Investigations of the Endocannabinoid System in Adipose Tissue

Effects of Obesity / Weight Loss and Treatment Options

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- Marianne F. Bennetzen, Maria P. Nielsen, Bjørn Richelsen, and Steen B. Pedersen. "Effects on food intake and blood lipids of cannabinoid receptor 1 antagonist treatment in lean rats" Obesity, November 2008, volume 16, issue 11, p2451-2455.
- Marianne F. Bennetzen, Thomas S. Nielsen, Søren K. Paulsen, Sanne Fisker, Jørgen Bendix, Niels Jessen, Steen Lund, Bjørn Richelsen, and Steen B. Pedersen. "Reduced levels of cannabinoid receptor 1 protein in subcutaneous adipose tissue of obese" European Journal of Clinical Investigation, February 2010, volume 40, issue 2, p121-126.
- Marianne F. Bennetzen, Niels W. Andersen, Sidrha S. Ahmed, Saira M. Ahmed, Thi A. Diep, Harald S. Hansen, Bjørn Richelsen, and Steen B. Pedersen. "Investigations of the Human Endocannabinoid System in Two Subcutaneous Adipose Tissue Depots in Lean Subjects and in Obese Subjects Before and After Weight Loss" International Journal of Obesity, Advanced Online Publication, February 2011.

INTRODUCTION

OBESITY

Obesity is becoming an increasing problem world wide; the number of people with obesity is skyrocketing. It is estimated, that globally more than 1 billion adults are overweight, and at least 300 million of them obese, and obesity is recognised as a chronic disease and the second leading cause of preventable deaths (exceeded only by cigarette smoking) (1). In Denmark at least 10-13 % of the adult population (30 -60 years of age) are obese and 40% men and 26% women are overweight, based on numbers from 2000 (2). BMI is weight in kilograms divided by squared height in meters (kg/m2), and according to the Global Database of Body Mass Index from World Health Organization (WHO), BMI above 25 is over weight.

Classification	BMI (kg/m2)
Normal range	18.5 - 24.9
Overweight	≥25
Pre-obese	25 - 29.9
Obese	≥30
Obese class I	30 – 34.9
Obese class II	35 - 39.9
Obese class III	≥40

Figure 1: **The International Classification of adult overweight and obesity according to BMI, Adapted from WHO (3)**

Abdominal circumference is more related to visceral fat than the subcutaneous depot (4), and the visceral AT (VAT) seems to be more metabolically active compared with the subcutaneous AT (SAT) (5). Therefore waist circumference (WC) or waist-hip-ratio (WHR) may be a superior measure of obesity with health complications(6).

Gender / Classification	WC	WHR
Men	94 cm	1
Women	80 cm	0.8

Figure 2: **Classification of visceral adiposity (Bruun, Ugeskr Laeger 2009) (6)**

The human body has not had enough time to keep up with the last 50 years industrialised development resulting in plenty and easy accessible food. But, adipose tissue (AT) is more than a mere storage organ for excess energy – it is also a setting for complex metabolic processes (7), and obese persons have increased incidences of many diseases, including gall stones, arthritis, some types of cancer, hypertension, sleep apnoea, and complications to wound healing after surgery (8) Obesity also seems to increase the risk of depression (9), and obesity, especially intra abdominal adiposity, increases the risk of diabetes mellitus type 2 (DM2) 6 times (10;11) and risk of death due to cardiovascular disease (CVD) 3-4 times (12;13).

Visceral adiposity is associated with the metabolic syndrome (MS) (14). MS is important because it helps to identify those at increased risk of developing DM2 and CVD (15). Several definitions exist of MS, but central adiposity relates to all of the components (4).

	The Metabolic Syndrome
1	Central obesity (BMI>30 or waist circumference>94/80)
2 & 3	+ 2 of the following: Elevated triglycerides (TG > 1.7 mmol/L) or Low high density lipoprotein cholesterol (HDL< 1.03 (males)or 1,29 (females)) or High blood pressure (systolic >130 or diastolic > 85) or Increased fasting plasma glucose (>5.6 mmol/L)

Figure 3: **The International Diabetes Federation's definition of the metabolic syndrome (MS), Lancet, 2005 (15)**

5-10% weight loss is associated with reduction in risk factors for CVD (besides reduced BMI) such as decreased low density lipoprotein cholesterol (LDL), increased high density lipoprotein cholesterol (HDL), reduced circulating cytokines and improved glucose and insulin parameters (16;17). In addition, weight loss has been shown to prevent DM2 (18), and the antiobesity effect may go beyond weight change to changes in adipocytokines that directly affect the risk of DM2 and CVD (19).

However, conventional treatment of obesity with life style modifications (hypocaloric diet and exercise) have limited effects and have not proved successful in maintaining large weight losses over prolonged periods of time.

Figure 4: **On the scales**

Intensive regiments with Very low calorie diets (VLCD) can yield a weight loss of 11% in 12 weeks (20) but the weight loss is not long-lasting. Pharmacotherapy can improve weight losses in obese patients. Weight loss obtained through behavioural programs is in general 5% to 10% after 6 to 12 months treatment (21). Orlistat, the only available medical treatment option in Denmark today, provides a mean weight loss of 3 kilos after one year of treatment and this is the same picture for previously approved weight loss drugs (22).

But most study participants starts to regain the lost weight within 1 to 2 years and often return to baseline weight within 5 years of completing therapy (23;24). This highlights that obesity is a chronic condition, and today, bariatric surgery is recommended for very obese individuals. 10 and 15 years follow up after bariatric surgery reveals an average weight loss of 16%, improvement in most risk factors associated with obesity and a smaller reduction in overall mortality in spite of the increased risk of the surgery procedure (25;26)

THE ENDOCANNABINOID SYSTEM (ECS)

Overconsumption and overweight might not be strictly pathological conditions. It may merely reflect the effective operation of natural mechanisms, which have evolved to promote positive energy balance during times of plenty, to prepare for times of starving. Looking not that many years back, many systems were needed to ensure plenty energy intake when food was present and less systems were needed to stop eating when satisfied. The endocrine system regulates appetite via approximately 40 known (and possible more unknown) orexigenic and anorexigenic hormones, neuropeptides, and other cell signalling molecules and enzymes (27).

In the 1990's the endocannabinoid system (ECS) was discovered and characterized with receptors and ligands, and research aimed at understanding this system and its implications in obesity exploded with the availability of tools, such as synthetic pharmacological agents and genetically manipulated mice. The ECS is one of the signalling systems that control feeding behaviour and it exerts its effect at several levels. It can attenuate or lower the desire of finding and consuming food (interacting with reward mechanism) and modulate orexigenic or anorexigenic mediators such as induce appetite after periods of fasting.

The ECS is implicated in many functions, such as control of locomotion, pain, memory, addiction, cardiovascular response, inflammation, gastric motility, and feeding, and could be considered a stress recovery system (28) with antitumoral- (29;29;30), neuro protective- (28); and cardioprotective effects (31;32) maintaining homeostatic balance. It also seems to integrate nutrient intake, metabolism and storage, having a net anabolic effect (33).

Soon after the discovery of the EC's, the first potent and selective CBR1-blocker, SR141716 or Rimonabant, was introduced by Sanofi-Aventis (34). It started as a compound against skizophrenia (35) and was then tested as a smoking cessation aid, and when it showed weight reducing capacity, was tried as a weight loss drug (36). This discovery sparked further interest in and research of the ECS and its significance for weight and metabolism.

AIM

The ECS is a recently discovered system, primarily involving the central nerve system, but far from all the pieces of the puzzle are assembled yet. In relation to obesity, blocking the CBR1 has been found to induce weight loss in both animal models and humans, which has mainly been suggested to be due to reduced food intake/reduced appetite. We and other groups have, however, found that the CB1-blocking-induced reduced food intake was rather short lasting which disappears after days to one week whereas the weight loss continues over time. Thus, our first aim was to

1) get more insight in the endocannabinoid system (ECS) in the treatment of obesity particularly in relation to development of tolerance/tachyphylaxia on food intake by the CBR1 antagonist, Rimonabant and

2) whether intermitted versus continuous treatment with a CB1 antagonist affects the development of tachyphylaxia and

3) whether reducing the tendency to tachyphylaxia would enhance the weight lossand other beneficial effects induced by a CB1-anatagonist.

From recent animal and human investigations it is suggested that the CBR1 antagonist, Rimonabant, may be able to induce weight loss independent of its central/cerebral effects. Moreover, it has been suggested that Rimonabant may have weight-loss independent effects on metabolic syndrome, lipids, and low-grade inflammation. Thus, in an attempt to answer whether these pe-

ripheral effects of Rimonabant may be mediated by the adipose tissue, the second aim was to get insight of the role of the ECS in the adipose tissue by
1) investigating the existence of the ECS in adipose tissue (receptors, enzymes, agonists etc.)
2) getting information about the regulation of the ECS in adipose tissue by the obese state, fat distribution etc.
Thus, the aim of this part of the investigation was to get insight into the ECS in adipose tissue, and whether the effect of Rimonabant on weight loss/metabolic aberrations may involve the ECS in the adipose tissue.

THE ENDOCANNABINOID SYSTEM - IMPLICATIONS IN OBESITY
The present review is based on a systematic literature search and my own studies. The focus is the ECS in AT and obesity. I chose to search in 6 medical literature databases and the Danish national database: Medline at PubMed, Embase, The Cochrane Library, Web of Science, Scopus, SveMed+ and bibliotek.dk. I searched using subject headings and free text searching combining the endocannabinoid system or Rimonabant with overweight or obesity. I sorted by choosing the ones covering obesity, food intake, adipose tissue, or energy metabolism and deselected the ones mainly related to pain, locomotion, fertility, memory, liver disease, atherosclerosis, addiction or specific cerebral locations.

CANNABINOID HISTORY
Cannabis, Marijuana, and Hash refers to a number of preparations from the plant Cannabis (C. Sativa). This plant has been known, grown and used for millenniums for leisure, clothes, and medicine; the earliest mention of hemp dates as far back as 2-4000 years BC (37;38). Cannabis seems to stimulate appetite, especially for sweet and palatable foods, and this effect of cannabis intoxication is commonly referred to as "the munchies" (39). Early, studies by Hollister, 1971 (40), and Abel, 1971 (41), did indeed show increased feeling of hunger and greater intake of chocolate milkshakes and marshmallows, respectively, after cannabis administration, and this effect was reviewed in 1975 by E.L. Abel (42). In 1976, more sophisticated studies with study participants admitted to research wards, confirmed that a transiently increased caloric intake followed by weight gain is commonly seen in marijuana smokers (43).

The psycho-active ingredient in cannabis, Δ9 THC (tetra-hydrocannabinol), was first identified in 1964 (44).

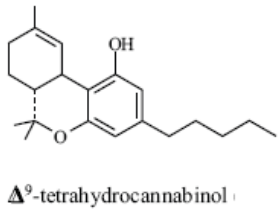


Figure 6: **Structure of Δ9 THC (Cota et al, Int J Obes 2003) (28)**

In 1985 "Dronabinol" (Δ9 THC) was approved by the Food and Drug Administration (FDA) for treatment of chemotherapy induced nausea; moreover, it has been shown that treating cancer patients with cannabis improves appetite, provides weight gain and improves quality of life (45). Marinol (Dronabinol) also proved safe and useful in the treatment of HIV-wasting-syndrome (46) and was shown to induce weight gain and reduce disturbed behaviour in Alzheimer patients (47).

Primary studies in the 1970's and 1980's indicated receptor mediated pharmacology; hence followed the search for and discovery of the cannabinoid receptors. This in turn prompted the search for internally produced ligands for these receptors, and led to the characterisation of at least seven endocannabinoid ligands (EC's) to date. Like cannabis, EC's stimulate appetite (48).

PubMed at Medline search for literature concerning the endocannabinoid system (ECS) shows that the number of articles about the subject has doubled in the previous five years as it had the five years before that, indicating knowledge in this area under rapid expansion. The latest "quick and dirty" search in PubMed August 2010, 5 years later, again retrieved almost the double amount of articles since 2005. Thus, it can be concluded that it continued to be an area of intense research.

Figure 5: Search words for searches in various database

Database	Date	Subject Headings and Free Text Words	Articles
Medline [Pubmed]	3.8.2010	((("Obesity"[Mesh] OR "Obesity, Morbid"[Mesh] OR "Bariatrics"[Mesh])) AND ("Endocannabinoids"[Mesh] OR "Receptors, Cannabinoid"[Mesh])) OR "rimonabant "[Substance Name]	1616
Embase	3.8.2010	obes* OR bariatric OR 'overweight'/exp OR overweight AND (endocannab* OR 'cannabinoid receptor'/exp OR 'cannabinoid receptor' OR 'rimonabant'/exp OR rimonabant)	2152
Cochrane	3.8.2010	obes* Or overweight OR bariatric AND enodcannabinoid OR endogenous cannabinoid OR rimonabant:	32
Web of science	3.8.2010	Topic=(obes* OR overweight OR bariatr*) AND Topic=(endocannabinoid* OR endogenous cannabinoid* OR rimonabant)	798
Scopus	3.8.2010	(TITLE-ABS-KEY(obes* OR overweight OR bariatr*) AND TITLE-ABS-KEY(endocannabinoid* OR endogenous cannabinoid* OR rimonabant))	609
SveMed+	3.8.2010	Obesity AND endocannabinoids	0
bibliotek.dk	3.8.2010	Fed? OG endocannab?	1

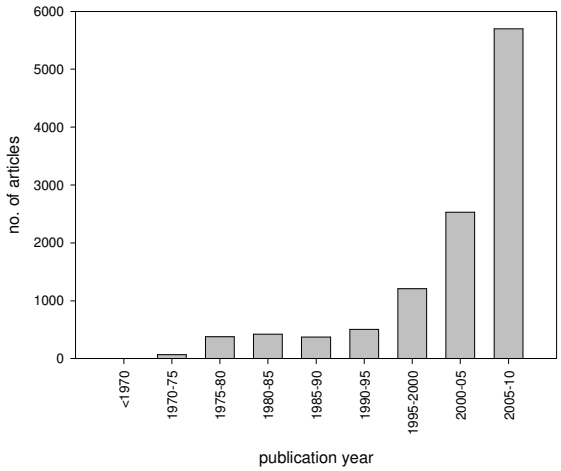


Figure 7: **PubMed search results for "cannabinoid* OR endocannabinoid*" with varying limits of date.**

STRUCTURE OF THE ECS

The ECS consists of the endogenous signalling lipids, biosynthesising and inactivating enzymes, and at least two membrane-bound receptors.

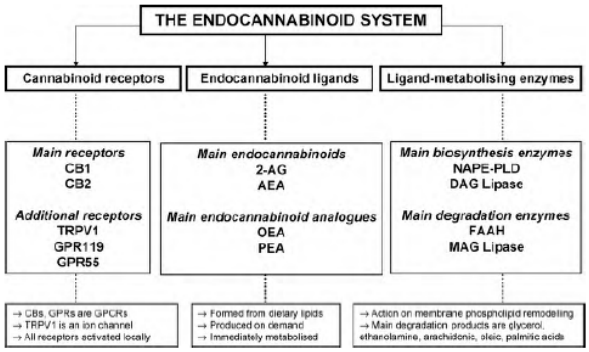


Figure 8: **Outline of the ECS (André et al., Int J Biochem Cell Biol 2010) (49)**

RECEPTORS

In 1988 Devane et al. were the first to discover and characterise rector binding sites for cannabinoids in rat brain (50). The first cannabinoid receptor (CBR) was cloned in 1990 in rat (51) and shortly thereafter in 1991 in human (52) and timely named CBR1. The CBR2 was identified in 1993 (53). There has also been sequenced a splicing variant of CBR1 termed CBR1A (54;55), and an orphan receptor, GPR55, is claimed to belong to the CBR family (56) Proof exists, that more receptors have yet to be discovered (57). The transient receptor potentially vanilloid 1 (TRPV-1) can also be characterised as cannabinoid, with AEA binding to it as a full agonist (58). Δ9 THC can also activate peroxisome-proliferator-activated receptor γ (PPAR-γ) (59), and this is supported by the finding that AEA induce PPARγ2 gene expression (60) .

CBR1 is primarily found in the central nervous system, especially in the hypothalamus, hippocampus, cortex, basal ganglia and cerebellum (61). It is also present in several peripheral tissues, specifically in all tissues related to energy metabolism (62). We and others have shown the presence of CBR1 and CBR2 in adipose tissue (AT) and isolated adipocytes (63;64), and other researchers have shown that human adipocytes contain the entire machinery to synthesise and degrade EC's (65).

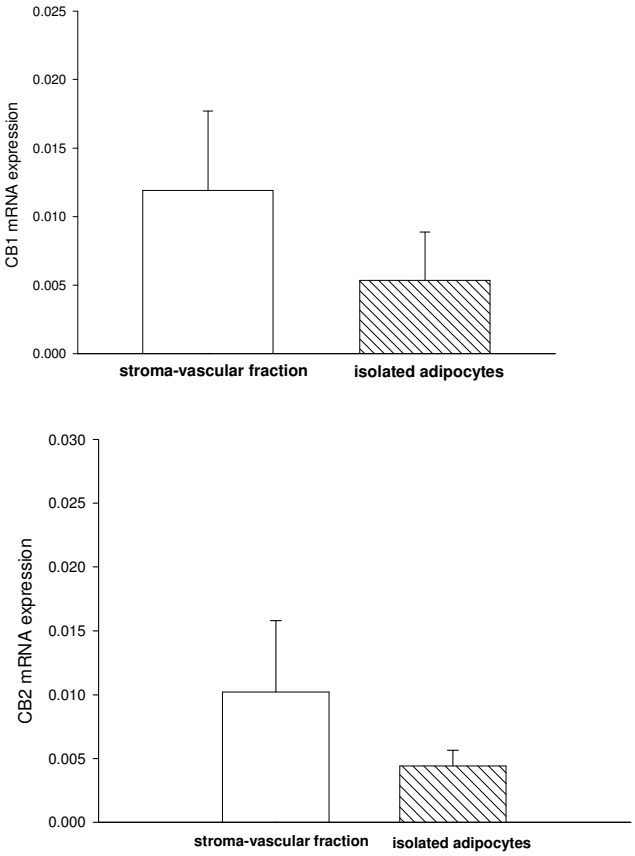


Figure 9: **CBR1 and CBR2 are present in the stroma-vascular tissue fraction and in isolated adipocytes (Bennetzen et al., Eur J Clin Invest, 2010 (64) and Bennetzen et al., unpublished results)**

Isolated hepatocytes from mice express CBR1 (66) and so does skeletal myocytes (67), and macrophages (68), pancreas (69) and the intestine (70). CBR1 is also present in many other tissues including the testes and immune cells (61). CBR2 is mainly present in cells belonging to the immune system and bone cells (61;71). In the CNS the CBR1 is found presynaptically and when EC's are released from the postsynaptic cell they signal in retrograde direction to influence/depress synaptic transmission (72). Thus EC's act as a brake that reduces transmitter flux across synapses (73;74).

Both CBR1 and CBR2 are 7 trans-membrane-domain proteins and are coupled to G-proteins (62). Both CBR1 and CBR2 agonists inhibit cAMP production and regulate ion channels - both calcium and potassium (61;75). Cannabinoids stimulate Adenosine monophosphate-activated protein kinase (AMPK) activity in the hypothalamus and the heart, while inhibiting AMPK in liver and adipose tissue (76). AMPK regulates energy balance in response to hormones and nutrient signals by inhibiting lipogenesis and TG synthesis, increasing fatty acid oxidation, and thereby improving insulin sensitivity (77).

Figure 12: Key points in ECS research

year	Discovery	reference
1964	Identification of the active constituent of marijuana (Δ9-THC)↓	Gaoni et al.
1988	Identification of CB binding sites in the brain	Devane et al.
1990	First cloning of CB1 receptor (in rat)	Matsuda et al.
1991	First cloning of CB1 receptor (in human)	Gerard et al.
1992	Identification of AEA	Devane et al.
1993	First cloning of CB2 receptor	Munro et al.
1994	Development of Rimonabant	Rinaldi-carmoni et al.
1995	Identification of 2-AG	Mechoulam et al.
1996	Identification of FAAH	Cravatt et al.
1998	First description of weight reduction by CB1-antagonism	Colombo et al.
1999	Creation of CB1-knockout mice	Zimmer et al. / Ledent et al.
2003	First description of peripheral effects of endocannabinoids	Cota et al./ Bensaid et al.
2003	Cloning of DAGL	Bisogno et al.
2004	Identification of NAPE-PLD	Okamoto et al.
2005	First publications from the RIO trial	Van gaal et al./ Despres et al.
2005	First publications on ECS activation in human obesity	Engeli et al.
2006	EMEA approves Rimonabant in Europe	
2007	FDA advises against approval in the USA	
2008	Sanofi-Aventis withdraws Rimonabant from the European market	

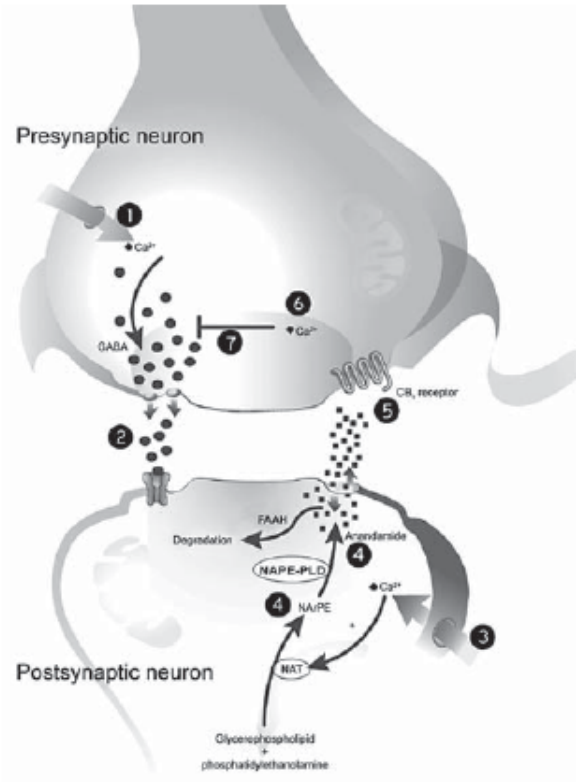
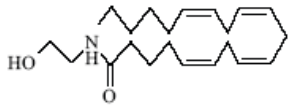


Figure 10: **Endocannabinoid transmission in nerve tissue. When a signal is transmitted through nerve terminals (1,2,3), EC's are synthesised and released from the postsynaptic neuron (4) affecting receptors on the presynaptic neuron (5), usually inhibiting ongoing transmission (6,7). (Rosenson, Cardiology, 2009) (78)**

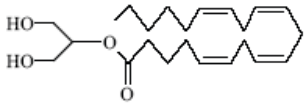
CBR1 knockout (CBR1(-/-)) mice are viable and leaner than their wild type littermates and show hypophagia when they are young (79). CBR1(-/-) mice are also resistant to diet induced obesity and show enhanced leptin sensitivity (80). Mouse strains have been created with CBR1 knockout restricted to a specific tissue or cell localisation (liver (81) and forebrain neurons (82)).

THE ENDOCANNABINOID LIGANDS (EC'S)

The first endogenous cannabinoids to be discovered were N-arachidonoylethanolamine or Anandamide (AEA) in 1992 (83) and 2-arachidonoylglycerol (2-AG) in 1995 (84). These two ligands are the EC´s most studied, but several others have been characterised, all are derivatives of arachidonic acid such as virodhamine, N-arachidonoylglycerol, N-arachidonoyltaurine, palmitoylethanolamide (PEA) (85), and noladin ether (86). One of them, Virodhamine is an endogenous antagonist (87). Recently, Hemopressin, which is a peptide not a fatty acid derivative, has also been shown to be an antagonist of the CBR1 (88).



N-arachidonoylethanolamine (anandamide)



2-arachidonoylglycerol (2-AG)

Figure 11: **Structure of AEA and 2-AG (Cota et al., Int J Obes 2003) (28)**

Both 2-AG and AEA activates both CBR1 and CBR2 receptors (89). The first to show that AEA and 2-AG are produced by human white subcutaneous adipocytes were Gonthier et al. (90). EC's can, because of their lipophilic nature, not be stored in intracellular vesicles and therefore have to be produced "on demand" from phospholipid precursors in the cell membranes (91). The enzymes that synthesise and degrade 2-AG are diacylglycerol lipases (DAGLs) and monoacylglycerol lipases (MGLs), respectively (92), while it can also be oxygenated by cyclooxygenase 2 (COX-2) (93). The ones that produce and degrade AEA are N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH), respectively

(94), but other pathways exist (95). FAAH can also degrade 2-AG (89;96). FAAH-2 and NAAA may also contribute to the termination of AEA function (91).

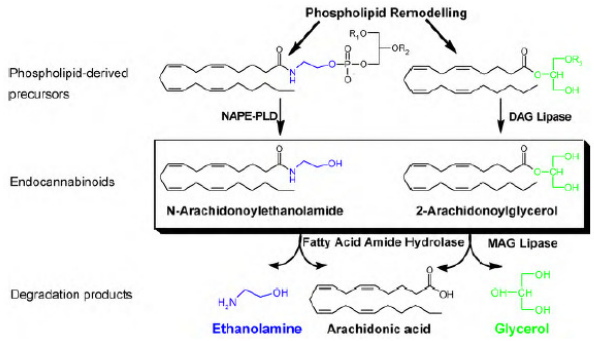


Figure 13: **Biosynthesis and inactivation of AEA and 2-AG. AEA and 2-AG are synthesised by NAPE-PLD and DAGL, respectively, from phospholipid precursors, and degraded by FAAH and MGL, respectively – though FAAH can degrade both EC's. (André et al., Int J Biochem Cell Biol 2010) (49)**

FAAH knockout (FAAH(-/-)) mice exhibit 15 fold increased AEA levels in the brain and are supersensitive to exogenous AEA administration due to lack of degradation showing CBR1-dependent effects including hypomotility, analgesia, catalepsy, and hypothermia (97). Another group of researcheres find similar results, which indicate that the lack of FAAH in FAAH(-/-) mice predominantly promotes energy storage by mechanisms independent of food intake, through the enhancement of AEA levels (98).

The ECS plays an integrative role in the regulation of food intake, both the homeostatic and the hedonistic aspects, and as a generalization one could say that the ECS can be considered to exert an overall anabolic tone in the nervous system, with EC's increasing energy intake and storage (73). AEA induce overeating in mice and rats (99;100). Food deprivation induces a sevenfold increase in AEA in the small intestine of rats and levels normalise with refeeding (101). Fasting also increases levels of 2-AG in the hypothalamus, where it falls again with eating (102). In mice, Δ9-THC increases food intake, while Rimonabant reduces it, but Rimonabant had no effect in CBR1(-/-) mice, whose food intake was already smaller compared with wild type mice (103). Wise et al. investigated exogenous AEAs effect in both CB1(-/-) mice, FAAH(-/-) mice, and mice with the two knockouts in combination and found, that AEA only elicited the expected behavioural effects in FAAH(-/-) mice, indicating that AEA exerts its effect through CB1 (104).

CROSS-TALK

EC's seem to regulate energy homeostasis through interaction with other hormones, neurotransmitters and neuropeptides involved in energy balance (105), and CBR1 blockage modifies adipokine synthesis and production (106). Leptin is a satiety hormone secreted by AT (107). It is believed to bring information about the status of the energy stores to hypothalamus; the level of leptin in the circulation correlates directly with the amount of body fat (108). Correspondingly, high fat diet in 3 weeks increases leptin in the hypothalamus (109). Leptin reduces food intake by up-regulating several anorexigenic neuropeptides and defective leptin signalling is associated with increased EC levels in the hypothalamus (110). Hypothalamic endocannabinoid level is under partial negative influence by leptin, and hypothalamic leptin suppresses AEA in AT (48). When this suppression of EC tone is prevented by systemic CBR1 activation, hypothalamic leptin fails to suppress AT lipogenesis (111). This may indicate that the increased endocannabinoid tone observed in obesity is linked to a failure of central leptin signalling.

Grehlin, on the other hand, is an orexigenic peptide synthesised by the stomach, and evidence indicates that Rimonabant blocks this effect (112). This also means that grehlin might mediate its effects on food intake through EC release. Rimonabant treatment in rats is followed by decrease in Neuropeptide Y (NPY), a peptide that increases feeding behaviour, and an increase in cocaine and amphetamine regulated transcript (CART) and alpha melanocyte-stimulating hormone (α-MSH) in the hypothalamic neurons activated by Rimonabant, consistent with its ability to reduce food intake and increase energy expenditure (113). In the CNS, CBR1 is co-localised with dopamine and serotonin receptors, and connected to noradrenaline (99), which suggests potential cross talk with these systems (114). Evidence also exists of cross-talk between the cannabinoid receptor CBR1 and the orexin-1 receptor (115), and CBR1 is coexpressed with corticotropin-releasing hormone (CRH), melanin-concentrating hormone (MCH), and preproorexin, neuropeptides known to modulate food intake (79). Opoids are implicated in the mediation of food reward, and functional cross talk exist between the opioid and EC systems (28). Nogueiras et al. have recently reviewed the current understanding about the hypothalamic control of peripheral lipid metabolism in a brain-AT cross-talk (116).

Through CBR1 the ECS may participate in the development of insulin resistance in human skeletal muscle by contributing to the cross-talk between AT and muscle (117). Lee et al. provided a thorough review of the cross talk between AT and other organs (118). All in all, the mechanisms by which the ECS can manipulate energy intake and metabolism, centrally and peripherally, are several and not yet fully elucidated, but it is clear that the ECS affects several other hunger and satiety signals.

CENTRAL AND PERIPHERAL EFFECTS

EC's seem to have both central and peripheral effects (119). The central effect is on several levels; one is the hypothalamic "hunger centre", where many signals on nutritional status seem to be integrated to control consumptive behaviour (120). From here, neurons project to the mesolimbic area, a centre involved in mediating reward (121). Peripherally, EC tone may mediate the effects of leptin on adipogenesis with induction of hepatic and AT lipogenesis, enhancement of fatty acid oxidation in muscle, and augmentation of circulating adiponectin (122). Leptin and insulin sensitivity, as well as AT metabolism, may be regulated by hepatic CBR1 (81). Direct effects on skeletal muscle are also possible; a recent investigation showing that CBR1 blockage or genetic silencing in skeletal muscle leads to increased glucose uptake (123). Taken together, the peripheral effects of the EC's are suggested to promote the storage of fat in adipocytes and liver and to decrease energy expenditure (124).

Nogueiras et al. tried to determine central from peripheral effects in lean and DIO rats (125). Specific central CBR1 blockade decreased body weight and food intake, but had no beneficial influence on peripheral lipid- and glucose metabolism, independent from this. Peripheral treatment with Rimonabant, leading to global CBR1 antagonism, reduced food intake and body weight but, also prompted lipid mobilization pathways in AT, cellular glucose uptake, insulin sensitivity, and skeletal muscle glucose

uptake, while decreasing hepatic glucose production. This indicates a peripheral effect which is only brought into play if peripheral receptors are also antagonised. However, they also studied the central concentration of Rimonabant following peripheral administration and this showed higher concentration in the brain compared with serum, so the differences in effects could be brought about by differences in dosage.

Also other researchers aimed to separate the central and peripheral effects without reaching clear answers (126-128). There is no doubt that CBR1 antagonism elicits peripheral effects in several tissues, but it is not yet clear whether these peripheral effects are mediated by the CNS. This raises the question whether a product with selectivity for the peripheral CBR1 would produce the same effects as Rimonabant? Recently, a research group evaluated the effect of a secret Rimonabant derivative dubbed "compound-1", whose blood-brain barrier penetration allegedly should be much lower than that of Rimonabant. Compound-1 showed dose-dependent antiobesity activities in DIO mice and it also completely suppressed the elevated hepatic SREBP-1 expression (129), indicating direct peripheral effects.

IMPLICATIONS IN OBESITY

THE ECS IN RODENT STUDIES

Adipose tissue

Adipocyte differentiation

CBR1 is located in multiple organs related to energy metabolism where they modify glucose and lipid metabolism. A few authors do not find CBR1 or CBR2 to be present in AT of rats (130), but other researchers find both receptors to be present in mouse 3T3-cells (131). It has been shown that AEA increase the differentiation of rat adipocytes (60), and studies in mouse 3T3 preadipocytes indicate that CB1-antagonism inhibits cell proliferation (132). CBR1 is more prominent in mature adipocytes with levels peaking before differentiation, and the expression of CBR1 is higher in mature adipocytes compared with preadipocytes (78;133); this indicates a role in metabolism rather than cell differentiation. Altogether it shows that EC's promote adipogenesis by differentiating adipocytes and stimulating their growth and this in turn helps to explain the anti-obesity effect of antagonising the system.

Adiponectin

CBR1 blockage results in increased production of adiponectin in some (106;134), but not all studies (135-137). Adiponectin is a protein with possible anti-inflammatory, anti-atherogenic and insulin-enhancing effects (138). It is secreted exclusively by adipocytes, but with plasma levels negatively correlated with obesity (139), and anti-inflammatory treatment in AT increases adiponectin expression (140). Research aimed to establish whether adiponectin is the key to some of the effects of Rimonabant has been inconclusive (141;142); adiponectin seems required to mediate the amelioration in insulin sensitivity with Rimonabant treatment but not the effect on body weight.

We measured adiponectin in our rat intervention study. It was unchanged in plasma of the lean rats with or without Rimonabant treatment. It could seem that Rimonabant only increases the adiponectin levels in obese subjects, whom a priori have reduced levels of adiponectin due to their adiposity, while it remains unaffected in lean animals which already exhibit normal levels. Data are, however, conflicting and further studies are necessary to elucidate the direct role of CBR1 antagonists on adiponectin.

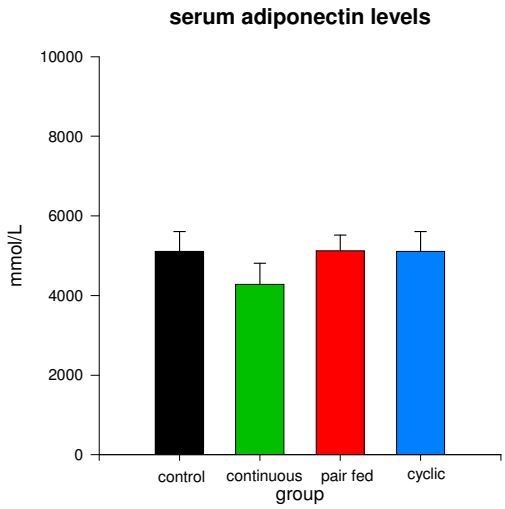


Figure 14: **Unchanged serum adiponectin in the four rat groups of study I. Control group (black bar), continuous Rimonabant treated group (green bar), pair fed to continuous group (red bar) and cyclic Rimonabant treated group (blue bar) (Bennetzen et al., Obesity 2008) (135)**

Adipose tissue metabolism

EC's influence several intracellular mechanisms to increase energy stores by stimulating lipogenesis and increasing insulin signalling and glucose uptake. Stimulation of CBR1 increases lipoprotein lipase activity in mouse adipocytes and this would enhance the flux of NEFA to the adipocytes and thereby TG synthesis (79;106). It also increases glucose access into the adipocytes, thereby supplying fat cells with energy (143), whereas antagonism by Rimonabant or other CB1 antagonists seem to modulate insulin sensitivity in adipocytes (144;145).

Epididymal AT from obese mice contains higher amounts of AEA and 2-AG than lean mice (133). In contrast, some researchers find a decrease in EC concentrations in SAT following high fat diet, although there was no diet-induced change in VAT (146). Several groups found increased CBR1 expression in AT from obese rodents compared with lean (106;147).

The exact role of the ECS in AT is probably a complex entity, and data are divisive. In rodents, the ECS is up regulated in AT in obesity, and stimulation or inhibition of CBR1 is followed by increase or decrease in lipoprotein lipase activity in AT, respectively. Recently published data does not show a direct lipolytic effect of Rimonabant treatment, but suggest an acute lipolytic effect by increasing noradrenaline via the sympathoadrenal system (148).

Other tissues

Muscle

Rimonabant treatment increases basal oxygen consumption and increases glucose uptake from isolated mouse soleus muscle independent of weight changes (149), but tolerance seems to develop to this possible effect on energy expenditure (150). CBR1 gene expression is lower in muscle of obese, insulin resistant rats compared with lean, insulin sensitive rats (151). However, for both nutritional states CBR1 antagonism directly improved glucose transport activity (152). This indicates that the ECS may be

implicated in regulating energy expenditure in skeletal muscle, but data are few and more research necessary.

Liver
CBR1 antagonism seems to reduce the degree of steatosis and elevated levels of TG seen in obesity thereby having a liver-protective effect (81;153). In vivo EC activation of CBR1 in mice increases the hepatic gene expression of enzymes involved in fatty acid synthesis (66). On a high fat diet, liver-specific CBR1 knockout mice develop a similar degree of obesity as that of wildtype mice, but have less steatosis, hyperglycemia and dyslipidemia than wildtype mice.

Pancreas
The ECS is also present in the endocrine pancreas, however data are divisive as to in which cells CBR1 are most prominent (133;154-156). AEA has been found to influence insulin secretion from mouse pancreatic islets (154), and research suggests that Rimonabant can have direct effects on islets to reduce insulin secretion, when secretion is elevated above normal levels by obesity.
(157). In mouse beta-cells production of EC's themselves are under negative control by insulin (133), and differential regulation is suggested in obesity (158). Bermudez-Silva et al. concluded in their review of the ECS in endocrine pancreas, that the ECS is present in the pancreas of both rodents and humans and that cannabinoid receptors can modulate insulin and glucagon secretion (159). The contradictory data makes it difficult to make more solid conclusions.

Other factors are suggested to influence EC levels, such as exercise (160) and gut flora (161), but the significance of this is unclear.

Food intake and body weight
The ability of CBR1 antagonist treatment to reduce food intake and induce weight loss in rats was initially reported in 1998 by Colombo et al. (162). In 1999 and 2002 three different strains of CBR1 knock-out mouse models (CBR1(-/-)) were created by three independent groups (163-165). These mice apparently develop and behave normally, but they show higher mortality throughout their life span compared with wildtype mice, and the deaths are both due to natural causes and unexplained sudden deaths (166). The CBR1(-/-) mice were leaner than their wild type littermates and this difference remained unaffected from pair feeding the adult animals (79), pointing to increased energy expenditure as a possible reason.

Food intake
Chronic administration of the CBR1 antagonist Rimonabant to rodents reduce food intake transiently while causing a lasting reduction in weight for the duration of treatment (162;167), suggesting a metabolic effect independent of the of food intake. The period of reduced food intake varies from 3 to 14 days, with the shorter period being in lean animals and the longer period in obese animals (127;162;167-169), indicating enhanced effect of CBR1 blockage on food intake in obese animals. This period, when food intake is suppressed I call food intake-sensitivity, and the time when food intake returns to baseline levels, is hereafter referred to as food intake-tolerance.

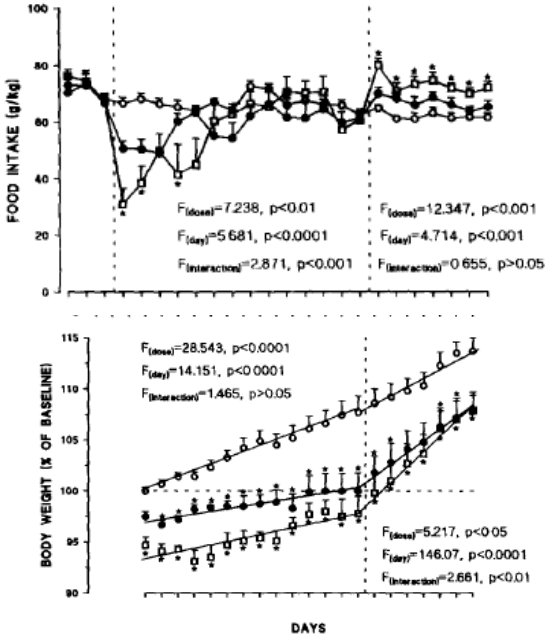


Figure 15: **Food intake and body weight changes during CBR1 antagonist treatment in lean rats. On the left is the food intake; the dotted lines are the beginning and termination of treatment, respectively. The control group consumes approximately the same amount of food throughout the experiment (open circles), whereas the Rimonabant treated rats reduce their food intake the first four days of treatment (open squares) and the pair fed group the same but with delay (black squares). On the right is the weight change; the control group showing a higher rate of weight gain than the Rimonabant treated animals (and pair fed) during treatment, but at the end of treatment (dotted line), the increase in weight are comparable. (Colombo et al., Life Sci 1998) (162)**

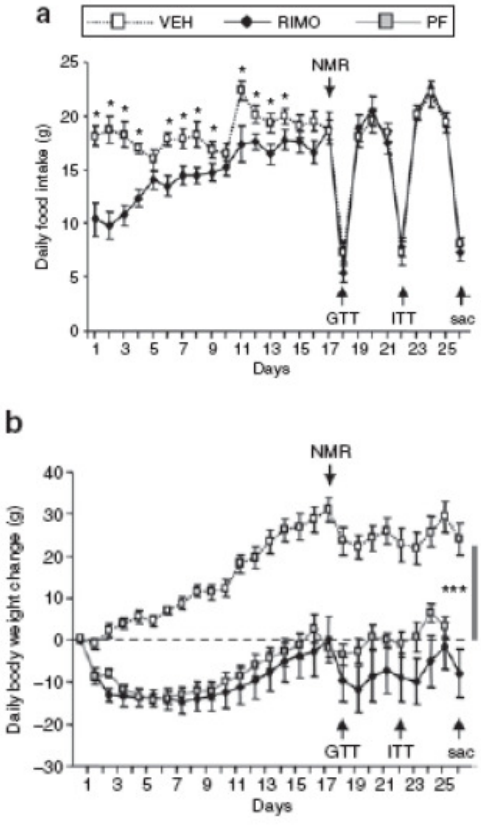


Figure 16: **Food intake and body weight changes during CBR1 antagonist treatment in DIO rats. Similar to figure 15, but in obese animals. On the left is the reduction in food intake induced by treatment (black circles), which is 10 days compared to controls (open squares) From day 17 and forward are various investigations and can be neglected here in both a and b. On the right are the body weight changes. The control group keeps gaining weight until day 17, while the Rimonabant treated group looses weight the first 4-5 days of the study and thereafter gains weight at a slower pace than control group. (Cota et al., Obesity 2009) (136)**

In a recent study performed by Martín-García et al. they investigated food intake in both lean and obese rats following chronic treatment with Rimonabant (126). They also showed the transient reduction in food intake and maintained lesser weight gain, and both effects were indeed more pronounced if the rats were obese. Food intake-sensitivity was only seen in two days in lean rats, but in 14 days in obese animals.

Other groups did not find any tolerance development on food intake. They found that a single administration of the CB1 antagonist AM 251 to reduce food intake for a total of 6 days, was accompanied by reductions in weight gain for 6 days (170). However, they did not seem to examine the rats for a longer period and therefore cannot know whether tolerance would have developed at day seven.

The difference in tolerance development reported may be caused by differences in nutritional status of the rats, various strains of rats or mice and different CBR1 antagonists and even various doses of Rimonabant when this was used. The development of food intake-tolerance could be caused by tachyphylaxia (reduced effect after prolonged ligand-receptor interaction, down-regulation of CBR-1 centrally etc.) but the direct mechanism remains to be elucidated.

We hypothesised that weight loss would improve, if this food intake-sensitivity could be preserved. It could be imagined, that by administering Rimonabant in 3 days and then pause before the food intake returned to normal, and repeating this cycle, the effects of treatment would enlarge. Hence, we set out to investigate this, by administrating Rimonabant in a cyclic manner to lean rats (135).

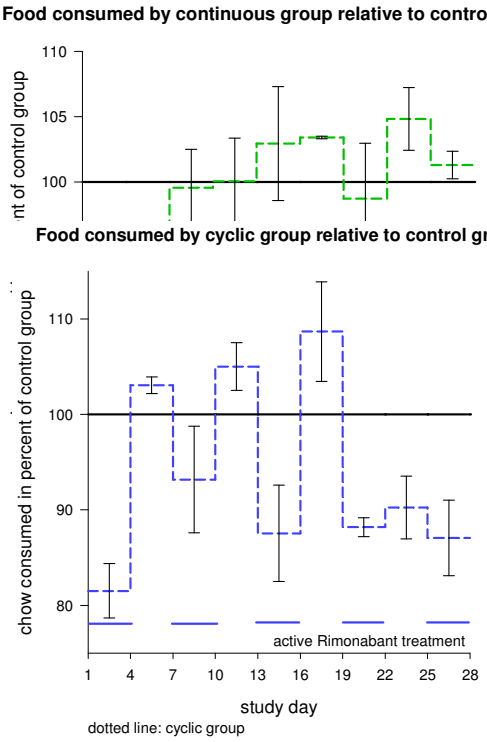


Figure 17: **Amount of food consumed by continuous Rimonabant (green line, left figure) and cyclic Rimonabant treated groups (blue line, right panel) relative to control group in study I. Study I showed reduced food intake in the first six days of treatment in the continuous treated group, but throughout the experiment in active treatment cycles of cyclic treated group. (Bennetzen et al., Obesity, 2008) (135)**

Consistent with previous studies, we found reduced food intake in 6 days for the continuous treated group. In the cyclic group, however, this depression in food intake was observed throughout the study (28 days) in the days of active treatment and resulted in lower body weight compared with the continuous treated group (135). Between the last two cycles of active treatment, the rats did not over consume as in the previous periods between active treatment. The reason is unknown and may account for the difference in food intake observed. It could be very interesting to elaborate further on this by repeating the study and extend the study period.

Food intake independent effects of CBR1 antagonists on body weight

The question remained, as to how the continuous effect on body weight was mediated, when the reduction in food intake was only transient? This implies a peripheral effect on metabolism, but the effect is clouded by the fact that metabolism change with weight changes. One attempt to answer this question could be by investigating animals that were pair feed so they lost the same amount of weight as the Rimonabant treated animals. Some groups of researchers found that the entire effect on weight loss could be accounted for by the food intake reduction (171;172). On the other hand, several groups found increased energy expenditure contributing to the weight loss (127;173-176), by increased energy expenditure due to various mechanisms such as increased fat oxidation driven by lipolysis from AT – possibly mediated via the sympathoadrenal system, increased temperature in brown AT by increased uncoupling protein 1, and differentiation of white fat towards brown fat.

We also included a pair fed group in our previously mentioned study to distinguish changes in metabolic parameters independent of the food intake and weight change. Our results indicated weight loss independent effects of CBR1 blockage on TG and NEFA levels in serum (135).

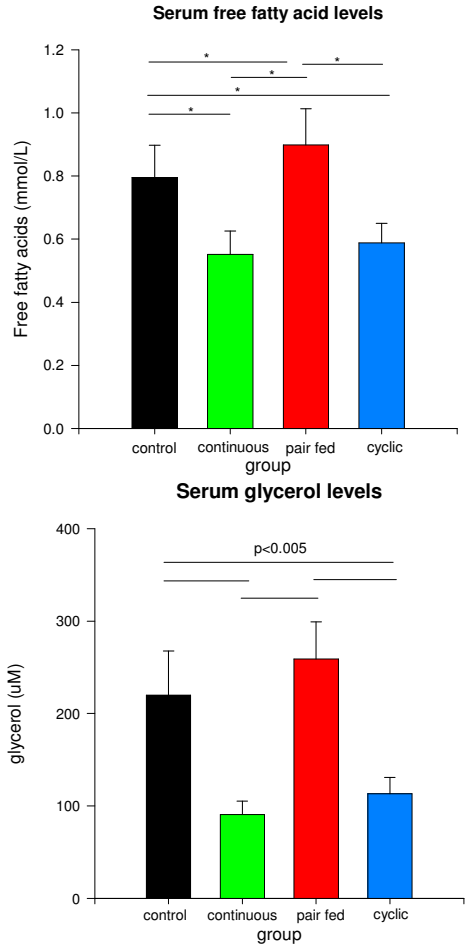


Figure 18: **Serum NEFA and glycerol in study I (Bennetzen et al, Obesity, 2008) (135)**
Cota et al. performed a study similar to ours but with DIO rats (136). Like in our lean rats, Rimonabant treatment also decreased circulating NEFA and TG levels, plus reduced TG content in oxidative skeletal muscle compared with the pair fed group, plus restored insulin sensitivity to that of chow-fed, lean controls during an insulin tolerance test. This confirms that CB1 antagonism has food intake independent effects on metabolism.

To conclude, clear indications of direct Rimonabant induced change in lipid oxidation independent of the food intake and weight loss. Differences in study results could be caused by investigations performed in different states of the feeding cycle, weight loss phases, and different AT depots investigated.

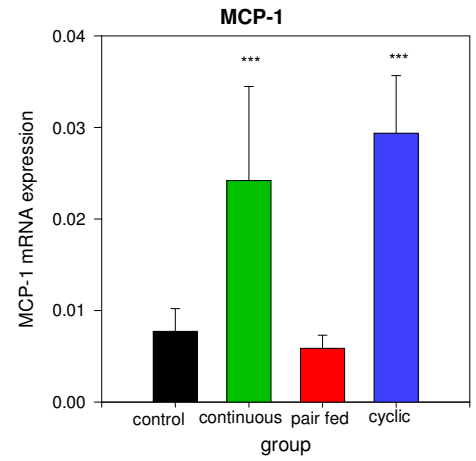
Effects of CBR-1 antagonists on food choices
As was the case for the first studies with cannabis, CBR1 antagonism seems to reduce food intake especially for palatable foods (177-179). Several studies confirm this, using various palatable options (180-182), but results vary as to which component of nutrients is avoided (183-185). The mechanism seems to be central, since exogenous administration of 2-AG into the area in the brain where gustatory signals are transmitted in rats, stimulates feeding of pellets high in content of fat and sucrose as opposed to standard chow (186) and the density of CBR1 is down regulated by high palatable diets in the areas involved with the hedonic aspects of food (187). Rimonabant treatment reduces extracellular dopamine release in the reward areas of the brain of rats when palatable foods are ingested (188). Similar to the question of tolerance development, findings by Rasmussen et al. suggest that obese rats may exhibit a heightened sensitivity to Rimonabant compared with lean (189).

Does this mean greater effect on the hedonic value of food than the nutritional effect? Or does endocannabinoids amplify the palatability of all foods? Food reward is a complex process that involves "liking" (an objective hedonic reaction), "wanting" (addictive component) and learning (associations), and their roles in obesity are just beginning to be understood (190). Kirkham systematically reviewed the "wanting" and "liking" aspects in relation to the ECS and concluded, that endocannabinoids may be essential for the reward anticipation and initiation of eating and that endocannabinoid activity contributes to the pleasure associated with food (191). This means that besides the homeostatic control of food intake, depending on energy stores and nutritional status integrated in the hypothalamus, the ECS can also influence food intake through the brain reward system – the mesolimbic system. This way both strict effects on palatable foods or general effects on food intake could be applicable depending on the circumstances.

Many other factors could be implicated in the regulation of energy intake and expenditure. Gastric emptying is also affected by the ECS (192) and fatty acid intake affects EC levels (193). This could also influence regulation of feeding status and energy metabolism, for instance by modifying the rate at which nutrients appear in the circulation, while other factors, such as exercise, enhances the effects of Rimonabant on food intake and weight loss and could also cause differential results (194).

Inflammation
Obesity is associated with a state of systemic low-grade inflammation and it has been suggested, that AT should be considered an immune organ besides endocrine organ and energy storage depot (195). Obesity-related ECS activation is accompanied by elevated expression of the pro-inflammatory cytokine TNF-α, which in turn stimulates ECS activation in vitro (196). CBR1 activation in endothelial cells may be involved in atherogenesis (197), and Rimonabant seems to reduce the inflammatory effects of macrophages, which may limit the development of atherosclerosis (198). This inflammatory condition could be the link between obesity and DM2, and endothelial dysfunction could play a role in the association of obesity with increased risk of CVD (199).

To our surprise inflammatory markers in AT seemed to rise following CBR1 antagonist treatment irrespective of administration. This effect on inflammation is not seen in human studies where CRP on the contrary is reduced with Rimonabant treatment (200). The ECS is associated to inflammation in several tissues but it is unclear whether it is a pro-inflammatory or an anti-inflammatory association (201). The reason could be, that it varies with tissues, and the positive effect on CRP in circulation in the Rimonabant in obesity (RIO) studies could be caused by the weight loss and increase in adiponectin, while in our lean rats, with no changes in adiponectin and no real weight loss but reduced weight gain, there may be a direct inflammatory effect of CBR1 antagonist treatment in AT.
A



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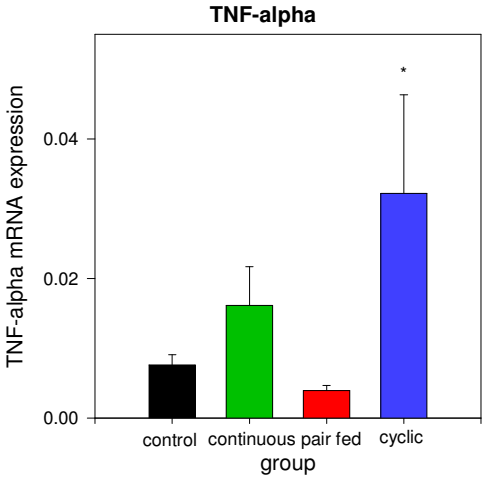

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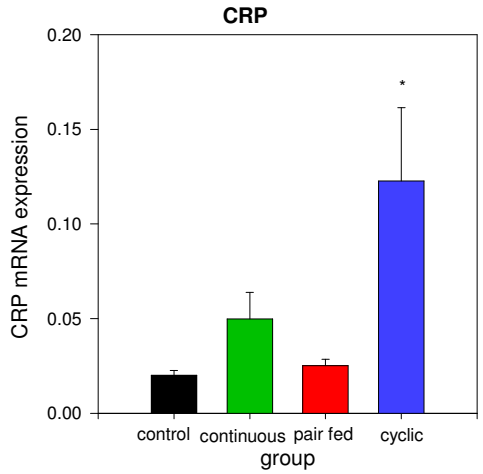


Figure 19: **Inflammatory markers, MCP-1 (fig. A), TNF-α (fig. B), and CRP(fig. C), in AT following CBR1 antagonist treatment in study I (Bennetzen et al., unpublished results)**

THE ECS IN HUMAN OBESITY

The EC's in the circulation

There is some evidence of an up regulation of the ECS in human obesity by measuring ECs in circulation. The first indication that the ECS was up regulated in obesity was from Engeli et al. in 2005, who found both EC's to be up regulated in obese women (202). Other groups found only 2-AG to be up regulated (203;204).

Recently however, Sipe et al. found no difference in EC levels between lean and obese (205). Peripheral endocannabinoid overactivity may explain why CBR1 blockage induces reduction in lipogenesis and enhancement in insulin secretion in obese subjects (158), but knowing that the EC's are produced "on demand" in various tissues it is difficult to establish the relevance of the levels in the circulation. It could be caused by "spill-over" from an active tissue – a relevant hypothesis in obesity is AT. Taken together, data points to increased 2-AG levels in the circulation of obese subjects but the origin and relevance of this is uncertain.

The ECS in AT

EC

Matias et al. found, that patients with obesity or hyperglycaemia had higher levels of 2-AG in VAT than controls, and that VAT of obese persons contained more 2-AG than SAAT, measured by LC-MS (133). Recently, Anuzzi et al. investigated SAAT levels of lean, obese, and obese with DM2 (206). He found higher AEA and reduced 2-AG levels in obese with DM2, but no difference between lean and obese.

We also investigated the peripheral levels of both 2-AG and AEA in VAT and SAAT of obese individuals and we found that levels of both EC's tended to be lower in VAT compared with SAAT at least for 2-AG (Bennetzen et al., study on gender differences). And for obese subjects we found women to have higher EC levels in both AT depots.

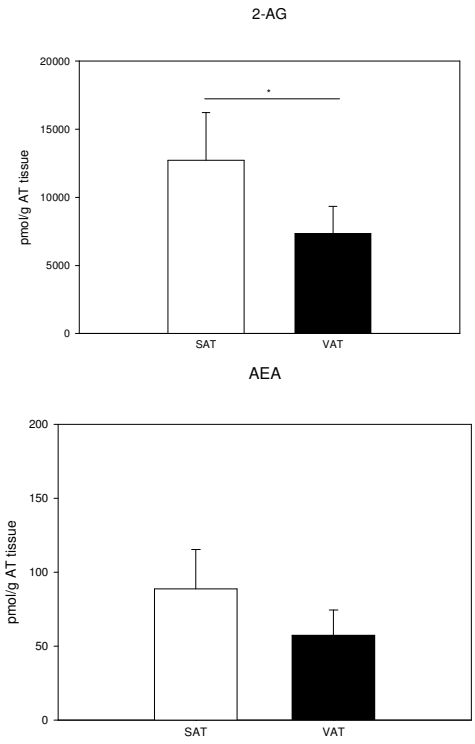


Figure **20: 2-AG and AEA levels in SAT and VAT of obese subjects. SAT: subcutaneous adipose tissue, VAT: visceral adipose tissue. (Bennetzen et al., unpublished results)**

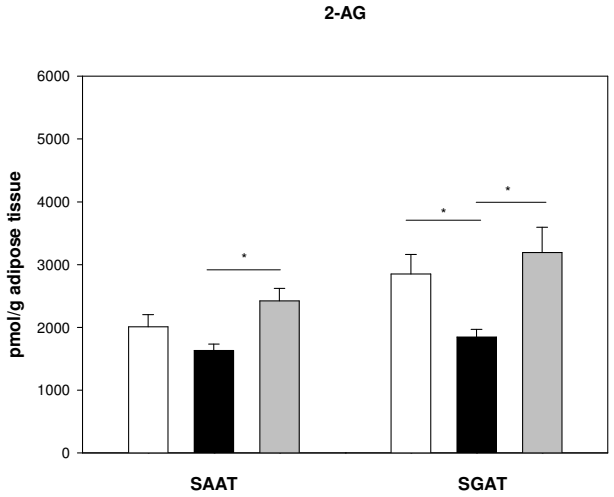


Figure 21: **2-AG levels in subcutaneous AT. Lean (white bars), Obese at baseline (black bars) and Obese after weight loss (grey bars). SAAT: subcutaneous abdominal adipose tissue, SGAT: subcutaneous gluteal adipose tissue (Bennetzen et al., Manuscript III)**

We also performed the same investigations in lean and obese before and after 10% weight loss, and measured EC's in two subcutaneous AT depots. The study revealed the same or lower 2-AG levels in obesity and a rise in both depots with weight loss (Bennetzen et al., manuscript III). The AEA levels in the two subcutaneous depots were unaffected by weight changes.

CBR1

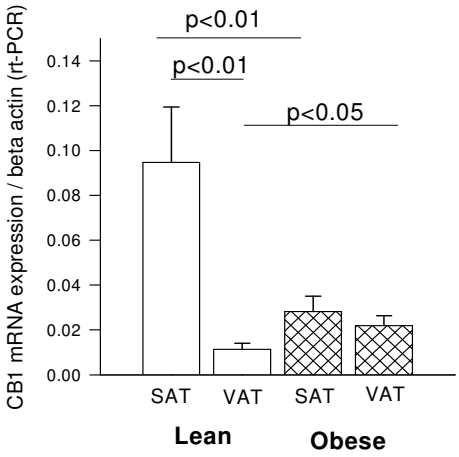
Data regarding changes in CBR1 in AT in obesity are divisive, ranging from lower, unchanged to increased levels. Most researchers find levels of CB1to be reduced in obesity in SAAT and VAT (196;202;204;207), but some research groups find unchanged levels (208;209) and a few research groups find increased levels in obesity (143;210).

We also investigated the CBR1 levels of lean and obese subjects and confirmed our results by using two different methods. We came to the conclusion, that in obesity CBR1 levels in SAAT was decreased and in VAT they were increased or similar, resulting in comparable levels in the two depots in obesity (64).

Moreover, we also discovered that for both gluteal and abdominal subcutaneous depots, CBR1 levels were the same or reduced in obesity, and increased with weight loss (Bennetzen et al., manuscript III). To conclude, in concordance with several previous studies we find CBR1 reduced or similar in obesity depending on depot.

The great discrepancy in the presented data could be caused by variation in the CBR1 gene. It could be strictly regulated and subjected to continuous change or depend on many factors we do not take into account when designing the studies.

Gene expression



Protein levels

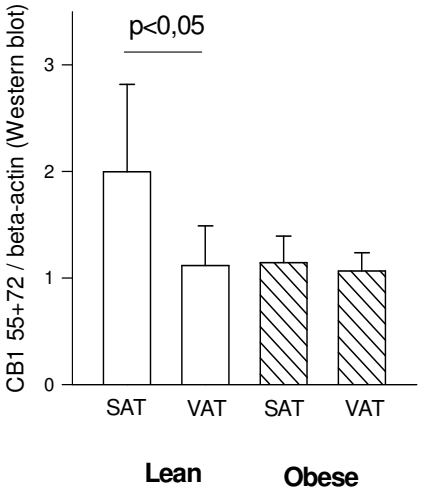


Figure 22: **CBR1 Levels in SAT and VAT of lean and obese. SAT: subcutaneous adipose tissue, VAT: visceral adipose tissue. (Bennetzen et al., Eur J Clin Invest 2010) (64)**

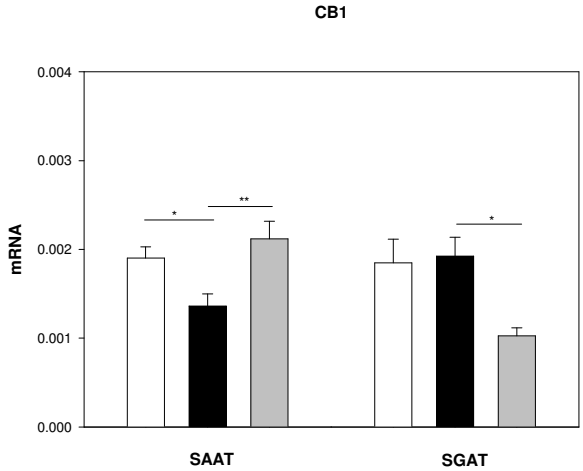


Figure 23: **CBR1 Levels in SAAT and SGAT with weight changes, lean (white bars), Obese at baseline (black bars) and Obese after weight loss (grey bars). SAAT: subcutaneous abdominal adipose tissue, SGAT: subcutaneous gluteal adipose tissue (Bennetzen et al., Manuscript III)**

Genetic variations in the CBR1 gene
A gene variation in the CBR1 gene (3813G) has both been associated with higher (191) and lower BMI (192). However in the presence of obesity, the polymorphism is associated with a better cardiovascular profile (193;194). The last mentioned group also investigated the same polymorphism in naïve diabetic patients and found no association to obesity (195). This indicates that there is no certain association to obesity with this specific polymorphism, but that in obesity this polymorphism may be protective against CVD.
For various other CBR1 polymorphisms, there is no congruent evidence for association with obesity (211-216), but a possible indication in lipid metabolism is emerging (217;218).

Synthesising and degrading enzymes
Data regarding changes in enzyme gene expression levels in obesity are even more divisive than for the receptor, but results concentrate on FAAH, which seem to be reduced in obesity (143;196;202;204) though some groups find it increased (143;209) depending on depot investigated. FAAH is a membrane bound enzyme and its inactivation by either genetic deletion or pharmacologic inhibition is followed by increased half-life and elevated levels of AEA in animal studies (219). In our investigations we also find FAAH and FAAH2 to be affected by weight changes, but data are inconsistent ((64) and Bennetzen et al., manuscript III). To our surprise we found correlation between 2-AG and FAAH2. The reduction in enzyme levels could explain the increase in 2-AG levels in SGAT following weight loss, but we find no increase in obesity to explain the rise in 2-AG, and the actions on the enzymes may not be as simple as previously anticipated (220). It is unknown whether gene expression is an adequate level of active enzyme levels in the tissues. They could be subject to strict regulation or variation unaffected by the expression levels.

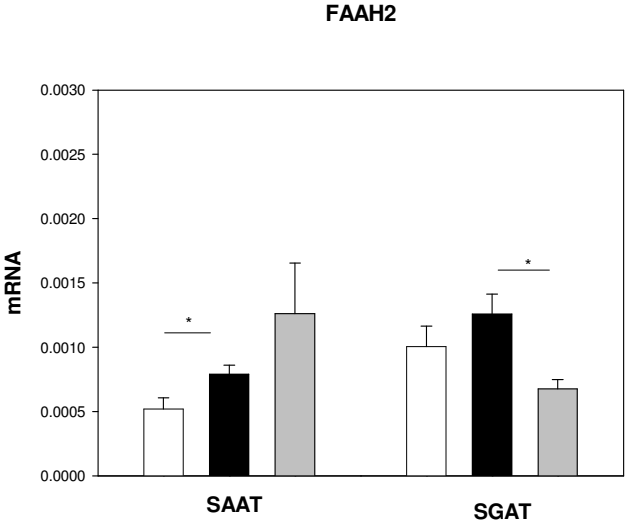


Figure 24: **FAAH2 levels Levels in SAAT and SGAT with weight changes, lean (white bars), Obese at baseline (black bars) and Obese after weight loss (grey bars). SAAT: subcutaneous abdominal adipose tissue, SGAT: subcutaneous gluteal adipose tissue (Bennetzen et al., Manuscript III)**

Genetic variations in the FAAH gene
The P129T mutation in the FAAH gene does not seem to be associated with obesity (221;222), while other groups find modest association to class III obesity (223). However it could be involved in lipid metabolism, since obese and dyslipidaemic carriers of the P129T mutation in the FAAH gene had a significantly greater decrease in triglycerides and total cholesterol as a consequence of 6 weeks low fat diet compared with wild type (224). Another variation in FAAH (rs324420) was found to be associated with increased BMI, increased TG, and reduced levels of HDL (225). The same variation (rs324420) and another (rs2295632) where found to be associated with early onset obesity, but not with adult obesity, indicating a weak connection (226).

The homozygous FAAH 385 A/A genotype was significantly associated with overweight and obesity in several studies (227;228). Not surprisingly, a previously mentioned study Sipe et al. find this FAAH-polymorphism to be associated with increased EC levels (205), but investigating only obese, the mutant type A358C of FAAH is associated with lower glucose, insulin and HOMA levels and higher visfatin levels than of the wild-type group in females (229), indicating a positive consequence of this polymorphism.

Some even investigated both CBR1 and FAAH variations, but they found no association to adiposity traits in their population (n= 2415 from the Framingham Offspring study) (230). Taken together, the data support the hypothesis of variants in the FAAH gene leading to increased EC levels. The direct association to obesity is more complex, and no general conclusion can be drawn from the present studies.

THE RIMONABANT STORY
As mentioned in the introduction, Rimonabant was discovered to reduce weight and tested in a concert of large clinical trials (fig. 26). Analogues of Rimonabant were also tested for various pharmaceutical companies.

Rimonabant is often described as a CBR1-selective antagonist (also in this review), but is, in fact, an inverse agonist to the CBR1. This means negative modulation of the CBR1 receptor from its constitutively active “on” state to a more inactive or “off” state (231).

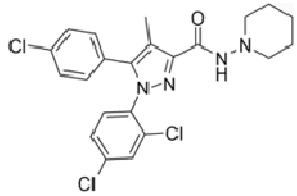


Figure 25: **Structure of Rimonabant (SR141716) (Cota et al., Int J Obes 2003) (28)**

Weight loss and possible weight-loss independent effects of Rimonabant
As shown in fig. 26 the mean weight loss after 1 year treatment with Rimonabant was about five to six kg higher than in the placebo-controlled group. This weight loss seemed to be maintained for up to 2 years. After quitting Rimonabant therapy, body weight increased rather fast, as seen in most other pharmacological-induced weight losses.

Pooled 1 year data from all four studies revealed improvement in metabolic parameters (HDL, TG, adiponectin and HbA1c for diabetic patients) beyond the effect caused by the weight loss alone according to the RIO study investigators (232).

Adverse effects of Rimonabant
The adverse events reported from the RIO studies were gastrointestinal and psychiatric. Also in STRADIVARIUS, SERENADE, ADAGIO-lipids, and ARPEGGIO, psychiatric adverse effects were more common in the Rimonabant group. The psychiatric side

effects were depressed mood disorders, anxiety and sleep disturbances - despite depressed mood being an exclusion criterion in these trials. Hence, the estimate of psychiatric side effects from these studies is likely conservative. Questions about ideation of suicide were not part of the RIO questionnaire, but FDA analysis of side effects found increased suicide rate with Rimonabant treatment (233).

The Comprehensive Rimonabant Evaluation Study of Cardiovascular ENDpoints and Outcomes (CRESCENDO) was a multinational trial of Rimonabant 20 mg for reducing the risk of major cardiovascular events in abdominally obese patients. The study was terminated preterm in 2008, when Rimonabant was withdrawn(234), and to my knowledge only a subset of the study has been published (205).

Figure 26: The major results on weight loss of great Rimonabant trials

Study	n	Publication Year	Body Weight Change	Other End Points
STRATUS -worldwide, -us, -eu 1 year	**3228**	**2005-6 (36)**	**Weight gain was significantly lower among the Rimonabant (20 mg) quitters but not reported in absolute kilos**	**(RR) for quitting the cigarettes with Rimonabant (20 mg) was 1,5 (CI: 1,1 to 2,05) and for remaining abstinent 1,29 (CI: 1,06 to 1,57)**
RIO - North America, -Europe, -diabetes, -lipids 1 year results	**6630**	**2005-6 (235) (200) (236)**	**-1,25kg (95% CI: -1,64 to -0,86) and -4,64kg (95% CI: -4,99 to -4,28) with 5 and 20 mg, respectively**	**Rimonabant 5 and 20mg was followed by reduction in WC (1,2 cm, 95% CI: -1,7 to -10,7 and 3,8 cm, 95% CI: -4,3 to -3,4, respectively). The 20 mg dose induced beneficial changes in HDL (7.2 to 8.9% increase), TG level (12,4 to 16.4% reduction), fasting insulin (1,1 to 2,8 µIU/ml reduction), fasting glucose (0,03 to 0,97 mmol/L reduction) and minor reductions in blood pressure (systolic: 0,2 to 2,4 mmHg reduction, and diastolic: 0,2 to 1,5 mmHg reduction, respectively). In RIO-lipids and-diabetes there was a reduction in CRP (0,5 to 1,4 mg/L). Change in adiponectin level was only reported for the RIO-lipids and amounted to 2,2 ± 2,5µg/ml (mean± SD) for 20 mg. Rimonabant. Change in HbA1c with 20 mg Rimonabant in RIO-diabetes was -0,6 ± 0,8% (mean ±SEM)**
STRADIVARIUS 18 months	**839**	**2008 (237)**	**-4.3kg (CI: -5.1 to -3.5)**	**No change in percent atheroma volume. Decrease with Rimonabant treatment (20 mg) on total atheroma value, WC, median triglyceride levels, and high sensitivity CRP, smaller increase in Hba1c levels and an increase in HDL**
SERENADE 6 months	**281**	**2008 (238)**	**-6.7kg (SD: ± 5.5kg)**	**Rimonabant also induced improvements from baseline in WC, fasting plasma glucose, triglycerides, and HDL. Reduction in Hba1C, with a larger Rimonabant effect in patients with baseline Hba1C > or = 8.5%.**
ADAGIO Lipids 1 year	**803**	**2009 (239)**	**-5.8kg (SD: ± 5.9kg)**	**20 mg Rimonabant increased HDL and decreased TG levels. Greater changes from baseline of LDL and HDL particle sizes, CRP, and adiponectin levels. Rimonabant decreased SAAT cross-sectional area, with a greater reduction in VAT, and reduced liver fat content, systolic and diastolic blood pressure.**
ARPEGGIO 42 weeks	**368**	**2010 (240)**	**-2,5kg (SD: ± 0.3kg)**	**It showed reduced Hba1C (8.2± 0.1%). Improved other cardiometabolic parameters (HDL, TG levels),**
STRATUS -worldwide, -us, -eu 1 year	**3228**	**2005-6 (36)**	**Weight gain was significantly lower among the Rimonabant (20 mg) quitters but not reported in absolute kilos**	**(RR) for quitting the cigarettes with Rimonabant (20 mg) was 1,5 (CI: 1,1 to 2,05) and for remaining abstinent 1,29 (CI: 1,06 to 1,57)**
RIO - North America, -Europe, -diabetes, -lipids 1 year results	**6630**	**2005-6 (235) (200) (236)**	**-1,25kg (95% CI: -1,64 to -0,86) and -4,64kg (95% CI: -4,99 to -4,28) with 5 and 20 mg, respectively**	**Rimonabant 5 and 20mg was followed by reduction in WC (1,2 cm, 95% CI: -1,7 to -10,7 and 3,8 cm, 95% CI: -4,3 to -3,4, respectively). The 20 mg dose induced beneficial changes in HDL (7.2 to 8.9% increase), TG level (12,4 to 16.4% reduction), fasting insulin (1,1 to 2,8 µIU/ml reduction), fasting glucose (0,03 to 0,97 mmol/L reduction) and minor reductions in blood pressure (systolic: 0,2 to 2,4 mmHg reduction, and diastolic: 0,2 to 1,5 mmHg reduction, respectively). In RIO-lipids and-diabetes there was a reduction in CRP (0,5 to 1,4 mg/L). Change in adiponectin level was only reported for the RIO-lipids and amounted to 2,2 ± 2,5µg/ml (mean± SD) for 20 mg. Rimonabant. Change in HbA1c with 20 mg Rimonabant in RIO-diabetes was -0,6 ± 0,8% (mean ±SEM)**

Other human studies

Effects of CBR-1 antagonists on food choices

Rimonabant treatment does not seem to affect intake of a specific macronutrient in obese humans (241). However high-fat intake may reduce CBR1 and MGL gene expression in skeletal muscle, having no effect in AT (242). This supports the previous finding of a small study investigating the effects of stimulating or blocking the CB1 in human skeletal muscle. Results implied that endocannabinoids regulate pathways affecting skeletal muscle oxidation, and these effects seemed to be emphasised in myotubes from obese individuals (243).

Effects on insulin levels

CBR1, CBR2, and the enzymes involved in synthesis and degradation of 2-AG are also present in the human pancreatic islets of Langerhans (155). Insulin may reduce EC levels inversely to BMI, waist circumference, SAT, fasting insulin and total glucose, and insulin areas under the curve during the OGTT (244). FAAH gene expression positively correlated with the fasting serum insulin concentration, and following hyperinsulinaemia, mRNA levels increased in the lean but not in the obese subjects (209). The ECS and insulin levels may affect each other, but studies on the impact of CB1 antagonist on insulin production are divisive and include both decrease (180) and increase (158). Insulin resistance may lead to increased EC tone and consequently increased adiposity, which again increases insulin resistance starting a vicious cycle (245).

ECS in human obesity and influence of weight loss

It would be interesting to know, whether the EC levels were different before obesity developed or whether it changed as a consequence of this state. An approximate measure of this was weight loss in order to return to a "closer to normal" state and investigate whether this would affect components of the ECS. Only a few studies addressed this issue. 5% diet induced weight loss did not affect EC levels in postmenopausal women (202), neither did 5% Sibutramin (a formerly approved weight loss drug) induced weight loss (246). Di Marzo et al. investigated the effects of 1 year life style intervention (including healthy eating and physical activity) in viscerally obese men (n=49) (247). The intervention led to a decrease in body weight, which reached 7% and was followed by reduction in WC, VAT and circulating AEA and 2-AG. Only the decrease in levels of 2-AG (not AEA) correlated with decreases in VAT and TG levels and with increase in HDL levels.

A weight loss of 10% is necessary to reduce markers of inflammation and increase serum adiponectin (248) and following, we hypothesised that such a weight loss was necessary to induce changes in the ECS. Hence, we performed an intervention study with 10% weight loss in obese subjects (n=42) and found the reduction in 2-AG seen in Subcutaneous AT in obesity normalised with weight loss (Bennetzen et al., manuscript III). Also both degrading enzymes (FAAH2 and MGL) were reduced with weight loss (see earlier figures of EC and FAAH2). Taken together these studies indicate that a certain weight loss is needed before changes appear in the ECS, but implies that the system is changed as a consequence of the obese state.

CONCLUSIONS

To summarise, the ECS is conserved across evolution. It interacts with many an- and orexigenic pathways both in the brain and the periphery, including leptin, adiponectin and insulin. Activation of CBR1 induces increased food intake and body weight. Blockage is followed by transiently reduced food intake and maintained body weight loss. The main effect on food intake is in my view mediated via interaction with central receptors. It remains to be established that direct, peripheral effects exist without being mediated from the central nervous system. But it could be possible that peripheral receptors could be affected by CBR1 antagonism and hence, by cross-talk with various appetite and satiety pathways, and not necessarily direct presence in the brain, has potential to mediate peripheral effects on energy metabolism.

Circulating levels of EC's (2-AG) are elevated in human obesity, but levels in AT are not the source, as they are reduced. A weight loss of 10% is necessary to normalise the changes in AT in the ECS. CBR1 antagonism by Rimonabant in rodents induce inflammatory changes in AT. The ECS seems to mediate obesity-induced changes in the liver and antagonism of the CBR1 is followed by improvement of this and of serum lipid levels. So like many other drugs, CBR1 antagonism could induce positive changes in the liver and on lipid metabolism but induce unwanted psychiatric effects and could intensify the inflammation seen in obesity, where the circulating levels may affect atherogenesis.

The presence of CBR1 in many tissues associated with energy metabolism is indicative of great potential. At present no medicinal product is approved to modulate this system, but if the psychiatric side effects can be circumvented, and the CBR1 antagonism could be limited to certain tissues a positive effect will be present.

STUDIES

STUDY I

"EFFECTS ON FOOD INTAKE AND BLOOD LIPIDS OF CANNABINOID RECEPTOR 1 ANTAGONIST TREATMENT IN LEAN RATS"

Aim

The aim of this study was to investigate whether the food intake-tolerance to Rimonabant could be circumvented and whether this would intensify results on body weight and blood parameters in lean rats. We also aimed to establish possible weight loss independent effects of treatment by using a pair fed group.

Materials & Methods

Study participants

We used approximately 7 weeks old male Wistar Hannover Galas rats (n=40) for the study. None of the rats died preterm or seemed sick throughout the experiment. The rats were randomly allocated to 4 groups, with 10 rats in each group, and for the 4 weeks of the study, the rats received either 2 mg Rimonabant (= 10 mg/kg) or vehicle depending on study group. Rimonabant was generously provided from Novo Nordisk (Syntagon, code: NN007-HCl, batch: 270/14) dissolved in dimethylsulfoxide (DMSO) at a concentration of 2 mg/50 µL (0,04g/ml). We injected Rimonabant and vehicle, or vehicle only, intra peritoneally (IP) every day on all 40 rats of the study.

1. Control group had free access to food and was daily injected with vehicle.
2. Continuous Rimonabant group was injected with Rimonabant every day and had free access to food.
3. Pair fed group was daily injected with vehicle and the group was pair fed with the continuous Rimonabant group (group 2) in order to obtain similar body weight as group 2.

4. Cyclic Rimonabant group was treated for 3 days with daily Rimonabant injections and then 3 days with vehicle and this cyclic alteration between Rimonabant and vehicle continued throughout the study period. The rats had free access to food.

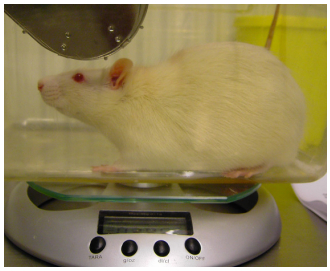


Figure 27: **One of the rats on the scales**

After 28 days of treatment, the rats were sacrificed and various tissue samples collected and frozen.
The study complied with Danish regulations for care and use of laboratory animals, and all animals used were recorded and reported. The protocols were approved by the Danish Council for Animal Research. Protocol no: 2206/561-1136.

Blood samples
Rat blood samples were analysed by enzyme-linked immunosorbent assay (ELISA). To all kits the company manual was used and the absorbance was measured with a spectra reader photometer. TG analyses were performed using no. 12016648-122, GPO-PAP, Roche/Hitachi. Range of standard curve: 0,05 mmol/L – 11,3 mmol/L, intra-assay coefficient of variation (CV) 1,5 %, and inter-assay CV: 1,8 %. NEFA analyses were done with no. 999-75406, NEFA C, Trichem. Range of standard curve: 0,01-4,00 mEq/L and intra-assay coefficient of variation (CV) 1,5 %. Adiponectin was analysed by B-bridge International, Biocat. Range of standard curve: 0,25 ng/ml – 8 ng/ml, intra-assay CV: 4,8 %, and inter-assay CV: 13,6 %. Glycerol was measured by a modified lipolysis assay from Novo Nordisk and luminescence measured on a luminometer, Luminoscan, Ascent Software, Labsystems, Finland.

Real time RT-PCR described in study II

Statistics
In all four studies we used Sigma plot 11.0 with incorporated sigma stat soft ware (Systat software, Inc., Richmond, CA, USA). Results are expressed as means ± standard error of the mean (SEM). Differences were considered significant if p<0,05 and marked with * on figures.

Results
Food intake
Comparing amount of food consumed during the first 6 days by control group and continuous Rimonabant treated group, the continuous Rimonabant group showed significantly less food intake (p<0,01). The cyclic Rimonabant group showed significantly less food intake during the active treatment periods compared to control group both in the first half of the study (p<0,05) and during the second half of the study (p<0,01). The food intake throughout the study period was significantly less in the cyclic Rimonabant group compared to the continuous Rimonabant group (592,4g versus 621,1g, p<0.05), and less only in the cyclic Rimonabant group.

Body weight
Groups one to three ended up with similar body weights (321,4g ±5,83g, 318,2g ±7,51g and 319,5g ±3,03g). Only the cyclic Rimonabant group had a significant less weight gain as compared with the control group (P< 0.05)

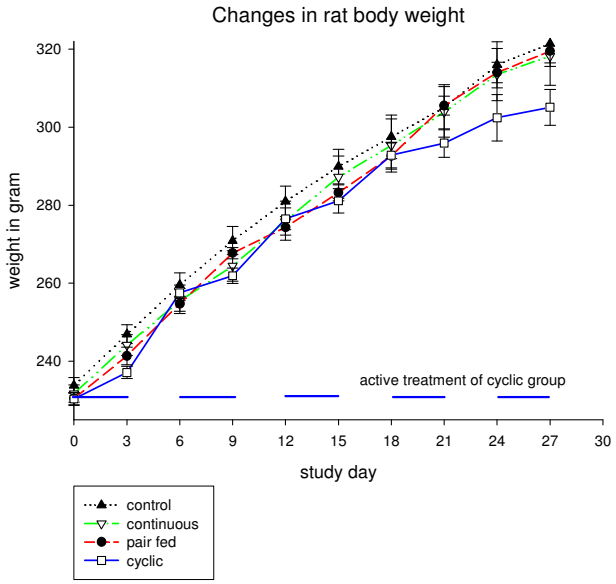


Figure 28: **Absolute body weight in gram during treatment. Only the cyclic Rimonabant group had a significant less weight gain as compared with the control group (Bennetzen et al., Obesity, 2008) (135)**

Blood samples
Serum TG, glycerol and NEFA levels were reduced in both treated groups, though only the cyclic treated group had less weight gain. Thus there were indications for weight loss independent effects. We did not find any change in circulating adiponectin levels with Rimonabant treatment.

Gene expressions
Methods described for study II. Target gene found stable in this specific intervention and used was GADPH. To our surprise we also found the previously mentioned indication of increased inflammation in both treated groups, measured by increased gene expression of MCP-1, CRP and TNF-α in AT.

Discussion
We used the dosage 10 mg pr. kilo body weight after a literature review. This is a higher dose than Rimonabant was registered at in human use, but makes the animal studies easier to compare.

Our result with tolerance development in six days in lean animals with continuous Rimonabant treatment is in concordance with previous studies. To my knowledge, no other studies have examined the effect of cyclic administration of Rimonabant. Because the food intake-curve seems to break in the end of the study period, it would have been interesting to see, what would have happened in the next weeks, had the study continued. We have no information as to whether the side effects are similarly enlarged as the positive effects.

We used lean rats in this study, because we did not want major differences in weight. Others have performed the same kind of study in DIO rats (excluding the cyclic treated group) and found results similar to ours (136). Their rats developed food intake tolerance in 14 days, as expected in obese animals. They found the same weight independent reduction in circulating levels of NEFA and TG and they neither found any change in plasma adiponectin. Like us, they found reduction in leptin only in the treated group. Furthermore, they clarified that the body weight loss was caused by fat mass loss as measured by MRI and this is confirmed by a third study in DIO mice by Koolman et al. by a simpler method (184). We found leptin to be reduced in both treated groups in our study and since the continuous treated group has the same body weight as controls, this raises the question of how leptin levels could be reduced without a reduction in surplus fat mass. We have not measured adipose tissue mass. It could have given a hint to simply weigh the fat dissected from the rats, and better to have subjected the rats to MRI's.

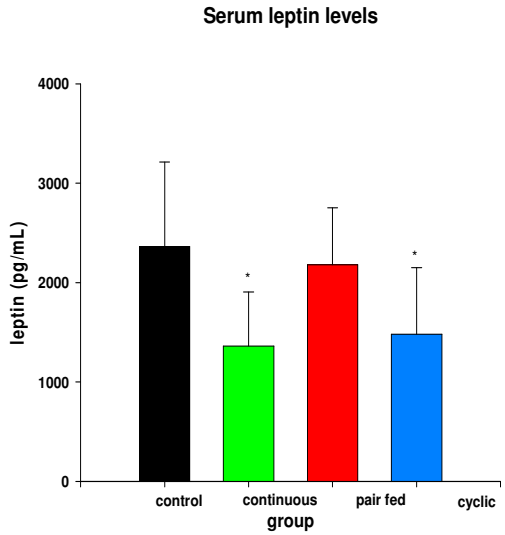


Figure 29: **Serum leptin levels in study I (Bennetzen et al., unpublished results)**

We know that EC's and leptin interact; maybe CBR1 antagonism exerts a direct effect on leptin levels independent of weight loss. Another explanation is the implication of the ECS in bone turnover. Various genetic knockouts of CBR1 yield varying results on bone mass. Tam et al. found a phaenotype with a lower bone mass (249), while Idris et al. found that CBR1(-/-) mice have increased bone mass (71). Recent data also implies a lowering effect of 2-AG on exercise motivation (250), and it could be imagined, that CBR1 antagonism increases the activity level. Chronic treatment with Rimonabant could therefore be followed by an insignificant increase in lean mass due to increased bone density or muscle mass or a combination, and this way mask a small amount of fat loss in our study, but the significance of this is questionable.

Conclusion

Tolerance to the hypophagic effect of continuous Rimonabant developed with 6 days of treatment in lean rats. This is in contrast to the cyclic Rimonabant treatment where tolerance was not observed for the whole study period (28 days). Moreover, we found indications that Rimonabant (both continuous and cyclic) has weight-independent effects on circulating levels of NEFA and glycerol (and inflammatory markers) and the effects on triglycerides showed the same tendency whereas no effects were observed on adiponectin. The direct effects on NEFA and glycerol may be mediated through peripheral CB1 in adipose and liver tissues or via leptin

STUDY II

"REDUCED LEVELS OF CANNABINOID RECEPTOR 1 PROTEIN IN SUBCUTANEOUS ADIPOSE TISSUE OF OBESE"

Aim

The aim here was to establish and to investigate the possible differences in the expression of the CBR1 gene in human SAT and VAT from lean and obese subjects. We also wanted to explore whether the gene expression of CBR1 is an adequate measure of corresponding protein levels

Materials & Methods

Study participants

The study participants for the gene expression analysis included 12 obese and 10 lean women (n=22). Paired samples of SAAT and VAT were taken during obesity surgery for the obese subjects and during gynaecological surgery for the lean women. For the protein analysis 10 other subjects were used (five obese/five lean) undergoing the same operation procedures. Six overweight women donated AT from cosmetic liposuction procedures to investigate the gluteal and the abdominal subcutaneous depot. This was also the case for the eight subjects donating AT for the investigations of isolated adipocytes and stroma-vascular AT fraction. These were cross sectional studies. The protocols were approved by the local ethical committee and all subjects had provided written informed consent. Journal no: 20070032.

Immunoblotting

Was performed by Dept. of medicine and endocrinology, MEA (at that time, M), Aarhus University Hospital, Denmark.

Analysis of mRNA levels

Isolation of RNA

AT samples were homogenized in TriZol reagent (Gibco BRL, Life Technologies, Roskilde, Denmark) and total RNA was extracted following the manufacturer's protocol. RNA was quantitated by measuring absorbency at 260 nm and 280 nm and the ratio was 1.8 or higher. The integrity of the RNA was checked by visual inspection of two ribosomal RNAs on an ethidium bromide stained agarose gel.

Real-time RT-PCR

Reverse transcription of RNA was performed using random hexamer primers at 42ºC for 30 minutes and 92ºC for two minutes followed by 4ºC as described by the manufacturer (versoTM cDNA Kit from ABgene, Surrey, UK). Then, PCR-mastermix containing the specific primers and Taq DNA polymerase (KAPATM SYBR® FAST qPCR Kit, Kapabiosystems, Boston, Massachusetts, USA) were added. The primers were designed using the primer analysis software Oligo version 6.64.

Real time quantitation of target gene to housekeeping gene was performed with a SYBR-Green real-time PCR assay using an ICycler from BioRad.

The target and housekeeping gene were amplified in separate tubes. The increase in fluorescence was measured in real-time during the extension step. The threshold cycle (Ct) was calculated, and the relative gene-expression was calculated essentially as described in the User Bulletin #2, 1997

Figure 30: **Primersequences used for RT-PCR in study II (bp: base pairs)**

Gene	Sequence	Product size
B-actin	Sense 5' ACGGGGTCACCCACACTGTGC 3' Antisense 5' CTAGAAGCATTTGCGGTGGACGATG 3'	658 bp
B-2-microglobulin	Sense 5' AATGTCGGATGGATGAAACC 3' Antisense 5' TCTCTCTTTCTGGCCTGGAG 3'	128 bp
CBR1	Sense 5' CATGGCATCCAAATTAGGGTA 3' Antisense 5' CTGGTCTGCTGGGACTAGCTG 3'	118 bp
DAGLa	Sense 5' TGCTAGTGTTGGCGCGTAT 3' Antisense 5' GGCAGGCAGGGCAGGTAGGGT 3'	92 bp
DAGLb	Sense 5' CGCGGAAGTCTATGTCTAAGC 3' Antisense 5' GTTGCGATGATGCCGTTTACA 3'	139 bp
FAAH	Sense 5' GGGCCGTCAGCTACACTATGC 3' Antisense 5' ATGTTCCATCTGGGCCTCGTC 3'	101 bp
FAAH2	Sense 5' CGCTAGGCTTTCTCATAGGC 3' Antisense 5' CCGAAAGCAGAAGCAATGGTT 3'	111 bp
NAAA	Sense 5' CGGACTGCCTCCTGGTCAAC 3' Antisense 5' CCACTGTCAGCTTGCGTAAGA 3'	141 bp
NAPE-PLD	Sense 5' TGGTGACCTCCCGTCTCT 3' Antisense 5' CCTCAGCCTCCCAAGTACCTG 3'	99 bp
MGL	Sense 5' GGTGTGCGCGGAGCTAGTTTC 3' Antisense 5' AGCGGCGCTGCGATTCTC 3'	117 bp
MGL2	Sense 5' CTGGCCCGCCGCAAACGA 3' Antisense 5' TCTTCAGGTCCGGGGCCACGA 3'	74 bp

from Perkin Elmer (Perkin Elmer Cetus, Norwalk, CT). Briefly the target gene (X0) to housekeeping gene (R0) ratio in each sample before amplification was calculated as X0/R0=kx1/((2**ΔCt)), ΔCt is the difference between Ct-target and Ct-reference, and k is a constant, set to 1. All samples were amplified in duplicate. The target gene (β-2-microglobulin) was tested for stability prior to further investigations.

Results

CBR1 in AT

Both Western blot and real time RT-PCR showed higher CBR1 levels in SAAT compared with VAT in lean ($p<0.01$ and $p<0.05$, respectively). In obese, we discovered a reduction in CBR1 levels in SAAT ($p<0.01$ and $p=0.058$ respectively) and for the visceral depot, there was a small increase for PCR ($p<0.05$) and similar levels with immunoblotting (see fig. 22).

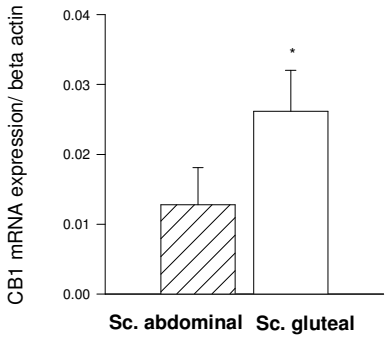


Figure 31: **Gene expressions levels of CBR1 relative to β-actin in gluteal and abdominal subcutaneous depots from overweight subjects (Bennetzen et al., Eur J Clin Invest 2010) (64)**

We also found differences in the subcutaneous depot from various locations, with higher levels in the gluteal compartment compared with the abdominal in over weight subjects.

Discussion

In this study we unfortunately do not have much information on study participants. It would have been interesting to see the CBR1 levels in relation to insulin levels for instance, but blood samples were unavailable.

Previous studies show wide variation in the results on CBR1 expression from different depots, as reassessed in section four of this dissertation. We used two different methods and found similar results, increasing the reliability of our results. It could be imagined that the CBR1 receptor is subject to strict and fast regulation, and that various study standards are followed by very differing conclusions.

Conclusion

Our data clearly demonstrate that obese subjects have a decrease in subcutaneous CBR1, which is followed by almost similar CBR1 expression in SAT and VAT in obese individuals. This is in contrast to the higher CBR1 expression in SAT compared to VAT of lean subjects, and does not indicate hyperactivity in AT at least at receptor level.

STUDY III

"NO INDICATIONS OF HYPERACTIVITY OF THE ENDOCANNABINOID SYSTEM IN SUBCUTANEOUS ADIPOSE TISSUE IN THE OBESE STATE. INVESTIGATIONS IN LEAN SUBJECTS AND IN OBESE SUBJECTS BEFORE AND AFTER WEIGHT LOSS"

Aim

Here we aimed to establish whether 10% weight loss would affect components of the ECS in AT in humans and to establish whether hyperactivity of the ECS in adipose tissue is present in the obese state.

Materials & Methods

Study participants

This study included 21 lean and 21 obese human volunteers (n=42). 47 subjects were screened. Four obese subjects left the

study before the investigations after intervention (one woman, three men).

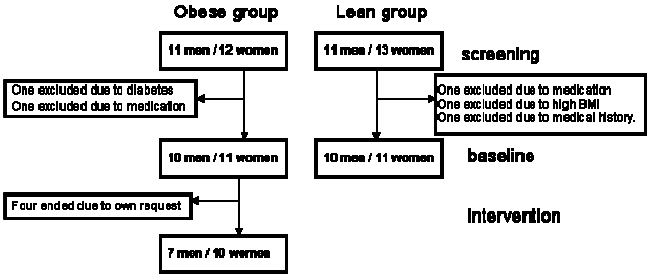


Figure 32: **Flow chart of study participants**

The obese group underwent clinical investigations at baseline and after 10 weeks (8 weeks diet followed by 2 weeks weight stabilising period with the study participants' normal diet adjusted to the new weight). The weight loss approximated 10% and was achieved by very low calorie diet (NUPO nutritional powder, Flex pack Industry A/S, Greve, Denmark) including 200 grams of fruit and vegetables daily and weekly motivating sessions with a dietician. The lean study participants were investigated only at baseline. Height, weight, waist- and hip circumference, and bioimpedance measurements were measured on all study participants. We collected AT samples by liposuction procedure on the abdomen (SAAT), just below the level of the umbilicus in the midline, and from the gluteal region (SGAT), between the greater trochanter and tuberositas ischii. This was done under local anaesthesia with Lidocain 10 mg/ml without adrenalin.

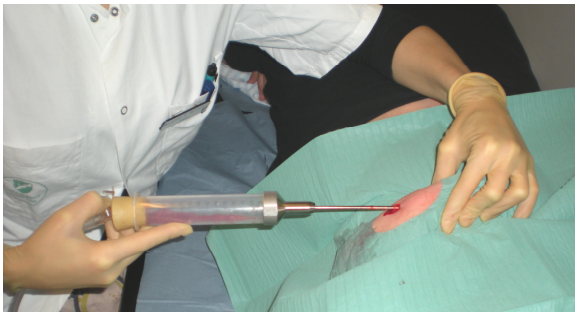


Figure 33: **Liposuction procedure**

Fasting blood samples were drawn from all study participants. The evening before clinical investigations they all had the same meal (calorically adjusted for gender), and they fasted in the morning until after the tests. They were also asked not to engage in excessive physical exercise or alcohol intake the day before and the morning of the clinical investigations.
The study was approved by the Central Denmark Region Committees on Biomedical Research Ethics and the Danish Data Protection Agency. Journal number: 21944, case number: M-20090058, and amendment protocol number: 23509. All participants signed a written informed consent. The Project was performed approximated to the guidelines of GCP.

Analysis of blood samples in human subjects
Total cholesterol, HDL, LDL, triglycerides, and fasting glucose were measured by the hospital clinical biochemical department according to standard procedure. Fasting insulin were measured by ELISA (DAKO K6219, Electra Box Diagnostics Aps, Rødovre, Denmark) according to manufacturer's manual. The assay precision as supplied by the manufacturer was intra-assay coefficient of variation (CV) of 7,5% (n=20) and inter-assay CV of 9,3% (n=20), with a lower detection limit of 32,6pmol/L. The absorbance was measured with a spectra reader photometer.
We used the homeostasis model assessment (HOMA) to evaluate insulin resistance according to Matthews et al. (251) by the following formula: (fasting serum insulin (µU/L) * fasting plasma glucose (mmol/L) /22.5. We used 6 as the conversion factor for insulin from pmol/L (SI unit) to µU/L (252).

Real-time RT-PCR
As described for study II.
Liquid chromatography mass spectrometry (LC–MS)
Performed by professor Harald S. hansen at Dept. of Pharmacolocy and Pharmacotherapy, Faculty of Pharmaceutical Sciences, University of Copenhagen.

Results
EC levels
This investigation surprisingly revealed lower 2-AG levels in the gluteal AT compartment with obesity ($p<0.05$) and a tendency for reduction in the SAAT. In both SAAT and SGAT, the 2-AG levels were increased with weight loss (SAAT $p<0.01$, SGAT: $p<0.001$, see figure 21). No difference was found in AEA levels between the three groups in neither SAAT nor SGAT.

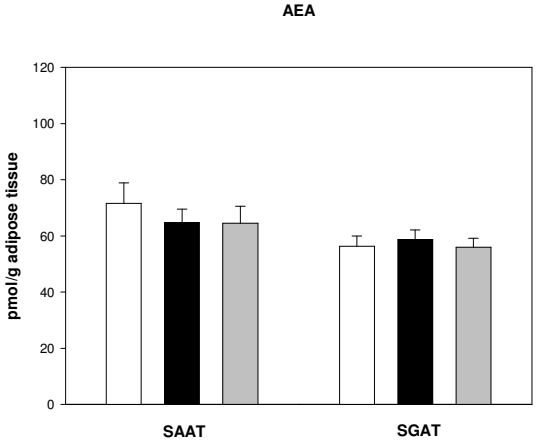


Figure 35: **AEA levels in SAAT and SGAT of lean (open bar), obese (black bar) and obese after weight loss (grey bar). SAAT: subcutaneous abdominal adipose tissue. SGAT: subcutaneous gluteal adipose tissue. No variation with weight changes. (Bennetzen et al., manuscript III)**

CBR1 levels
Supporting study II, we found lower CBR1 in SAAT from obese compared with lean, and this was normalised with weight loss. In SGAT, CBR1 levels were similar in lean and obese, but were reduced with weight loss. SAAT and SGAT CBR1 levels were similar in lean, but, again confirming previous results in over weight subjects, reduced in SGAT compared with SAAT of obese (see figure in section four).

FAAH levels
We found similar gene expression levels of FAAH and FAAH2 between lean and obese at baseline except from an increase in FAAH2 in SAAT of obese at baseline compared with lean ($p=0.003$, figure 4a and b). In SGAT for both FAAH and FAAH2, we found a significant decrease in gene expression with weight loss ($p<0.001$ and $p=0.02$, respectively).

Discussion

In the human intervention study, we lost 4 participants to follow up. This was expected due to the large drop out rate in obesity trials. Unfortunately it was one woman, who was replaced, and tree men who were not. This was due to the fact that the woman went out of the study just after it had started and was replaced, while two of the men chose not to participate in the final investigations just before the study ended were it was not possible to replace them. The difference between groups is regrettable but unpredictable in the planning of the study.

Magnetic Resonans would have been a superior measure of lean and fat body mass compared with bio-impedans. However this was only used to rule out reduction in muscle mass with weight loss, and we chose the simple way to do it.

We found a reduction in the degrading enzymes for both 2-AG (MGL, MGL2, FAAH and FAAH2) and AEA (FAAH and FAAH2) in SGAT with weight loss, but interestingly only an association between 2-AG and FAAH2. The reduction in enzyme levels could explain the increase in 2-AG levels following weight loss, but we would have expected AEA to rise as well.

EC's show variation within studies, and data are emerging, that EC's may show a circadian rhythm with highest levels of AEA in the morning compared with before bedtime (253), and therefore differences could be caused by measurements being performed in different times of the day – or individual participants getting in and out of bed at various times.

Conclusion

To summarise, the amount of 2-AG was reduced in subcutaneous AT from obese subjects and 2-AG increased/normalized after weight loss. Moreover, the expression of CBR1 was reduced in subcutaneous AT in obese individuals and increased following weight loss. Thus, both one of the important endogenous ligands, 2-AG, and the main EC receptor were down regulated in the obese state, indicating that simple obesity is not associated with ECS hyperactivity in subcutaneous AT.

STUDY ON GENDER DIFFERENCES

Aim

In this study we aimed to study possible gender differences in peripheral AT EC levels of the CBR1 and enzymatic machinery between men and women of comparable levels of adiposity.

Study participants

For this study, 10 men and 10 women undergoing gastric obesity surgery (n= 20) volunteered AT samples. Paired samples from SAT and VAT were obtained during abdominal surgery procedure. Subjects had fasted overnight. None of the subjects suffered from inflammatory or malignant diseases. These were also cross-sectional studies. The protocols were approved by the local ethical committee and all subjects had provided written informed consent. Journal no: 20070032.

Methods

Liquid chromatography mass spectrometry (LC–MS) and Real-time RT-PCR

Results

There were increased levels of both endocannabinoids in SAAT and VAT of women compared with men, where both 2-AG and AEA were measured by LC-MS. In SAAT, 2-AG was almost 4 fold higher in women ($p<0.05$) and AEA 3 fold higher ($p<0.05$) and in VAT, both 2-AG and AEA were 3 fold higher in women ($p<0.05$).

Both endocannabinoids tended to be higher in the subcutaneous depot compared with the visceral depot in both genders, but this difference was only statistically significant for women (2-AG: $p<0.05$ and AEA: $p<0.01$).

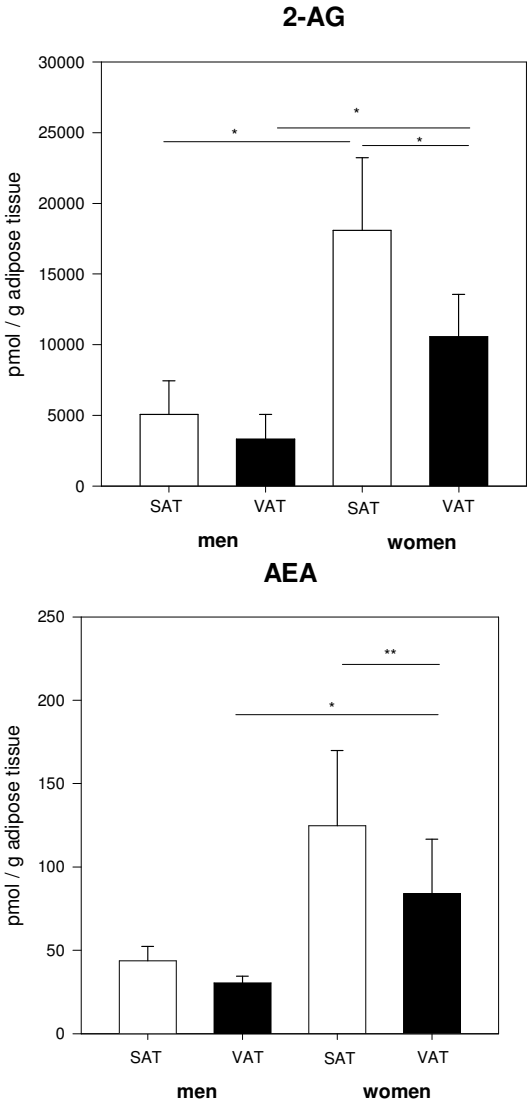


Figure 36: **Levels of 2-AG and AEA in SAT and VAT from men and women (Bennetzen et al., unpublished results)**

The gene expression of FAAH2 was elevated in VAT by 50% compared with SAT in both men and women ($p<0.05$), while none of the other synthesising and degrading enzymes seemed significantly affected by the weight change.

Discussion

For the EC study in obese subjects, we would have liked to include a lean control group, but it was not possible at the time. Because differences exist in the two groups in diagnoses and medications leading to confounding, it would have been interesting to see the correlation of EC's to insulin levels in an OGTT or clamp situation, but this was not possible.

Conclusion

We found higher AT EC levels in women compared with men and higher EC levels in SAT compared with VAT in both genders. Our study suggests differential regulation in the two depots and sexes, which imply that the effects of ECs in the two depots might differ.

CONCLUSIONS AND PERSPECTIVES

GENERAL CONCLUSIONS

Our results clearly show the presence of all the components of the ECS (the EC's, receptors and synthesising and degrading enzymes) in human AT, confirming a role of the ECS in the peripheral tissues, in this case the AT. Moreover, it was demonstrated that this EC system in AT was influenced by the obese state, by weight loss, and that subcutaneous AT (abdominal versus gluteal) does not behave similar to weight loss.

The results obtained do not support the hypothesis of an overactive ECS in human obesity. Our findings are more compatible with the suggestion that the ECS in subcutaneous AT is under-active, not over-active, in simple obesity, which is obesity without complications. Thus EC levels in AT in obesity cannot explain the increased circulating levels observed in obesity.

We found it possible to circumvent the tolerance development on food intake following CBR1 antagonist treatment in rodents. Moreover, we found indications that Rimonabant (both continuous and cyclic) has food intake and weight independent effects on lipid metabolism and inflammatory markers in rodents.

PERSPECTIVES & FURTHER RESEARCH

The control of food intake and energy homeostasis is a complex setting of nutritional status, emotional status, and the environment surrounding us (254). The regulation depends on both peripheral and central, orexigenic and anorexigenic, humoral and cellular signals and the cross-talk and interaction between these circuits makes it difficult to shed light on, or even interfere with, one mechanism without touching upon several others.

Despite the fact that Rimonabant has been withdrawn from the marked as an anti-obesity tool, the considerations of the endocannabinoid system in the maintenance of energy homeostasis and feeding tone is still necessary to understand the basic actions of the system. Because CBR1 is distributed throughout the body, it still represents a desirable target for potential treatment. It may have useable effects on the cardiovascular system, inflammation and atherogenesis, if it is possible to create a compound with all the beneficial effects without the psychiatric side effects. Maybe it will be a neutral agonist, a tissue-specific CBR1-blocker, a compound not able to cross the blood-brain-barrier, or an ECS enzyme modulator (255). If this happens, it may be tested whether the attenuation of tolerance development by cyclic administration may improve the benefits and increase the weight loss in a clinical setting as well.

Development of obesity may change the expression of the ECS in various AT depots, but future studies are needed in order to determine whether this phenomenon is beneficial or malignant.

We are at present conducting further investigations of the inflammatory effects of CBR1 ago- and antagonists in AT.

SUMMARY

Obesity is a world wide epidemic; it is becoming more usual to be overweight or obese than to be normal weight. Obesity increases the risk of an extensive range of diseases such as cardiovascular disease, diabetes mellitus type 2, hypertension, depression and some types of cancer. Adipose tissue is more than a storage organ for surplus energy – it is also a setting for complex metabolic processes and adipose tissue releases substances that interact with other parts of the body to influence several systems including food intake and energy metabolism.

The endocannabinoid system (ECS) is one of the signalling systems that control feeding behaviour. The ECS is implicated in many functions, such as pain, memory, addiction, inflammation, and feeding, and could be considered a stress recovery system. It also seems to integrate nutrient intake, metabolism and storage maintaining homeostatic balance.
The ECS is a recently discovered system, and research indicates hyperactivity in obesity. The aim of this thesis is to elaborate on the relationships of this widespread system and its elements in adipose tissue in obesity.

Study I is a 4 weeks rat intervention study to investigate whether weight independent effect of Rimonabant treatment exists. We found that food intake-tolerance development could be circumvented by cyclic administration of Rimonabant and implications of weight independent effects of treatment.
Study II is a cross-sectional study to establish the expression of cannabinoid receptor 1 from various adipose tissue depots of lean and obese persons. In this study we conclude, that the subcutaneous adipose tissue express more CBR1 than the visceral depot in lean, but comparable levels in obese.
Study III is a 10 weeks human intervention study to asses the effects on the ECS of 10% weight loss. We found reduction in the ECS in obesity that normalised with weight loss.

Our results clearly show the presence of all the components of the ECS in human AT, and suggest that the ECS is reduced in AT in obesity. Our results do not support the hypothesis of hyperactivity of the endocannabinoid system in human obesity. Possible future treatment of obesity with CBR1 antagonist could involve cyclic treatment of specific peripheral compounds.

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