department of Psychiatry, University of Bonn, Germany. Heterozygous breeding couples were obtained by breeding male WT C57BL/6j with female $CB_1^{-/-}$ mice. Mice were housed as breeding couples in a light-controlled (lights on 4 AM- 4 PM) and temperature-controlled (21°C) facility. Mice were allowed tap water and food (standard chow) *ad libitum*. Experimental protocols were approved by the local Experimental Ethical Committee for Animal Experiments. Retrospectively, we determined genotype distribution in offspring of our heterozygous breeding couples and, over a four-month period while we maintained the breeding colony, we registered mortality of weaned pups that were included in a series of experiments.

Experimental procedures

Pups of 41 heterozygous $CB_1^{+/-}$ breeding couples were followed throughout lactation. Pregnant dams were daily checked for the presence of litters. At day two of life, pups were coded (by toe clips), genotyped, sexed and weighed. Litters were culled to six pups within one week after birth. Pups were weighed again at day 6 and day 13. At weaning, on day 19, 20 or 21, pups were weighed and then sacrificed by cardiac puncture under inhalation anaesthesia followed by cervical dislocation. Epididymal fat pads were removed and stored at -80°C until further analysis. Carcasses were stored at -20°C until carcass analysis.

Body composition

In specific cohort of offspring, anal-chin length was measured in defrosted carcasses, after which carcasses were dried to constant weight at 103 °C, and fat was extracted by

petroleum ether (Boom BV, Meppel, The Netherlands) in a soxlet distillation apparatus. Fat mass of carcasses was determined from weight differences before and after the fat extraction procedure, including the weight of removed fat pads.

Cnr_1-expression (CB_1-expression)

Total RNA was isolated from frozen epididymal adipose tissue using the TRI-reagent method (Sigma). Using random primers, RNA was converted to cDNA with M-MuLV Reverse Transcriptase (Sigma) according to the manufacturer's protocol. For realtime-PCR, cDNA was amplified using the primers and probes listed in Supplemental Table 1. Relative gene expression levels were normalized to *β-actin*.

Statistical analysis

All values in the tables and figures represent means ± standard error of the mean for the number of animals indicated in the table and figure legends. Data were statistically analyzed by the ANOVA test with *post hoc* Bonferroni correction. Statistical significance of differences was accepted at a *P*-value of less than 0.05. Analyses were performed using SPSS 16.0 for Windows software (SPSS, Chicago, IL).

Results

Genotype distribution and mortality

Genotypes were distributed according to Mendelian segregation, indicating no difference in intra-uterine mortality between different genotypes (Table 1). Our evaluation of mortality of male pups included in several experiments demonstrated an increased mortality of $CB_1^{-/-}$ mice, especially in the first week after weaning (Table 2).

Table 1. Genotype distribution

	$CB_1^{+/+}$	$CB_1^{-/-}$	$CB_1^{+/-}$
n	204	184	399
genotype distribution (%)	25,9	23,4	50,7

Body weight during lactation

No significant difference in body weight of pups was found at day 2 and 6 (Figure 1). However, from day 13 onwards (*i.e.* at peak lactation) body weights of male $CB_1^{-/-}$ mice

were significantly lower compared to male WT mice (day 13, $p = 0.011$ and day 20, $p < 0.001$) (Figure 1a). Female mice showed a similar pattern as males, although the difference between $CB_1^{-/-}$ mice and WT mice was not significant until day 20 ($p = 0.031$) (Figure1b). At day 20, body weights of male and female $CB_1^{-/-}$ mice were also reduced compared to $CB_1^{+/-}$ mice ($p < 0.001$ and $p < 0.005$). The decrease in body weight at day 20 in male $CB_1^{+/-}$ mice compared to male WT mice was not significant and there was no difference in body weight at day 20 between female $CB_1^{+/-}$ mice and female WT mice.

Body composition

Male $CB_1^{-/-}$ mice showed a significant reduction in anal-chin length ($p = 0.006$) on day 20 compared to WT mice (Figure 2). Also, male $CB_1^{-/-}$ mice showed a significant reduction in lean mass ($p = 0.008$) on day 20 compared to WT mice (Figure 3a). Furthermore, male $CB_1^{-/-}$ mice showed a significant reduction in fat mass ($p < 0.01$) on day 20 compared to WT mice (Figure 3b). Thus, reduction in body weight of male $CB1^{-/-}$ mice compared to WT mice, was caused by a decrease in lean mass and in fat mass, indicating that not only adiposity but also changes in structural body size contributed to the reduced body weight. Lean mass and fat mass of male $CB_1^{-/-}$ mice tended to be reduced compared to $CB_1^{+/-}$ mice, however this did not reach statistical significance. Differences between $CB_1^{+/-}$ mice and WT mice were neither significant. Remarkably, no significant differences in anal-chin length and in lean mass were found in female mice (Figures 2 and 3a). Therefore, the observed reduction in body weight in female $CB_1^{-/-}$ mice compared to WT mice was solely based on a reduction in fat mass (Figure 3b).

Cnr_1-receptor expression (CB_1-receptor expression)

Obviously, Cnr_1-expression (Cb_1-expression) in $CB_1^{-/-}$ mice was undetectable. Cnr_1-expression in female WT mice was lower compared to Cnr_1-expression in male WT mice (Cnr_1-expression in female WT mice 9.9 *versus* 12.7 in male WT mice), however this difference did not attain significance. Cnr_1-expression was significantly reduced in female $CB_1^{+/-}$ mice compared to female WT mice (female $CB_1^{+/-}$ mice 4.0 *versus* 9.9 in female WT mice, $p = 0.001$). The reduction in Cnr_1-expression in male $CB_1^{+/-}$ mice compared to WT mice did not reach statistical significance (male $CB_1^{+/-}$ mice 8.4 *versus* 12.7 in male WT mice, $p = 0.074$). In male and female $CB_1^{-/-}$ mice, Cnr_1-expression was significantly reduced compared to both $CB_1^{+/-}$ mice and WT littermates (female $CB_1^{-/-}$ mice 0.0 *versus* 4.0 in female $CB_1^{+/-}$ mice, $p = 0.018$, female $CB_1^{-/-}$ mice 0.0 *versus* 9.9 in female WT mice, $p < 0.001$ and male $CB_1^{-/-}$ mice 0.0 *versus* 8.4 in male $CB_1^{+/-}$ mice,

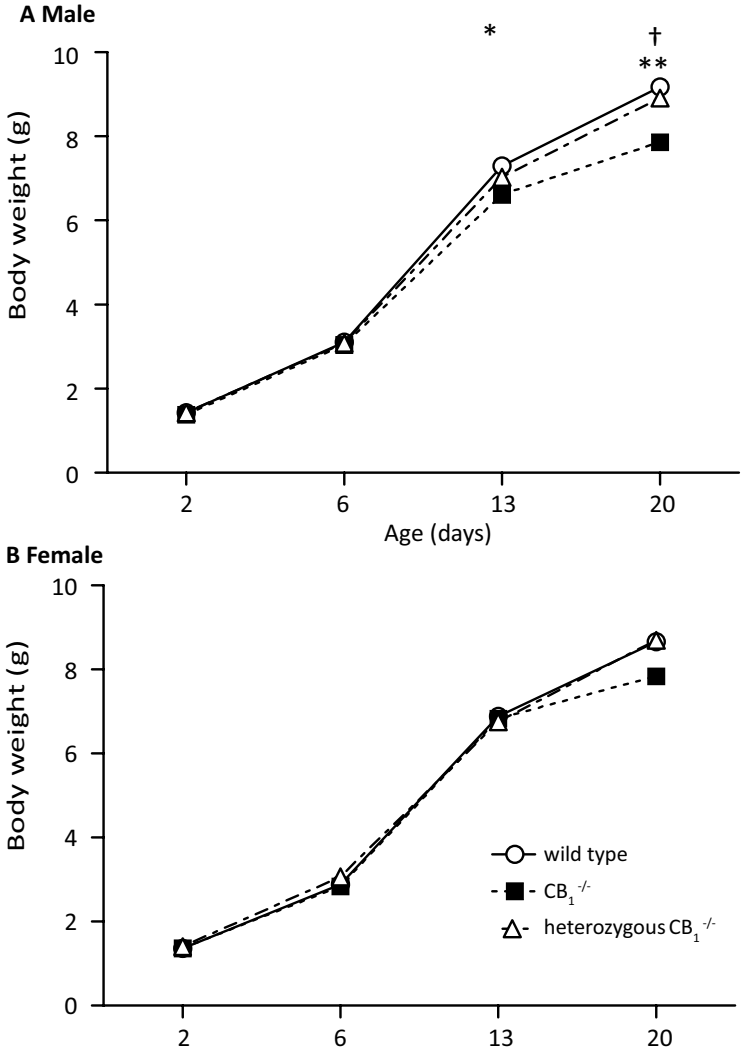

Figure 1. Body weight gain during lactation. A) Growth curves during lactation in male CB_1-deficient ($CB_1^{-/-}$), wild type (WT) and heterozygous ($CB_1^{+/-}$) mice. Continuous lines, WT mice; irregular dotted lines, $CB_1^{+/-}$ mice; dotted lines, $CB_1^{-/-}$ mice. Values are means; n = 27-47, * WT *versus* $CB_1^{-/-}$ p = 0.011 at day 13 and ** WT *versus* $CB_1^{-/-}$ p = 0.000 and † $CB_1^{-/-}$ *versus* $CB_1^{-/+}$ p = 0.000 at day 20. B) Growth curves during lactation in female $CB_1^{-/-}$, WT and heterozygous mice. Continuous lines, WT mice; irregular dotted lines, $CB_1^{+/-}$ mice; dotted lines, $CB_1^{-/-}$ mice. Values are means; n = 17-45, * WT *versus* $CB_1^{-/-}$ p = 0.031 and † $CB_1^{-/-}$ *versus* $CB_1^{-/+}$ p = 0.003 at day 20.

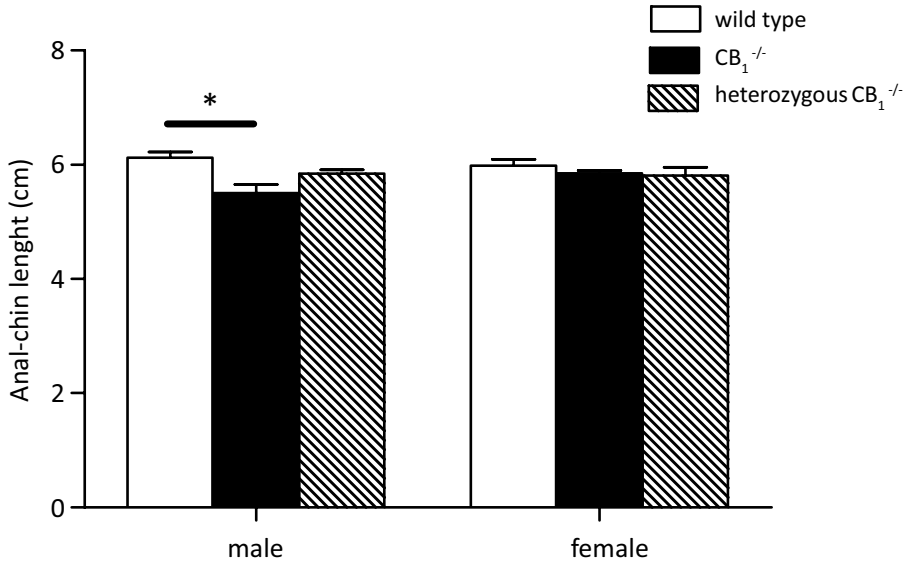

Figure 2. Anal-chin length at weaning. Anal-chin length in male and female CB_1-deficient ($CB_1^{-/-}$), wild type (WT) and heterozygous ($CB_1^{+/-}$) mice. Open bars, WT mice; gray bars, $CB_1^{+/-}$ mice; closed bars, $CB_1^{-/-}$ mice. Values are means +/- SEM; n = 5-13; * p = 0.006 male WT *versus* $CB_1^{-/-}$.

p = .005, male $CB_1^{-/-}$ mice 0.0 *versus* 12.7 in male WT mice, $p < 0.001$). *Cnr*$_1$-expression in heterozygous mice was approximately two-third of the expression level found in WT mice. *Cnr*$_1$-expression in female heterozygous mice was significantly reduced compared to *Cnr*$_1$-expression in male heterozygous mice.

Table 2. Mortality after inclusion

	Male $CB_1^{+/+}$	**Male $CB_1^{-/-}$**
Total number of mice included in experiments	88	85
Mortality after inclusion	0	16
within 1 week after weaning	0	13
more than 1 week after weaning	0	3

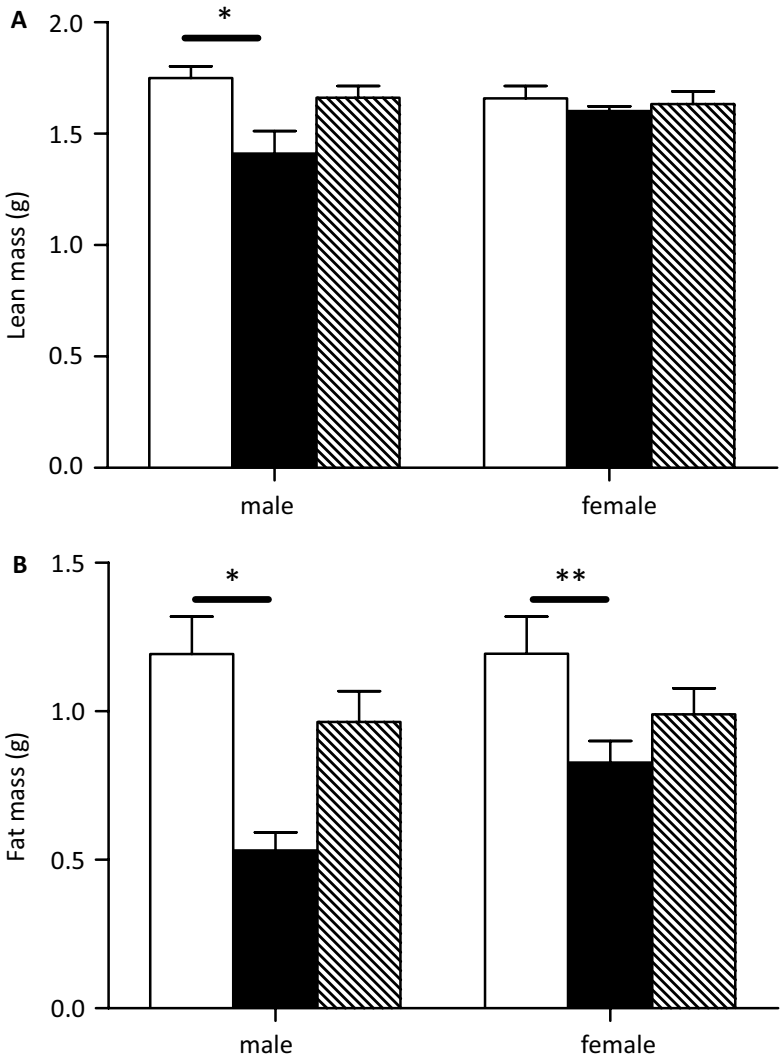

Figure 3. Body composition at weaning. A) Lean mass in male and female CB_1-deficient ($CB_1^{-/-}$), wild type (WT) and heterozygous ($CB_1^{+/-}$) mice. Open bars, WT mice; gray bars, $CB_1^{+/-}$ mice; closed bars, $CB_1^{-/-}$ mice. Values are means +/- SEM; n = 5-13; * p = 0.008 male WT *versus* $CB_1^{-/-}$. B) Fat mass in male and female $CB1^{-/-}$, WT and heterozygous mice. Open bars, WT mice; gray bars, $CB_1^{+/-}$ mice; closed bars, $CB_1^{-/-}$ mice. Values are means +/- SEM; n = 5-13; * p = 0.009 male WT *versus* $CB_1^{-/-}$, ** p = 0.063 female WT *versus* $CB_1^{-/-}$.

Discussion

The results of our study show that the CB_1-receptor contributes to body weight gain during lactation in both male and female mouse pups, potentially in a gene-dose effect relationship, and they rule out that these effects are mediated through differences in the "maternal environment". Secondly, these results show that male $CB_1^{-/-}$ pups have a more pronounced lean phenotype compared to female $CB_1^{-/-}$ mice.

Results of our experiments confirm previous observations of Fride and colleagues demonstrating that $CB_1^{-/-}$ mice have decreased body weight gain compared to WT mice during lactation.[8] While Fride *et al.* showed in their experiments already from day 5 postpartum a significantly lower body weight of newborn pups of $CB_1^{-/-}$ mothers compared to pups of WT mothers, we first observed from day 13 *postpartum* a significantly lower body weight in $CB_1^{-/-}$ pups compared to their WT littermates.[8] Based on this discrepancy between results of Fride *et al.* and results of these experiments, our results do not exclude maternal contribution to reduction in body weight gain in $CB_1^{-/-}$ pups during lactation. However, since most of the difference in body weight between $CB_1^{-/-}$ pups and WT pups is preserved in our experiments comparing $CB_1^{-/-}$, heterozygous and WT littermates of heterozygous mothers, the effect on body weight is mostly independent of maternal factors. This implies that endocannabinoid signalling is crucial for body weight development in the neonatal period.

In view of the crucial role of the ECS in the neonatal period, impaired endocannabinoid signalling was already suggested by Fride *et al.* to be a pathophysiological substrate for non-organic FTT.[9,10] They suggested that CB_1-antagonist (SR141716)-treated pups had severe oral-motor impairment based on a series of experiments showing that SR141716-treated pups approached the nipple of an anaesthetized nursing dam like vehicle-injected pups did, but they did not suckle adequately.[10] When SR141716-treated pups were exposed to a dish with milk, they were able to ingest the same amount of milk as controls suggesting that suckling, but not ingestion is impaired. Fride and colleagues speculated that treatment with a CB_1-antagonist at birth leads to incomplete synaptogenesis of the hypoglossal nerve with resulting impairment of suckling.[10] Interestingly, there are also reports on oral-motor dysfunction in children with non-organic FTT.[11,12] In a large whole-population survey conducted by Reilly and colleagues, 17 children of a group of 47 children with non-organic FTT were shown to have significant oral-motor dysfunction without global developmental delay.[11] Ramsay *et al.* reported that feeding difficulty at 10 months of age is related to non-organic FTT

but they could not confirm a relation between maternal depression and non-organic FTT.[13] Impaired endocannabinoid signalling could be a possible explanation for the observed vulnerability to non-organic FTT.

Another indication for a maternal contribution to the results obtained by Fride *et al.* is the fact that we did not observe increased perinatal mortality as they described for $CB_1^{-/-}$ pups derived from $CB_1^{-/-}$ mothers.[8] Absence of mortality in the $CB_1^{-/-}$ pups in our experiments is supported by the Mendelian 1:1:2 distribution of $CB_1^{-/-}$, $CB_1^{+/+}$, $CB_1^{+/-}$ genotypes that we observed in offspring of $CB_1^{+/-}$ x $CB_1^{+/-}$ breedings across a large number of studies. Several interacting mechanisms could contribute to the increased mortality in $CB_1^{-/-}$ pups observed by Fride and colleagues (i.e., stunted growth by insufficient lactation, cannibalism of pups by the $CB_1^{-/-}$ mothers). We noticed, however, an increased mortality in male $CB_1^{-/-}$ pups immediately after weaning and speculate that this is a consequence of the impaired capacity of the $CB_1^{-/-}$ pups to adjust to solid food. Feeding difficulties in children with non-organic FTT have been reported to involve problematic transition from one kind of food to another, and these effects could be homologous to those resulting from deficient endocannabinoid signalling observed in the present study.

Results of our experiments suggest a gene-dose effect relationship in the contribution of the CB_1-receptor to body weight gain. This is in contrast with results reported by Cota *et al.* that did not show differences in body weight between male $CB_1^{+/-}$ and $CB_1^{+/+}$ at any age.[14] During lactation we did observe reduced body weight in male heterozygous $CB_1^{+/-}$ mice compared to their male WT littermates. Moreover, in males as well as females we observed reduced fat mass in heterozygous $CB_1^{+/-}$ mice compared to their WT littermates. Differences between WT and heterozygous littermates in body weight and fat mass did not reach significance, but there was a clear trend towards a gene-dose response. Selley *et al.* showed an increase in efficiency of CB_1-receptor binding and CB_1-receptor activation in neural tissue, partially compensating for the decrease in receptor density in heterozygous mice.[15] In agreement with a gene-dose effect, we showed a decrease in Cnr_1-receptor expression in white adipose tissue of heterozygous CB_1-deficient mice compared to WT littermates. The observed gene-dose effect on body weight and fat mass in these pups does illustrate the crucial role of endocannabinoid signalling early in life.

As mentioned, our results show a significant decrease in body weight at weaning (day 20) in male as well as in female $CB_1^{-/-}$ pups compared to WT pups. Remarkably, the difference in fat mass between male $CB_1^{-/-}$ and WT pups was larger than between

female $CB_1^{-/-}$ and WT pups; in the latter case the difference between $CB_1^{-/-}$ and WT pups was not statistically significant. This gender-specific effect was even more pronounced for lean mass. Male $CB_1^{-/-}$ pups showed a more pronounced phenotype compared to female $CB_1^{-/-}$ pups in this respect. Cota *et al.* also reported on gender–specificity in adult mice, with a less pronounced lean phenotype in female $CB_1^{-/-}$ mice than in male $CB_1^{-/-}$ mice.[14]

A possible explanation for this gender-specificity could lie in an interaction of endocannabinoid signalling with the hypothalamic-pituitary-gonadal axis. There are several lines of evidence supporting such an interaction. First of all, gonadal steroids have been extensively linked to adipogenesis.[16,17] In evaluating gene expression of adipogenic factors in relation to gender and CB_1-genotype in white adipose tissue of mice sacrificed at weaning, however, we did not observe any differences in expressions of genes encoding AP2, PPAR alpha2, CEBP alpha, PEPCK (chapter 5). Instead, central mechanisms could be involved in the differential modulation of metabolic phenotype of impaired endocannabinoid signalling by gonadal steroids. Gonadal steroids are recognized to modulate central nervous system effectors of energy homeostasis that are also influenced by endocannabinoids. For example, cannabinoid effects on gene expression of corticotrophin-releasing hormone and proopiomelanocortin were found to be differentially regulated in male and female rats[18] and endocannabinoid modulation of neurotransmission was found to be influenced by gonadal steroids.[19,20] Moreover, there are several reported studies describing gonadal steroid influence on endocannabinoid tone.[21-23] González *et al.* showed that expression of the CB_1-receptor gene in the anterior pituitary gland of rats is regulated by gonadal steroids in both males and females. They found that whereas estradiol had a stimulatory effect on Cb_1-expression in males, it had an inhibitory effect on Cb_1-expression in females. They also showed that female rats had higher anandamide levels in the anterior pituitary gland and in the hypothalamus and concluded that there are sex dependent differences in ligand-receptor ratios. It is possible that these ligand-receptor ratio differences are responsible for the observed gender-specificity resulting in a more pronounced lean phenotype in male $CB_1^{-/-}$ mice.

In conclusion, our results show that the CB_1-receptor contributes to body weight gain during lactation in both male and female mice, potentially in a gene-dose effect relationship and these effects are not mediated through differences in the prenatal environment. Moreover, we demonstrated gender-specificity for the role of the CB_1-receptor with a more pronounced lean phenotype in male $CB_1^{-/-}$ pups. Impaired

endocannabinoid signalling could be involved in failure to thrive in humans and future research is needed to further address this possibility.

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CHAPTER 3

Metabolic responses to long-term pharmacological inhibition of CB_1-receptor activity in mice in relation to dietary fat composition

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International Journal of Obesity, 2010; 34: 374-84

Abstract

The anti-obesity effects of suppressed endocannabinoid signalling may rely, at least in part, on changes in lipid fluxes. As fatty acids exert specific effects depending on their level of saturation, we hypothesized that the dietary fatty acid composition would influence the outcome of treatment with a CB_1-receptor antagonist (rimonabant). Therefore we performed a controlled study in mice in which we analyzed the effects of treatment with rimonabant on (markers of) lipid fluxes and obesity-associated derangements in relation to differences in dietary fatty acid composition. Mice were treated with rimonabant (10 mg kg^{-1} body weight per day) or vehicle while equicalorically fed either a low-fat diet (chow, LF), a high-fat (HF) diet or a HF diet in which 10% of the saturated fatty acids (SFA) were displaced by polyunsaturated fatty acids (PUFA) from fish oil (FO). Food intake and body weight were registered daily. Indirect calorimetry was performed and feces were collected. After three weeks, mice were sacrificed for blood and tissue collection.

Results showed that relative to the LF (chow) diet, the HF diet caused anticipated metabolic derangements, which were partly reversed by the HF/FO diet. The HF/FO diet, however, was most obesity-promoting despite inhibiting lipogenesis as indicated by low gene expression levels of lipogenic enzymes. On all three diets, rimonabant treatment improved metabolic derangements and led to significantly lower body weight gain than their respective controls. This latter effect appeared largest in the HF/FO-group, but occurred without major changes in nutrient absorption and energy expenditure.

Conclusion: The effects of chronic rimonabant treatment on body weight gain occurred irrespective of diet-induced changes in lipogenic activity, food intake and daily energy expenditure, and were, in fact, most pronounced in the HF/FO mice. The effects of dietary PUFA replacement in an HF diet on expansion of adipose tissue might allow the favourable effects of dietary PUFA on dyslipidemia and hepatic steatosis. In light of other disadvantageous effects of weight gain, this might be a risky trade-off.

Introduction

Detailed understanding of the mechanisms involved in regulation of energy balance is crucial for treatment and prevention of obesity and associated comorbidities. The endocannabinoid system (ECS) has recently been implicated in the regulation of energy balance.[1-3] The psychoactive substance, Δ^9-tetrahydrocannabinol (THC), as well as endogenous ligands of the ECS, anandamide and 2-arachidonoylglycerol, stimulate food intake by activation of CB_1-receptors.[4-7] CB_1-receptor-null mice are lean and resistant to diet-induced obesity.[8-11] Consistent with these findings, the CB_1-receptor antagonist SR141716, also known as rimonabant, was shown to be able to reduce food intake and body weight in obese humans.[12,13] In humans as well as in rodents the effects of CB_1-receptor antagonism on food intake are generally transient, whereas changes in body fat content upon treatment are more persistent,[7,14-16] suggesting a role for the ECS in energy fluxes independent of food intake.

In line with this are several reports to indicate that CB_1-receptor activity affects lipid mobilization and utilization. With respect to fat tissue, for example, Matias *et al.*[7] observed direct effects of endocannabinoids on lipid droplet formation in mouse and human adipocytes, and these effects could be prevented by CB_1-receptor antagonism.[2] Furthermore, Cota *et al*.[10] found that stimulation of CB_1-receptors of primary adipocytes increases lipoprotein lipase activity, an effect that could be blocked by co-administration of the CB_1-receptor antagonist rimonabant. Jbilo *et al.*[17] found that treatment with this CB_1-receptor antagonist increases gene expression of enzymes involved in lipolysis and ϐ-oxidation in white and brown adipose tissue. Herling *et al*.[18] and Osei-Hyiaman *et al*.[19] independently reinforced these results by showing increased rates of lipid oxidation (by indirect calorimetry) following bolus administration of rimonabant, particularly during the post-ingestive phase. Finally, CB_1-receptor antagonist in obese patients consistently improved plasma lipid profiles.[12,13,20] With respect to the liver, Osei-Hyiaman *et al.*[11] observed that feeding a high-fat diet increases hepatic levels of anandamide and CB_1-receptor-density, and showed that pharmacological activation of CB_1-receptors by HU210 in mice increases hepatic gene expression of *Srebp-1c* (*srebf1*), *Acc1* (*Acaca*) and *Fasn*, all indicative of increased lipogenesis. While CB_1-receptor-null mice have reduced lipogenesis,[19] which was suggested to explain their lean phenotype, data on the effects of chronic rimonabant-treatment on markers of lipogenesis are currently lacking. Taken together, these data indicate that CB_1-receptor antagonism is a useful

tool against disturbance in lipid fluxes known to be underlying, or at least associated with, the 'metabolic syndrome'.

It is generally accepted that not only the quantity of dietary fat intake, but also its quality affects lipid fluxes in the body.[21] Thus, while a high-saturated-fat diet stimulates the expression of genes encoding lipogenic enzymes in the liver such as *Srebp-1c* and *Fasn* expression,[22] a diet with a high content of poly-unsaturated fatty acids (PUFA) has the opposite effects.[23,24] Since changes in lipid fluxes are suggested to be, at least in part, responsible for the anti-obesity effect of rimonabant treatment, it can be hypothesized that efficacy of rimonabant treatment may depend on changes in lipid fluxes resulting from differences in dietary fat composition. Up till now, relatively little attention has been paid to the role of dietary fat composition in relation to the efficacy of rimonabant treatment. Humans consume food that varies considerably in fat content and in the ratio of saturated fatty acids (SFA) to PUFA. In fact, because of presumed favourable effects on lipid profiles and on the risk for atherosclerosis, PUFA supplementation is popular in those at risk of obesity-associated comorbidities.

As different fatty acids have specific effects on lipid fluxes and compartmentalization of triglycerides in the body, we hypothesized that differences in dietary fatty acid composition will influence the outcome of treatment with a CB_1-receptor antagonist like rimonabant. Therefore we performed a controlled study in mice in which we analyzed the effects of dietary fatty acid composition in relation to treatment with rimonabant on obesity-associated derangements.

Methods

Animals

Eight-week-old male C57BL/6J mice were obtained from Harlan (Zeist, The Netherlands) and were individually housed in a light-controlled (lights on 8 AM – 8 PM) and temperature-controlled (21°C) facility. Mice were allowed tap water and food *ad libitum*. Experimental protocols were approved by the local Experimental Ethical Committee for Animal Experiments. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

Experimental diets

All experimental diets were obtained from Abdiets bv, Woerden, The Netherlands. Mice received either a low-fat (LF) diet (standard laboratory chow RMH-B 2103), a high-fat

diet (HF) containing 36 weight % fat consisting of bovine fat (custom synthesis, diet number 4031.45) or a high-fat fish-oil-enriched diet (HF/FO) containing 36 weight % fat consisting for 58% of bovine fat and for 42% of fish oil (FO), (custom synthesis, diet number 4031.54). After preparing pellets, diets were stored at -20°C. The HF/FO diet was replaced every two days to prevent oxidation of fatty acid species. For diet composition, see Table 1.

Table 1. Fatty acid profiles of experimental diets in mg g^{-1}.

	chow (LF)	HF	HF/FO
C14:0	0,5	12,2	16,1
C16:0	8,4	92,5	79,5
C16:1	0,7	11,5	18,0
C18:0	3,7	76,3	50,5
C18:1	13,7	133,2	101,0
C18:2	16,9	11,5	9,7
C18:3	1,9	2,9	15,2
C20-22	0,4	4,0	53,3
C16 desaturation index	0,1	0,1	0,2
C18 desaturation index	3,7	1,7	2,0
ratio n-6/n-3	12,0	ND*	0,4
total dietary fat content	6%	36%	36%

Abbreviations: FO, fish oil; HF, high-fat; LF, low-fat; ND, not detectable.
The level of n-6 PUFA in the HF diet are non-detectable ND. Composition of experimental diets.

Experimental procedures

The effect of dietary PUFA and fat intake, the effect of treatment with a CB_1-receptor antagonist and the interaction between diet and this treatment were studied in a Latin square design. On arrival, mice were divided into three dietary groups: chow, HF, HF/FO. Mice were matched for plasma lipids, glucose and body weight. Half of the animals in each dietary group were subjected to CB_1-receptor antagonist (rimonabant) treatment. Thus, six groups of eight mice were compared in this experiment.

Intake and body weight were registered daily. Rimonabant (reference compound kindly provided by Solvay) was administered orally at a dose of approximately 10mg kg^{-1} body weight per day by thoroughly mixing it through the diet. Oral rimonabant treatment was started after 3 weeks on the various diets. Indirect 24-h calorimetry was performed

before start of treatment with the CB_1-antagonist and 2 weeks after start of treatment. Feces were collected before start of treatment and after 18 days of treatment. After 3 weeks of oral rimonabant treatment (and thus 6 weeks after start of the diet), treated and control mice were sacrificed, blood and tissue were collected.

Indirect calorimetry

Mice were placed in an open-circuit, indirect calorimeter system for 24h with access to water and food. Gas-exchange measurements were performed in an eight-channel, open flow system. Flow rates were measured and controlled with a mass flow controller. O_2 and CO_2 concentrations of dried inlet and outlet air from each chamber were measured every 10 min with a paramagnetic O_2 analyzer and an infrared CO_2 gas analyzer. Data were collected from each metabolic cage separately. The respiratory quotient (RQ) was defined as CO_2 production (l)/ O_2 consumption (l). Energy expenditure was calculated according to Brouwer[25] using the following equation: $(16.18*VO_2*0.001)+(5.02*VCO_2*0.001)$. Lipid oxidation and carbohydrate oxidation were calculated according to Lusk using the following equations:[26]

Lipid oxidation (g h^{-1}): $38.461*(VO_2 \text{ (mol } h^{-1}) - VCO_2 \text{ (mol } h^{-1}))$

Carbohydrate oxidation (g h^{-1}): $94.017*VCO_2 \text{ (mol } h^{-1}) - 66.239*(VO_2 \text{ (mol } h^{-1})$

Feces

The energy content of dried, homogenized feces was determined using a bomb calorimeter (CBB 330, standard benzoic acid 6320 cal g^{-1}, BCS-CRM No.90N)

Plasma lipids and adipokines

Plasma lipids were measured using commercially available kits from Roche (Mannheim, Germany) for triglycerides and cholesterol. Plasma leptin, resistin and tumor necrosis factor-α levels were determined using a commercially available adipokine lincoplex kit (Linco Research, St Charles, MO, USA). Adiponectin was measured using a commercially available RIA kit (Linco Research).

Hepatic lipids

Livers were removed and freeze-clamped. Before further analysis, livers were crushed on liquid nitrogen and stored at -80°C. For hepatic lipid extraction, frozen crushed livers were homogenized in ice-cold saline. Hepatic lipids were extracted according to Bligh and Dyer[27] and hepatic triglycerides and total cholesterol were measured

using commercially available kits from Roche; free cholesterol was measured using commercially available kit from Wako Chemicals (Neuss, Germany). Phospholipid content of the liver was determined according to Böttcher *et al.*[28]

Gene expression in liver and epididymal adipose tissue

Total RNA was isolated from frozen liver and epididymal adipose tissue using the TRI-reagent method (Sigma, Zwijndrecht, The Netherlands). Using random primers, RNA was converted to cDNA with M-Mulv Reverse Transcriptase (Sigma) according to the manufacturer's protocol. For realtime-PCR, cDNA was amplified using the appropriate primers and probes. The sequences of the primers and probes for *β-actin, Srebp-1c*, *Fasn*, *Acc1* and *Acc2* have been published (http://www.labpediatricsRUG.nl Realtime Primers Datalist Pediatrics UMCG). Relative gene expression levels were normalized to *β-actin*.

Body composition

Carcasses were eviscerated and stored at -20°C. Carcasses and organs were dried to constant weight at 103 °C, and fat was extracted by using petroleum ether (Boom BV, Meppel, The Netherlands) in a soxlet apparatus. Percentage fat of carcasses and organs were determined from weight differences before and after the fat extraction procedure.

Statistics

All values in the figures and in the tables represent means ± standard errors of the means for the number of animals indicated in the figure and table legends. To evaluate effects of diet, rimonabant treatment, and their interactions, data were statistically analyzed using a general linear model with Bonferroni *post hoc* analyses. Treatment effects were further analyzed by Student's *t*-test. In case of non-parametric distribution, Mann-Whitney *U*-test was used for statistical analysis. Statistical significance of differences was accepted at a *P*-value of less than 0.05. Analyses were performed using SPSS 16.0 for Windows software (SPSS, Chicago, IL)

Results

Effects of dietary fat composition on body weight gain, adiposity and food efficiency in C57BL/6J mice

Mice were matched for serum levels of glucose, triglycerides and total cholesterol, as well as for body weight at the onset of the experiment. Figure 1a shows changes in body weight relative to initial weights from the start of the experimental diets. In the control groups on all the three experimental diets, body weights increased over the course of the experiment, but most pronounced in HF/FO-fed mice. At day 22, body weights of mice fed HF/FO were significantly increased compared to mice fed chow and mice fed the HF diet (Figure 1b). Data on body composition shown in Table 2 illustrate that increased body weight in the HF/FO fed mice was associated with increased adiposity in these mice as compared to that in HF-fed mice and the chow-fed mice. There was a significant increase in fat mass in mice fed the HF/FO diet as compared with that in mice fed chow as well as that in mice fed the HF diet. Dietary fat composition did not affect body fat distribution as is shown in Figure 1c.

Increased weight gain in the HF/FO group could not be explained by higher food intake. In fact, mice fed HF/FO had slightly reduced caloric intake; however, this trend did not reach statistical significance (Figures 1d and e). To assess whether increased absorption could contribute to increased body weight gain in mice fed HF/FO, residual energy contents in feces collected over a period of 48 h were determined using bomb calorimetry (Figure 2a). Fecal energy excretion was significantly lower in the HF/FO control group (1.58 kcal $24h^{-1}$ ± 0.45) as compared with that in the chow control group (3.11 kcal $24h^{-1}$ ± 0.47; p = 0.000) and the HF control group (2.22 kcal $24h^{-1}$ ± 0.37; p = 0.037). Reduction of fecal energy excretion in the HF control group as compared with that in the chow control group was also significant (p = 0.008). However, calculated as absorbed energy (food intake in kcal $24h^{-1}$ minus residual fecal energy in kcal $24h^{-1}$), the amount of absorbed energy was not affected by diet as shown in Figure 2b.

Food efficiency (body weight gain in gram per kilocalorie intake) calculated for the treatment period is shown in Figure 2c. Food efficiency of mice in the HF/FO control group was significantly higher as compared with food efficiency in the chow control group (113.7% higher in the HF/FO control group compared with levels calculated in the chow control group; p = 0.006) and in the HF control group (99.4% higher in the HF/FO control group compared with levels calculated in the HF control group; p = 0.010).

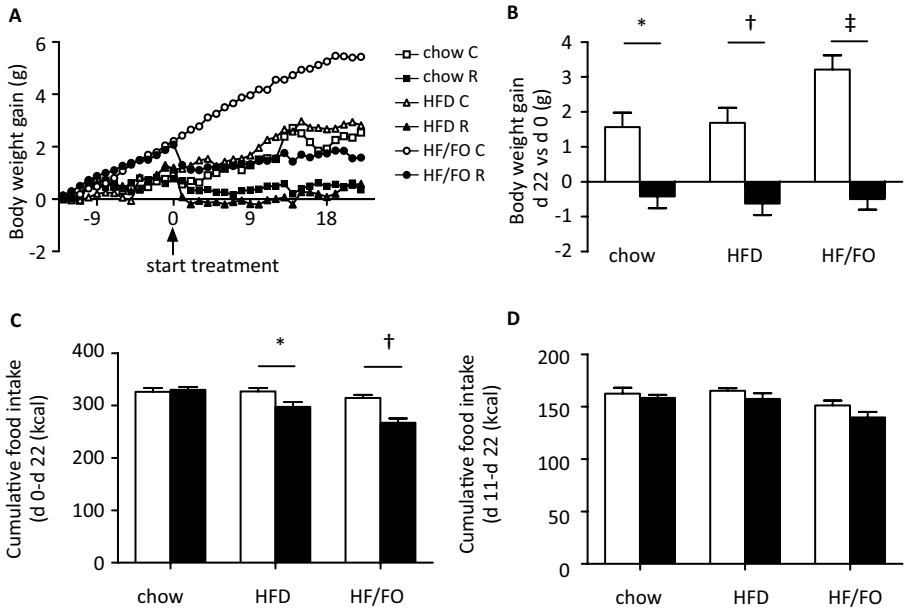

Figure 1. Body weight gain and food intake. A) Body weight gain compared to body weight at start of diet expressed in grams per day. On day 0, treatment with rimonabant was started as indicated by the arrow. Open squares, chow C (low-fat control group); black squares, chow R (low-fat treatment group); open triangles, HF C (high-fat control group); black triangles, HF R (high-fat treatment group); open circles, HF/FO C (HF/FO control group); black circles, HF/FO R (HF/FO treatment group). Values are means for n = 8 mice per group. Body weight at day 0 (means ± SD for n = 16 mice per diet group) chow 25.6 ± 1.4, HF 28.3 ± 2.0, HF/FO 27.0 ± 2.2. B) Body weight gain between day 0 and day 22. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± SEM for n = 8 mice per group. * chow R *versus* chow C $p = 0.002$, † HF R *versus* HF C $p = 0.001$, ‡ HF/FO R *versus* HF/FO C $p = 0.000$. Trend towards significant interaction between treatment and diet $p = 0.063$. C) Cumulative intake day 0 until day 22 expressed in kilocalories. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± SEM for n = 8 mice per group, * HF R *versus* HF C $p = 0.020$, † HF/FO R *versus* HF/FO C $p = 0.000$. Significant interaction between treatment and diet $p = 0.003$. D) Cumulative intake day 11 until day 22 expressed in kilocalories. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± SEM for n = 8 mice per group. No significant differences. FO, fish oil; HF, high-fat.

Table 2. Body composition in mice fed chow (LF), HF and HF/FO diets either with or without adding a CB1-receptor antagonist.

		chow (LF)		HF		HF/FO	
		control	**rimonabant**	**control**	**rimonabant**	**control**	**rimonabant**
Body mass (g)		29,09 ± 0,62	26,05 ± 0,63§	30,56 ± 0,55	28,84 ± 0,82	33,63 ± 1,14†,‡	29,19 ± 1,38**
Lean carcass (g)		4,00 ± 0,08	4,03 ± 0,15	3,76 ± 0,04*	3,73 ± 0,06	4,21 ± 0,18‡	3,89 ± 0,13
Fat mass (g)		3,65 ± 0,44	2,56 ± 0,25§	6,53 ± 0,51*	4,86 ± 0,51#	9,15 ± 0,79†,‡	6,09 ± 0,88**
Muscular fat (g)		1,07 ± 0,14	0,84 ± 0,07	1,26 ± 0,09	1,13 ± 0,14	2,32 ± 0,20†,‡	1,40 ± 0,24**
Visceral fat (g)		1,19 ± 0,11	0,81 ± 0,09§	2,26 ± 0,17*	1,77 ± 0,14	2,84 ± 0,25†	1,99 ± 0,24**
	epididymal	0,69 ± 0,05	0,51 ± 0,04§	1,49 ± 0,10*	1,21 ± 0,09#	1,81 ± 0,21†	1,22 ± 0,15**
	retroperitoneal	0,29 ± 0,03	0,19 ± 0,03§	0,45 ± 0,04*	0,28 ± 0,03#	0,71 ± 0,06†,‡	0,46 ± 0,07**
	mesenteric	0,20 ± 0,05	0,12 ± 0,04	0,32 ± 0,04	0,29 ± 0,05	0,32 ± 0,09	0,31 ± 0,08
Peripheral fat (g)	subcutaneous	1,33 ± 0,16	0,78 ± 0,11§	2,89 ± 0,26	2,18 ± 0,18	3,81 ± 0,45†	2,50 ± 0,39**

Abbreviations: FO, fish oil; HF, high-fat; LF, low-fat. Values are mean ± SEM for n=7-8, * $p < 0,05$ HF control vs chow (LF) control, † $p < 0,05$ HF/FO control vs chow control, ‡ $p < 0,05$ HF/FO control vs HF control, § $p < 0,05$ chow control vs chow rimonabant-treatment, # $p < 0,05$ HF control vs HF rimonabant-treatment, ** $p < 0,05$ HF/FO control *vs* HF/FO rimonabant-treatment

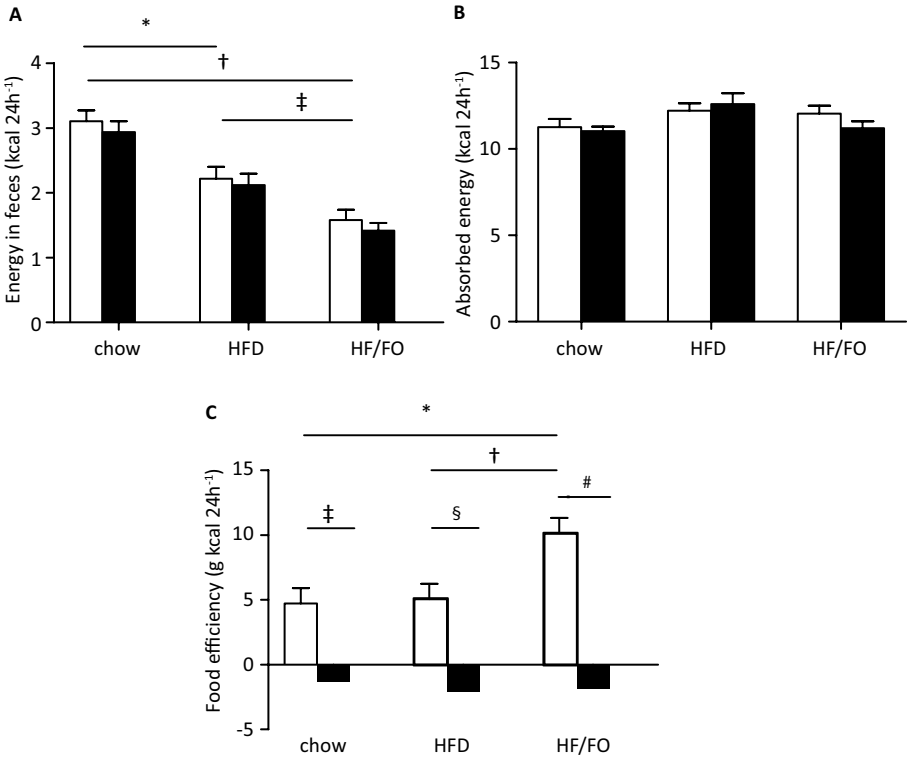

Figure 2. A) Fecal energy excretion. Residual energy in feces collected during 48 h, measured by bomb calorimetry. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± SEM for n = 4-8 mice per group, HF C *versus* chow C * $p = 0.008$, HF/FO C *versus* chow C † $p = 0.000$, HF C *versus* HF/FO C ‡ $p = 0.037$. B) Energy absorption. Absorbed energy calculated from food intake in kilocalories per day minus residual fecal energy in kilocalories per day. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± SEM for n=4-8 mice per group. No significant differences. C) Food efficiency day 0 – day 22 expressed in gram body weight gain per kilocalorie intake. Open bars, control groups; black bars, rimonabant treatment groups. Values are means ± SEM for n = 8 mice per group, * HF/FO C *versus* chow C $p = 0.006$, † HF/FO C *versus* HF C $p = 0.010$, ‡ chow R *versus* chow C $p = 0.002$, § HF R *versus* HF C $p = 0.001$, HF/FO R *versus* HF/FO C $p = 0.000$. Significant interaction between treatment and diet $p = 0.027$. FO, fish oil; HF, high-fat.

Effects of dietary fat composition on lipid profile and lipogenic markers in C57BL/6J mice

Plasma concentrations of triglycerides were significantly lower in the HF/FO control group as compared with that in the chow and HF control groups (Table 3). In the HF control group as well as HF/FO control group, plasma cholesterol concentrations were significantly elevated as compared with that in the chow fed control group. Hepatic triglycerides, hepatic total cholesterol, free cholesterol and cholesterol esters were significantly elevated in the HF group as compared with that in the chow group (Table 3). The hepatic levels of total cholesterol, free cholesterol and cholesterol esters normalized in mice fed HF/FO as compared with that in the HF group, and became indistinguishably from those in chow controls. Hepatic triglyceride levels were decreased in the HF/FO control group as compared with that in the HF control group, but remained significantly elevated compared with that in the chow control group. Both plasma leptin and adiponectin levels were significantly increased in the HF/FO control group as compared with that in the other control groups (Table 3).

Consistent with previous data from our laboratory, hepatic expression of genes encoding lipogenic enzymes (shown in Figure 3) were significantly elevated in the HF control group as compared with that in the chow control group, and in the HF/FO group expression levels were similar to the chow control group or even lower.

Effects of dietary fat composition on RQ and oxygen consumption in C57BL/6J mice

As expected, calorimetry data showed higher RQs for mice in the chow group as compared with those in the HF group and the HF/FO group (Table 4). In the dark phase and in the light phase, oxygen consumption and calculated energy expenditure were significantly increased in the HF-fed mice as compared with that in the chow control group and the HF/FO control group. There were no significant differences between the HF/FO-fed mice and the chow-fed mice regarding oxygen consumption or energy expenditure. There was a significant reduction in CO_2 production in the HF/FO-fed mice as compared with that in chow-fed mice and HF-fed mice.

Lipid oxidation was significantly increased in the HF control group as compared with that in the chow control group in the dark as well as light phase. This increase was even more pronounced in the HF/FO control group. HF/FO-fed mice had significantly elevated lipid oxidation compared with that in HF-fed mice. In the light and in the dark phase, HF/FO-fed mice had significantly decreased carbohydrate oxidation compared

with both the HF control group and the chow control group. In the HF-fed mice carbohydrate oxidation was also significantly decreased in the light phase and in the dark phase as compared to the chow control group.

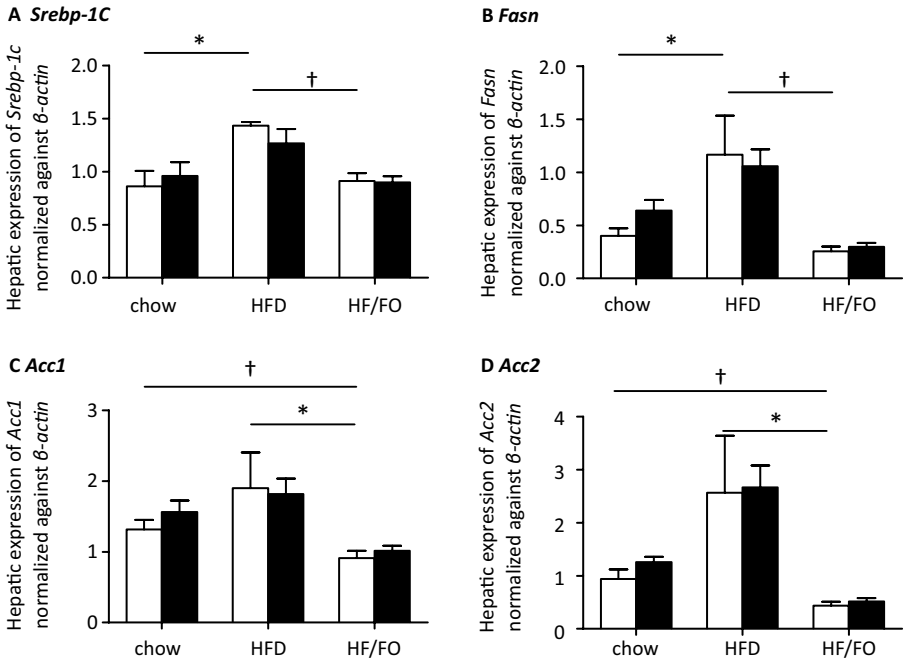

Figure 3. Hepatic lipogenic gene expression. A) Relative hepatic mRNA expression of *Srebp-1c* (*srebf1*) in control mice (white bars) and mice treated with rimonabant (black bars) on either chow (LF), HF or HF/FO. Results are normalized to *β-actin*. Values are means ± SEM for n=3-8 mice per group. HF C *versus* chow C * $p = 0.027$, HF/FO C *versus* HF C † $p = 0.003$. B) Relative hepatic mRNA expression of *Fasn* in control mice (white bars) and mice treated with rimonabant (black bars). Results are normalized to *β-actin*. Values are means ± SEM for n=3-8 mice per group. HF C *versus* chow C * $p = 0.019$, HF/FO C *versus* HF C † $p = 0.001$. C) Relative hepatic mRNA expression of *Acc1* (*Acaca*) in control mice (white bars) and mice treated with rimonabant (black bars). Results are normalized to *β-actin*. Values are means ± SEM for n=3-8 mice per group. HF C *versus* HF/FO C * $p = 0.012$, HF/FO C *versus* chow C † $p = 0.034$. D) Relative hepatic mRNA expression of *Acc2* (*Acacb*) in control mice (white bars) and mice treated with SR141716 (black bars). Results are normalized to *β-actin*. Values are means ± SEM for n=3-8 mice per group. HF C *versus* HF/FO C * $p = 0.007$, HF/FO C *versus* chow C † $p = 0.013$. FO, fish oil; HF, high-fat; LF, low-fat.

Table 3. Hepatic and plasma parameters in mice fed chow (LF), HF, HF/FO diets either with or without adding a CB_1-receptor antagonist

		chow (LF)		HF		HF/FO	
		control	**rimonabant**	**control**	**rimonabant**	**control**	**rimonabant**
Plasma							
	Triglycerides (mmol l^{-1})	0,92 ± 0,14	0,68 ± 0,10	1,07 ± 0,12	1,03 ± 0,17	0,70 ± 0,06	0,48 ± 0,10
	Cholesterol (mmol l^{-1})	3,59 ± 0,18	3,09 ± 0,21	5,93 ± 0,26*	5,94 ± 0,19	6,16 ± 0,22†,‡	6,14 ± 0,36
	Leptin (ng ml^{-1})	5,38 ± 2,59	3,27 ± 0,68	10,25 ± 1,93	9,35 ± 1,40	32,75 ± 5,17†	11,68 ± 3,44**
	Resistin (ng ml^{-1})	3,43 ± 0,55	3,03 ± 0,16	5,87 ± 0,59*	6,69 ± 0,57	7,21 ± 0,72†,‡	4,13 ± 0,75**
	TNF-α (pg ml^{-1})	2,51 ± 0,62	2,20 ± 0,29	1,68 ± 0,44	1,33 ± 0,27	2,75 ± 0,32	2,28 ± 0,43
	Adiponectin (µg ml^{-1})	10,72 ± 0,97	12,36 ± 2,42	9,03 ± 0,35	10,04 ± 0,53	23,07 ± 2,29 †	15,94 ± 1,55**
Liver							
	Triglycerides (nmol mg^{-1} liver)	17,29 ± 1,30	14,61 ± 0,67	40,79 ± 0,88*	38,03 ± 1,28	24,82 ± 1,41†,‡	26,32 ± 1,99
	Total cholesterol (nmol mg^{-1} liver)	4,88 ± 0,27	5,24 ± 0,35	7,23 ± 0,21*	7,32 ± 0,29	5,07 ± 0,20‡	5,72 ± 0,14**
	Free cholesterol (nmol mg^{-1} liver)	4,35 ± 0,29	4,61 ± 0,35	5,66 ± 0,15*	5,47 ± 0,26	4,41 ± 0,14‡	4,90 ± 0,11**
	Cholesterolesters (nmol mg^{-1} liver)	0,54 ± 0,10	0,64 ± 0,13	1,57 ± 0,09*	1,86 ± 0,12	0,66 ± 0,09‡	0,82 ± 0,06
	Phospholipids (nmol mg^{-1} liver)	24,98 ± 1,10	24,40 ± 0,66	24,48 ± 0,34	25,39 ± 0,23 #	26,65 ± 0,57	27,13 ± 0,43

Abbreviations: FO, fish oil; HF, high-fat; LF, low-fat; TNF, tumor necrosis factor. Blood samples taken on the day of sacrifice. Values are mean ± SEM for n=6-8, * $p < 0,05$ HF control *vs* chow (LF) control, † $p < 0,05$ HF/FO control vs chow control, ‡ $p < 0,05$ HF/FO control vs HF control, § $p < 0,05$ chow control vs chow rimonabant-treatment, # $p < 0,05$ HF control vs HF rimonabant-treatment, ** $p < 0,05$ HF/FO control vs HF/FO rimonabant-treatment

Table 4. Indirect calorimetry data in mice fed chow (LF), HF and HF/FO diets either with or without adding a CB1-receptor antagonist

	chow (LF)		HF		HF/FO	
	control	**rimonabant**	**control**	**rimonabant**	**control**	**rimonabant**
O2 consumption (ml h^{-1})						
dark phase	22,73 ± 0,76	22,08 ± 0,72	26,51 ± 0,54*	26,02 ± 0,39	23,04 ± 0,77‡	24,36 ± 0,27
light phase	19,92 ± 0,74	17,82 ± 0,76	22,84 ± 0,53*	22,08 ± 0,39	19,71 ± 0,53‡	20,36 ± 0,45
CO2 production (ml h^{-1})						
dark phase	22,39 ± 0,86	20,29 ± 0,65	21,70 ± 0,41	20,95 ± 0,26	17,72 ± 0,62†,‡	18,47 ± 0,22
light phase	18,32 ± 0,95	15,22 ± 0,66§	18,80 ± 0,42	17,87 ± 0,31	15,08 ± 0,41†,‡	15,47 ± 0,34
RQ						
dark phase	0,98 ± 0,01	0,92 ± 0,01§	0,82 ± 0,00*	0,81 ± 0,00#	0,77 ± 0,00†,‡	0,76 ± 0,00**
light phase	0,92 ± 0,02	0,85 ± 0,01§	0,82 ± 0,00*	0,81 ± 0,00#	0,77 ± 0,00†,‡	0,76 ± 0,00
EE per gram lean mass (kJ h^{-1} g^{-1})						
dark phase	0,48 ± 0,02	0,46 ± 0,01	0,54 ± 0,01*	0,53 ± 0,01	0,46 ± 0,02‡	0,49 ± 0,01
light phase	0,41 ± 0,02	0,36 ± 0,02	0,46 ± 0,01*	0,45 ± 0,01	0,39 ± 0,01‡	0,41 ± 0,01
Lipid oxidation (mg h^{-1})						
dark phase	1,3 ± 1,1	10,4 ± 0,9§	28,1 ± 1,0*	29,4 ± 0,7	35,2 ± 0,8†,‡	36,1 ± 1,3
light phase	9,2 ± 1,9	15,8 ± 0,8§	23,6 ± 0,6*	24,4 ± 0,6	30,8 ± 0,9†,‡	30,0 ± 0,8
Carbohydrate oxidation (mg h^{-1})						
dark phase	101,5 ± 4,2	76,6 ± 4,3§	46,4 ± 1,7*	40,2 ± 1,2#	26,4 ± 1,3†,‡‡	21,7 ± 1,4**
light phase	68,9 ± 6,8	43,4 ± 2,9§	41,5 ± 1,0*	35,5 ± 0,9#	21,4 ± 1,5†,‡	18,5 ± 1,2

Abbreviations: EE, energy expenditure; FO, fish oil; HF, high-fat; LF, low-fat; RQ, respiratory quotient. Values are mean ± SEM for n=7-8, * $p < 0,05$ HF control vs chow (LF) control, † $p < 0,05$. HF/FO control vs chow control, ‡ $p < 0,05$ HF/FO control vs HF control, § $p < 0,05$ chow control vs chow rimonabant-treatment, # $p < 0,05$ HF control vs HF rimonabant-treatment, ** $p < 0,05$. HF/FO control vs HF/FO rimonabant-treatment

Effects of rimonabant treatment on food intake and 'food efficiency' and fecal energy excretion in C57BL/6J mice on different diets

We observed no effect of administering the CB_1-receptor antagonist on food intake in the chow-fed treatment group as compared with the chow-fed control group. However, after start of treatment, food intake was significantly lower in the HF treatment group and in the HF/FO treatment group. The effects on food intake diminished during the first week of treatment. At day 4, there was no difference in food intake between the HF treatment group and the HF control group anymore, and after 10 days the difference in food intake had also disappeared between the HF/FO treatment group and the HF/FO control group (data not shown). The cumulative caloric intake calculated for the treatment period was significantly reduced in the HF/FO treatment group as compared with that in the HF/FO control group (HF/FO treatment *versus* HF/FO control $p < 0.001$), and in the HF treatment group compared with that in the HF control group (HF treatment *versus* HF control $p = 0.02$), as is illustrated in Figure 1c. However, there were no significant differences in the cumulative caloric intake calculated from day 11 until day 22 (Figure 1d), illustrating the observation that the effect of treatment with a CB_1-receptor antagonist on food intake is transient.

To assess if decreased absorption could contribute to decreased weight gain in treated mice, we measured residual energy contents in feces by bomb calorimetric analysis of feces collected over a period of 48 h. Treatment with CB_1-receptor antagonist had no significant effect on fecal energy excretion on either diet (Figure 2a) and calculated as absorbed energy (food intake in kcal $24h^{-1}$ minus residual fecal energy in kcal $24h^{-1}$) the amount of absorbed energy was not affected by treatment (Figure 2b).

On all three diets, rimonabant treatment led to a significant reduction in food efficiency (Figure 2c; chow treatment *versus* chow control, $p = 0.002$; HF treatment *versus* HF control, $p = 0.001$; HF/FO treatment *versus* HF/FO control, $p < 0.001$). There was a significant interaction between diet and treatment ($p = 0.027$). Indeed, effect of treatment was most prominent in the HF/FO treatment group as compared with that in the HF/FO control group. Again, this was a consequence of higher food efficiency in the HF/FO control group rather than of lower food efficiency in the HF/FO treatment group.

Effects of rimonabant treatment on RQ and oxygen consumption in C57BL/6J mice on different diets

Table 4 shows that treatment with rimonabant significantly reduced the RQ during both the light phase and the dark phase in the chow-fed mice (light-phase chow treatment

versus chow control $p = 0.004$, dark-phase chow treatment *versus* chow control $p < 0.001$) and in the HF-fed mice (light-phase HF treatment *versus* HF control $p = 0.003$, dark-phase HF treatment *versus* HF control $p = 0.033$). In the HF/FO treatment group, a significant reduction in RQ as compared with that in the HF/FO control group was only observed in the dark phase (HF/FO treatment *versus* HF/FO control $p = 0.011$). A significant increase in lipid oxidation and a significant decrease in carbohydrate oxidation was observed in the chow-fed mice upon rimonabant treatment in the light phase as well as in the dark phase. There was no treatment effect on lipid oxidation in the HF groups nor in the HF/FO groups. Yet, there was a significant decrease in carbohydrate oxidation in the HF treatment group as compared with that in the HF control group in the light phase and in the dark phase, and in the HF/FO treatment group compared with that in the HF/FO control group only in the dark phase.
Rimonabant treatment did not affect oxygen consumption or energy expenditure as calculated from indirect calorimetry data, neither in the light phase nor in the dark phase in any of the groups (Table 4).

Discussion

In the present study, we compared the effects of treatment with rimonabant, a selective endocannabinoid CB_1-receptor antagonist, on food intake and on hormonal and metabolic characteristics, in young adult mice fed diets with differences in the total amount and ratio of SFA *versus* PUFA. Despite notable differences in hormonal and metabolic profiles among mice fed the different experimental diets, we found that rimonabant treatment prevented weight gain and improved metabolic derangements, without major differences in energy intake and expenditure. This highlights a role for the ECS in triglyceride deposition in adipose tissue as well as in the liver irrespective of dietary fat content or composition.

Compared with mice fed a fibered chow, carbohydrate-rich diet, feeding a diet with HF content caused increase in body fat mass and hyperleptinemia, and a massive increase in hepatic triglyceride content in mice. These well-known derangements are major risk-factors for development of hepatic steatosis and type-2 diabetes mellitus.[29-31] The increased hepatic fat deposition – being a hallmark of the metabolic syndrome - appeared to be abolished when mice were fed a diet with a fat content similar as the aforementioned HF diet, but in which 10 % of the SFA were replaced by PUFA derived from FO (HF/FO diet). We observed, however, that lipid deposition inside adipose tissue

as well as plasma leptin levels were greatly augmented in HF/FO-fed mice as compared with that in those fed the HF diet.

The finding that a 10% displacement of SFA by PUFA caused a reduction of hepatic triglyceride deposition almost back to the level observed in the chow-fed mice fits well with the documented effects of PUFA to stimulate hepatic lipid utilization,[32] and to reduce hepatic lipogenesis.[23] Indeed, mice in the present study fed the HF/FO diet showed reduced hepatic gene expression levels of lipogenic enzymes *Srebp-1c*, *Acc1, Acc2* and *Fasn* compared with those observed in the HF group. In addition, the rate of lipid oxidation assessed by indirect calorimetry was clearly highest in the HF/FO group. For these reasons, it is counterintuitive that ingestion of a HF/FO diet potentiated the level of body adiposity well above the levels observed in the HF and chow group. Mice fed the HF/FO diet showed significantly elevated plasma levels of adiponectin, which could have, in part, prevented fat deposition in extra-adipose tissues, and at the same time, mediated the increased expansion of adipose tissue in this group. Such an effect has been proposed by Kim *et al*.,[33] who showed that overexpression of adiponectin leading to elevated plasma adiponectin levels, also led to increased adipose tissue mass. Like in our study, the increased plasma adiponectin levels together with expanded fat mass were associated with normalization of hepatic and plasma lipid profiles. Another issue relevant to the beneficial effect of the HF/FO diet on metabolic endpoints while enhancing fat mass (that is, relative to those observed in the HF diet condition), is the observation that the HF/FO diet seems to augment, percent-wise, the subcutaneous fat depot (subcutaneous adipose tissue) more than the visceral fat depot (visceral adipose tissue) as compared with the HF diet, and with respect to the corresponding values at day 22 in the chow diet. While both diets augment subcutaneous adipose tissue more than visceral adipose tissue, the difference is approximately 20% higher with the HF/FO diet. In view of the well-established strong connections between visceral adipose tissue and some metabolic disorders,[34] this perhaps may have contributed to the higher adiponectin levels (despite the stronger increase in body weight) and also to the beneficial effects of on hepatic lipogenesis observed with the HF/FO diet.

The exaggerated expansion of adipose tissue of HF/FO-fed mice as compared with the other groups could not be attributed to an increase in food intake or to an increased gastrointestinal absorption rate, which indicates that these animals were more ‘food efficient’ than those fed the HF and chow diet. Indeed, calculation of body weight gain per absorbed energy content revealed a doubling of food efficiency in the HF/FO group as compared with that in the HF and chow groups. Food effciency

depends on energy expenditure (EE), which in turn is comprised of resting metabolic rate, activity thermogenesis, and thermic effects of food. Resting metabolic rate comprises thermogenesis, detoxification, maintenance of membrane potentials and tissue differentiation and tissue maintenance. EE during the light and the dark phase and resting metabolic rate calculated from indirect calorimetry were indeed lower in the HF/FO group as compared with that in the levels found in the HF group, which may have underlied the increased food efficiency and adipose tissue expansion in the HF/FO group relative to the HF group. Compared to the chow-fed mice, however, this lowering of EE and resting metabolic rate in the HF/FO group was not significant. This discrepancy of increased food efficiency and weight gain in the HF/FO group *versus* the chow group without alterations in EE may be explained by a shift between the various components of energy expenditure without affecting total energy expenditure. For example, a decrease in the thermic effects of food could make more energy available for storage and tissue expansion. We are currently performing experiments to specifically address these possibilities.

As pointed out above, rimonabant treatment prevented weight gain and ameliorated adiposity in all three experimental diet groups despite marked differences in adiposity and metabolic profiles between the diet groups. After start of treatment with the CB_1-receptor antagonist, there was a slight and temporal reduction of food intake in the HF and HF/FO group, which explains the reduction in body weight by rimonabant-treatment only to a limited degree. Since total absorbed energy (as assessed by bomb calorimetry of feces) was not different among groups, this indicates that body weight loss induced by treatment with rimonabant was mediated by changes in metabolism. In fact, rimonabant treatment rendered mice remarkably food inefficient, such that the diet-induced differences in food efficiency were entirely lost upon rimonabant treatment. However, the effects of rimonabant could not be explained by differences in metabolic rate since EE measured by indirect calorimetry was similar in treated *versus* non-treated mice in the respective diet groups. It is tempting to speculate that metabolic coupling was affected by rimonabant treatment in the different diet groups, which contributed to prevent weight gain. While efficiency of metabolic coupling as a potential mechanism has to be addressed, our results regarding discrepancies between weight loss by blockade of CB_1-signalling, without changes in energy intake and expenditure corresponds with findings in several other recent studies.[10,35] Reports that, in contrast to these results, do demonstrate an increase in EE in animals and humans treated with a CB_1-receptor antagonist [19,36-38] frequently employ an acute rather than continuous

treatment. Kunz *et al.*[36] clearly demonstrated rapid development of tolerance to rimonabant towards induction of oxygen consumption, stressing differences in effects of acute *versus* chronic treatment. Moreover, investigators frequently normalize EE and oxygen consumption to total body weight, thereby ignoring fat-free *versus* fat mass, which causes an over-estimation of EE when fat mass is lost without changes in lean mass.[19,37]

The effect of CB_1-receptor antagonism to prevent weight gain and to ameliorate adiposity levels in the chow, HF, as well as HF/FO-fed mice corresponds with other studies showing treatment effects irrespective of diet composition.[14-17,20] It is, however, rather surprising in light of the findings by others showing that HF diets given to mice cause dysregulation of the ECS, either *via* alterations in the expression of CB_1-receptors, *via* alterations in the levels of endogenous ligands, or both.[7,39-41] Also the fatty acid composition of dietary fat may influence endocannabinoid action.[42] For example, the presence or absence of n-3 PUFA in the diet affects endocannabinoid levels in the brain, as shown by Berger *et al.*[43] and Watanabe *et al.*[44] Batetta *et al.*[45] recently found that dietary n-3 PUFA can reduce inflammatory markers and liver triglyceride levels, and these effects were associated with lower levels of endocannabinoid ligands in peripheral organs. Finally, there is an *in vitro* study showing that n-3 PUFA can reduce the levels of both anandamide and 2-arachidonoylglycerol in differentiated mouse adipocytes, whereas n-6 PUFA (that is, arachidonic acid) were found to increase endocannabinoid levels.[46] The efficacy of an antagonist such as rimonabant should depend on the extent of tonic activation of CB_1-receptors by, among other things, endocannabinoids. Since n-6 PUFA content is relatively high in FO (that is, with a ratio of n-6/n-3 of 0.4 in the HF/FO diet, and no n-6 at all in the HF diet), this may provide an explanation for the finding that rimonabant is more efficacious in mice fed the HF/FO diet to induce weight loss in the present study. Associated with reversal of HF-diet-induced obesity by rimonabant, studies generally reported lowering of hepatic triglyceride accumulation and plasma leptin levels, and increased plasma adiponectin levels by rimonabant treatment.[11,15,17,20] The reversal of these comorbidities by rimonabant was not observed in the present study. It is possible that the discrepancy between the results of our study and others is due to differences in the age of mice and experimental duration. In our study, endpoint measurements were performed at 14 weeks of age, whereas in all other mice used were 6 months or older, and mice in those studies were subjected to experimental diets for months.[11,15,17,20] nevertheless, we did find profound inhibitory effects of the HF/FO diet on markers for hepatic lipogenesis relative to the HF diet condition. It was previously

suggested by Osei-Hyiaman *et al.* [11] that HF-diet-induced obesity was a consequence of increased lipogenesis (by increased hepatic *Srebp-1c* expression), with a permissive role for hepatic CB_1-signalling in these effects. This is in contrast with our results that showed reduction of body weight gain in mice treated with rimonabant on all three diets, and, in fact, reduction of body weight gain was most pronounced in treated mice on the HF/FO diet in which lipogenesis was suppressed. Thus, our results suggest that suppression of hepatic lipogenesis does not play a role in the effect of rimonabant on body weight, at least in young adult mice. This fits with the most recent observation of Osei-Hyiaman *et al.*[19] that liver-specific $CB_1^{-/-}$ mice are not resistant to diet-induced obesity, whereas total $CB_1^{-/-}$ mice are.

In conclusion, our results show that treatment with a CB_1-receptor antagonist is not only effective in reducing body weight gain in mice fed a HF diet based on SFA, but that a significant reduction in body weight is achieved irrespective of dietary fat quality and quantity. Furthermore, the effect of chronic rimonabant treatment to limit body weight gain occurred independent of basic lipogenic activity and, in fact, appeared to be most pronounced in mice fed a diet with a mixed SFA/PUFA composition, by which lipogenic activity was lowest. Finally, we demonstrated that SFA replacement in a HF diet by PUFA not only results in improvement of HF-diet-induced metabolic derangements, but also in augmentation of adipose tissue stores. While the effects of dietary PUFA replacement in a HF diet on expansion of adipose tissue might allow the favourable effects of dietary PUFA on dyslipidemia and hepatic steatosis, this might be a risky trade-off in light of other disadvantageous effects of weight gain. Thus, if FO supplements are used to prevent the adverse metabolic effects of a Western-type diet in children and adolescents, based on the findings in the present study, the outcome will be an exacerbation of the development of obesity in young people. If other preventive measures (for example, exercise programs, avoiding Western-type diets, and so on) turn out to be fruitless, improvement of pharmacotherapy might remain the last resort for the treatment of obesity at exceedingly young age.

Acknowledgement

We thank Solvay for kindly providing SR141716. This study was financially supported by a grant from Kruidvat and UMCG to the Groningen Expert Centre on Childhood Obesity (to AHK) and from the Dutch Diabetes Foundation (to GvD). We thank Mark Doornbos for technical assistance.

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CHAPTER 4

Changes in hepatic lipid metabolism underlying resistance of CB_1-deficient mice to diet-induced hepatic steatosis

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Abstract

Hepatic expression of cannabinoid (CB) receptors is markedly upregulated in several liver diseases and may play a role in development of hepatic steatosis. To elucidate mechanisms underlying resistance of CB_1-receptor-deficient ($CB_1^{-/-}$) mice to diet-induced hepatic steatosis, we measured hepatic lipogenic fluxes in $CB_1^{-/-}$ mice and wild type (WT) littermates fed 1) a low-fat control diet, 2) a high-saturated fat diet (HF) which induces lipogenesis and steatosis, or 3) a HF diet in which part of the saturated fat was replaced by fish oil (HF/FO) that inhibits hepatic lipogenesis. Despite resistance of $CB_1^{-/-}$ mice to HF diet-induced hepatic steatosis, we did not observe any suppression of *de novo* lipogenesis as assessed by incorporation of ^{13}C-acetate into hepatic fatty acids in $CB_1^{-/-}$ mice as compared with that in their WT littermates. In addition, we could confirm that VLDL-triglyceride production by the liver is not affected by ablation of the CB_1-receptor. Since hepatic pools of C16:0 and C18:1 as well as hepatic C18:2 were decreased in HF diet-fed $CB_1^{-/-}$ *versus* WT mice, resistance of $CB_1^{-/-}$ mice to hepatic steatosis must be caused by decreased fatty acid delivery to the liver and/or increased fatty acid oxidation. Results of indirect calorimetry, showing significantly decreased respiratory quotients in $CB_1^{-/-}$ mice fed either chow or HF diet as compared with that in their respective controls, are consistent with increased fatty acid oxidation.

Conclusions: Resistance of $CB_1^{-/-}$ mice to a high-fat diet-induced hepatic steatosis cannot be attributed to suppressed hepatic lipogenesis nor to increased VLDL-production. A decrease in fatty acid uptake and/or an increase in fatty acid oxidation by the liver in $CB_1^{-/-}$ mice more likely attribute to resistance to hepatic steatosis in $CB_1^{-/-}$ mice.

Introduction

The metabolic syndrome comprises a cluster of risk factors (obesity, glucose intolerance, dyslipidemia and hypertension) for cardiovascular diseases and type 2 diabetes mellitus.[1] Non-alcoholic fatty liver disease (NAFLD), which predisposes to hepatic fibrosis and cirrhosis, is considered to represent the hepatic feature of the metabolic syndrome.[2] The endocannabinoid system, consisting of cannabinoid receptors (CB_1 and CB_2-receptor) and their endogenous ligands called endocannabinoids, has been recognized as a potential target for treatment and prevention of aspects of the metabolic syndrome. Anti-obesity effects of CB_1-receptor antagonists are established in both animals and in humans.[3,4] Moreover, in rodents and in humans, pharmacological blockade of the CB_1-receptor also favourably affects other risk factors of the metabolic syndrome in part independent of the concomitant weight loss.[4-6] Targeted deletion of the CB_1-receptor ($CB_1^{-/-}$) in mice results in an obesity-resistant phenotype[5-8] and $CB_1^{-/-}$ mice are resistant to high-fat diet-induced hepatic fat accumulation.[8] Treatment with a CB_1-antagonist reduces obesity and hepatic steatosis in Zucker rats.[9] The role of the endocannabinoid system in the pathophysiology of liver disease has only recently become a focus of attention. Hepatic expression of cannabinoid receptors is relatively low in healthy subjects, but appeared to be markedly up regulated in patients with several liver diseases.[10] CB_1-receptors are not only implicated in the development of hepatic steatosis but also in the progression to fibrosis.[10,11] Osei-Hyiaman and colleagues showed that liver-specific $CB_1^{-/-}$ mice were not resistant to diet-induced obesity but that these mice did show an improved hepatic lipid profile suggesting that endocannabinoid signalling is involved in the development of hepatic steatosis independent of its effects on body weight.[6] Since Osei-Hyiaman and colleagues showed induction of hepatic lipogenic genes and increased hepatic fatty acid synthesis upon pharmacological CB_1-activation (by administration of the CB_1-receptor agonist HU210), the endocannabinoid system has been implicated in control of *de novo* lipogenesis in the liver.[6,8] However, other studies show opposing results. Noguieras *et al.* reported decreased mRNA expression of *Scd-1* (*desaturation*) and observed no effect on lipogenic gene expression in adipose tissue and livers of rats treated with a CB_1-receptor antagonist compared with controls.[12] For a better understanding of the role of endocannabinoids in the development of hepatic steatosis, it is essential to directly evaluate the relevant metabolic fluxes under physiological conditions since mRNA levels do not always reflect the magnitude of metabolic processes.[1] Therefore, we directly quantified *de novo* lipogenesis and chain elongation in $CB_1^{-/-}$ mice under various dietary

conditions. Effects of genetic disruption of CB_1-receptors might be different in mice on diets that differ in fatty acid composition since it has been suggested that feeding high-fat diets modifies endocannabinoid system activity in peripheral tissues.[13,14] It should be realized, however, that suppression of lipogenesis is not the only mechanism by which CB_1-blockade can prevent hepatic steatosis. For instance, reduced hepatic uptake of circulating fatty acids might also contribute as well as increased fatty acid oxidation and/or stimulation of VLDL-triglyceride production.[2]

To elucidate mechanisms underlying resistance of $CB_1^{-/-}$ mice to diet-induced hepatic steatosis, we measured hepatic lipogenic fluxes (by determination of incorporation of ^{13}C-acetate into fatty acids followed by mass isotopomer distribution analysis) in $CB_1^{-/-}$ mice and wild type (WT) littermates fed either 1) chow, 2) a high-saturated fat diet (HF) known to induce steatosis and lipogenesis, or 3) a HF diet in which part of the saturated fat was replaced by fish oil (HF/FO) as a lipogenesis-suppressing diet.[15,16] Furthermore, we determined expression of genes involved in hepatic fatty acid uptake, transport and hepatic fatty acid oxidation and we quantified VLDL-triglyceride production *in vivo* to determine the contribution of these mechanisms to the reported resistance of $CB_1^{-/-}$ mice to diet-induced steatosis. Finally, indirect calorimetry was performed to determine potential involvement of changes in fatty acid oxidation.

Experimental procedures

Animals

$CB_1^{-/-}$ on a C57BL/6J background and their WT littermates were bred in our facility. Initial breeding pairs were kindly provided by Prof. Dr. A. Zimmer, Laboratory of Molecular Neurobiology, University of Bonn, Germany. Mice were individually housed in a light-controlled (lights on 4 AM- 4 PM) and temperature-controlled (21^{o}C) facility. Mice were allowed tap water and food *ad libitum*. Experimental protocols were approved by the local Experimental Ethical Committee for Animal Experiments.

Experimental diets

All experimental diets were obtained from Abdiets BV, Woerden, The Netherlands. Mice received either standard laboratory chow (RMH-B), a high-fat diet (HF) containing 36 weight % fat consisting of bovine fat (diet number 4031.45), or a high-fat diet containing 36 weight % fat consisting for 58% of bovine fat and for 42% of fish oil (diet number 4031.54). Composition of these experimental diets has been published before

(Supplemental Table 2).[15] The HF/FO diet was replaced every two days to prevent oxidation of fatty acid species.

Methods

Four-week-old $CB_1^{-/-}$ mice and WT littermates were divided into three groups by matching for body weight. For six weeks, each group was fed one of the three experimental diets. Intake and body weight were registered twice a week.

Mice were sacrificed under inhalation-anaesthesia by cardiac puncture, followed by cervical dislocation. Livers were removed, weighed, two small parts were kept apart for histology (frozen in isopenthane), the remainder of the liver was freeze clamped and stored at -80°C. Separate experiments were conducted for determination of lipogenic fluxes and quantification of VLDL-TG production rates *in vivo*.

Hepatic and plasma lipid analyses

Before further analysis, freeze-clamped frozen livers were crushed on liquid nitrogen and stored at -80°C. For hepatic lipid extraction, frozen crushed livers were homogenized in ice-cold PBS. Hepatic lipids were extracted according to Bligh and Dyer[17] and hepatic triglycerides and total cholesterol were measured using commercially available kits from Roche (Mannheim, Germany) and free cholesterol was measured using a commercially available kit from Wako Chemicals (Neuss, Germany). Phospholipid content of the liver was determined according to Böttcher *et al*.[18] Protein concentrations in livers were determined according to Lowry *et al*. using bovine serum albumin as standard (Pierce).

Liver histology

Liver morphology was visualized by standard Hematoxilin Eosin (HE)-staining. Liver histology was further examined on frozen liver sections after standard Oil-Red-O staining for neutral lipids.

Hepatic gene expression

Total RNA was isolated from frozen crushed liver using the TRI-reagent method (Sigma). Using random primers, RNA was converted to cDNA with M-MuLV Reverse Transcriptase (Sigma) according to the manufacturer's protocol. For realtime-PCR, cDNA was amplified using the appropriate primers and probes. The sequences of the primers and probes for *Srebp-1c* (*Srebf1*), *Fas* (*Fasn*), *Acc1* (*Acaca*), *Acc2* (*Acacb*), *Scd1*, *Cd36*, *Lcad* (*Acad1*), *Hmgcs2*, *Aox* (*Acox1*) and *Elovl6* have been published (http://www.

labpediatricsRUG.nl Realtime Primers Datalist Pediatrics UMCG). For other primers and probes used in this study, sequences are listed in Supplemental Table 1. Relative gene expression levels were normalized to *cyclophilin G* expression.

Determination of hepatic lipogenic fluxes *in vivo*

During the fifth week of the dietary manipulation, they were equipped with a permanent heart catheter *via* the right jugular vein, as described previously.[19] Mice recovered their pre-operative body weights after 3-6 days following surgery, after which the actual experiment took place (week 6). They were kept on the experimental diets until the actual experiment. A baseline blood sample was taken from the tail of the mise after a three hour fasting period, before infusion of [1-^{13}C] acetate was started (0.18 mmol/hr). After an infusion period of 6 hours, mice were anaesthetized with isoflurane, followed by cervical dislocation and removal of the liver. The liver was freeze clamped and stored at -80^{o}C until further analyses.

Liver homogenates were prepared in PBS and C17:0 was added as internal standard. Lipids were hydrolyzed in HCl/acetonitrile (1:22 v/v) for 45 minutes at 100 °C. Fatty acids were extracted in hexane and derivatized for 15 minutes at room temperature using α-Br-2,3,4,5,6-pentafluorotoluene (PFB) /acetonitrile /triethanolamine (1:6:2 v/v). Derivatization was stopped by adding HCl and fatty acid-PFB derivatives were extracted in hexane. The fatty acid-PFB isotopomer patterns were analyzed using a Agilent 5975 series GC/MSD (Agilent Technologies, Santa Clara, CA). Gas chromatography was performed using a ZB-1 column (Phenomenex, Torrance, CA). Mass spectrometry analysis was performed by electron capture negative ionization using methane as moderating gas.

To determine *de novo* fatty acid synthesis, mass isotopomer distribution analysis (MIDA) was applied. The theoretical background of MIDA is described in detail elsewhere.[20] Briefly, the normalized mass isotopomer distributions measured by GC-MS (m_0-m_x) were corrected for the fractional distribution due to natural abundance of ^{13}C by multiple linear regression as described by Lee *et al*.[21] to obtain the excess fractional distribution of mass isotopomers (M_0-M_x) due to incorporation of the infused labelled compound *i.e.* [1-^{13}C]-acetate. This distribution was used in MIDA algorithms to calculate isotope incorporation and dilution according to Hellerstein *et al*.[20] Incorporation of [1-^{13}C]-acetate into palmitate was assumed to solely result from *de novo* lipogenesis *via* the malonyl-CoA/FAS pathway. Measured M_1 and M_3 isotopomers of palmitate were used to calculate the acetyl-CoA precursor pool enrichment ($p_{acetate}$)

and fractional palmitate synthesis ($f_{C16:0}$). Stearate is synthesized by chain elongation of *de novo* synthesized and/or pre-existing palmitate. The M_1 mass isotopomer of stearate represents the sum of these two processes, while the M_3 mass isotopomer solely results from chain elongation of labelled palmitate. $p_{acetate}$ and M_1 and M_3 of palmitic, stearic and oleic acid were used to calculate fractional synthesis rates and to asses the contribution of *de novo* lipogenesis and chain elongation using the formulas as described previously by Oosterveer *et al.*[15]

Fatty acid profiles

Hepatic fatty acid composition was analyzed by gas chromatography after transmethylation using C17:0 as internal standard.[22]

VLDL-triglyceride production and VLDL-composition

In a separate experiment, in WT and $CB_1^{-/-}$ mice, after being fed one of the three experimental diets for 6 weeks and after a fasting period of 3 hours, plasma lipolysis was blocked by intraperitoneal injection of Poloxamer 407 (0,1 mg/g body weight as a saline solution).[23] Blood samples were obtained from the retro-orbital plexus under inhalation anaesthesia, before, 30 minutes, 120 minutes and 240 minutes after Poloxamer 407 injection. To assess VLDL-TG-production rate, triglycerides were determined in these blood samples, using a commercially available kit (Roche, Mannheim, Germany). VLDL-production rate was calculated from the slope of the triglyceride concentration *versus* time curve. Immediately after the last blood draw, mice were sacrificed by terminal cardiac puncture under inhalation anaesthesia followed by cervical dislocation and followed by removal of liver and adipose tissue. From the cardiac plasma sample, the plasma VLDL-fraction was isolated by ultracentrifugation.

VLDL-isolation and analyses

Plasma VLDL/intermediate-density lipoprotein (density <1.006 g/ml) was isolated by density gradient ultracentrifugation. Hereto, 300 µl plasma was adjusted to 700 µl with a NaCl-solution of density 1.006 g/ml and centrifuged at 108,000 rpm for 125 minutes in a Optima LX tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA). The top layer containing the VLDL-fraction was isolated by tube slicing, and the volume was recorded by weight. VLDL-TG and cholesterol content of the nascent particles were determined as described.[23] Phospholipid content was determined using a commercial

kit (Wako Chemicals). VLDL-particle diameter was estimated according to Fraser *et al*.[24] VLDL-particle volume was subsequently derived from its diameter.

Indirect calorimetry

Mice were placed in an open circuit indirect calorimetry system for 24 hours with access to water and food. Gas exchange measurements were performed in an eight-channel open flow system. Flow rates were measured and controlled with a mass flow controller. O_2 and CO_2 concentrations of dried inlet and outlet air from each chamber were measured every 10 minutes with a paramagnetic O_2 analyzer and an infrared CO_2 gas analyzer. Data were collected from each metabolic cage separately. The RQ (respiratory quotient) was defined as CO_2 production (L)/ O_2 consumption (L).

Statistics

All values in the figures and in the tables represent means ± standard errors of the means for the number of animals indicated in the figure and table legends. To evaluate effects of diet, genotype, and their interactions data were statistically analyzed using a general linear model with Bonferroni *post hoc* analyses. Treatment effects were further analyzed by Student's *t*-test. In case of non-parametric distribution, Mann-Whitney *U*-test was assed for statistical analysis. Statistical significance of differences was accepted at a p-value of less than 0.05. Analyses were performed using SPSS 16.0 for Windows software (SPSS, Chicago, IL).

Results

$CB_1^{-/-}$ mice are resistant to high-fat diet-induced hepatic steatosis

Exposure of WT mice to the HF diet for 6 weeks resulted in significantly increased hepatic triglyceride (TG) contents as compared with that in mice fed either chow or HF/FO (Figure 1A). Hepatic total cholesterol and hepatic cholesterol ester contents were also elevated in WT mice fed HF diet as compared with that in WT mice fed chow or HF/FO diet (Table 1). There were no significant differences in liver weights between the WT mice fed either one of the three experimental diets. HF-fed $CB_1^{-/-}$ mice did show a decreased hepatic TG content and a significantly decreased liver weight as compared with that in HF-fed WT controls (Figure 1A and Table 1). Thus, as expected, $CB_1^{-/-}$ mice are resistant to HF diet induced hepatic steatosis. Although genetic ablation of the CB_1-receptor had a significant effect on liver TG content, hepatic morphology/histology were

still minimally affected as illustrated by Figure 1B. The absence of significant differences in plasma transaminase levels indicate that feeding the HF diet to WT mice at this age, despite the presence of hepatosteatosis, did not damage hepatocytes (Table 1).

No effects of CB_1-deficiency on hepatic lipogenesis

In WT mice, key genes of lipogenesis showed a small increase in hepatic expression, i.e., *Srebp-1c*, *Fas*, *Acc2* and *Scd1*, when fed a HF diet compared with chow: only differences in *Acc2* and *Scd1* were statistically significant (Figure 2). Expression of lipogenic genes was suppressed in HF/FO-fed mice, as expected.[16]

Based on the previously suggested involvement of endocannabinoids in regulation of hepatic lipogenesis,[6,8] we expected to find effects of genotype on hepatic expression of lipogenic genes and on fractional synthesis of fatty acids. However, compared with WT mice, we did not observe any specific difference in the expression of lipogenic genes in $CB_1^{-/-}$ mice fed either one of the three diets (Figure 2).

In agreement with gene expression data, the results of MIDA analysis revealed no significant differences in lipogenic pathways between $CB_1^{-/-}$ mice and WT mice fed either one of the three experimental diets. Figure 3 and Table 2 illustrate the effects of both diet and genotype on hepatic *de novo* lipogenesis. Calculated enrichments of the acetyl-CoA pools showed no dietary effects, however, values in $CB_1^{-/-}$ mice were higher in the HF-condition as well as in the HF/FO condition, suggesting a decreased turnover of the acetyl-CoA pool in the $CB_1^{-/-}$ mice. The fractional contribution of *de novo* lipogenesis to hepatic palmitic acid and to hepatic stearic acid were increased in WT mice fed HF diet as compared with that in WT mice fed chow whereas WT mice fed HF/FO showed a decrease in the fraction of *de novo* synthesized palmitate but no effect for stearic acid (Table 2). In parallel with our results on hepatic lipogenic gene expression, we did not observe any effect of genotype on fractional contribution to *de novo* lipogenesis of hepatic palmitic acid and hepatic stearic acid. Thus, hepatic lipogenesis was not suppressed in $CB_1^{-/-}$ mice.

In agreement with our results on expression of genes involved in elongation showing an induction of *Elovl6* in mice fed HF diet (Figure 2f), fractional contribution of chain elongation of existing palmitate to stearic acid, in particular in case of *de novo* synthesized stearic acid, was increased in WT mice fed HF diet (Table 2). Despite the observed significantly decreased expression of *Elovl6* in $CB_1^{-/-}$ mice fed HF diet (Figure 2f), we found no genotype effect in fractional contribution of chain elongation of existing palmitate to stearic acid (Table 2 and Figure 3).

Table 1. General characteristics (body weight, hepatic and plasma parameters) after 3 hours of fasting in cannabinoid-1 receptor deficient (CB1-/-) mice and in wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet.

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Body weight (g)	25,0 ± 0,6	22,6 ± 0,3	26,8 ± 1,1	23,4 ± 0,4	26,9 ± 0,8	23,2 ± 0,8
Liver weight (g)	1,13 ± 0,04	1,01 ± 0,07	1,06 ± 0,05	0,87 ± 0,05#	0,90 ± 0,05†	0,83 ± 0,05
Liver total cholesterol (nmol mg^{-1} liver)	4,82 ± 0,14	5,48 ± 0,47	6,77 ± 0,36*	6,61 ± 0,71	5,49 ± 0,46‡	5,77 ± 0,33
Liver free cholesterol (nmol mg^{-1} liver)	4,16 ± 0,09	4,45 ± 0,20	4,50 ± 0,24	4,71 ± 0,41	4,52 ± 0,25	5,04 ± 0,25
Liver cholesterolesters (nmol mg^{-1} liver)	0,66 ± 0,09	1,04 ± 0,30	2,27 ± 0,18*	1,90 ± 0,32	0,96 ± 0,27‡	0,73 ± 0,11
Liver phospholipids (nmol mg^{-1} liver)	28,1 ± 1,3	29,1 ± 1,6	25,0 ± 1,3	27,5 ± 2,3	30,8 ± 2,2‡	30,7 ± 1,6
C14:0 (µmol g^{-1} liver)	0,7 ± 0,1	0,6 ± 0,1	1,4 ± 0,1*	0,7 ± 0,1#	0,8 ± 0,3‡	0,3 ± 0,0
C16:0 (µmol g^{-1} liver)	39,7 ± 4,3	34,4 ± 3,2	51,5 ± 5,1	25,2 ± 1,9#	30,7 ± 5,2‡	22,3 ± 1,2
C16:1 (µmol g^{-1} liver)	4,4 ± 0,6	3,2 ± 0,5	7,6 ± 0,9	3,0 ± 0,4#	3,1 ± 1,7†‡	0,9 ± 0,1
C18:0 (µmol g^{-1} liver)	14,6 ± 0,3	14,8 ± 0,6	17,2 ± 2,2	13,6 ± 0,3	16,0 ± 1,1	14,3 ± 0,6
C18:1 (µmol g^{-1} liver)	32,3 ± 3,3	26,3 ± 2,9	90,0 ± 15,4	44,9 ± 3,4#	28,2 ± 6,9‡	17,4 ± 1,8
C18:2 (µmol g^{-1} liver)	35,2 ± 3,6	29,5 ± 3,6	12,2 ± 2,6*	6,3 ± 0,4#	9,0 ± 0,7†	8,2 ± 0,6
C18:3 (µmol g^{-1} liver)	4,5 ± 0,7	3,9 ± 0,6	1,1 ± 0,2*	0,6 ± 0,0#	1,2 ± 0,3†	0,7 ± 0,1
C20-22 (µmol g^{-1} liver)	28,5 ± 0,9	28,4 ± 1,2	32,2 ± 4,0	23,7 ± 0,6	32,3 ± 3,5	25,3 ± 1,4
ALAT (U l^{-1})	5,48 ± 0,17	5,43 ± 0,23	6,14 ± 0,25	6,63 ± 0,44	6,56 ± 0,74	5,91 ± 0,46
ASAT (U l^{-1})	9,10 ± 1,15	9,28 ± 1,27	11,53 ± 1,30	12,50 ± 1,41	12,31 ± 0,77	10,33 ± 1,00

Values are means +/- SEM; n = 6-9; * $p < 0.05$ CH *vs* HF, † $p < 0.05$ CH *vs* HF/FO, ‡ $p < 0.05$ HF *vs* HF/FO, # HF $CB_1^{-/-}$ *vs* HF WT

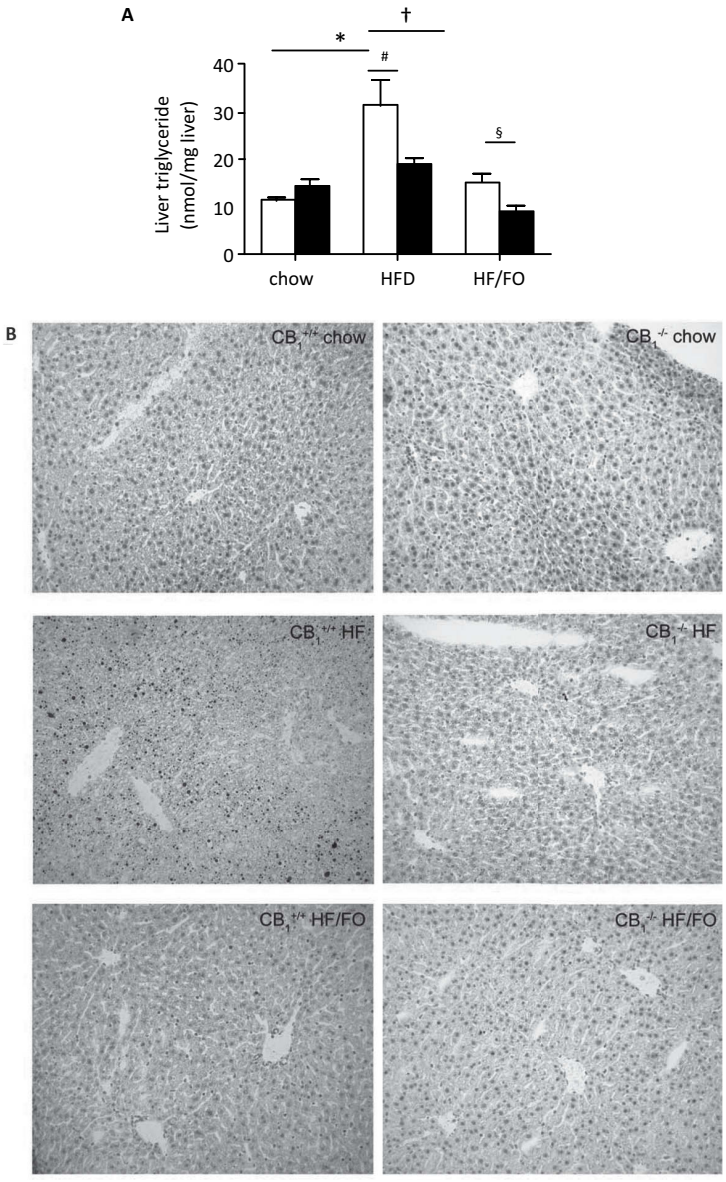

Figure 1 A) Hepatic triglyceride content after 3h of fasting in cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice and in wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet. Open bars, WT mice; closed bars, $CB_1^{-/-}$ mice. Values are means +/- SEM; n = 6-8; * $p < 0.05$ chow *versus* HF, ‡ $p < 0.05$ HF *versus* HF/FO, # $p < 0.05$ HF $CB_1^{-/-}$ *versus* HF WT, § $p < 0.05$ HF/FO $CB_1^{-/-}$ *versus* HF/FO WT. **B) Liver histology.** Oil-Red-O staining of frozen liver sections of WT mice and $CB_1^{-/-}$ mice fed chow, HF or HF/FO.

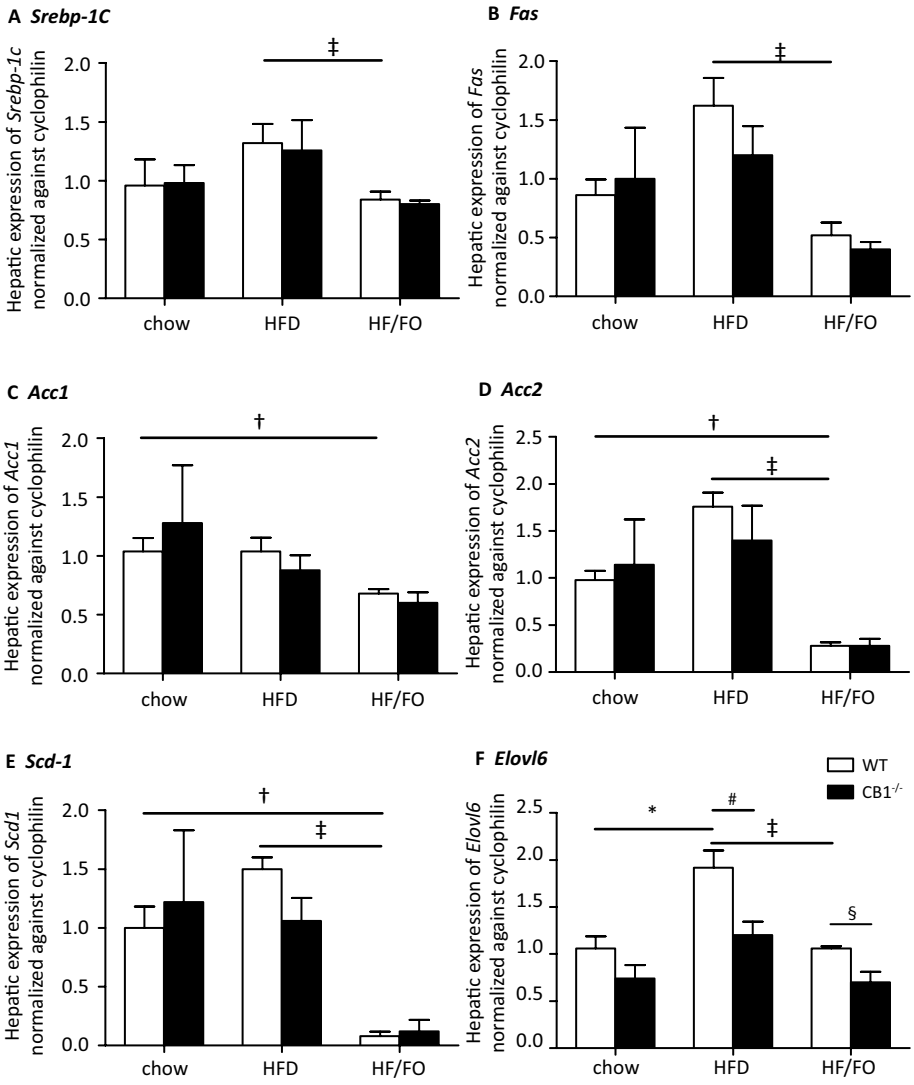

Figure 2. Hepatic expression of genes involved in lipogenesis and elongation. Relative hepatic mRNA expression of a) *Srebp-1c*, b) *Fas*, c) *Acc1*, d) *Acc2,* e) *Scd1*, and f) *Elovl6* in cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice (closed bars) and in wild type (WT) mice (open bars) fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet. Results are normalized to *cyclophilin G*. Values are means +/- SEM; n=5; * $p < 0.05$ chow *versus* HF, † $p < 0.05$ chow *versus* HF/FO, ‡ $p < 0.05$ HF *versus* HF/FO, # $p < 0.05$ HF $CB_1^{-/-}$ *versus* HF WT, § $p < 0.05$ HF/FO $CB_1^{-/-}$ *versus* HF/FO WT.

Table 2. Hepatic lipogenesis assessed by measuring labelled acetate incorporation in cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice and in wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
p-value (%)	12,9 ± 0,5	13,8 ± 0,3	14,2 ± 0,7*	17,1 ± 0,5#	11,0 ± 0,6‡	13,5 ± 0,6$
Fractional palmitate synthesis (%)	1,7 ± 0,3	2,3 ± 0,2	3,9 ± 0,6*	5,4 ± 0,9	1,5 ± 0,3†‡	1,1 ± 0,2
Fractional stearate synthesis (%)						
from de novo synthesized palmitate	0,4 ± 0,3	0,8 ± 0,2	1,3 ± 0,3	2,0 ± 0,6	0,3 ± 0,1	0,2 ± 0,1
from pre-existing palmitate	7,3 ± 1,5	11,2 ± 0,6	10,7 ± 1,1	11,9 ± 0,9	5,1 ± 0,5 †‡	3,2 ± 0,6
Fractional oleate synthesis (%)						
from de novo synthesized palmitate	0,1 ± 0,0	0,2 ± 0,1	0,1 ± 0,0	0,3 ± 0,1	0,1 ± 0,1	0,1 ± 0,0
from pre-existing palmitate	0,3 ± 0,2	1,2 ± 0,5	1,1 ± 0,4	1,8 ± 0,5	ND	ND

Values are means +/- SEM; n=5-7; * $p < 0.05$ CH *vs* HF, † $p < 0.05$ CH *vs* HF/FO, ‡ $p < 0.05$ HF *vs* HF/FO, # HF $CB_1^{-/-}$ *vs* HF WT, $ HF/FO $CB_1^{-/-}$ *vs* HF/FO WT

Table 3. Hepatic expression of genes involved in fatty acid uptake by the liver and in fatty acid oxidation in cannabinoid-1 receptor deficiënt ($CB_1^{-/-}$) mice and in wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet

	chow		**HF**		**HF/FO**	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Hepatic clearance of fatty acids						
Cd36	1,26 ± 0,23	1,27 ± 0,14	1,14 ± 0,24	0,80 ± 0,09	1,19 ± 0,21	1,43 ± 0,24
Fatp5 (Slc27a5)	1,69 ± 0,21	1,60 ± 0,12	1,42 ± 0,14	1,33 ± 0,14	1,13 ± 0,09	1,46 ± 0,07
L-fabp (Fabp1	1,64 ± 0,13	1,43 ± 0,26	1,79 ± 0,10	1,51 ± 0,20	1,24 ± 0,09	1,44 ± 0,09
Fatty acid oxidation						
Cpt1a	0,93 ± 0,07	1,10 ± 0,18	1,28 ± 0,05	1,24 ± 0,12	0,99 ± 0,07	1,02 ± 0,09
Lcad (Acadl)	1,02 ± 0,08	1,12 ± 0,17	1,01 ± 0,04	0,85 ± 0,11	0,98 ± 0,03	1,05 ± 0,05
Hmgcs2	1,33 ± 0,08	1,34 ± 0,26	1,21 ± 0,09	1,07 ± 0,12	1,39 ± 0,06 *	1,60 ± 0,07
Aox (Acox1)	1,32 ± 0,15	1,18 ± 0,29	1,33 ± 0,14	1,15 ± 0,14	1,40 ± 0,17	1,37 ± 0,14

Results are normalized to cyclophilin G. Values are means +/- SEM; n = 5-7; * $p < 0.05$ HF *vs* HF/FO

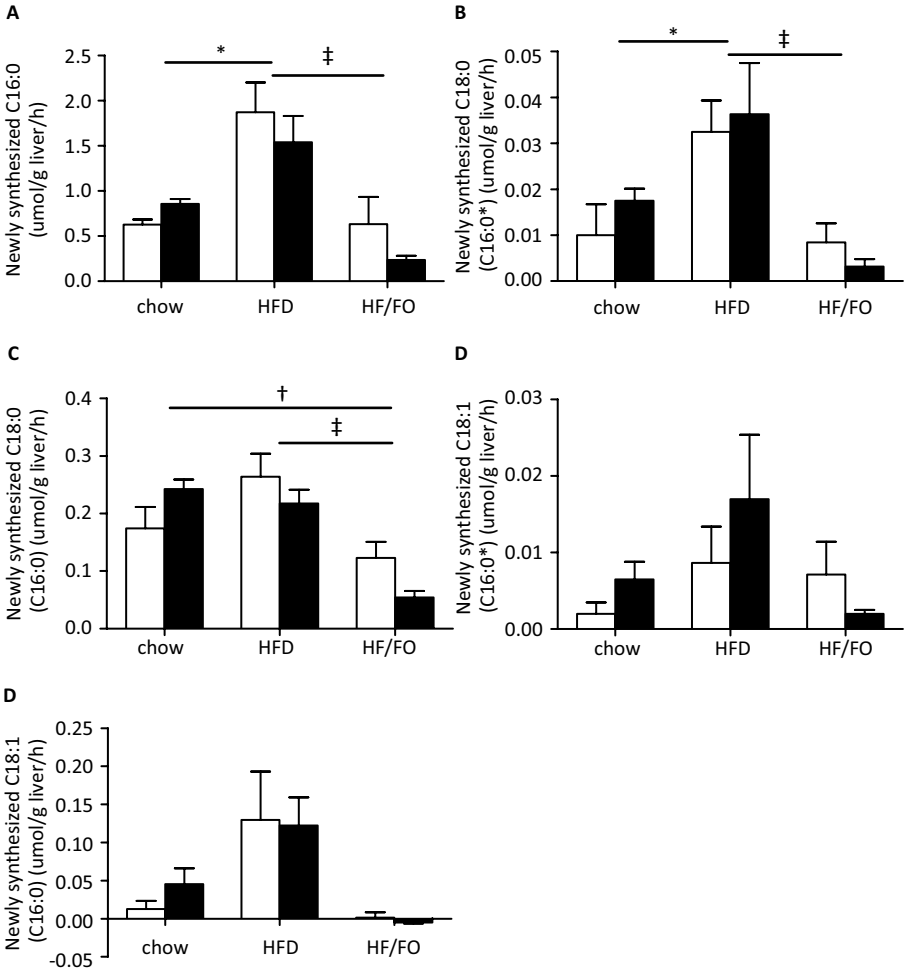

Figure 3. Hepatic lipogenesis in cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice (closed bars) and in wild type (WT) mice (open bars) fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet, assessed by incorporation of labelled acetate. Absolute synthesis rates are expressed as micrograms per gram liver per hour. Values are means +/- SEM; n = 6-7; * $p < 0.05$ chow *versus* HF, † $p < 0.05$ chow *versus* HF/FO, ‡ $p < 0.05$ HF *versus* HF/FO. A) Absolute palmitate synthesis from de novo lipogenesis, B) Absolute stearate (C18:0) synthesis from elongation of *de novo* synthesized (C16:0*) palmitate, C) Absolute stearate (C18:0) synthesis from elongation of pre-existing (C16:0) palmitate, D) Absolute oleate (C18:1) synthesis from elongation of *de novo* synthesized (C16:0*) palmitate, E) Absolute oleate (C18:1) synthesis from elongation of pre-existing (C16:0) palmitate.

Reduced uptake of circulating fatty acids or increased fatty acid oxidation indicated by hepatic fatty acid profiles of $CB_1^{-/-}$ mice

Hepatic lipid accumulation may also be prevented in $CB_1^{-/-}$ mice by a decreased uptake of circulating fatty acids into the liver and/or an increased hepatic fatty acid oxidation. In parallel with reduced TG content in livers of $CB_1^{-/-}$ mice fed HF diet, fatty acid profiles of livers of $CB_1^{-/-}$ mice fed HF diet revealed reduced pools of the three major fatty acids comprising the hepatic TG pool; C16:0, C18:1 and C18:2 (Table 1). Since C18:2 is an essential fatty acid, reduction in hepatic pool of C18:2 suggests that reduced TG content in livers of $CB_1^{-/-}$ mice fed HF diet as compared with that in WT littermates on the same diet is either due to decreased uptake of fatty acids or increased fatty acid oxidation. However, we did not find any genotype effect on expression of genes involved in hepatic uptake of fatty acids from the circulation nor on expression of genes involved in fatty acid oxidation to support this suggestion (Table 3).

Decreased respiratory quotients in $CB_1^{-/-}$ fed chow or HF diet are in support of a contribution of increased fatty acid oxidation to resistance to hepatic steatosis in $CB_1^{-/-}$ mice

Figure 4 shows respiratory quotients (RQ) in the dark and in the light period. As expected, in both the dark and light period RQ were significantly higher in mice fed chow as compared with that in mice fed either HF diet or HF/FO diet. Interestingly, $CB_1^{-/-}$ mice fed chow and HF diet exhibited significantly decreased RQ in the light period as compared with WT controls, indicating an increase in the ratio of fatty acid oxidation to carbohydrate oxidation.

VLDL-TG production is not affected by CB_1-receptor-deficienc.

VLDL-TG production rates were similar in WT mice fed chow and WT mice fed HF diet. VLDL-TG production rate was significantly decreased in mice fed HF/FO diet as compared with that in mice fed the HF diet, as expected. CB_1-deficiency did not affect VLDL-TG production rate (Table 4). CB_1-deficiency also did not affect VLDL-composition as shown in Table 4. Particle size was increased in WT mice fed HF diet as compared with that in WT mice fed HF/FO diet.

Table 4. Composition and size of nascent VLDL-particles and VLDL-TG production of cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice and in wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Triglycerides (%)	84,8 ± 1,2	84,4 ± 0,5	88,5 ± 0,9*	87,6 ± 0,9	83,1 ± 1,1‡	82,2 ± 0,9
Phospholipids (%)	10,3 ± 0,8	10,4 ± 0,4	8,4 ± 0,6	8,7 ± 0,7	11,5 ± 0,8†‡	13,1 ± 0,8
Cholesterol (%)	4,9 ± 0,5	5,2 ± 0,4	3,1 ± 0,4*	3,7 ± 0,4	5,4 ± 0,7‡	4,8 ± 0,2
TG:PL ratio	8,7 ± 1,1	8,2 ± 0,3	11,0 ± 1,2	10,7 ± 1,3	7,5 ± 0,6‡	6,4 ± 0,5
Particle size (nm)	126,1 ± 14,0	119,6 ± 4,4	154,9 ± 14,6	151,5 ± 17,1	110,6 ± 8,2‡	97,7 ± 6,2
VLDL-TG prod. ($\mu mol\ kg^{-1}h^{-1}$)	274 ± 16	234 ± 18	295 ± 13	290 ± 20	229 ± 20‡	189 ± 25

VLDL-TG production (VLDL-TG prod.) rate in $\mu mol\ kg^{-1}\ h^{-1}$ calculated from plasma TG versus time curves normalized for body weight. Values are means +/- SEM; n = 5-7; * $p < 0.05$ CH vs HF, † $p < 0.05$ CH *vs* HF/FO, ‡ $p < 0.05$ HF *vs* HF/FO

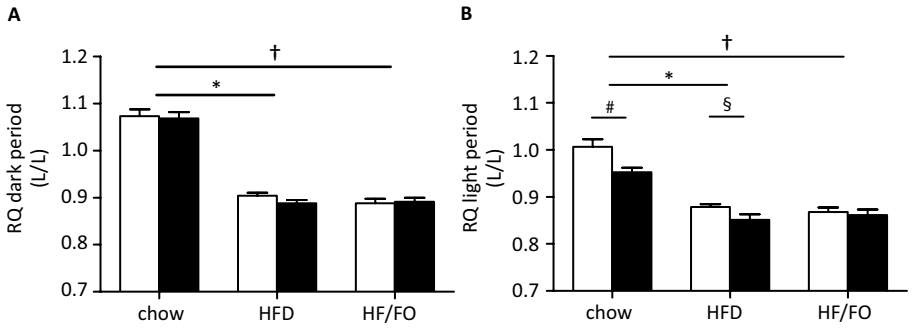

Figure 4. Respiratory quotients (RQ) in A) the dark phase or B) the light phase in cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice (closed bars) and in wild type (WT) mice (open bars) fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet. Values are means +/- SEM; n = 6-7; * $p < 0.05$ chow *versus* HF, † $p < 0.05$ chow *versus* HF/FO, # $p < 0.05$ chow $CB_1^{-/-}$ *versus* chow WT, $ $p < 0.05$ HF $CB_1^{-/-}$ *versus* HF WT.

Discussion

The presented data confirm earlier findings from our laboratory that lipogenesis is increased in mice fed a HF diet and that it is suppressed by feeding a HF/FO diet.[15,16] WT mice fed a HF diet showed hepatic lipid accumulation, whereas $CB_1^{-/-}$ mice were resistant to this diet-induced hepatic steatosis, as reported by Osei-Hyiaman *et al.*[6,8] However, our data unequivocally demonstrate that this resistance to development of hepatic steatosis cannot be attributed to a failure to induce hepatic lipogenesis as proposed by Osei-Hyiaman *et al.* Using an adapted MIDA procedure, we showed that both lipogenesis as well as chain elongation were unaffected by CB_1-receptor deficiency.[15] Likewise, an enhanced production of TG-containing VLDL particles was shown not to be the cause of the resistance to diet-induced hepatic steatosis in the absence of CB_1-signalling. Instead, our results indicate that altered fat distribution may contribute to absence of the diet-induced phenotype in $CB_1^{-/-}$ mice.

The anticipated significant increase in hepatic TG content in WT mice fed HF diet (as compared with that in WT mice fed chow) and the blunting of this HF diet effect in $CB_1^{-/-}$ mice correspond with findings of others showing that rodents treated with a CB_1-receptor antagonist as well as $CB_1^{-/-}$ mice are resistant to HF diet-induced hepatic steatosis.[6,8,9] Pair-feeding studies have shown that beneficial effects of CB_1-blockade

on hepatic steatosis are independent of food intake and body weight.[9] Furthermore, Osei-Hyiaman and colleagues demonstrated that liver-specific $CB_1^{-/-}$ mice had reduced hepatic steatosis as compared with WT mice, but this appeared to be independent of the persistence of HF-diet induced obesity in these liver-specific $CB_1^{-/-}$ mice. This suggests that the endocannabinoid tone affecting resistance/proneness to diet-induced hepatic steatosis is localized in the liver and is presumably independent of changes in body weight.[6] The implication of CB_1-receptors in development of hepatic steatosis and the established contribution of CB_1-receptors to the progression of hepatic steatosis to hepatosteatitis, fibrosis and cirrhosis, makes the ECS an interesting target for the development of drugs aimed to prevent or treat NAFLD. Osei-Hyiaman and colleagues demonstrated that hepatocytes express CB_1-receptors, activation of which by a synthetic ligand induced hepatic lipogenic gene expression and increased hepatic *de novo* fatty acid synthesis *in vivo*.[8] These authors suggested accordingly that endocannabinoid activation of hepatic CB_1-receptors likely contributes to HF diet-induced steatosis *via* stimulation of hepatic lipogenesis.[6,8] However, others have found no involvement of CB_1-signalling in the control of hepatic lipogenesis.[12] To resolve the controversy regarding the role of CB_1-receptors in hepatic lipogenesis, we re-evaluated findings of Osei-Hyiaman and colleagues with the additional differentiation between *de novo* lipogenesis and elongation of pre-existing fatty acids. This differentiation is not possible using the methodology of Osei-Hyiaman *et al.*[6,8] The relative contribution of circulating fatty acids accounts for ~60% to hepatic triglyceride content in NAFLD patients, while that of dietary and *de novo* synthesized fatty acids is ~10% and ~30%, respectively. It is therefore of great interest to assess whether induction of lipogenesis and the role of the endocannabinoid system herein represents increased *de novo* lipogenesis, increased chain elongation, or both.[25]

In agreement with our earlier observations and results of others,[15,16] we found an overall induction in hepatic lipogenic gene expression (*Srebp-1c*, *Fas*, *Acc2*) paralleled by an increased *de novo* hepatic lipogenesis in HF-fed WT mice as compared with that in WT mice fed chow. A suppression of these parameters was observed in WT mice fed HF/FO diet as compared to WT mice fed chow. We did not observe, however, an effect of genotype on any of these parameters. In part, these results correspond to findings of Nogueiras and colleagues, who reported no effect of CB_1-blockade on hepatic lipid metabolism in diet-induced obese rats.[12] Consistently, we did not find a lower hepatic *de novo* lipogenesis in $CB_1^{-/-}$ mice fed HF diet compared with WT littermates receiving the same diet and there was no genotype effect on fractional contribution of chain

elongation of pre-existing palmitate to hepatic stearic acid and oleate synthesis. HF/FO-fed $CB_1^{-/-}$ mice showed a significant decrease in hepatic triglyceride content, similar to what was observed in WT mice fed HF/FO diet. Taken together, this argues against the idea that inhibited lipogenesis plays a major role in the resistance of $CB_1^{-/-}$ mice against hepatic steatosis.

The discrepancy between our results and findings reported by Osei-Hyiaman and colleagues on the role of CB_1-receptors in *de novo* hepatic lipogenesis seems to be related to methodological differences.[6,8] Osei-Hyiaman *et al.* determined tritiated water incorporation into the total hepatic lipid pool (including cholesterol) following an overnight fast. In the current study, we quantified *de novo* lipogenic and elongation fluxes upon incorporation of the labelled C2 fatty acid precursor acetate into particular hepatic fatty acids (*i.e.*, C16:0, C18:0, C18:1), which provides a more specific measure for lipogenic fluxes. Our results reflecting postprandial conditions showed no genotype effect on fatty acid synthesis, whereas Osei-Hyiaman *et al.* did find decreased total lipid synthesis in overnight-fasted $CB_1^{-/-}$ mice fed HF diet compared with WT littermates receiving the same diet. Altogether, these data suggest a temporal effect, possibly as a consequence of a differential regulation of lipogenesis under postprandial and fasted conditions. Lipogenesis is regulated by many different factors (*i.e.*, SREBP-1c, ChREBP, LXR, FXR, PPARs) and, apparently the mechanisms regulating hepatic *de novo* lipogenesis interact with CB_1-signalling under specific metabolic conditions (*i.e.*, after an overnight fast). Finally, an effect of differences in duration of dietary intervention in our study (*i.e.*, 6 weeks) and the one performed by Osei-Hyiaman and colleagues (*i.e.*, 3 weeks) cannot be ruled out, although this is likely less explanatory than the previous arguments.

LC-PUFA (long chain polyunsaturated fatty acids) diets have been reported to affect concentrations of endocannabinoid ligands. Therefore, it has been suggested that LC-PUFA-rich diets might have similar effects as (pharmacological) CB_1-antagonism.[14,26] In our experiments, HF/FO diet and CB_1-inactivation indeed exerted similar effects on hepatic lipid deposition, but this appeared to be mediated *via* distinct mechanisms. For example, lipogenesis was found to be suppressed by feeding HF/FO diet, but not by CB_1-ablation. In fact, data presented in Table 2 and Figure 3 seem to indicate that the effect of feeding a HF/FO diet on hepatic *de novo* lipogenesis is even stronger in the absence of CB_1-signalling, indeed suggesting mechanisms acting parallel to signalling *via* CB_1. Thus, these findings indicate to our opinion that suppression of hepatic lipogenesis by dietary PUFA does not involve modifying endocannabinoid tone as was suggested

earlier. Moreover, endocannabinoid tone in itself possibly reduces suppression of hepatic lipogenesis by LC-PUFA.

The fatty acid profile we observed showed reduced hepatic levels of three predominant TG-derived fatty acids (16:0, 18:1 and 18:2) in livers of $CB_1^{-/-}$ mice as compared with that in WT littermates receiving the same diet. Since C18:2 is an essential fatty acid, either decreased uptake from the circulation or increased oxidation has to contribute to the smaller hepatic C18:2 pool in $CB_1^{-/-}$ mice. The mechanism underlying this putative decrease in uptake is expected to be related to the liver itself since Osei-Hyiaman *et al.* showed that HF-fed liver-specific CB_1-deficient mice are resistant to hepatic steatosis despite their obese phenotype. We speculated that down-regulation of transporters of fatty acids in the liver could be involved in this, but we did not observe differences between $CB_1^{-/-}$ mice and WT littermates in hepatic expression of *Cd36*, *Fatp5* and *L-Fabp*, all involved in hepatic fatty acid uptake and transport (Table 3). We cannot exclude that CB_1-receptor-deficiency is associated with post-transcriptional regulation of the expression of these proteins.

Both VLDL-production rate and VLDL-composition were not altered by CB_1-deficiency. Therefore a change in VLDL-production cannot explain resistance of $CB_1^{-/-}$ mice to the development of hepatic steatosis. This corresponds with data of Ruby *et al.* who quantified VLDL-production rate in mice treated with a CB_1-receptor antagonist.[27] Another possible mechanism of resistance to hepatic steatosis is through increased fatty acid oxidation. Increased fatty acid oxidation has indeed been proposed to contribute to the protection against lipid accumulation in $CB_1^{-/-}$ mice. Herling and colleagues demonstrated increased energy expenditure and increased calculated fat oxidation upon treatment with a CB_1-receptor antagonist in female rats receiving candy diet compared with pair-fed controls.[28] Osei-Hyiaman and colleagues demonstrated a decreased CPT-1 (carnitine palmitoyltransferase-1) activity in mice treated with a CB_1-receptor agonist and this decrease was absent in both global and liver-specific $CB_1^{-/-}$ mice.[6] Although we did not observe an induction of fatty acid oxidation genes in livers of $CB_1^{-/-}$ mice compared with WT mice, on whole body level indirect calorimetry did reveal a significant decrease in respiratory quotient in the light phase in $CB_1^{-/-}$ mice fed HF diet as well as in $CB_1^{-/-}$ mice fed chow as compared with that in their respective littermates. This finding suggest increased fatty acid oxidation in $CB_1^{-/-}$ mice fed HF diet or chow, however, this is measured on whole body level. Therefore, no conclusions on liver-specific alterations in fatty acid oxidation can be drawn from such analysis.

In conclusion, resistance of $CB_1^{-/-}$ mice to diet-induced hepatic steatosis can not be attributed to suppression of hepatic lipogenesis or to an increase in VLDL-TG production. Our data suggest that reduced hepatic entry of fatty acids may contribute to resistance to develop hepatic steatosis in HF-fed $CB_1^{-/-}$ mice; however, increased fatty acid oxidation during certain phases of the day may be a contributing factor. Our data indicate that future research on the relation between hepatic steatosis and endocannabinoid signalling should focus on the effect of endocannabinoid signalling on hepatic uptake of circulating fatty acids and fatty acid oxidation.

Acknowledgement

The authors thank Pieter de Boer, Gerard Overkamp, Theo S. Boer, Ingrid A. Martini and Juul F.W. Baller for excellent technical assistance.

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CHAPTER 5

Resistance to diet-induced obesity in CB_1-receptor deficient mice is not due to disturbances in lipogenesis or lipoprotein lipase action in adipose tissue

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Abstract

Overactivity and/or dysregulation of the endocannabinoid system (ECS) contribute to the development of obesity. *In vitro* studies indicate a regulatory role for the cannabinoid receptor 1 (CB_1) in adipocyte function and CB_1-receptor deficient ($CB_1^{-/-}$) mice are resistant to high fat diet-induced obesity. Whether this phenotype of $CB_1^{-/-}$ mice is related to altered fat metabolism in adipose tissue is unknown. In the face of expected changes in energy balance we evaluated adipose tissue differentiation/proliferation markers and quantified lipogenic and lipolytic activities in fat tissues of $CB_1^{-/-}$ and $CB_1^{+/+}$ mice that were fed a high-fat (HF) or a high-fat/fish oil (HF/FO) diet as compared to animals receiving a normal low-fat chow diet. The comparison between the HF diet and the HF/FO diet allowed us to investigate the influence of dietary fat quality on adipose tissue biology in relation to CB_1-functioning. The obesity-resistant phenotype of the $CB_1^{-/-}$ mice was characterized by a lower adiposity index and reduced adipocyte size in HF and HF/FO-fed $CB_1^{-/-}$ as compared to $CB_1^{+/+}$ mice. This was not associated with changes in expression of adipocyte differentiation/proliferation markers. Lipogenic gene expression levels were comparable in $CB_1^{-/-}$ and $CB_1^{+/+}$ mice fed either of the three diets and fatty acid synthesis rates were unaffected. Whole-body and adipose-derived lipoprotein lipase (LPL) activities were also not altered in $CB_1^{-/-}$ mice. Energy expenditure, however, was slightly but significantly increased in $CB_1^{-/-}$ mice fed the HF and HF/FO diets.

In conclusion, these data indicate that protection against diet-induced obesity in CB_1-deficient mice is not related to changes in adipocyte function *per se*, but may rather result from an increased energy loss by oxidative and non-oxidative pathways.

Introduction

The endocannabinoid system (ECS) comprises the endogenous cannabinoids (endocannabinoids), the cannabinoid receptors and the enzymes involved in the synthesis and degradation of endocannabinoids.[1] The two most abundant endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are amides and esters, respectively, of long-chain polyunsaturated fatty acids (LC-PUFA).[2] To date, two G protein-coupled cannabinoid receptors through which endocannabinoids exert their actions have been identified. Because of its role in the central regulation of food intake and energy balance, the cannabinoid 1 (CB_1)-receptor has emerged as an interesting drug target for treatment of obesity, dyslipidemia and insulin resistance. CB_2-receptors, on the other hand, are mainly involved in immune function.[3] Administration of (endo)cannabinoids increases food intake, while CB_1-receptor antagonism results in hypophagia.[4] CB_1-receptor-deficient ($CB_1^{-/-}$) mice are lean and have less fat stores compared to their wild type (WT) littermates.[5] This reduction in adiposity appears to be independent of food intake,[5] suggesting that CB_1-deficiency alters the balance between energy intake, utilization and storage.

CB_1-receptors are localized in the brain, peripheral nerves and several peripheral organs including liver and adipose tissue.[3] Particularly, adipose tissue represents an important peripheral node of the ECS and several *in vitro* studies indicate that CB_1-receptor activity is critical for adipocyte function.[5-7] CB_1-expression has been reported to increase during adipocyte maturation[8] and endocannabinoids are required for adipocyte differentiation and growth,[7,9,10] supposedly by promoting lipid storage[7,11-13] *via* the induction of *Fas*, *Scd1* and *Dgat2* expression and/or by increasing lipoprotein lipase (LPL)-mediated fatty acid uptake.[5,7] In line with these results, CB_1-receptor antagonism has been shown to arrest adipocyte proliferation and to reduce lipogenic gene expression.[7,13] Interestingly, growth retardation in $CB_1^{-/-}$ mice is already apparent during the first weeks of life.[5] Appropriate CB_1-receptor functioning may therefore be critical for adipose tissue development *in vivo*.Overactivity and/or dysregulation of the EC system have been proposed to contribute to the development of obesity.[6,11,14] High-fat (HF) feeding induces obesity in rodents and has been reported to increase the endocannabinoid tone in their adipose tissue[11,15] and CB_1-deficient mice are resistant to HF diet-induced obesity.[16] Interestingly, different types of dietary fats exert specific effects on body weight regulation[17] and n-3 PUFA have been shown to decrease endocannabinoid levels in adipocytes.[18] *In vitro* studies indicate that CB_1-receptor

signalling directs metabolism towards fat storage[7,11] while reducing fat catabolism.[6,11,19] The protection against diet-induced obesity in CB_1-deficient mice may therefore, at least in part, be related to changes in fat cell metabolism in these animals. However, very few studies have evaluated the consequences of CB_1-ablation for fat cell biology *in vivo*. Furthermore, it is unknown whether CB_1-receptor mediated changes in adipose tissue function contribute to HF diet induced obesity in rodents.

In the face of the expected changes in energy balance, we determined the expression of differentiation/proliferation markers and quantified the rate of fatty acid synthesis and LPL-activity *in vivo* in the epididymal fat pads of young WT and $CB_1^{-/-}$ mice receiving a standard, low-fat chow diet, a high-fat (HF) or a HF diet in which part of the fat was replaced by fish oil (HF/FO). The comparison between the two different HF diets allowed us to investigate the influence of dietary fat quality in relation to CB_1-functioning in adipose tissue.

Methods

Animals and experimental design

Male WT and $CB_1^{-/-}$ mice on a C57BL/6J background were bred within our own facility from heterozygous crossing. Initial breeding pairs were kindly provided by prof. dr. A. Zimmer, Laboratory of Molecular Neurobiology, Department of Psychiatry, University of Bonn, Germany. Animals were housed under light- and temperature-controlled conditions (lights on 4:00 AM-4:00 PM, 21°C) with free access to food and drinking water. From four weeks of age onwards, they were divided into groups and fed three different diets during six weeks. All diets were obtained from Abdiets, Woerden, The Netherlands. One group received normal laboratory chow (RMH-B), the second group received HF (beef tallow) diet and the third group received a diet in which 42% (w/w) of the beef tallow was replaced by fish oil (HF/FO diet; menhaden oil). For diet composition see Supplemental Table 2. The HF/FO diet was renewed three times per week to prevent oxidation. Body weight and food intake were registered regularly. During the 5th week of dietary intervention, feces were collected over a 72-hour period. Fecal energy content was determined as previously described.[20] Prior to all experiments, mice were subjected to a short postprandial fasting period of 3 hours (6-9 AM) with drinking water available to exclude acute postprandial effects without the induction of a fasting response. Experimental procedures were approved by the Ethics Committees for Animal Experiments of the University of Groningen.

Fat cell histology, plasma metabolite and gene expression analysis

Mice were sacrificed by cardiac puncture under isoflurane anaesthesia. Adipose tissue was quickly removed, snap-frozen in liquid nitrogen and stored at -80°C. Part of the epididymal adipose tissue was fixed in 4% paraformaldehyde in PBS and embedded in paraffin. For adipocyte histology, 3 µm paraffin sections were stained with hematoxylin and eosin and analyzed at 10x magnification. The area of 240-420 fat cells per group was quantified using image analysis software (Qwin, Leica, Wetzlar, Germany). Fat cell area data were analyzed using the percent relative cumulative frequency (PCRF) approach and EC50 values were calculated according to Riachi *et al*.[21] Blood was centrifuged (4000 x g for 10 minutes at 4°C) and plasma was stored at -20°C. Plasma leptin, resistin and adiponectin concentrations were determined by Luminex® Multiplex technology (Luminex Corporation, Austin, TX) using Multiplex Immunoassays (Millipore, Amsterdam, The Netherlands). Adipose tissue-derived LPL-activity was determined as described below. RNA was extracted from adipose tissue using Tri reagent (Sigma-Aldrich, St. Louis, MO) and subsequently converted into cDNA by a reverse transcription procedure using M-MLV and random primers according to the manufacturer's protocol (Sigma-Aldrich). For quantitative PCR (qPCR), cDNA was amplified using the appropriate primers and probes. Primer and probe sequences for Acetyl-CoA carboxylase 1 (*Acc1/Acaca*), Carnitine palmitoyltransferase-1a (*Cpt-1a*), Fatty acid synthase (*Fas*/*Fasn*), Lipoprotein lipase (*Lpl*), Phosphoenolpyruvate carboxykinase (*Pepck*/*Pck1*), Stearoyl-CoA desaturase 1 (*Scd1*) and Sterol regulatory element binding protein-1c (*Srebp-1c*/*Srebf*) have been published (www.LabPediatricsRug.nl). The sequences for all other primers and probes are given in Supplemental Table 1. All mRNA levels were calculated relative to the expression of *cyclophilin G* and normalized for expression levels of chow-fed WT mice.

Indirect calorimetry

Separate groups of mice were place in an indirect calorimeter chamber with free access to food and drinking water. During 24 hours, gas exchange measurements were performed using an eight-channel open flow system. Flow rates were measured and controlled by a mass flow controller. O_2 and CO_2 concentrations of dried inlet and outlet air from each chamber were measured at 10-minute intervals using a paramagnetic O_2 detector and an infrared CO_2 detector. Estimated energy expenditure in kcal per 24 hours was calculated using the following equation:[22]

$(24 * (16.18*VO_2*0.001) + (5.02*VCO_2*0.001)) / 4.184$

Data were normalized for lean body mass, which was determined as described earlier.[20]

Determination of de novo lipogenesis and chain elongation in adipose tissue

Separate groups of mice received sodium [1-^{13}C]-acetate (99 atom %, Isotec/Sigma-Aldrich) *via* the drinking water (2%) during the final 72 hours of the dietary period. After a postprandial fast, mice were sacrificed by cardiac puncture under isoflurane anaesthesia. Epididymal adipose tissue was quickly removed, snap-frozen in liquid nitrogen and stored at -80°C. Lipids were hydrolyzed in HCl/acetonitrile (1:22 v/v) for 45 minutes at 100°C. Fatty acids were extracted in hexane and derivatized for 15 minutes at room temperature using α-Br-2,3,4,5,6-pentafluorobenzyl (PFB)/acetonitrile/triethanolamine (1:6:2 v/v). Derivatization was stopped by adding HCl and fatty acid-PFB derivatives were extracted in hexane. The fatty acid-PFB mass isotopomer distributions were measured using an Agilent 5975 series GC/MSD (Agilent Technologies, Santa Clara, CA). Gas chromatography was performed using a ZB-1 column (Phenomenex, Torrance, CA). Mass spectrometry analysis was performed by electron capture negative ionization using methane as moderating gas.

The normalized mass isotopomer distributions measured by GC-MS (m_0-m_x) were corrected for natural abundance of ^{13}C by multiple linear regression[23] to obtain the excess fractional distribution of mass isotopomers (M_0-M_x) due to incorporation of [1-^{13}C]-acetate. This distribution was used in mass isotopomer distribution analysis (MIDA) algorithms to calculate the acetyl-CoA precursor pool enrichment ($p_{acetate}$), fractional palmitate synthesis rates ($f_{C16:0}$) and the fraction of palmitate and oleate generated by elongation of *de novo* synthesized palmitate ($f_{C18:0/1(C16DNL)}$), or by elongation of pre-existing palmitate ($f_{C18:0/1(C16PE)}$) as described.[24]

Lipolytic activity

Plasma LPL-activity was determined using a separate group of animals. Following the postprandial fast, a baseline blood sample was drawn by retro-orbital bleeding under isoflurane anaesthesia into heparinized capillaries. Mice subsequently received an intra-orbital injection of heparin in saline (0.1 U/g). After 10 minutes, a post-heparin blood sample was drawn by retro-orbital bleeding. Mice were sacrificed by cardiac puncture and blood was centrifuged (4000 x g for 10 minutes at 4°C). Total lipase activities were determined as described in 10 µl of plasma.[25] To determine post-heparin LPL-activity,

post-heparin hepatic lipase activity was subtracted from the total post-heparin lipase activity.

Adipose tissue specific LPL-activity was determined in biopsies collected after a postprandial fast. Tissue was first homogenized in 1 mL of a buffer containing sucrose (0.25 M), EDTA (1 mM), Tris.Hcl (10 mM) and Deoxycholate (12 mM), pH 7.4.[26] Tissue homogenates were centrifuged (20 min at 12000 x g for at 4°C) and the fraction between the upper fat layer and the bottom sediment was collected. Lipase activities were determined as described using 50 µl of tissue fraction. LPL-activity was calculated by subtracting lipolytic activity determined in a final NaCl concentration of 0.83 M (non-LPL) from total lipolytic activity measured without NaCl. Data obtained in adipose tissue were normalized for protein concentration of the tissue fraction.[26]

Statistics

All data are presented as mean values ± SEM. Statistical analysis was performed using SPSS for Windows software (SPSS 12.02, Chicago, IL, USA). To test dietary effects, genotype, and their interactions data were analyzed using a general linear model and Bonferroni *post hoc* analyses. Treatment effects were further analyzed by Student's *t*-test. In case of non-parametric distribution, Mann-Whitney *U*-test was applied. The null hypothesis was rejected at the 0.05 level of probability.

Results

The resistance against high-fat diet-induced obesity in $CB_1^{-/-}$ mice is associated with a reduced adipocyte size

We first evaluated the adiposity-inducing effects of two different HF diets in WT and $CB_1^{-/-}$ mice. As expected, HF and HF/FO feeding resulted in a comparable increase in adipose tissue mass (Figure 1A) and a slightly higher body weight (Figure 1B) in WT mice, while $CB_1^{-/-}$ mice remained lean on the HF and HF/FO diet. The leanness in $CB_1^{-/-}$ mice was independent of caloric intake since we did not observe differences in energy consumption between WT and $CB_1^{-/-}$ mice during the dietary interventions (Figure 1C). Fecal energy loss was also similar in WT and $CB_1^{-/-}$ mice, indicating that intestinal nutrient absorption was comparable (Figure 1D). Fish-oil replacement, however, increased nutrient absorption in HF-fed mice, since fecal energy loss was reduced in WT and $CB_1^{-/-}$ mice that were fed the HF/FO diet. Estimated energy expenditure was increased in CB_1^{-}

$^{-/-}$ mice compared to WT mice, however, the difference reached statistical significance for the HF and HF/FO groups only (Figure 1E; $p = 0.069$ in chow-fed WT and $CB_1^{-/-}$ mice).

$CB_1^{-/-}$ mice had smaller adipocytes (Figure 2A), which is also indicated by the separate 95%-confidence intervals of the fat cell area EC50s in $CB_1^{-/-}$ *versus* WT mice fed either of the three diets (Figure 2B). Adipose tissue expression of *Faah*, *Pparγ2*, *Cebp/α, Ap2* and *Adiponectin* were not affected by CB_1-deficiency or HF and HF/FO feeding, while *Napepld* expression was increased in HF-fed mice of both genotypes (Table 1). Plasma leptin concentrations were elevated with HF or HF/FO induced adiposity in WT mice while adiponectin and resistin levels were comparable in all mice (Table 2).

Fatty acid synthesis in adipose tissue is not affected in $CB_1^{-/-}$ mice

To assess whether lipogenesis was reduced in $CB_1^{-/-}$ mice, we determined fatty acid synthesis in adipose tissue using ^{13}C-acetate incorporation. Acetyl-CoA pool enrichments were increased in HF-fed WT and $CB_1^{-/-}$ mice as compared to chow-fed animals (Table 3) and normalized by FO replacement. No differences between WT and $CB_1^{-/-}$ mice were observed. Fractional palmitate synthesis from *de novo* lipogenesis was similar in WT and $CB_1^{-/-}$ mice, but was reduced in mice fed the HF and HF/FO diets as compared to chow-fed animals (Table 3). Furthermore, fractional synthesis of stearate and oleate from elongation of *de novo* synthesized palmitate was reduced in WT and $CB_1^{-/-}$ mice fed the HF and HF/FO diets as compared to chow-fed animals (Table 3) with no differences between WT and $CB_1^{-/-}$ mice. In contrast to the lower fractional synthesis rates fromelongation of *de novo* synthesized palmitate, fractional stearate and oleate synthesis from elongation of unlabeled palmitate were maintained in WT and $CB_1^{-/-}$ mice fed the HF and HF/FO diets as compared to chow-fed animals (Table 3). The unaltered fatty acid synthesis rates in $CB_1^{-/-}$ mice were supported by gene expression analysis in adipose tissue: we did not observe any changes in lipogenic gene expression between WT and $CB_1^{-/-}$ mice under the different dietary conditions (Table 1). HF and HF/FO feeding did not affect *Fatp4, Acc1, Fas* and *Pepck* (Table 1) expression. HF feeding induced *Scd1* expression in both WT and $Cb_1^{-/-}$ mice while we observed a tendency for an increased *Srebp-1c* expression. These inductions were normalized in HF/FO-fed WT and $CB_1^{-/-}$ mice (Table 1).

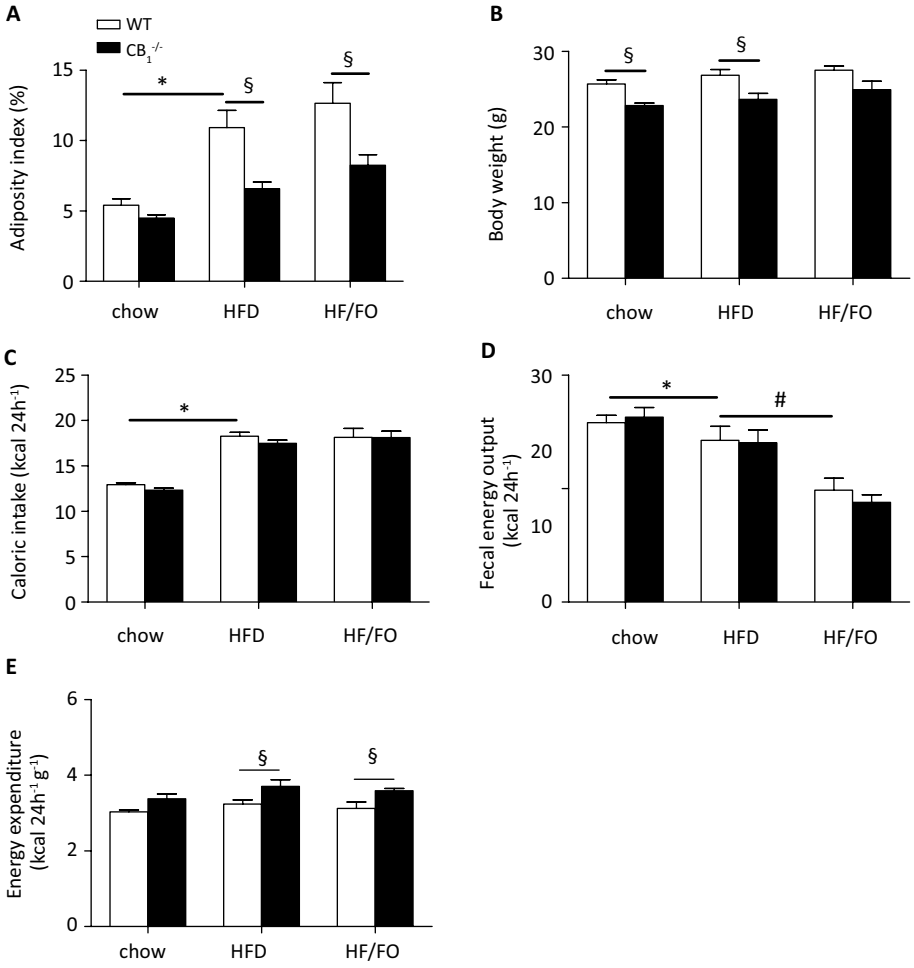

Figure 1. Body weight and energy balance in 4 week old CB_1-deficient ($CB_1^{-/-}$) and wild type (WT) mice fed chow, HF or HF/FO diet during 6 weeks. A) Adiposity index, representing the percentage fat mass over total body mass. B) Body weights at the end of the dietary intervention. C) Caloric intake. D) Fecal energy output and E) Energy expenditure, derived from indirect calorimetry. Values are given as means ± SEM for n = 5-8. Open bars, WT mice; closed bars, $CB_1^{-/-}$ mice. * $p < 0.05$ chow *versus* HF, # $p < 0.05$ HF *versus* HF/FO (Bonferroni). § $p < 0.05$ $CB_1^{-/-}$ *versus* WT (Student's *t*-test).

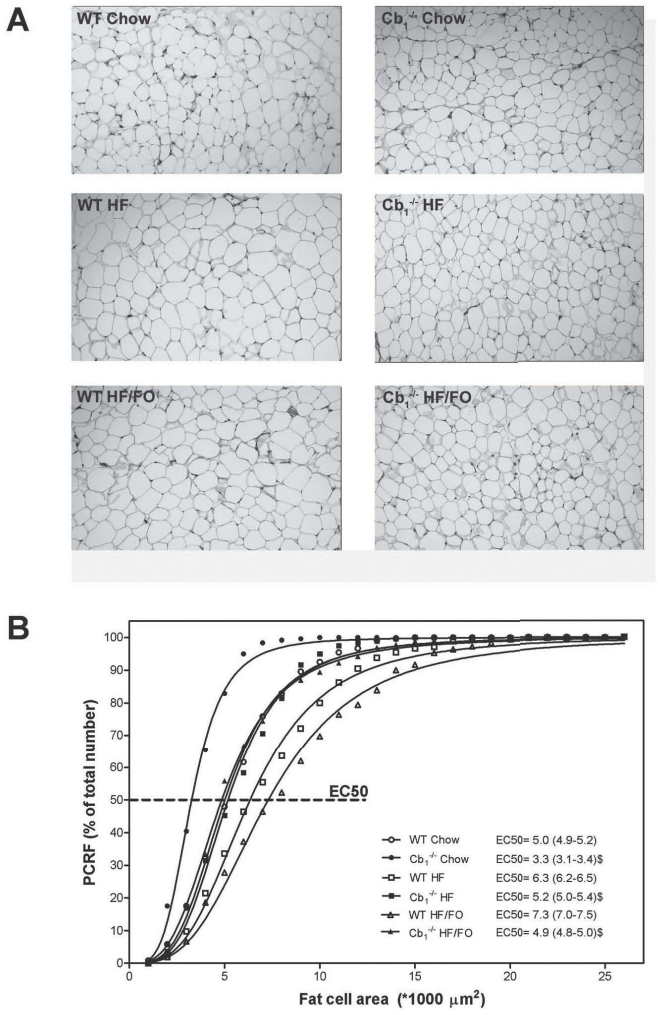

Figure 2. Fat cell area in 4 week old CB_1-deficient ($CB_1^{-/-}$) and wild type (WT) mice fed chow, HF or HF/FO diet during 6 weeks. A) Representative pictures of 3 µm paraffin hematoxylin and eosin-stained sections and B) percent relative cumulative frequency (PCRF) curves of 240-420 fat cell areas. Inset: EC50 values of the PCRF curves and their 95%-confidence intervals. $ $p < 0.05$ $CB_1^{-/-}$ mice *versus* WT.

Table 1. Adipose tissue expression of genes in cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice and in wild type (WT) mice.

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Endocannabinoid system						
CB1 (Cnr1)	1.0 ± 0.1	ND	1.0 ± 0.1	ND	1.1 ± 0.2	ND
Nape-pld	1.0 ± 0.1	0.9 ± 0.1	1.4 ± 0.1*	1.3 ± 0.2*	1.2 ± 0.1	1.1 ± 0.1
Faah	1.0 ± 0.3	1.8 ± 0.5	1.1 ± 0.6	1.1 ± 0.4	1.0 ± 0.5	1.3 ± 0.6
Adipocyte proliferation and differentiation						
Pparγ2	1.0 ± 0.2	1.0 ± 0.1	1.4 ± 0.1	1.2 ± 0.3	1.0 ± 0.1	0.9 ± 0.1
Cebp/α	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.3 ± 0.3	1.0 ± 0.1	0.9 ± 0.1
Ap2	1.0 ± 0.1	0.9 ± 0.1	1.5 ± 0.0	1.2 ± 0.2	1.4 ± 0.1	1.2 ± 0.1
Adiponectin (Arcp30)	1.0 ± 0.2	0.9 ± 0.2	1.3 ± 0.1	1.3 ± 0.4	1.3 ± 0.1	1,00 ± 0.1
Fatty acid uptake and synthesis						
Lpl	1.0 ± 0.3	0.9 ± 0.2	1.2 ± 0.1	0.9 ± 0.2	1.4 ± 0.2	1.0 ± 0.1
Fatp4 (Slc27a4)	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.4 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
Srebp-1c (Srebf)	1.0 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.8 ± 0.4	1.1 ± 0.0#	1.1 ± 0.1#
Acc1 (Acaca)	1.0 ± 0.2	1.1 ± 0.3	0.9 ± 0.1	0.9 ± 0.3	0.6 ± 0.1	0.5 ± 0.1
Fas (Fasn)	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.0 ± 0.3	0.7 ± 0.1	0.4 ± 0.0
Scd1	1.0 ± 0.1	0.9 ± 0.1	2.8 ± 0.1*	3.1 ± 0.7*	0.9 ± 0.1#	1.1 ± 0.1#
Pepck (Pck1)	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	1.3 ± 0.4	1.1 ± 0.1	0.9 ± 0.1
Adipocyte lipolysis						
Hsl	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
Atgl/desnutrin (pnpla2)	1.0 ± 0.2	1.1 ± 0.2	1.5 ± 0.2	1.4 ± 0.4	1.2 ± 0.1	1.2 ± 0.2
Fatty acid oxidation						
Cpt-1a	1.0 ± 0.1	1.2 ± 0.2	1.6 ± 0.2	1.2 ± 0.2	1.3 ± 0.1	1.1 ± 0.1

Expression levels were normalized to cyclophilin G expression. Values are means +/- SEM; n=4-8; * $p < 0.05$ chow *vs* HF/FO, # $p < 0.05$ HF *vs* HF/FO. $CB_1^{-/-}$, cannabinoid-1-receptor deficient mice; FO, fish-oil; HF, high-fat; ND, not detectable.

Table 2. Plasma adipokine concentrations in cannabinoid-1 receptor deficient (CB1-/-) mice and in wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet.

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Leptin (ng ml^{-1})	0.7 ± 0.1	0.6 ± 0.1#	2.1 ± 0.5*	1.0 ± 0.2*#	2.2 ± 0.5	1.7 ± 0.3
Adiponectin (µg ml^{-1})	14.3 ± 1.3	18.5 ± 2.4	15.4 ± 1.5	17.0 ± 2.9	18.1 ± 2.2	19.1 ± 2.2
Resistin (ng ml^{-1})	2.3 ± 0.2	2.5 ± 0.4	3.0 ± 0.5	2.9 ± 0.3	2.1 ± 0.2	2.6 ± 0.4

Values are means +/- SEM; n=6-8; * $p < 0.05$ chow *vs* HF, # HF $CB_1^{-/-}$ *vs* HF WT.

Lipolytic activity is maintained in $CB_1^{-/-}$ mice

CB_1-receptor agonist treatment enhances lipolytic activity *in vitro*.[5] As a consequence, CB_1-deficiency may result in a reduction of lipoprotein triglyceride lipolysis, thereby limiting fat storage in adipose tissue. Yet, adipose tissue *Lpl* and *Fatp4* expression were not affected by CB_1-deficiency or HF and HF/FO feeding (Table 1). To test whether an increased lipid clearance contributed to the obesogenic effects of the HF diets, we also determined LPL-activity. Post-heparin plasma LPL-activity as well as adipose tissue-derived LPL-activity were similar in all groups (Figure 3A and 3B). In addition, we did not observe significant differences in adipose tissue expression of *Hsl*, *Atgl* and *Cpt-1a* among the different groups (Table 1).

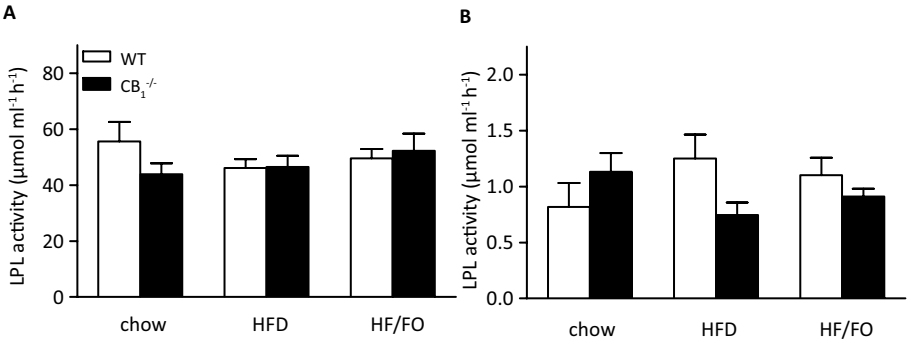

Figure 3. Lipolytic activity in 4 week old CB_1-deficient ($CB_1^{-/-}$) and wild type (WT) mice fed chow, HF or HF/FO diet during 6 weeks. A, Post-heparin plasma lipoprotein lipase (LPL) activity and B, Adipose tissue-derived LPL-activity. Values are given as means ± SEM for n = 5-6. Open bars, WT mice; closed bars $CB_1^{-/-}$ mice.

Table 3. Fractional fatty acid synthesis in adipose tissue in cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice and in wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Acetyl-CoA pool enrichment (%)	12.9 ± 0.3	11.8 ± 0.5	14.9 ± 0.4*	14.3 ± 0.6*	11.4 ± 0.6#	10.7 ± 0.7#
Fractional palmitate synthesis (%) ($f_{C16:0}$)	3.8 ± 0.6	4.5 ± 0.4	1.8 ± 0.2*	1.7 ± 0.2*	2.1 ± 0.2	1.6 ± 0.2$
Fractional stearate synthesis (%)						
from de novo synthesized palmitate ($f_{C18:0(C16DNL)}$)	1.9 ± 0.3	2.1 ± 0.2	0.3 ± 0.0*	0.3 ± 0.0*	0.5 ± 0.1	0.5 ± 0.0
from pre-existing palmitate ($f_{C18:0(C16DNL)}$)	0.8 ± 0.3	1.1 ± 0.6	0.4 ± 0.0	0.7 ± 0.1	0.7 ± 0.3	0.4 ± 0.1
Fractional oleate synthesis (%)						
from de novo synthesized palmitate ($f_{C18:0(C16DNL)}$)	0.8 ± 0.2	0.9 ± 0.2	0.1 ± 0.0*	0.1 ± 0.0*	0.1 ± 0.0	0.1 ± 0.0
from pre-existing palmitate ($f_{C18:0(C16DNL)}$)	3.8 ± 1.0	5.2 ± 0.9	4.8 ± 0.9	6.3 ± 0.9	6.0 ± 1.2	4.9 ± 1.2

Values are means +/- SEM; n = 4-7; * $p < 0.05$ Chow *vs* HF, ‡ $p < 0.05$ HF *vs* HF/FO, # HF $CB_1^{-/-}$ *vs* HF WT, $ HF/FO $CB_1^{-/-}$ *vs* HF/FO WT

Discussion

Overactivity and/or dysregulation of the ECS may contribute to the development of obesity.[6,11,14] Several *in vitro* studies suggest an involvement of the CB_1-receptor in controlling adipocyte development and function.[5-13] To our knowledge, we are the first to report a reduction in fat cell size in CB_1-deficient mice. Yet, we also show that the protection against HF diet-induced obesity in $CB_1^{-/-}$ mice is not related to alterations in adipocyte differentiation and proliferation markers or to changes in the rates of *in vivo* lipogenesis and lipoprotein lipolysis in fat tissue. Impaired CB_1-signalling has been shown to protect against HF diet-induced obesity in adult mice,[16,27] however, underlying mechanisms have not been resolved. The ECS has been implicated in the regulation of adipocyte proliferation and differentiation *in vitro*[5-13] and CB_1-deficient mice are retarded in growth from an early age on.[5] We therefore decided to study the metabolic consequences of HF feeding in young $CB_1^{-/-}$ mice, *i.e.* in a phase when the body weight of the mice is still increasing. Six weeks of HF and HF/FO feeding induced obesity in WT mice which was accompanied by an increased adipocyte size. This effect was clearly blunted in $CB_1^{-/-}$ mice, however, the $CB_1^{-/-}$ mice did become heavier on the HF and HF/FO diets as compared to chow-fed $CB_1^{-/-}$ mice. Gene expression analysis indicated that this is unlikely due to an impaired ability of adipocyte differentiation and/or proliferation in $CB_1^{-/-}$ mice, since we did not observe differences in *Pparγ2*, *Cebp/α* and *Ap2* expression. The expression of these genes was also not different prior to the start of the HF diet interventions (at three weeks of age, data not shown). PPARγ and CEBP/α play key roles in controlling adiponectin transcription.[28,29] We did not observe a differences in *adiponectin* mRNA levels, indicating that, in parallel to their unaltered expression levels, the transcriptional activity of PPARγ and CEBP/α was not impaired by CB_1-deficiency. This is supported by the comparable plasma adiponectin concentrations among the different groups studied. HF feeding has been reported to decrease plasma adiponectin concentrations in WT mice.[27,30] In those studies, 6-10 week old mice were fed a HF diet for 14 weeks, whereas in the current study, 4-week old mice were subjected to a 6-week dietary challenge. These experimental differences may underlie the discrepant findings in the current study as compared to others.[27] Although adiponectin expression and release have been suggested to be modulated by CB_1-receptor activity,[6,11] pharmacological CB_1-receptor activation and blockade do not directly affect adiponectin expression in cultured adipocytes.[12,31] The observed changes in adiponectin expression and secretion may rather be attributed to acute changes in food intake.[32] Similar to

what has been found by others,[27] we did not observe major differences in voluntary caloric intake between WT and $CB_1^{-/-}$ mice. Interestingly, isocaloric replacement of the saturated fat in the HF diet for PUFA increased intestinal fatty acid uptake in both genotypes, as indicated by the lower fecal energy loss. This can presumably be ascribed to upregulation of fatty acid transport proteins and consequently increased nutrient uptake in animals on the HF/FO diet.[33] However, no differences in fecal energy loss were observed between $CB_1^{-/-}$ mice and WT mice fed either of the three diets. Altogether, these data indicate that reduced adiposity in $CB_1^{-/-}$ mice does not result from an intrinsic disability of adipocytes to expand or from a different nutrient availability.

Pharmacological CB_1-receptor activation promotes lipid storage in adipocytes[7,11-13] and induces the expression of lipogenic genes in fat tissue.[7] We therefore studied the lipogenic flux *in vivo* in adipose tissue of WT and $CB_1^{-/-}$ mice that were fed diets differing in fat quantity and quality. Fractional fatty acid synthesis rates were not affected by CB_1-deficiency, paralleled by an unaltered expression of lipogenic genes. In both genotypes, HF and HF/FO feeding resulted in a reduction of the fractional fatty acid synthesis from *de novo* lipogenesis (indicated by lower fractional palmitate synthesis as well as reduced stearate and oleate synthesis from *de novo* synthesized palmitate). This is in agreement with a recent report showing that fatty acid synthesis from glucose is in the fat tissue of HF- fed mice compared to animals fed a low-fat diet.[34] These observations indicate that HF feeding promotes adiposity by accelerating elongation of dietary fatty acids. Fractional fatty acid elongation of pre-existing palmitate, on the other hand, was maintained.

Dietary fatty acids enter the body after their incorporation into triglycerides that are subsequently packaged into chylomicron particles in the intestine. Tissue-specific LPL- activity catalyzes the liberation of fatty acids from chylomicron-triglycerides, after which the fatty acids can be taken up by organs and tissues. To assess whether the protection against HF diet-induced adiposity in $CB_1^{-/-}$ mice was secondary to a reduced lipolytic activity, we determined whole-body and adipose tissue-specific LPL-activity. WT and $CB_1^{-/-}$ mice fed either of the three diets exhibited comparable LPL-activities. Previous studies have shown that CB_1-receptor antagonism inhibits LPL-activity, only when cells were co-treated with a CB_1-receptor agonist.[5] Furthermore, elevated plasma endocannabinoid concentrations were found to be associated with increased LPL-activity.[35] On the other hand, CB_1-receptor antagonist treatment by itself did not inhibit LPL-activity *in vitro*.[5] Consistent with previous work,[27] we did not observe an increase in LPL-activity upon HF feeding. Thus, the adiposity-resistant phenotype of

the CB_1-deficient mice was not related impaired LPL-mediated lipolysis of circulating triglycerides or to reduced adipocyte lipogenesis. The comparable expression levels of *Hsl* and *Atgl* and *Cpt-1a* in adipose tissue of $CB_1^{-/-}$ mice suggest that triglyceride hydrolysis and fatty acid oxidation were also not altered in this tissue.

Altogether, the protection against diet-induced adiposity in $CB_1^{-/-}$ mice may not be related to intrinsic alterations in adipocyte biology due to absence of the receptor. The reduced accumulation of energy as fat in $CB_1^{-/-}$ mice reflects a less positive energy balance as compared to their WT littermates. Since energy intake was similar in both genotypes,the reduction in fat mass must therefore be due to a higher energy loss in $CB_1^{-/-}$ mice. Indirect calorimetry indeed revealed higher energy expenditure in $CB_1^{-/-}$ mice. This difference was mainly attributable to increased fat utilization in the light phase (chapter 6). CB_1-receptor deficiency may furthermore increase energy dissipation as heat. CB_1-receptor blockade induces a mitochondria-rich, thermogenic phenotype in adipocytes.[36] It furthermore increases brown adipose tissue temperature concomitantly with an increase in *Ucp-1* expression, which is attenuated upon denervation.[37] CB_1-receptor activation has also been reported to reduce *Ucp-2* expression.[7] Both pharmacological CB_1-blockade and CB_1-deficiency have recently been shown to increase mitochondrial function.[38,39] In addition, CB_1 antagonism enhanced substrate oxidation and increased mitochondrial uncoupling, thereby increasing oxidative and non-oxidative energy expenditure.[38]

In summary, our data indicate that the obesity-resistant phenotype of $CB_1^{-/-}$ mice is not due to changes in adipose tissue differentiation/proliferation, lipogenesis or or fat-derived LPL-activity *in vivo*. Energy expenditure was increased in $CB_1^{-/-}$ mice compared to their WT littermates. Dietary energy intake and fecal energy loss remained unaffected, which resulted in lower adiposity and fat cell size upon HF feeding. We therefore propose that the protection against diet-induced obesity in $CB_1^{-/-}$ mice is not related to functional alterations in fat tissue *per se*, but rather results from of an increased energy loss by oxidative and potentially non-oxidative pathways.

Acknowledgements

This work was supported by the Groningen Expert Centre on Childhood Obesity and the Dutch Diabetes Foundation. The authors thank Nanda Gruben, Rick Havinga, Gerard Overkamp, Theo S. Boer, Jan Keijzer, Jan Bruggink and Juul F.W. Baller for excellent technical assistance.

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CHAPTER 6

Circadian rhythm of energy expenditure and substrate oxidation in CB_1-receptor deficient mice

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Abstract

The endocannabinoid system (ECS), comprising cannabinoid receptors and endogenous ligands for these receptors, not only plays a pivotal role in the regulation of feeding behaviour but also in the modulation of fuel metabolism and partitioning. The resistance of cannabinoid-1 receptor deficient ($CB_1^{-/-}$) mice to diet-induced obesity compared to pair-fed wild type (WT) mice was recently attributed to increased daily energy expenditure. It has been suggested that increased energy expenditure in mice with impaired CB_1-signalling is driven by increased fatty acid oxidation (FAO). Previous data of our laboratory showed increased FAO in mice treated with rimonabant, a CB_1-receptor antagonist. Based on physiological diurnal fluctuations in FAO, CB_1-receptor deficiency should affect energy expenditure primary in the inactive phase, when FAO predominates over carbohydrate oxidation. For this reason we investigated substrate oxidation patterns in WT and $CB_1^{-/-}$ mice subjected to chow or a high-fat (HF) diet or to a HF diet supplemented with fish oil (HF/FO). Results showed that energy expenditure expressed per lean mass is increased in $CB_1^{-/-}$ mice compared to WT in both the inactive and the active phases. This increased expenditure is fuelled by an increased FAO, expressed per lean body mass, in the inactive phase and increased carbohydrate utilization in the active phase. Free fatty acid (FFA) levels are lower in $CB_1^{-/-}$ mice than in WT mice in the middle of the light phase, *i.e.*, at a time when FAO is elevated in $CB_1^{-/-}$ mice compared to WT mice. Diurnal patterns of food intake did not differ between $CB_1^{-/-}$ mice fed chow and WT controls, indicating that the pronounced substrate shift towards FAO in the inactive phase in $CB_1^{-/-}$ mice and towards carbohydrate utilization in the active phase can not be attributed to a changed food intake pattern.

Introduction

The recently discovered endocannabinoid system (ECS), consisting of cannabinoid receptors (CB_1 and CB_2-receptor), endogenous ligands for these receptors and enzymes involved in synthesis and degradation of these so-called endocannabinoids, has been shown to be an important regulator of energy metabolism. The ECS not only plays a role in the regulation of feeding behaviour but also in the modulation of fuel utilization. This can be inferred from the fact that mice lacking the CB_1-receptor (*i.e.*, $CB_1^{-/-}$ mice) are resistant to diet-induced obesity when compared with pair-fed wild type (WT) controls.[1] This could be due to a higher energy expenditure in $CB_1^{-/-}$ mice and, indeed, this is what we (chapter 5) and others[2,3] have recently demonstrated. Increased fatty acid oxidation in $CB_1^{-/-}$ mice has been put forward as a mechanism underlying resistance to high-fat diet-induced obesity and associated derangements such as hepatic steatosis (chapter 4). Since carnitine palmitoyltransferase-1 (*Cpt1*) activity is reduced in mice treated with a CB_1-receptor agonist, an effect that is not observed in $CB_1^{-/-}$ mice as reported by Osei-Hyiaman *et al.,*[3] these data appear to indicate that signalling of cannabinoids *via* the CB_1-receptor plays a pivotal role in controlling energy expenditure.

Fluctuations in fat and carbohydrate oxidation in rodents are linked to the diurnal cycle – with fat oxidation predominating during the inactive phase and carbohydrate utilization during the active phase. Since mice are nocturnal animals, the light period is the inactive phase whereas the dark period is the active phase. When differences in fat oxidation are relevant for differences in energy expenditure between WT and $CB_1^{-/-}$ mice, one might predict that these differences are particularly present during the light period. It is therefore of interest to establish temporal circadian patterning of fuel oxidation and energy expenditure in WT and $CB_1^{-/-}$ mice. Since differences in energy expenditure between WT and $CB_1^{-/-}$ mice were found to be largest in mice subjected to high-fat diets, we determined circadian substrate utilization patterns by indirect calorimetry in WT and $CB_1^{-/-}$ mice subjected to either a mainly carbohydrate-containing laboratory chow diet or to an obesogenic high-fat (HF) diet consisting of saturated fat, or a HF diet supplemented with fish oil (HF/FO). The latter comparison is of interest because effects of CB_1-antagonism on energy balance regulation were found to be largest in mice subjected a HF/FO diet (chapter 3-5). Carcass analysis was performed to relate energy expenditure and fuel oxidation patterns to lean tissue mass.

Methods and Procedures

Animals

CB_1-receptor deficient mice ($CB_1^{-/-}$) on a C57BL/6J background and their WT littermates (WT) were bred in our own facility. Initial breeding pairs were kindly provided by Prof. Dr. A. Zimmer, Laboratory of Molecular Neurobiology, Department of Psychiatry, University of Bonn, Germany. Mice were individually housed in a light-controlled (lights on 4 AM- 4 PM) and temperature-controlled (21°C) facility. Mice were allowed tap water and food *ad libitum*. Experimental protocols were approved by the local Experimental Ethical Committee for Animal Experiments. These regulations are consistent with the guidelines for the care and use of laboratory animals as described by the U.S. National Institutes of Health.

Experimental diets

All experimental diets were obtained from Abdiets BV, Woerden, The Netherlands. Mice received either standard laboratory chow (RMH-B), a high-fat diet (HF) containing 36 weight % fat consisting of bovine fat (custom synthesis, diet number 4031.45), or a high-fat diet containing 36 weight % fat consisting for 58% of bovine fat and for 42% of fish oil (custom synthesis, diet number 4031.54). Composition of experimental diets has been published previously (Supplemental Table 2).[4,5] The HF/FO diet was replaced every two days to prevent oxidation of fatty acid species.

Experimental procedures

Four-week-old $CB_1^{-/-}$ mice and WT littermates were divided into groups by matching for body weight and fed one of the three diets during six weeks. Intake and body weight were registered twice a week. Indirect 24h-calorimetry was performed between four and five weeks after start of diets. When mice were six weeks on experimental diets, they were sacrificed by cardiac puncture under inhalation anaesthesia followed by cervical dislocation after a fasting period of three hours. Blood and tissues were collected. Carcasses were stored at -20°C until carcass analysis. We choose to sacrifice at age of ten weeks because of the described increase in mortality in $CB_1^{-/-}$ mice from the tenth week of life onwards.[6]

Body composition

Carcasses were eviscerated and stored at -20°C. Carcasses and organs were dried to constant weight at 103°C, and fat was extracted by using petroleum ether (Boom BV, Meppel, The Netherlands) in a soxlet apparatus. Percentage fat of carcasses and organs were determined from weight differences before and after the fat extraction procedure.

Indirect calorimetry

Mice were placed in an open circuit indirect calorimetry system for 24 hours with access to water and food. Gas-exchange measurements were performed in an eight-channel open flow system. Flow rates were measured and controlled with a mass flow controller. O_2 and CO_2 concentrations of dried inlet and outlet air from each chamber were measured every 10 minutes with a paramagnetic O_2 analyzer and an infrared CO_2 gas analyzer. Data were collected from each metabolic cage separately.

The respiratory quotient (RQ) was defined as CO_2 production (l)/ O_2 consumption (l). Energy expenditure (kJ h^{-1}) was calculated according to Brouwer using the following equation: (16.18*VO_2(ml h^{-1})*0.001)+(5.02*VCO_2(ml h^{-1})*0.001).[7] Energy expenditure was expressed per lean body mass. Lipid oxidation and carbohydrate utilization (expressed per lean body mass) were calculated according to Lusk using the following equations:[8]

Lipid oxidation (g h^{-1}): 38.461*(VO_2 (mol h^{-1}) - VCO_2 (mol h^{-1}))

Carbohydrate utilization (g h^{-1}): (94.017*VCO_2 (mol h^{-1})) - (66.239*VO_2 (mol h^{-1}))

Measurement of plasma free fatty acids, ketone bodies, blood glucose and insulin

Plasma free fatty acids (FFA), β-hydroxybutyrate (β-HB) and lactate concentrations were determined using commercially available kits from Wako Chemicals (Neuss, Germany) for FFA and from Roche Diagnostics (Mannheim, Germany) for β-HB and lactate. A Euroflash glucose meter (Lifescan Benelux, Beerse, Belgium) was used to measure blood glucose concentrations. Plasma insulin concentrations were determined by Luminex Multiplex technology (Luminex Corporation, Austin, TX, USA) using multiplex Immunoassays (Millipore, Amsterdam, The Netherlands).

Circadian patterning of food intake

To rule out differences in circadian rhythm of food intake as an underlying cause of differences in fat oxidation in the light phase, we added an experiment to determine

circadian patterning of food intake. Since difference in fat oxidation in the light phase were largest between WT and $CB_1^{-/-}$ fed chow, we measured food intake every two hour over a 24-hour-period in $CB_1^{-/-}$ and WT controls fed chow.

Statistics

All values in Figure 1 and in the tables represent means ± standard errors of the means for the number of animals indicated in the figure and table legends. All values in Figures 2, 3 and 4 represent means. Data were analyzed using General Linear Model (GLM) Repeated Measures for temporal patterning of RQ, energy expenditure, fatty acid oxidation and carbohydrate utilization. Periods analyzed were defined as period A, ZT12-16 (Zeitgeber Time, ZT0 is the start of the dark phase, *i.e.*, 4 PM); period B, ZT16-20; period C, ZT0-12; period D, ZT12-20; and period E, ZT0-20. To evaluate effects of diet, genotype, and their interactions on body weight, weight gain, results concerning body composition and average values per period on energy expenditure, lipid oxidation and carbohydrate utilization, and plasma parameters, these data were statistically analyzed using a General Linear Model Univariate Analysis with Bonferroni *post hoc* analyses. Treatment effects were further analyzed by Student's *t*-test. In case of non-parametric distribution, Mann-Whitney *U*-test was assed for statistical analysis. Statistical significance of differences was accepted at a *P*-value of less than 0.05. Analyses were performed using SPSS 16.0 for Windows software (SPSS, Chicago, IL).

Results

Induction of obesity and adiposity of WT mice fed HF diet or HF/FO diet

After the six week dietary intervention, body weights of WT mice fed HF diet or HF/FO diet were comparable to those of mice fed chow (Figure 1a). When expressed as body weight gain from start of diet manipulations, the HF/FO diet was more weight-promoting in WT mice compared with chow and there was a trend towards a higher body weight gain in WT mice fed HF diet compared to WT mice fed chow (Figure 1b). Total fat mass as well as visceral fat mass and subcutaneous fat mass were significantly higher in WT mice fed either HF diet or HF/FO diet compared to mice fed chow (Table 1).

$CB_1^{-/-}$ mice are resistant to diet-induced obesity

After being on the experimental diets for 6 weeks $CB_1^{-/-}$ mice on all three diets weighed significantly less than their WT littermates (Figure 1a). This difference in body weight was attributed to a significant reduction in adiposity in $CB_1^{-/-}$ mice as compared with that in WT littermates on all three experimental diets (Table 1). In mice fed chow, HF diet or HF/FO diet, total fat mass was respectively 21.3%, 41.8% and 41.4% lower in $CB_1^{-/-}$ mice as compared with that in WT mice fed the same diet.

Caloric intake was similar among $CB_1^{-/-}$ mice and WT littermates as was energy absorption as determined by bomb calorimetry of feces of these mice (chapter 5 of this thesis). So, as compared with WT littermates, we found a lean phenotype (meaning a phenotype characterized by reduced adiposity) in $CB_1^{-/-}$ mice on all three experimental diets and we confirmed that $CB_1^{-/-}$ mice are resistant to diet-induced obesity. Reduction in body weight and adiposity could not be attributed to decreased energy intake or decreased absorption.

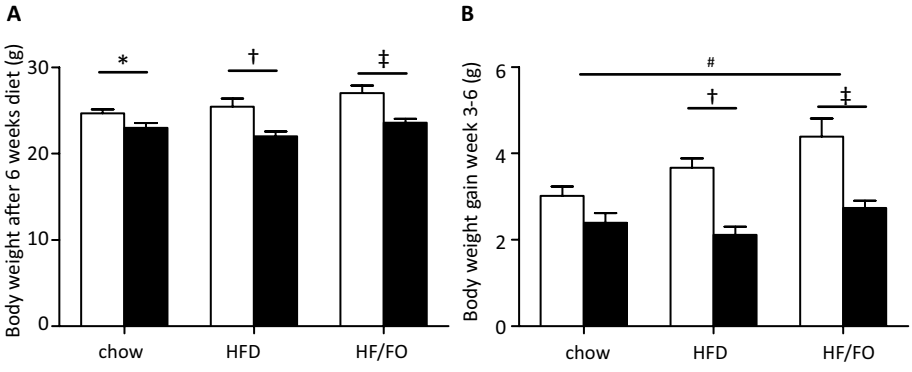

Figure 1. A) Body weight at sacrifice in male CB_1-deficient ($CB_1^{-/-}$) mice and wild type (WT) mice fed chow, high-fat diet (HF) or high-fat fish-oil enriched diet (HF/FO). Open bars, WT mice; closed bars, $CB_1^{-/-}$ mice. Values are means +/- SEM; n =6-7, * $p < 0.05$ chow $CB_1^{-/-}$ *versus* chow WT, † $p < 0.05$ HF $CB_1^{-/-}$ *versus* HF WT, chow *versus* HF/FO, ‡ $p < 0.05$ $CB_1^{-/-}$ HF/FO *versus* HF/FO WT, # $p < 0.05$ chow *versus* HF/FO. **B) Body weight gain during week 3-6 in male $CB_1^{-/-}$ mice and WT mice fed chow, HF or HF/FO diet**. Open bars, WT mice; closed bars, $CB_1^{-/-}$ mice. Values are means +/- SEM; n =6-7, † $p < 0.05$ HF $CB_1^{-/-}$ *versus* HF WT, chow *versus* HF/FO, ‡ $p < 0.05$ $CB_1^{-/-}$ HF/FO *versus* HF/FO WT, # $p < 0.05$ chow *versus* HF/FO

Table 1. Body composition of cannabinoid-1-receptor deficient ($CB_1^{-/-}$) mice and wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil (HF/FO) diet.

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Total fat mass (g)	2,35 ± 0,17	1,85 ± 0,09#	4,55 ± 0,59*	2,65 ± 0,15$	5,22 ± 0,67†	3,06 ± 0,26&
Lean mass (g)	5,18 ± 0,09	4,64 ± 0,08#	5,04 ± 0,12	4,70 ± 0,08	5,05 ± 0,08	4,74 ± 0,14
Visceral fat mass (g)	0,63 ± 0,07	0,44 ± 0,04#	1,24 ± 0,17*	0,65 ± 0,05$	1,28 ± 0,17†	0,74 ± 0,09&
Epididymal fat mass (g)	0,32 ± 0,04	0,23 ± 0,02	0,72 ± 0,11*	0,36 ± 0,03$	0,64 ± 0,08†	0,41 ± 0,06
Retroperitoneal fat mass (g)	0,12 ± 0,02	0,07 ± 0,01#	0,22 ± 0,03*	0,11 ± 0,01$	0,30 ± 0,05†	0,14 ± 0,02&
Subcutaneous fat mass (g)	0,92 ± 0,09	0,72 ± 0,04	2,07 ± 0,30*	1,07 ± 0,08$	2,52 ± 0,37†	1,38 ± 0,14&

Values are means +/- SEM; n=6-8; * $p < 0.05$ chow vs HF, † $p < 0.05$ chow *vs* HF/FO, # chow $CB_1^{-/-}$ *vs* chow WT, $ HF $CB_1^{-/-}$ *vs* HF WT, & HF/FO $CB_1^{-/-}$ *vs* HF/FO WT

Circadian rhythm of energy expenditure in $CB_1^{-/-}$ mice

Accordingly, increased energy output was expected to contribute to the observed lean phenotype of $CB_1^{-/-}$ mice. Figure 2a shows circadian patterning of energy expenditure in $CB_1^{-/-}$ mice compared to WT mice fed the three experimental diets. Analysis of these data by overall GLM repeated measures demonstrated no significant interactions between diet and genotype on energy expenditure. A significant diet effect irrespective of genotype on energy expenditure was observed in both the dark and light period and analysing sources of significance with *post hoc* tests revealed a significant reduction in energy expenditure in mice fed chow as compared with that in HF-fed mice (in all periods $p < 0.05$) and HF/FO-fed mice (period B, C and E $p < 0.05$). With respect to genotype, energy expenditure was only found to differ in the middle of the light period (period B, ZT 16-19), with $CB_1^{-/-}$ mice having an increased energy expenditure compared to WT mice ($p = 0.016$). From the majority of mice that had participated in indirect calorimetry measurements, we also obtained data on body composition (n = 4-6 instead of n = 5-7). When results of this subgroup of mice were expressed per lean mass, the observed differences were generally more pronounced (Figure 2b). GLM repeated measures on data of energy expenditure expressed per lean mass revealed no interaction between genotype and diet. After normalization, diet effects in the dark (active) phase were lost, whereas in the light (inactive) phase energy expenditure expressed per lean mass was higher in mice fed chow as compared with that in HF-fed or HF/FO-fed mice. Effects of genotype on energy expenditure expressed per lean mass became significant for all periods analyzed ($p < 0.001$ for all analyzed periods).

Substrate shift towards lipid oxidation in $CB_1^{-/-}$ mice in the light phase and towards carbohydrate utilization in the dark phase

Figure 3 shows circadian patterning of respiratory quotients (RQ). GLM repeated measures demonstrated no significant interactions between diet and genotype on RQ in the light phase or the dark phase. GLM repeated measures revealed that RQ was significantly higher in chow-fed mice as compared with that in HF-fed or HF/FO-fed mice in both the light period and the dark period. $CB_1^{-/-}$ mice exhibited significantly decreased RQ in the light period compared to WT controls ($p = 0.004$), indicating an increase in the ratio of fatty acid oxidation (FAO) to carbohydrate utilization (Figure 3). Indeed, GLM repeated measures revealed an increase in lipid oxidation calculated from oxygen consumption and carbon dioxide production (Figure 4a) in the light period in

$CB_1^{-/-}$ mice compared to WT mice (p = 0.026). Rates of carbohydrate utilization were not different between mice of either genotype (Figure 4b).

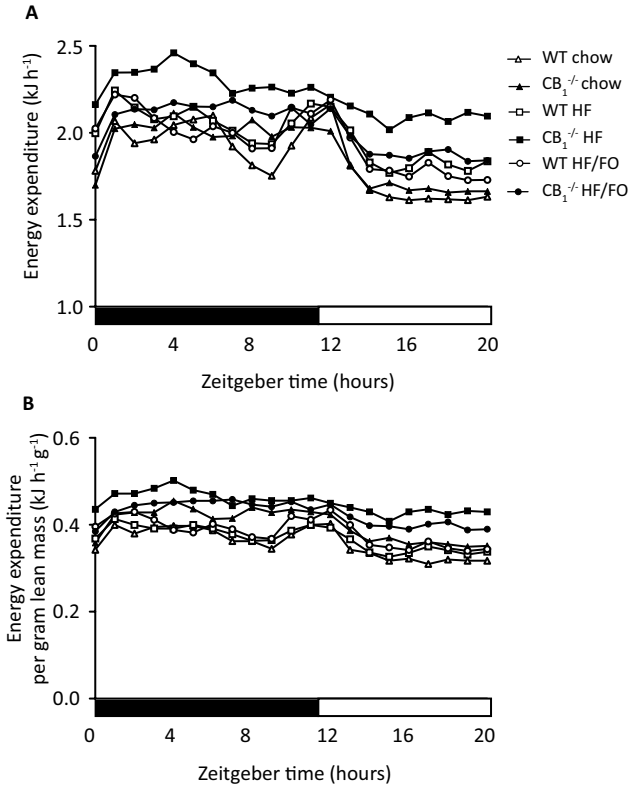

Figure 2. A) Energy expenditure (EE) in male CB_1-deficient ($CB_1^{-/-}$) mice and wild type (WT) mice fed chow, high-fat diet (HF) or high-fat fish-oil enriched diet (HF/FO). Open symbols, WT mice; closed symbols, $CB_1^{-/-}$ mice; triangles, chow; squares, HF; circles, HF/FO. Each data point represents mean for n =5-7 mice. The dark bar on the X-axis represents the dark period. ZT0 (Zeitgeber time) is start of the dark period (*i.e.*, 4PM). **B) Energy expenditure (EE) expressed per gram lean body mass.** Open symbols, WT mice; closed symbols, $CB_1^{-/-}$ mice; triangles, chow; squares, HF; circles, HF/FO. Each data point represents mean for n =4-6 mice. The dark bar on the X-axis represents the dark period. ZT0 (Zeitgeber time) is start of the dark period (*i.e.*, 4PM).

Indirect calorimetry revealed a shift in RQ in mice fed chow during transition from the light period to the dark period and *vice versa*, since FAO predominates in the light period and carbohydrate utilization predominates in the dark period. Chow-fed or HF-

fed $CB_1^{-/-}$ mice showed a pronounced shift in RQ during transition from dark period to the light period and *vice versa* as compared with that in WT mice fed these diets. In the dark period, mice of either genotype had similar RQ with values depending on the diet. In the light period, however, $CB_1^{-/-}$ mice fed chow or HF-diet exhibited significantly lower RQ, resulting in greater shift in RQ in $CB_1^{-/-}$ mice as compared with WT mice (Figure 3). This effect was most pronounced in $CB_1^{-/-}$ mice fed chow. As compared with that in WT controls, chow-fed $CB_1^{-/-}$ mice not only exhibited a faster decline in RQ in the light period, but they also showed a slower return to the levels of RQ in the dark phase (Figure 3). Thus, in the transition from dark to light phase, $CB_1^{-/-}$ mice demonstrated an increased shift towards FAO compared to WT mice.

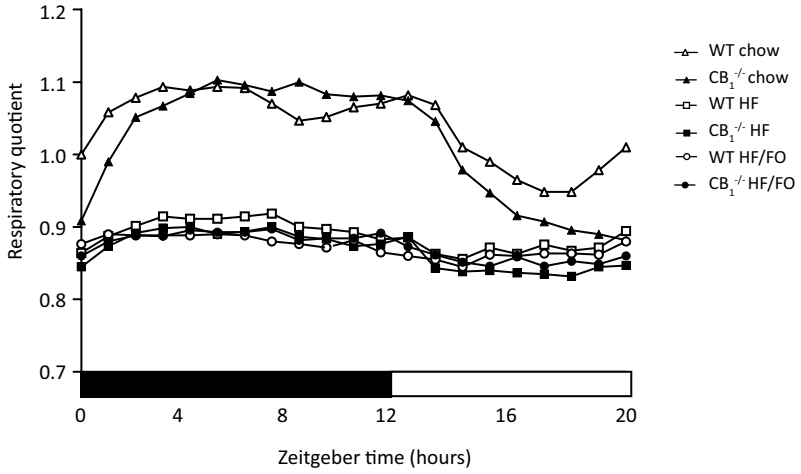

Figure 3. Respiratory quotient (RQ) in male CB_1-deficient ($CB_1^{-/-}$) mice and wild type (WT) mice fed chow, high-fat diet (HF) or high-fat fish-oil enriched diet (HF/FO). Open symbols, WT mice; closed symbols, $CB_1^{-/-}$ mice; triangles, chow; squares, HF; circles, HF/FO. Each data point represents mean for n =6-8 mice. The dark bar on the X-axis represents the dark period. ZT0 (Zeitgeber time) is start of the dark period (*i.e.*, 4PM).

When corrected for lean body mass, besides the still significant increase in FAO in $CB_1^{-/-}$ mice as compared with that in WT mice in the light phase, GLM repeated measures analysis revealed a significant increase in carbohydrate utilization in the dark phase. Again this effect was most pronounced in the chow-fed mice (Figure 4c and d).

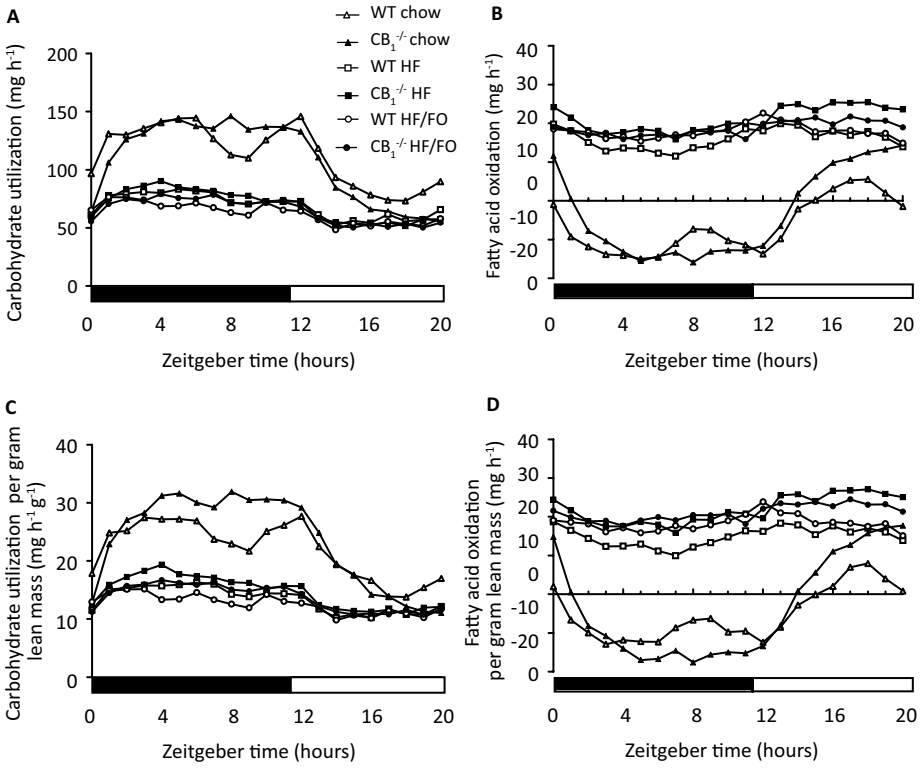

Figure 4. A) Fatty acid oxidation (FAO) calculated from oxygen consumption and carbodioxide production in male CB_1-deficient ($CB_1^{-/-}$) mice and wild type (WT) mice fed chow, high-fat diet (HF) or high-fat fish-oil enriched diet (HF/FO). B) Carbohydrate utilization (CHO ox) calculated from oxygen consumption and carbodioxide production in male $CB_1^{-/-}$ mice and WT mice fed chowHF or HF/FO. Open symbols, WT mice; closed symbols, $CB_1^{-/-}$ mice; triangles, chow; squares, HF; circles, HF/FO. Each data point represents mean for n =5-7 mice. The dark bar on the X-axis represents the dark period. ZT0 (Zeitgeber time) is start of the dark period *(i.e.*, 4PM). **C) Fatty acid oxidation (FAO) expressed per lean body mass. D) Carbohydrate utilization (CHO ox) expressed per lean body mass**. Open symbols, WT mice; closed symbols, $CB_1^{-/-}$ mice; triangles, chow; squares, HF; circles, HF/FO. Each data point represents mean for n =4-6 mice. The dark bar on the X-axis represents the dark period. ZT0 (Zeitgeber time) is start of the dark period (*i.e.*, 4PM).

Blood glucose, plasma insulin, FFA and ketone bodies levels in the middle of the light phase in $CB_1^{-/-}$ mice

Table 2 shows blood glucose levels, and plasma levels of FFA, β-HB, lactate, and insulin. Plasma FFA levels were significantly decreased in mice fed HF/FO compared to mice fed HF diet (p = 0.000) and plasma FFA levels were significantly decreased in $CB_1^{-/-}$ fed chow compared to WT controls (p = 0.029). We found no differences between diet groups or genotypes in ß-HB and lactate. For glucose no significant effect of diet was observed. However, $CB_1^{-/-}$ mice fed HF diet showed decreased glucose concentrations as compared with that in WT controls (p = 0.002). Glucose levels of HF/FO-fed $CB_1^{-/-}$ mice were also decreased; however this difference did not reach statistical significance. Insulin levels did not differ between diet groups or genotypes.

Changes in fuel partitioning in $CB_1^{-/-}$ mice can not be attributed to shifted circadian pattern in food intake

Energy expenditure corrected for lean body mass appears increased in $CB_1^{-/-}$ mice in the light phase as well as the dark phase and, apparently this increase is accounted for in the light phase by increased fatty acid oxidation whereas in the dark phase it is accounted for by increased carbohydrate utilization. Since this changed pattern in fuel partitioning might be based on a shifted pattern in food intake, we analyzed 24 hour food intake pattern in $CB_1^{-/-}$ mice and WT mice fed chow, the latter being the diet with the most pronounced differences in RQ and substrate oxidation. Measurements of 2h-intake of food over a 24h- period did not reveal changes between $CB_1^{-/-}$ and WT mice in temporal patterning of food intake (Figure 5).

Table 2. Plasma free fatty acids, lactate, β-hydroxybutyrate, glucose and insulin in cannabinoid 1-receptor deficient ($CB_1^{-/-}$) mice and wild type (WT) mice fed chow, high-fat (HF) diet or high-fat fish-oil enriched (HF/FO) diet between ZT17 and ZT19 (between 9 AM and 11 AM) after 3 hours fasting

	chow		HF		HF/FO	
	WT	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**	**WT**	**$CB_1^{-/-}$**
Plasma free fatty acids (uM)	588 ± 47	422 ± 45#	598 ± 33	588 ± 51	438 ± 33‡	370 ± 26
Plasma lactate (mM)	6,92 ± 0,88	5,80 ± 0,45	5,14 ± 0,38	5,93 ± 0,72	5,38 ± 0,52	4,67 ± 0,50
Plasmaß-hydroxybutyrate (mM)	0,17 ± 0,02	0,19 ± 0,04	0,18 ± 0,03	0,21 ± 0,03	0,25 ± 0,01	0,23 ± 0,02
Blood glucose (mM)	9,3 ± 0,5	7,7 ± 0,8	9,1 ± 0,3	7,4 ± 0,4$	9,0 ± 0,4	8,1 ± 0,3&
Plasma insulin (pg ml^{-1})	334 ± 53	356 ± 86	325 ± 39	270 ± 64	396 ± 79	271 ± 73

Values are means +/- SEM; n=6-8; ‡ $p < 0.05$ HF *vs* HF/FO, # $p<0.05$ CH $CB_1^{-/-}$ vs CH WT, $ HF $CB_1^{-/-}$- *vs* HF WT, & HF/FO $CB_1^{-/-}$ *vs* HF/FO WT ZT, zeitgebertime; ZT0 is the start of the dark period (*i.e.*, 4PM)

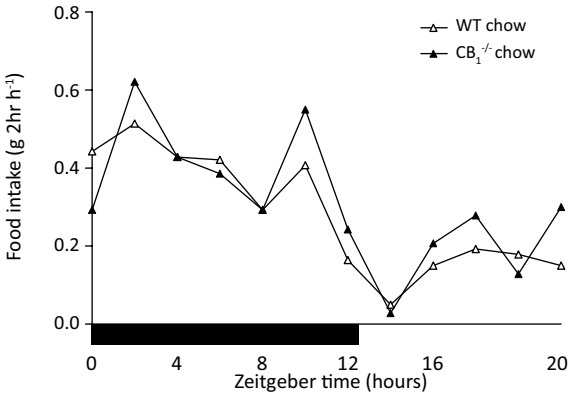

Figure 5. **Circadian rhythm of food intake in male CB_1-deficient ($CB_1^{-/-}$) mice and wild type (WT) mice fed chow.** Food intake was estimated every 2 hours for a 24-hour period. Open symbols, WT mice; closed symbols, $CB_1^{-/-}$ mice. Each data point represents mean for n =7 mice; n =7 mice. ZT0 (Zeitgeber time) is start of the dark period (*i.e.*, 4PM). No significant differences.

Discussion

Results of our experiments demonstrate that increased energy expenditure corrected for lean body mass, in the dark period as well as in the light period, contributes to the observed lean phenotype of fat-fed $CB_1^{-/-}$ mice. According to our expectation, a significantly increased FAO during the light period as calculated from oxygen consumption and carbon dioxide production was observed to underlie this effect in $CB_1^{-/-}$ mice compared to WT mice. In the dark period, however, differences in energy expenditure could not be accounted for by fatty acid oxidation, but by increased carbohydrate utilization instead. Finally, our data suggest involvement of CB_1-mediated endocannabinoid signalling in substrate shift during the transition from the active to the inactive phase and *vice versa*.

Our data are consistent with previous reports describing a phenotype of $CB_1^{-/-}$ mice on both chow and HF-diet that is characterized by a lower body weight gain and decreased adiposity compared to WT mice, independent of caloric intake.[1,9-12] Previously, we found no increases in fecal energy loss in mice treated with rimonabant compared to controls (chapter 3)[5] or in $CB_1^{-/-}$ mice compared to WT mice (chapter 5), disqualifying a potential role for intestinal malabsorption. In chapter 5, evaluating energy balance of $CB_1^{-/-}$ mice compared to WT mice fed chow, HF or HF/FO, we did

observe an increase in daily energy expenditure. Results described in this chapter show that energy expenditure expressed per lean mass is increased in $CB_1^{-/-}$ mice compared to WT in both the light period and the dark period. A remarkable finding of this study is the effect of genetic CB_1-ablation on circadian variation in RQ and, thus in substrate oxidation. In the light period, FAO expressed per lean mass is increased in $CB_1^{-/-}$ mice compared to WT whereas carbohydrate utilization expressed per lean mass is increased in the dark period. Thus, the overall increased energy expenditure in $CB_1^{-/-}$ mice compared to WT mice was found to be fuelled differently during the light (i.e., by increased FAO) *versus* the dark phase (i.e., by increased carbohydrate utilization). Consequently, there is a more pronounced substrate shift in $CB_1^{-/-}$ mice compared to WT controls during transition from the light period to the dark period. Since these observed CB_1-mediated effects on diurnal patterns in fuel utilization might be a reflection of a different pattern in food intake in the $CB_1^{-/-}$ mice compared to WT mice, we investigated circadian rhythm of food intake. This study was conducted in mice fed chow, because the largest differences in diurnal patterns in fuel utilization were found in mice fed chow. Diurnal patterns of food intake did not differ between $CB_1^{-/-}$ mice and WT controls, indicating that the more pronounced substrate shift from carbohydrate utilization in the dark period towards fat oxidation in the light period in $CB_1^{-/-}$ mice can not be attributed to a changed pattern in food intake.

Herling *et al*. suggested that increased energy expenditure in $CB_1^{-/-}$ mice is due to enhanced oxidation of fatty acid which is permitted by increased lipolysis of triglycerides in adipose tissue.[13] If this chain of events indeed occurs, one would expect that temporal patterns of energy expenditure observed in $CB_1^{-/-}$ mice would be synchronized with changes in fatty acid oxidation. The results of our experiments are only in agreement with this view during the light period, in which fatty acid oxidation prevails. Involvement of CB_1-mediated signalling specifically related to carbohydrate utilization during the dark period should also be considered. Indeed, in another report by Herling *et al.,*[14] levels of liver glycogen exhausted more rapidly in rats treated with a CB_1-receptor antagonist than in untreated controls.

An important question is whether low activity of the ECS leads to an increased mitochondrial fatty acid availability driving fatty acid oxidation or to an increased fatty acid oxidation that reduces fatty acid availability for accumulation. In the first scenario, a possible mechanism could be imagined allowing increased entry of fatty acids in mitochondria and/or decreased entry of fatty acids into adipose tissue. Data described in chapter 5 do not support a decreased uptake of fatty acids in adipose tissue, while

Flamment *et al.*[15] did demonstrate increased entry of fatty acids in mitochondria from rats treated with rimonabant. The next issue then is whether the increased entry into metabolically active tissue is "pushing" fatty acid oxidation or whether increased fatty acid oxidation is "pulling" fatty acid translocation (schematically depicted in Figure 6).

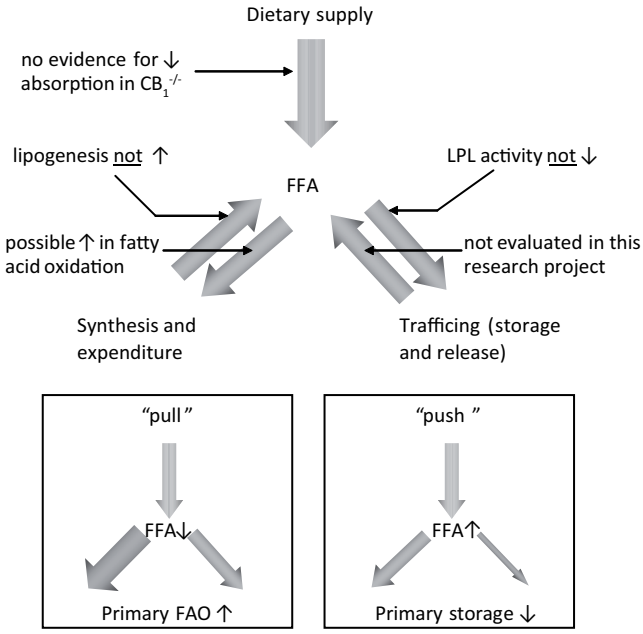

Figure 6. Schematic representation of the possible relations between fatty acid oxidation (FAO) and levels of free fatty acids (FFA) as influenced by FAO itself, lipogenesis, fatty acid storage and release of fatty acids from adipose tissue.

This issue was also raised by Flamment *et al.*[15] They suggested that the rimonabant-induced increased entry of fatty acids into mitochondria may attribute to the increase in fatty acid oxidation. In a simplified model depicting potential mechanisms underlying increased lipid oxidation, increased "push" of fatty acid would be expected to result in elevation of available circulating free fatty acid (FFA), whereas increased "pull" would result in decreased availability of circulating FFA. In our studies, we observed decreased availability of circulating FFA (reflected by plasma FFA levels) in HF/FO-fed WT mice as compared to WT mice fed either chow or HF diet. No differences in plasma FFA levels were found between WT mice and $CB_1^{-/-}$ mice fed HF diet or HF/FO diet. However,

plasma FFA levels in chow-fed $CB_1^{-/-}$ mice were significantly decreased as compared with that in chow-fed WT littermates which also corresponds to the fact that chow-fed $CB_1^{-/-}$ mice and WT mice showed the largest differences in diurnal shift of substrate oxidation. Collectively, our data suggest a primary “pull” of fatty acids through increased fatty acid oxidation at least in $CB_1^{-/-}$ mice fed chow.

Taken together, the increased energy expenditure in $CB_1^{-/-}$ mice as compared with that in WT littermates is apparently not driven by increased fatty acid oxidation during the entire circadian cycle. In fact, our data suggest that increased energy expenditure in $CB_1^{-/-}$ mice is fuelled differently with with an increased fatty acid oxidation in the light period and an increased carbohydrate utilization in the dark period. Future experiments should be designed to investigate these specific effects.

Acknowledgements

The authors thank Gerard Overkamp, Juul F.W. Baller, Pieter de Boer, Stefano Guidotti, Gretha Boersma and Tineke Jager for excellent technical assistance.

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CHAPTER 7

Summary and general discussion

Summary

In most mammalian species, including humans and rodents, the pattern of food intake is characterized by specific meals that are interspaced by periods of varying duration. This is allowed because ingested nutrients can be stored in the form of glycogen and triglycerides which buffer the flow of fuels that maintain ongoing cellular oxidative phosphorylation. Several signalling and effector systems are essential to maintain energy balance and fuel homeostasis over prolonged periods of time. Over the last two decades, knowledge on mechanisms that regulate energy balance and fuel compartmentalization and metabolism has truly exploded. A recently discovered system involved in this regulation is the endocannabinoid system (ECS). This system consists of cannabinoid receptors (*i.e.*, CB_1- and CB_2-receptors), endogenous ligands for these receptors called endocannabinoids and enzymes involved in synthesis and degradation of endocannabinoids. Among many functions, the ECS was found to be involved in regulation of food intake *via* the CB_1-receptor. Studies in animals and humans showed that blockade of CB_1-signalling causes temporal reduction in food intake and sustained reduction in body weight gain. When the reduced daily food intake of CB_1-receptor deficient ($CB_1^{-/-}$) mice was forced onto wild type (WT) mice for several weeks, $CB_1^{-/-}$ mice were still leaner than WT mice. This demonstrates that CB_1-mediated effects on body weight are partly independent of food intake and suggests that the ECS not only plays a pivotal role in regulation of feeding behaviour, but also in modulation of peripheral metabolism. Moreover, CB_1-blockade causes prevention of lipid accumulation in the bloodstream and in the liver, which are risk factors for development of hepatic steatosis, atherosclerosis, and insulin resistance, all components of the metabolic syndrome.[1-3] Since these effects are observed independently of weight loss, they support the notion that regulation of peripheral metabolism contributes to the metabolic effects of endocannabinoid signalling. The aim of the work described in this thesis was to explore involvement of the ECS in modulation of energy balance in mice and, more specifically, the effects of endocannabinoid signalling in fuel partitioning and metabolism. The main results of this research are summarized and discussed below.

Cannabinoid-1 receptor deficient mice

Since metabolic effects of endocannabinoid activation are found to be mostly CB_1-mediated, $CB_1^{-/-}$ mice (i.e., generated by Zimmer *et al.*) represent a valid animal model to study contribution of endocannabinoid signalling to fuel partitioning and metabolism.

The first study was designed to determine whether the ECS system plays a role in neonatal energy balance regulation by evaluating the phenotype of $CB_1^{-/-}$, $CB_1^{+/-}$, and $CB_1^{+/+}$ pups born from heterozygous ($CB_1^{+/-}$) breeding couples (**chapter 2**). Compared to WT littermates, $CB_1^{-/-}$ pups at three weeks of age not only showed significantly lower body weights but also reduced fat mass. These results reinforce a role for CB_1-signalling in neonatal thrift, as has been pointed out by Fride *et al.*[4] In addition to the results published by Fride *et al.*, the data in Chapter 2 demonstrate that the phenotypic differences between $CB_1^{-/-}$ and WT mice cannot be attributed to maternal genotype, an issue left unresolved in previous research. Moreover, the attenuation of neonatal thrift was more pronounced in male pups than in female pups. Findings described in chapters 3, 4, 5 and 6 are based on results derived from experiments conducted in male mice only.

The endocannabinoid system and lipid metabolism

The weight-reducing effects of suppressed endocannabinoid signalling were previously suggested to rely, at least in part, on changes in lipid fluxes. Considering that endocannabinoids are lipid signalling molecules derived from long chain polyunsaturated fatty acids (LC-PUFA) and that specific fatty acids exert pronounced effects on lipid fluxes, it was expected that not only dietary fat content but also dietary fatty acid composition would influence the outcome of impaired CB_1-signalling. For this reason, the effects of the receptor antagonist SR141716, with the generic name rimonabant, were investigated in mice fed either standard laboratory chow (*i.e.*, low-fat, high-carbohydrate fibered diet), a high saturated fat (HF) diet, or a HF diet in which part of the saturated fat was replaced by fish oil (HF/FO) (**chapter 3**). Since feeding a HF diet was found previously to increase hepatic lipogenesis and feeding a HF/FO was found to suppress hepatic lipogenesis, we were especially interested in the interaction between dietary supplementation of LC-PUFA (*i.e.*, abundant in fish oil) and treatment with rimonabant. As expected, feeding a HF diet to control mice induced dyslipidemia and hepatic steatosis which were not found in control mice fed the HF/FO diet. However, at the same time, the HF/FO diet was found to be most adiposity-promoting and effects of treatment with rimonabant to prevent weight gain and adiposity were the largest in the HF/FO group. This is in contrast with the previously suggested role of hepatic lipogenesis in the anti-adiposity effects of CB_1-blockade since lipogenesis is already remarkably suppressed in mice on HF/FO enriched diet. In mice receiving a HF diet as well as in mice receiving either chow or HF/FO diet, rimonabant-treatment led

to significantly lower body weight gain than their respective controls without major changes in nutrient absorption or energy expenditure.

Although it was argued that suppressed hepatic lipogenesis is not responsible for the anti-adiposity effect of CB_1-blockade, suppressing hepatic lipogenesis was not excluded as a mechanism responsible for resistance to diet-induced hepatic steatosis after CB_1-blockade.

To determine the contribution of hepatic lipogenesis to CB_1-blockade-mediated resistance to diet-induced hepatic steatosis, hepatic lipogenesis in $CB_1^{-/-}$ mice and WT littermates on either chow, a HF diet or HF/FO diet was evaluated (**chapter 4**). Based on the results described in this chapter, three arguments can be put forward to exclude the possibility that suppressed lipogenesis is a major factor in resistance to diet-induced hepatic steatosis in $CB_1^{-/-}$ mice. First of all, on all three diets, no suppression of hepatic lipogenesis was found in $CB_1^{-/-}$ mice compared to WT mice by measuring incorporation of labelled acetate into fatty acids. Secondly, compared to WT littermates, $CB_1^{-/-}$ mice fed HF/FO diet also showed a significant reduction of hepatic triglyceride content, also suggesting that hepatic lipogenesis does not contribute to this effect since lipogenesis is already suppressed in WT mice fed HF/FO diet. Finally, hepatic fatty acid profiles of $CB_1^{-/-}$ mice fed any of the three experimental diets not only showed decreased levels of C16:0 and C18:1 compared to WT littermates, but also decreased levels of C18:2. Since C18:2 is an essential fatty acid, this indicates that not suppressed *de novo* lipogenesis, but rather decreased fatty acid uptake or increased fatty acid oxidation is involved in resistance to diet-induced hepatic steatosis in $CB_1^{-/-}$ mice. Furthermore, results described in **chapter 4** confirmed that VLDL-production is not affected by genetic ablation of CB_1-receptors. Thus, results of **chapter 3 and 4** indicate that the anti-adiposity effect of treatment with rimonabant can not be attributed to an effect on hepatic lipogenesis, nor can resistance to diet-induced hepatic steatosis in $CB_1^{-/-}$ mice be attributed to suppressed hepatic lipogenesis or increased VLDL-production. Results so far suggested a decrease in fatty acid uptake by the liver or an increase in fatty acid oxidation as a contributor to resistance to hepatic steatosis in $CB_1^{-/-}$ mice.

To further explore the relation between endocannabinoid signalling and lipid fluxes, we evaluated effects of CB_1-deficiency on adipogenesis, adipose tissue lipolysis and adipose tissue lipogenesis in relation to dietary fat intake (**chapter 5**). On all three experimental diets, $CB_1^{-/-}$ mice were resistant to obesity and showed a reduced fat mass compared to WT mice. Adipocyte size was reduced in $CB_1^{-/-}$ mice compared to WT littermates under comparable dietary conditions. No differences were observed

between $CB_1^{-/-}$ mice and WT mice in expression of differentiation and proliferation markers in adipose tissue. Rates of adipocyte fatty acid synthesis were only stimulated by HF feeding only and CB_1-deficiency did not reduce fatty acid synthesis in adipose tissue. Post-heparin and adipose tissue-derived LPL activity were comparable in all groups. It is therefore concluded that the resistance of $CB_1^{-/-}$ mice to high-fat diet-induced obesity is not related to altered adipocyte function *per se* but rather results from a lower amount of energy available for storage due to an increase in energy expenditure.

The endocannabinoid system, energy expenditure and substrate utilization

In **chapter 6** the effects of endocannabinoid signalling on energy expenditure and substrate utilization were explored in relation to circadian rhythm. Based on previous results, showing decreased RQ in mice treated with rimonabant compared to controls, and based on reported data suggesting increased fatty acid oxidation in $CB_1^{-/-}$ mice underlying increased energy expenditure, we expected energy expenditure in $CB_1^{-/-}$ mice to be temporally related to increased rate of fat oxidation. Since carbohydrate and fat oxidation are known to fluctuate over the diurnal cycle - with high levels of fat oxidation during the inactive (*i.e.* light) phase and increased carbohydrate oxidation during the active (*i.e.*, dark) phase – we focussed on diurnal variations in energy expenditure and substrate utilization in $CB_1^{-/-}$ mice and wild type littermates subjected to either chow, a HF diet or HF/FO diet (**chapter 6**). The results showed that energy expenditure normalized for lean mass is increased in $CB_1^{-/-}$ mice compared to WT in both the inactive phase and the active phase. Remarkably, this increased expenditure is fuelled differently during the day with an increased fatty acid oxidation expressed per lean mass in the inactive phase and increased carbohydrate oxidation expressed per lean mass in the active phase. Thus, these data do not support a role for fatty acid oxidation in driving energy expenditure in CB_1-deficient mice. This is supported by significantly decreased plasma free fatty acid (FFA) availability in $CB_1^{-/-}$ mice halfway the inactive phase. Diurnal patterns of food intake did not differ between $CB_1^{-/-}$ mice fed chow and WT controls, indicating that the more pronounced substrate shift towards fatty acid oxidation in the inactive phase in $CB_1^{-/-}$ mice and towards carbohydrate oxidation in the active phase can not be attributed to a changed pattern in food intake.

General discussion

The endocannabinoid system and lipid metabolism

Data described in this thesis demonstrate that both pharmacological blockade of the CB_1-receptor and targeted deletion of the CB_1-receptor result in decreased body weight gain and reduced body fat mass compared to respectively vehicle-treated or WT controls, confirming the association between a lean phenotype and disruption of CB_1-signalling (**chapter 2-6**). However, contrary to the hypothesis suggested by others that this lean phenotype is based on primary changes in adipose tissue functioning itself (**chapter 5**), a reduced availability of fatty acids left for storage appears to be a key issue in the effect of CB_1-disruption. This reduced availability of fatty acids is not a consequence of changes in lipogenesis, since lipogenesis *in vivo* neither in adipose tissue nor in the liver differed between $CB_1^{-/-}$ and WT mice (**chapter 4 and 5**). Potentially, increased fatty acid oxidation, as suggested by results described in this thesis (**chapter 4 and 6**) and by results reported by others, might contribute to decreased availability (Figure 1).

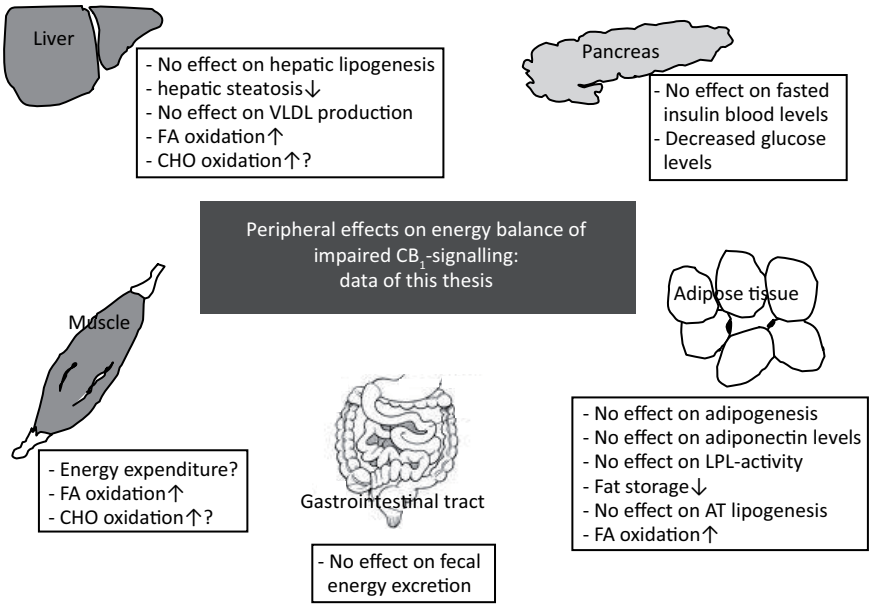

Figure 1. Summarized of peripheral effects of impaired CB_1-signalling on energy balance based on data described in this thesis. Abbreviations; VLDL, very low density lipoproteins; FA, fatty acid; CHO, carbohydrate; LPL, lipoprotein lipase; AT, adipose tissue.

An important question is whether low activity of the ECS leads to an increased mitochondrial fatty acid availability driving fatty acid oxidation (for example as a consequence of decreased uptake of fatty acids by adipose tissue) or an increased fatty acid oxidation reducing fatty acid availability for fat accumulation. Data described in **chapter 5** do not support a decreased uptake of fatty acids in adipose tissue. An increased entry of fatty acids into mitochondria was put forward by Flamment *et al.*[5] leading in turn to the discussion whether this increased entry is either the consequence of increased fatty acid oxidation or causing increased fatty acid oxidation (**chapter 6 figure 6**). Flamment *et al.,* based on their data on mitochondrial function in rats treated with rimonabant, suggested that the rimonabant-induced increased entry of fatty acids into mitochondria may contribute to the increase in fatty acid oxidation. Our data showing significantly decreased plasma FFA levels in $CB_1^{-/-}$ mice fed compared to WT littermates suggest that increased fatty acid oxidation is the primary factor (**chapter 6**). In this event, FFA are pulled into mitochondria away from plasma to fuel fatty acid oxidation.

Part of the controversy on primary and secondary mechanisms by which reduced activity of CB_1-signalling contributes to leanness is explained by short-term *versus* chronic manipulation of the CB_1-signalling. For example, Herling *et al.* reported an immediate increase in plasma FFA level after a single dose of rimonabant administered to rats in the postprandial state and concluded that rimonabant induces lipolysis *in vivo*.[6] By contrast they observed that long-term treatment with rimonabant reduced plasma FFA levels and they stated that this reflected decreased fat mass. They hypothesized that treatment with rimonabant results in mobilization of fat stored in adipocytes *via* stimulation of lipolysis. According to their hypothesis this mobilization of fat initially results in increased plasma FFA levels, whereas later on, when total fat mass is reduced, plasma FFA levels will decrease during treatment with rimonabant.[6] Although acute administration of compounds can easily induce non-physiological changes, the point raised by Herling *et al.* has to be taken into account to fully understand the driving forces of CB_1-mediated changes in lipid metabolism. Taken together, increased fatty acid oxidation appears to contribute to the lean phenotype of $CB_1^{-/-}$ mice. To establish in animal models the contribution of fatty acid oxidation to diet-induced increases in adiposity and diet-induced hepatic steatosis it is essential to determine changes in lipid fluxes over time. This is a challenging task since several intermediates of lipid metabolism are involved and have to be taken into account.

Interaction of dietary LC-PUFA intake and endocannabinoid tone

Long chain polyunsaturated fatty acids (LC-PUFA), abundant in fish oil, are suggested to favourably affect metabolic risk factors like hepatic steatosis and insulin resistance compared to saturated fatty acids. They improve plasma lipid profiles and suppress *de novo* lipogenesis. Because of the suppression of hepatic lipogenesis demonstrated in mice fed HF/FO diet and the suggested role of CB_1-mediated hepatic lipogenesis in the development of obesity and hepatic steatosis, we expected mice fed HF/FO 1) to have a reduced fat mass; 2) to be resistant to diet-induced hepatic steatosis; and 3) to be less sensitive to the effects of treatment with a CB_1 receptor antagonist. The work described in this thesis demonstrates indeed favourable effects on dyslipidemia and hepatic steatosis after supplying a HF/FO diet to young male mice. However, this HF/FO diet was found to be remarkably obesity-promoting and although anti-obesity effects of pharmacological CB_1-blockade and CB_1-deficiency were observed in mice on either chow or HF diet or HF/FO diet, the most prominent effects of CB_1-blockade to cause body weight loss and reduced adiposity were observed in the HF/FO group. A possible explanation for the obesity-inducing effects of dietary LC-PUFA replacement in a HF diet might involve expansion of adipose tissue *via* increased adiponectin as was proposed by Kim *et al.*[7] This adipose tissue expansion might allow the favourable effects of dietary LC-PUFA to ameliorate dyslipidemia and hepatic steatosis , and this is apparently mediated *via* the CB_1-receptor (this thesis).

Another explanation for an interaction between dietary LC-PUFA content and treatment effect of a CB_1-receptor antagonist would be an effect of dietary LC-PUFA content on endocannabinoid tone. As endocannabinoid precursors, LC-PUFA could theoretically influence activity of the endocannabinoid system. There are reports suggesting decreased endocannabinoid levels following dietary LC-PUFA supplementation.[8,9] In contrast, results of others point in the direction of a stimulating effect of LC-PUFA on the ECS.[10] Overactivity of the endocannabinoid system is associated with increases in body adiposity. Therefore, an interesting explanation for the significantly increased body weight and fat gain in mice fed HF/FO diet compared to mice fed chow and mice fed HF diet would be a stimulation of the endocannabinoid system by dietary LC-PUFA supplementation. If dietary fish oil stimulates endocannabinoid activity, treatment with a CB_1-receptor antagonist would have a stronger effect in mice on HF/FO diet than in mice fed a HF diet without LC-PUFA supplementation. Although we were not able to detect an effect of HF/FO diet on expression of CB_1, FAAH (responsible for degradation of anandamide) or NAPE-PLD

(responsible for synthesis of anandamide) (data not shown), these data do not exclude an interaction between fish-oil supplementation and activity of the endocannabinoid system. To reveal a potential interaction it is necessary to directly determine the effect of dietary fish oil on endocannabinoid levels in relevant tissues. A pitfall of investigating effects of fish-oil supplementation on endocannabinoid signalling is that the ratio n-3 to n-6 PUFA can vary between various forms of fish oil. This ratio could be responsible for differential effects on endocannabinoid tone with n-6 PUFA presumably increasing endocannabinoid levels *via* increased precursor availability and n-3 PUFA suppressing endocannabinoid levels *via* competing with n-6 for location in membranes. This could explain controversy in reported findings.

It is especially relevant to further investigate an interaction between fish-oil supplementation and activity of the endocannabinoid system also in relation to fish-oil supplementation-induced expansion of adipose tissue mass, since fish oil is nowadays broadly advertised as being beneficial for health. In fact, LC-PUFA are being added to many food products (*i.e.*, butter, bread, oil), before having evaluated all advantages and disadvantages thoroughly.

Endocannabinoid interference with energy balance

Weight gain and weight loss result from an imbalance between energy intake and energy expenditure. Consequently, reduced body weight gain is either caused by decreased food intake or increased energy expenditure or both. As such, indirect calorimetry in **chapters 5 and 6** identified differences in energy expenditure between $CB_1^{-/-}$ and WT mice, which could contribute to the resistance of (diet-induced) weight gain in the first. However, results of experiments described in **chapter 3** of this thesis revealed a dramatic reduction in body weight gain and adiposity in rimonabant-treated mice *versus* controls despite a transient and small reduction in food intake and no increase in oxygen consumption (Figure 2).

Since differences in absorption were also excluded by measuring fecal energy contents, these results suggest a form of energy dissipation in mice treated with rimonabant independent from oxygen consumption. Indirect calorimetry offers an estimation of energy expenditure based on oxygen consumption whilst oxygen consumption might result in different amounts of ATP-production. The latter is, for example, the case when the coupling between ongoing oxidative phosphorylation and the proton gradient across the mitochondrial membrane is disrupted. This example

illustrates the limitation of indirect calorimetry in estimating energy expenditure *in vivo*.

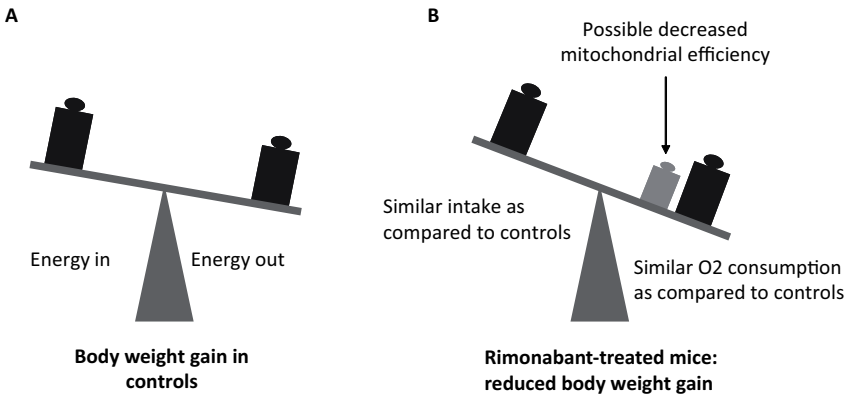

Figure 2. Schematic representation of presumed effect of impaired CB1-signalling on energy balance.

Several studies indicate that decreased mitochondrial efficiency is indeed involved in resistance to weight gain in mice treated with rimonabant. First, microarray analysis of transcriptional patterns in adipose tissue after treatment with rimonabant in WT and $CB_1^{-/-}$ mice fed HF diets indicated increased energy expenditure mainly through futile cycling (*via* simultaneous induction of glycogen phosphorylase and glycogen synthase and *via* induction of expression of calcium-binding proteins that transport calcium across membranes in opposite directions).[11] Secondly, Shearman *et al.* showed induction of *uncoupling protein 1* and *uncoupling protein 3* mRNA expression in brown adipose tissue of mice with diet-induced obesity that were treated for 9 days with a CB_1-antagonist.[12] Moreover, Tedesco *et al.* described that rimonabant increased mitochondrial DNA amount, mRNA levels of genes involved in mitochondrial biogenesis, mitochondrial mass and function, *via* eNOS induction as discussed in **chapter 6**.[13] Finally, Flamment *et al*. determined mitochondrial enzyme activity in isolated liver mitochondria, mitochondrial DNA quantity and oxygen consumption with various substrates in Sprague Dawley rats on HF diet treated with rimonabant for three weeks compared to vehicle-treated, pair-fed controls. They showed that treatment with rimonabant results in improvement of mitochondrial function *via* facilitation of substrate oxidation and *via* increasing fatty acid entry into mitochondria and increasing proton leak (*i.e.*, uncoupling).[5]

In case of uncoupling or other forms of energy dissipation, energy is thought to be lost as heat. We therefore conducted a pilot experiment to evaluate whether mice generate more heat when treated with rimonabant. We hypothesized that, if decreased mitochondrial efficiency contributes to the lean phenotype upon CB_1-blockade, these mice should generate more heat and radiate this heat to the environment. Hence, these mice should become hyperthermic under enthermic conditions. In contrast to our hypothesis, we found no differences in body temperature between rimonabant-treated mice and vehicle-treated controls when room temperature was maintained at 33°C for 12 hours. Clearly, further studies are needed to determine the role of thermogenesis and mitochondrial efficiency in CB_1-mediated effects on energy balance.

As mentioned above, results of experiments described in **chapter 5 and 6**, comparing $CB_1^{-/-}$ mice to WT mice, did show small elevations in energy expenditure in $CB_1^{-/-}$ mice compared to WT mice. It is, however, conceivable that decreased mitochondrial efficiency also contributes to the observed lean phenotype in $CB_1^{-/-}$ mice. A possible contribution of mitochondrial function to resistance of $CB_1^{-/-}$ mice compared to WT mice to diet-induced weight gain and adiposity-related metabolic derangements needs to be further explored.

If it is believed that hyperactivity of the ECS predisposes to weight gain and related metabolic profiles, then what is the relevance of this system that is so abundantly present in the nervous system and so well-preserved throughout evolution? In 1962, Neel proposed that obesity and associated diseases could be attributed to the existence of 'thrifty genes'.[14,15] According to his hypothesis a "thrifty genotype" is responsible for a phenotype that is exceptionally efficient in intake and utilization of food and hence to be of high selective value in pre-historic times when most species had to survive periods of food deprivation. Based on this theory, most species are biased towards conserving energy, because starvation used to be a major threat for survival. Since the environment has changed dramatically for humans in Western society over a relative short time, this initially successful biologically adaptive mechanism has become maladaptive and contributes to the current pandemic of obesity and associated metabolic derangements.[14,16] Since the function of the ECS in the regulation of fuel metabolism and partitioning can be qualified as "energy-conserving", one can consider endocannabinoid signalling to be an explicit thrifty mechanism. Thus, according to Neel's hypothesis, overactivity of the endocannabinoid system could have been of survival advantage in pre-historic times whereas it is considered "dysfunctional" in many scientific evaluations.[17-20]

A totally different view, but not necessarily excluding the previous one, is that conserving energy serves a purpose in the sense of protecting from oxidative stress as was put forward by Nunn *et al.*[21] These authors stated that endocannabinoids play a role in storing fat safely to prevent toxic effects of fatty acid overload. According to this view, thriftiness should not be considered as a "remnant adaptation following survival advantage to past periods of starvation", but as a pivotal mechanism in dealing with oxidative stress.

Clinical relevance of manipulating the endocannabinoid system in relation to regulation of energy balance

Shifting from fundamental research on the involvement of the ECS in the regulation of energy balance in animal models to clinical implications seems a giant leap. However, the obesity epidemic has led to an unprecedented search for new targets for weight loss medication. Worldwide, 1.6 billion adults (over 15 years of age) are overweight and 400 million adults are obese according to the definition of the World Health organization. In addition, 20 million children under the age of 5 are overweight globally.[22] According to Statistics Netherlands (Centraal Buro voor de Statistiek), based on the criteria of the World Health Organization, one out of nine children is overweight in the Netherlands.[23] Obese patients are at risk for cardiovascular disease and type II diabetes and obesity has been associated with other metabolic risk factors such as hypertension, hepatic steatosis, dyslipidemia, and insulin resistance. Obesity-related diseases like diabetes type II are diagnosed at continuously younger age. These dramatic numbers stress the urgent need to develop strategies to prevent and treat obesity and related derangements. Consequently, it is important to identify mechanisms underlying disturbances in energy balance.

The emerging role of the ECS in the regulation of energy balance and fuel homeostasis seems to offer new possibilities. Interestingly, the endocannabinoid system appears to be involved in several aspects of the complex pathophysiology of obesity and its comorbidities. It is not only involved in rewarding and satiety aspects of food intake, but also in regulation of lipid and glucose metabolism, adipocyte proliferation and differentiation, gastro-intestinal function and energy expenditure. Despite recent registration of anti-obesity drugs based on antagonizing endocannabinoid activity, many aspects of endocannabinoid regulation of energy balance and beyond are still unknown today. Thus, although the CB_1-receptor antagonist rimonabant has already been extensively tested in large clinical trials and was already approved in many

European countries, registration was recently withdrawn due to concerns related to adverse psychological effects. These latter issues are to be taken seriously since subjects with obesity and related metabolic risk factors more frequently suffer from mood disturbances than healthy subjects. So, it is relevant to discuss clinical implications to make the connection between current basal knowledge and clinical practice. Clinical relevant findings hitherto in combination with findings described in this thesis could be summarized as follows:

1. Increased CB_1-mediated endocannabinoid signalling drives obesity and obesity-related diseases.
2. CB_1-mediated endocannabinoid signalling affects weight gain *via* central and peripheral modulation of energy balance.
3. Endocannabinoid signalling *via* CB_1-receptors is involved in many physiological functions including cognitive processes, nociception, emotion, and neuroprotection thus increasing the risk of adverse effects when interfering with endocannabinoid signalling.
4. Increased mortality of $CB_1^{-/-}$ mice and the fact that the ECS is a phylogenetically old system that is well preserved throughout evolution, suggest that endocannabinoid activity is pivotal for survival for example *via* protection against oxidative stress.
5. Males, as compared to females, show a more pronounced phenotype with respect to effects of CB_1-blockade.

Since the recognition of the regulatory role of the ECS in energy balance, it was identified as a promising target for weight loss medication. However, in line with the many physiological functions of the ECS, there is a high risk of adverse, especially centrally-evoked side effects. Targeting future CB_1-antagonists/ inverse agonists to bypass central effects while maintaining peripheral effects on obesity and its comorbidities is a logical next step.[24] Further elucidation of peripheral effects of endocannabinoids on metabolic functioning and fuel partitioning is therefore still relevant.

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APPENDICES

Nederlandse samenvatting (Summary in Dutch)

List of abbreviations

Supplemental material

Dankwoord

Curriculum Vitae

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Nederlandse samenvatting

Obesitas ofwel ernstig overgewicht is een wereldwijd gezondheidsprobleem, niet alleen bij volwassenen maar in toenemende mate ook bij kinderen. Obesitas speelt een grote rol in het ontwikkelen van andere aandoeningen als suikerziekte en hart- en vaatziekten. Aan de basis van het ontstaan van de huidige epidemie van overgewicht ligt een overvloedige inname van nutriënten in combinatie met een minder actieve of passieve levensstijl. Hoewel deze gevolgtrekking op het eerste gezicht eenvoudig is, blijkt de werkelijke achtergrond van de obesitas-epidemie veel complexer te zijn en interacties tussen genetische, psychologische, pedagogische, socio-economische, dieet- en epigenetische factoren te omvatten. Opmerkelijk is dat individuen die ogenschijnlijk blootgesteld zijn aan dezelfde omgevingsfactoren een grote variatie kunnen vertonen in de ontwikkeling van overgewicht. Zowel vanuit fundamenteel als klinische oogpunt is het interessant om meer inzicht te verkrijgen in de intrinsieke regelmechanismen die deze variatie kunnen verklaren. Een veelbelovende kandidaat op dit vlak is het endocannabinoïd systeem (ECS).

De naam 'endocannabinoïd systeem' is afkomstig van cannabis. Cannabinoïd-receptoren worden gestimuleerd door o.a. Δ9-THC, het belangrijkste psychoactieve bestanddeel van cannabis. Twee decennia geleden werden deze cannabinoïd receptoren geïdentificeerd, aanvankelijk in dieren maar later ook in mensen. Vervolgens werden lichaamseigen stoffen ontdekt die op deze receptoren aangrijpen: deze worden endocannabinoïden genoemd. Er zijn verschillende cannabinoïd-receptoren waarvan de cannabinoïd-1-receptor (CB_1) en de cannabinoïd-2-receptor (CB_2) het bekendst zijn. CB_1-receptoren komen vooral voor in het centraal zenuwstelsel, maar ook buiten de hersenen, bijvoorbeeld in lever-, in spier- en in vetweefsel. CB_2-receptoren komen vooral voor op cellen van het afweersysteem. De CB_2-receptor valt buiten het bestek van dit proefschrift, aangezien het niet of nauwelijks betrokken is bij regulatie van de energiebalans. Opmerkelijk is dat endocannabinoïden gevormd worden uit langketen meervoudig onverzadigde vetzuren (LCPUFA, *o.a.* omega-3 en omega-6 vetzuren). Deze vetzuren komen veel voor in visolie. Verondersteld wordt dat de hoeveelheid omega-3 en omega-6 vetzuren in het dieet van invloed is op de activiteit van het endocannabinoïd systeem.

Van cannabis is al sinds de oudheid bekend dat het een stimulerend effect heeft op eetlust en tegenwoordig wordt Δ9-THC gebruikt om eetlust te stimuleren bij oncologische patiënten. Op grond van deze observatie is remming van de activiteit

van het endocannabinoïd systeem onderzocht als een mogelijk aangrijpingspunt voor het ontwikkelen van medicijnen voor de preventie en behandeling van obesitas. De eerste CB_1-antagonist, rimonabant genaamd, bleek in studies bij zowel dieren als mensen gewichtstoename te voorkomen. Aanvankelijk werd verondersteld dat het gewichtsverminderende effect van blokkeren van de CB_1-receptor een exclusief gevolg is van het remmen van eetlust. Echter, onafhankelijk van het effect op gewicht werden ook gunstige effecten van behandeling met rimonabant op andere risicofactoren van overgewicht gevonden zoals verlaging van plasmacholesterol en verbeterde insulinegevoeligheid. Ook bleek uit dierproeven dat wanneer 'normale' muizen (wild type (WT) muizen) net zoveel te eten krijgen als muizen die de CB_1-receptor missen – zogenaamde CB_1-knockout muizen ($CB_1^{-/-}$) – de laatste minder dik worden dan WT muizen. Dit laat zien dat het ECS effecten heeft op energiebalans die onafhankelijk zijn van de effecten via de voedselinname. Hoewel rimonabant aanvankelijk veelbelovend leek in de behandeling van obesitas, is de registratie van rimonabant als medicijn voor obesitas opgeschort op grond van het optreden van ernstige bijwerkingen (depressie en verhoogd risico op suïcide).

Het doel van het onderzoek beschreven in dit proefschrift is inzicht verkrijgen in hoe het ECS betrokken is bij de regulatie van energie balans. Daarbij is tevens gekeken naar de interactie met visolie aangezien endocannabinoïden gevormd worden uit langketen meervoudig onverzadigde vetzuren die als zodanig de activiteit van het systeem zouden kunnen beïnvloeden.

Een aantrekkelijk model om het endocannabinoïd systeem te bestuderen is de eerder genoemde $CB_1^{-/-}$-muis. In **hoofdstuk 2** wordt beschreven dat de CB_1-receptor al vroeg in de ontwikkeling een rol speelt in de energiehuishouding. Eerder onderzoek bij muizen had aangetoond dat pups van $CB_1^{-/-}$-moeders een lager lichaamsgewicht hebben dan pups van WT moeders. Uit deze eerdere studies kon echter niet worden geconcludeerd of dit het effect was van maternale factoren (bijvoorbeeld lactatie of zorg voor pups) dan wel van het ontbreken van de CB_1-receptor in de pup. Voor de experimenten beschreven in **hoofdstuk 2** zijn heterozygote $CB_1^{+/-}$ muizen paartjes gekruist waarbij in overeenstemming met de verwachting op basis van erfelijkheidsprincipes ongeveer 25% van de pups $CB_1^{-/-}$, 25% WT en 50% heterozygoot bleek te zijn. Op deze manier konden, van dezelfde moeder, pups met en zonder de CB_1-receptor met elkaar vergeleken worden. De gevonden significante verschillen in lichaamsgewicht en vetpercentage op de leeftijd van 3 weken tonen aan dat de CB_1-receptor een rol speelt in groei van jonge muizen en dat een effect op lichaamsgewicht

en vetgehalte dus onafhankelijk van het maternale genotype gevonden wordt. Uit de data beschreven in **hoofdstuk 2** bleek verder dat het verschil tussen het $CB_1^{-/-}$- en WT genotype groter is in mannelijke pups dan in vrouwelijk pups. Resultaten beschreven in **hoofdstuk 3, 4, 5 en 6** zijn gebaseerd op studies in mannelijke muizen.

Een kerngedachte in dit onderzoek was dat metabole verandering door manipuleren van activiteit van het endocannabinoïd systeem met name gezocht moeten worden in specifieke effecten op het vetmetabolisme. Aangezien endocannabinoïden uit meervoudig onverzadigde vetzuren (PUFA) worden gevormd en aangezien bekend is dat deze vetzuren het vetmetabolisme beïnvloeden, zou kunnen worden aangenomen dat de hoeveelheid PUFA in het dieet van invloed is op effecten van blokkeren van CB_1-activiteit. In **hoofdstuk 3** zijn resultaten beschreven van studies in muizen die een van de volgende diëten kregen toegediend: 1) een standaard vezelrijk en vetarm dieet, 2) een hoog-vet dieet op basis van verzadigde vetzuren, of 3) een hoog-vet dieet waarbij een gedeelte van het vet afkomstig was uit visolie. Van elke dieetgroep werd de helft van de muizen behandeld met rimonabant. De rimonabant-behandelde dieet groepen namen significant minder toe in gewicht dan de controlegroepen. Opmerkelijk was dat deze relatieve gewichtsvermindering niet gepaard ging met een verminderde energie inname, noch met verminderde absorptie in de darm of een meetbaar verschil in energieverbruik. In overeenstemming met de verwachtingen lieten resultaten van **hoofdstuk 3** leververvetting en dyslipidemie zien in de controlegroep op het hoog-vet dieet, terwijl dit niet werd gevonden in de controlegroep op het hoog-vet visolie dieet. Van het hoog-vet dieet is bekend dat het de vetzuuraanmaak in de lever verhoogd, terwijl van diëten rijk aan PUFA (zoals visolie) bekend is dat het de vetzuuraanmaak in de lever juist verminderd. Eerder was gesuggereerd dat het effect van blokkering van CB_1-activiteit op het voorkomen van gewichtstoename een gevolg zou zijn van remming van de vetzuuraanmaak in de lever. Op basis van deze suggestie werd verwacht dat behandeling met rimonabant minder effect zou hebben op gewichtstoename van muizen die het hoog-vet visolie dieet kregen toegediend aangezien dit dieet de vetzuuraanmaak in de lever zelf al onderdrukt. Echter, de resultaten beschreven in **hoofdstuk 3** laten het tegenovergestelde zien, namelijk dat het grootste effect van behandeling met rimonabant op gewichtstoename te vinden is in de hoog-vet visolie groep. Deze resultaten zijn dus in tegenspraak met een rol voor onderdrukking van de vetzuuraanmaak in het effect van behandeling met rimonabant op overgewicht. De studie beschreven in **hoofdstuk 4** was opgezet om effecten van manipulatie van het endocannabinoïd systeem op vetzuuraanmaak in de lever nader te bepalen en

mogelijke mechanismen verantwoordelijk voor de ongevoeligheid van $CB_1^{-/-}$-muizen voor leververvetting te evalueren.

In **hoofdstuk 4, 5 en 6** is gekeken naar verschillen tussen jonge $CB_1^{-/-}$-muizen en WT muizen afkomstig uit dezelfde nesten. Na verspenen kregen deze muizen gedurende 6 weken één van de drie boven genoemde diëten toegediend. $CB_1^{-/-}$-muizen op een hoog-vet dieet kregen geen leververvetting in tegenstelling tot WT muizen op hetzelfde dieet (**hoofdstuk 4**). De vetzuuraanmaak in de lever bleek echter niet te verschillen tussen $CB_1^{-/-}$-muizen en WT muizen en ook de hoeveelheid vet die de lever verliet bleek niet te verschillen. Dit wijst op een verschil in aanvoer van vetzuren naar de lever (opname vanuit het bloed) dan wel een verschil in verbruik van vetzuren in de lever (vetzuuroxidatie). Ontwikkeling van het vetweefsel (adipogenese), aanmaak van vetzuren in het vetweefsel (lipogenese) en opname van vetzuren in het vetweefsel (lipolyse door LPL-activiteit) bleken niet beïnvloed door afwezigheid van de CB_1-receptor (**hoofdstuk 5**). Wel bevestigden resultaten beschreven in **hoofdstuk 5** dat $CB_1^{-/-}$-muizen ongevoelig zijn voor het ontwikkeling van obesitas op een hoog-vet dieet en een verminderd vetgehalte hebben in vergelijking tot WT muizen uit dezelfde nesten. Tevens werden in $CB_1^{-/-}$-muizen significant kleinere vetcellen gevonden vergeleken met die in WT muizen. Dit lijkt dus niet een gevolg te zijn van veranderingen in functioneren van het vetweefsel zelf maar eerder een gevolg van veranderingen in aanbod van vet voor opslag. Mogelijk speelt een verhoogd verbruik van vetzuren hierin een rol. Op chow en op hoog-vet dieet werd zowel in muizen behandeld met rimonabant (**hoofdstuk 3**) als in $CB_1^{-/-}$-muizen (**hoofdstuk 6**) in vergelijking tot de respectievelijke controlegroepen, een significante verlaging van de respiratoire quotiënt (RQ) gevonden, hetgeen aansluit bij een toegenomen verbruik van vetzuren als energiebron (substraat). **Hoofdstuk 6** gaat in op de vraag of een verhoogd vetzuurverbruik de stuwende kracht is achter verhoogd energieverbruik of – vice versa – dat verhoogd energieverbruik juist de oorzaak is van een verhoogd vetzuurverbruik. Het laatste lijkt het geval aangezien wel een verhoogd energieverbruik werd gevonden in $CB_1^{-/-}$-muizen ten opzichte van WT muizen maar geen relatie in de tijd bleek te bestaan tussen toegenomen vetzuurverbruik (alleen overdag, dus in gevaste toestand) en verhoogd energieverbruik (hele dag).

Concluderend bevestigen deze resultaten dat onderdrukking van activiteit van het endocannabinoïd systeem resulteert in gewichtsafname en een gunstiger metabool profiel (verlaagd vetgehalte, minder leververvetting, 'minder ongunstige vetten in het bloed'). Deze effecten van blokkering van de CB_1-receptor blijken niet gerelateerd te zijn aan veranderingen in het functioneren van het vetweefsel en er lijkt geen sprake

te zijn van onderdrukking van vetzuuraanmaak in de lever, noch in het vetweefsel. Mogelijk is sprake van een toegenomen energieverbruik m.n. door metabolisme van vetzuren. Interessant is daarbij ook de rol van efficiëntie in omgaan met beschikbare energie, hetgeen nader onderzoek verdient. Daarnaast verdient de rol van visolie in het dieet in bovengenoemde regulatiemechanismen extra aandacht, zeker gezien de bevindingen in dit proefschrift dat visolie suppletie in het dieet van muizen – naast gunstige metabole effecten – een ongewenste toename in vetgehalte en gewicht tot gevolg had. In hoeverre inzicht in de werking van het ECS uiteindelijk klinische toepasbaar is, is op dit punt moeilijk in te schatten. Aan de ene kant benadrukken de hier beschreven resultaten dat het ECS een veelbelovend aangrijpingspunt vormt voor preventie en behandeling van obesitas en comorbiditeit, echter de keerzijde is dat het ECS betrokken is bij de regulatie van uiteenlopende functies met name gerelateerd aan ‘preventie van schade’ waarbij manipuleren van de activiteit van dit systeem dus een groot risico op bijwerkingen met zich mee zou kunnen brengen. Lange termijn studies zijn essentieel om alle laatstgenoemde negatieve effecten te evalueren tegenover de positieve uitkomsten die manipulatie van het ECS kan hebben op de pathofysiologie van obesitas.

List of frequently used abbreviations

2-AG	2-arachidonoyl glycerol
AA	arachidonic acid
AEA	anandamide
AT	adipose tissue
CB	cannabinoid
CB_1-receptor	cannabinoid-1-receptor
CB_2-receptor	cannabinoid-2-receptor
$CB_1^{-/-}$	CB_1-receptor deficient
$CB_1^{+/-}$	heterozygous for the CB_1-receptor
CH	chow
CHO	carbohydrate
DAGL	diacylglycerol lipase
DHA	docosahexaenoic acid
EC	endocannabinoid
ECS	endocannabinoid system
EE	energy expenditure
FAAH	fatty acid amide hydrolase
FAO	fatty acid oxydation
FFA	free fatty acids
FO	fish oil
FTT	failure to thrive
GLM	general linear model
HF	high-fat
HF/FO	high-fat fish-oil enriched
LC-PUFA	long-chain polyunsaturated fatty acid(s)
LF	low-fat
LPL	lipoprotein lipase
MAGL	monoacylglycerol lipase
MIDA	mass isotopomer distribution analysis
NAFLD	non-alcoholic fatty liver disease
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
PCRF	percent relative cumulative frequency
PUFA	polyunsaturated fatty acid(s)

RQ	respiratory quotient
TG	triglycerides
VLDL	very low-density lipoprotein(s)
WT	wild type
ZT	Zeitgeber time

Abbreviations of genes

Gene		Official abbreviation
Acc1	acetyl-Co-enzyme A carboxylase alpha	Acaca
Acc2	acetyl-Co-enzyme A carboxylase beta	Acacb
Arcp30	adiponectin	Adipoq
Aox	acetyl-Co-enzyme A oxidase 1, palmitoyl	Acox1
Ap2	fatty acid binding protein 4	Fabp4
Atgl/desnutrin	adipose triglyceride lipase	Pnpla2
B-actin	actin, beta	Actb
Cb1	cannabinoid receptor 1	Cnr1
Cd36	CD36 antigen (fatty acid translocase)	Cd36
Cebpα	CCAAT/enhancer binding protein α	C/ebpα
Chrebp	MLX interacting protein-like	Mlxipl
Cpt1a	carnitine palmitoyltransferase 1a, liver	Cpt1a
Cyclophilin G	peptidyl-prolyl isomerase G	Ppig
Dgat2	diacylglycerol O-acyltransferase 2	Dgat2
Elovl6	ELOVL family member 6, elongation of long chain fatty acids	Elovl6
Faah	fatty acid amide hydrolase	Faah
Fas	fatty acid synthase	Fasn
Fatp4	solute carrier family 27 (fatty acid transporter), member 4	Slc27a4
Fatp5	solute carrier family 27 (fatty acid transporter), member 5	Slc27a5
Fxr	farnesoid x receptor	Nr1h4
HmgCoas	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2

Hsl	hormone sensitive lipase	Lipe
Lcad	acyl-Coenzyme A dehydrogenase, long chain	Acadl
L-fabp	fatty acid binding protein 1, liver	Fabp1
Lpl	lipoprotein lipase	Lpl
Lxr	liver x receptor	Nr1h3
Nape-pld	N-acyl phosphatidylethanolamine phospholipase D	Napepld
Pparγ2	peroxisome proliferator activated receptor gamma, isoform 2	Pparg
Pepck	phosphoenolpyruvate carboxykinase 1	Pck1
Scd1	stearoyl-Coenzyme A desaturase 1	Scd1
Srebp1c	sterol regulatory element binding transcription factor 1	Srebf1
Ucp-1	uncoupling protein 1 (mitochondrial, proton carrier)	Ucp1
Ucp-2	uncoupling protein 2 (mitochondrial, proton carrier)	Ucp2

Supplemental Table 1

Primers and probes used for realtime PCR

Gene		Sequences (5' to 3')	GenBank™ accession no.
Adiponectin	Forward	AGG ACA TCC TGG CCA CAA TG	NM_009605
	Reverse	CTT AGG ACC AAG AAG ACC TGC AT	
	Probe	CTC TCC AGG AGT GCC ATC TCT GCC A	
Ap2 (Fabp4)	Forward	CAC CAT CCG GTC AGA GAG TAC TT	NM_024406.1
	Reverse	TCT AGG GTT ATG ATG CTC TTC ACC T	
	Probe	CAT CGA ATT CCA CGC CCA GTT TGA	
Atgl/desnutrin	Forward	AGC ATC TGC CAG TAT CTG GTG AT	NM_025802
	Reverse	CAC CTG CTC AGA CAG TCT GGA A	
	Probe	ATG GTC ACC CAA TTT CCT CTT GGC CC	
Cb1	Forward	ACA AGC TTA TCA AGA CGG TGT TTG	NM_007726.3
	Reverse	TGC TCC TCA GAG CAT AGA TGA TG	
	Probe	CTC TGC CTG CTG AAC TCC ACC GTG	
C/ebpα	Forward	CCA AGA AGT CGG TGG ACA AGA A	NM_004364.2
	Reverse	AGG CGG TCA TTG TCA CTG GT	
	Probe	CGC AAC AAC ATC GCG GTG CG	
Cyclophilin G	Forward	CAG ATC GAG GGA TCG ATT CAG	NM_001081086
	Reverse	TCA CCA CTT GAC ACC CTC ATT C	
	Probe	CTC CTC CAC ATT GGA GAC AAG AGA TGC A	
Faah	Forward	CAG AAG CTG TGC TCT TTA CCT ACC	NM_010173.2
	Reverse	CAG ATA GGA GGT CAC ACA GTT GGT	
	Probe	CTT TGT TCA CTT CCC AGG CCT TTC CC	
Fatp4	Forward	CCA GAC AAG GGT TTT ACA GAT AAG CT	NM_011989
	Reverse	ACC TGC TGT GCA CCA CAA TG	
	Probe	CGG GCA CCA CGG GGC TAC CC	
Fatp5	Forward	GTG CTG ATT GTG GAT CCA GAC	NM_009512
	Reverse	GAA TGT TCT CAG CTA GCA GCT TG	
	Probe	CCA GGA GAA CCT GGA AGA AGT CCT TCC	
Hsl	Forward	GAG GCC TTT GAG ATG CCA CT	NM_010719
	Reverse	AGA TGA GCC TGG CTA GCA CAG	
	Probe	CCA TCT CAC CTC CCT TGG CAC ACA C	
L-fabp	Forward	GAA CTT CTC CGG CAA GTA CCA A	NM_017399
	Reverse	TGT CCT TCC CTT TCT GGA TGA G	
	Probe	CCA TTC ATG AAG GCA ATA GGT CTG CCC	

Gene		Sequences (5' to 3')	GenBank™ accession no.
Nape-pld	Forward	GGC CTT GGA GTC GAT TCT TCT	NM_178728.3
	Reverse	GTA TTT CAT AAA CCA CCT TGG TTC AT	
	Probe	AGG TCA AAA GGA CCA AAC CTT TTT CCA ATC TC	
Pparγ2	Forward	CTA TGA GCA CTT CAC AAG AAA TTA CCA	U09138.1
	Reverse	CAC AGA GCT GAT TCC GAA GTT G	
	Probe	ACA CAG AGA TGC CAT TCT GGC CCA C	

Supplemental Table 2

Fatty acid profiles of experimental diets in mg g^{-1}

	chow (LF)	HF	HF/FO
C14:0	0,5	12,2	16,1
C16:0	8,4	92,5	79,5
C16:1	0,7	11,5	18,0
C18:0	3,7	76,3	50,5
C18:1	13,7	133,2	101,0
C18:2	16,9	11,5	9,7
C18:3	1,9	2,9	15,2
C20-22	0,4	4,0	53,3
C16 desaturation index	0,1	0,1	0,2
C18 desaturation index	3,7	1,7	2,0
ratio n-6/n-3	12,0	ND*	0,4
total dietary fat content	6%	36%	36%

Abbreviations: FO, fish oil; HF, high-fat; LF, low-fat; ND, not detectable.

* The level of n-6 PUFA in th HF diet are non-detectable ND. Composition of experimental diets.

Dankwoord

Onderzoek doe je niet alleen en ik heb de afgelopen jaren dankbaar alle mogelijke hulp aanvaard. Zowel in Haren als in het Laboratorium Kindergeneeskunde hebben veel collega's bijgedragen aan de totstandkoming van dit proefschrift. Niet alleen kwam ik volstrekt onwetend het lab ingewandeld in 2005, maar ook zijn de meeste experimenten beschreven in dit proefschrift simpelweg niet alleen uit te voeren. En dat is dan dus nog afgezien van het feit dat input van anderen onontbeerlijk is.

Allereerst wil ik mijn promotores bedanken. Gertjan, jij bent met name betrokken geweest bij de dagelijkse gang van zaken, zoals de uitvoering van de experimenten en het oplossen van de daarbij onvermijdelijke problemen. Echter in jouw wereld zijn geen problemen maar uitdagingen en zie hier het uiteindelijke resultaat van zoveel enthousiasme. Altijd toegankelijk, geen zee te hoog, praktische/creatieve oplossingen, waarom niet 'Science' proberen!? Hartelijk dank! Folkert, goed tegenwicht, om explosies aan ideeën enigszins te beteugelen. Ruimte geboden, op de rails gehouden, er een punt aan weten te draaien, nuchtere kijk, "no nonsense", "als je geen effect ziet is het er ook niet", maar tot in de puntjes/komma's redigeren. Hartelijk dank! Professor Sauer, toen ik nog dacht een oriënterend gesprek te gaan voeren over mogelijkheden om onderzoek te doen tijdens de specialisatie in het UMCG, had u Folkert al uitgenodigd om gelijk spijkers met koppen te kunnen slaan. Ik ben u dankbaar voor de mogelijkheid dit onderzoek te doen, voor het vertrouwen, maar met name ook voor de steun in de periode waarin combineren kliniek en onderzoek toch heel lastig bleek.

Bijzonder belangrijk gedurende het hele traject bleken ook mijn kamergenoten, zowel in het UMCG als in Haren (Angelique, Izabella en Simon). Zij brachten mij de basisbeginselen van onderzoek doen, laboratoriumwerk en promoveren bij, maar relativeerden ook waar nodig en bleven vriendelijk ondanks frequente onvrijwillige opsluiting en andere warrige acties. Vincent de Noordeling (dat is een compliment ja), labgids, vraagbaak, "signifier", literatuurleverancier, mooi dat ik de afgelopen jaren op je kon rekenen, die kussenslopen heb je nog te goed. Collega Mulder, rots in de branding bij onderzoeksleed, jij kan alles uitleggen en nog begrijpelijk ook, onwaarschijnlijk strak georganiseerde aanpak, op elk dekseltje kun je een verhaal kwijt, reversed pipetteren triplo, veelvuldig op jouw kennis geleund. Ook van jouw manier van werken heb ik geprobeerd te leren en de les "hoe bewaar ik monsters" heeft mij waarschijnlijk uiteindelijk veel ellende gescheeld. Ik blijf erbij, "Kool" was leuk geweest, en dan de volgende "Boer", maar goed bij "Thomas" kan ik me neerleggen.

Theo, het is je vergeven dat je mijn mooie plekje ingepikt hebt. Volgens mij past F1.12 je als gegoten. Het heeft zeker ook geholpen om samen de laatste fase van het promoveren te doorstaan en ik hoop dat jij 26 mei een geweldige dag hebt. Nicolette en Janny, niet lang geduurd, maar wel gezellig. En dan de laatste aanwinst van F1.12: Maaike, en het is goed gekomen. Je was niet alleen kamergenoot maar vooral sister in crime. We hebben elkaar mooi op weten te jutten, maar waar nodig ook op de been weten te houden, kortom teamwerk (met cappuccino's, champagne, koekjes en ijsjes). Jij gaat het in Zwitserland helemaal maken dat weet ik zeker en dan weer mooi terug naar Groningen want wat moet dit lab zonder Oosterveer. Die stukjes sturen we nog wel even weg…

Ook in Haren heb ik dankbaar gebruik kunnen maken van de kennis en ervaring van mijn kamergenoten en bovendien een hele leuke tijd gehad. Angelique, gedreven, nuchtere, en gezellige collega, met veel meer onderzoekservaring zeker ten aanzien van dierexperimenten wat vooral in het eerste jaar zeer vaak broodnodig bleek. Izabella, always interested, always willing to help, even with less pleasant work like carcass analyses, always nice company.

In mijn eerste jaren in Haren kreeg ik bovendien hulp van Kristin (indirecte calorimetry koningin), Rolinka (Science bleek toch net te hoog gegrepen, maar ik vond de poging zeer geslaagd, en ik denk oprecht dat het je nog weleens gaat lukken ook.. laat maar weten) en Mark Doornbos (waanzinnig veel werk verzet om materiaal van specifieke breinregio's te verkrijgen en eveneens geholpen bij karkasanalyse).

In 2008 moest het project in een hogere versnelling nadat veel tijd verloren was gegaan na ziek worden van de muizenfok. De gezamenlijke experimenten met Maaike, vanuit haar hoedanigheid als visolie-koningin en op grond van andere expertise en gewoon gezelligheid, bleken een ware marathon. Dit "Haren-avontuur" was alleen mogelijk dankzij de inzet van Trijnie (de rust zelf in alle hectiek, altijd gezellig en ja ik kom straks echt een keer langs in Friesland), Nanda (perfectionist, vuurdoop heet dat, veel van je gevraagd, maar hopelijk pluk je daar nu vruchten van!), Pieter (enthousiast *in vitro* werk en, waarschijnlijk iets minder enthousiast, genotyperen) en Rick (mooie tijden, mocht ik ooit een roddel willen verspreiden dan weet ik waar ik wezen moet).

De verzorging van de dieren heeft altijd aandacht nodig maar was bij tijden een intensieve toestand. Auke, Jaap, Linda, Henk en Wanda bedankt voor jullie ondersteuning bij alles wat te maken had met de verzorging, fok en registratie van de muizen.

Ook al houdt deel uit maken van twee verschillende laboratoria in dat je nergens voor 100% mee doet, dan nog heeft dit er niet toe geleid dat ik me niet thuis heb gevoeld in zowel het kindergeneeskunde lab van het UMCG als in Haren. Alle collega's in het Laboratorium Kindergeneeskunde en in Haren wil ik bedanken voor de hulp (van praktische adviezen tot hulp bij experimenten) maar natuurlijk ook de gezelligheid: Marijke Schreurs (van eerste kennismaking met het lab tot eerste kennismaking met dierproeven tot alle mogelijke ondersteuning), Gretha (oftewel de Marijke van Haren), Anke (uitstapjes Haren, ware held), Juul (bijdrage aan experimenten, breinen verzamelen, muizenfokken en genotyperen voor gevorderden tot histologie), Esther (volgens die beslisdocumenten waren wij toch begin vorig jaar al gepromoveerd..), Henk (labvraagbaak: "in welk vat gooi ik dit dan weg"), Gerard en Paul (gedogen van mijn bom-calorimetrie-activiteiten op jullie territorium), Fjodor (primer en probe koning), Theo B, Ingrid (onontbeerlijke hulp bij vetzuurpiekjes), Albert (die proef met de orosboros komt nog wel), Renze (voorzitter bom-calorimetrie-genootschap), Frank Perton, Dirk-Jan, Jan Bruggink en Ramon (alle mogelijk ondersteuning in Haren), Jan Keijzer (meten van adipocyten en ander klein leed), Gerard Overkamp (onmisbare schakel in het indirecte calorimetrie gebeuren), Jan Strubbe, Hester (~visualiseren~), Aldo, Alinda, Anja, Annelies, Arne, Caroline, Deepa, Frans C, Frans S, Gemma, Girste, Harmen, Henriette, Hermi, Hilde, Jaana, Jan Freark, Janine, Jelske, Jurre, Karin, Klary Niezen, Leonie, Margot, Marije, Maxi, Niels, Pauline, Roelina, Riejanne, Robert, Sabina, Simon, Terry, Thierry, Thomas, Uwe, Wytse en Wytske. Anniek Werner, het heeft zeker mee aan jouw enthousiasme gelegen dat ik alsnog promotieonderzoek ben gaan doen en je praktische adviezen (dan geef je een slinger aan de snor) hebben me het eerste jaar goed op weg geholpen. Dank! Ik had graag je commentaar op dit boekje gehoord. En natuurlijk Stefano, jij neemt het stokje deels over, maar hebt ook al flink bijgedragen aan de laatste experimenten, zorg goed voor de CB_1-tjes en ik ben benieuwd naar verdere resultaten.

Veel dank ben ik ook verschuldigd aan degenen die wel grip weten te houden op regeldingen; Joke, Hilde, Gea, Han, Jannie, Marieke en Riekje Banus (GUIDE). Bedankt voor de ondersteuning en hier en daar het nodige geduld.

Daarnaast kon ik in Leiden terecht om een uitstapje te maken naar mTOR, Dr. Margriet Ouwens en medewerkers van haar laboratorium, bedankt voor de gastvrijheid en de bereidheid om ook later nog opnieuw met mijn materiaal aan de slag te gaan.

Tevens wil ik de “GECKO raad van advies” bedanken voor hun inbreng, met name in het eerste deel van het project. Ook de andere GECKO-onderzoekers wil ik natuurlijk bedanken voor hun inbreng en natuurlijk voor de gezelligheid.

Leden van de beoordelingscommissie, prof. dr. Bert Groen, prof. dr. Anton Scheurink en prof. dr. R.F. Witkamp, hartelijk dank voor uw bereidheid dit proefschrift te beoordelen. Anton, bedankt voor de belangstelling voor mijn project en de inbreng met name wat betreft het anorexia verhaal, maar natuurlijk ook voor de rondleiding in Stockholm! Bert, helaas niet op 9 juni, maar wellicht komt er een andere gelegenheid om te discussiëren over de beperkingen en mogelijkheden van indirecte calorimetrie.

Hartelijk dank ook aan alle anderen die hebben bijgedragen aan de totstandkoming van dit proefschrift, in het bijzonder collega’s van de kindergeneeskunde, bedankt voor de belangstelling en het geduld ondanks eindeloos gezeur over die laatste loodjes. Joke, bedankt dat je me op het juiste moment weer op het juiste spoor wist te krijgen.

Degenen die de grootste offers hebben gebracht zijn niet collega’s maar vrienden en familie. Wat de vrienden aan gaat wil ik vooral zeggen: dit feestje is voor jullie en hopelijk is er nu eindelijk meer tijd. Wat betreft de familie kan ik ook kort zijn; zonder jullie geen proefschrift. Of het nu “data invoeren omdat je toch niks anders kan met gescheurde kniebanden”, “eerste hulp bij computerleed” of “begrip voor de zoveelste keer afhaken” was, uiteindelijk heeft jullie steun gemaakt dat ik dit project heb weten af te ronden. En Marijke, ik heb het goed begrepen je breekt mijn benen als ik nu gelijk weer aan een nieuw project begin…

Ton en Janny, de afgelopen jaren heb ik zeer frequent een beroep op jullie gedaan: hartelijk dank voor jullie bijdrage aan dit boekje!

Pap, mam, ik wist al dat ik ten alle tijde op jullie alles omvattende ondersteuning kan rekenen en het was dan ook niet de bedoeling om dat nogmaals uit te testen, maar in praktijk hebben jullie je opnieuw in alle mogelijke bochten gedraaid om het voor ons makkelijker te maken. Voor Giel en Jort was dit zeker geen straf en ik hoop dat jullie uiteindelijk ook voornamelijk goede herinneringen over houden aan deze periode. Mochten Robert en ik denken dat we het druk hebben met twee kleine kinderen, jullie hebben het pas druk met vijf volwassen dochters. Dat Carolien en Marleen naast mij zullen staan als paranimfen maakt de cirkel wat mij betreft rond.

Lieve Robert, bedankt.. maar dat weet je wel en op ons! Robert, Giel en Jort, dit boekje is voor jullie!

Curriculum Vitae

Anniek Koolman werd 1 november 1973 geboren te Warffum. Na het behalen van het VWO-diploma in 1992 aan het Nienoordcollege te Leek en de propedeuse Psychologie aan de Rijksuniversiteit Groningen studeerde zij Geneeskunde aan de Rijksuniversiteit Groningen. In november 2000 behaalde zij haar artsexamen cum laude. Vanaf februari 2001 was zij werkzaam als arts-assistent in het Wilhelmina Kinderziekenhuis te Utrecht op de afdeling Kinderhematologie en -oncologie en vervolgens vanaf 2002 in de Isala klinieken te Zwolle als arts-assistent Kindergeneeskunde. In april 2003 startte zij haar opleiding tot kinderarts in het Beatrix Kinderziekenhuis (opleider prof. dr. P.J.J. Sauer). In het kader van deze opleiding was zij van oktober 2004 tot oktober 2005 werkzaam als arts-assistent Kindergeneeskunde in het Medisch Spectrum Twente te Enschede (opleider dr. R.F.H.M. Tummers). Per oktober 2005 deed zij promotieonderzoek naar de effecten van het endocannabinoid systeem op de regulatie van de energiehuishouding, deels in het Researchlaboratorium Kindergeneeskunde van het UMCG en deels op in het Instituut voor Gedrag- en Neurowetenschappen, afdeling Neuroendocrinologie van het Biologisch Centrum Haren (promotores prof. dr. F. Kuipers, prof. dr. G. van Dijk en prof. dr. P.J.J. Sauer). In januari 2009 hervatte zij de opleiding Kindergeneeskunde in het Beatrix Kinderziekenhuis te Groningen (opleider mr. dr. A.A.E. Verhagen).

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