Brain neuropeptide Y and CCK and peripheral adipokine receptors: temporal response in obesity induced by palatable diet

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Running head: NPY, CCK and adipokines in dietary obesity

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Abstract

Objective: Palatable food disrupts normal appetite regulation, which may contribute to the etiology of obesity. Neuropeptide Y (NPY) and cholecystokinin play critical roles in the regulation of food intake and energy homeostasis, while adiponectin and carnitine palmitoyl-transferase (CPT) are important for insulin sensitivity and fatty acid oxidation. This study examined the impact of short and long term consumption of palatable-high-fat-diet (HFD) on these critical metabolic regulators.

Methods: Male C57BL/6 mice were exposed to laboratory chow (12% fat), or cafeteria-style palatable-HFD (32% fat) for 2 or 10 weeks. Body weight and food intake were monitored throughout. Plasma leptin, hypothalamic NPY and cholecystokinin, and mRNA expression of leptin, adiponectin, their receptors, and CPT-1, in fat and muscles were measured.

Results: Caloric intake of the palatable-HFD group was 2-3 times greater than control, resulting in a 37% higher body weight. Fat mass was already increased at 2 weeks; plasma leptin concentrations were 2.4 and 9 times higher than control at 2 and 10 weeks respectively. Plasma adiponectin was increased at 10 weeks. Muscle adiponectin receptor1 was increased at 2 weeks, while CPT-1 mRNA was markedly upregulated by HFD at both time points. Hypothalamic NPY and cholecystokinin content were significantly decreased at 10 weeks.

Conclusion: Palatable-HFD induced hyperphagia, fat accumulation, increased adiponectin, leptin, and muscle fatty acid oxidation, and reduced hypothalamic NPY and cholecystokinin. Our data suggest that the adaptive changes in hypothalamic NPY and muscle fatty acid oxidation are insufficient to reverse the progress of obesity and metabolic consequences induced by a palatable-HFD.

Key words: hyperphagia, adiponectin, adiponectin receptor 1, CPT-1, leptin receptor

Introduction

The increasing prevalence of overweight and obesity has become a crucial worldwide health problem. The epidemic of obesity on an unchanging genetic background suggests powerful environmental forces can affect energy balance. Throughout evolution our concern was with food shortages, and our genetic makeup reflects adaptive responses set in train to ensure survival [1]. Starvation and famine exerted a strong selection effect on the human genome, whereby adaptations that selected to promote fat deposition in times of plenty had a survival advantage in periods of food shortage and famine, a concept that has been coined the "thrifty-genotype" [1]. One of the major public health concerns in this century is the medical consequences stemming from the ready availability of high-fat, energy-dense foods, as this is one of the fundamental causes of the obesity epidemic [2]. Physiological experiments illustrate that energy-dense diets can potentially compromise normal appetite regulation in humans [3]. This study aimed to specifically explore the impact of palatable foods on appetite and metabolic regulation.

There has been a substantial increase in knowledge of the neural regulatory systems involved in regulating appetite and energy balance, specifically in the hypothalamus that responds to nutritional signals and plasma hormones, such as leptin and insulin, enabling accurate regulation of food intake and body weight [4]. The roles of orexigenic neuropeptides, including neuropeptide Y (NPY) and agouti-related protein, and anorexigenic peptides, including melanocyte-stimulating hormones and cocaine- and amphetamine-regulated transcript, have been widely studied in dietary obese animal models. NPY, a 36 amino acid member of the pancreatic polypeptide family, is of interest due to its powerful orexigenic effect [5], and critical role in periodic eating behavior and maintenance of body weight [6]. Central administration of NPY was shown to induce hyperphagia even under conditions of satiation,

to increase fat deposition, to decrease energy expenditure, and to promote obesity [7, 8]. However, in addition to these CNS neuropeptides, other peptides may also play a role. Cholecystokinin (CCK), which induces satiety by both peripheral and central means, is secreted from the duodenal mucosa when ingested fat reaches the duodenum (reviewed in [9]). CCK is also found in high concentrations in the cortex and hypothalamus [10]. Administration of CCK into the hypothalamus and hindbrain can cause direct anorectic effects [10]. Although fat is the most potent stimulus for CCK secretion, CCK-induced reduction in food intake and gastric emptying are both attenuated in rats maintained on a high-fat diet (HFD) [11]. The hyperphagic phenotype displayed in CCK2 receptor knockout mice suggests that lack of CCK signaling can lead to over consumption [12]. However, no study has investigated the involvement of endogenous central CCK in appetite regulation.

The discovery of proteins produced in the adipose tissue and skeletal muscles that are involved in feeding, metabolism, and insulin sensitivity, such as leptin, adiponectin and carnitine palmitoyl-transferase (CPT)-1, indicate additional peripheral regulatory systems. Circulating leptin, as well as insulin, directly access the arcuate nucleus (Arc) in the hypothalamus to inhibit neurons expressing orexigenic peptides and activate neurons expressing anorexigenic peptides [4]. Diabetes, obesity, and the metabolic syndrome susceptibility locus have been mapped to chromosome 3q27. This region encodes adiponectin, a 30KDa protein secreted exclusively by white adipose tissue (WAT), that is capable of increasing insulin sensitivity, stimulating fatty acid oxidation, and inhibiting the vascular inflammatory process [13, 14]. Skeletal muscle plays an important role in the clearance of blood free fatty acids and triglyceride, and in whole-body fatty acid oxidation, and CPT is the rate-limiting step for fatty acid oxidation and ketogenesis [15]. Dysregulation of these cytokines can accelerate the process of fat accumulation and glucose intolerance [16-19].

Consumption of palatable food disrupts normal appetite regulation. Over-eating is stimulated by the ready availability of food rich in fat and sugar [20]. Thus the aim of this study is to look at appetite regulation under palatable HFD and the contribution of central and peripheral metabolic mediators. The hypothesis is that animals will display marked over-consumption in the face of a palatable HFD, and this will be associated with a decrease in the putative satiety factors and an increase in the appetite stimulant NPY. We also hypothesized that uncontrolled fat accumulation in dietary obesity is related to dysregulation of peripheral leptin, adiponectin, their receptors, and CPT-1. As adaptive changes in response to palatable HFD are developed over time [21, 22], we aimed to examine both short term and long term effects.

Materials and methods

1. Dietary intervention

Animals were housed at 20 ± 2°C in micro-isolator cages with 8 in each, and maintained on a 12:12 h light/dark cycle (lights on at 06:00h). Male C57BL/6 mice (aged 5 weeks, n = 60) were assigned to four groups of equal average body weight. The control groups were exposed to standard laboratory chow (GlenForrest stockfeeders, Perth, Australia) which provided 3.54 kcal/g of diet (12% energy as fat), while the palatable HFD fed groups (HFD group) were presented with HFD providing 4.23 kcal/g of diet (32% energy as fat), for a period of 2 or 10 weeks. Briefly, the HFD consists of modified laboratory chow made on-site by combining, as a percent of total weight, 60% powdered chow, 33% sweetened condensed milk, and 7% saturated animal fat. The high-fat modified chow was supplemented with highly palatable cafeteria style food such as cakes and biscuits (4-5 different food types per day) of known caloric content. Body weight was measured between 16:00 and 17:00 h twice a week. Fresh food was provided daily. For the measurement of caloric intake, preweighed food was put

inside a container into a clean cage and the remainder collected 24 hours later. The current study was approved by the Animal Experimentation Ethics Committee of the University of Melbourne.

2. Sample collection

Animals were given *ad libitum* access to food before dissection. At the end of the experiment, mice were deeply anesthetized (ketamine/xylazine 180/32 mg/kg, intraperitoneal). After measurement of naso-anal (N-A) length and girth (waist and groin circumferences), blood was collected by cardiac puncture and separated plasma was stored at -80°C for hormone measurements. Mice were killed by decapitation and the brain was rapidly dissected on ice into regions containing paraventricular nucleus (PVN), Arc, anterior and posterior hypothalamus (AH and PH), and medulla. Brain regions were weighed, frozen on dry ice, and stored at -80°C for determination of NPY and CCK peptide content. Body fat (testicular WAT, retroperitoneal (Rp) WAT, leg subcutaneous WAT, visceral WAT, and brown adipose tissue (BAT)) was collected as well as various organs (heart and liver) and skeletal muscles (mixture of adductor magnus, gracillis posticus, semimembranosus) to provide further markers of growth and metabolism. Tibia length was measured.

3. Assays

3.1 Brain NPY, plasma NPY, leptin, and adiponectin assays

Endogenous NPY from the various brain regions was extracted by 0.5M acetic acid, lyophilized and reconstituted with assay buffer. NPY-like immunoreactivity was measured by a specific radioimmunoassay using synthetic NPY as standard (10-1280 pg/tube, Auspep, Australia) as described before [23]. NPY in each brain region was calculated as pmol NPY per mg tissue. Plasma leptin and adiponectin concentrations were measured using commercially

available radioimmunoassay kits (Linco, Missouri, USA), according to the manufacturers' instructions.

3.2 Brain CCK assay

CCK-like immunoreactivity in the various brain regions was measured by a specific radioimmunoassay using synthetic sulphated CCK-8 as standard (0.2-200 fmol/tube) [24]. Briefly, acid extracts from the 10 week diet cohort were dehydrated and reconstituted in water. Samples were incubated with antiserum 92128 (1:60,000, a generous donation by Jens Rehfeld, University Hospital, Copenhagen, Denmark) and ^{125}I-Bolton-Hunter-CCK-8 (Amersham International, Bucks, UK). CCK in each brain region was calculated as fmol CCK per mg tissue.

4. Real-time PCR

Total RNA was isolated from 10 mg of both skeletal muscle and WAT using ToTALLY RNA™ kit (Ambion Inc., Austin, USA) protocol and reagents. Total RNA concentration was determined spectrophotometrically at 260 and 280 nm. RNA was reverse transcribed to synthesize first strand cDNA using AMV reverse transcriptase (Promega, Madison, WI). The cDNA was stored at -20°C for subsequent analysis [25]. Primers (Table 1) were purchased from GeneWorks (Adelaide, Australia). Real-time PCR was performed using the GeneAmp® 5700 Sequence Detection System (Applied Biosystems). The expression of the gene of interest (adiponectin, adiponectin receptor (AdipoR)1, leptin, leptin receptor, CPT-1 was standardized to rat cyclophilin.

5. Statistical analyses

Results are expressed as mean ± S.E.M. Body weight of HFD and chow fed mice, and 24h

food intake, were analyzed using ANOVA with repeated measures, followed by a post hoc LSD test. Differences in fat and organ masses, plasma hormone concentrations, brain NPY and CCK concentrations and content, and mRNA expression were analyzed using *Student's* unpaired *t*-test.

Results

Effects of palatable HFD on energy intake and body weight

Prior to the introduction of the palatable HFD, no difference in energy intake or body weight was observed between the two groups. Caloric intake was almost doubled during the first 24h of HFD feeding, and this was progressively increased to nearly 3 times within the first 5 days of dietary intervention ($p < 0.05$ for all time points, Fig 1A). Thereafter, the hyperphagic state of the HFD group was maintained throughout the experimental period ($p < 0.05$ for all time points, Fig 1A).

The average body weight of the HFD group began to increase more rapidly than chow fed animals shortly after the diet began, and was significantly higher than the chow fed group after 3 days of diet (Fig 1B, $p < 0.05$). Over time, this difference became more pronounced (Fig 1B). After 2 weeks of HFD, the average weight gain was 40% greater than the control mice (ΔBW 7.7 ± 0.3 g in the HFD group vs. 5.4 ± 0.3 g in the control group, $p < 0.05$). After 10 weeks of diet, the average weight gain of the HFD group (ΔBW 22.3 ± 0.5 g) was 86% more than the control group (ΔBW 12.0 ± 0.5 g, $p < 0.05$).

Effects of palatable HFD on body size, organ and fat masses

Both N-A length and girth (waist and groin circumferences) were significantly increased by HFD at 10 weeks, while tibia length was not changed (Table 2). Liver, heart, and BAT weights were only significantly increased at 10 weeks (Table 2). After 2 weeks of dietary intervention, in addition to testicular and leg subcutaneous WAT, RpWAT mass was also markedly increased by nearly 80% when data were considered as net mass, or more than 60% when standardized as a percentage of body weight. The sum of WAT masses sampled (Sum) of the HFD fed mice was increased by 53% ($p < 0.001$). After the longer period of diet

intervention, the increase in both BAT and WAT mass of all locations sampled was more pronounced, with total WAT mass sampled more than four times greater than control mice ($p < 0.001$, Table 2).

Effects of palatable HFD on plasma hormones

After 2 weeks of HFD, plasma leptin concentration was more than doubled in the mice fed a HFD compared to the mice on the control diet ($p < 0.05$); furthermore, with an additional 8 weeks of HFD, plasma leptin concentration was 9 times greater than in control mice ($p < 0.05$, Table 3). Plasma leptin concentration was positively correlated with RpWAT mass at both 2 weeks ($p < 0.0001$, $r = 0.77$, $n = 30$), and 10 weeks ($p < 0.0001$, $r = 0.96$, $n = 30$). Plasma NPY concentration was increased by HFD, with a more marked effect at 10 weeks (50% increase) than 2 weeks (25% increase) ($p < 0.05$, at both time points, Table 3). After two week diet, no difference in plasma adiponectin levels was observed between chow and HFD fed mice (Table 3). However at 10 weeks, plasma adiponectin concentration was increased by 24% in the HFD fed mice compared with the control animals ($p < 0.05$, Table 3).

Effects of palatable HFD on brain NPY and CCK peptides

No difference in NPY concentration in hypothalamic subregions was observed at 2 weeks (data not shown), and the total hypothalamic NPY content was also similar in both groups (14.9 ± 0.3 vs. 15.2 ± 0.4 pmol in HFD and control groups, respectively). However, in the longer term, a significant reduction in NPY concentrations in the AH, Arc and PH was observed in HFD fed animals ($p < 0.05$, Fig 2). Total hypothalamic NPY content was significantly reduced to 10.3 ± 0.5 pmol compared with 13.8 ± 0.6 pmol in control mice ($p < 0.05$).

At 10 weeks, CCK concentrations in the AH, Arc, PH and medulla were significantly decreased by 16%, 15%, 22% and 15% respectively in the HFD group ($p < 0.05$, Fig 3). No obvious change was observed in the PVN between the two dietary groups. The total hypothalamic CCK content was also lower in the animals fed a HFD (347.5 ± 18.3 fmol) compared with control animals (446.4 ± 25.7 fmol, $p < 0.05$).

Effects of palatable HFD on gene expression in skeletal muscle and WAT

In skeletal muscle, AdipoR1 expression was only upregulated after 2 weeks of diet ($p < 0.05$, Fig 4A). The mRNA expression of the leptin receptor was significantly reduced in the HFD group at 10 weeks ($p < 0.05$, Fig 4B). CPT-1 mRNA expression was significantly increased at both 2 and 10 weeks ($p < 0.05$, Fig 4C).

In WAT, no obvious changes in adiponectin mRNA expression were observed at 2 and 10 weeks (Fig 5A). WAT leptin mRNA expression was increased by 8 fold after 10 weeks of HFD ($p < 0.05$, Fig 6B), while its receptor expression in WAT was reduced by 40% and 71% at 2 and 10 weeks respectively by HFD ($p < 0.05$, Fig 5C). There was a negative correlation between the expression of leptin and leptinR mRNA in WAT at 10 weeks ($P < 0.05$, $r = 0.50$, $n = 24$). WAT CPT-1 mRNA expression was only significantly increased at 2 weeks ($p < 0.05$), and tended to decrease at 10 weeks ($P = 0.09$, Fig 5D). There was no significant change in mRNA expression of the house keeping gene cyclophilin in skeletal muscle and WAT at either 2 or 10 weeks.

Discussion

In this study, obesity was induced in mice by using a palatable cafeteria-style HFD, which was accompanied with markedly increased fat accumulation. An important observation in the current study is that the counter-regulatory mechanisms apparently set in train by sustained overfeeding, increased leptin and reduced hypothalamic NPY, failed to impact on appetite. The reduced endogenous CCK observed in the hypothalamus may have contributed to the hyperphagia. Mice exposed to our palatable diet appeared to gain more weight than animals of the same age and strain given non-cafeteria diet with even higher fat composition (59-60% from fat [26-29]), suggesting a greater response to the palatable HFD, possibly related to hedonic-type eating.

The energy dense diet used here was of a different nature from pelleted HFDs. Exposure to this diet caused an immediate and sustained increase in caloric intake, and a significant increase in body weight within 3 days of diet inception. The weight gain appeared to accelerate after 6 weeks, possibly due to decreased energy expenditure relative to increased energy intake as shown by Ghibaudi and colleagues [30]. Overconsumption of palatable food gradually shifts the set point for energy balance and body weight, similar to the adaptation observed in drug addiction [31, 32]. This change in energy balance set point may explain the prolonged hyperphagia. The increase in percentage WAT mass induced by HFD suggests adipose accumulation largely contributed to the weight gain. In addition, we postulated that the increased liver weight might be mainly due to lipid deposit, as liver is the systemic buffer, largely increasing its lipid content to help avoid an excessive increase in circulating lipids.

Feeding is not only controlled by homeostatic mechanisms which theoretically would allow an individual to maintain an ideal body weight but also by a brain reward system [33]. Palatable

food activates the reward system, to reinforce motives without homeostatic value [20].

A number of Arc NPY neurons express the "long" isoform of the leptin receptor, and these cells respond to alterations in circulating leptin levels, thus regulating the synthesis and release of NPY [34-36]. Although leptin resistance was reported in obesity [37], reduced Arc NPY content in our mice at 10 weeks may represent a possible response to the hyperleptinemia. The decreased NPY concentrations in the AH and PH may reflect a decrease in NPY release from the Arc. This is supported by our previous study, in which baseline NPY release was reduced by long term exposure to palatable HFD in rats [38]. Although NPY was not changed in the PVN, changes in the AH and the PH would still affect feeding behavior.

In contrast to the reduction in Arc NPY peptides observed here in the face of chronic obesity, increased Arc NPY mRNA was observed in dietary obese prone rats [39], and the activity of hypothalamic neurons expressing NPY is greatly elevated in *ob/ob* mice that are morbidly obese because of their inability to produce leptin [35, 40]. Fasting or chronic food restriction increases NPY mRNA expression, and refeeding restores it. Both increases and decreases in brain NPY have been observed in previous short and long term studies using pelleted HFD [27, 41-43]. Similarly the contribution of NPY to palatable diet induced obesity remains unclear. We previously observed a gradual downregulation of hypothalamic NPY over the development of dietary obesity in Sprague Dawley rats using the same diet [21, 38], a similar pattern observed in the present study with C57BL/6 mice. Interestingly, changes in weight gain, caloric intake, and hypothalamic NPY peptide were greater in mice than in rats [21, 38].

The previously reported transient increase in hypothalamic NPY expression in pre-obese Otsuka Long Evans Tokushima Fatty rat suggests that there may be a threshold for NPY to

initiate hyperphagia, but NPY is not necessary to maintain the hyperphagic state [44]. One interpretation of our result is that the positive energy balance and satiety due to HFD, reduced NPY synthesis in the Arc and therefore whole hypothalamic NPY content. Perhaps this suggests an attempt to restrain overconsumption of palatable food. The altered NPY production may modulate NPY receptor expression, as increased hypothalamic Y_2 and Y_5 receptor expression was observed in dietary obese rodents [43, 45]. In keeping with this, we have previously found exaggerated responses to exogenous NPY administration in dietary induced obese rats [38]. Furthermore, previously we observed a reduced PVN alpha-melanocyte-stimulating hormone (α-MSH), together with reduced NPY peptide, after chronic high fat feeding in the rat [21] which may explain the prolonged hyperphagia. Chronic hyperphagia and obesity may alter the balance of hypothalamic peptidergic systems controlling feeding.

The current study demonstrated for the first time that long term palatable HFD downregulates central CCK content. CCK, a satiety signal released from the intestine after ingestion of nutrients, leads to meal termination [9, 46]. However locally generated hypothalamic CCK may also have a role, since direct injection of CCK into this area can alter food intake [10]. Although reduced CCK in the cortex and increased hypothalamic CCK receptor and binding were found during fasting [47], the changes induced by prolonged hyperphagia have not been previously studied. Decreased brain CCK was found in the cerebral cortex but not in the hypothalamus of *ob/ob* mice [48, 49]. Our data suggest that the inhibition imposed by CCK on the hypothalamic feeding circuitry might be attenuated, and this may contribute to the sustained hyperphagia; however confirmation of this proposal would require further work. Our findings taken together with the observation that high-fat diets attenuate the inhibitory effect of exogenous CCK [11] suggest a loss of both peripheral and central satiety effects of CCK.

In the periphery, NPY is released from the sympathetic nerves and the adrenal medulla, by intense or prolonged sympathetic activation [50-53]. The persistent increase in plasma NPY levels in HFD fed mice reported in this study may represent hyperactivity of the sympathetic nervous system. There is also evidence of increased sympathetic activity in obese humans [54-56]. Since NPY has potential angiogenic functions and stimulates cardiac hypertrophy [51], the increase in circulating NPY if sustained, may lead to changes in blood pressure or vascular function.

It has been recognized that decreased plasma adiponectin concentrations is linked to both animal and human obesity, which is thought to largely contribute to the insulin resistance, vascular dysfunction, and atherosclerosis that often accompany obesity [13]. Interestingly, in the current study plasma adiponectin level was significantly increased at 10 weeks by HFD without an obvious change in adipose adiponectin mRNA expression. While most reports suggested adiponectin concentration was reduced in human obesity, increased adiponectin has been observed in morbidly obese individuals, and attributed to disturbed endocrine loops [57]. In addition, postprandial hypertriglyceridemia and hyperglycemia may mediate the increased adiponectin levels as shown in obese but not lean subjects [58]. The mice in the current study were not fasted before harvest, which could explain increased adiponectin levels at 10 weeks. Increased plasma adiponectin has also been observed in Sprague Dawley rats after 12 weeks of HFD in our laboratory [59].

In skeletal muscle, adiponectin plays a key role in insulin sensitivity, glucose utilization, and fatty acid oxidation [60-62]. The action of adiponectin on skeletal muscle was found to be blunted in obesity, which may contribute to reduced adiponectin sensitivity and eventually

insulin resistance [62, 63]. Two adiponectin receptors, AdipoR1 and AdipoR2, have been identified recently [64]. Fasting increased the expression of muscle AdipoR1 to increase fatty acid oxidation, which was restored to baseline after refeeding [65]. In the current study, an early increase in AdipoR1 might be a compensatory reaction to increase glucose uptake and lipid oxidation in the skeletal muscle.

Skeletal muscle is an important contributor to whole-body energy expenditure, and has a key role in determining systemic insulin sensitivity due to major proportion of glucose utilization, including redirecting spared glucose towards *de novo* lipogenesis in adipose tissue, occurring in skeletal muscle under insulin-stimulated conditions [66]. Any reduction of metabolic rate in muscle would reduce glucose utilization, thereby leading to hyperinsulinaemia. The reduced leptinR mRNA expression observed in both skeletal muscle and WAT is likely a physiological response to the increased leptin production. Leptin may increase glucose uptake by an insulin-independent mechanism in skeletal muscle [67], and stimulate fatty acid oxidation in both adipocyte and muscle, via signalling pathways involving phosphatidylinositol 3 kinase and AMP-activated protein kinase, which can be inhibited by a CPT-1 inhibitor [66]. Therefore, the reduction in leptinR mRNA expression in both adipose tissue and skeletal muscle might impair glucose uptake and fatty acid oxidation in these organs.

Increased fatty acids in obese individuals can blunt the ability of muscle to oxidize fat which is thought to be related to the pathogenesis of insulin resistance [68, 69]. In the fed state when insulin is elevated and glucagon levels are low, CPT-1 activity is inhibited to increase fatty acid synthesis; while in the catabolic state, CPT-1 is activated to stimulate fatty acid oxidation [15]. The increased CPT-1 mRNA expression in muscle at both 2 and 10 weeks suggests the catabolic activity was higher in obese animals to reduce the excessive adipose store. However,

in WAT CPT-1 mRNA expression was only increased in the short term, suggesting impaired function in adipose tissue after long term hypertrophy, which could not be compensated by increased CPT-1 mRNA in skeletal muscle. We speculate that, in the obese state, particularly in the long term, the fat cells act more as a place to store excessive triglycerides, and lose their ability to maintain homeostasis; while the contribution of skeletal muscles to fatty acid oxidation and glucose metabolism might increase. However, the compensation is not sufficient.

In conclusion, obesity was readily induced by a palatable high-fat western cafeteria diet in mice with a sustained doubling of caloric intake. The hyperleptinemia was accompanied by reduced leptinR mRNA in both muscle and adipose tissue. Moreover, with excessive fat accumulation and hyperleptinemia, fatty acid oxidation was upregulated, and the orexigenic peptide NPY was downregulated, modest reduction in of the CCK peptide was observed in the hypothalamus. However our data suggest that these adaptive changes in the hypothalamic neuropeptide system and fatty acid oxidation pathway are not sufficient to correct the excessive caloric intake and fat accumulation.

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Titles and legends to figures

Fig 1 Energy intake (A) and body weight (B) of chow (□ n=15) and HFD (■ n=15) fed mice during the experimental period. Results are expressed as mean ± S.E.M. Energy intake were analysed by *Student's* unpaired *t* test. Body weight were analysed by ANOVA with repeated measures followed by a LSD post hoc test.

* Significantly different from chow fed mice ($p<0.05$).

Fig 2 NPY-like immunoreactivity in chow (□ n = 15) or HFD (■ n = 15) fed mice at 10 weeks. Areas shown are the anterior hypothalamus (AH), paraventricular nucleus (PVN), arcuate nucleus (Arc), posterior hypothalamus (PH) and medulla (Med). Results are expressed as mean ± S.E.M. Data were analysed by *Student's* unpaired *t* test.

* Significantly different from chow fed mice ($p< 0.05$).

Fig 3 CCK-like immunoreactivity in chow (□ n = 15) or HFD (■ n = 15) fed mice at 10 weeks. Areas shown are the anterior hypothalamus (AH), paraventricular nucleus (PVN), arcuate nucleus (Arc), posterior hypothalamus (PH) and medulla (Med). Results are expressed as mean ± S.E.M. Data were analysed by *Student'*s unpaired *t* test.

* Significantly different from chow fed mice ($p< 0.05$).

Fig 4 mRNA expression of AdipoR1 (A), leptin receptor (B), and CPT-1 (C) in skeletal muscle of chow (□ n = 12) or HFD (■ n = 10) fed mice at 2 and 10 weeks. Data are expressed as mean ± S.E.M; results are relative to cyclophilin. Data were analysed by *Student'*s unpaired *t* test.

* Significantly different from chow fed mice ($p< 0.05$).

Fig 5 mRNA expression of adiponectin (A), leptin (B), leptin receptor (C), and CPT-1 (D) in WAT of chow (□ n = 12) or HFD (■ n = 10) fed mice at 2 and 10 weeks. Data are expressed as mean ± S.E.M; results are relative to cyclophilin. Data were analysed by *Student's* unpaired *t* test.

* Significantly different from chow fed mice ($p < 0.05$).

Table 1 Primer used for real-time PCR

Gene	GenBank Accession No.	Forward Primer (5' → 3')	Concentration (mM)	Reverse Primer (5' → 3')	Conccentration (mM)
Adiponectin	NM_009605	TCCTGGAGAGAAGGGAGAGAAAG	9	CAGCTCCTGTCATTCCAACATC	9
Adiponectin receptor 1	NM_028320	GACTGGCAACATCTGGACACAT	3	ATATTTGGTCTCAGCATCGTCAAG	3
CPT-1	AF029875.1	CCTTGGCTACTTGGAACGAATTCT	3	GCGCATGCAGTGGGACAT	3
Cyclophilin	M19533	CTGATGGCGAGCCCTTG	4	TCTGCTGTCTTTGGAACTTTGTC	4
Leptin	NM_008493	TCTATTGCATTTACATACCGCATTTC	3	TTATTTTTACGACCCCAGGTATCC	3
Leptin receptor	NM_010704	GCAGTCCAGCCTATACGCTTGT	3	TAGTAACGTCCCCATCCATTTTTC	3

CPT-1: carnitine palmitoyl-transferase-1.

Table 2 Effect of 2 weeks and 10 weeks of HFD on body weight, length, girth, organ mass, and adiposity

	2 weeks		10 weeks	
	Control (n=15)	**HFD (n=15)**	**Control (n=15)**	**HFD (n=15)**
Body weight (g)	22.1 ± 0.3	23.5 ± 0.3*	27.2 ± 0.4	37.9 ± 0.7*
N-A Length (cm)	8.8 ± 0.1	8.7 ± 0.1	9.4 ± 0.1	9.9 ± 0.1*
Waist (cm)	8.8 ± 0.1	8.7 ± 0.1	7.5 ± 0.1	8.9 ± 0.1*
Groin (cm)	7.5 ± 0.1	7.6 ± 0.1	7.9 ± 0.1	9.4 ± 0.2*
Tibia (cm)	2.07 ± 0.03	2.04 ± 0.02	2.03 ± 0.03	2.00 ± 0.03
Liver (mg)	1140.2 ± 43.3	1101.0 ± 33.4	1318.9 ± 41.0	1773.5 ± 60.6*
Heart (mg)	123.0 ± 3.7	128.5 ± 2.3	137.3 ± 4.4	159.8 ± 2.8*
BAT (mg)	54.4 ± 2.9	64.9 ± 4.0	70.1 ± 3.2	192.0 ± 12.4*
RpWAT (mg)	41.7 ± 2.7	74.0 ± 6.4*	81.3 ± 4.8	453.2 ± 20.3*
Testicular WAT (mg)	262.1 ± 9.9	470.4 ± 28.8*	446.0 ± 16.3	2161.0 ± 88.4*
Visceral WAT (mg)	359.6 ± 13.0	399.3 ± 15.8	453.2 ± 9.9	1110.2 ± 61.6*
Leg WAT (mg)	248.5 ± 15.7	446.9 ± 35.1*	250.8 ± 14.6	1613.8 ± 92.4*
Sum of WAT (mg)	935.7 ± 35.3	1464.7 ± 82.1*	1392.3 ± 39.3	6186.4 ± 310.8*

Results are expressed as mean ± S.E.M. Data were analysed by *Student's* unpaired *t* test.

* Significantly different from control mice of that dietary period ($P < 0.05$).

BAT: brown adipose tissue; N-A: naso-anal; Rp: retroperitoneal; WAT: white adipose tissue.

Table 3 Plasma leptin, NPY and adiponectin concentrations at 2 and 10 weeks

	2 weeks		10 weeks	
	Control (n=15)	**HFD (n=15)**	**Control (n=15)**	**HFD (n=15)**
Plasma leptin (ng/ml)	2.3 ± 0.3	5.5 ± 0.6 *	2.9 ± 0.4	26.0 ± 1.6 *
Plasma NPY (ng/ml)	3.73 ± 0.16	4.67 ± 0.35 *	3.76 ± 0.44	5.58 ± 0.56 *
Plasma adiponectin (µg/ml)	6.45 ± 0.65	6.02 ± 0.44	5.75 ± 0.48	7.15 ± 0. 39 *

Results are expressed as mean ± S.E.M. Data were analysed by *Student's* unpaired *t* test.

* Significantly different from control mice ($P < 0.05$).

HFD: HFD; NPY: neuropeptide Y.