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THE EFFECTS OF HIGH FAT DIET ON THE BASAL ACTIVITY OF THE HYPOTHALAMUS-PITUITARY-ADRENAL AXIS IN MICE

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ABSTRACT

Alterations of the hypothalamus-pituitary-adrenal (HPA) axis activity have been linked to the development of metabolic syndrome (MetS). Common features of the MetS, like insulin resistance and obesity, are reproducibly induced by high fat diet (HFD) in animal models of diet-induced obesity (DIO). These models, hampered by methodological differences, reveal conflicting results with respect to HPA axis activation.

This study was aimed to evaluate in detail non-stressed diurnal HPA axis activity in mice during obesity development. Male C57Bl/6J mice were fed high or low fat diet for 12 weeks. HPA axis activity was evaluated by plasma corticosterone concentrations (at 0700, 1200, and 1800 h), corticotropin-releasing hormone (CRH) and glucocorticoid receptor (GR) mRNA expression in the hippocampus, amygdala, and hypothalamus, and 11β-hydroxysteroid dehydrogenase type-1 and -2 (11β-HSD-1 and -2) expression in adipose tissue and liver. Within one week, HFD induced obesity and decreased corticosterone levels at 1200 and 1800 h, which persisted throughout the experiment. 12 weeks of HFD decreased CRH mRNA in the PVN and amygdala and GR mRNA in the PVN at 0900 h. At 1800 h, CRH mRNA expression increased in PVN and amygdala, and GR mRNA increased in the CA1 region. 11β-HSD-1 expressions decreased in gonadal, visceral and subcutaneous adipose tissue at 0900 h and at 1800 h, whereas hepatic 11β-HSD-1 expression increased at 1800 h whereas 11β-HSD-2 expression was unaffected. HFD induces complex changes in the diurnal regulation of the different components of the HPA axis. These changes are not unequivocally characterized by increased, but rather by decreased HPA axis activity.

INTRODUCTION

Glucocorticoids (GC) (cortisol in humans and corticosterone in rodents) are secreted by the adrenals in response to stimulation of the hypothalamus-pituitary-adrenal (HPA) axis by a stressor, and induce behavioral and metabolic adaptations enabling the host to adequately coping with the stressor (fight or flight). Increased activity of the HPA axis has been linked to the development of the Metabolic Syndrome (MetS) (1). The metabolic effects of GC are directed both towards recruitment of energy stores for gluconeogenesis (peripheral effects), and towards augmentation of energy stores by adjusting feeding behavior and intake of palatable foods to compensate for the energy loss (central effects). Increased GC exposure will increase food intake and insulin levels, facilitating the development of obesity and the MetS (2, 3). In accordance, patients with Cushing's syndrome (CS), which is caused by prolonged excessive exposure to GC, exhibit many features of the MetS (4) associated with increased cardiovascular morbidity and mortality (5). Finally, manipulation of cortisol exposure at the tissue level in mice, through stimulation or abrogation of 11β-hydroxysteroid dehydrogenase type-1 and -2 (11-β-HSD-1 and 11-β-HSD-2) activity can increase, or regress, visceral fat accumulation, as well as other features of the MetS (6, 7).

Nonetheless, it is still controversial how the development of the MetS and its complications affect the activity of the HPA axis. Common features of the MetS, like insulin resistance and obesity, are reproducibly induced in mouse models of diet-induced obesity (DIO) by feeding of high fat diet (HFD), but the effects on the activity of the HPA axis have been evaluated in only a minority of these studies, and their results are conflicting. Many factors, like differences in mouse strains, housing and sampling conditions, but also the content and duration of the diets affect the activity of the HPA axis and preclude simple comparisons after induction of DIO. In addition, the evaluation of the HPA axis in most studies was restricted to a single measurement of plasma corticosterone levels, which was combined with either 11-β-HSD-1 enzyme activity in peripheral tissues or in the central nervous system in only a few of these studies (8).

Therefore, the aim of the present study was to evaluate the effects of HFD in mice in detail on basal, non-stressed activity of the HPA axis, using standardized evaluations that control for housing and sampling conditions. For these studies, we used C57Bl/6J mice that develop obesity and insulin resistance upon HFD (9-12).

MATERIAL AND METHODS

Mouse strain, housing, and diets

In the current study we used male C57Bl/6J mice (Charles River, Maastricht, The Netherlands), which develop, obesity and insulin resistance, specific features of the MetS (9-12). Twelve-week-old mice (n=36) were single housed in a separate room from other experimental animals in the facility to minimize environmental stressors, and maintained on a 12 h:12 h light-dark cycle (lights on 7 a.m.) at controlled room temperature (21-22°C) and fed *ad libitum* with free access to drinking water. Mice were weight-matched and randomly assigned to the following diets for 12 weeks: high fat diet (45 energy % lard fat, D12451, Research Diet Services, Inc, New Brunswick, US) (HFD) (n=18) or low fat diet (10 energy % lard fat, D12451B, Research Diet Services, Inc, New Brunswick, US) (LFD) (n=18). All mice were fed LFD for three weeks before starting the HFD.

All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures from the Leiden University Medical Center approved the protocol.

Sampling of corticosterone

Mice on LFD (n=18) and HFD (n=18) were divided into two groups of 9 mice, and blood for measurement of plasma corticosterone levels was sampled in week 1, 5, and 9 (first group), or at week 3, 7 and 11 (second group). Blood samples were collected during the first light hour at 0700 h, at 1200 h, and during the last light hour at 1800 h. To establish that the peak plasma corticosterone peak had not 'shifted' toward the dark hours of the light/dark cycle, at week 11, plasma corticosterone was measured at 1900 h, 2000 h and 2100 h during the dark phase in red light conditions. All corticosterone samples were obtained within 90 seconds from disturbing the cage, via tail incision, allowing the mouse to move freely on top of the home cage (13). Plasma insulin was measured after a 4-hour fast in the same weeks as corticosterone. Plasma leptin levels were determined from the trunk blood after decapitation. After 12 weeks, the mice were decapitated within 90 seconds from disturbing the cage, either in the morning (0900-1000 h) or during the last light hour (1800-1900 h). After decapitation, the trunk blood was collected, the brain was harvested and snap frozen in isopentane and stored at −80°C. Liver, muscle, gonadal, abdominal visceral and subcutaneous fat pads were dissected, snap-frozen in liquid nitrogen and stored at −80°C.

Plasma hormone measurements

Plasma corticosterone levels were determined by radioimmunoassay (MP Biomedicals LLC, Orangeburg, NY; intra-assay variation 7.3%, inter-assay variation 6.9%,).

Insulin and leptin were measured with ELISA (Crystal Chem Inc., Downers Grove, IL, USA; intra-assay precision coefficient of variation ((CV)≤10% and inter-assay precision CV≤10% for both kits). All measurements were assayed according to the manufacturer's instructions.

Evaluation of HP- axis activity in the central nervous system (in situ hybridization)

Brain sections of 16 µm of the paraventricular nucleus (PVN) (Bregma -0.70mm), amygdale (Bregma -0.70 mm) and hippocampus (Bregma -1.70 mm) were cut according to the brain atlas of Paxinos and Franklin 2001 (14) on a cryostat and mounted on polysine microscope slides (Menzel-Gläzer, Braunschweig, Germany) and stored in -80°C until further use. The hybridization was performed as described previously by Meijer *et al*. 1997 (15) with minor adjustments. Briefly, sections were fixed in 4% paraformaldehyde, further permeabilized by proteinase K treatment, acetylated twice with 0.25% acetic anhydride in 0.1 M triethanolamine and dehydrated in a graded ethanol series.

Riboprobes were generated from linearized constructs containing the respective cDNAs in pBluescript. A 500-bp SalI–HindIII fragment of exon 2 of the mouse gene was used for GR (16). The cRNA from CRH was transcribed from a 1-kb cDNA insert in pGEM 4 containing full-length coding region of rat CRH (17). ^{35}S-UTP labeled antisense probes were generated using the appropriate polymerase using a standard protocol.

A hybridization mix was prepared containing 60% deionized formamide, 10% Dextran SO_4, 2xSSC, 0.1 mg/ml yeast tRNA, 0.1 mg/ml sssDNA, 10 mM dithiothreitol, 0.05 M PBS. All radiolabeled probes were diluted to 16.7x10^6 cpm/ml. Of these mixtures 120 µl was applied to each slide and then covered with a cover slip. The sections were hybridized overnight in a moisturized chamber at 55°C.

The next day, the cover slips were removed carefully and sections were washed in 2xSSC for 10 min at room temperature. After washing, sections were treated with RNAse A (2 mg/100 ml in 0.5 mol/l NaCl, 0.1 mol/l Tris, pH 7.5) at 37°C for 10 min and subsequently washed at 55°C in 2xSSC for 10 min, 1xSSC for 10 min, 0.1xSSC for 2x30 min and, finally, at room temperature in 0.1xSSC for 5 min. Sections were dehydrated in an ethanol series (70%, 80%, 96% and 100% ethanol) and dried on air. Signal was visualized with exposure of Kodak Biomax MR films, scanned and quantified by using Image J software (National Institutes of Health) and related to standard curves of ^{14}C (RPA 504 microscales; Amersham, Buckinghamshire, UK). Two sections per mouse per brain area were quantified**.** For CRH mRNA in PVN and amygdala and for GR mRNA in the PVN, the values represent a sum of the two areas measured. For GR mRNA in the hippocampus, the values represent the average of the two measurements.

11β-HSD-1 and 11β-HSD-2 expression analysis in liver and adipose tissue

Total RNA was extracted from liver and adipose tissues using the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality was examined with lab-on-a-chip technology using Experion Std Sens analysis kit (Biorad, Hercules, CA). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and the obtained cDNA was purified with Nucleospin Extract II kit (Macherey-Nagel). Real-time PCR for 11β-HSD-1 (forward primer: CAGCAAAGGGATTGGAAGAG; reverse primer: CTTTCCCGCCTTGACAATAA) and 11β-HSD-2 (forward primer: TTTGGTGCACTTGAGCTGAC; reverse primer: AGCCGAATGTGTCCATAAGC) were carried out on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of cyclophilin (forward primer: CAAATGCTGGACCAAACACAA; reverse primer: GCCATCCAGCCATTCAGTCT) and hypoxanthine guanine phosphoribosyl transferase (forward primer: TTGCTCGAGATGTCATGAAGGA reverse primer: AGCAGGTCAGCAAAGAACTTATAG).

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using Mann-Whitney test for non-parametric data, with GraphPad Prism, version 5.01. (GraphPad Software, Inc.La Jolla, CA), $P<0.05$ was considered as statistically significant.

RESULTS

High fat diet increases plasma insulin and leptin levels and body weight without affecting thymus weight

As anticipated, HFD feeding resulted in a greater increase in body weight as compared to LFD, which already reached significance within 1 week, and this difference remained significant throughout the experiment (Fig. 1A). HFD did not affect the thymus weight (Fig. 1B). HFD increased plasma insulin concentrations already within one week (Fig. 1C), which remained significantly increased throughout the experiment. Plasma leptin levels were significantly increased after 12 weeks of HFD both at 0900 h and 1800 h Fig 1D).

High fat diet decreases diurnal peak plasma corticosterone levels both acutely and in the long term

A diurnal corticosterone rhythm was observed in all animals, with a nadir in the morning (0700 h) and with peak values during the last light hour before the dark phase (1800 h). HFD decreased plasma

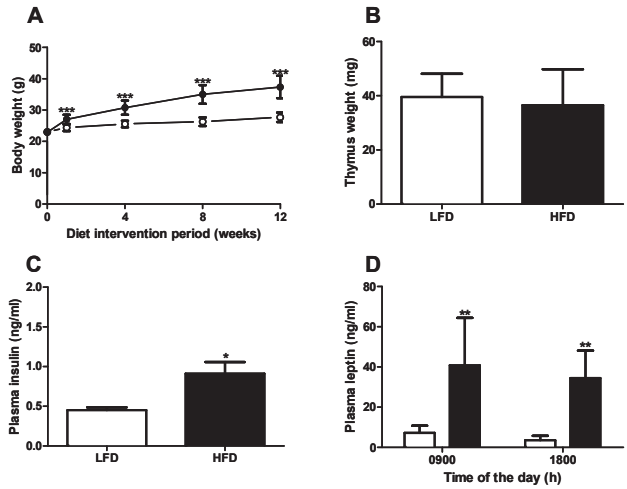

Figure 1. Effect of diet on male C57Bl/6J mice fed a low fat diet (open circles and bars) or high fat diet (closed circles and bars) on body weight (A), thymus weight at week 12 (B), plasma insulin concentrations at week 1 (C) and plasma leptin levels at week 12 (D), Mann-Whitney test, *P<0.01, **P<0.001, ***P<0.0001.

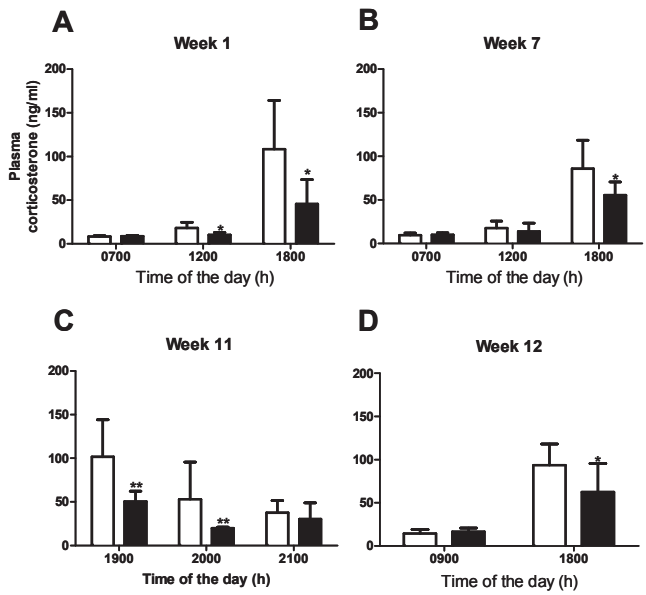

Figure 2. Effect of high fat diet on circadian plasma corticosterone. Male C57Bl/6J mice were fed a LFD (open bars) or HFD (closed bars), and plasma corticosterone was determined in the morning (0700), at noon (1200) or at the evening peak (1800) after 1 week (A), 7 weeks (B). At 11 weeks (C) corticosterone was determined at 1900, 2000 and 2100 (n=6 per time point), and at 12 weeks (D) at 0900 and 1800. Mann-Whitney test, *P<0.05, **P<0.01.

corticosterone levels within one week by 44% at 1200 h, and by 52% at 1800 h in the evening (Fig. 2A). This decrease in evening peak corticosterone levels persisted throughout the experiment at weeks 7 and 12 (Fig. 2B and D). Furthermore, peak corticosterone levels were not 'shifted' toward the dark hours of the light/dark cycle but declined from the 1800 to 1900 h time points in both LFD and HFD groups. The suppression of plasma corticosterone levels was also evident in HFD group during the beginning of the dark phase (at 1900 h, 2000 h) at week 11 when compared to the LFD group (Fig. 2C).

High fat diet induces changes in mRNA expression of CRH and GR in the brain

HFD significantly decreased CRH mRNA expression in the PVN and amygdala at 0900 h in the morning (Fig. 3A and B). Moreover, HFD decreased GR mRNA in the PVN at 0900 h in the morning basal period (Fig. 3C). HFD increased CRH mRNA expression at 1800 h in the evening in both PVN and amygdale (Fig. 3A and B). Furthermore, HFD increased GR mRNA in the evening in the CA1 region (Fig. 3D), but not in the other regions (CA3 and dentate gyrus (DG) of the hippocampus (Fig. 3E and G).

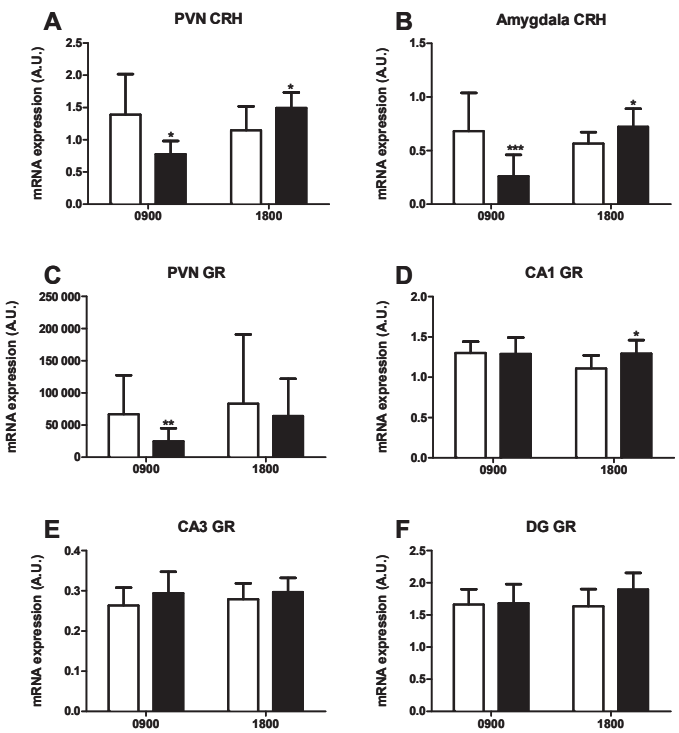

Figure 3. Effect of high fat diet on mRNA expression of CRH and GR in the hypothalamus and hippocampus. Male C57Bl/6J mice were fed a LFD (open bars) or HFD (closed bars) for 12 weeks, and mRNA expression was determined: CRH in the PVN (A) and amygdale (B), and GR in the PVN (C), CA1 (D), CA3 (E) and DG (F) region of the hippocampus in the morning (7.00) and evening (18.00) (black bars). Mann-Whitney test, $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

High fat diet feeding induces opposite changes in 11β-HSD-1 mRNA expression in adipose tissue and liver, whereas 11β-HSD-2 mRNA expression remains unaffected

HFD decreased 11β-HSD-1 expression in gonadal, visceral and subcutaneous adipose tissues both in the morning by 65%, 37% and 66%, respectively (Fig. 4B, C and D), and in the evening by 62%, 47% and 67%, respectively, whereas no changes were observed in 11β-HSD-2 expression in the same tissues at both time points (Fig 4B, C and D). In contrast, in the liver, HFD increased 11β-HSD-1 expression at 1800 h by +23% whereas 11β-HSD-2 expression was not affected (Fig. 4A).

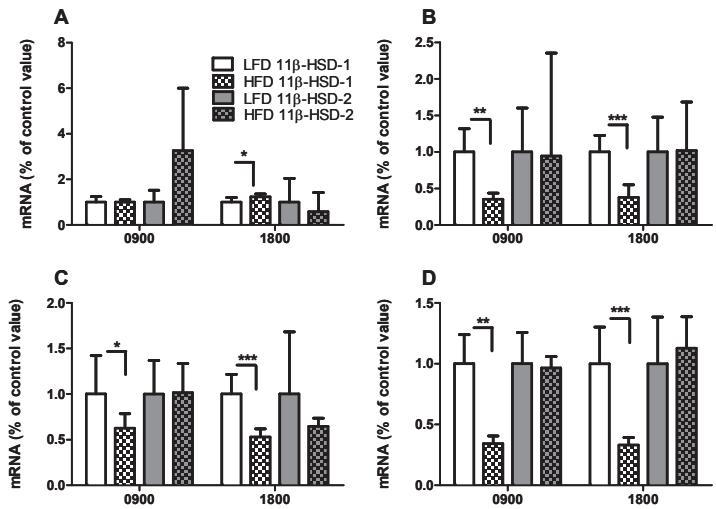

Figure 4. Effect of high fat diet on 11β-HSD-1 and 11β-HSD-2 expression in peripheral tissues. Male C57Bl/6J mice were fed a LFD (open bars) or HFD (chequered bars) for 12 weeks, and 11β-HSD-1 (white bars) and 11β-HSD-2 (grey bars) mRNA expression was determined in the liver (A) and gonadal (B), visceral (C) and subcutaneous (D) adipose tissues in the morning and evening. Mann-Whitney test, *P<0.05, **P<0.01, ***P<0.001.

DISCUSSION

This study aimed to characterize in detail, using standardized evaluations that control for housing and sampling conditions, the diet-induced changes that occur in basal activity of the HPA axis in the C567Bl/6J mouse model that develops obesity and insulin resistance, distinct features of the MetS. HFD feeding resulted in down regulation of the activity of the HPA axis, as reflected in decreased diurnal corticosterone concentrations, decreased 11β-HSD-1-enzyme expression in peripheral tissues, and altered CRH and GR expression in the CNS (decreased in the morning and increased in the evening). These observations indicate that HFD induces complex changes in the diurnal regulation of the different components of the HPA axis. These changes are not unequivocally characterized by increased, but rather by decreased, HPA axis activity.

As expected, HFD significantly increased both body weight and plasma insulin concentrations already within one week and HFD reduced diurnal corticosterone levels already within one week that persisted throughout the experiment. This persistent reduction in circulating corticosterone levels was not due to a shift in the diurnal peak of corticosterone and was evident from 1800-2000 h in the evening.

Several mechanisms may explain the early decrease in diurnal corticosterone peak levels upon HFD. First, hypercortisolism in human obesity has not been established and cortisol secretion is increased in obese humans, primarily because of increased clearance and increased distribution volume of the circulating cortisol resulting in secondary central activation of the HPA axis (18, 19). Second, this decrease in diurnal corticosterone peak levels may reflect counteracting mechanisms directed towards prevention of further progression in insulin resistance, both centrally and in peripheral tissues. Third, circulating leptin concentrations increase proportionally to the fat mass gained (20) and leptin and insulin resistance have been documented to develop already within three days of high fat feeding (21).

In accordance, HFD increased in CRH mRNA expression in the PVN and an increase in GR mRNA expression in the CA1 region of the hippocampus in the evening representing reduced negative feedback by decreased circulating corticosterone levels. This increase in GR mRNA in the CA1 region of the hippocampus also indicates that CNS areas important for specific types of learning and memory are relatively preserved in the presence of HFD and subsequent dampening of the HPA axis. In accordance, reduction of glucocorticoid levels in a specific mouse model of insulin resistance (*db/db*) reverses the cognitive impairment related to hippocampal neurons induced by insulin resistance (22). These findings imply that dampening of the HPA axis in the presence of insulin resistance, induced by HFD, might be a mechanism to rescue hippocampal neurons from impairments and to maintain normal cognitive function. In addition, both leptin and insulin can activate pro-opiomelanocortin (POMC) neurons (23), which produce ACTH. However, glucose sensing by POMC neurons is impaired in obese mice (24), which may result in insufficient adrenal stimulation by ACTH (diminished forward coupling between ACTH and corticosterone) resulting in decreased diurnal peak corticosterone and reduced negative feedback. In addition, the potency of ACTH is decreased in obesity (18).

Intriguingly, there is a disparity between the effects of HFD on the activity of the HPA axis in the morning as compared to the evening. Whereas the central activation in the evening can be easily explained by reduced negative feedback as a result of reduced peak corticosterone levels, the central inhibition of CRH and GR expression found in the morning in the presence of unaltered the circulating corticosterone levels, is very difficult to explain. To the best of our knowledge, such a disparity has not been previously documented or investigated. This further exemplifies that HFD induces complex changes in the diurnal regulation of the different components of the HPA axis. It is also likely that other factors outside the individual components of the HPA axis influence these complex effects of dietary intervention. Reduction of hypothalamic CRH expression in the morning might include the following mechanisms. For instance, induction of leptin resistance in the presence of obesity induces an increase in endocannabinoid tone (25, 26), which could lead to suppression of CRH. However, leptin resistance induced by high fat feeding is selective and does not impact on autonomic nervous system activity (27). Thus it could be speculated that hyperleptinemia dampens CRH expression and release in the face of selective sensitivity of the central HPA axis. It has also been shown in *in vitro* studies that leptin can reduce the sensitivity of adrenal cortex cells to ACTH,

thereby reducing circulating corticosterone levels (28, 29). However, the present data do not permit firm conclusions in this respect. In addition, a true effect of the diet *per se* can not be excluded as no differences were observed in plasma corticosterone levels in the morning, although it is well possible that small changes in circulating corticosterone levels at the moment of the diurnal nadir are not detected. The observed decreased expression of hypothalamic GR expression, however, is not explained because it suggests increased corticosterone exposure.Down regulation of CRH mRNA in the morning, was accompanied by downregulation of CRH mRNA and in the amygdala. Activation of the amygdala promotes HPA-activation and previous studies in rats have shown that increase in circulating glucocorticoids increase (30) whereas adrenalectomy decreases (31) CRH in the amygdala. Furthermore, increased CRH in the amygdala mediates anxiety-like behavior during stress (32) and high fat diet decreases anxiety-like behaviors facilitating stress recovery (33, 34). Thus, HFD-induced reduction of CRH expression in the amygdala enables protection from further exposure to systemic glucocorticoids.

In accordance, the effects of HFD on the peripheral activity of the HPA axis were characterized by reduced mRNA expression of 11β-HSD-1 in adipose tissues. It has been proposed that this may reflect a mechanism to counteract tissue-specific insulin resistance (35). These differential, fat depot-specific effects are in agreement with a recently- proposed, dynamic and depot-selective relationships between adipose tissue 11β-HSD-1-activity and fat mass (35). HFD increases lipoprotein lipase activity (36), which would direct circulating triglycerides towards peripheral adipose tissues for storage. Corticosterone impairs glucose tolerance (37) and is a lipolytic hormone (38). Therefore down regulation of 11β-HSD-1 expression would protect the adipose tissue from further insulin resistance. These observations are further strengthened by the fact that 11β-HSD-2 expression in the same tissues was not affected, resulting into overall reduction of glucocorticoid exposure of the tissues.

In conclusion, we found that HFD has profound effects on the basal, thus non-stressed, activity of the HPA axis, reflected in reduced diurnal corticosterone concentrations, distinct expression of CRH in the CNS across the diurnal rhythm, and reduced 11β-HSD-1-enzyme expression in peripheral fat tissues. The observed effects in this study, both in the CNS and peripheral adipose tissues (downregulation of CRH mRNA and 11β-HSD-1 enzyme mRNA, respectively), are in agreement with the well-known metabolic effects of glucocorticoids, that are directed towards recruitment and augmentation of energy stores. In the presence of HFD induced obesity or palatable foods, the HPA axis adapts.

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