Mechanisms and Pathways in Adaptation of the Detection of Dietary Fat

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"A journey of a thousand miles starts with a single step."

(Mao Tse-tung (Zedong), 1893-1976)

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BACKGROUND

67%. This is the proportion of the US population that is either overweight (25<BMI<30) or obese (BMI>30). This number includes about 17% of children aged from 6 to 19 according to Time magazine (June 11, 2007). Even with its healthy fancy cuisine and the French paradox, France has not been spared by this epidemic. In 2003, 41.6% of the French population was overweight or obese. This proportion increased from 31.6% in 1997 to 41.6% within 6 years. We can easily imagine that this number increased since then and the proportion could be reaching 50% pretty soon. And this trend is followed in every single western country.

Obesity has now become first cause of mortality in the US before smoking, and adiposity has been positively correlated as a risk factor in mortality among adults (Singh, Lindsted et al. 1999). Surprisingly in developing countries where most of the people used to starve decades ago a similar trend is appearing. However this epidemic hits different socio-economical group; in developed countries people with lower income will tend to be more obese when in developing countries people with higher income will tend to have a higher BMI (Ball and Crawford 2006).

So what are the causes of obesity since it seems to be spreading like a pandemic? Is obesity the result of nature or nurture, or both? Can we think about a model broad enough to include all social, economical, biological factors that can influence obesity so we can try to point out who is responsible? (Egger, Swinburn et al. 2003).

After the class-action filled against the four big fast-food corporations in 2002, which from the point of view of the world seemed ridiculous, and after the success of the movie “Super Size Me”, people started to realize that maybe widely available cheap and processed food might not be good for your health and that maybe having a sedentary life, watching TV 8 hours a day with brain washing commercials might not be good as well. But here is the fact: 1 on 4 Americans visits a fast food everyday. So do you think the fast-food should take the blame for obesity?

Most people will blame it on genetics, on sedentary life; billions are spent on research trying to understand the mechanisms underlying obesity. Just by looking at the number of publications on Pubmed, over 100.000 papers have been published since the first publication of the post-mortem description in a case of Extreme Obesity (Oliver 1880). That is more than 2 papers a day for almost 150 years. And yet no solution has been found, or should I say no solution want to be found. Can you imagine the amount of money spend under the name of obesity? Between the drug companies, the diet companies, the books, the DVDs, the personal trainers, the gyms, the food industry everything that involves obesity sells, so why would we want to find a cure to obesity or should I say why would we want to find the cause to obesity when it is clear and simple. Eat healthy, avoid cheap and processed foods and exercise and you won’t get obese.

Of course there are some genetics factors in obesity, some single nucleotide polymorphisms (SNPs) that are going to affect your decision to eat or your ability to store or burn fat. Of course there is an interaction between gene and diet since as any other

animals we evolved in parallel with what we ate, according to what was available in our geographical area. This is irrefutable. But what is the proportion of the responsibility of our genes when we intoxicate our body with food that we know will make us obese. If the real will of our governments is to fight obesity, then the solution should be simple. Let's act directly to the root of the problem by educating our kids about nutrition and let's focus all our efforts in providing cheap healthy food to the mass.

But instead our governments will urge to pass a bill to ban lawsuits against fast-food companies or will fund research in hope to find new targets to cure the symptoms of obesity but not the cause so drug companies can make millions on the sales of their new miracle drug and downstream HMOs too. Health is no longer a moral priority but has become a business. If we get rid of the major problem then maybe we can focus on the remnants obese people that would still be. Who is to blame? The people who would rather die than quit going to their favorite fast-food? The lobbying of the food and drug industries that would do anything to make millions on the back of the population? The government who would rather protect his interest in keeping alive those industries than trying to solve the problem from the source? I guess the responsibility is shared by all of them.

In that case, my part of responsibility was to understand how our decision to eat can be influenced by our diet and especially by a high-fat diet which is pretty representative of our Western Diet. The first aim of this study was therefore to prove on an animal model that at a behavioral level, the decision to eat can be modified by a high-

fat diet. The second aim was to identify the part of responsibility of a well-known receptor involved in the control of food intake the cholecystokinin 1 receptor (CCK1-R). The final aim was to understand how the communication between this receptor and others receptors and hormones involved in the decision to eat was modified by a high-fat diet.

In the humongous wall of research on obesity, this is the brick I am adding…

INTRODUCTION

I/ DIETARY FAT: FROM THE PLATE TO OXIDATION

A. WHAT IS DIETARY FAT?

Dietary fat or lipids are one of the four major classes of biologically essential organic molecules found in all living organisms (the other classes are proteins, carbohydrates, and nucleic acids). They have intermediate molecular weights that range between 100 and 5000 kDa and they include many chemical classes such as hydrocarbons, steroids, soaps, detergents or even more complex molecules such as waxes, triacylglycerols (fats and oils), phospholipids, sphingolipids, fat-soluble vitamins, and lipopolysaccharides). They function as barriers, receptors, antigens, sensors, electrical insulators, biological detergents, membrane anchors for proteins, and, last but not least, a major energy source.

Because of its lipophilic nature, dietary fat is important in the body to compartment space from inside to outside but also within the body between the different organs, or at a more microscopic level within a cell between the different microstructures such as cytoplasm, the reticulum, the Golgi apparatus, the nucleus or the mitochondria.

a) The chemical classes of lipids

- Hydrocarbons: They contain only hydrogen and carbon. They may be saturated or unsaturated, branched or unbranched, cyclic or aliphatic, or they may exhibit a combination of these characteristics.

- Substituted hydrocarbons: Alcohols (R-CH_2OH), aldehydes (R-CHO), acids (R-COOH), and amines (R-CH_2NH_2) are examples of substituted hydrocarbons. Such molecules are usually found in low concentrations in cells, as they are rapidly metabolized. Fatty acids are the most abundant of the substituted hydrocarbons. The major saturated fatty acids in higher animals are palmitic (16 carbons) and stearic (18 carbons), followed by smaller amounts of 12-, 14-, and 20-carbon fatty acids. The major monounsaturated fatty acids are oleic acid (18:1ω9), which has 18 carbon atoms and a cis double bond at carbon 9; vaccenic acid (18:1ω7), which has the double bond at carbon 11; and palmitoleic acid, (16:1ω7), which has a cis double bond at carbon 9. The major polyunsaturated fatty acids in plasma and tissues are linoleic (C18:2ω6), arachidonic (C20:4ω6), eicosapentaenoic (EPA; C20:5ω3), and docosahexaenoic (DHA; C22:6ω3). Fatty acids are essential building blocks for membrane lipids.

- Waxes, Esters, and Ethers: Waxes are long-chained, rather nonpolar compounds found on the surfaces of plants and animals, many are esters of long-chain alcohols, R'-CH_2OH, and long-chain fatty acids, R-COOH, (e.g., R-$COOCH_2$-R'). These tend to be

solid at ambient temperature. Ethers are good barriers against water loss which explains their high concentration in skin lipids.

- Acylglycerol and Fats: Many complex lipids have a backbone of glycerol, a 3-carbon polyalcohol. When two fatty acids are reacted with glycerol to form ester bonds, a diacylglycerol or diglyceride is formed. Diacylglycerol are biochemical intermediates in many lipolytic reactions. They are critical building blocks used in the synthesis of more complex phospholipids and triacylglycerols, and second messengers for some membranes triggered reactions. A triacylglycerol or triglyceride is formed when all three hydroxyls of glycerol form ester bonds with fatty acids. Triacylglycerols are the major storage lipids of plants and animals. In animals, adipose tissue is the main source of fat, but skeletal muscle, heart, liver, skin, and bone marrow often contain appreciable amounts of triacylglycerols in intracellular oil droplets.

- Glycerophospholipids: They have a phosphate at the 3 position of glycerol and acyl or alkyl groups on at least one (usually both) of the other glycerol carbons. The phosphate esterified to the sn-3glycerol may be free (phosphatidic acid) or esterified to other small molecules.

- Sphingolipids: They are formed by the addition of fatty acids to the base of sphingosine (Bell et al., 1993). The sphingolipid precursors or breakdown products as sphingosine and ceramide may have important roles as cellular second messengers

(Hannun 1996). Sphingomyelin may also have a role in the intracellular movement of cholesterol.

- Steroids: They are defined as “all those substances that are structurally related to the sterols and bile acids to the extent of possessing the characteristic perhydro-1,2-cyclopentano-phenanthrene ring system.” (Gunstone 1994). Steroid hormones (i.e. testosterone, androgens, estrogens, progesterones, cortisol, cortisone, aldosterone, and vitamin D hormone) are formed from cholesterol. These molecules exert major effects in regulating metabolism in higher animals. The alkali metal salts of hydroxylated bile acids conjugated with taurine or glycine are natural detergents synthesized in the liver and secreted into bile. They solubilize phospholipids and cholesterol in the bile of higher animals, thus permitting secretion of cholesterol into the gut. The excretion of both cholesterol and bile acids is the major way cholesterol is removed from the body. Bile acids also aid in the digestion and absorption of fat and fat-soluble vitamins in the intestine.

- Other Lipids: Eicosanoids are oxygenated fatty acids principally derived from the 20-carbon fatty acids, arachidonic acid, eicosatrienoic acid, and eicosapentaenoic acid. Acyl coenzyme A and acylcarnitine are key intermediates in fatty acid metabolism. Lipopolysaccharides (endotoxin) are a large class of bacterial glycolipids, present in the outer leaf of the outer membrane of gram negative organisms.

b) What is dietary lipid specifically?

Dietary lipids have been described as that part of the diet that can be extracted by organic solvents (Borgstrom, 1986), mostly non polar lipids such as triacylglycerols and polar lipids such as phospholipids. By far the greatest quantity of dietary lipids is in the form of triacylglycerols (triglycerides). Furthermore most of these triglycerides contain predominantly long-chain fatty acids (chain lengths of 14 to 20 carbons) esterified to the glycerol backbone. Dietary triacylglycerol is a major source of energy, with a higher caloric density than the other macronutrients. Other dietary lipids include the fat-soluble vitamins A, D, E and K, cholesterol and cholesteryl esters, and phospholipids, but we will not focus our study in those types of dietary lipids since they are not the most abundant.

B. DIGESTION AND ABSORPTION OF DIETARY FAT

a) Luminal digestion of lipids

The digestion of triacylglycerols begins in the stomach, with the action of gastric lipase secreted by the gastric mucosa and the lingual lipase secreted by the serous Van Ebner glands of the tongue. Acid lipases hydrolyze triacylglycerols that contain medium-chain fatty acids faster than they hydrolyze those containing long-chain fatty acids. Although the enzyme works well in the stomach, it probably continues to digest triacylglycerol in the upper duodenum where the pH is between 6 and 7. Acid lipase preferentially cleaves the fatty acid at the sn-3 position of the triacylglycerol molecule,

regardless of the fatty acid esterified to this position. The 1,2-diacylglycerols (diglycerides) and fatty acids produced as a result of the action of acid lipases may promote the emulsification of dietary fat in the stomach. Grinding and mixing of the gastric contents also contribute to dispersion of the lipid droplets. The combined action of bile and pancreatic juice brings about a marked change in the chemical and physical form of the digested lipid emulsion. Most of the digestion of triacylglycerol is brought about by pancreatic lipase in the lumen of the upper part of the intestinal tract. Pancreatic lipase works at the interface between the oil and aqueous phases. Pancreatic lipase acts mainly on the sn-1 and sn-3 positions of the triacylglycerol molecule to release 2-monoacylglycerol and free fatty acids. In order to be efficient, the lipase needs the colipase factor synthesized and secreted by the pancreas as procolipase and activated to colipase in the small intestine by proteolytic cleavage by trypsin. The binding of the colipase to the triacylglycerol/aqueous interface allows the binding of lipase to the lipid/aqueous interface.

b) Absorption of lipids by enterocytes

The digestion products of triacylglycerols are predominantly monoacylglycerols and fatty acids. Although these lipid digestion products are somewhat polar, they have very limited capacity to dissolve in water. The epithelial surface of the small intestine is surrounded by a layer of water called the unstirred layer, and the thickness of the unstirred water layer depends on how vigorously the small intestinal contents are mixed. Increased mixing reduces the thickness of the unstirred water layer. This unstirred water

layer represents a barrier that lipids must cross before they can be absorbed by the enterocytes (small intestinal epithelial cells). To overcome this barrier, lipid digestion products are first solubilized in mixed bile salt micelles which provide an efficient mechanism for transport across the unstirred water layer and the subsequent uptake of these lipid molecules by the enterocytes. At least two different mechanisms have been proposed for the uptake of lipid digestion products by the small intestine: passive uptake versus carrier-mediated uptake. Once digested, lipids are presented to the surface of the brush border membrane of the enterocytes; the products of lipid digestion can dissolve in the lipids of the brush border membrane. The concentration gradient between the lipids in the brush border and those in the intracellular compartment of the enterocytes favors initial diffusion of these products into the cell. The rapid reesterification of the intracellular lipids to form triacylglycerols, phospholipids, and cholesteryl esters by enzymes of the endoplasmic reticulum helps maintain low intracellular concentrations of these lipids, thus favoring the continued uptake or diffusion of these lipids into the intracellular compartment of the enterocytes. The more water-soluble products of lipid digestion, such as glycerol and short-chain fatty acids, if present, are efficiently taken up by diffusion.

Specific binding proteins have been identified that may participate in the uptake processes for some lipids, including fatty acids and cholesterol. These binding are located at the apical membrane of the small intestinal epithelial cells.

A difference in the abilities of the proximal and distal small intestine to absorb fat had been described in rats (Sabesin and Holt 1975; Wu, Clark et al. 1980). Not only was the distal intestine much less efficient than the proximal intestine in chylomicron production (Sabesin and Holt 1975), but also the chylomicrons produced by the distal intestine were larger (Wu, Clark et al. 1980). The investigators suggested that this difference between intestinal segments could be due to the availability of phospholipids for the coating of pre-chylomicrons or to altered intracellular membrane lipid composition. Most phospholipids are absorbed before the chime reaches the distal small intestine. Although the proximal intestine is supplied with biliary phospholipids, the distal small intestine has to meet most of its phospholipids requirements by either de novo synthesis or uptake of lipoproteins from the plasma.

C. WHAT HAPPENS TO FAT AFTER ABSORPTION?

Once absorbed in the enterocyte, lipid compounds are again in aqueous environment. Lipid digestion products need to be transported from the apical membrane to the reticulum endoplasmic where they are largely reesterified for export in chylomicrons. 2-monoacylglycerols and fatty acids are reconstituted to form triacylglycerol, mainly via the monoacylglycerol pathway. The other pathway present in intestinal mucosa for the formation of the triaclyglycerol is called the glycerol 3-phosphate pathway. During normal lipid absorption, the monoacylglycerol pathway is much more important than the glycerol phosphate pathway in enterocytes because of the abundant supply of 2-monoacylglycerol and fatty acid and their efficient conversion to

triacylglycerol, and also because 2-monoacylglycerol inhibits the glycerol phosphate pathway.

The formation of chylomicrons needs the assembly of intestinal lipoproteins. Lipoproteins are lipid-protein complexes formed by the small intestine and the liver for the export of lipids from these organs. The small intestine secretes the following lipoproteins: (1) chylomicrons; (2) intestinal very low density lipoproteins (VLDLs, small chylomicrons); and (3) high density lipoproteins (HDLs). Both chylomicrons and intestinal VLDLs are triacylglycerol-rich lipoproteins.

During fasting, the major lipoproteins secreted by the small intestine are the intestinal (apo B-48-containing) VLDLs. Chylomicrons are the major lipoproteins secreted by the small intestine following a lipid-rich meal.

Only the small intestine secretes chylomicrons. The major apolipoproteins associated with chylomicrons are apo A-I, apo A-IV, and apo B-48. Traces of apo E and apo C are also added to the chylomicrons after their entry into the circulation. The fatty acid composition of the triacylglycerol of chylomicrons closely resembles that of the dietary lipid consumed. The intestinal triacylglycerol-rich lipoproteins are transported from the endoplasmic reticulum to the Golgi apparatus. The Golgi apparatus serves as the final site of assembly for many proteins and also lipoproteins where they receive their final glycosylation. The Golgi vesicles containing the pre-chylomicrons migrate toward the basolateral membrane of the enterocytes, and pre-chylomicrons are discharged into

the intercellular space through exocytose. Chylomicrons are the major lipoproteins produced by the small intestine following a lipid-reach meal.

The majority of absorbed fatty acids are transported by intestinal lymph as chylomicrons and intestinal VLDLs. However, there is evidence for portal transport of long-chain fatty acids, and this transport is increased when there is a defect in the intracellular esterification of fatty acids to form triacylglycerol or impairment in chylomicron formation.

II/ DETECTION OF DIETARY FAT

A. DUODENAL PHASE OF THE DETECTION

Lipids, after being digested by the lipase into monoglycerides and free fatty acids, are taken up into the enterocytes and incorporated into chylomicrons with lipoproteins. The increase in the synthesis of ApoB, Apo AIV and lipids triggers the assembly and the secretion of the lipoproteins necessary for the chylomicron formation in the lumen of the endoplasmic reticulum (Hussain, Fatma et al. 2005). Lipids in the intestine are a potent inhibitor of gastric secretomotor function, the existence of this feedback response implies the existence of chemosensors that can detect the presence of nutrients in the intestinal wall. Only long-chain free fatty acids are able to produce a secretomotor response of the small intestine. Long-chain triglyceride requires chylomicron formation for absorption, and there is strong evidence that the formation of chylomicron is required for sensory transduction in the intestinal wall (Raybould 1999). The detection of lipids in the duodenum wall has several functional effects such as a decrease in gastric motility, increase in gastric acid secretion and decrease in food intake. These processes are tightly regulated to match the digestive and absorptive capacities of the intestine with the entry of food from the stomach. Food intake is also regulated to limit entry of nutrients into the intestine.

There are strong evidence that the apolipoprotein A-IV (Apo A-IV), a glycoprotein synthesized by the human intestine and by both the small intestine and liver

in rodents, is necessary in the transduction of the lipid signaling to the brain through the vagal afferent pathway (Whited, Lu et al. 2005). Intestinal apo A-IV synthesis is markedly stimulated by fat absorption and does not appear to be mediated by the uptake or reesterification of fatty acids to form triglycerides (Phan and Tso 2001). Rather, the local formation of chylomicrons acts as a signal for the induction of intestinal apo A-IV synthesis. The stimulation of intestinal synthesis and secretion of apo A-IV by lipid absorption are rapid; thus apo A-IV likely plays a role in the short-term regulation of food intake (Tso and Liu 2004; Tso, Sun et al. 2004). The formation of chylomicrons with apolipoprotein A-IV released from the intestinal mucosa during lipid absorption stimulates the release of endogenous CCK that activates CCK(1) receptors on vagal afferent nerve terminals, initiating feedback inhibition of gastric motility (Glatzle, Wang et al. 2003; Glatzle, Darcel et al. 2004). In order for the CCK pathway to be active, the neural afferents of the vagus nerve need to be intact, otherwise the feed-back control is abolished (Holzer, Turkelson et al. 1994). At the level of the small intestine it is now clear that in order to be detected lipids or long chain fatty acids (>12 Carbons) need to be signaled through the formation of chylomicrons, which trigger a release of CCK which will bind to the CCK1-R on the vagal afferents.

B. ILEUM PHASE OF THE DETECTION

Most of the lipids are absorbed in the small intestine. However if some lipids were to appear in the distal gut, there are some additional feedback control in the ileum. Peptide YY is a 36-amino acids peptide discovered from the porcine gut in 1982 for its

inhibition on the exocrine function of the pancreas (Tatemoto 1982). Since it has been identified as a gut-hormone, its effect on gastric motility, gastric secretion and food intake have been widely studied. It is synthesized in the L-type endocrine cells of the GI mucosa. It is stimulated by intraluminal nutrients, including glucose, bile salts, lipids, short-chain fatty acids and amino acids (Ballantyne 2006). PYY inhibits many GI functions, including gastric acid secretion, gastric emptying, small bowel and colonic chloride secretion, mouth to caecum transit time, pancreatic exocrine secretion and pancreatic insulin secretion which is generally called the ileal brake (Van Citters and Lin 2006). Although the release of PYY from lipids is now clear, the modulation of PYY secretion by others gut-hormones and its own action on other gut-hormone peptide is still controversial.

PYY seem to be regulated by CCK production from the proximal gut to the distal gut. Administration of a CCK-1 receptor antagonist; dexoloxiglumide abolished the effect of long chain fatty acids, on plasma levels of both ghrelin and PYY. This shows that generation of long chain fatty acids through hydrolysis of fat is a critical step for fat-induced inhibition of ghrelin and stimulation of PYY in humans; the signal is mediated via CCK release and CCK-1 receptors (Lin, Chey et al. 2000; Degen, Drewe et al. 2007). Therefore CCK serves as a foregut signal linking fat in the proximal gut with the release of distal gut PYY (Whited, Tso et al. 2007). Also the release of PYY has been shown to have an effect on the release of intestinal apolipoprotein A-IV via a posttranscriptional mechanism of action (Kalogeris, Qin et al. 1998).

C. From detection within the gut to signaling in the brain: The gut-brain Axis

Once the dietary lipids have been broken down and absorbed by the enterocytes, they need to be signaled to the brain in order to adapt the gastrointestinal function with the bowel for a cost-effective absorption. In this function, the gut-brain axis is particularly important. The gut-brain axis is composed of "the gut, the pancreatic islets of Langerhans, elements in the portal vasculature, and even visceral adipose tissue communicating with the controllers of energy balance in the brain by means of neural and endocrine pathways" (Badman and Flier 2005). In our case we will focus on a few gut-hormones and on the neural pathway from the gut to the hindbrain: the vagus nerve. There are more than 15 gut-hormones with anorexigenic effects described but the point of this introduction is to focus on the ones important in dietary fat detection and the one that could be involved in a potential adaptation to dietary lipid.

Most of afferent vagal fibers pass through nodose ganglia to terminate into the nucleus tracti solitari (NTS) (Konturek, Konturek et al. 2004). The NTS shows a viscerotopic fibers distribution from esophagus and stomach ending at its rostral-lateral part, from stomach at caudal-medial part and from intestines at central and rostral part of the NTS. The vagal afferent neurons with cell bodies in the nodose ganglia enter the brain stem, which is an important crossroads for information signaled from the gut to CNS and required to control gut functions via long vago-vagal-reflexes (Konturek, Konturek et al. 2004). Information from the vagal afferents ends in the NTS; some signals from the gut

are transmitted to higher center of integration in the hypothalamus such as the paraventricular nuclei (PVN) or the arcuate nucleus (ARC). CNS projection initiated by gut-brain signaling run through vagal afferents. Expression of c-Fos, that serves as a marker of neuron activation by vagal signaling from gut to brain, confirmed an increase in the NTS after administration of CCK (Rinaman, Hoffman et al. 1994) hence by lipids as well (Lo, Ma et al. 2007). These mechanisms will induce a feed-back control on food intake and gastric secretion through vagal efferents. Although this pathway seem to be the most important in the short-term regulation of food intake (meal to meal) and digestive system there is some evidence that gut-hormones also have a direct effect on the hypothalamus by crossing the blood brain barrier.

The inhibition of food intake by apo A-IV can also be mediated centrally (Liu, Doi et al. 2001). Other evidence suggests that apo A-IV may also be involved in the long-term regulation of food intake and body weight, as it is regulated by both leptin (Morton, Emilsson et al. 1998) and insulin (Attia, Touzani et al. 1997). PYY (3-36) acting through Y2-receptors on NPY-containing cells in the arcuate nucleus inhibits NPY release and, thereby, decreases appetite and promotes weight loss (Brunetti, Orlando et al. 2005).

III/ SENSING LIPIDS WITHIN THE BODY

Since lipids are highly calorific, they are the major source of energy of the body and they are stored into adipocytes to form the adipose tissue. The adipose tissue was for a long time considered as a simple storage organ but since the discovery of leptin in 1994 it is now described as a complex endocrine organ (Fischer-Posovszky, Wabitsch et al. 2007).

A. LEPTIN: A BIOMARKER OF ADIPOSITY

Leptin is a 16 kDa protein hormone produced by the white adipose tissue discovered in 1994 by Jeffrey M. Friedman and colleagues at the Rockefeller University (Maffei, Fei et al. 1995). It is synthesized in adipocytes in response to an increase in adiposity. It is therefore a good biomarker of fuel storage available for the body. Leptin binds to its receptor Ob-R which is found in many tissues in several alternatively spliced forms raising the possibility that leptin exerts effects on many tissues including the hypothalamus. Ob-R is a member of the gp130 family of cytokine receptors which are known to stimulate gene transcription via activation of cytosolic STAT proteins specifically STAT3 in the hypothalamus nuclei (Vaisse, Halaas et al. 1996). Leptin is secreted from the adipocytes into the blood stream; it then circulates until it reaches its receptor in target tissues. Transport of leptin into the brain is reduced by fasting. The transport system for leptin shows a diurnal rhythm which is partially saturated in mice with normal weight and even more in obese mice (Pan and Kastin 2007). Secretion of leptin is insulino-dependent since insulin increase triglyceride stores in adipocytes,

therefore after a meal there is a significant increase in leptin secretion in rodents and humans (Dallongeville, Hecquet et al. 1998; Leibowitz, Chang et al. 2006) and a steadily decrease in a fasted state (Levitt Katz, Abraham et al. 2006).

B. ROLE OF LEPTIN IN THE CONTROL OF FOOD INTAKE

The major site of action of leptin is the hypothalamus. The hypothalamus receives and integrates neural, metabolic, and humoral signals from the periphery. In particular, the arcuate nucleus contain two population of neurons that are the most leptin-sensitive (Cone 2005). The first population of neurons expresses two potent orexigenic peptides, the melanocortin antagonist Agouti-related peptide (AgrRP) and the Neuropeptide Y (NPY). The second population expresses the peptide cocaine and amphetamine-related transcript (CART) and the large precursor peptide pro-opiomelanocortin (POMC). Both sets of neurons project to second-order, melanocortin 4 receptor (MC4R) expressing neurons within the hypothalamus and elsewhere in the brain (Coll, Farooqi et al. 2007).

In the arcuate nucleus leptin inhibits NPY/AgRP neurons, and fasting significantly upregulates the expression of NPY and AgRP which will in turn increases food intake. In contrast to NPY/AgRP neurons, POMC/CART neurons are stimulated by leptin and fasting decreases POMC expression which will increase food intake (Meister 2000).

Leptin receptors are also found outside of the arcuate nucleus, although the importance of leptin's activity at these other sites has not been clear until recently. The caudal brainstem contains both leptin-responsive, Ob-Rb-expressing neurons (Grill,

Schwartz et al. 2002) and a population of POMC neurons, just like the arcuate nucleus. Although fasting induced a fall in POMC mRNA in both regions, in contrast to the arcuate, the reduction seen in the brainstem was not reversed by leptin administration. Furthermore, leptin did not cause STAT-3 phosphorylation or c-*fos* activation within brainstem POMC neurons, suggesting that leptin signaling via POMC-derived peptides in the CNS occurs entirely via hypothalamic POMC neurons (Huo, Grill et al. 2006).

Leptin receptors have also been detected on the vagus nerve and apart of being activated by adipocyte-derived leptin they could also been activated by leptin produced by the stomach (Buyse, Ovesjo et al. 2001). It seems that leptin acts directly on vagal afferent neurons to trigger acute influxes of extracellular calcium and that there is cooperation between leptin and CCK in the activation of some vagal afferent neurons. This could contribute in the modulation of visceral reflexes and control of food intake. Acute activation of vagal afferents by leptin alone and in combination with CCK may contribute to modulation of visceral reflexes and control of food intake (Peters, Karpiel et al. 2004; Peters, Ritter et al. 2006). Recent studies even pointed out that the vagal afferent sensitivity to CCK and leptin is concentrated in neurons that innervate the stomach and duodenum in order to enhance satiation (Peters, Ritter et al. 2006).

Leptin receptors are also present on adipocytes and have an important role in regulation of adiposity by a negative feed-back of leptin secretion directly on adipose tissue through the binding to the leptin receptor (Huan, Li et al. 2003).

IV/ CONCLUSION

Through this introduction we described how dietary fat in a non-pathological state were first digested, then absorbed and finally detected and signaled to the brain in order to modulate and tune gastro-intestinal function and food intake. We also pointed out the role of the central nervous system and the adipose tissue in the energy homeostasis.

The next step was to go trough all these processes and study the effect of a chronic ingestion of a high-fat diet on the lipid digestion and absorption, but more interestingly on the adaptation of the lipid signaling from the gut to the brain and within the body through the adipose tissue.

Therefore we focused our review on the major sites of control of food intake: the GI tract, the gut-brain axis and adipose tissue.

CHAPTER 1

ADAPTATION TO DIETARY FAT

(*status: in preparation*)

"All our science, measured against reality, is primitive and childlike--and yet it is the most precious thing we have."

(Albert Einstein)

ABSTRACT

This review focuses on the most recent findings on adaptation to dietary fat at the level of the digestion, absorption and signaling within the gut and the brain and also at the peripheral level. It is now clear that chronic ingestion of dietary fat will lead to changes in the digestion process of lipids by generally increasing the enzymes needed to breakdown the triglycerides. Most recent findings have shown that anatomical and physiological permeation of the gut wall was modified with chronic ingestion of fat. In addition, the normal signaling of fat through the brain-gut axis feed-back is attenuated leading to an over consumption of this hyper caloric nutrient. Adaptive changes occur at the peripheral level such as the gastrointestinal tract, the adipose tissue and the liver but also at the central level from the vagus nerve to the hypothalamus. We will therefore present in this review the most obvious changes in the adaptation to high-fat diet at the level of the central and peripheral system in order to understand how this adaptation can be involved in the modification of the control of food intake and in a long-term lead to obesity.

KEYWORDS: adaptation, lipid detection, high fat diet, food intake regulation, nodose ganglia.

I/ ADAPTATION OF THE GASTROINTESTINAL TRACT

A. STOMACH

It is known for 25 years that the enzymatic systems responsible for hydrochloric acid secretion in the stomach show a response to a high-fat diet by increasing the HCO^-_3 ATPase activity (Khramtsov and Chernikov 1982). More recently a high-fat diet has been shown to decrease the expression of gastric procolipase (enzyme responsible of preparing the lipase-catalyzed fat digestion already in the stomach), which releases colipase and enterostatin involved in the onset of early satiety (Winzell, Lowe et al. 1998).

The stomach also has an endocrine role with the secretion of the orexigenic hormone: ghrelin (Kojima, Hosoda et al. 1999). Ghrelin is released from the stomach and plasma levels of ghrelin reach a peak right before a meal and is decreased after ingestion of food (Tschop, Smiley et al. 2000). It binds to the Growth Hormone Secretagogue Receptor (GHS-R) on the vagus nerve (Date, Murakami et al. 2002) and acts by stimulating gastric motility (Inui, Asakawa et al. 2004). Little is known about the effect of a chronic consumption of a high-fat diet on the ghrelin system. So far, it is known that fasted plasma ghrelin is decreased in obese human (Tschop, Weyer et al. 2001), and in fasted rats on a high-fat diet (Beck, Musse et al. 2002) but it is not associated with a difference in mRNA expression on a fed state (Lindqvist, de la Cour et al. 2005). This decrease was also described in fasted mice on a high-fat diet (Lindqvist, de la Cour et al. 2005), this was associated with a decrease in gene expression at the level of the fundus of

the stomach on a fasted state. On the contrary, Moesgaard *et al.* have shown that plasma ghrelin was decreased in mice fed a high-fat diet on a fed state but not a fasted state (Moesgaard, Ahren et al. 2004).

The stomach also presents other endocrine cells. Despite the development of obesity in DIO rats there was no significant difference in the number of gastric PYY positive cells between DIO and DR tissues (Hyland, Pittman et al. 2007). Secretion of gastric leptin does not seem to be modified by high-fat feeding but overall plasma leptin changes upon high-fat feeding. The change in plasma leptin will be discussed further in the chapter.

Concerning the motor function of the stomach, it has been shown that adaptation to an high-fat diet increases gastric emptying in human (Cunningham, Daly et al. 1991; Castiglione, Read et al. 2002). In rodents gastric emptying is inhibited by a high-fat diet on a short-term exposure (Whited, Hornof et al. 2004) but is reduced after adaptation to the diet (Covasa and Ritter 2000).

B. SMALL INTESTINE

Once fat exits the stomach, it is released in the small intestine through the pylorus and start to be digested by the lipase coming from the exocrine pancreas helped by the bile acids coming from the gall bladder to emulsify the triglycerides. Adaptation to a high-fat diet can modify the enzyme content in the small intestine which will change the

biodigestibility of the triglycerides and therefore their absorption. After 4-week on a high-fat diet, the capacity for lipid digestion and absorption was greater than rats fed a chow diet due to an increase in uptake and reesterification capacity of the ileum and jejunum (Singh, Balint et al. 1972). In humans, the mouth to caecum time was increased in volunteers fed a high-fat diet for 14 days (Cunningham, Daly et al. 1991). More recently, Petit et al. showed an increase in lipid absorption capacity with an induction of the intestinal mitotic index and in the expression of genes involved in fatty acid uptake, trafficking and lipoprotein synthesis in mice (Petit, Arnould et al. 2007).

Concerning the endocrine role of the small intestine, it was reported that there were no differences in plasma CCK secretion between lean and obese people in a fasting state as well as in response to the ingestion of a mixed meal (Lieverse, Masclee et al. 1994) however other studies showed an increase in plasma CCK in humans fed a high-fat diet for 14 days at 90 and 120 min after the meal (French, Murray et al. 1995) and in rats (Spannagel, Nakano et al. 1996). Both rodents and humans data suggest that intestinal apo A-IV synthesis and secretion become less responsive to fat following its chronic consumption (Tso and Liu 2004). In rats fed a high fat-diet for 15 weeks the concentration of plasma PYY was significantly higher in Diet Induced Resistant (DR) group than in Diet Induced Obese (DIO) or Chow Fed (CF) group, while no significant difference was found between DIO and CF group ($P<0.01$). The expression of PYY mRNA in the ileum and colon was increased in DIO-HF refed a chow diet for 8 weeks compared to DIO rats kept on a HF diet (Yang, Wang et al. 2005). The same decrease in plasma PYY has been described in a mice model after 22 weeks on a high-fat diet. DIO

mice had a significant lower level of plasma PYY compared to DR or LF mice (Rahardjo, Huang et al. 2007). There are some evidence that obese human also have a decreased plasma PYY after a meal (le Roux, Batterham et al. 2006).

C. PANCREAS

The changes in the digestion of a high-fat meal are dependent on the enzyme synthesized by the exocrine pancreas and the bile acids coming from the gall bladder. It has been shown that amylase activity is decreased in rats fed a high-fat diet for 52 weeks compared to rats fed a chow diet. They conclude that the reduction of intracellular and secretory amylase activity may be a long-term adaptive response of the pancreas to a reduced carbohydrate component, and this long-term adaptation of the pancreatic secretion of amylase may be due to the changes in the intracellular amylase activity rather than to the altered sensitivity of the acinar to CCK (Lee, Ahn et al. 2006).

D. GALL BLADDER

Bile salts are also really important for fat digestion and fat absorption. In rats, fat feeding stimulated bile salt synthesis (Botham and Boyd 1983). In humans, both low-fat and high-fat diets decrease the bile acids turnover and synthesis and, consequently, impair removal of cholesterol from the body via bile acids (Bisschop, Bandsma et al. 2004). Bile acids are either excreted or reabsorbed into the circulation. Watanabe et al. (Houten, Watanabe et al. 2006; Watanabe, Houten et al. 2006) demonstrated that bile

acids increase the metabolic rate in brown fat tissue by binding to a G-coupled protein receptor (TGR5) that increases cAMP content and induces type 2 deiodinase enzyme (D2) expression, thereby enhancing local conversion of thyroxine (T4) to the active triiodothyronine (T3) and downstream increasing metabolic rate in the brown fat of rodents for sustaining body temperature in cold conditions (adaptive thermogenesis). So bile acids seem to block fat accumulation by inducing thyroid hormone signaling in brown fat. On a high-fat diet, the D2 response is more sensitive to bile acids through an unknown mechanism. In humans, a similar mechanism in skeletal muscle has been described (Baxter and Webb 2006)

E. COLON

PYY expression in the ascending colon was increased in obese mice but the mRNA expression was not significantly different from lean mice in the same tissue (le Roux, Batterham et al. 2006). These findings suggest that the plasma PYY deficiency observed in obese mice may result from an impaired PYY release rather than a decreased synthesis, although the possibility of an enhanced clearance rate or reduction in mRNA translation can not be excluded.

The colon is also really important in energy intake and lipid metabolism since it contains about 10^{14} bacteria and yeasts cells, representing about ten times the number of cells in our body. The human intestinal microbiota genome ("microbiome") contains at least 100 times as many genes as our own genome. Thus, humans are superorganisms

whose metabolism represents an amalgamation of microbial and human attributes (Gill, Pop et al. 2006). Recent evidence have shown that germ free mice were not able to become diet-induced obese suggesting an important role of the gut microflora in the offset of obesity and that this phenotype was associated with increased levels of AMPK in muscle and liver and their downstreams target involved in fatty acid oxidation such as acetyl-CoA carboxylase or carnithine-palmitoyltransferase. In addition to this phenomenon, germ free knockout mice for the gene of fasting-induced adipose factor Fiaf (a circulating lipoprotein-lipase inhibitor normally selectively suppressed in the gut epithelium by the microflora (Backhed, Ding et al. 2004)) are not protected from diet-induced obesity. The plasma Fiaf is decreased by chronic high fat feeding (Kersten, Mandard et al. 2000). In conclusion, germ free animals are protected from diet induced obesity by two complementary but independent mechanisms that result in increased fatty acid metabolism; first by elevated levels of Fiaf, which induces Pgc-1α; and second by increased AMPK activity (Backhed, Manchester et al. 2007). Furthermore the study of the obese microflora showed a difference in the proportion of the two bacterial dominant division Firmicutes and Bacteroidetes with different capacities in energy harvesting in favor of the Firmicutes. This trait was transmissible: colonization of germ-free mice with an ‘obese microbiota’ results in a significantly greater increase in total body fat than colonization with a ‘lean microbiota’ (Turnbaugh, Ley et al. 2006) These findings suggest that the gut microbiota can influence both sides of the energy balance equation (energy intake and energy expenditure).

II/ ADAPTATION OF THE GUT-BRAIN AXIS

A. VAGUS NERVE

Once dietary fat has been absorbed, it needs to be signaled to the brain in order to adapt the GI function to the bowel. This is the role of the vagus nerve: to carry gut signals to the brain. The signals coming out of the GI tract are modified by chronic consumption of a high-fat diet but is there any adaptation at the level of their chemoreceptors on the vagal afferents?

In the nodose ganglia, where all the cell bodies from the sensory fibers in the vagus nerve are concentrated, 33% of the neuron profiles (NPs) contained CCK1-R mRNA. CCK mRNA was not found in normal nodose ganglia. No significant difference in the number of NPs labeled for CCK1-R mRNA was detected following 48 h food deprivation or a high-fat content diet (Broberger, Holmberg et al. 2001).

Recent studies from Burdyga *et al.* suggest a regulation of the receptor expression with nutritional status. In fasted rats, the expression of the CB1 receptor is increased and decreased after refeeding. This can be mimicked by peripheral CCK injection or abolished by injection of lorglumide, a CCK1-R antagonist, demonstrating a effect of the anorexigenic CCK pathway on the orexigenic cannabinoids pathway (Burdyga, Lal et al. 2004). Similarly, they demonstrated an interaction between the orexigenic ghrelin pathway with the anorexigenic CCK pathway. The actions of CCK and ghrelin are

mediated by a common population of vagal afferent neurons. Ghrelin may act to limit the action of CCK in depressing expression of CB-1 and MCH-1 receptors and other orexigenic receptors (Burdyga, Varro et al. 2006).

Little is known about the effect of the diet on the expression of gut-hormones receptors at the level of the vagal afferents. However it seems to be an important question since vagal afferents are the first players in the short-term regulation of food intake. Therefore if their activity can change with diet, the short-term regulation will also and that could evolve to long-term changes in adiposity and body weight and finally lead to obesity.

B. HINDBRAIN

The hindbrain is the part of the brain that controls unconscious processes. It is composed by the cerebellum, the pons and the medulla oblongata in humans. It is really important in the control of food intake since it receives information from the gut through the sensory fibers in the vagus nerve. These fibers terminate on dorsal hindbrain neurons of the nucleus of the solitary tract and area postrema. Dorsal hindbrain expression of Fos-like immunoreactivity (Fos) following intraperitoneal CCK injection or oleate infusion into the duodenum of rats maintained on high fat was completely abolished compared to rats fed a low fat diet suggesting a diminution of vagal CCK responsiveness (Covasa, Grahn et al. 2000; Covasa, Grahn et al. 2000). This neural activation is dependent on the CCK1-R, therefore an antagonist of the CCK1-R stops the activation in the nucleus of the solitary tract (Webb, Gulley et al. 2005; Lo, Ma et al. 2007).

III/ ADAPTATION OF THE CENTRAL NERVOUS SYSTEM

A. HYPOTHALAMUS

The hypothalamus links the nervous system to the endocrine system via the pituitary gland, therefore it regulates metabolism and it plays a major role in the control of food intake and energy balance. Since the hypothalamus is really important in the long-term regulation of food intake, many studies have been published on the changes that occur at the level of the different nuclei within the hypothalamus.

Rats fed a high fat diet had a significantly lower level of hypothalamic apo A-IV mRNA than rats fed a low fat or chow diet after 10 wk. Intragastric infusion of lipid emulsion to animals that were fasted overnight significantly stimulated hypothalamic apo A-IV mRNA in low fat and chow rats but had no effect in high fat rats. This demonstrates that chronic consumption of a high fat diet significantly reduces apo A-IV mRNA levels and the response of apo A-IV gene expression to dietary lipids in the hypothalamus (Liu, Shen et al. 2004).

What are the effects of a high-fat diet or obesity on the expression of the two different populations of neurons in the arcuate nucleus?

- AgRP/NPY: The agouti-related peptide (AgRP) is a peptide expressed only in the arcuate nucleus in the hypothalamus with orexigenic properties by being a natural

competitive antagonist of the MC4-R receptor (Ollmann, Wilson et al. 1997; Tritos and Maratos-Flier 1999). Neuropeptide Y is a brain peptide with structural similarities to peptide YY and pancreatic polypeptide (Tatemoto, Carlquist et al. 1982).

Their expression is decreased with a chronic high-fat feeding in mice (Ziotopoulou, Mantzoros et al. 2000; Wang, Storlien et al. 2002; Huang, Han et al. 2003; Staszkiewicz, Horswell et al. 2007). This decrease has also been described in rats (Archer, Rayner et al. 2004). However some data suggest that elevated AgRP mRNA along with reduced proopiomelanocortin (POMC) mRNA is associated with many types of obesity (Mizuno, Makimura et al. 2003). The level of NPY mRNA expression in the arcuate and dorsomedial hypothalamus was decreased in rats fed a high fat diet but was only decreased in the arcuate in mice (Bi, Chen et al. 2007; Chen, Hansen et al. 2007). However this decrease is still controversial since recent study showed an increase in NPY and its receptor in DIO rats (Wang, Yang et al. 2007).

- **CART/POMC:** cocaine and amphetamine-regulated transcript (CART) is a brain-located peptide with satiety properties and is closely associated with the actions of two important regulators of food intake, leptin and neuropeptide Y (Kristensen, Judge et al. 1998). Similarly the proopiomelanocortin (POMC) is a complex polypeptide precursor which is cleaved in smaller peptides such as the melanocortins, alpha-, beta- and gamma-melanocyte-stimulating hormone (Coll 2007).

After 2 weeks on a high-fat diet the level of CART/POMC mRNA was not changed in mice (Heijboer, Voshol et al. 2005) but after thirteen weeks they became obese and had a concurrent reduction of POMC mRNA expression in the arcuate nucleus (Huang, Xin et al. 2004).

DIO rats have a decrease in the number of neurons carrying alpha-MSH and CART peptide in the arcuate nucleus of the hypothalamus (Tian, Li et al. 2004)

- **MC4-R:** The melanocortin 4 receptor exerts a tonic inhibition of feeding behavior by bonding to its natural ligand α-MSH in the secondary neurons in the hypothalamus (Fan, Boston et al. 1997). In mice fed a high-fat diet, a 40% higher level of MC4-R mRNA expression in the ventromedial hypothalamic nucleus (VMH) has been described compared to mice fed a low fat diet (Huang, Han et al. 2003; Enriori, Evans et al. 2007).

The expression of these neurons is regulated by leptin. Despite high levels in leptin in DIO rats or DIO rodents in general, they have a reduced leptin sensitivity at the level of the hypothalamus (Ricci and Levin 2003). In sheep, gene expression for Ob-Rb was higher in Lean animals than in Fat animals but there was no difference in expression between Fat and Normal animals (Kurose, Iqbal et al. 2005). In rats chronically fed a high-fat diet there was no difference in the hypothalamic Ob-R mRNA compared to the rats fed a low fat diet (Peiser, McGregor et al. 2000; Sahu, Nguyen et al. 2002). The same observation has been made in mice (Enriori, Evans et al. 2007) but an increase in SOCS-

3, a potential suppressor-of-cytokine-signaling mediating central leptin resistance (Bjorbaek, Elmquist et al. 1998), levels was observed.

In conclusion, these data altogether show an overall central leptin resistance due to an increase in the suppressing pathway of leptin signaling within the primary neurons in the melanocortin system which in turns reacts by an increase in MC4-R in secondary neurons to compensate the lack of α-MSH produced.

There is also a theory where leptin permeability through the blood-brain barrier would be dependent upon triglycerides concentration in the plasma. Therefore a hypertriglyceridemia would decrease the permeability of leptin leading to a leptin resistance in the hypothalamus. This would solve the paradox of why obesity should induce resistance to an anorectic signal because hypertriglyceridemia is also observed with starvation; therefore the triglyceride-induced resistance to leptin transport across the blood brain barrier was first evolved to limit the signal of leptin in the brain during starvation (Banks, Farr et al. 2006).

B. MESOLIMBIC REWARD CENTER

The mesolimbic reward center is the region of the brain that processes pleasurable feelings. The mesolimbic pathway is one of the neural pathways in the brain that links the ventral tegmentum in the midbrain to the nucleus accumbens. It is one of the four major pathways where the neurotransmitter dopamine is found. The mesolimbic pathway is

thought to be involved in producing pleasurable feeling, and is often associated with feelings of reward and desire, particularly because of the connection to the nucleus accumbens, which is also associated with these states.

It is well known that preference for high-fat food is higher than other food because of the palatability of the food. This preference tend to override the physiological mechanisms affecting fat intake, therefore fat consumption is often determined by the amount of fat in the food (Drewnowski 1997). Behind those orosensory aspects, some molecular effects of high-fat food on the mesolimbic system have been demonstrated.

After 6 weeks on high-fat diet, DIO mice had a significantly higher level of Dopamine 2 (D2) receptor mRNA expression in the core of the nucleus accumbens and ventral parts of caudate putamen compared to the DR and LF mice. Dopamines 4 (D4) receptor mRNA expression in the ventromedial hypothalamic nucleus (VMH) and the ventral part of the lateral septal nucleus were also significantly higher in the DIO mice compared to the DR and LF mice. D4 receptors may play an important role influencing satiety via the mesohypothalamic pathway while the D2 receptor may regulate reward and motor centers via mesolimbic and nigrostriatal pathways (Huang, Yu et al. 2005). An experiment on rats trained on either a chow diet or a high-fat diet showed the implication of the dopamine-3 receptor in the hyperphagia of an unexpected high fat meal (Davis, McQuade et al. 2006). The study of the mesolimbic system in humans is more complicated and involves psychological effects which can enhance or inhibit this pathway but an interesting model describing how sensitivity to reward can influence your preference to high-fat/high-sugar food and therefore increase your BMI has been described (Davis, Patte et al. 2007).

IV/ ADAPTATION OF THE PERIPHERAL SYSTEM

A. ADIPOSE TISSUE

Eating a high-fat diet will also modify the peripheral system of an organism especially if the organism has the capability to store fat in adipose tissue. By eating a high-fat diet, rodents and humans increase their adiposity. Leptin is secreted proportionally to adiposity. However in DIO rodents and in obese human there is an increase in the ob gene expression and plasma leptin which induces a state of resistance to leptin as seen previously (Guerre-Millo 1997). Leptin production is increased in rodent and human by insulin and corticosterone. Leptin expression increased linearly with age but only under a high fat diet despite body weight gain under both diets (Staszkiewicz, Horswell et al. 2007). At the same time, the response to hyperleptinemia in the adipose tissue is different in rats fed chronically a high-fat diet than rats fed a low-fat diet. As seen in the hypothalamus there is an increase in SOCS-3 mRNA in adipocytes. This increase is associated with a decrease in mRNA expression of the leptin receptor, therefore the STAT-3 was less activated in rats fed the 60% high-fat diet than rats fed a 4% low-fat diet. These data altogether show that leptin resistance at the level of the WAT inhibit the fat depletion effect of leptin through a decrease in Ob-R expression and an increase in SOCS-3 expression (Wang, Orci et al. 2005).

We can find in the adipose tissue the endocannabinoid system (EC). The EC system has been shown to have an important role in energy homeostasis and control of

food intake (Cota, Marsicano et al. 2003). On fasted state, there is an increase of endocannabinoids in the small intestine (Gomez, Navarro et al. 2002) and an increase in CB1 receptor in the nodose ganglia (Burdyga, Lal et al. 2004) which leads to an increase in food intake. FAAH is the hormone that degrades endocannabinoids (Giang and Cravatt 1997). Preliminary recent study indicate higher levels of circulating endocannabinoids in obese women, in addition a reduction of FAAH in the adipose tissue has been described compared to lean women (Bluher, Engeli et al. 2006). Mutation on this enzyme has been pointed out as potential cause of obesity (Sipe, Waalen et al. 2005).

B. LIVER, PANCREAS, MUSCLE AND BONES

It is well known and described that the lipid metabolism is modified in obese versus lean animal and human. The purpose of this review was to describe the changes in the centers of regulation of food intake on a chronic high-fat feeding.

It is evident that understanding all the mechanisms that are modified on a high-fat diet in every organ will help to have a better understanding the energy balance and homeostasis as a whole but this is not the focus of our study here since we are essentially interested in the adaptation of the detection of dietary fat.

Briefly, consumption of a high fat diet will lead to a non-alcoholic fatty liver and to an insulin-resistance in rodents and in humans. A recent study even showed the role of the skeleton in the energy metabolism. They demonstrate that bones have also an

endocrine role by producing osteocalcin. This hormone seems to play a role in fat and glucose metabolism. Mice knocked out for the gene of osteocalcin tend to be more obese and insulin resistant than mice producing a lot of osteocalcin (Lee, Sowa et al. 2007).

It would be too ambitious to try to describe what is happening at the level of every organ when rodents and humans are fed a high-fat diet. This is why our review only focused on how the system that controls short-term regulation of food intake can be modified by chronic ingestion of a high-fat diet. Obviously, studying how the metabolism adapts to the high-fat diet is interesting also and is an important part of the equation in the energy homeostasis.

V/ CONCLUSION

Despite many years of study of the control of food intake, the mechanisms underlying the adaptation to dietary fat are still unclear. There are many evidences on the adaptation of the long-term regulation of food intake such as the leptin-resistance of the hypothalamus but little is known about the adaptation of the short-term regulation at the level of the vagus nerve. However it seems important to understand if there is a modification of the short-term control of food intake since short-term regulation is at the base of the decision to eat. So if the system after being adapted will tend to be orexigenic, then it is more likely that you would become obese. It is therefore essential to understand what is happening at the level of the vagus nerve after chronic ingestion of a high-fat diet.

FIGURE LEGENDS

Figure 1: Recapitulative Table of the effect of a chronic ingestion of a high-fat diet on different organs involved in the short-term regulation of food intake. ↑ means an increase, ↓ means a decrease and → means no change in the hormone, the receptor or the function compared to a chronic ingestion of a chow diet. The same symbols mean an increase or a decrease in food intake in a non-pathological state caused by the hormone or receptor.

Figure 1

System	Organ	Function	Enzyme/Hormone/Receptor or Motor function	Effect of a chronic ingestion of a high-fat diet compared to chow	Usual effect on food intake
GI tract	***Stomach***	*Motor*	Gastric emptying	↑	
		Exocrine	HCO3-	↑	
			Procolipase	↓	↓
		Endocrine	Ghrelin	↓	↑
			PYY	→	↓
			Leptin	→	↓
	Small Intestine	*Motor*	Absorption	↑	
			Uptake & Reesterification	↑	
			Mouth to Caecum time	↑	
		Endocrine	CCK	→	↓
				↑	↓
			Apo A-IV	↓	↓
			PYY	↓	↓
	Pancreas		Amylase	↓	
	Gall Bladder		Bile acids	↓	
	Colon	*Endocrine*	PYY	↓	↓
			mRNA PYY	→	
		Microflora	Fiaf	↓	↓
			Firmicutes	↑	better harvesting
Brain-Gut Axis	***Vagus Nerve***		CCK1-R	→ (after acute ingestion of lipid) no data after chronic ingestion	
	Hindbrain		Fos	↓	↓
CNS	***Hypothalamus***		Apo A-IV	↓	↓
			AgRP/NPY	↓	↓
			POMC/CART	↓	↑
			MC4-R	↑	↓
			Ob-Rb	↓	↓ in sheep
			Ob-Rb mRNA	→	↓
			SOCS-3	↑	↑
			Leptin permeability	↓	↓
	Mesolimbic		D2 receptor mRNA	↑	↑
			D4 receptor mRNA	↑	↑
Peripheral	***Adipose Tissue***		Leptin	↑	↓
			SOCS-3 mRNA	↑	↑
			Ob-Rb	↓	↑
			Endocannabinoids	↑	↑
			FAAH	↓	↓

CHAPTER 2

ADAPTATION OF LIPID-INDUCED SATIATION IS NOT DEPENDENT ON CALORIC DENSITY IN RATS

(*status: published*)

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny . . .'"

(Isaac Asimov)

ABSTRACT

Food intake is modulated by ingestive (gastrointestinal) and postingestive signals; ingested fat is potent to produce short-term satiety (satiation) but this can be modified by long-term ingestion of a high fat diet. AIM: Determine whether altered lipid-induced satiation is dependent on the fat content of the diet, rather than increased caloric density or changes in adiposity. METHODS: Initial experiments determined the differences in the microstructure of meal patterns in rats fed a high fat diet (HF: 38% fat kcal) and in rats pair-fed an isocaloric, isonitrogenous low fat diet (LF: 10% fat kcal) and changes in meal patterns measured after long-term maintenance on the HF diet. RESULTS: Rats fed the HF diet had a significant 50% increase in meal frequency compared to rats fed the LF diet; in addition, there was a significant reduction in meal size (32%) and inter meal interval (38%) consistent with induction of satiation. After 8 weeks on the HF diet, these parameters tend to approach those of rats maintained on the LF diet. There was a significant 56% decrease in the activation of neurons in the NTS in response to intragastric gavage of lipid in rats maintained for 8 weeks on the HF compared to LF diet. CONCLUSION: Dietary fat alters meal patterns consistent with induction of a short-term satiety signal. This signal is attenuated with long-term exposure to dietary lipid, in the absence of ingestion of additional calories or changes in body weight. This adaptation of short-term satiety might contribute to diet induced obesity.

KEYWORDS: high fat, meal pattern analysis, satiation, diet adaptation, food intake

INTRODUCTION

In addition to its role as a digestive and absorptive organ, the gut is also a sensing and signaling organ. In order to accomplish this sensing role, the gut uses neural and endocrine pathways to communicate with controllers of energy balance in the hypothalamus and hindbrain (Strader and Woods 2005). When nutrients are detected within the intestinal lumen, information is sent to the brain via either neural pathway, for example the vagal afferent pathway, or via humoral pathways. The gastrointestinal tract is primarily involved in the short-term factors that regulate food intake such as satiation, the physiological process of meal termination, whereas the long term control of body weight and food intake is a function of the central nervous system (Burton-Freeman, Gietzen et al. 1997).

It is well known that dietary fat has a satiating effect (Greenberg, McCaffery et al. 1999; Lucas and Sclafani 1999). The mechanism by which dietary lipid is detected in the intestine involves the formation and secretion of chylomicrons and apolipoprotein A-IV (Whited, Lu et al. 2005), and subsequent activation of CCK_1Rs, likely those expressed on vagal afferents terminating in the gut wall (Moran, Ladenheim et al. 2001; Reidelberger, Hernandez et al. 2004). This leads to lipid-induced inhibition of gastric emptying, gastric acid secretion and inhibition of food intake (Tso, Liu et al. 1999; Glatzle, Wang et al. 2003; Whited, Thao et al. 2006). This feedback system allows the organism to match ingested nutrients with the digestive and absorptive capacity of the small intestine.

It is accepted that there is a positive relationship between the level of fat in the diet and the body weight, possibly contributing in the long-term to obesity (Lissner and Heitmann 1995). Recent evidence suggests that changes in lipid detection in the gut may contribute to diet-induced obesity. Recently Covasa M. *et al*. showed that dietary fat sensing within the small intestine was reduced in rats adapted to a high fat, high energy diet (Covasa and Ritter 1999). Persistently elevated plasma levels of CCK accompanying long-term HF diet consumption, leads to several adaptive changes such as increased food consumption due to reduced sensitivity to peripheral CCK (Savastano and Covasa 2005).

The endocannabinoid system, consisting of the CB1 receptor and its natural ligands (anandamide and 2-arachidonoyl glycerol), is known to be an important regulator of food intake (Cota, Marsicano et al. 2003; Vickers and Kennett 2005). On a fasted state, there is an increase in anandamide in the small intestine but not the hypothalamus leading to an increase of food intake (Gomez, Navarro et al. 2002; Kirkham, Williams et al. 2002). If the nutritional state can influence the quantity of endocannabinoids produced, we can imagine a similar regulation of its receptor: CB1. We know that injection of CCK will decrease CB1 expression in the nodose on a fasted state (Burdyga, Lal et al. 2004). Therefore we hypothesize that CB1 expression can also be modified by nutritional state but also by dietary history since there is some evidence that endocannabinoids level can be increased by high-fat diets (Matias, Bisogno et al. 2006).

Although there is evidence to show rats freely fed a high fat diet result in diet-induced obesity and diminished sensitivity to detection of lipid in the intestine, little is

known about how the feeding behavior, in terms of meal patterns, changes with adaptation to a high fat diet, nor whether the change is dependent on the increase in body weight or adiposity. The purpose of the present study was to clearly identify whether the response to a high fat diet and its effects on feeding behavior and body weight is due to calorie content or to the dietary fat itself. This was accomplished by developing two precision pellet diets: one high in fat (38% fat/kcal HF), the other low in fat (10% fat/kcal LF), both having the same energy density. The first aim of the present study was to determine the effects of dietary fat on meal patterns. The second aim was to demonstrate how detection of dietary fat changes with sustained stimulation by analyzing meal patterns during long term maintenance on an isocaloric, isonitrogenous high fat diet. The third aim was to establish that the endocannabinoid system can be regulated by nutritional status and by dietary history and therefore contribute to the development of hyperphagia.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats weighing 245-320g at the start of the experiments were housed individually in cages and adapted to a 12-h light:dark cycle (lights off at 09:00 am) in a temperature-controlled room. Water was freely available throughout the experiments, and body weight was recorded daily. All experiments were performed in protocols reviewed and approved by the Institutional Animal Care and Use Committee, UC Davis.

Rats were maintained on either a 38% of energy fat diet (high fat diet: HF) or a 10% of energy fat diet (low fat diet: LF). The composition of the diets can be found in **Table 1**. Note that the LF diet is 263% higher in carbohydrate in the form of corn starch than the HF diet, and that the HF diet is 214% higher in fiber in the form of cellulose than the LF diet. Both diets were isonitrogenous (21% of energy) and isocaloric (3.4 kcal/g). The HF diet was offered *ad libitum* and LF rats were fed based on HF daily caloric intake.

Food intake analysis

Feeding patterns (number of meals, duration of meal, meal size, inter meal interval) were continuously measured from 09:00 am to 03:00 pm using food intake

monitoring cages (The Habitest® System, Coulbourn Instruments) delivering 45 mg pellets (Bioserv Custom Dustless Precision Pellets). The pellet dispensers were controlled by infrared pellet-sensing photo beams. Individual pellets were delivered in response to removal of the previous pellet. Data was recorded and analyzed using Spike2 (version 5.07, Cambridge Electronic Design 1988-2004) and by SigmaStat (version3.11, Systat Software Inc. 2004) and Graph Prism® (version 3.02, GraphPad Software Inc. 1994-2000). A meal was defined as an acquisition of at least 5 pellets within 600s preceded or followed by 10 mins of no feeding. According to the literature Levitsky and Collier, 1968 and Castonguay et al. 1986 (Castonguay, Kaiser et al. 1986), 10 min of no interruption of the beam was used to define a meal. In addition, meal size and intermeal interval were optimized by varying the definition of meal size from 3 to 6 pellets per meal and observing the resulting food intake graph. The number of meals calculated by the software was compared to the actual number of meals determined by visually examining the food intake plot. Following each change in meal size and IMI definition, the meal parameters calculated by the analysis program (Spike2, Cambridge Electronic Design, Cambridge, England) was examined and compared to the food intake plot. The parameters were optimized by empirical comparison between the food intake plot and the meal definition criteria.

Body composition

At the end of the experimental period, rats were euthanized using an overdose of sodium pentobarbital (Nembutal, Abott Laboratories; 100mg/kg IP). Epididymal,

mesenteric and retroperitoneal fat pads were dissected. Fat-pads weights were measured to 0.01 g.

Experimental Protocols

Experiment 1: Effect of a high fat diet on meal patterns.

Rats (n=16) were maintained on the feeding protocol as described above for up to 21 days in order to determine the short-term effects of a high fat diet compared to a low fat diet on meal patterns.

Experiment 2: Effect of a long-term exposure to a high fat diet on meal patterns.

Rats (n=16) were maintained on the same feeding protocol as described above for 8 weeks in order to determine the long term effects of a high fat diet on the meal patterns. There was no significant difference in the weight of the LF versus the HF group at the beginning of the experiment (LF: 284 ± 7 g, n=8; HF: 280 ± 8 g, n=8).

A subpopulation of these rats (n=8) were fed the LF diet for 9 days before randomizing them into low fat and high fat groups. Meal patterns were then analyzed according to the method described above. Data was analyzed using a one way-ANOVA with individual rat as an independent variable. After making sure there was no significant

difference between rats (data not shown), we randomized the rats into two groups and we subjected the parameters to a one-way ANOVA with the diet as an independent variable.

Measurement of c-Fos protein expression in the NTS

After 8 weeks on the respective diets, rats (HF=5, LF=5), were gavaged with 1.5 mL lipid (Intralipid 20%; Baxter HealthCare Corp., Deenfield). After 90 min, animals were deeply anesthetized with sodium pentobarbital (Nembutal, Abott Laboratories; 100mg/kg IP), heparin was also injected (1 ml/kg IP) and rats transcardially perfused with phosphate buffered saline (PBS) buffer (0.1 M, pH 7.4) followed by 4% paraformaldehyde. The brains were removed, postfixed, and processed for DAB immunocytochemistry for c-Fos protein according to the protocol described by (Whited, Lu et al. 2005).

Free-floating brains sections (100 µm), obtained using a vibratome, were washed 3 times with PBS. Primary antibody (anti c-Fos, Santa Cruz) was applied at a dilution of 1:2,000 with 2% goat normal serum, 0.2% Triton X-100, 0.1% bovine serum albumin in PBS (GS-PBS) for 60 min at room temperature and at 4°C overnight with gentle agitation. The biotinylated secondary antibody (goat anti-rabbit immunoglobulin G; Vector Laboratories, Burlingame, CA) diluted 1:200 into GS-PBS was applied for 120 min at room temperature. Tissue was incubated for 3 hr in ABC solution (Standard *Elite* Vectastain ABC Kit, Vector Labs, Burlingame, CA, USA). DAB solution (Sigma, St. Louis, MO, USA) was added for a 5 minute incubation and then 50 µL H_2O_2-PBS (0.1 ml

30% H_2O_2: 10 mls PBS) was added to catalyze the DAB reaction; the reaction was stopped with a PBS wash. Tissue was thoroughly washed between each incubation period. Images were taken on a Provis microscope and analyzed using Paint Shop Pro, Edition 7. A stereotaxic rat brain atlas was used to determine the location of the NTS in each section of tissue (Paxinos & Franklin, 2001). A region of interest was drawn around the nucleus of the solitary tract (NTS) and the area postrema (AP) and all activated neurons in the NTS region of interest were counted. Neurons were determined to be immunopositive (above threshold) by their color and size. Representative sections were chosen to represent regions of the NTS: caudal NTS (Bregma -14.6 to -14.08 mm), mid-NTS (-14.08 to -13.30 mm) and rostral NTS (-13.30 to -12.80 mm). Three sections were chosen for each region for a total of nine sections per rat. The numbers of labeled neurons per section were averaged for each region for each rat; this value was used in subsequent statistical analyses.

Western Blot Analysis

On week 4 and 8, 2 HF and 2 LF rats were gavaged with 1.5 mL of intralipid 20% (Baxter HealthCare Corp., Deenfield) when 2 HF and 2 LF were gavaged with 0.9 mL of saline. 30 min after gavage, animals were anesthetized with 0.5 mL of Nembutal Sodium Solution (Abott Laboratories). Duodenum, ileum, colon, adipose tissue, nodose ganglion, NTS and Hypothalamus were collected and stored at -80 °C. Tissues were processed to be run for proteins assays. Suspension buffer (3.03 g Tris Base 112.7 mL of 0.5M EDTA dissolved in 500mL ddH2O, 1 % Triton-X 100, 1% Protease inhibitor cocktail (Sigma)

and PMSF (100 μg/mL in 100% Ethanol) has been prepared up to 5 μL per mg of tissue. Tissues have been homogenized, sonicated and centrifuged at 10,000 rpm at 4°C 10 min. the supernatant were collected and protein concentration were determined by DC protein assay (Biorad). The concentrations were read at 590 nm on Perkin Elmer HTS 7000 plate reader using HT Soft 2.0. Tissue proteins extracts were analyzed on 10% Tris-Acetate Gels with MOPS Running Buffer using the Invitrogen X Cell SureLock Mini-Cell for 60 min at 200 V and 125 mA and transferred on polyvinylidene difluoride (PVDF) membranes for 75 min at 30 V and 140 mA. The following steps were performed at room temperature. The membranes were then wet in methanol, washed in 20 mL of Tris-Buffered Saline + 0.1% Tween-20 (50 mL 10x Tris Buffered Saline and 0.5 mL Tween-20 in 449.5 mL ddH2O) (TBST), blocked for 60 min in Blocking Buffer (100 mL Tris Buffered saline + 3mg casein + 0.1 mL Tween 20). Primary Antibody (Rabbit polyclonal Anti-Cannabinoid CB1 Receptor Unconjugated, Biosource Camarillo, CA USA) was applied at a dilution of 1:1000 for 60 min at room temperature and then overnight at 4°C. The membranes were washed three times with 20 mL of 5% milk-TBST. Secondary antibody (anti-rabbit conjugated with HRP) at a 1:2000 dilution in milk TBST was applied on the membranes for 60 min at room temperature. Membranes were washed three times with 20 mL of TBST and then reacted with 10 mL of Lumiglo for 1 min (0.5 mL 20x Lumiglo reagent + 0.5 mL 20x H2O2, 9 mL ddH2O).

Experiment 3: Relative preference for the low-fat food in rats fed high-fat or low-fat diets.

A further group of rats (n=8) was adapted to their respective maintenance diets (HF or LF) for 1 week before preference testing began. Rats were fed *ad libitum* from 09:00 am to 03:00 pm and food deprived from 03:00 pm to 09:00 am during this period. Total food intake and weight were recorded daily. To prevent neophobia before the initiation of the preference testing, all rats were given access to each of the two diets (HF or LF) on 2 different occasions as previously described (Savastano and Covasa 2005). After the last day of diet exposure, 6-h 2-choice preference testing began from 09:00 am to 03:00 pm. For all rats, the 2 different diets were presented in two different cups containing 20 g of each diet during two consecutive days with the position of the diets within the cage (left or right side) randomized and alternated each test day. The food intake was measured at the end of the first, third, fifth and sixth hour.

Statistical Analysis

In Experiment 1, data from day 5 to day 18 were analyzed for the number of meals, the meal size, the eating rate, the inter meal interval, the meal duration and the total food intake per day and per rat; data were averaged for each day and each diet group. The parameters were subjected to a one-way ANOVA, with maintenance diet as independent variable.

In Experiment 2, the parameters from 16 rats fed for 8 weeks were subjected to a two-way ANOVA with maintenance diet and time as independent variables, data were not geometrically connected, and some subjects were missing data for some levels so we could not perform a Two Way Repeated Measures ANOVA. Data were compared between Week 2 and Week 8 in order to show an adaptation. All analyses were conducted using SigmaStat (version 3.11, Systat Software, Inc.) Differences among group means were analyzed using multiple comparison procedures (Holm-Sidak method), with overall significance level $P<0.05$.

In Experiment 3, we did the preference testing on 8 rats, 4 rats maintained on a HF diet and 4 rats maintained on a LF diet. Mean intakes for each rat were subjected to a two-way ANOVA, with maintenance and exposure diets as independent variables. Paired samples t-test did not reveal any significant side difference for any of the preference test; therefore, the left- and right-side intakes were pooled for further analysis. Mean intake at each time point for each rat was subjected to a two-way ANOVA, with maintenance and exposure diets as independent variables. The relative-preference was determined by the total amount consumed for each diet divided by the total amount of food consumed across all preference tests, multiplied by 100 to yield a percentage.

RESULTS

Experiment 1: *Effect of high vs. low fat diet on meal patterns*

There was no significant difference in the quantity of food ingested in the first meal between the LF and the HF fed groups. However, the size of the 2nd and 3rd meal was significantly 34% and 31% smaller in the HF compared to LF group, respectively ($P<0.001$) (**Fig. 1**).

The meal frequency in the HF group was significantly 50% higher compared to the LF group (4 ± 0.2 vs. 6 ± 0.2, $P<0.001$). This increase in meal frequency was due to a significant shorter inter meal interval which was reduced by 36 % and 41 % for meal 1 and meal 2, respectively, in the HF compared to the LF fed rats (**Fig. 2**).

Experiment 2*: Effect of long term exposure to a high fat diet on body weight, food intake and meal patterns*

After 2 weeks, rats fed an isocaloric low fat diet gained more weight than rats on the isocaloric high fat diet ($P<0.001$); the difference was significant from week 2 to week 8 (**Fig. 3**). There was no significant difference in the adiposity index (calculated as the sum of the peritoneal, mesenteric and epididymal fat pads divided by the body weight) between the two groups at 4 weeks or at 8 weeks (4 weeks: LF: 2.51 ± 0.28 % vs. HF 2.16 ± 0.28%, n=4 in each group, NS; 8 weeks LF: 2.40 ± 0.39 % vs. HF: 2.98 ± 0.39,

n=4 in each group, NS). There was a 62% increase in the fecal pellet output in the HF rats compared to the LF rats throughout the experiment (1.2 ± 0.1 vs. 3.2 ± 0.2 g/day, LF vs. HF, n=8 in each group, P <0.001).

There was no significant difference in food intake (expressed as percent of food intake per g of body weight) between the HF and LF fed rats at week 2. However, after 8 weeks on the LF or HF diet, there was a significant 27% increase in the food intake in HF fed but not in the LF group (p<0.05) (**Fig. 4**).

After maintenance on the HF diet for 8 weeks, the size of the meal 2 was significantly increased in the HF group compared to week 2 (P<0.05) (**Fig. 5**). In addition, there was a significant increase in the IMI after meal 2 in the HF fed rats (P<0.001) (**Fig. 6**). However, these parameters did not change from week 2 to week 8 in the rats fed the LF diet (**Fig. 5 and 6**).

Effect of HF vs. LF diet on lipid-induced c-Fos protein expression in the NTS

In rats fed the low fat diet for 8 weeks, there is a significant increase in the number of neurons expressing c-Fos in the NTS in response to intragastric gavage with lipid, the region in the brainstem where vagal afferents terminate, as previously described. However, in rats fed the HF diet for 8 weeks, the number of c-Fos immunoreactive in the NTS is significantly decreased compared to the LF fed rats (n=5 in each group, p<0.001) (**Fig. 7a and 7b**).

Effect of HF vs. LF diet on CB1 expression in GI tract, the nodose ganglia and the hypothalamus

GI tract. The CB1 receptor was detected in the colon but not in the duodenum or ileum of any groups, HF or LF, fasted or fed (data not shown).

Nodose ganglia. There is an overall increase in the receptor on a fasted state. There is an overall decrease in the receptor in all groups after 8 weeks on the diet. On a fed state, the difference in the expression of the nodose between 8 weeks and 2 weeks was 3-fold bigger in the HF group than in the LF group. The difference between the fasted and fed state expression of CB1 is 2-fold higher in the HF group than in the LF group after 8 weeks (**Fig. 8**).

Hypothalamus. There was a 10-fold decrease difference in the delta of the expression of CB1 in the HF group fasted vs. fed compared to LF fasted vs. fed. The expression of CB1 was 4 times bigger in the fasted state in the HF group compared to the LF group (**Fig. 9**).

Experiment 3: Relative preference for the low-fat food in rats fed high-fat or low-fat diets.

During preference testing, rats were presented with the two different diets on two different occasions to test the effect of the position of the food in the cage. There was no significant difference between the two different positions allowing us to pool the food

intake between the two days. When LF-maintained rats were presented with the HF and the LF diets at the same time, rats ingested 5.0 ± 2.7 g of the HF diet and 18.9 ± 1.7 g of the LF diet within the 6-h test intake period. Similarly the HF-maintained rats presented with the HF and LF diets at the same time ingested 5.1 ± 3.2 g of the HF diet and 17.6 ± 3.0 g of the LF diet. Only the cumulative 6-h food intake is presented because the relative preference did not differ among the test. Rats both exhibited a significant preference for the LF diet when exposed to both of the diets at the same time. In addition, when exposed to a single diet during the acclimatization period the total food intake did not differ between the two maintained groups. There was even a tendency in the HF group to eat more than the LF group ($P = 0.068$).

DISCUSSION

The results of the present study show that feeding a high fat diet compared to an isocaloric low fat diet elicits different feeding behavior, characterized by an increase in meal frequency and a decrease in meal size. This is consistent with the higher lipid content of the meal eliciting short-term satiety (smaller and more frequent meals). The results also show the satiating effect of the high fat diet is modified with time and decreases when rats are maintained on the diet. Thus, meal size and IMI increase over the 8 weeks on which the rats are maintained on the high fat diet. In addition, there was a significant increase in the amount of diet eaten in the high fat fed rats. This adaptation occurred in the absence of any change in adiposity or in body weight in the high fat fed group. Taken together, this data suggests that dietary fat is responsible for the changes in the feeding behavior observed with long-term adaptation to a diet of high fat content and that a deregulation of the cannabinoid system could be involved in the change in feeding behavior.

After the first meal, rats on the high fat diet ate smaller and more frequent meals than rats fed the low fat diet. This suggests that a signal generated within the meal involving the detection of dietary fat in the ingested food terminates a meal sooner. However, after the cessation of the meal, this decrease in meal size results in the initiation of the next meal sooner than in the LF fed rats, either due to the more rapid fading of the short-term satiety signal in the HF group or due to another signal that detects the overall calorie content of the prior meal. This finding is in contrast with previous findings where

ingestion of a high fat isocaloric diet increased the meal size in rats (Warwick, McGuire et al. 2000). However, this study used liquid diets as opposed to the solid diet used in the present study. Our data from the third experiment shows that rats prefer the LF to the HF diet, suggesting that the HF diet is less palatable than the LF diet. In addition, the eating rate was reduced in the HF group compared to the LF, again suggesting a less palatable food (Swiergiel and Cabanac 1989). No significant difference was shown when both diets were presented to rats sequentially. There is evidence that the meal patterns of two diets with differing palatability depend upon the method of presentation with differences appearing when the diets are offered simultaneously but not when they are offered sequentially (Sunday, Sanders et al. 1983). Therefore it is more likely that the HF diet is not as palatable as the LF diet, but it is unlikely that the rats fed a HF diet are experiencing an aversion to this diet. Thus, it is unlikely that palatability alone can account for the differences in meal patterns observed between the two groups.

The main differences were observed after the first meal; there was no significant difference in the size of the first meal, presumably due to the overnight fast and a relatively high orexigenic drive in all the rats, regardless of the fat content of the maintenance diet. We predicted that HF rats would be more satiated because they were ingesting more calories in the form of lipid resulting in a longer inter meal interval, they do a shorter IMI. However, we observed a decrease in inter meal interval. This shorter inter meal interval in rats ingesting high fat diets has already been described (Warwick, McGuire et al. 2000). We have done an analysis of pre and post-meal correlation between meal size and IMI and we have not found any type of correlations. Furthermore, in the

literature the correlation between meal size and IMI remains controversial. Le Magnen and colleagues consider that there is a correlation between meal size and IMI using long end-of-the-meal definition supporting the depletion-repletion idea of food intake (Le Magnen and Devos 1984). However, when Castonguay and colleagues used a log-survivorship analysis of different end-of-the-meal definition, they concluded that there is no correlation between meal size and intermeal interval (Castonguay, Kaiser et al. 1986) Differences in dietary fiber between the isocaloric HF and LF diets are unlikely to account for the reduced inter meal interval effect as it has been demonstrated in a previous paper; rats fed HF diets, either high or low in fiber content, drank more oil than rats fed the HC diet implying that fiber does not affect postprandial satiety (Reed and Friedman 1990). The fact that the meal size is decreased supports the hypothesis that fat by itself modifies the meal patterns by increasing satiation but not satiety.

In this study we demonstrate for the first time that fat is not by itself responsible for the increase of body weight, at least for a relatively short period of 8 weeks. In previous studies using high fat, high energy diets *ad libitum* there is a significant increase in body weight and adiposity in the high fat fed rats. In the present study, we controlled the caloric content and the fat content intake in both groups so that we could separate the effect of fat from that of calories or adiposity. During the 8 weeks period on the diet, the increase in body weight in the HF group is smaller than the LF group. This has been described in a previous paper were rats were fed an isocaloric HF diet (Ramirez and Friedman 1990). Palatability of the food could also be responsible for the difference between the two groups as palatable food can offset normal appetite regulation and

therefore body weight control (Erlanson-Albertsson 2005), the high fiber content of the HF food also prevent overeating and excessive weight gain (Van Itallie 1978) and finally it has been shown that daily fat excretion was greater in high fiber food compared to normal fiber food (Munakata, Iwane et al. 1995)**,** thus this over excretion of fat in the HF group combined with the higher palatability of the food in the LF group could explain the difference in body weight increase.

The second aim of the study was to demonstrate that feeding behavior can be modified after a long-term exposure to a HF diet. We found that the intrameal satiety or satiation (reduced meal size) of meal 2 and meal 3 was reversed after 8 weeks on the HF diet. In addition, there is no significant difference in the inter meal interval before the third meal between the two groups. This suggests that adaptation to the short term satiety signal has occurred in the absence of any increase in body weight or adiposity compared to the LF fed rats. Thus it would seem that the adaptation occurs solely in response to the level of dietary fat content. It is likely that this adaptive response involves the vagal afferent pathway and CCK, as activation of neurons in the NTS following an intragastric lipid challenge, as revealed by immunoreactivity for c-Fos, is markedly reduced in the HF compared to the LF rats. Thus, although the only variable in these experiments is the level of ingested fat, the adaptation to ingested lipid involves the same deficit as seen in other studies employing high energy high fat diets (Covasa, Grahn et al. 2000).

It has already been described that maintenance of rats on high-fat diets reduces sensitivity to some satiety peptide signals. Reduced sensitivity to satiety signals might

contribute to overeating and obesity often observed when rats are maintained on high-fat diets (Covasa and Ritter 1998) which is often associated with a decrease sensitivity to CCK (Covasa, Marcuson et al. 2001; Savastano and Covasa 2005) or PYY deficiency that would reduce satiety and could thus reinforce obesity (le Roux, Batterham et al. 2006). Little evidence has been shown concerning a potential adaptation of the neural center of the control of food intake: the hypothalamus, even though it has been shown that after a long-term exposure there was a reduction in the Apo A-IV mRNA production in the hypothalamus after a lipid-preload (Liu, Shen et al. 2004). The increase in the IMI had already been described in the hyperphagia of a Sprague-Dawley rat model of chronic diet-induced obesity (Farley, Cook et al. 2003). This confirms that the changes we see are due to adaptation to the high-fat content of the diet and not the high fiber. Taking these data altogether, it is more likely that the changes we observed with time are due to a decrease in the sensitivity of the vagal-afferent to detect fat in the high fat group by decreased sensitivity to CCK or by a decrease in the release of PYY.

Finally these changes could be due to an adaptation of the digestion or the absorption of the dietary lipid. It has been shown that there is an effect of the amount of fat on the adaptive response of rat pancreatic lipase to dietary fat (Sabb, Godfrey et al. 1986). Pancreatic lipase activity adapts primarily to the amount of dietary fat and though increases in HF rats but some results indicate that a source of dietary fiber, cellulose, can affect the availability of enzymes and bile acids in the small intestine (Forman and Schneeman 1980; Schneeman and Gallaher 1980; Sommer and Kasper 1984). Although fiber reduces the absorption rate of fat and carbohydrate because it decreases the

availability of the enzymes and bile to digest fat there is no significant effect of cellulose on oleic acid absorption (Vahouny, Satchithanandam et al. 1988). Fat could therefore still be sensed through free fatty acids like oleic acid within the small intestine even though triglycerides are not broken down yet.

The third aim of the study was to incriminate the endocannabinoid system as responsible for the change in feeding behavior. Therefore we looked at the expression of the CB1 receptor in two important center of regulation of food intake: the nodose ganglia and the hypothalamus. We wanted to prove that the changes in behavior due to a chronic ingestion of a high-fat diet were correlated with changes in expression of the CB1 receptor. Although the changes were not significant because the power of the test was weak due to a small sample size (n=2), there could be a significant effect of the diet on the CB1 expression (P=0.0520 in the nodose and P=0.0530 in the hypothalamus).

In the nodose ganglia, we observed an overall decrease of the cannabinoid receptor between 2 weeks and 8 weeks. Rats are still on their growth curve and need therefore more nutrients than adult rats. The increase in orexigenic factors would encourage the rats to eat more to supply the body with the necessary nutrients. In both groups, at 2 and 8 weeks, the increase on a fasted state is consistent with the fact that anandamide is increased during fasting (Gomez, Navarro et al. 2002), therefore we expected to see an increase in its receptor. The decrease on a fed state is consistent with the data showing a decrease in CB1 receptor after injection of CCK (Burdyga, Lal et al. 2004) since ingestion of a high fat preload (intralipid) is going to increase CCK production in the

small intestine and then inhibit the expression of the CB1 receptor. Surprisingly on a fed state, the decrease in CB1 expression in the HF group after 8 weeks was 3-fold higher than the decrease in the LF group. Furthermore the decrease between the fasted state and fed state is 2-fold higher in the HF group than in the LF after 8 weeks on the diet. Even if any explanation may be impossible so far, it is interesting to notice that the expression of one of an important receptor involved in the control of food intake can be regulated by long-term adaptation to a high-fat diet in the nodose ganglia.

These changes occurred in the center of short-term regulation of food intake but they also occurred in a different manner in the hypothalamus: center of long-term regulation of energy homeostasis. After 8 weeks, the difference in expression in the HF group between the fed and fasted state is not noticeable when in the LF group we observe a 10-fold decrease in expression between the fed and fasted state. Kirkham and al. showed that on a fed state the production of 2-arachidonoylglycerol (a natural ligand of CB1) was smaller than when rats were food deprived (fasted state). If the changes we observed are true then we could imagine that the ratio endocannabinoid/receptor is more important in the decision to eat than one or the other by itself. In fact, if on a fasted state there is a decrease in the expression of the receptor and an increase of the production of the ligand, the chances that the ligand would bind to its receptor would be bigger than in the opposite situation (increase of receptor and decrease in ligand). Therefore the downstream biological activity would be increased in a bigger ratio Ligand/Receptor than in the second case. These are only supposition but it could explain why we observe those changes in the hypothalamus in the LF diet. Concerning the HF diet, since the

endocannabinoid system is involved in the decision to eat then we can imagine that as the feeding behavior change between feeding and fasting state, the expression of the receptor also change in the hypothalamus. CB1 is coexpressed with corticotrophin-releasing hormone (CRH) and cocaine-amphetamine regulated transcript (CART) in the hypothalamus suggesting a direct influence of the endocannabinoids on the expression or function of these neuropeptides (Cota, Marsicano et al. 2003). Since these neuropeptides are at the basis of the decision to eat by tuning their expression in function of the body needs maybe a deregulation of the CB1 in HF rats after 8 weeks could result in a constant drive to eat which would explain the change in feeding behavior and the hyperphagia.

In conclusion, this study demonstrates for the first time that fat by itself can signal and trigger satiation by a mechanism not dependent on the caloric content of the food. In addition, this data show that adaptation to dietary fat does not involve an increase in adiposity or body weight. Finally, we demonstrated that the endocannabinoid system was dysregulated during this adaptation at the level of the nodose ganglia and the hypothalamus. This adaptive response to dietary fat may contribute to altered body weight regulation with a high fat diet.

FIGURE LEGENDS

Figure 1: Effect of isocaloric low and high fat diets on meal size. Rats ingesting the high fat diet were fed for 6 hours each day and rats ingesting the isocaloric low fat diet pair-fed. There was no significant difference in the size of the first meal; however, the size of the second and third meal is significantly reduced in the HF group compared to the LF fed rats. Values are means ± SEM (n=8 animals per group); # $p<0.001$ * $p<0.01$.

Figure 2: Effect of diet on inter meal interval. The inter meal interval following meal 1 and meal 2 was significantly shorter in the rats ingesting the high fat diet compared to those ingesting the isocaloric low fat diet. Values are means ± SEM (n=8 animals per group) **$p<0.05$ ***$p<0.001$

Figure 3: Increase in body weight in rats fed either isocaloric low fat or high fat diets over an 8 week period. Data expressed as the percent of body weight as mean ± SEM (n=8 animals per group, percent of body weight increase compared with body weight at time=0 week). LF vs. HF * $p<0.05$.

Figure 4: Food intake in isocaloric low and high fat fed rats at week 2 compared to week 8. At week 2, there is no significant difference in the daily food intake in either group of rats. However, after 8 weeks on the two different diets, those rats fed the high fat diet had a significantly increased daily food intake. Data is expressed as percent of

total food intake/g body weight. Values are means ± SEM (n=8 animals in each group) **p<0.05.

Figure 5: The meal size of the second meal in isocaloric high fat and low fat fed rats at week 2 and week 8. The smaller meal size in the high fat fed in the first two weeks on the diet is abolished after 8 weeks on the diet. Values are means ± SEM (n=8 animals per group). *** p<0.001.

Figure 6: The inter meal interval between meals 2 and 3 is significantly increased in the HF group after 8 weeks on the high fat diet. Values are means ± SEM (n=8 per group). ***p<0.001.

Figure 7a: The number of neurons activated in the NTS by intragastric gavage of lipid is significantly decreased in rats fed a HF diet for 8 weeks than rats fed a LF diet for 8 weeks. ***p<0.001 (n=5 in each group).

Figure 7b: Sections of the NTS at the Area Postrema demonstrating c-fos immunoreactivity by DAB staining in rats gavaged with intralipid (1.5 mL) after 8 weeks on a LF or a HF diet, 90 min after gavage. The HF group shows a decrease in c-fos immunoreactivity compared to the LF group. ap= area postrema; nts= nucleus tractus solitarius.

Figure 8: Expression of the CB1 receptor in the nodose ganglia in HF and LF groups after two weeks (A) or eight weeks (B) on the diet, 30 mins after gavaging 1.5 mL 0.9% saline (fasted state) or gavaging 1.5 mL intralipid (fed state). Western blots showing the positive control and the housekeeping protein actin (C). There is an overall increase in the receptor on a fasted state. There is an overall decrease in the receptor in all groups after 8 weeks on the diet. On a fed state, the difference in the expression of the nodose between 8 weeks and 2 weeks was 3-fold bigger in the HF group than in the LF group. The difference between the fasted and fed state expression of CB1 is 2-fold higher in the HF group than in the LF group after 8 weeks. Values are means ± SEM (n=2 in 8 weeks group; n=1 in 2 weeks group). The predicted size of CB1 was 64 kDa.

Figure 9: Expression of the CB1 receptor in the hypothalamus in HF and LF groups after eight weeks (A) on the diet, 30 mins after gavaging 1.5 mL 0.9% saline (fasted state) or gavaging 1.5 mL intralipid (fed state). Western blots showing the positive control and the housekeeping protein actin (B). There was a 10-fold decrease difference in the delta of the expression of CB1 in the HF group fasted vs. fed compared to LF fasted vs. fed. The expression of CB1 was 4 times bigger in the fasted in the HF group compared to the LF group. Values are means ± SEM (n=2 in each group). The predicted size of CB1 was 64 kDa.

Table 1: Composition of the two isocaloric, isonitrogenous Low Fat and High Fat Diets

Ingredients	Low Fat Diet (g/kg)	High Fat Diet (g/kg)
Casein	*175*	*175*
DL-Met or L-cys	*3*	*3*
Corn Starch	*415*	*158*
Maltodextrin	*100*	*100*
Sucrose	*80*	*80*
Cellulose	*136*	*291*
Oil (Soy)	*38*	*140*
MM TD.79055 Ca-P defic.	*16*	*16*
$CaHPO_4$	*15*	*15*
$CaCO_3$	*7*	*7*
Vitamin mix AIN-93G	*12*	*12*
Choline Bitatrate	*3*	*3*
Total	*1,000*	*1,000*

Figure 1

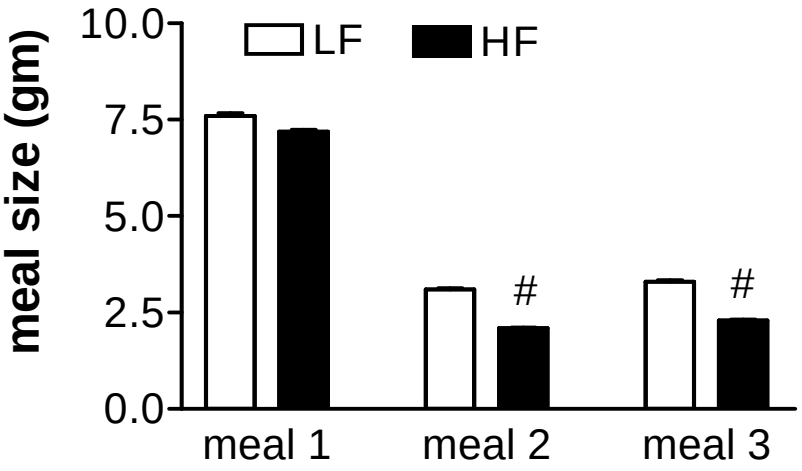
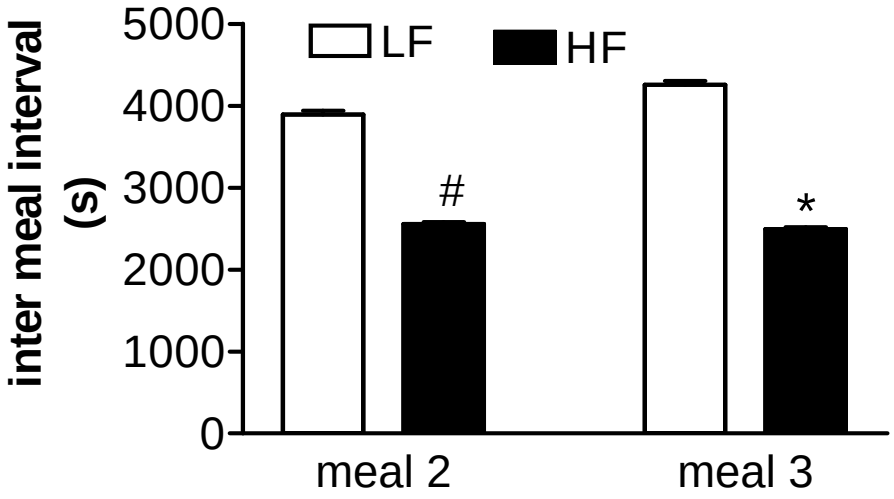


Figure 2



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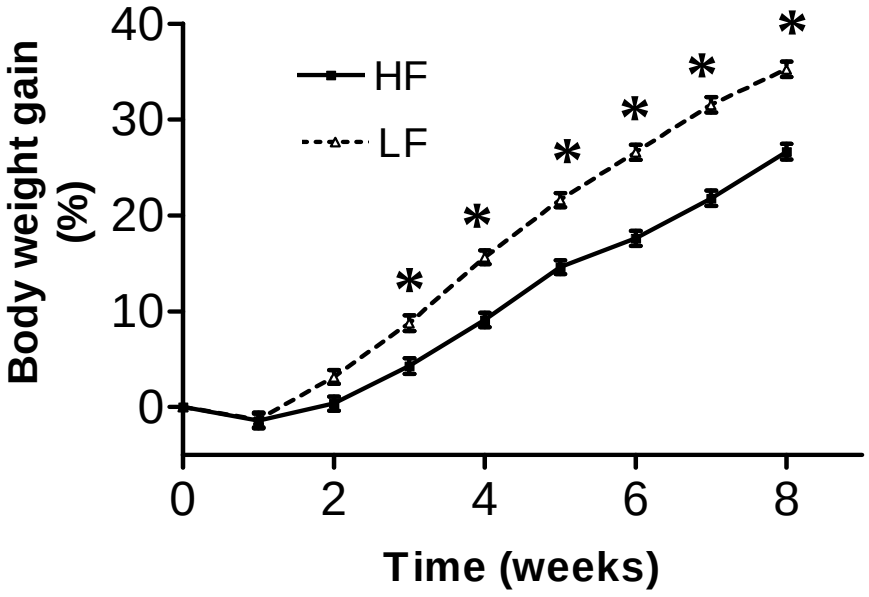

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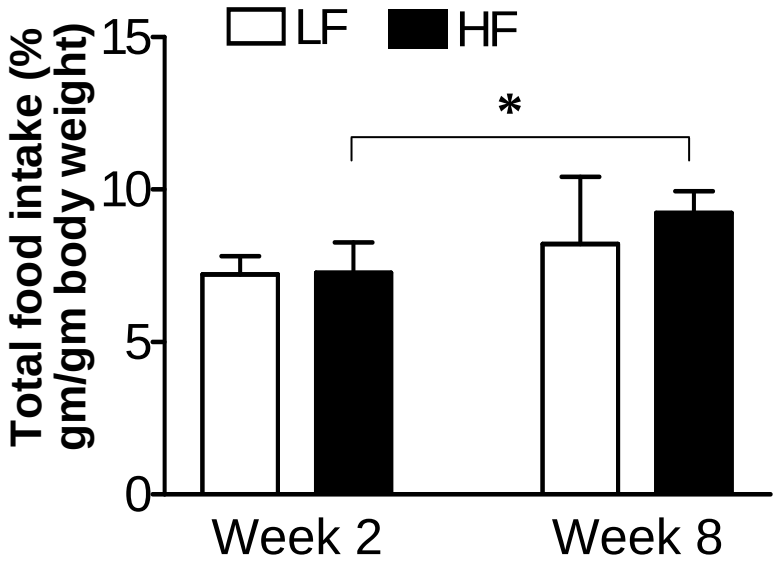

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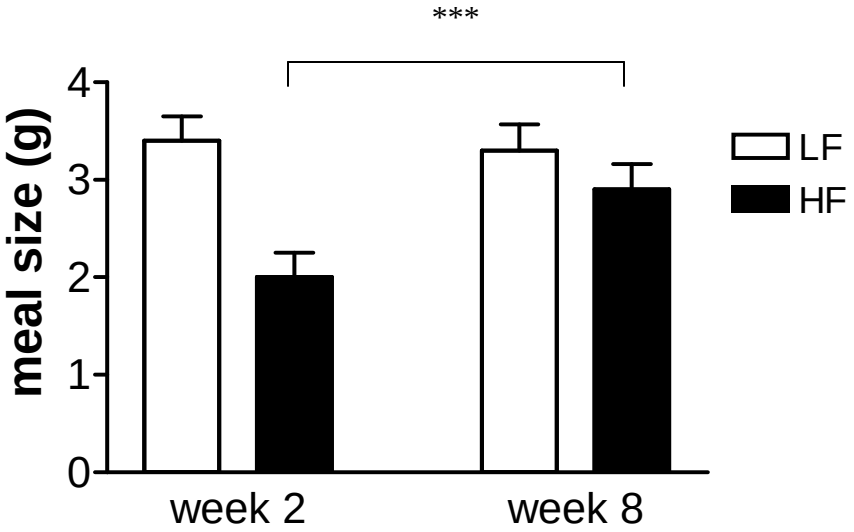

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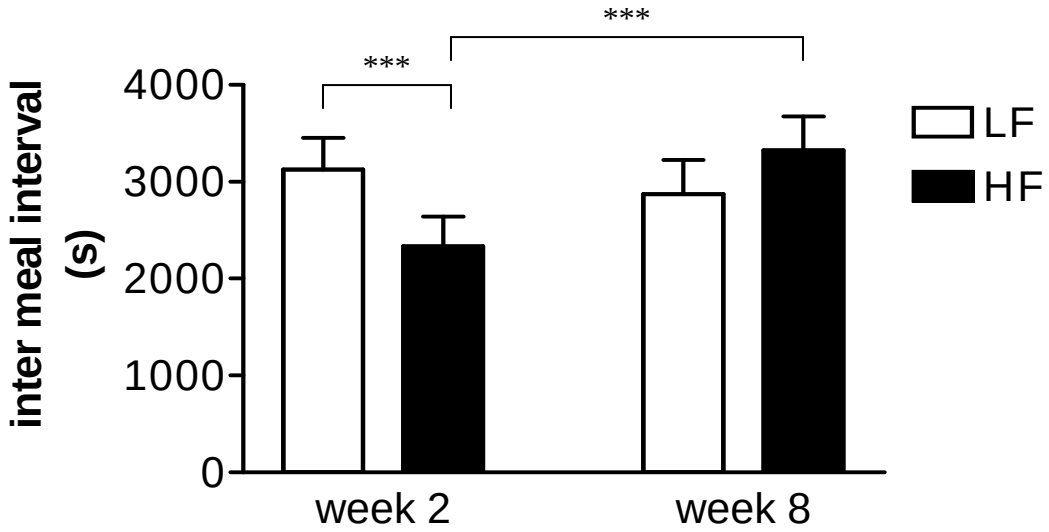


Figure 7a

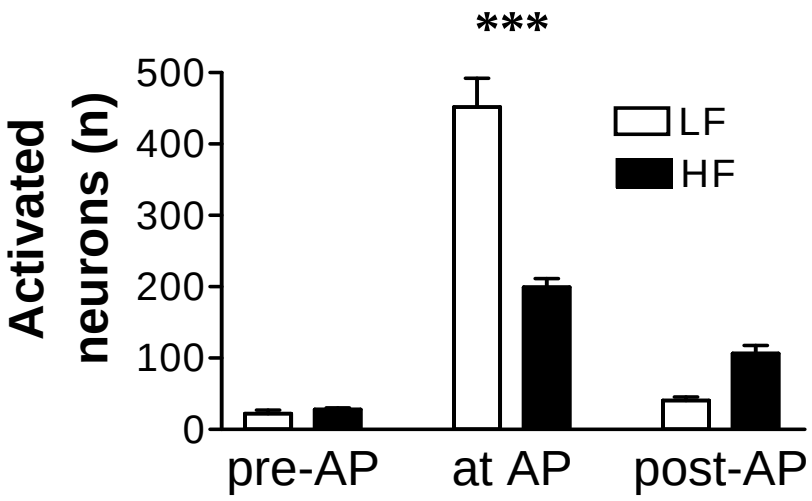


Figure 7b

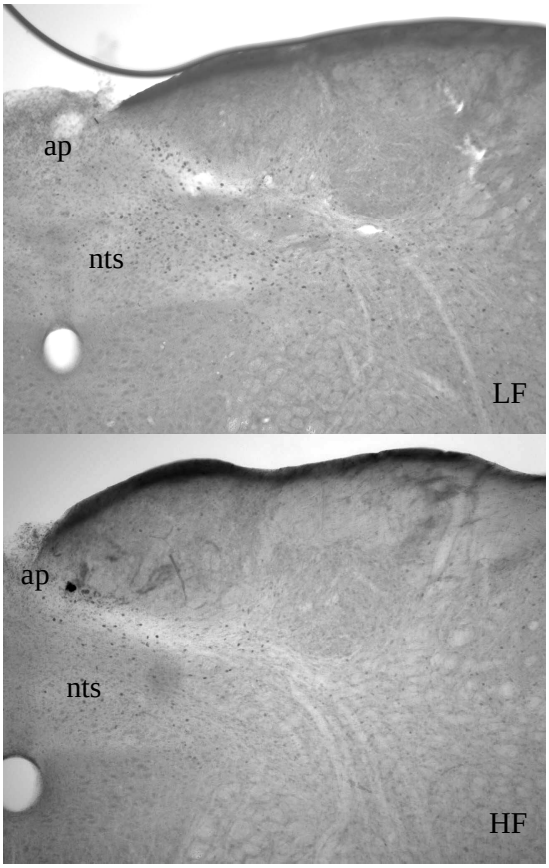


Figure 8

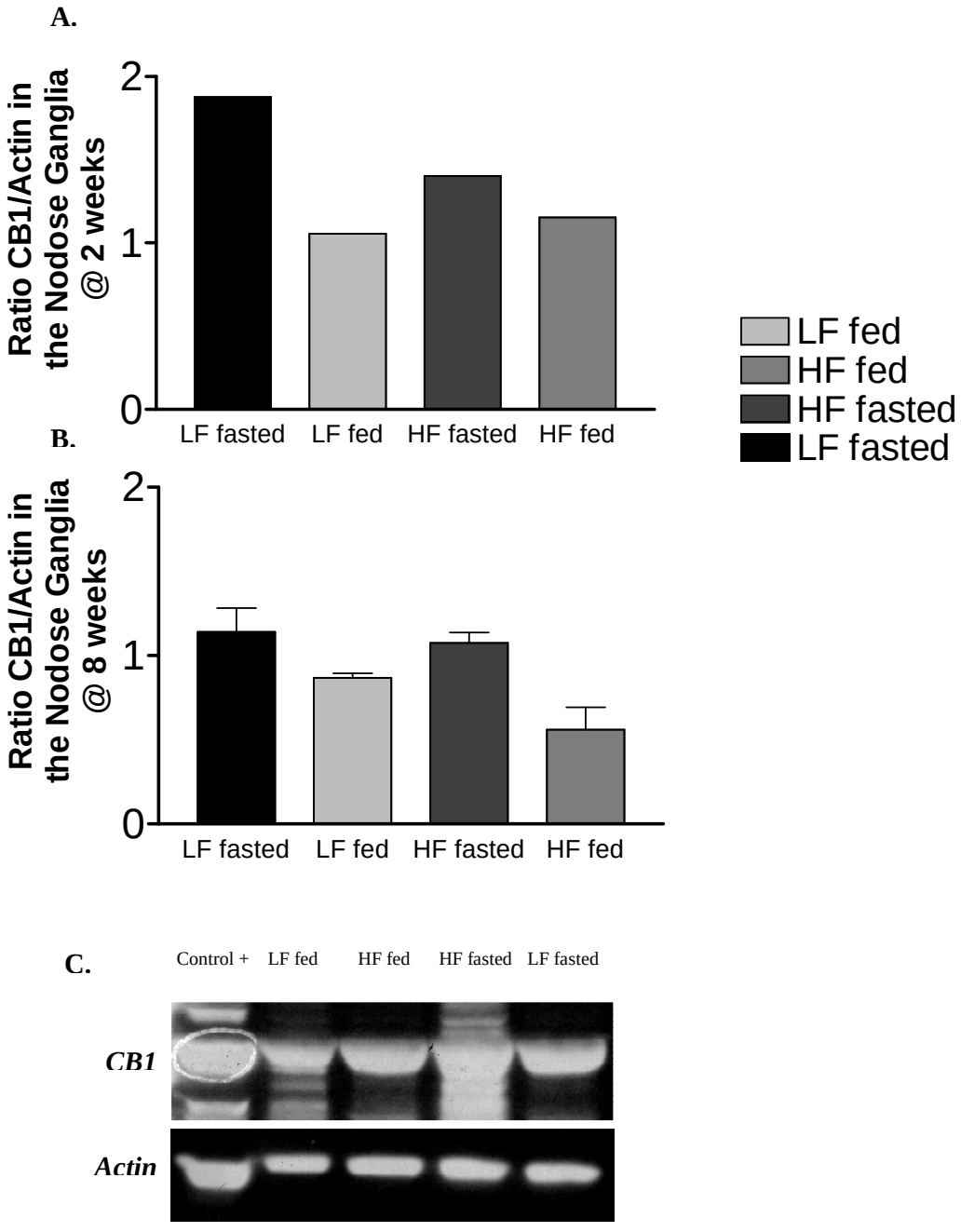
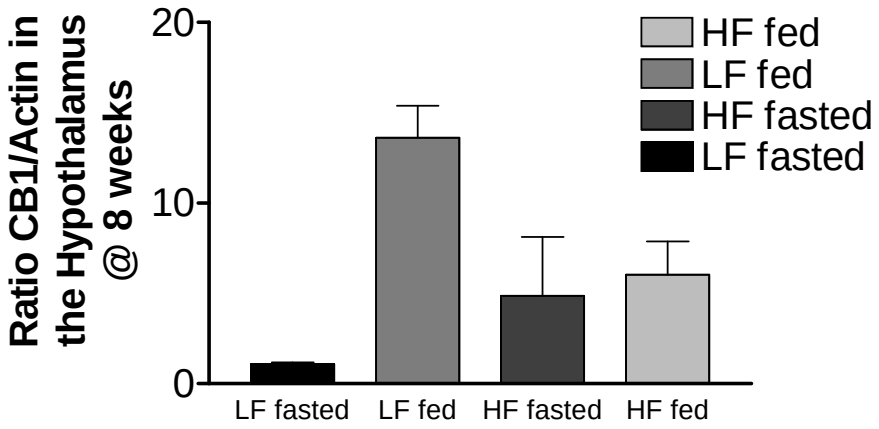
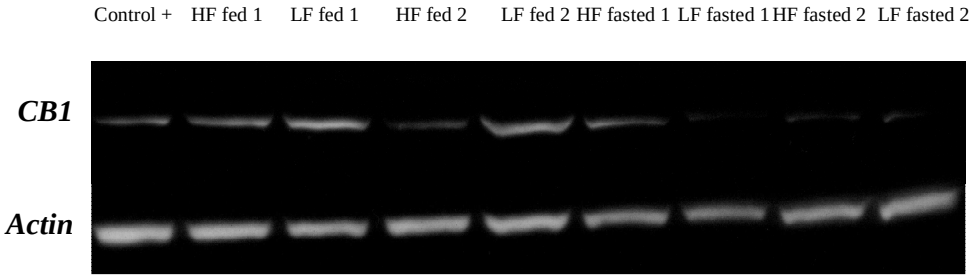

A.
Ratio CB1/Actin in the Nodose Ganglia @ 2 weeks
2
1
0
LF fasted
LF fed
HF fasted
HF fed
LF fed
HF fed
HF fasted
LF fasted
B.
Ratio CB1/Actin in the Nodose Ganglia @ 8 weeks
2
1
0
LF fasted
LF fed
HF fasted
HF fed
C.
Control +
LF fed
HF fed
HF fasted
LF fasted
CB1
Actin

Figure 9


Ratio CB1/Actin in the Hypothalamus @ 8 weeks
20
10
0
HF fed
LF fed
HF fasted
LF fasted
LF fasted
LF fed
HF fasted
HF fed


Control +
HF fed 1
LF fed 1
HF fed 2
LF fed 2
HF fasted 1
LF fasted 1
HF fasted 2
LF fasted 2
CB1
Actin

CHAPTER 3

CCK_1 RECEPTOR IS ESSENTIAL FOR NORMAL MEAL PATTERNING IN MICE FED HIGH FAT DIET

(*status: published*)

"Somewhere, something incredible is waiting to be known."

(Blaise Pascal)

ABSTRACT

Cholecystokinin (CCK), released by lipid in the intestine, initiates satiety by acting at cholecystokinin type 1 receptors (CCK_1Rs) located on vagal afferent nerve terminals located in the wall of the gastrointestinal tract. In the present study, we determined the role of the CCK_1R in the short term effects of a high fat diet on daily food intake and meal patterns using mice in which the CCK_1R gene is deleted. $CCK_1R^{-/-}$ and $CCK_1R^{+/+}$ mice were fed isocaloric high fat (HF) or low fat (LF) diets *ad libitum* for 18 hours each day and meal size, meal frequency, intermeal interval, and meal duration were determined. Daily food intake was unaltered by diet in the $CCK_1R^{-/-}$ compared to $CCK_1R^{+/+}$ mice. However, meal size was larger in the $CCK_1R^{-/-}$ mice compared to $CCK_1R^{+/+}$ mice when fed a HF diet, with a concomitant decrease in meal frequency. Meal duration was increased in mice fed HF diet regardless of phenotype. In addition, $CCK_1R^{-/-}$ mice fed a HF diet had a 75% decrease in the time to 1st meal compared to $CCK_1R^{+/+}$ mice following a 6 hr fast. These data suggest that lack of the CCK_1R results in diminished satiation, causing altered meal patterns including larger, less frequent meals when fed a high fat diet. These results suggest that the CCK_1R is involved in regulating caloric intake on a meal to meal basis, but that other factors are responsible for regulation of daily food intake.

KEYWORDS: meal pattern analysis; cholecystokinin type 1 receptor; high fat diet.

INTRODUCTION

The presence of nutrients in the lumen of the gastrointestinal tract modulates food intake through the release of regulatory peptides, such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Sommer and Kasper 1984) and activation of the vagal afferent pathway (Berthoud and Neuhuber 2000). It is well established that CCK functions as a satiety signal. CCK is released from enteroendocrine cells in response to the presence of lipid or protein in the gut (Liddle, Green et al. 1986). Lipid, especially long chain fatty acids, is a particularly potent stimulator of CCK release (McLaughlin, Lomax et al. 1998). CCK acts by binding to the CCK_1R on vagal afferent nerve terminals located in the intestinal mucosa (Moran, Baldessarini et al. 1997). The vagal afferent fibers, with cell bodies located in the nodose ganglion, project to the nucleus of the solitary tract (NTS) in the dorsal vagal complex, and from there, transmit information on meal content to the hypothalamus to terminate feeding (Berthoud and Neuhuber 2000; Broberger and Hokfelt 2001).Exogenous CCK was first shown to decrease food intake in rats in 1973 by Gibbs *et al.* (Gibbs, Young et al. 1973), and since then has been shown to be effective in decreasing food intake in humans (Kissileff, Pi-Sunyer et al. 1981; Lieverse, Jansen et al. 1994). Duodenal nutrient infusion inhibits food intake in rats and this is reversed by administration of a CCK_1 receptor antagonist, devazepide (Yox, Brenner et al. 1992; Strohmayer and Greenberg 1996). In addition, continuous intravenous infusion of devazepide increases *ad libitum* intake of chow in freely feeding rats, demonstrating a role for CCK and the CCK_1R in the physiological regulation of food intake (Reidelberger and O'Rourke 1989). Despite this evidence that CCK plays a role in the regulation of food intake, mice lacking the CCK_1R have

normal daily food intake and normal body weight (Kopin, Mathes et al. 1999)**.** There is evidence that $CCK_1R^{-/-}$ mice have altered meal patterns compared to wildtype controls on a standard rodent chow diet (Bi, Scott et al. 2004). $CCK_1R^{-/-}$ mice had significantly increased average meal size and decreased meal frequency compared to wild type mice; this is consistent with the known effects of CCK on food intake and short-term satiety.

It is well established that diet composition has a significant effect on food consumption. Ingestion of a high fat, high energy diet has been associated with increased food intake and weight gain compared to a low fat, high carbohydrate (LF) diets in laboratory animals (Warwick and Schiffman 1992) and in humans (Lissner, Levitsky et al. 1987)**.** The changes in meal patterns that account for this overall increase in food intake may involve increases in meal size, meal duration or meal frequency. It remains unclear how CCK regulates free feeding of food containing various nutrient compositions. Little is known about how feeding behavior of $CCK_1R^{-/-}$ mice is altered by diet composition. The CCK_1R is involved in the detection of dietary lipid within the intestine in mice (Whited, Thao et al. 2006), but it is not clear whether these mice are able to respond to the satiating effects of lipid in the diet. We hypothesized that the CCK_1R is involved in meal termination, particularly in the presence of high lipid content of the ingested diet.

The first aim of this study was to determine the changes in meal patterns in response to an increase in the amount of lipid in the diet in mice. Mice were fed either isocaloric low fat (10% fat, LF) or high fat (38% fat, HF) solid diets *ad libitum* for 18 hours a day and food intake was monitored continuously using cages with automatic

feeders. The second aim of this study was to determine if the CCK_1R is essential for the regulation of meal patterns during ingestion of either the LF or HF diet. To address this question, we used mice in which the gene for the CCK_1R is deleted (Kopin, Mathes et al. 1999).

MATERIALS AND METHODS

Animals

Adult male $CCK_1R^{-/-}$ mice (n=24) were used in all experiments. These mice were generously provided by Alan Kopin, MD, Tufts University School of Medicine. These mice are congenic with 129sv mice, which were used as wildtype controls. Aged matched 129Sv mice (8 weeks) (n=24) were obtained from Taconic (Oxnard, CA). All mice were individually housed on wire bottom cages and maintained on a 12-h light, 12-h dark cycle (lights on at 3:00 am) at 23^{o}C in a temperature-controlled room. Water was freely available throughout the experiments and body weight was recorded daily. All procedures were approved by the Institutional Animal Care and Use Committee at University of California Davis.

Food intake analysis

Mice were maintained on either a high fat diet (HF; n=24) with 38% of energy fat derived from fat or a low fat diet (LF; n=24) with 10% of energy from fat. Diets were isonitrogenous (21% of energy) and isocaloric (3.4 kcal/g) (Table 1). Mice were fasted daily in wire bottom cages for 6 hours during the light cycle (9:00 am to 3:00 pm). Body weight was measured at 3 pm, prior to placement in the meal pattern analysis cages. Feeding patterns (meal frequency, meal duration, meal size, intermeal interval) were continuously measured daily from 3:00 pm to 9:00 am using food intake monitoring cages (The Habitest® System, Coulbourn Instruments, Allentown, PA) delivering 20 mg pellets (Bioserv Custom Dustless Precision Pellets,

Frenchtown, NJ). The pellet dispensers were controlled by infrared pellet-sensing photo beams; individual pellets were delivered in response to removal of the previous pellet.

Each experiment lasted 15 days. Mice were acclimated to the diets and the feeding paradigm for 5 days. By the end of the acclimation period, spillage was less than 8 pellets per mouse per day. Following the acclimation period, meal pattern data was recorded for 10 consecutive days.

Data analysis

Data was recorded from EZ count software and analyzed using Spike2 (version 5.07, Cambridge Electronic Design 1988-2004), SigmaStat (version3.11, Systat Software Inc. 2004) and Graph Prism® (version 3.02, GraphPad Software Inc. 1994-2000). The parameters were based on a previously published analysis (Castonguay, Kaiser et al. 1986). A meal was defined as the acquisition of at least 4 pellets within 10 min, preceded or followed by 10 min of no feeding. Data from day 6 to day 15 was analyzed for the number of meals, meal size, intermeal interval, and meal duration. Due to occasional technical error with the feeders, some data collection days were excluded from the final analysis. Days were chosen for analysis based on habitual total food intake. Generally, all mice in this study ate a minimum of 2 grams per day. Consequently, if the total daily food intake recorded was less than 1.5 grams, that entire day excluded from analysis. Based on these criteria, data from 5 individual days was randomly chosen for each mouse for additional data analysis. From these 5 randomly chosen days, every meal for each mouse was analyzed for size and duration,

and average meal size, average intermeal interval, and average meal duration were calculated for each animal over the 5 days. Data for each mouse were averaged from the 5 randomly chosen days; this mean value was used to generate the average data for each group.

Statistical Analysis

A two-way ANOVA was performed with diet and strain as independent variables. All analyses were conducted using SigmaStat (version 3.11, Systat Software, Inc.). Differences among group means were analyzed using multiple comparison procedures (Holm-Sidak method) and considered significant if $P<0.05$.

RESULTS

Body weight

There was no difference in body weight between any of the groups at the start or at the end of the experimental period (start of experiment: $CCK_1R^{+/+}$ 22.7 ± 0.9g, n=24, $CCK_1R^{-/-}$ 22.5 ± 0.7g, n=24; end of experiment: $CCK_1R^{+/+}$ 20.6 ± 0.5g, n=24, $CCK_1R^{-/-}$ 20.1 ± 0.8g, n=24).

Total food intake

There was no significant difference in daily food intake between any groups (total food intake: $CCK_1R^{+/+}$ LF and. HF diet; 5.4 ± 1.5g and 5.2 ± 1.4g, respectively; $CCK_1R^{-/-}$ LF and HF; 4.9 ± 1.2g and 5.2 ± 1.4g, respectively, NS, n=24 per group).

Meal pattern analysis

Individual meals were analyzed for differences in meal duration, size and frequency, and intermeal interval. With the exception of the size of the first meal, there were no significant differences found for any parameter between individual meals for each animal over the course of each day, therefore values from all meals were averaged for each mouse and are presented as mean ± SEM.

Data from the first meal was analyzed separately as parameters differed significantly from all subsequent meals in all groups. The size of the first meal was

larger in mice maintained on HF diet compared to LF diet, regardless of genotype. In $CCK_1R^{+/+}$ mice ingesting the HF diet, the 1st meal was 18% larger than mice ingesting LF diet although this did not reach significance (596 ± 50 mg vs. 705 ± 39 mg LF vs. HF, respectively, NS, n=24). In $CCK_1R^{-/-}$ mice ingesting the HF diet, the 1st meal was 66% larger than in mice ingesting the LF diet (548 ± 45 vs. 907 ± 59 mg LF vs. HF, respectively, $p<0.001$).

Meal size

Ingestion of the HF diet decreased meal size in wildtype mice, but had no effect on average meal size in $CCK_1R^{-/-}$ mice (Fig 1a). There was a significant decrease in meal size in $CCK_1R^{+/+}$ mice when ingesting the HF compared to the LF diet (360 ± 6 mg vs. 339 ± 4 mg LF vs. HF, respectively, $p<0.05$). There was no difference in meal size in $CCK_1R^{-/-}$ mice on the HF diet compared to the LF diet (meal size: LF: 362 ± 6 mg vs. 375 ± 8, LF vs. HF, respectively, NS). However, there was a significant effect of genotype on the meal size on the HF diet; meal size was increased by 10% in $CCK_1R^{-/-}$ mice compared to $CCK_1R^{+/+}$ fed the HF diet. ($p<0.05$) (**Fig. 1a**).

Meal frequency

There was a decrease in the number of meals in $CCK_1R^{+/+}$ mice fed the HF diet compared to the LF diet (**Fig. 1b**); ingestion of the HF diet resulted in a 10% decrease in meal frequency compared to LF diet in $CCK_1R^{+/+}$ ($p< 0.05$). There was no difference in meal frequency in the $CCK_1R^{-/-}$ mice on LF or HF diet. However, there

was a significant genotype difference in meal frequency; $CCK_1R^{-/-}$ mice ate significantly fewer meals compared to $CCK_1R^{+/+}$ mice, irrespective of diet ($p< 0.001$).

Meal Duration

Ingestion of the HF diet resulted in a significantly longer meal duration in both $CCK_1R^{+/+}$ and $CCK_1R^{-/-}$ mice (Figure 2). Average meal duration was 35% longer in $CCK_1R^{+/+}$ mice on the HF diet compared to the LF diet ($p<0.001$). Similarly, average meal duration was 32% longer in $CCK_1R^{-/-}$ mice on the HF compared to the LF diet ($p<0.01$). However, there was no effect of genotype on meal duration in mice fed either the LF or HF diet (NS) (**Fig. 2**).

Intermeal Interval

Diet composition or genotype had no significant effect on the average intermeal interval **(**IMI) (NS, n=24). IMI in $CCK_1R^{+/+}$ mice on LF and HF diet was 2116 ± 58s vs. 2242 ± 78s, respectively. Similarly, average IMI in $CCK_1R^{-/-}$ mice on LF diet and HF diet was 2283 ± 73s vs. 2351 ± 86s. There was a tendency for the intermeal interval to be longer in the $CCK_1R^{-/-}$ mice regardless of the diet on which they were maintained; however, this did not reach statistical significance.

Delay to start of the first meal

The time to the start of the first meal was significantly decreased in $CCK_1R^{-/-}$ mice fed the HF diet compared to the LF diet (**Fig. 3**; $p<0.05$, n=24). There was also a

42% decrease in time to the first meal between LF and HF diet groups in the $CCK_1R^{+/+}$. There was no significant difference between $CCK_1R^{+/+}$ mice and $CCK_1R^{-/-}$ mice in the time to initiation of the first meal when mice were maintained on a LF diet (NS, n=24). However, there was a significant decrease in the time to the start of the first meal in the $CCK_1R^{-/-}$ compared to the $CCK_1R^{+/+}$ mice on HF diet. Thus, the $CCK_1R^{-/-}$ mice had 51% shorter time to 1st meal compared to $CCK_1R^{+/+}$ mice when fed the HF diet ($p<0.05$, n=24).

DISCUSSION

The results from the present study demonstrate that the CCK_1R is a major determinant in the regulation of meal patterns. Mice lacking the CCK_1R ate larger and longer meals when ingesting a high fat diet compared to wildtype mice, while meal frequency and overall daily food intake were unaffected These data suggest that the absence of the CCK_1R impairs the ability to terminate meals and support the hypothesis that both CCK and the CCK_1R are involved in the determination of meal size. Thus, in normal mice, the presence of nutrients, in particular lipid when ingesting a HF diet, acts via release of CCK, and subsequent activation of CCK_1Rs, to terminate a meal. In the absence of the CCK_1R, detection of lipid in the intestinal lumen is reduced and there is diminished intestinal feedback regulation of meal size. As a result, the alteration of meal patterns in CCK_1R null mice is more marked when ingesting a high fat diet, consistent with the role of the receptor in detection of lipid in the intestinal lumen (Whited, Thao et al. 2006). However, overall daily food intake and intermeal interval remain unchanged, suggesting that other mechanisms regulate food intake over the course of several meals. Furthermore, these data also show that the $CCK_1R^{-/-}$ mice ingesting the HF diet have significantly decreased latency to first meal after a short term fast. These data are consistent with increased hunger and urgency to feed and suggest a role for the CCK_1R in meal initiation. Collectively, these results support the hypothesis that the CCK_1R is essential for dietary lipid sensing within the intestine and normal meal patterning, especially when ingesting a HF diet.

The first aim of the study was to determine how dietary fat influences meal patterns in $CCK_1R^{+/+}$ mice. In order to determine the effect of lipid in the absence of increased caloric density of a high fat diet, we used isocaloric diets. There was no overall change in the total daily food intake; however, the daily pattern of ingestion was markedly altered. There was a significant increase in meal size and an increase in meal duration in $CCK_1R^{+/+}$ mice ingesting the HF diet compared to the isocaloric LF diet; which is consistent with previous observations in rodents and humans (Warwick, McGuire et al. 2000; Synowski, Smart et al. 2005). In addition, there was a concomitant decrease in meal frequency in $CCK_1R^{+/+}$ mice fed the HF diet compared to the isocaloric LF diet. This is in accordance with other rodent studies that showed an inverse relationship between meal size and meal frequency (West, Fey et al. 1984; Mathis, Johnson et al. 1995). Studies examining meal patterns have revealed that an increase in meal size results in a decrease in meal frequency (Clifton 2000; Chi, Fan et al. 2004).

Satiety is sometimes measured by the correlation between meal size or meal duration and intermeal interval. In the present study there was no significant correlation between IMI and meal size or meal duration. Although several labs have documented a correlation between meal size and postprandial intermeal interval (Davies 1977; Thomas and Mayer 1978; Rosenwasser, Boulos et al. 1981), this concept has been disputed and remains controversial (Johnson, Ackroff et al. 1986). Additionally, other factors secreted by ingested food may act to extend the intermeal interval. Experiments using exogenous CCK injections found that CCK does not extend postprandial interval between meals (Gibbs, Young et al. 1973; Strohmayer and Smith 1981; Miesner, Smith et al. 1992), while bombesin and GRP do (Rushing,

Henderson et al. 1998). This indicates that CCK may be most influential in regulating satiety for the first meal, while additional factors predominate subsequently.

The second aim of the study was to determine if the CCK_1R is a major determinant of meal patterning in mice in response to dietary fat. Previous work has shown that the CCK_1R is important in the regulation of food intake, but the role of the receptor in overall regulation of food intake has been hampered by limitations in the pharmacological tools available, particularly the duration of action of CCK_1R antagonists. Experiments using rats have shown that continuous infusion of the CCK_1R antagonist results in increase meal size (Miesner, Smith et al. 1992). Otsuka Long Evans Tokushima Fatty (OLETF) rats, which lack the CCK_1R, are hyperphagic due to an increase in meal size and an insufficient decrease in meal frequency to compensate for this increase in meal size (Moran and Bi 2006). The results from the present study show that the lack of the CCK_1R reversed the decrease in meal size in response to HF diet, but had no effect on mice fed LF diet. $CCK_1R^{+/+}$ mice ate smaller meals of HF diet compared to the mice fed LF diet, but the $CCK_1R^{-/-}$ mice ate the same size meals of LF and HF diet. This finding is inconsistent with the results observed by Bi et al. who showed that $CCK_1R^{-/-}$ mice consumed 35% more chow per meal compared to wild-type mice. However, this difference in meal size could be due to differences in diet composition because $CCK_1R^{+/+}$ mice ate smaller meals of HF diet compared to the mice fed LF diet, but $CCK_1R^{-/-}$ mice ate the same size meals of LF and HF diet.

In addition, both groups of mice on the HF diet had significantly longer meal duration compared to mice fed the LF diet. These data suggest that, in CCK_1R null

mice, there is an inability to end meals and the mice fed HF diet eat larger meals over a longer time. It is well established that fat is a potent inhibitor of food intake via CCK release (Liddle, Goldfine et al. 1985; Matzinger, Gutzwiller et al. 1999). We conclude that in the absence of the CCK_1R, the detection of the presence of dietary nutrients, notably fat, in the intestinal lumen is impaired and the pathway mediating intestinal feedback leading to satiation has been interrupted. It is possible that other satiety factors released from the GI tract and associated organs such as PYY, GLP-1 or amylin may act to terminate the meal; the time required for release of these other factors results in prolonged meal duration. Together, these results provide evidence that the CCK_1R is required for regulation of meal size and intrameal satiation in mice. The onset of satiation is the major determinant of meal size, thus delaying satiation will cause an increase in meal size. Mice lacking the CCK_1R may have larger meal size on HF diet due to faster gastric emptying time due to lack of lipid-induced intestinal feedback. Prior studies have shown more rapid gastric emptying in $CCK_1R^{-/-}$ mice compared to wildtype controls (Whited, Thao et al. 2006).

One of the most striking findings in the present study was that $CCK_1R^{-/-}$ mice ingesting a HF diet had significantly shorter latency to the start of the first meal after a 6 hour fast. This suggests that lack of the CCK_1R reduces intrameal satiation, and additionally influences hunger. The association between CCK and initiation of feeding was established by Foltin and Moran who showed a dose dependent increase in the onset of feeding in adult male baboons using the CCK analog U-67827E (Foltin and Moran 1989). The mechanism is unclear, but may involve orexigenic peptides, such as ghrelin or cannabinoids, both of which have been shown to increase hunger, and receptors for these peptides are expressed on vagal afferents (Burdyga, Lal et al.

2004; Burdyga, Varro et al. 2006). It is likely that there is an intrinsic difference between wild type mice and those lacking the CCK_1R in terms of receptor expression in the gut-brain pathway. It has been suggested that CCK modulates additional anorexigenic and orexigenic factors and it is probable that in the absence of the CCK pathway, expression of these factors or their receptors is altered (Burdyga, Lal et al. 2004).

$CCK_1R^{-/-}$ mice ate fewer meals compared to $CCK_1R^{+/+}$ mice, irrespective of diet. This confirms previous results by Bi *et al* (Bi, Scott et al. 2004) who saw a slight decrease in meal frequency in $CCK_1R^{-/-}$ mice fed standard rodent chow and other published data, as discussed above. The mechanism by which meal frequency is regulated remains unclear, but most likely involves other feeding peptides such as ghrelin. Degraaf *et al*. reported that exogenous administration of CCK decreased the stimulatory effects of ghrelin on meal size and meal initiation (de Graaf, Blom et al. 2004). Additionally, it was reported that injection of sulfated CCK-8 blocked the orexigenic effect of ghrelin (Kobelt, Tebbe et al. 2005).

Consequently, it appears that the CCK_1R pathway is involved with meal termination in response to HF diet but does not directly regulate meal frequency. Interconnected with meal frequency, intermeal interval was also not changed by diet or phenotype, suggesting that other satiety factors such as leptin or PYY may be involved in regulating food intake over multiple meals. It has been previously reported that exogenous CCK decreased meal size without changing the duration of the intermeal interval (Strohmayer and Smith 1981). Moreover, foraging studies using

rats have shown little correlation between meal size and intermeal interval (Johnson, Ackroff et al. 1986).

We observed no difference in total food intake in $CCK_1R^{-/-}$ mice compared to $CCK_1R^{+/+}$ mice regardless of diet. This is in accordance with previous studies have shown that there was no difference in 24 hour food intake between $CCK_1R^{-/-}$ and $CCK_1R^{+/+}$ mice fed chow (Kopin, Mathes et al. 1999; Bi, Scott et al. 2004). It is well established that rodents can regulate their caloric intake by eating less of a high calorie diet and more of a low calorie diet (Treit and Spetch 1986). In the present study the caloric density was identical for both diets, which could account for the observation that total food intake was uniform across all groups.

Our results support the hypothesis that meal composition and the CCK_1R are involved in regulating meal size. The present study provides evidence that $CCK_1R^{-/-}$ mice have decreased satiation compared to wild type mice when fed a HF diet. These results support the idea that the CCK_1R is required for satiation, but has little effect on satiety in mice. It is evident that the ability to regulate body weight and energy balance over days and weeks is independent of the CCK_1R in mice. It is well established that leptin and CCK act in synergy to reduce food intake and it has been shown that CCK enhances weight loss, but not the anorexic response to leptin (Matson, Reid et al. 2002). Consequently, it is probable that normal expression of long term satiety signals such as leptin are responsible for the maintenance of normal body weight in the absence of the CCK_1R.

CONCLUSION

While previous studies have shown that the CCK_1R is involved in regulating short term food intake, no studies have examined the role of the CCK_1R in 129sv mice in conjunction with feeding a high fat diet. In this study we have shown that mice lacking the CCK_1R have diminished satiation causing altered meal patterns including longer, larger meals. Furthermore, we have shown that this reduction in satiation is accentuated by feeding a high fat diet. This effect is possibly due to diminished detection of dietary lipid within the intestine. Our results suggest that lipid detection in the CCK_1R-/- mice may be impaired resulting in altered meal patterns. However, due to the action of other long term satiety peptides such as leptin and PYY these animals are able to maintain a normal body weight. The observed feeding patterns on HF diet suggest that the CCK_1R is involved in regulating caloric intake on a meal to meal basis, and other factors are responsible for regulation over multiple meals.

FIGURE LEGENDS

Figure 1: $CCK_1R^{-/-}$ mice ate larger meals compared with $CCK_1R^{+/+}$ mice on both HF and LF diet. Mice maintained on the HF diet ate larger meals compared to those maintained on LF diet regardless of genotype. Data represent the average quantity of food ingested per meal over the entire 18 hour feeding period. (Values are means ± SEM, n =8, values with different letters denote significant difference between groups, $p<0.05$)

Figure 2: $CCK_1R^{-/-}$ mice fed the HF diet had a 20% longer average meal duration compared to $CCK_1R^{+/+}$ mice (n=8 per group, $p<0.05$). There was no difference in average meal duration between $CCK_1R^{+/+}$ and $CCK_1R^{-/-}$ mice maintained on the LF diet. ($p<0.05$)

Figure 3: $CCK_1R^{-/-}$ mice on HF diet had significantly shorter latency to first meal compared to $CCK_1R^{+/+}$ mice and compared to -/- mice maintained on LF diet. There was no difference between -/- and $^{+/+}$ on LF diet (NS, n=8).

Figure 1a

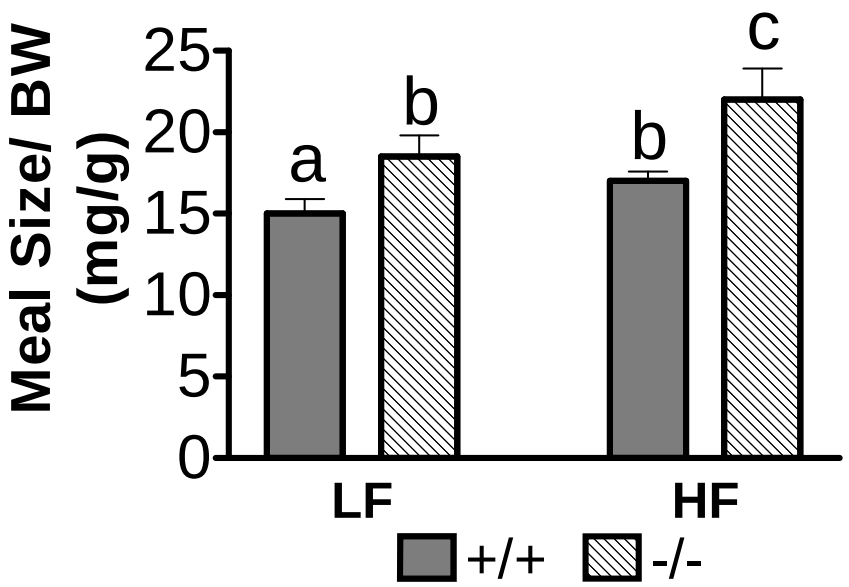


Figure 1b

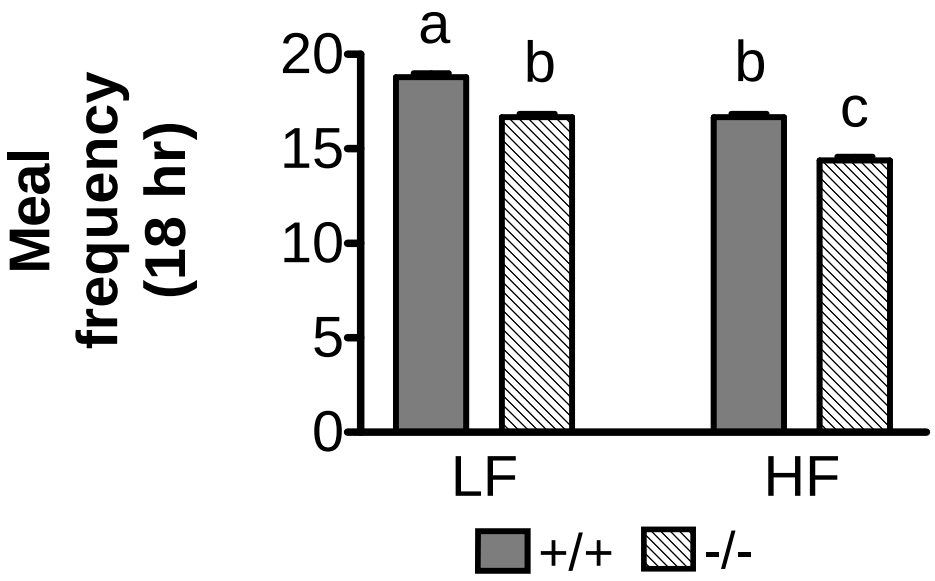


Figure 2

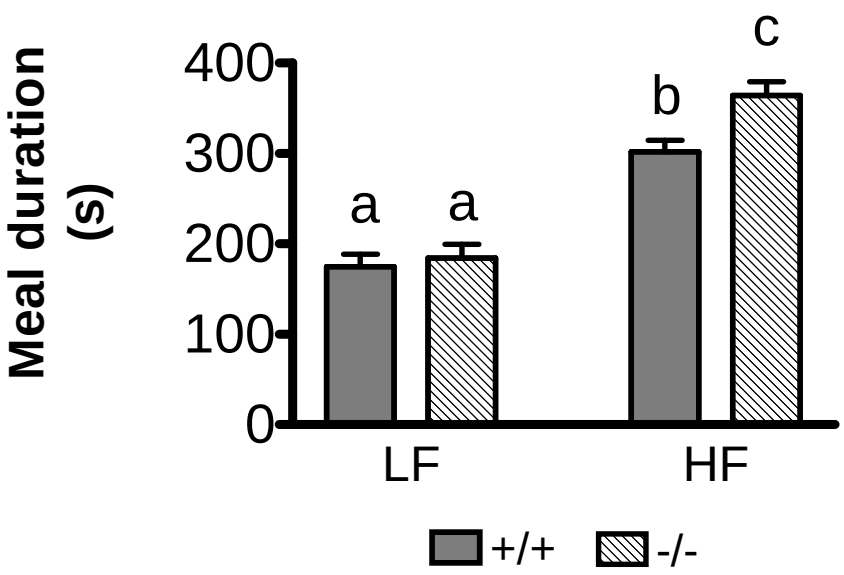
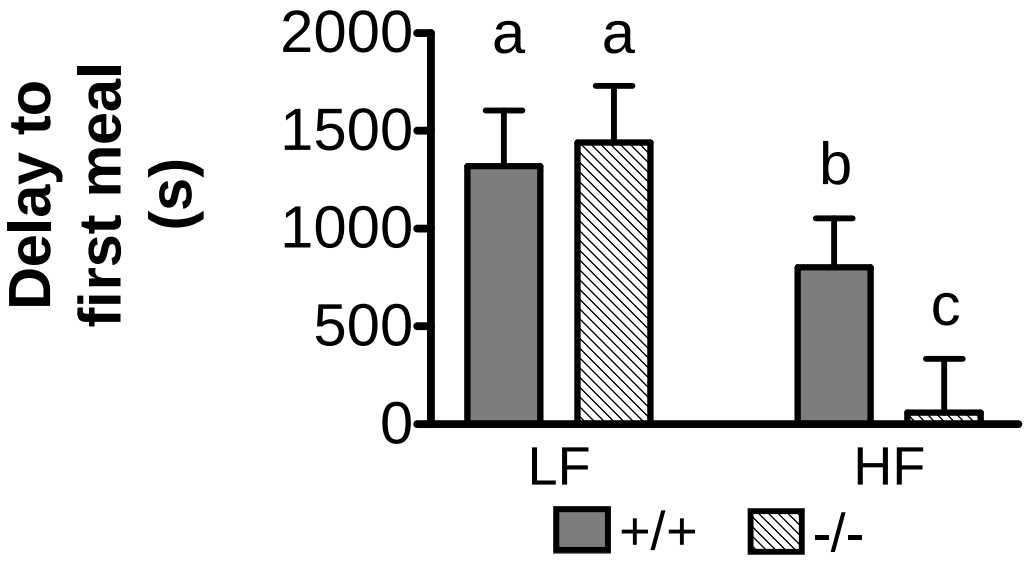


Figure 3



CHAPTER 4

ADAPTATION TO A HIGH FAT DIET ALTERS THE NORMAL RECEPTORS EXPRESSION INVOLVED IN THE DETECTION OF DIETARY FAT

(*status: in preparation*)

"I haven't failed: I just found 100,000 ways that don't work."

(Albert Einstein)

ABSTRACT

Food intake is modulated by both humoral and neuronal pathways; there is evidence that maintenance on a high fat diet leads to hyperphagia, via a peripheral, CCK-dependent mechanism, involving the gut-brain axis. Our hypothesis was rats fed a high-fat diet become hyperphagic because the expression of the genes involved in the short-term regulation of food intake is modified and tend to an orexigenic system. The first aim of this study was to describe metabolic changes occurring in rats fed either a low-fat (LF) or a high-fat diet (HF). The second aim was to highlight key receptors involved in the control of food intake and show that their expression was modified by a chronic ingestion of a HF diet. Rats fed a high fat diet and prone to obesity presented hyperleptinemia, hyperinsulinemia accompanied with an increase in ObR in the adipose tissue. The expression profile of receptors involved in appetite was modified in the nodose ganglia (decrease in ObR, increase in CB1) leading to an orexigenic system after a chronic ingestion of a HF diet. A more systemic and integrative investigation of the hormones and receptors expression involved in humoral and neuronal pathways of appetite is needed to describe what is really hunger and to understand how can a dysregulation lead to obesity.

KEYWORDS: high fat diet, adaptation, nodose ganglia, CCK, Leptin, CB1, Ob-R.

INTRODUCTION

The control of food intake is complex, involving both neural and endocrine pathways and short-term and long-term regulation (Coll, Farooqi et al. 2007). It allows the body to maintain its weight within a range even though intake is organized into discreet events. This implies a complex control with incremental feedback in order to minimize the spikes in body weight or in nutrients absorbed by the body. For example, the mechanism by which dietary lipid is detected in the small intestine involves the formation of chylomicrons containing the apolipoprotein AIV, which allows the release of the satiety hormone CCK (Glatzle, Darcel et al. 2004). In the distal gut, lipids are detected through the release of PYY3-36. This release appears to be a synergistic effect of both lipids and CCK release (Whited, Tso et al. 2007). These hormones are part of the short-term regulation of food intake and are released in response to nutrient in order to terminate a meal, known as satiation. On the other hand, some molecules released by the gastrointestinal tract can stimulate appetite. These include ghrelin, peptide released from the gastric corpus (Kojima, Hosoda et al. 1999), or endogenous cannabinoids such as anandamide (Gomez, Navarro et al. 2002). These hormones or metabolites are released during the inter meal interval and are at their highest level right before a meal. But the control of food intake also requires a long-term regulation. Leptin, a hormone released from the adipocytes, reflects adiposity. Therefore it is a good signal of the energy storage available in the body (Barinaga 1995). Leptin secretion is insulino-dependent and both leptin and insulin acts at the level of the arcuate nucleus in the hypothalamus to decrease food intake (Baskin, Figlewicz Lattemann et al. 1999). These mechanisms interact on a non pathological state of the body in order to regulate food intake and energy homeostasis.

However these systems seem to be altered by a chronic ingestion of a high fat (HF) diet. It is now clear that chronic ingestion of a HF diet lead to obesity and co morbid cardio-vascular diseases. There is some evidence that adaptation to lipids modify the vagal-sensitivity to the gut-hormone CCK (Savastano and Covasa 2005). It has also been shown that in Diet-Induced Obese Rats, the release of PYY is decrease (Yang, Wang et al. 2005), increasing therefore their food intake. Numerous studies have shown an adaptation at the level of the hypothalamus after chronic ingestion of a HF diet. Specifically, leptin-resistance at the level of the arcuate nucleus by increased expression of the suppressor of cytokine signaling 3 (SOCS3) could explained diet-induced obesity in rodents (Enriori, Evans et al. 2007). However this adaptation occurs in the center of regulation of homeostasis. Little is known about the adaptation of the center of short-term regulation of food intake: the nodose ganglia. Although there is evidence that the gut-hormone profile is modified in pathological state such as bolemia or anorexia (Baranowska, Radzikowska et al. 2000), few studies have looked at the level of the receptors. Most of the important gut-hormones involved in the detection of nutrient possess a receptor that is synthesized in the nodose ganglia and transported to the vagal afferent innervating the gastrointestinal tract from stomach to colon. The effect of hormone receptors level on short-term regulation is now more understood. Leptin receptor Ob-R and CCK1-R are both present on the vagus nerve and cooperate to enhance satiation in rats (Peters, Karpiel et al. 2004; Peters, Ritter et al. 2006). CCK inhibits the expression of CB1 receptor through CCK1-R in the nodose and CB1 is upregulated after food deprivation (Burdyga, Lal et al. 2004). These three receptors and their natural ligands are differently regulated upon nutritional status. So far, receptors expression in the nodose ganglia has only been studied under acute ingestion of a high fat diet (Broberger, Holmberg et al. 2001).

They have shown no change in CCK1 receptor expression in normal nodose ganglia after a 48h food deprivation or after a high-fat meal. Our interest in this study is to describe and to understand how a high-fat diet can influence the gene expression of the receptors involved in the short-term regulation of food intake and how adiposity can play a role in the neural pathway of appetite and how it can lead to diet-induced obesity.

Although there is evidence to show that rats fed a HF diet become obese and present pathological metabolic response such as insulin-resistance, leptin-resistance and dislipidemia, little is known about how the complex mechanism by which dietary lipids are detected by the vagus nerve, through the receptors on the vagal afferents, is modified after a chronic ingestion of a HF diet. We first wanted to investigate the metabolic profile after a HF load in rats adapted to the HF diet versus rats fed a low fat (LF) diet. Therefore, we measured plasma triglycerides, insulin and leptin levels after an intralipid gavage. Secondly we wanted to identify if the adaptation to a HF diet alters the production of the receptors to the gut-hormones (CCK1R, Y2 receptor) but also to leptin (Ob receptor) and CB1 receptor at the level of the vagal afferents. Therefore, nodose ganglia were collected and quantitative real-time RT-PCRs were performed.

MATERIALS AND METHODS

Animals: Diet and Experimental Procedures

Male Sprague Dawley rats (6 weeks of age, Harlan) were fed *ad libitum* a low fat diet (LF: Research Diets D12450B) or a high fat diet (HF: Research Diets D12451) for 2 or 8 weeks. The LF diet provided 3.85 kcal/g of energy (70%carbohydrate, 20%protein, 10%fat) and the HF diet provided 4.73 kcal/g of energy (35%carbohydrate, 20%protein, 45%fat). The experiment was repeated 3 times, with n=10 rats per group in each cohort (total n=30 rats per group) and the rats were housed in pairs for the first 2 weeks and then individually in a temperature-controlled room. The animals were kept under regular light conditions (lights on at 06:00 am and off at 06:00 pm). Water was freely available throughout the experiments, and body weight was recorded daily. The measure of food intake was done estimated for the first two weeks by dividing the total food intake of the cage by two. The HF group weighted 265.2 ± 8.8 g when the LF group weighted 269.2 ± 9.2 g at the beginning of the experiment, the difference was not significant. All experiments were performed in protocols reviewed and approved by the Institutional Animal Care and Use Committee, UC Davis.

Blood Sampling for Insulin, Leptin and Triglycerides Assay

Blood samples were obtained after a 12h fast during the light cycle, at week 2 and 8. Rats were then gavaged with 1mL/100g of intralipid (Intralipid 20%; Baxter HealthCare Corp., Deenfield) and blood samples were collected 1 and 2 hours after

gavage. The blood samples were then centrifuged at 1,000g for 10 mins and plasma layers were recovered into a new tube and store at -80 C. Triglycerides were assayed at 0, 1 and 2 hours (Serum Triglyceride Determination Kit, Sigma, Saint-Louis, MI), Insulin and Leptin were measured by ELISA at 0 and 2 hours (ALPCO Diagnostics, Salem, NH).

Surgeries and Tissue Collection

After a 12h fast, rats were gavaged with 1 mL/100g intralipid (Intralipid 20%; Baxter HealthCare Corp., Deenfield) and anesthetized with Nembutal (0.2mL/100g) after 2 hours. After taking a 2mL blood sample from the aorta, rats were decapitated and tissues from the brain (nodose ganglia and hypothalamus) and from the visceral cavity (stomach, duodenum, ileum, colon, liver, muscle, epididymal, mesenteric and retroperitoneal fat pads) were collected quickly with instruments cleaned with RNA Zap (Ambion, Austin, TX) to minimize degradation of RNA from the tissue. Tissues were collected in 2 mL Heppendorf tubes previously decontaminated with RNA Zap, and were flash frozen in liquid nitrogen and then stored at -80 C.

Body Composition

Epididymal, mesenteric and retroperitoneal fat pads were weighted individually and an adiposity index consisting of the sum of the three fat pads divided by the body weight was calculated.

Quantitative real-time PCR on Nodose Ganglia

Nodose ganglia were pooled from 5 rats for the first 2 cohorts, and from 2 (HF-DIO) and 3 (HF-DR) rats from the third cohort to distinguish sub-groups in the HF 8 weeks group. The samples were grinded with a mortar and pestle under a constant flow of nitrogen liquid to avoid thawing. Once powdered, samples were weighted and RNA extraction was then executed with the RiboPure Kit (Ambion, Austin, TX). Samples were bioanalyzed on a RNA 6000 Nano chip kit (Agilent 2100 Bioanalyzer, Agilent Technologies, Inc., Palo Alto, CA) and with a NanoDrop to check for integrity and concentration.

cDNA was synthesized by reverse transcription from 1 μg total RNA using a cDNA Synthesis Kit Superscript III First Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using Taqman® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The Taqman® Gene Expression Assay consisted of a FAM™ dye-labeled TaqMan® MGB probe and two PCR primers formulated into a single tube for each target gene (GenBank Accession Number: CCK1-R: M88096, Y2-R: AY004257, CB1: X55812, FAAH: U72497, Fa: D84550, GHS-R: AB001982, Sncγ: X86789, Tusc5: AB218813 and 18S ribosomal: X03205). 6 μL of cDNA diluted 30-fold after reverse transcription were added to each well and let dried out overnight. Each PCR was run with 8 μL of a mix containing 2*PCR Mix TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 20* TaqMan® Gene Expression Assay on the he ABI PRISM 7700

Sequence Detection System according to the manufacturer (Applied Biosystems, Foster City, CA, USA).

Analysis of relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Briefly, the C_T (threshold cycle when fluorescence intensity exceeds 10 times the S.D. of the baseline fluorescence) value for the target amplicon (CCK1-R, Y2-R, CB1, FAAH, GHS-R, Sncγ, Tusc5) and endogenous control (18S) are determined for each PCR reaction. Each PCR reaction was repeated in triplicates for each target gene and each sample to make sure of the repeatability of the measure. An average Ct is then chosen for each sample and each gene by normalization against one of the replicate if the replicates seemed similar. The differences in C_T for the target and the C_T for the endogenous control were determined (ΔC_T). This normalizes the amount of nucleic acid in each reaction and the reverse transcription step. The group LF 2 weeks was used as a calibrator. The ΔC_T value for each sample is then subtracted from the calibrator. This difference is called the $\Delta\Delta C_T$. Finally, the relative amount of mRNA in each sample normalized to the endogenous control and relative to the calibrator is calculated by $2^{-\Delta\Delta CT}$. The $2^{-\Delta\Delta CT}$ value for each sample was then adjusted such that the mean relative mRNA level in the LF 2 weeks nodose ganglion was 1. Therefore the samples are expressed as an *n*-fold difference relative to the LF 2 weeks nodose ganglion (Yang, Wang et al. 2004). Data are presented as the adjusted mean $2^{-\Delta\Delta CT} \pm$ S.E.M. Statistical analysis of the differences in mRNA levels was performed by two-way ANOVA with Time and Diet as independent factors followed by a Bonferroni post-test.

Statistical Analysis.

Body Weight and Body composition. A two-way ANOVA Repeated Measure was performed with diet and time as independent variables and the rat as the subject repeated. All analyses were conducted using SAS (version 8.02, SAS Institute, Inc, Cary, NC.). Differences among group means were analyzed using multiple comparison procedures (Holm-Sidak method) and considered significant if $P<0.05$.

Plasma Assays. A repeated-measure three-way ANOVA was performed with diet, time and time after gavage as independent variables and the rat as the repeated subject. All analyses were conducted using SAS (version 8.02, SAS Institute, Inc, Cary, NC). Differences among group means were analyzed using multiple comparison procedures (Bonferroni method) and considered significant if $P<0.05$.

Quantitative Real-Time PCR. A two-way ANOVA was performed with diet and time as independent variables. All analyses were conducted using SAS (version 8.02, SAS Institute, Inc, Cary, NC). Differences among group means were analyzed using multiple comparison procedures (Holm-Sidak method) and considered significant if $P<0.05$.

RESULTS

Body weight and body composition.

After 3 weeks, rats fed a high fat diet gained more weight than rats on the low fat diet ($P<0.05$); within the HF diet group we could distinguish rats that were diet-induced obese (DIO) beyond the median of the HF group and rats that were diet-induce obese resistant (DR) above the median of the HF group. The HF-DIO group was significantly different from the HF-DR from week 3 to 8, and the HF-DR group was not significantly different from LF diet (**Fig. 1**). There was no significant difference in the adiposity index (calculated as the sum of the peritoneal, mesenteric and epididymal fat pads divided by the body weight) between the two groups at 2 weeks. At 8 weeks the HF-DIO group had a significantly higher adiposity index than the HF-DR which had a significantly higher adiposity index than the LF group (2 weeks LF: 2.37 ± 0.16% vs. HF: 2.87 ± 0.15%, n=15 in each group, NS; 8 weeks LF: 2.58 ± 0.09% n=15 vs. HF-DR: 3.14 ± 0.11% n=8 vs. HF-DIO: 4.15 ± 0.22% n=7, $p<0.001$) (**Fig. 2**)

Plasma Assays.

Triglycerides assay. In the fasted state there was an increase in true triglycerides (TG) level in the LF group compared to HF group at either 2 weeks or 8 weeks (2 weeks LF: 0.63 ± 0.06 g/L vs. HF: 0.30 ± 0.03 g/L, n=10; 8 weeks LF: 0.76 ± 0.15 g/L, n=10 vs. HF-DR: 0.36 ± 0.03 g/L, n=6 vs. HF-DIO: 0.36 ± 0.05 g/L, n=4. After 1 or 2

hours the TG level was still higher in the LF group than in the HF group $p<0.001$ (**Fig. 3**).

Leptin assay. On a fasted state there was no difference in leptin secretion after 2 weeks on either a LF or HF diet. After 8 weeks on their respective diet, HF-DIO had a significantly higher level of circulating leptin than LF or HF-DR rats (2 weeks LF: 397.90 ± 170.30 pg/mL vs. HF: 382.800 ± 176.90 pg/mL, n=15 in each group, $p>0.05$; 8 weeks LF: 720.900 ± 261.50 pg/mL, n=15 vs. HF-DR: 463.700 ± 157.6000 pg/mL, n=8 vs. HF-DIO: 1303.00 ± 244.100 pg/mL, n=7, $p<0.001$) (**Fig. 4**).

Insulin assay. On a fasted state there was no difference in insulin secretion after 2 weeks on either a LF or HF diet. After 8 weeks on their respective diet, HF-DIO had a significantly higher level of circulating insulin than LF or HF-DR rats (2 weeks LF: 0.9439 ± 0.3247 μg/mL vs. HF: 0.498 ± 0.2072 μg/mL, n=15, $p>0.05$; 8 weeks LF: 1.103 ± 0.5681 μg/mL, n=15 vs. HF-DR: 1.135 ± 0.3631 μg/mL, n=8 vs. HF-DIO: 2.001 ± 0.3725 μg/mL, n=7, $p<0.001$) (**Fig. 5**).

Quantitative Real-time PCR.

There was no significant difference in the expression of any of the gene in any of the group. However we can notice some trends in some gene expression. We did not see any difference in the expression of CCK1-R,Y2-R or FAAH in any of the groups, however if we look at the expression of CB1 we can see a 2-fold increase of the CB1 receptor in HF-DIO compared to LF 8 weeks and a 30% increase compared to HF-DR (**Fig. 6**).

The expression of the Ob-R gene was decreased by 36% in the HF-DIO group compared to the HF-DR group in the nodose ganglia but was 2-fold bigger in the retroperitoneal adipose tissue compared to LF or HF-DR (**Fig. 6 and 7**).

DISCUSSION

The results from the present study demonstrate that rats fed chronically a high fat diet develop different metabolic responses from rats fed a low fat diet and this is associated with changes in the expression of the receptors involved in the short-term regulation of food intake in the nodose ganglia and in the retroperitoneal adipose tissue. These changes are consistent with previous studies showing metabolic profile changes in rats fed a high-fat diet (hyperleptinemia and hyperinsulinemia), but bring new evidence of the adaptation of the gut-brain axis in the expression of the receptors implicated in the short-term regulation of food intake. There is an increase in the expression of the CB1 receptor and a decrease in the expression of the Ob-R receptor which might explain the hyperphagic behavior of rats fed a high fat diet that display the diet-induced obese (HF-DIO) phenotype. We also present in this study evidence of different metabolic and neural changes between rats prone to obesity (HF-DIO) and rats resistant to the effects of an obesigenic diet (HF-DR). These changes occurred with increased adiposity and body weight for the HF-DIO group but not for the HF-DR or LF group. Taken altogether these data suggest that the gut-brain axis, in addition to metabolic changes associated with a chronic ingestion of a high-fat diet, can also be modified by the diet, leading the altered short-term control of food intake towards an orexigenic system.

After 2 weeks on their different diets, rats fed a HF diet had a significant increase in body weigh than rats fed a LF diet. Also within the HF group, two groups of rats can be identified: rats in which body weight was above the median were significantly heavier than rats below the median. From this time point, rats were classified into two

groups: HF-DIO for the rats above the median and HF-DR for rats below the median. After 8 weeks on the diet the difference was even more pronounced between HF-DIO and HF-DR or LF. This change in body weight was associated with an increased adiposity in the HF-DIO. Although there was no significant difference in body weight between HF-DR and LF, rats resistant to the diet had a significant increased adiposity after 8 weeks compared to the LF fed rats. This was already observed in a study looking at body weight, adiposity and meal patterns in rats fed a LF vs. HF diet (Farley, Cook et al. 2003).

The changes in body weight and adiposity were accompanied by different metabolic response between LF, HF-DR and DIO rats. After 2 weeks on their respective diet, HF and LF had the same plasma level of leptin and insulin in the fasted state. After 8 weeks, HF-DIO had a significantly higher level of leptin and insulin than either HF-DR or LF. Although HF-DR rats have an increased adiposity compared to LF, this data suggests that they secrete the same level of insulin and leptin. This suggests that there is a difference at the level of the adipose tissue in secreting leptin. Furthermore, when we correlate the leptin level with the insulin level we obtain positive correlations for all the groups except HF-DR (data not shown). Leptin secretion is insulino-dependent (Cammisotto and Bukowiecki 2002; Lee, Wang et al. 2007), thus a positive correlation between plasma leptin and insulin in all groups is expected. However in the HF-DR, no correlation was found between insulin and leptin level even though HF-DR had an increased adiposity. This indicates that the insulino-dependent leptin secretion is somehow dysfunctional in HF-DR. This metabolic difference between HF-DIO and HF-DR is interesting; both groups are eating the same diet but differ in their response to the diet, with only the HF-DIO becoming

hyperphagic. Although the lower level of leptin associated with a resistance and the higher level associated with obesity is counterintuitive, studies have shown leptin-resistance at the level of the hypothalamus may explain why hyperleptinemic rats become obese. Similarly, we could imagine a non-responsiveness of HF-DR rats to insulin to secrete leptin which somehow would make them leaner, or the difference in adiposity between LF and HF-DR is just not so different that a difference in leptin secretion was not detectable.

Rats fed a LF diet presented a fasted hypertriacylglycerolaemia compare to rats fed a HF diet either resistant or prone to obesity. This change in lipid profile has been described in previous studies (Chong, Fielding et al. 2007), however it is still not clear why de novo lipogenesis is increased in rats fed a low fat diet. It is interesting to note that despite a difference in adiposity, leptin and insulin secretion, the lipid profile of HF-DIO and HF-DR did not differ.

These changes in metabolic profile between LF, HF-DR and HF DIO were also accompanied with changes in expression of the receptors involved in the short-term regulation of food intake. The expression level of CCK-1R (CCK receptor), Y2R (PYY receptor), CB1 (Endonnabinoid receptor), FAAH (Enzyme hydrolyzing endocannabinoids) and ObR (Leptin receptor) in the nodose and in the retroperitoneal white adipose tissue were quantified relatively to the LF group after 2 weeks fed on their diet. We looked at the expression after an overnight fast followed by a gavage of Intralipid (1.0 ml/100g) followed by a 2-hour digestion. We wanted to assess the changes observed in feeding behavior in a previous study (Paulino G. et al., 2007) with changes at the level of the nodose ganglia which plays an essential role in nutrient detection and short-term regulation of food intake. From the previous study,

we have shown that rats fed a HF diet became hyperphagic after chronically ingesting the diet for 8 weeks by prolonging their meal size compared to rats fed a LF diet. Therefore we wanted to determine whether the gut-hormone receptors profile at the level of the nodose ganglia was modified with chronic ingestion of a HF diet. We looked at the receptors that we thought played a major role in the detection of lipids.

It is important to note that this is preliminary data and no significant difference was found between any of the groups for any of the gene because the sample size was small (n=3 for each group and n=1 for HF-DIO and HF-DR) since we had to pool nodose from 5 rats to create one RNA sample. Nonetheless, we can detect some tendencies in the differential gene expression. There was no change observed in CCK1-R, Y2R or FAAH expression between the 5 groups. However, it is interesting to note the expression of FAAH in the nodose ganglia meaning that there must be a regulation of the endocannabinoids at the level of the vagal afferents in conjunction with the CB1 receptor, which could have an important physiological role in the regulation of other hormones or receptors in the GI tract. On the other hand, CB1 and ObR expression seem to be altered by the diet. After 8 weeks, there was a 58% increase in CB1 expression in HF-DIO compared to the LF and a 34% increase compared to the HF-DR. ObR expression was increased by 41% in HF-DR compared to LF but decreased by 10% in HF-DIO. These changes in receptor expression are consistent with the orexigenic drive observed in the feeding behavior. Indeed, the cannabinoid system is associated with an increase in food intake when leptin has anorexigenic properties, therefore an increase in the CB1 receptor in HF-DIO associated with a decrease in ObR (leptin receptor) are not counterintuitive and could explain why rats after ingesting chronically a HF diet become hyperphagic. Indeed there is some evidence that leptin and CCK interact synergistically in suppressing

food intake (Buyse, Ovesjo et al. 2001; Burdyga, Spiller et al. 2002; Peters, Simasko et al. 2006) and that CCK inhibits CB1 expression in the nodose ganglia (Burdyga, Lal et al. 2004). Therefore we can hypothesize that the inhibition of CB1 receptor expression by the synergistic effect of leptin and CCK is reduced because the expression of the leptin-receptor is decreased after a chronic ingestion of a HF diet. The result is a hyperphagic behavior observed in rats fed a HF diet (**Fig. 8**). However, we need to be cautious in interpreting these data until we increase our sample size.

These changes in receptor expression occurred in the nodose ganglia but some other changes took place in the retroperitoneal adipose tissue. The expression of the Ob-R was 133% higher in the HF-DIO compared to LF or HF-DR. This is in contradiction with the literature where rats fed a HF diet presented a decreased in ObR expression and an increase in the mRNA of the postreceptor leptin inhibitor, suppressor of cytokine signaling-3 (SOCS-3) (Wang, Orci et al. 2005). Again this results need to be taken carefully since the sample size is too small making the power of the test low. Therefore, we can not make any conclusion on whether or not there is an effect of the diet on the gene expression at the level of the nodose or the white adipose tissue.

When studying the effect of a hormone on a behavior, it is important to look at its receptor at the same time in the tissue where the hormone has an action. Indeed, there could be an increase of the hormone but the receptor could be decreasing so the conclusions that could be made only from the information on the ligand would be biased. Too many studies look at a hormone level and draw conclusions from there without showing any data on the receptor level. The ratio hormone/receptor seems therefore a better indicator of the physiological effect of the ligand. In addition, when

we study a system as complex as the regulation of food intake, we tend to reduce our problem by either looking at the anorexigenic side of the system (CCK, PYY. GLP-1, Leptin, etc…) or by looking at the orexigenic side of it (Ghrelin, Endocannabinoids, Orexin, etc…). Few studies actually look at both sides at the same time. However it seems important when you are studying food intake to look at the balance of both systems and how they interact in order to define the breakpoint of the decision to eat. By looking at the same time to the ligand/receptor ratio and to the anorexigenic and orexigenic system balance we would get a better understanding of the control of food intake.

In conclusion, this study demonstrates that rats fed a high-fat diet develop a metabolic profile and neural response to a meal that leads to a hyperphagic behavior. This occurs by a global decrease of the responsiveness to anorexigenic signals (decrease in leptin receptor even though insulin and leptin are oversecreted) and by an increase of the orexigenic pathway (increase in CB1). This switch in the short-term regulation of food intake may contribute to the hyperphagic behavior observed in HF-DIO and therefore to obesity.

FIGURES LEGENDS:

Figure 1: Rats fed a high fat-diet and diet-induced obese have a significant body weight increase after 2 weeks compared to rats fed low-fat or diet-induced obese resistant. Data expressed as the percent of body weight as mean ± SEM (n=30 animals for LF and n=15 for HF-DR and HF-DIO, percent of body weight increase compared with body weight at time=0 week). Diet Induced Obese rats have been selected above the median of weight gain when Diet Resistant were selected below the median of weight gain. HF-DIO vs. HF-DR or LF* $p<0.05$.

Figure 2: Rats fed a high-fat diet and diet-induced obese had a significantly higher adiposity index compared to rats fed a low-fat diet or to rats resistant to obesity after 8 weeks. At 2 weeks there was no significant difference in adiposity. Data expressed as the sum of the epididymal, mesenteric and retroperitoneal fat pads divided by the body weight in percentage. Groups with different letters are significantly different $p<0.05$.

Figure 3: Rats fed a low-fat diet had a significantly higher level of triglycerides in the plasma compared to rats fed a high-fat diet either obese prone or resistant. This difference was also true at 2 weeks. Data expressed are mean ± SEM (n=15 for LF 2 weeks, LF 8 weeks and HF 2 weeks, n=8 for HF-DR and n=7 for HF-DIO).

Figure 4: Fasted plasma leptin concentration was significantly higher in HF-DIO rats than in LF or HF-DR rats after 8 weeks. Data expressed are mean ± SEM

(n=15 for LF 2 weeks, LF 8 weeks and HF 2 weeks, n=8 for HF-DR and n=7 for HF-DIO). Groups with different letters are significantly different $p<0.05$.

Figure 5: Fasted plasma insulin concentration was significantly higher in HF-DIO rats than in LF or HF-DR rats. Data expressed are mean ± SEM (n=15 for LF 2 weeks, LF 8 weeks and HF 2 weeks, n=8 for HF-DR and n=7 for HF-DIO). Groups with different letters are significantly different $p<0.05$.

Figure 6: Ob-R expression tended to decrease in HF-DIO compared to LF or HF-DR rats. CB1 expression tended to increase in HF-DIO rats compared to LF or HF-DR rats. FAAH, CCK and Y2R expression did not seem to be modified by chronic ingestion of a high-fat diet. Receptor expressions in the nodose ganglia are relative to rats fed a low fat diet for 2 weeks, 2 hours after a 1.0 mL/100 g gavage of intralipid. Data expressed are mean ± SEM (n=3 for LF 2 weeks, LF 8 weeks and HF 2 weeks, n=1 for HF-DR and n=1 for HF-DIO).

Figure 7: Ob-R expression in the retroperitoneal adipose tissue tended to increase in HF-DIO rats but not in LF or HF-DR rats. Ob-R expression is relative to the low fat diet group fed for 2 weeks, 2 hours after a 1.0 mL/100 g gavage of intralipid. Data expressed are mean ± SEM (n=3 for LF 2 weeks, LF 8 weeks and HF 2 weeks, n=1 for HF-DR and n=1 for HF-DIO).

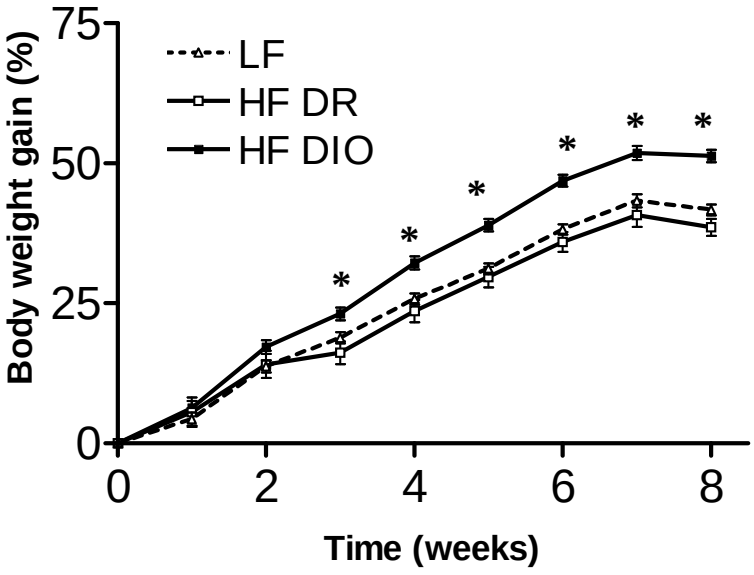
Figure 8: Simplified pathway of the expression modification at the level of the nodose ganglia. Leptin through Ob-R and CCK through CCK1-R act synergistically on the inhibition of CB1 to decrease food intake. After a chronic

ingestion of a high-fat diet in HF-DIO rats, Ob-R expression is decreased which diminishes the synergistic inhibition of CB1 allowing its expression to increase thus increasing food intake.

 Means a decrease in expression of the receptor in HF-DIO rats.

 Means an increase in expression of the receptor in HF-DIO rats.

Figure 1:



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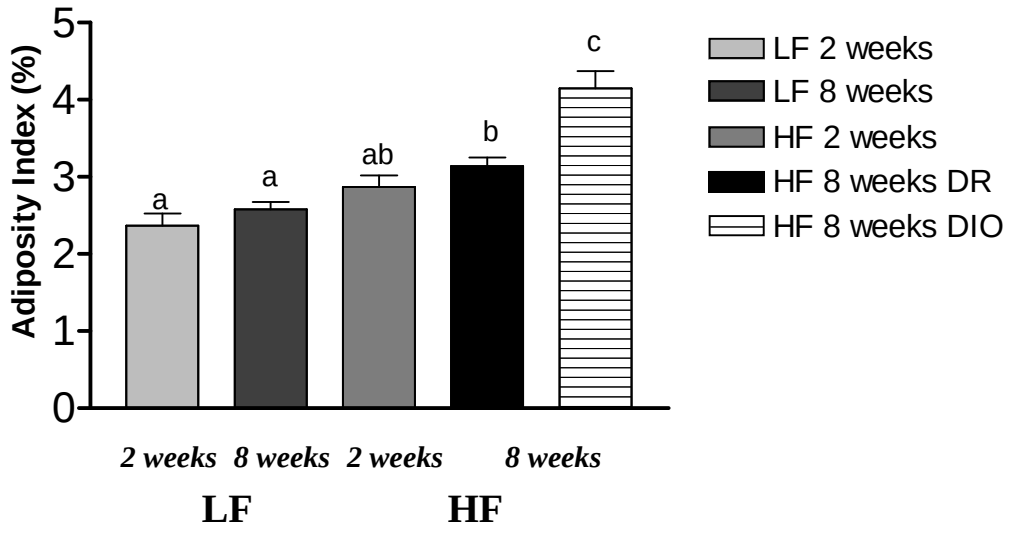

Adiposity Index (%)
5
4
3
2
1
0
a
a
ab
b
c
LF 2 weeks
LF 8 weeks
HF 2 weeks
HF 8 weeks DR
HF 8 weeks DIO
2 weeks
8 weeks
2 weeks
8 weeks
LF
HF

Figure 3 :

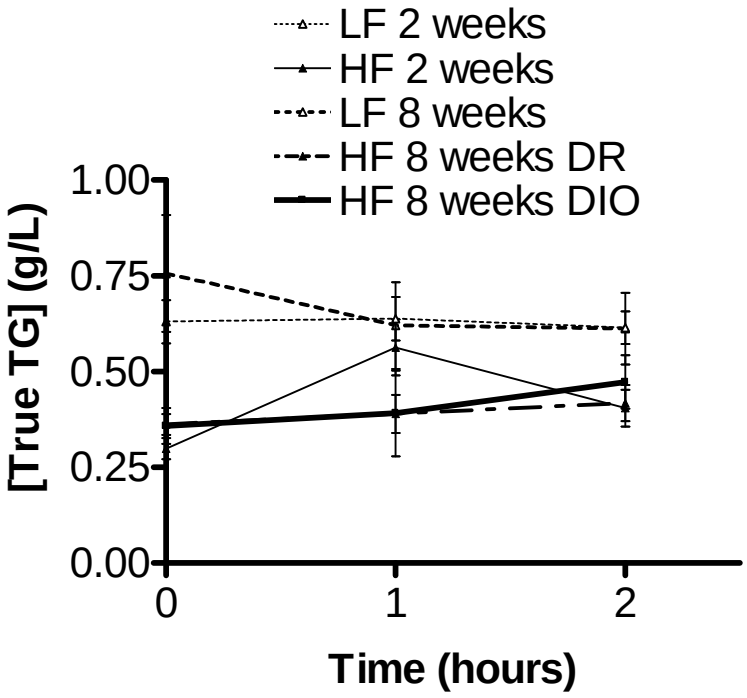

LF 2 weeks
HF 2 weeks
LF 8 weeks
HF 8 weeks DR
HF 8 weeks DIO
[True TG] (g/L)
1.00
0.75
0.50
0.25
0.00
0
1
2
Time (hours)

Figure 4:

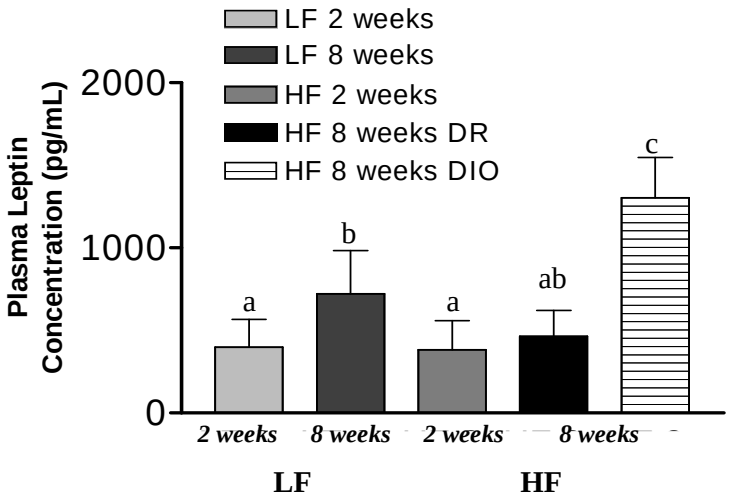

LF 2 weeks
LF 8 weeks
HF 2 weeks
HF 8 weeks DR
HF 8 weeks DIO
Plasma Leptin Concentration (pg/mL)
2000
1000
0
a
b
a
ab
c
2 weeks
8 weeks
2 weeks
8 weeks
LF
HF

Figure 5:

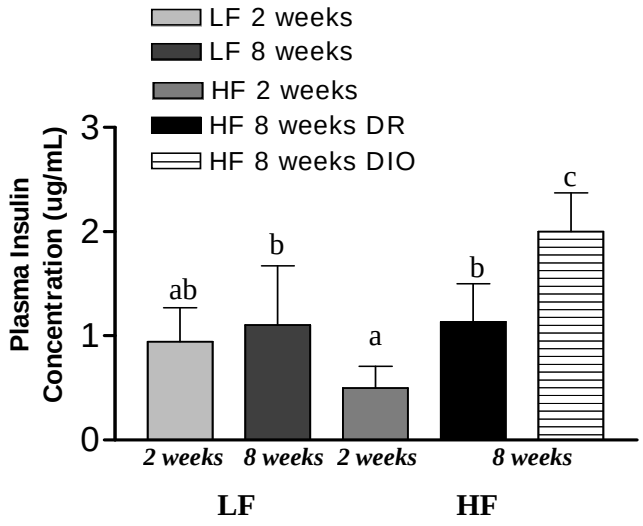

LF 2 weeks
LF 8 weeks
HF 2 weeks
HF 8 weeks DR
HF 8 weeks DIO
Plasma Insulin Concentration (ug/mL)
0
1
2
3
ab
b
a
b
c
2 weeks
8 weeks
2 weeks
8 weeks
LF
HF

Figure 6:

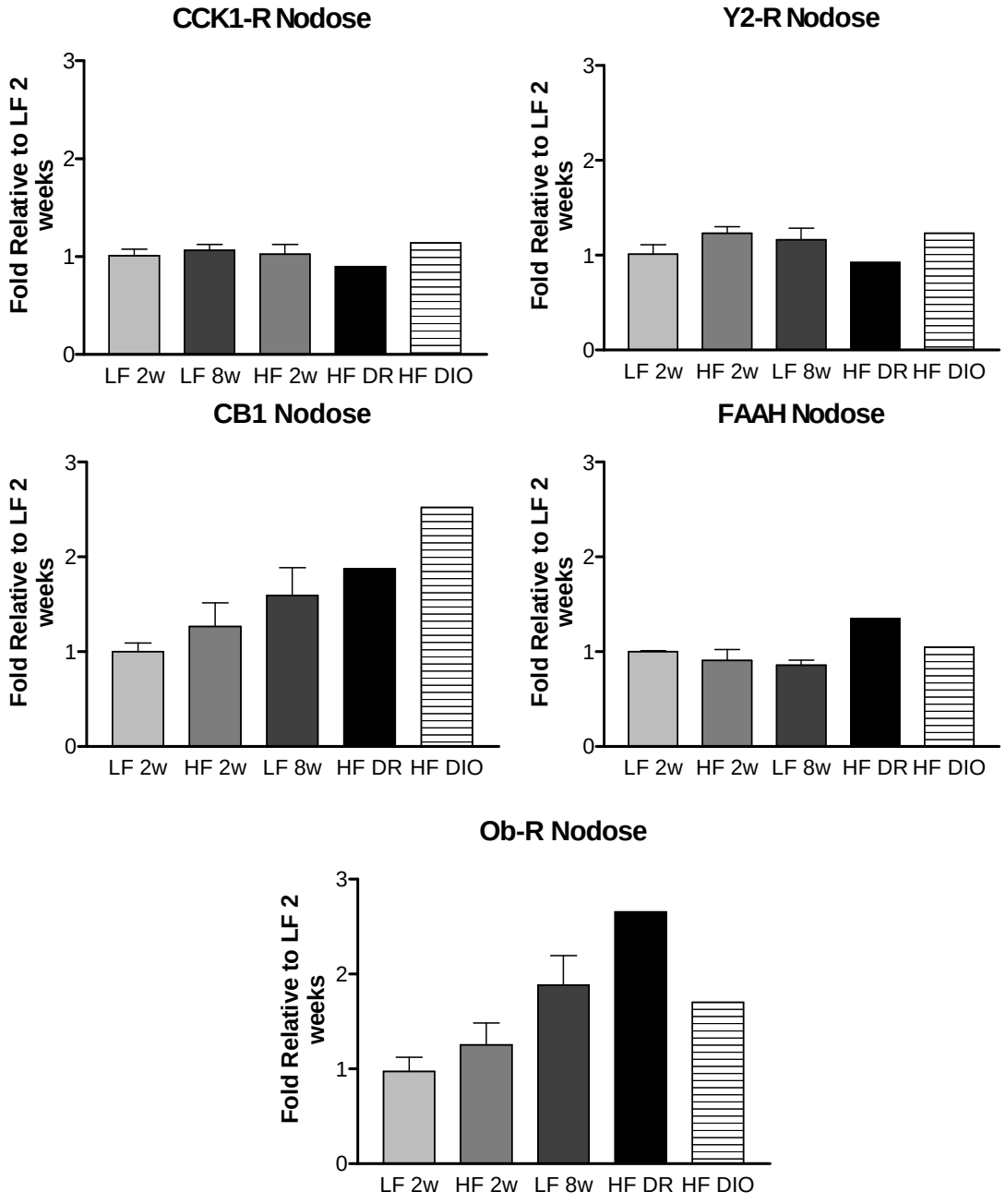

CCK1-R Nodose
Fold Relative to LF 2 weeks
0
1
2
3
LF 2w
LF 8w
HF 2w
HF DR
HF DIO
Y2-R Nodose
Fold Relative to LF 2 weeks
0
1
2
3
LF 2w
HF 2w
LF 8w
HF DR
HF DIO
CB1 Nodose
Fold Relative to LF 2 weeks
0
1
2
3
LF 2w
HF 2w
LF 8w
HF DR
HF DIO
FAAH Nodose
Fold Relative to LF 2 weeks
0
1
2
3
LF 2w
HF 2w
LF 8w
HF DR
HF DIO
Ob-R Nodose
Fold Relative to LF 2 weeks
0
1
2
3
LF 2w
HF 2w
LF 8w
HF DR
HF DIO

Figure 7:

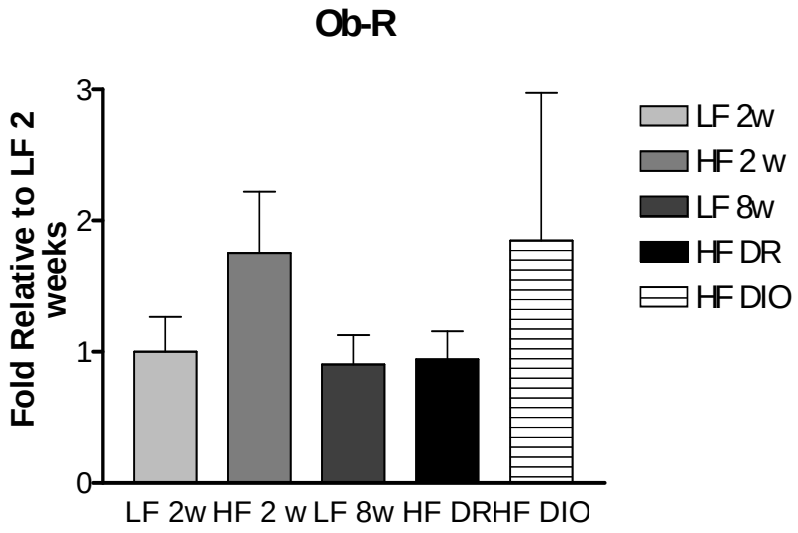
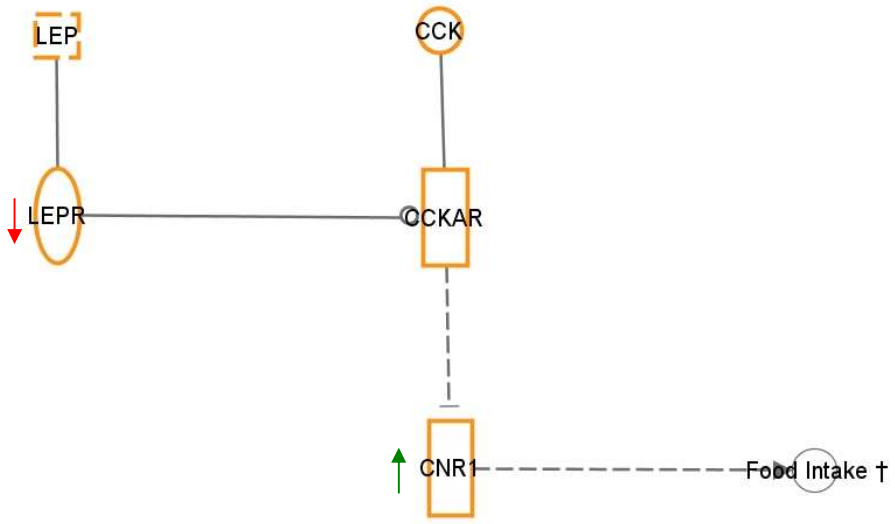

Ob-R
Fold Relative to LF 2 weeks
3
2
1
0
LF 2w
HF 2 w
LF 8w
HF DR
HF DIO
LF 2w HF 2 w LF 8w HF DR HF DIO

Figure 8:



CHAPTER 5

FUTURE EXPERIMENTS

Despite our findings on how dietary fat can modify the basal expression of receptors and hormones involved in the control of food intake, many experiments still need to be conducted in order to have a better understanding of the complex interactions between diet and gene expression. The following is a brief description of experiments that would need to be done in order to have a better picture of what is happening.

Circadian cycle of receptors in the nodose

We are just starting to understand that detection of nutrients is not something static but completely dynamic. The expression of receptors and hormones involved in the detection and therefore in the process of the decision to eat is dependent on the genetic background but also influenced by the dietary history. Since we have shown that expression of receptors was modified at 1 time point (2 hours after gavage of intralipid), it seems important to look at the basal expression of receptors involved in the control of food intake on a normal diet (chow). A first experiment would consist of looking at the expression of the receptors in the nodose in different nutritional status (fed ad libitum, fasted 24h, fasted 12h, fasted and refed, and fasted and refed at different time point after refeeding). Such experiment has already been done but on ghrelin receptor only (Sato, Nakahara et al. 2007). With advancements in techniques such as genomics, transcriptomics, proteomics and metabolomics it would be

interesting to get a picture of what is happening at the level of the interface vagal-afferent/GI tract in terms of gut-hormones/receptors ratio in different nutritional status.

Effect of hormones on receptors expression

Once we get an idea of what is happening on a regular basis we can test the effect of hormones on the expression of different receptors. For instance, we know that CCK decreases the expression of the CB1 receptor (Burdyga, Lal et al. 2004). We also know that leptin enhances the satiating effect of CCK (Peters, Simasko et al. 2006). It would be interesting to describe how any of the different hormones or metabolites we are interested in such as ghrelin, leptin, CCK, PYY and Endocannabinoids have an effect on the different receptors expression or directly on the others gut-hormones and metabolites in the nodose ganglia first, but also in different organs involved in the control of food intake such as the WAT, the hypothalamus or the GI tract.

Effect of nutrients on receptors and hormones expression

Once we described the effect of different hormones on each others and on the expression of their receptors in different organ we can move on to the effect of different nutrients. First, we would study the effect of an acute ingestion of a single macronutrient such as lipid, carbohydrate or protein. Then, we would look at the expression of these receptors and hormones after a chronic ingestion of high-fat diet on different nutritional status (fed ad libitum, fasted 24h, fasted 12h, fasted and refed,

and fasted and refed at different time point after refeeding) in order to compare to the control of a chow diet. We would expect an overall increase in expression of the orexigenic pathways by blunting of anorexigenic ones which could explain the hyperphagia observed in obese people. Also we could test the effect of different type of macronutrient. Does saturated fat induce the same response and adaptation than unsaturated fat?

Adaptation at the cellular level

After describing *in vivo* how gut-hormones and receptors expression are modified by diet. We need to understand the cellular pathways behind this adaptation occurring in the nodose ganglia. In order to answer that question we need to use a cellular model. We could do a cell culture of neurons from the nodose ganglia and expose these neurons to a chronic flow of cholecystokinin (which would imitate a chronic ingestion of dietary fat). We would then look at the cascade of activation within neurons and try to find if there is a decrease in the intracellular signaling by showing a decrease in phosphorylated proteins (Westerns on p38MAPK, PIK3K or PKC) or decrease in depolarization (Calcium assays before and after chronic exposition to CCK). We could look at changes in other cell cultures such as enteroendocrine cell (STC1 cell or BON cells) or adipocytes. We could also look at cross-talk between cells in co-cultures of neurons/adipocytes or adipocytes/enteroendocrine cells or neurons/enteroendocrine cells.

Many experiments could be done to describe the changes occurring in adaptation to high-fat diet. The broader the model is, the better our understanding will be of the changes occurring in the decision to eat in obesity.

Adaptation of the microflora

With progress in microbiological ecology techniques and better understanding of the gut microflora, it really seems important to look at the changes within the gut in terms of changes in microbiological population but also in terms of interactions with the epithelium cells. The composition of our microflora has evolved with our genome from the beginning. If you think at the evolutionary level, there must be an explanation why this specific population has been selected compared to other ones. The challenge is to understand how diet can influence this ecosystem and how the changes in ecosystem interact with our body in order to modify our response to this diet. Food intake is no longer an interaction of our diet and our genome but an even more complex interaction between our diet, our genome and our microbiome.

Adaptation to a high-protein diet

Finally we have been interested in the adaptation to a high-fat diet because it seems to be a good model for obesity but it would be interesting to see how the control of food intake is modified by a high-protein diet since some studies have described an increase in the sensitivity of the vagus-nerve by chronic ingestion of a high-protein diet (Darcel, Fromentin et al. 2005). The experiments described above

can be adapted to a high-protein diet but with some focus on gut-hormones and receptors involved in protein detection (for example Pept1).

CONCLUSION

"Yes, I am a dreamer. For a dreamer is one who can find his way by moonlight, and see the dawn before the rest of the world."

(Oscar Wilde)

Food intake, Energy Homeostasis and Body Weight Regulation form a complex network that is tightly regulated yet delicate. It involves myriad of hormones, receptors and metabolites, which, depending on their relative abundance, will have a different downstream effect on appetite. At the end it always comes back to the simple binary algorithm: to eat or not eat?

We have seen through this work that first, feeding behavior can be modified by the diet. More specifically, we have shown that a high-fat diet can lead to hyperphagia and obesity in the rodent model. Additionally, we have incriminated the role of the CCK1-R receptor as a key player in the control of fat intake. Finally, we proved that receptor expression in the nodose ganglia, a critical center regulating short-term of food intake, could be modified by chronic exposure to a high-fat diet. We have proven that short-term regulation of food intake is not a static system but a dynamic one that integrates dietary history and energy storage.

It is necessary to execute a more systemic study looking at short-term regulation of food intake. This will lead us to a better and more accurate model to

understand what is behind the decision to eat in terms of gut-hormones, receptors, metabolites and neural networks. With progress in integrative techniques such as genomics, transcriptomics, proteomics, lipidomics and glycomics, and the aid of statistical and bioinformatic tools such as the Bayesian network, it is now possible to describe an organism as a complex matrix containing information about a particular tissue during a specific nutritional state with a unique dietary history. The study of nutrigenomics and the integration of the microbiome in this model seem to be the next step in order to gain a better understanding of how an organism is able to react to its environment. Integration of this information in different nutritional states with different dietary histories will lead us to a more complex yet accurate model of appetite regulation. The question: “to eat or not to eat?” can only be answered upon resolution of the integrative model of the organism.

Are we on the brink of a revolution by the way we are doing biology? Will we be able to unify a model as complex as Diet*Gene*Microflora interactions to explain Phenotype? Will we be able to assess and prescribe personalized nutrition and medicine to individuals, knowing their specific “superorganism” genome?

These are the biological challenges that we will be facing in the twenty-first century.

§

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ABSTRACT :

The global population is getting obese. From developed to developing countries the pandemic is now irrefutable. Research focusing on obesity has increased exponentially over the past few decades but yet no solution has been found. Dietary fat has been blamed for this epidemic because people adapted to a high-fat diet develop hyperphagic behavior and therefore become obese. The aim of this work was to understand the mechanisms by which a high-fat diet can induce hyperphagia starting from behavorial studies to the molecular biology behind it. These studies have been conducted in two animal models: *Rattus norvegicus* and *Mus musculus*. The results of these studies have shown that rats subjected to a chronic high-fat diet become hyperphagic upon vagal insensitivity to dietary fat compared to rats fed a low-fat diet. Also, we demonstrated that the cholecystokinin receptor 1 plays an important role in the detection of dietary fat. Finally, we proposed a molecular model of the adaptation of the nodose ganglia by which a decreased expression of the leptin receptor (anorexigenic) was associated with an increased expression of the cannabinoid receptor (orexigenic). This suggests one of the many mechanisms underlying hyperphagic behavior in rats fed a chronic high-fat diet. In conclusion, we have shown that diet is able to interact with genes involved in short-term regulation of food intake. These findings are critical in understanding the potential causes of obesity. The human genome has evolved from the direct interaction between environment and diet; it is not counterintuitive to think that diet can influence gene expression. Why does a high-fat diet induce a hyperphagic response in the organism? Can we find answers by looking back in time and observe how people, diet and environment evolved together? Does the thrifty gene theory make sense in this context? These are questions that need to be answered in order to find a solution to obesity.

KEYWORD: *Dietary lipid, adaptation, detection, nodose ganglia, cannabinoids, leptin, hyperphagia, obesity.*

RESUME:

La population mondiale devient obèse. Des pays développés aux pays en voie de développement, la pandémie est maintenant irréfutable. La recherche sur l'obésité a exponentiellement explosé et pourtant aucune solution n'a encore été trouvée. Les lipides alimentaires ont très vite été montrés du doigt et rendus responsables de cette épidémie car les personnes adaptées à un régime hyper-gras développent un comportement alimentaire hyperphagique et de ce fait deviennent obèses. Le but de ce travail était de comprendre quels sont les mécanismes par lesquels un régime hyper-gras pouvait induire un comportement hyperphagique en commençant à l'aide d'études du comportement alimentaire jusqu'aux processus cellulaires impliqués. Nous avons pour ceux-ci utiliser deux modèles animaux: *Rattus norvegicus et Mus musculus*. Les résultats de cette étude ont montré que des rats exposés de manière chronique à un régime hyper-gras développent un comportement alimentaire hyperphagique parce que leur sensitivité vagale aux lipides alimentaires est diminuée. De plus, nous avons démontrés que le récepteur à la cholecystokinin 1 était important dans la détection des lipides alimentaires. Finalement, nous avons proposé un modèle moléculaire de l'adaptation du ganglion nodal dans lequel l'expression du récepteur à la leptin était diminuée (aux propriétés anorexigéniques) et celle du récepteur aux endocannabinoides était augmentée (aux propriétés orexigeniques). Ceci suggère un des nombreux mécanismes pouvant expliquer le comportement hyperphagique observé chez les rats nourris un régime hyper-gras de manière chronique. En conclusion, nous avons montré qu'il existe une interaction entre gènes impliqués dans le contrôle de la prise alimentaire et le régime. L'étude de cette interaction est donc importante dans la compréhension des causes de l'obésité. Sachant que les hommes ont évolué en interaction directe avec leur environnement et leur régime, il semble intuitif de penser que le régime ait un effet sur l'expression des gènes. La question à se poser maintenant est pourquoi un organisme deviendrait hyperphagique sous un régime hyper-gras ? Peut-on trouver des réponses en observant et étudiant les coutumes alimentaires ancestrales au temps où l'homme, la nature et le régime évoluait en même temps et à la même vitesse ? La théorie du « gène économe » prend-elle sens dans ce contexte ? Voilà les questions auxquelles nous devons répondre afin de comprendre la cause même de l'obésité et afin de lutter efficacement contre celle-ci.

MOTS-CLES : *Lipides alimentaires, adaptation, détection, ganglion nodale, cannabinoides, leptin, hyperphagie, obésité.*