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Regulation of Adipose Tissue in Three Models of Obesity

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Summary

The present work investigated adipose tissue (AT) mass regulation in three rat models of obesity. The most prevalent view of AT mass regulation posits that endocrine “adiposity signals” such as insulin, leptin, ghrelin and amylin convey information about the level of AT mass to the brain, which adjusts eating and energy expenditure (EE) appropriately if AT mass is increased or decreased. Insulin and leptin are thought to act on neurons in the Arcuate nucleus of the hypothalamus (Arc) to initiate such regulatory responses. These Arc neurons express NPY and α -MSH, which are considered to be crucial signaling molecules in the neural network. Similarly, downstream signaling molecules, including PVN CRH neurons, are thought to be involved.

The first experiments investigated the dynamic compensatory changes in eating and the return to near-normal levels of AT mass following experimentally induced obesity, done here by chronic intragastric (IG) overfeeding. Such compensatory responses provide probably the strongest evidence for active regulation of AT mass. Additional experiments used ovariectomy (OVX) to model the increase in AT mass, in particular intra-abdominal AT (IAAT), associated with the loss of estrogens at menopause. In fact, however, whether OVX increases IAAT in rats has not been measured directly yet. These experiments also included rats with “dietary obesity,” i.e., obesity induced by the availability of palatable, energy-dense foods, which is thought to be the main cause of the present obesity epidemic. Each study involved measurement of AT mass and regional AT distribution by computed tomography (microCT). Such a direct measure of AT has not been applied to these models before.

Despite the strong evidence for dynamic AT regulation in rats recovering from forced overweight, changes in adiposity signals and associated brain mechanisms during such dynamic regulation has not been extensively investigated. Therefore in **study 1** I tested whether basal plasma levels of insulin, leptin, amylin and ghrelin encode information about AT mass during dynamic AT mass regulation. Rats were IG overfed for 23 d, resulting in excess BW and AT mass. When the IG overfeeding regimen was terminated, rats underate for at least 23 d, lost excess AT mass and BW. Basal levels of insulin, leptin and ghrelin, but not of amylin, changed linearly with AT mass during the overfeeding phase, but during the subsequent phase of dynamic AT regulation, none of the hormones accurately reflected AT mass. Plasma levels of insulin returned to control levels on d 1 of recovery and decreased below control levels thereafter. Leptin also decreased disproportionately to AT mass, but was still elevated on d 5 and 8 and was no longer higher than the control level by d 17 of recovery. Neither ghrelin nor amylin changed reliably during recovery. These data indicate

that basal plasma levels of insulin, leptin, ghrelin, and amylin do not provide sufficient information to guide dynamic regulation of AT mass following forced overweight and that changes in them are not necessary for such regulation.

In **study 2** in situ hybridization assays of mRNA were used to test whether hypothalamic levels of NPY, α -MSH or CRH might be involved during dynamic regulation of AT mass. Rats were similarly IG overfed but killed on d 9 of recovery, when they were undereating by ~40% and had ~94 g excess BW and ~75 g excess AT mass. Arc and DMH NPY mRNA and Arc POMC mRNA were not significantly changed. In contrast PVN CRH mRNA was significantly increased. Plasma corticosterone on d 9 was not significantly different between groups, indicating that the increase in CRH mRNA was neurocrine rather than neuroendocrine. Insulin and leptin levels were consistent with Study 1. These data indicate that compensatory undereating and AT mass loss is not mediated by down-regulation of Arc or DMH NPY or upregulation of Arc α -MSH, but that upregulation of PVN CRH mRNA might be involved.

In **study 3** OVX and sham-operated rats were fed normal chow or chow plus Ensure (Abbott) liquid diet and treated with subcutaneous injections of 2 ug estradiol benzoate / 4 d or the oil vehicle for 41 d. MicroCT indicated that OVX led to increased SAT, but little or no increase in IAAT. Chemical carcass analysis in chow-fed rats revealed the OVX increased fat mass by ~24 g, of which ~10 g was outside the AT, and increased fat-free mass ~33 g. Ensure feeding markedly increased BW (~65 g) and both SAT (~28 g) and IAAT (~23 g); the additional effect of OVX was similar to those in chow-fed rats. Estradiol treatment prevented all the effects of OVX. These data indicate that OVX in rats and human menopause differ in several respects that are likely to affect energy homeostasis: OVX selectively increases SAT rather than IAAT, increase rather than decreases fat-free body mass, and probably increases deposition of fat outside the AT more. Thus, OVX in rats is unlikely to be a homologous model for menopause-related metabolic changes and health risks. In contrast, dietary obesity in female rats parallels the AT profile of common obesity in humans.

These experiments should contribute to a better understanding of the regulation of AT mass, which in the future may be useful in efforts to develop more effective, physiologically based, non-surgical therapies for obesity.

Zusammenfassung

In dieser Arbeit wurde die Regulation der Fettgewebsmasse in drei Modellen für Adipositas untersucht. Gemäss der vorherrschenden Meinung bezüglich der Regulation der Fettgewebsmasse fungieren die basalen Plasmakonzentrationen von verschiedenen Hormonen als Feedbacksignale, welche dem Gehirn Informationen über die Masse des Fettgewebes liefern („Adipositassignale“). Wenn diese Signale eine Veränderung der Fettgewebsmasse anzeigen, steuert das Gehirn die Nahrungsaufnahme und den Energieverbrauch adäquat. Es werden hauptsächlich Insulin und Leptin, aber auch Amylin und Ghrelin als Adipositassignale vorgeschlagen. Dabei wird angenommen, dass Insulin und Leptin auf Neurone des Nucleus arcuatus (Arc) im Hypothalamus wirken, welche Neuropeptid Y (NPY) und α -Melanozyten-stimulierendes-Hormon (α -MSH) produzieren. NPY und α -MSH gelten als wichtige Signalmoleküle im neuronalen Netzwerk der Steuerung von Nahrungsaufnahme und Energieverbrauch. Ausserdem ist eine Reihe von nachgeschalteten Signalmolekülen involviert; dazu gehört das Corticotropin-Releasing Hormon (CRH), welches im Nucleus paraventricularis des Hypothalamus (PVN) gebildet wird.

Die ersten Experimente untersuchten die dynamische Regulation der Fettgewebsmasse und Kompensationen in der Nahrungsaufnahme bei Ratten, die chronisch intraagastral überfüttert wurden, um sie übergewichtig zu machen. Solche kompensatorischen Reaktionen liefern wohl den stärksten Hinweis auf eine aktive Regulation des Fettgewebes. Zusätzlich wurden Experimente an Ratten nach Ovariectomie (OVX) durchgeführt, welche als Modell für die Zunahme der Fettgewebsmasse nach der Menopause gelten. Ausserdem wurde noch eine Gruppe von intakten und OVX-Ratten in die Studie aufgenommen, denen zusätzlich zu ihrem normalen Futter als Modell für eine diätinduzierte Adipositas eine schmackhafte, energiedichte Nahrung angeboten wurde. Jede Studie nutzte die Messung der Fettgewebsmasse und der Fettgewebsverteilung mittels Computertomographie (CT) für Nager, was bisher in diesem Zusammenhang noch nie gemacht wurde.

Trotz der starken Hinweise für eine dynamische Regulation der Körperfettmasse als Reaktion auf experimentell induziertes Übergewicht, gibt es kaum Informationen über Veränderungen von Adipositassignalen oder deren nachgeschalteten Mechanismen im Gehirn in dieser Situation. Aus diesem Grund wurde in **Studie 1** getestet, ob basale Plasmakonzentrationen von Insulin, Leptin, Amylin und Ghrelin während dieser dynamischen Regulation als Informationsträger bezüglich der Masse des Fettgewebes fungieren. Die Ratten wurden dazu für 23 Tage intraagastral überfüttert, so dass sie ~130 g

Körpergewicht und ~100 g Fettgewebsmasse mehr zunahmen als Kontrolltiere. Nach dem Ende der Überfütterung frassen die Ratten für mehr als 23 Tage deutlich weniger als die Kontrollen und verloren Körpergewicht und Fettgewebsmasse. Während der Überfütterung veränderten sich die basalen Plasmakonzentrationen von Insulin, Leptin und Ghrelin, aber nicht Amylin, linear mit der Fettgewebsmasse. Während der folgenden Phase der dynamischen Regulation des Fettgewebes reflektierte keines dieser Hormone die Fettgewebsmasse akkurat. Die Plasmakonzentrationen von Insulin unterschieden sich bereits am Tag 1 nicht mehr von denen der Kontrollgruppe und sanken im weiteren Verlauf unter die Kontrollwerte. Leptin sank ebenfalls disproportional im Vergleich zur Fettgewebsmasse, war jedoch am Tag 5 und 8 noch erhöht, aber nicht danach. Weder Ghrelin noch Amylin veränderten sich während dieser Phase. Diese Ergebnisse sprechen dafür, dass basale Plasmakonzentrationen von Insulin, Leptin, Ghrelin oder Amylin nicht genügend Informationen für die dynamische Regulation der Fettgewebsmasse nach experimentell induziertem Übergewicht liefern und dass Veränderungen der basalen Plasmaspiegel für diese Regulation damit nicht notwendig sind

In **Studie 2** wurde in situ-Hybridisierung für mRNA eingesetzt um zu testen, ob NPY, α -MSH oder CRH in die dynamische Regulation der Fettgewebsmasse involviert ist. Dafür wurden Ratten ähnlich wie in Studie 1 überfüttert, aber am Tag 9 der Kompensation getötet. An diesem Tag war die Futterraufnahme um 40% reduziert, die Tiere hatten aber immer noch ein gegenüber der Kontrollgruppe um ~94 g erhöhtes Körpergewicht und ~75 g mehr Fettmasse. Die mRNA Konzentrationen von NPY sowie diejenige von Pro-opiomelanocortin (POMC, Vorläuferpeptid von α -MSH) im Arc waren nicht signifikant verändert. Im Gegensatz dazu war die mRNA von CRH im PVN erhöht. Plasmakonzentrationen von Corticosteron am Tag 9 wiesen keine Unterschiede zwischen den Gruppen auf. Die Ergebnisse bezüglich der Plasmakonzentrationen von Insulin und Leptin waren konsistent zu Studie 1. Diese Ergebnisse weisen darauf hin, dass die kompensatorische Hypophagie im Rahmen der dynamischen Regulation der Fettgewebsmasse nicht durch eine Abnahme von NPY im Arc oder DMH oder eine Erhöhung von POMC vermittelt wird. Hingegen könnte die Erhöhung der mRNA Konzentrationen von CRH im PVN beteiligt sein.

In **Studie 3** wurde getestet, ob OVX bei Ratten zur Bildung von mehr intraabdominalem Fettgewebe führt, ähnlich wie das für die Menopause angenommen wird. Dazu wurden weibliche Ratten ovariectomiert und anschliessend mit Östradiol (2 μ g alle 4 Tage) oder dem Öl (Träger) behandelt. Weitere weibliche Ratten wurden schein-operiert und erhielten entweder nur Standardfutter oder zusätzlich Ensure® (Abbott) ad libitum für 41 Tage. Die MicroCT zeigte, dass OVX-Ratten mehr subkutanes Fettgewebe (12 g), aber nur wenig

intraabdominales Fettgewebe zunehmen. Eine chemische Analyse der Körperzusammensetzung zeigte, dass OVX bei Ratten mit Standardfutter zu einer Zunahme der Fettmasse um 24 g führte, wovon 10 g ausserhalb des Fettgewebes waren. Die fettfreie Masse nahm um 33 g zu. Die Zufütterung von Ensure® führte zu einer deutlichen Zunahme der Körpermasse (≈ 65 g) sowie der subkutanen (≈ 28 g) und intraabdominalen Fettgewebsmasse (≈ 23 g). Die zusätzlichen Effekte der OVX waren ähnlich denen bei Standardfütterung. Die Behandlung mit Östradiol verhinderte alle OVX-Effekte. Diese Ergebnisse zeigen, dass OVX bei Ratten und die Menopause bei Menschen sich in verschiedenen Punkten unterscheiden, die vermutlich für die Energiehomöostase von Bedeutung sind: OVX bei Ratten führte zu einer selektiven Zunahme des subkutanen Fettgewebes und erhöhte die fettfreie Körpermasse und wahrscheinlich auch die Anlagerung von Fett ausserhalb des Fettgewebes. Hingegen geht man davon aus, dass die Menopause bei Frauen insbesondere das intraabdominale Fettgewebe vermehrt. Ausserdem kommt es nach der Menopause eher zu einem Abbau fettfreier Körpermasse. Deshalb ist OVX bei Ratten wahrscheinlich kein homologes Modell für die metabolischen und gesundheitsrelevanten Effekte der Menopause bei Frauen, was bei entsprechenden Untersuchungen berücksichtigt werden sollte. Im Gegensatz dazu gibt es durchaus Parallelen zwischen der diät-induzierten Adipositas bei weiblichen Ratten und Menschen.

Diese Experimente sollten dazu beitragen, die Physiologie der Regulation der Fettgewebsmasse zu verstehen. Dieses könnte genutzt werden um effizientere, auf Physiologie basierende Therapien für die Adipositas zu entwickeln.

1. Introduction

Fat in the adipose tissue (AT) is the body's main way to store energy. Changes in AT mass arise from imbalances between energy intake, i.e., food intake, and energy expenditure (EE). The last 50 years has seen a worldwide increase in the prevalence of people, both children and adults, with excessive amounts of adipose tissue (AT), which is sometimes called an obesity epidemic [1, 2]. The obesity epidemic is associated with increased prevalence of diabetes, cardiovascular disease, and other health problems and with increased mortality [3]. These health risks depend on the site of as well as the amount of AT deposition, with intra-abdominal AT (IAAT) being more deleterious than subcutaneous AT (SAT) [4-7]. Overconsumption of palatable, energy-dense foods is thought to be the main cause of the obesity epidemic ("dietary obesity") [8-11]. Lack of exercise, genetic factors, sex and age contribute importantly as well. For example, IAAT increases after menopause, apparently mainly due to the loss of estrogens, leading to increased health risks [12, 13].

Despite the increasing prevalence of obesity, there are several indications that AT mass is actively regulated. The strongest evidence for such regulation is provided by the dynamic compensatory responses in eating and EE that restore AT mass toward normal following experimentally induced increases or decreases in AT mass in humans and animals [14-19]. Such regulatory responses have been demonstrated in humans as well as animals. The brain plays a critical role in this regulation by controlling eating and EE. The most prevalent current view is that the brain receives feedback signals, so-called adiposity signals, which encode information about the level of AT mass and, if increases or decreases are detected, adapts eating and EE appropriately [20-25]. The details of this physiological mechanism, however, remain very poorly understood.

Unfortunately, non-surgical therapeutic options to prevent or reverse obesity are only minimally effective. In this context, understanding the physiological mechanisms regulating AT mass and AT distribution are crucially important scientific issues. This thesis attempted to contribute to this issue by examining the physiological regulation of AT mass in three rat obesity models. The primary focus was on the roles of hypothesized endocrine adiposity signals and brain signaling molecules during dynamic regulation of AT mass following obesity induced by chronic intragastric overfeeding. In addition, some of the work involved the role of estrogens in regulating AT mass in normally fed and dietary-obese female rats.

2. Regulation of adipose tissue

Homeostasis

Walter B. Cannon coined the term homeostasis (Greek for similar and steady) in 1929. He described that in order to maintain life, physiological variables are regulated. Cannon stressed the fact that to keep a steady state of the body by active regulation does not imply fixity, but that body states vary within tolerated limits. An important forerunner of this concept was Bernard (about 1878), who postulated the law of the “constancy of the interior biological environment” [26, 27].

In their classic *Textbook of Medical Physiology*, Guyton and Hall write that “essentially all organs and tissues of the body perform functions that help maintain ... constant conditions” and that homeostasis is a fundamental organizing principle of physiology. The nervous, endocrine and immune systems are the main regulatory units that control our body functions. For the functions of the different organs in the body to correspond to the homeostatic needs of the body, the organs have to be under some control. This control requires the ability to register the current state, compare it to the desired value, and to correct discrepancies if necessary. Most control systems of the body operate by negative feedback, but more complex systems and even anticipatory systems exist. In addition, regulation can take place via auto-regulatory mechanisms; that is, whole-body regulatory effects can result from mechanisms contained within individual single cell [28, 29].

Energy homeostasis

Energy homeostasis describes the maintenance of body energy status within a certain range. The concept of energy homeostasis involves the brain as key player in the regulation of body energy status by controlling energy expenditure (EE) and energy intake. The brain receives information about the body energy status, which is related to available energy substrates in the blood stream and stored energy [21, 30]. Maintaining a continuous energy supply for body functions by regulating energy substrates in the blood, e.g., glucose and fatty acids, requires regulation of energy storage because energy intake, i.e., food intake, occurs in discrete meals. Stored energy can then be mobilized if food is not available. Some energy is stored as glycogen. In the long term however, the primary energy store is triacylglycerides (TAG) in AT, because the capacity to store glucose as glycogen is limited.

Thus in the situation of ample energy, the brain processes this information with the result that energy intake and e.g. hepatic glucose production will be inhibited and EE will be increased [21]. If energy intake exceeds EE in the longer term, excess energy will be stored as fat. In the situation of chronic energy deficiency, the opposite occurs. The source of energy is food. Eating, however, is a complex behavior that is rarely a pure consequence of energy status. Other factors that influence eating are time of the day, habits, social situations, smell and taste of food, reward and emotions [30, 31]. The regulation of body AT is thought to be a main component of energy homeostasis. Evidence for regulation of body AT is presented in the next section.

Evidence for regulation of adipose tissue

If body AT is regulated there should be evidence that the regulation is constant and precise. The relative stability of body and AT masses during adulthood suggests the existence of regulation by a homeostatic system. The level of AT remains relatively constant in adults, despite the facts that energy intake and energy expenditure vary daily and that within one day, fluctuations of energy storage and release occur, according to eating and non-eating periods [20, 32]. In the long-term energy intake and energy expenditure agree closely [33]. The concept of energy homeostasis allows regulation of AT within a certain given, constant range. The age related gain in AT during adulthood [34] might be described as a regulated trajectory of AT gain [35]. The term homeorhesis is a concept, scarcely used in this context, which takes into account that a physiological system does not return to a particular state but to a trajectory (Bessesen SSIB Pittsburgh 2009, [36]).

Already in ~1950, the precision with which body weight is regulated was emphasized. Even small discrepancies between energy intake and EE, if consistent, would result in weight gain or loss. This notion is maintained by contemporary authors. Whereas it was the example of "a little deeper cut into the pat of butter" in former times, it is the extra potato chip per day nowadays that would gradually result in weight gain [31, 32, 37, 38].

Early studies on the regulation of body weight focused on the body's capability to adjust food intake in response to different environmental (e.g. different diets) or physiological conditions (e.g. lactation) [39, 40]. The strong relationship between food intake and body weight in several situations was described by Kennedy (1953), he showed for example, that this relationship is not different between normal and increased ambient temperatures, but changes proportionately. That is in hot environments, rats reduce their food intake and body weight, and in cold environments they increase their food intake and body weight. He also

described the phenomena of diet-resistant and diet-induced obese rats, meaning that some rats easily develop obesity on high-fat diets whereas others are resistant and not hyperphagic [40]. In other experiments it was shown that rats adjust the amount eaten of more or less diluted diet to maintain energy intake and body weight [32, 41].

The strongest piece of evidence that AT mass is regulated comes from demonstrations , that experimentally induced increases in AT and body weight lead to coordinate decreases in eating and increases in EE, and vice versa, in rats [14, 16, 17, 42, 43] and humans [44-47] until AT mass or BW is restored (example see **Figure 1**). Furthermore, the magnitudes of the compensatory responses often parallel the degree of perturbation. Such data strongly support the hypothesis that AT mass is dynamically regulated by an active, negative-feedback mechanism. Such active regulation may also be one reason obese people tend to re-gain lost body weight [35].

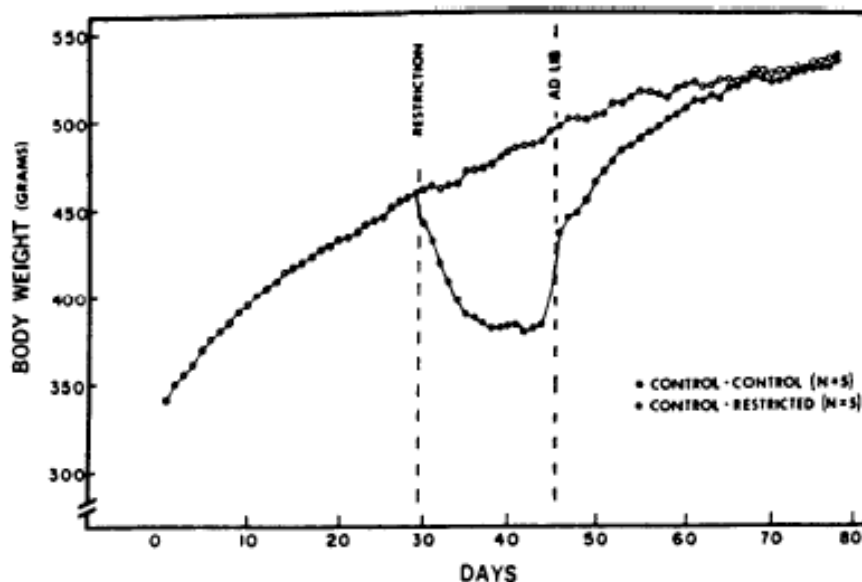


Figure 2. Example of body weight recovery after forced underweight by chronic food restriction in rats [16]. Rats were chronically food restricted, which induced underweight (control restricted). When rats were returned to ad libitum access to food (right dashed vertical line saying AD lib) rats returned to control body weight.

Body weight is often used as measure for body adiposity. But body weight changes often do not simply reflect AT mass [38]. Indeed, following experimental obesity in rats, body weight often recovers much more precisely than AT, which typically remains elevated [18, 42, 48]. Such findings suggest that lean body mass decreases below control levels. This, however, does not always occur. Harris et al. showed that BW and AT mass both remained elevated

and that protein content was not different from control rats [14]. Lean mass and AT mass also seem to recover at different rates. During recovery from underweight in rats, AT mass was recovered faster than protein content, whereas during recovery from overweight, protein content recovered faster than AT mass [14].

Lipectomy studies provide mixed support for regulation AT mass [49]. Seasonal animals, such as the Siberian hamster, return to control fat content after lipectomy [49, 50]. In contrast lipectomy in non-seasonal animals, such as rats and mice, the effects of lipectomy are inconsistent, with some studies reporting recovery of the fat loss whereas others do not [38, 49, 51-54]. Transplanting donor fat into mice resulted in compensatory reductions in the endogenous fat in one study [55]. Few studies of this type have been reported, probably because of the numerous limitations of the method, including that only small quantities of fat can be transplanted, that revascularization is inconsistent, and that re-innervation is limited or absent [49].

Traditionally, energy expenditure (EE) was regarded as less important than food intake in the regulation of AT, but this has changed in the last 40 years [15, 27, 56]. EE includes energy that is expended for metabolic costs of physiological processes such as the transmembrane ion gradients and resting cardiopulmonary activity (ca. 60% resting EE, REE), digesting and metabolizing food (ca. 10%, diet-induced thermogenesis, DIT), and remainder, mostly physical activity (30%) [57]. Overeating- or food restriction-induced weight gain or loss provide indirect evidence that EE is involved in the regulation of AT because the changes in weight are less than what can be predicted from the changes in energy intake [58]. More direct evidence that adjustments in EE contribute to regulation are provided by findings that oxygen consumption increases during overfeeding and decreases during food restriction more than predicted by the weight change per se [16, 58, 59]. Under steady-state conditions, EE and REE are linearly related to fat-free mass and fat-free mass explains ca. 80% of the individual differences in EE [57, 60]. During ongoing weight loss or gain, however, EE decreases more than what would be predicted by the change in FFM [59]. Furthermore, although the increases or decreases of EE that occur during periods of overeating or food restriction, become less during subsequent periods of weight stability, they do not disappear entirely [59]. The persistence of reduced EE in weight-reduced subjects favors the regain of weight [61, 62]. Thus adaptations in EE take place according to the changes in energy intake, in a way that minimize the effects on energy stores.

EE can be adjusted by altering resting metabolic rate, the efficiency and amount of physical activity (non-resting EE) or diet-induced thermogenesis. There is some evidence that these components of EE are differently affected during experimentally decreased or increased BW.

In humans, the decreased EE during food restriction and the maintenance of a reduced body weight was shown to be due to both a reduction in REE that was more than what was predicted by the loss of FFM and due to an increase in muscle work efficiency [59, 62]. In contrast increased EE during overeating and maintenance of an increased body weight was due to decreased skeletal muscle work efficiency at low levels of physical activity and increased DIT, but without a change in REE [59, 63]. Not all studies, however, have detected disproportionate increases in REE during experimentally induced weight gain [64, 65] and an increase in REE corrected for lean mass was also reported in one study [45]. This is consistent with some reports in rats that resting oxygen consumption changed disproportionately to weight during weight loss, but not during weight gain [16, 66]. Such data suggest that energy homeostatic response to weight loss is stronger than that to weight gain.

Despite the evidence for AT regulation by adjustments in EE and food intake, environmental factors, e.g. physical inactivity, stress, high-fat diets, and the palatability and availability of food can affect AT mass [67-69]. Given the increasing prevalence of obesity in the last decades, it seems as if the body is biased towards weight gain. From an evolutionary perspective, energy surfeits were rare and energy scarcity common, so it seems likely that there might be comparatively less selective advantage for a system that limit weight gain than for a system that opposes weight loss. Even though these teleological arguments seem plausible, the underlying mechanisms behind still have to be discovered [70].

Sensing AT mass

The lipostatic hypothesis put forward by Kennedy (1953) postulates that metabolites whose plasma levels are associated with body AT mass are sensed by the brain and control eating. The lipostatic hypothesis also supposes that there is a designated mass of AT, a lipostatic set point. In case of deviations from the set point (current state does not equal reference value), the brain initiates compensatory changes in food intake and EE until the designated AT mass is recovered [16, 27, 32, 71]. Evidence for circulating feedback signals in the blood that affect food intake came from studies on parabiotic rats, which share a peritoneal plasma filtrate. If one of them was made obese by lesions of the hypothalamic ventromedial nucleus, the partner reduced its body weight, and if one was overfed by stomach tube, its partner reduced body fat specifically [32, 72, 73].

An important step in identifying such a circulating factor was provided by parabiosis studies with the obese ob/ob and db/db mice and lean wild type mice. If a wild-type mouse or an

ob/ob mouse was paired with a db/db mouse, it reduced food intake and body weight, whereas the db/db mouse remained obese [74, 75]. This suggested that ob/ob mice have a mutated form of a hormone that signals AT mass and db/db mice have a mutated form of its receptor.

The subsequent identification of the ob gene, named leptin, in 1994 by Zhang et al. [76] a peptide hormone produced by white AT had a tremendous impact on research in the field of body weight regulation and is in large part responsible for the model of AT regulation that is described in the next section.

Prevalent model of AT regulation

The prevalent model of AT regulation is derived from the lipostatic hypothesis and in the wake of progress related to the discovery of leptin evolved to an "adiposity negative-feedback model" [23, 30, 76]. According to this model circulating factors inform the brain about the level of AT mass, and the brain compares the signal with the desired state and adjusts eating and EE in order to correct any errors; i.e., to maintain energy homeostasis (**Figure 2**).

Three main criteria that such "adiposity signals" should fulfill are: (1) they circulate in the blood in proportion to AT mass and have access to the brain, (2) physiological changes in their levels in the brain lead to eating and EE responses that promote weight and AT mass loss, and (3) inhibition of their central actions have the opposite effects, i.e. increase of AT mass by stimulating eating and reducing EE. Circulating factors including nutrients (e.g., glucose), cytokines (e.g., interleukin-6) and hormones (e.g., glucocorticoids) have been proposed to act as adiposity signals. Leptin and insulin are the most researched endocrine "adiposity signals" and are thought by many to fulfill these criteria [20, 23].

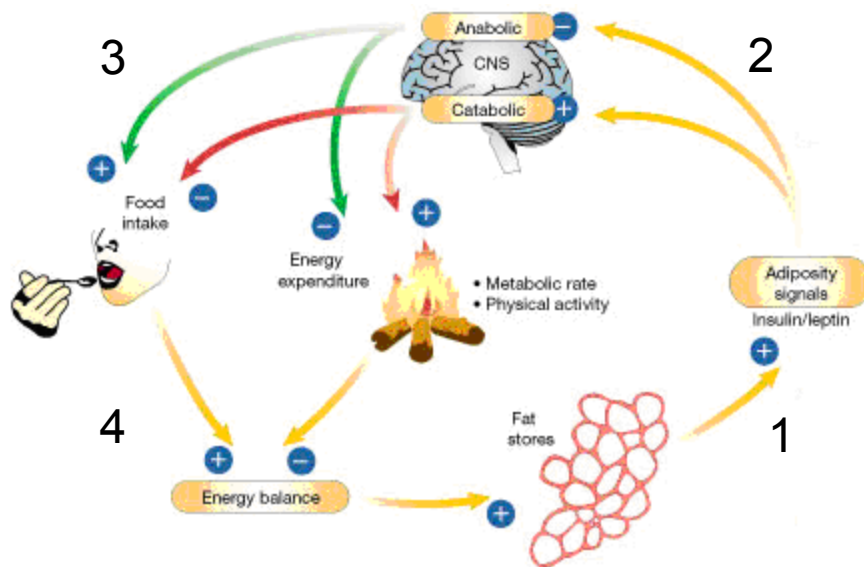


Figure 2. Model of how insulin and leptin act as adiposity signals. 1: Leptin and insulin circulate in the blood in concentrations proportional to body fat content and energy balance. 2: Leptin and insulin act on central effector pathways in the hypothalamus, repressing brain anabolic neural circuits that stimulate eating and inhibit energy expenditure, while simultaneously activating catabolic circuits that inhibit food intake and increase energy expenditure. 3: Low leptin and insulin levels in the brain during weight loss increase activity of anabolic neural pathways that stimulate eating and suppress energy expenditure, and decrease activity of catabolic pathways that cause anorexia and weight loss. 4: Ingestion of food generates neural and hormonal satiety signals to the hindbrain. Leptin/insulin-sensitive central effector pathways interact with hindbrain satiety circuits to regulate the meal size, thereby modulating food intake and energy balance [20].

The adjustment of eating consequently affects meal pattern. Meal-related signals that are thought to contribute to satiation are also integrated by the brain. These meal signals are thought to interact with "adiposity signals" and to be processed differently, depending on the state of AT mass. That is, in the case of increased fat stores the brain mediates reduced food intake by being more responsive to meal-related satiety signals, such as GLP-1, CCK and other gastrointestinal hormones (see **Figure 3**) [23, 77, 78].

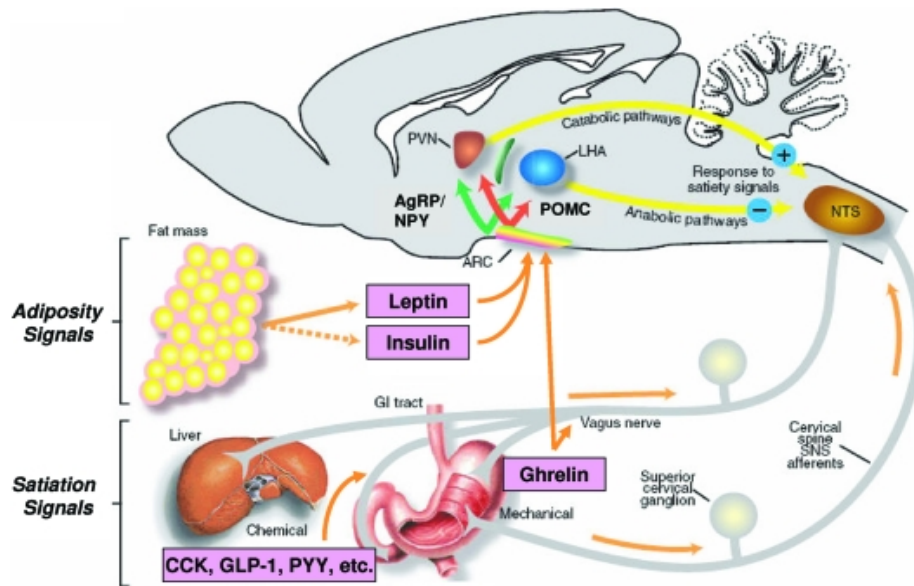


Figure 3. Model of the integration of satiety signals in the regulation of adipose tissue [78]. The arcuate nucleus of the hypothalamus (ARC) receives signals that reflect AT mass (adiposity signals) and the hindbrain (NTS, nucleus tractus solitarii) receives meal-related signals that are thought to contribute to satiety (satiety signals). In the case of increased AT mass the brain mediates reduced food intake by being more responsive to satiety signals, such as glucagon like peptide 1 (GLP-1), cholecystinin CCK and others.

3. The central neural network regulating adipose tissue mass

Brain areas involved in the regulation of adipose tissue mass

As described above, the principal characteristic of energy homeostasis is usually considered to be the regulation of AT mass. Because the brain controls eating and EE, it is crucial for the regulation of AT mass. Several brain areas are involved. It is thought that the hypothalamus is the key integrator of regulatory control signals [20, 23].

The hypothalamus receives various inputs related to the state of AT mass, called adiposity signals (described in detail below). These are processed in several hypothalamic areas and initiate signals to caudal brainstem areas that organize the motor controls of eating and EE. Adiposity signals, however, represent only one of many types of information that reach the hypothalamus. Additional inputs important for eating are neural and hormonal signals from the gastrointestinal tract, which are integrated in the caudal brainstem and project from there to the hypothalamus. Other signals, including learned controls and signals related to the palatability of the food, are processed in various forebrain areas that also project to the hypothalamus. Finally, genetic influences and other physiological conditions, such as pregnancy and lactation, provide inputs to the hypothalamus [20, 23, 25].

Early clinical reports and brain-lesion studies in rats demonstrated the importance of certain hypothalamic nuclei for the maintenance of "normal" body weight (for anatomic orientation see **Figure 4**). Rats with lesions of the lateral hypothalamic area (LHA) maintain a reduced body weight throughout life. The reduction in body weight is a result of a period of hypophagia, after which rats resume eating and weight gain with a similar rate as non-lesioned rats. These animals do not lose their ability to regulate body weight, because when challenged with food restriction they recover body weight like non-lesioned rats [79, 80]. Dorso-medial hypothalamic (DMH)-lesioned rats also have reduced body weight. An interesting difference between the two lesions is that whereas LHA lesions primarily affect body fat, DMH lesions do not change body composition [81-83]. Lesions of the ventromedial hypothalamus (VMH) or paraventricular hypothalamus (PVN) produce overeating and increase body weight [84-86]. Based on these studies, it was originally proposed that the brain contains certain hunger (e.g. LHA) and satiety (e.g. VMH) centers. This was soon

rejected as too simplistic, given the complexity of central pathways involved in the control of food intake and EE (reviewed in [38]).

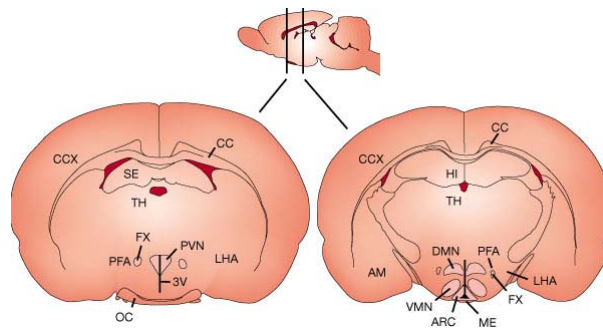


Figure 4. Cross-sections of a rat brain at two levels (indicated by vertical lines), showing hypothalamic nuclei involved in eating and EE that respond to peripheral signals related to energy status [20]. Cross-sections of the rat brain are shown at two levels, indicated by the vertical lines in the mid-sagittal view on the rat's brain on top of the figure. Hypothalamic structures that respond to adiposity signals are located in the arcuate nucleus (ARC), paraventricular nucleus (PVN) as well as the perifornical area (PFA) adjacent to the fornix (FX) and the lateral hypothalamic area (LHA), ventromedial nucleus (VMN) and dorsomedial nucleus (DMN). Abbreviations of other brain structures: AM, amygdala; CC, corpus callosum; CCX, cerebral cortex; HI, hippocampus; ME, median eminence; OC, optic chiasm; SE, septum; TH, thalamus; 3V, third cerebro-ventricle.

Undoubtedly the most influential current model (see **Figure 5**) of the neural regulation of AT mass is centered on another hypothalamic area, the arcuate nucleus (Arc). The model posits that the Arc neurons receive information about the state of AT mass via blood-borne signals (adiposity signals, e.g. leptin), in response alter synthesis and release of neurotransmitters that project to other hypothalamic and hindbrain areas involved in the control of eating, leading to tonic anabolic (increased eating, decreased EE; mediated especially by Arc NPY neurons) or catabolic (decreased eating, increased EE; mediated especially by α -MSH neurons) responses. These control signals converge on the dorsal vagal complex (DVC; NTS, nucleus tractus soletarii; AP, area postrema; DMX, dorsal motor nucleus of the vagus) of the caudal brainstem. The DVC integrates these signals as well as sensory inputs of gustatory (e.g. glossopharyngeal nerve in taste buds) and gastrointestinal (e.g., the vagus nerve) afferents, and endocrine input (e.g. amylin). Consequently the brainstem coordinates the organization and reflex control of ingestive behavior. Sympathetic nerve outflow from the brainstem changes EE and is a principal initiator of lipolysis in white AT [23, 87-89].

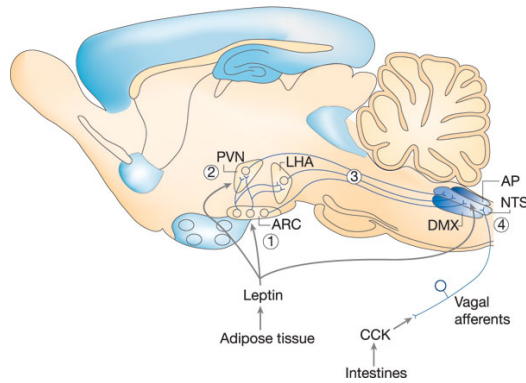


Figure 5: Prevalent model for integration of satiety signals (e.g. CCK) and adiposity signals (e.g. leptin) by the brain [23]. The brain coordinates the information on the state of adipose tissue mass (e.g. leptin) with meal related signals (e.g. CCK); increased levels of adipose tissue mass and leptin would enhance the satiating effect of CCK. (1) Leptin acts on the ARC, (2) other activated brain areas (3) hypothalamic nuclei which project to the caudal brainstem, (4) modulation of CCK's effect; leptin can also directly act on the NTS. ARC, arcuate nucleus of the hypothalamus; AP, area postrema; DMX, dorsal motor nucleus of the vagus nerve; LHA, lateral hypothalamic area; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus.

Although this hypothalamic-centric model is extremely influential, it is not without challenges. For example, many of the same neuropeptides and adiposity-signal receptors expressed in the Arc are also expressed in the caudal brainstem, and administration of those peptides or hormones into the 4th cerebral ventricle (cv) has similar effects on body weight as administration into the 3rd cv (reviewed in [89]). The extent to which adiposity signals act on the caudal brainstem is under current investigation, as well as the coordination of the brainstem and hypothalamus in response to the state of adiposity and the flux of energy substrates (reviewed in [9]).

Another perspective on energy homeostasis suggests that adiposity sensors are only one of several types of 'metabolic' receptors, and that these receptors are found in a widely distributed set of brain areas, so that the Arc plays no unique role in energy homeostasis [90]. This view is illustrated in **Figure 6**. Note, first, that this model includes sensors of many types located in many central and peripheral areas, and second, no specific brain area is assigned a role as the key integratory site.

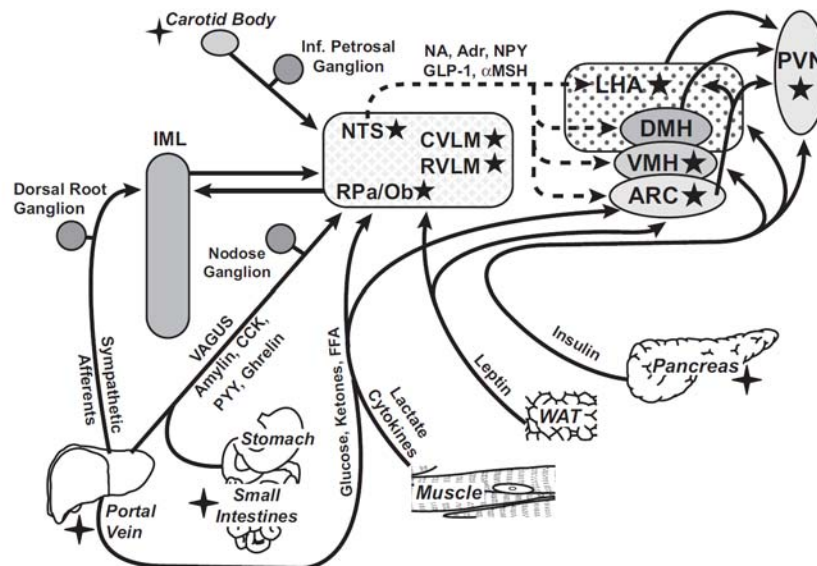


Figure 6. Model of convergence of a variety of peripheral signals (neuronal, energy metabolites, hormones) related to energy metabolism by neurons in the hypothalamus or hindbrain [90]. Metabolic-sensing neurons are in the caudal (CVLM) and rostral ventrolateral medulla (RVLM), contain noradrenaline, adrenaline, neuropeptide Y (NPY), glucagon-like peptide (GLP-1), and/or (α -melanocyte stimulating hormone (α -MSH), and project to the arcuate nucleus (ARC), lateral hypothalamic area (LHA), ventromedial nucleus (VMN) and dorsomedial nucleus (DMN) of the hypothalamus. Metabolic-sensing serotonin neurons in the raphé pallidus and obscurus (RPa/Ob) project to sympathetic preganglionic neurons in the intermediolateral (IML) cell column of the spinal cord.

The following section describes neurochemicals that are produced in the hypothalamus. As described in greater detail below, **study 2** of this thesis investigates the potential involvement of three neurochemicals produced in the Arc and PVN during dynamic regulation of AT mass in rats compensating for experimentally induced overweight, namely neuropeptide Y (NPY), α -melanocyte stimulating hormone (α -MSH) and corticotropin-releasing hormone (CRH).

Neurochemicals involved in the regulation of adipose tissue

This section provides an overview of several neurochemicals that have been implicated in the neural networks mediating the regulation of body weight and AT mass. Definitive evidence for a normal physiological role of neurochemical signals requires fulfilling a number of criteria that remain very difficult experimentally [24], and in each of the cases reviewed here, the evidence is incomplete. The majority of studies investigating the effects of neurochemicals on body weight or AT mass use pharmacological approaches, i.e.,

administration of the neurochemicals` agonists or antagonists. Another commonly used strategy is to study their expression patterns in response to experimentally induced increases or decreases in body weight or AT mass, in transgenic animals, etc. Many of these signals also produce short-term effects on food intake or EE without effects on body weight or respond themselves to short term manipulations of feeding state, e.g. fasting and refeeding. Such short-term studies, however, provide only weak and insufficient support for their hypothesized roles in long-term regulation of AT [19, 24]. Mutant animals, of course, do display long-term effects, but, as the review indicates, these often seem to be influenced by compensatory developmental changes rather than being pure lesion effects.

Neuropeptide Y (NPY)

NPY is a 36 amino acid peptide that belongs to the pancreatic polypeptide family and is produced throughout the brain [91]. Other members of the same family are peptide YY and pancreatic polypeptide, which influence energy balance as well [92]. In the hypothalamus, the Arc and DMH produce NPY [93, 94]. Arc neurons co-express NPY together with Agouti-related peptide (AgRP), which has similar effects on eating and EE [95]. Arc NPY neurons project to the PVN, LHA, DMH, VMN, median preoptic area and extrahypothalamic sites, e.g. dorsal vagal complex [9, 20, 93, 96, 97]. DMH NPY neurons also project to the DVC [98]. In addition, NPY is produced in the brainstem, with ascending projections to the PVN, DMH, median eminence, and median preoptic area [99].

There are 6 different NPY receptors (Y1 to 6 R). Y1R and Y5R are thought to mediate the effects on eating and EE [100]. Y1R are widely distributed throughout the brain, including both hypothalamic (PVN, DMH, VMH, Arc, LH) and extra-hypothalamic sites [101].

NPY administration into third, lateral cv or directly into the PVN of rats and mice stimulates eating by increasing meal size and meal duration, decreases energy expenditure and increases body weight and body fat [102-110]. Furthermore, specific up-regulation of DMH NPY gene expression by adeno-associated virus (AAV) injection increased food intake and body weight [98]. In addition, NPY injections into the perifornical area and VMH stimulated eating [100, 111]. Further, 4th cv injections increase eating and increase c-Fos expression in the PVN, which supports the idea that a bidirectional connection between the PVN and brainstem mediates NPY's effects on eating [112, 113].

There is also evidence supporting a role of endogenous NPY in the control of eating. Antagonism of NPY by administration of NPY antibodies into the 3rd cv [114] or by local

injections in the PVN [115] and ventromedial (VMN) nuclei [116] reduced food intake. Similarly AAV-mediated antagonism of NPY mRNA in the Arc results in decreased food intake and body weight [117].

Finally, NPY-induced increased in food intake can be reversed by intraperitoneal administration of Y1R or Y5R antagonists [100, 118, 119].

In contrast to the weight-increasing effect of chronic NPY administration, NPY knockout mice maintain normal body weight and body fat, which questions its necessity in AT mass regulation [120, 121]. It is possible, however that the genetic deficiency of NPY elicits compensatory mechanisms, masking a normal role in body-weight regulation [20].

In rodents rendered underweight by chronic food restriction, NPY mRNA levels in the Arc and DMH and NPY release in the PVN increase [122-127]. Similarly, NPY mRNA levels increased in the NTS [126]. Results from studies of experimentally induced overweight, however, do not produce such clear support. NPY mRNA levels and NPY content were decreased or unchanged in the Arc and PVN in rats fed a high-fat diet [128-131], and DMH NPY mRNA levels were increased [132].

In many rodent models of genetically induced obesity, e.g. db/db or MC 4 receptor knockout mice and fa/fa Zucker rats, Arc or DMH NPY expression or PVN NPY levels are elevated, which may explain the hyperphagia in these animals [100, 133, 134]. Interestingly, Arc and DMH NPY expression respond differently to high-fat diet feeding in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which are hyperphagic and obese due to a lack of the CCK-A receptor. That is, similar to genetically intact rats on high-fat diet, in which Arc NPY expression was decreased, and DMH NPY expression was increased [135].

Because of the apparent role of hypothalamic NPY in eating, EE and body weight regulation, in **study 2** of this thesis I used in situ hybridization to investigate the potential involvement of Arc and DMH NPY in the dynamic regulation of AT mass in rats compensating for experimentally induced overweight.

Agouti-related peptide (AgRP)

AgRP is expressed in Arc neurons together with NPY and, consequently, has the same projection targets [136]. AgRP is an inverse agonist for melanocortin (MC) 3/4 receptors, thus reversing their constitutive activity [137].

Administration of AgRP into the lateral or 3rd cv increased food intake [138]. Furthermore, ectopic overexpression of AgRP in the Agouti mouse lead to obesity [139]. Similar to NPY, however, knockout of AgRP did not result in increased weight and fat gain [121].

Underweight induced by chronic food restriction was associated with only a trend of increased AgRP mRNA levels measured in the entire hypothalamus or in the Arc [122, 123, 140]. In contrast, short-term food deprivation increased AgRP mRNA levels significantly, underlining the importance to differentiate short and long-term manipulations of body weight [122, 123].

Pro-opiomelanocortin (POMC)

POMC is a precursor for several peptides, with tissue-specific post-translational processing producing, among other, different melanocortins, beta-endorphin, and ACTH. POMC producing neurons are present in the Arc and NTS [136, 141]. The cleavage product alpha-melanocyte stimulating hormone (α -MSH) is thought to be involved in AT regulation. Neural α -MSH acts on melanocortin 3 and 4 receptors (MC3/4R) [21, 30, 142-146]. Arc α -MSH neurons project to the PVN, LH and NTS [147]. POMC is also produced by NTS neurons, especially in the DMX, which also expresses MC4R (reviewed in [136]).

Administration of α -MSH or the MC3/4R agonist MTII into the 3rd cv decreases food intake, increases EE, and decreases body weight [105, 146, 148-150]. Conversely, administration of the MC3/4R antagonist SHU9119 into the 3rd cv has the opposite effects [137, 150-154]. Lateral cv administration and 4th cv administration of MTII or SHU9119 had similar effects [155, 156]. Thus, hypothalamic and brain stem melanocortin system may act in coordinately in AT regulation (reviewed in [89, 136]).

Knockout of the POMC gene in mice [157] and POMC gene defect in humans [158] is associated with obesity. Knockout of the MC3/4R is associated with obesity in mice [159, 160]. In humans MC4R haploinsufficiency is the most common genetic cause for obesity and perhaps the most common genetic disease of any type [161, 162].

Underweight induced by chronic food restriction is associated with decreased Arc POMC mRNA levels [122, 125, 140], and overweight induced by intragastric-overfeeding is associated with increased Arc POMC mRNA levels [163]. Although high-fat diet feeding did not affect Arc POMC mRNA levels in one study, it decreased Arc α -MSH content in another [130, 164, 165]. In genetically obese, leptin-deficient ob/ob mice and Zucker fa/fa rats, Arc POMC mRNA levels and α -MSH contents were decreased [166].

To extend these data, in **study 2** of this thesis I investigated the potential involvement of Arc α -MSH, as indicated by POMC mRNA, in the dynamic regulation of AT mass in rats compensating for experimentally induced overweight.

Cocaine- and amphetamine-regulated transcript (CART)

CART is co-expressed with POMC in Arc neurons. CART is also expressed in the PVN, retrochiasmatic nucleus, DMH, zona incerta, LHA, perifornical area, premammillary nucleus, nucleus accumbens, pituitary, gut and vagus nerves [167]. In some of these areas CART is co-expressed with other neuropeptides, e.g. with melanin-concentrating hormone (MCH) in the LHA [168]. Arc CART/POMC neurons project mainly to the LHA.

Interestingly, CART seems to predominately affect eating, with little effect on EE. Lateral cv administration of recombinant CART reduced food intake and, similar to direct administration of CART into the PVN, also decreased NPY-induced eating [169-171]. Chronic lateral cv CART administration did not affect EE, but reduced food intake and body weight in obese Zucker (*fa/fa*) or high-fat diet fed rats and lean rats [172, 173]. Lateral cv delivered AAV leading to CART overexpression delivered into the lateral reduced food intake and body weight gain in rats fed a palatable, energy-dense diet, but this was due to reduction in lean mass rather than in fat mass [174]. CART overexpression did not affect EE. Finally, lateral cv administration of CART antibodies increased food intake [169, 170]. CART knockout mice fed a high-fat diet had increased body weight and fat mass and were hyperphagic, but did not change EE [175]. In genetically leptin-deficient animals, such as obese Zucker (*fa/fa*) rats or *ob/ob* mice, Arc CART mRNA levels were decreased [169]. In humans, polymorphism in the CART gene may be associated with obesity [167].

Chronic food-restriction-induced underweight does not seem to be associated with a decrease in hypothalamic CART mRNA levels, although fasting rats for 1 or 2 days is [123, 169, 176], which re-emphasizes that fasting is not adequate to test the involvement of a neurochemical in AT mass regulation. In high-fat-diet-fed rats, overweight and increased body fat are associated with increased hypothalamic or Arc CART mRNA levels [172, 177], which may contribute to the hyperphagia on such palatable diets.

Serotonin (5HT)

Serotonin synthesized from tryptophan, inhibited eating when administered into the 4th cv [178] and analogs are used as weight-loss agents in humans (reviewed in [179]). Effects on food intake are thought to be mediated by Arc neurons. Arc POMC neurons receive serotonergic input via 5HT-2C receptors and NPY neurons via 5HT-1B receptors from the NTS, see **Figure 7** (reviewed in [9]). Activation of these receptors via D-fenfluramine, a 5HT agonist, led to a reduction in food intake and further activation of MC4R on PVN neurons [178]. Similarly, administration of the MC3/4 receptor antagonist SHU reduced the reduction of food intake by fenfluramine (reviewed in [179]).

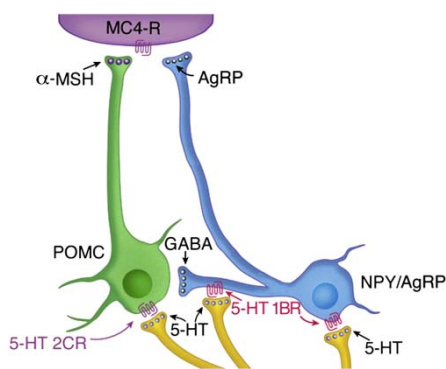


Figure 7. Model of the connectivity of NPY/AgRP and POMC neurons in the Arc that act on MC4 R neurons in the PVN. POMC neurons receive serotonergic input via 5HT-2C receptors and NPY neurons via 5HT-1B receptors from the NTS. Within the Arc NPY can inhibit POMC neurons via either Y1R or release of gamma-amino butyric acid (GABA) [9, 178].

Corticotropin-releasing hormone (CRH)

CRH is a classical neuroendocrine hormone and is part of the hypothalamic-pituitary-adrenal axis (HPA): it is synthesized by PVN neurons, released from nerve terminals in the median eminence, enters the posterior pituitary via hypophysial portal circulation via diffusion, is carried to the anterior pituitary, and acts there to stimulate adrenocorticotrophic hormone (ACTH) secretion, which then stimulates the synthesis and release of corticosteroid hormones (mainly corticosterone in rodents and cortisol in humans). Among other effects, stress-induced activation of the HPA axis is also thought to affect eating and EE [180]. In addition, independent of the stress response and activation of the HPA axis, CRH and related peptides such as urocortin have neurocrine actions, i.e., function as neurotransmitters in numerous brain areas. Hypothalamic CRH is thought to be involved in the control of eating and EE [181-183].

PVN CRH neurons project to several nuclei in the midbrain and hindbrain, and also to other nuclei within the hypothalamus [20, 182-185] and exert their effects on eating and EE mainly

via CRH 2 receptors (CRH2R) [186, 187]. In contrast, CRH's neuroendocrine effects are mediated primarily by CRH 1 receptors [183, 188].

Administration of CRH into the lateral or 3rd cv or directly into the PVN inhibits eating, increases EE and reduces body weight [105, 182, 184, 186, 189-192]. The reduction in body weight is predominately due to a reduction in body fat [193]. Higher doses of CRH inhibit eating non-specifically, as indicated by the formation of conditioned taste aversions; lower doses, however, appear to act specifically [184, 194, 195].

The catabolic effects of CRH on eating and EE contrast to the anabolic effects of glucocorticoids [183]. The interplay between the HPA axis, eating and EE can be demonstrated in adrenalectomized (ADX) rats. ADX reduces food intake and body weight, which can be reversed by systemic administration of glucocorticoids or administration of a CRH2R antagonist into the lateral cerebral ventricle [196, 197]. Central administration of CRH also increases levels of circulating glucocorticoids [193, 198], so a clear distinction between the effects of CRH and of glucocorticoids is difficult. Because centrally administered CRH reduces body weight despite stimulating corticosteroid release, it seems that the effects of CRH predominate.

A demonstration that administration of CRH2R antagonists into the lateral cerebral ventricle increased food intake and body weight [186] suggests that endogenous CRH controls eating and body weight. CRH and CRH2R knockout mice, however, had unaltered phenotypes [199, 200]. As in the case of NPY discussed above, this may be due to compensatory developmental effects rather than indicating that CRH signaling is not normally involved in eating and weight regulation.

Few data exist on chronic effects of experimentally altered body weight on CRH expression. In one study, whole hypothalamic CRH mRNA levels decreased during underweight, but in another they did not change [123, 201]. PVN CRH mRNA levels increased during intragastric overfeeding-induced overweight rats [164]. In genetic models of obesity, CRH production remains relatively unaffected [202]. For instance hypothalamic CRH mRNA levels were not different in obese Zucker (*fa/fa*) rats compared to wild-type rats [203] and Arc and PVN CRH concentrations were not different in *ob/ob* mice compared to their lean counterparts [204].

CRH may be a downstream mediator in the neural network driven by Arc melanocortin neurons. PVN CRH neurons express MC3/4R, and administration of a MC3/4R agonist into the 3rd cv increased PVN CRH expression. Furthermore, the potency of the MC3/4R agonist

alone to reduce food intake was attenuated by co-administration of a CRH receptor antagonist [205].

Because of the apparent importance of neurocrine CRH in energy homeostasis, in **study 2** of this thesis I use in situ hybridization of PVN CRH mRNA and radioimmunoassay of plasma corticosterone to assess the roles of neurocrine and neuroendocrine CRH in the dynamic regulation of AT mass in rats compensating for experimentally induced overweight.

Melanin concentrating hormone (MCH)

MCH is expressed mainly in the LHA and zona incerta [206, 207] and acts on MCH receptor 1 (MCH-1R), which are expressed in numerous brain sites, including cortical areas, olfactory bulb, hippocampus, NTS, DMX and reticular formation [208]. MCH neurons receive input from Arc NPY, AgRP and α -MSH expressing neurons [9].

MCH administration into the lateral or 3rd cv increases food intake, body weight, and AT and decreases EE [138, 206, 209-212]. Interestingly, unlike all the other neurochemicals reviewed here, 4th cv administration of MCH did not affect food intake [213]. Whether it affects EE is unknown.

Overexpressing MCH in the lateral hypothalamus resulted in hyperphagia and increased body weight and AT mass [214]. MCH knockout mice were hypophagic and hyperactive [215, 216]. Antagonizing endogenous MCH by administration of a MCH 1 receptor antagonist, however reduced food intake and body weight without affecting activity [217, 218]. In contrast, MCH R1 knockout mice were slightly hyperphagic, although lean, perhaps as a consequence of their hyperactivity [138].

One study of chronically food-restricted underweight rats failed to detect any change in hypothalamic MCH mRNA [123].

Orexin-A. Orexin-A expressing neurons are present in the LHA. They receive input from Arc NPY, AgRP and α -MSH neurons [9] and project to Arc POMC and NPY neurons [219]. Chronic administration of orexin-A into the PVN resulted in body weight loss, but had no effect on food intake [220]. Acute administration of orexin-A into the 3rd cv, however was shown to increase both food intake and EE [105]. Administration of orexin-A into the 4th cv had a small but significant effect on food intake [221]. Very few data are available on expression of orexin in situations of altered body weight. In underweight rats hypothalamic orexin –A mRNA levels were not changed [123].

Summary. A variety of evidence strongly supports, but according to the most rigorous criteria [24] does not prove, roles for the hypothalamic neurochemicals reviewed here in the physiological control of eating and EE. Similar evidence exists for other neurochemicals, including GABA, or galanin [9]. That several of these neurochemicals change with body weight supports the hypothesis that they contribute to energy homeostasis. Most studies, however, involve steady-state conditions, which fail to establish whether changed expression levels or activity are related to the dynamic compensatory changes in eating and EE. Thus, **study 2** of this thesis, although very limited in scope, makes a substantial contribution to the physiological understanding of the regulation of body weight and AT mass.

4. Adiposity signals in the regulation of adipose tissue mass

Morton et al. [23] proposed three main criteria for endocrine adiposity signals: 1) Adiposity signals circulate in the blood in proportion to AT adipose mass and have access to the brain; 2) Adiposity signals promote weight and AT mass changes by acting on the neural networks controlling eating and EE; and 3) Inhibition of their central actions promotes weight and AT gain.

Leptin and insulin are the most researched endocrine adiposity signals and are thought by some to fulfill these criteria [15, 20, 21, 23]. Amylin [222], ghrelin [223], peptide YY [224] among others, have also been proposed as endocrine adiposity signals, although the evidence for their involvement is less strong. The following sections review the status of insulin, leptin, amylin and ghrelin, all of which were tested in this thesis, as endocrine adiposity signals with respect to the criteria given above.

Insulin and leptin

Insulin, as is well known, is essential for the regulation of glucose levels. Insulin is produced by pancreatic beta cells and secreted into the circulation, where it has a half-life of 2-3 minutes [225]. Insulin inhibits hepatic gluconeogenesis and glycogenolysis and stimulates glucose uptake by insulin-sensitive tissues. Furthermore, insulin induces the liver, muscles and AT to store glucose either as glycogen or indirectly as fat [226]. In the 1970s, Woods and colleagues discovered that insulin acts in the brain to inhibit food intake and decrease body weight. They developed the concept of endocrine adiposity signals [35, 227].

As described above in mice single-gene mutations of a gene later discovered to encode leptin (*ob/ob* mice) and a gene encoding its receptor (*db/db*) were long-known models of obesity that prompted the search resulting in the discovery of leptin in 1994 [228]. Leptin has been considered as an adiposity signals since its discovery [76]. Genetic leptin deficiency in humans also results in hyperphagia and obesity, which can be reversed by leptin treatment [229, 230]. Apart from its function in body weight regulation, leptin is also involved in the control of the thyroid axis, reproductive axis, glucose homeostasis, immune function, growth, and other functions [231].

Leptin is produced by adipocytes [76, 232]. Its production and secretion are regulated by various factors, which are mostly related to the metabolic activity of the adipocyte [233-235]. When secreted into the blood stream the half-life of leptin is 45 minutes [225].

Insulin is transported across the blood-brain barrier via a receptor-mediated mechanism [35, 225, 227]. A rise in plasma insulin is followed by a rise in CSF insulin levels with a time lag of 60-90 min. After a meal or glucose bolus, the half-life of insulin in the CSF is about 140 minutes [236]. The entry of insulin into the brain is reduced during fasting or in dietary-induced obesity (reviewed in [237]).

Insulin receptors are highly expressed in the hypothalamus, especially in the Arc, and are also present in the DMH, PVN nuclei, and choroid plexus [77, 238]. Insulin receptors are also expressed in the DVC [239]. Activation of insulin receptors initiates a similar intracellular signaling cascade compared to peripheral tissues utilizing insulin receptor substrate-phosphatidylinositol 3-OH kinase. In the hypothalamus Arc neurons use the IRS-2, whereas the majority of cells use IRS-1 (reviewed in [237]).

Leptin has access to the brain via a saturable transport mechanism at the blood-brain barrier [240]. This is consistent with a cross-sectional study that revealed a strong correlation between plasma and cerebrospinal fluid (CSF) leptin levels in obese and lean subjects, with the plasma : CSF concentrations related by a log function so that the higher the plasma leptin level, the greater the relative difference between plasma and CSF insulin [241] .

The leptin receptor (LepR , formerly ob-R) belongs to the cytokine receptor family receptor. Several isoforms exist. The long form of the leptin receptor (LepR^b) mediates its physiological actions [242]. The mutation in the db/db mice is in LepR^b. LepR^b is densely expressed in the Arc and is also expressed in the choroid plexus, VMH, LH, DMH, and PVN [243, 244]. LepR^b are also present in the NTS, area postrema (AP) and the dorsal motor nucleus of the vague (DMX) of the dorsal vagal complex and the parabrachial nucleus [245, 246]. Binding of leptin to its receptor activates the JAK-STAT signaling pathway, which is a very common signal transduction pathway for cytokines (reviewed in [247]). Leptin was also shown to activate insulin receptor substrate-2 (IRS-2), which is an example for the converging effects of insulin and leptin on intracellular mechanisms [248].

Basal plasma insulin and leptin levels reflect AT mass under many conditions.

Numerous cross-sectional studies indicate that basal plasma levels of insulin and leptin are significantly correlated with BW or estimates of AT, such as BMI, resected AT mass, or AT volume estimated by computed tomography (CT) or magnetic resonance imaging in humans [19, 249-251] and rats [252-254]. Numerous studies in human subjects also show that in

situations of experimentally induced increases or decreases in body weight, leptin and insulin levels increase and decrease appropriately in both humans [46, 255-257] and rats [140, 232, 258-261]. Taken together, in such steady-state situations, plasma insulin and leptin reflect AT mass.

Basal plasma leptin levels are also proportional to body weight during ongoing weight gain, but dissociate from body weight during ongoing weight loss [19, 255]. For instance plasma leptin decreases markedly during early food restriction, before weight changes become evident [262, 263]. During ongoing weight loss, leptin levels are lower than during maintenance of the reduced body weight [19, 255]. Such dissociations from AT mass, in which leptin seems to be more closely related to the chronic negative energy balance, supports the hypothesis that leptin works rather as a starvation signal than an adiposity signal [15, 31]. Another dissociation of plasma insulin, leptin, and AT mass occurs in obese Roux-en-Y gastric-bypass surgery patients, who have significantly reduced leptin and insulin levels compared to BMI-matched controls and almost identical levels compared to lean subjects [264]. A recent study showed that leptin (by 50%) and insulin plasma levels fell markedly within the first week after bariatric surgery; by then weight loss was only 4% [265].

There are very few descriptions of levels of adiposity signals during dynamic regulation of AT mass or body weight. As described above, dynamic regulation refers to situations characterized by compensatory responses in eating and EE following experimentally induced changes in weight and AT mass. Furthermore, in most published studies of adiposity signals during dynamic regulation, design weaknesses prevent clear interpretations. For example, in several, manipulations were too short, i.e., only 1-3 d, to clearly distinguish the influences of changed AT mass on hormone levels from influences of acute changes in energy metabolite flux, gut contents, etc. In others, AT mass was not measured, the induced changes in AT mass or BW were not sufficient to induce longer-lasting compensatory responses, or the responses were measured when AT mass or BW was no longer significantly changing. For example, White et al. recently reported that plasma concentrations of insulin, leptin and PYY returned to control levels within 2 d after rats that had been rendered 45 g overweight by chronic IG infusions were returned to ad libitum feeding [266]. At the 2 d time point, however, rats were no longer losing BW and AT was not measured, so it was not clear if any effective compensation was occurring. Only for insulin do clear studies exist. In two studies insulin was tracked during longer periods of dynamic compensation for substantial increases in AT mass [14, 267], and neither supported the hypothesis that basal plasma insulin functions as an adiposity signal. Rather, insulin returned to control levels during recovery while AT was still significantly increased and while eating was still decreased.

Chronic insulin and leptin administration decreases AT mass. Insulin reduces food intake and body weight when administered into the lateral cv of baboons, into the 3rd cv or directly into the ventral hypothalamus of rats, or via a nasal spray in humans [225, 227, 268-270]. Studies of the effects on eating of peripheral administration of insulin are complicated by insulin's hypoglycemic actions, which can stimulate eating. Nevertheless, intravenously administered insulin has been shown to reduce food intake and body weight without inducing hypoglycemia (reviewed in [35]). Central administration of insulin also affects peripheral blood glucose levels, suggesting that similar brain mechanisms might be involved in the regulation of body weight and of blood glucose levels [271, 272]. Central administration of insulin suppresses hepatic glucose production and increases pancreatic insulin secretion, which is mediated by the vagus nerve, and dysfunction of the hypothalamic insulin receptor signaling pathway was shown to result in hyperglycemia (reviewed in [227, 272, 273]).

Antagonizing the effects of endogenous insulin is difficult because receptor antagonists do not exist [225]. Studies in transgenic mice with altered insulin signaling, however, provide some support for the adiposity signal hypothesis. Female mice that lack brain insulin receptors (NIRKO mice) were hyperphagic and obese, although male mice were not [274]. Conversely, mice with increased insulin signaling through genetic disruption of an intracellular insulin receptor inhibitor, reduced diet-induced weight gain [275]. In contrast specific insulin receptor gene disruption in POMC and AgRP neurons in the Arc of the hypothalamus did not alter food intake and body weight [276].

Intraperitoneal, subcutaneous, and 3rd, lateral, or 4th cv administration of leptin increases EE and reduces food intake and body weight in mice and rats [154, 230, 244, 246, 277-280]. Lateral and 4th cv administration affect body weight similarly, which suggests that both hypothalamus and brainstem are similarly important in mediating leptin's effects [246]. Evidence for an important role for endogenous leptin in the regulation of body weight comes from a study that administered a leptin antagonist over 7 days into the lateral cv. The leptin antagonist increased food intake and body weight and reversed exogenous leptin's effects on food intake, EE and body weight [281].

The potencies of insulin and leptin to decrease eating and body weight seem to depend on the state of AT mass, such that the hormones' actions are decreased in obese compared to lean animals and humans [228, 237]. This is referred to as central insulin and leptin resistance, respectively. Central insulin resistance can be observed in obese Zucker rats, which are insensitive to the eating inhibitory effect of centrally administered insulin compared to lean Zucker rats (reviewed in [227]). Type 2 diabetes, which is one of the most frequent negative health consequences associated with obesity, is characterized by high plasma

insulin and glucose levels. Not only peripheral but also central resistance to the action of insulin and other nutrient- or adiposity-related signals are thought to contribute to the progressive development of type 2 diabetes [272].

Peripherally administered leptin failed to reduce food intake and to activate STAT-3 in diet-obese mice [282, 283]. The potency of centrally administered leptin was not reduced as much, suggesting that part of the resistance was due to reduced passage across the blood-brain barrier. Conflicting results have been reported in tests of leptin in diet-induced obese rats [130, 284]. Finally, leptin monotherapy in obese humans was effective only if the doses were repeatedly increased [285]. Such loss of central sensitivity to leptin when circulating leptin levels are high suggests leptin resistance. Leptin resistance thought to have several causes, including: (1) defects of leptin transport across the blood-brain barrier; indeed obese compared to lean subjects transport a smaller portion of the plasma leptin into the CSF, thus are less efficient in leptin uptake [241]; (2) disturbances in receptor and intracellular-signaling function; and (3) alterations in the responsiveness of downstream neuronal circuits [247].

Combining leptin with amylin or other adiposity signals appear more promising than leptin monotherapy for the treatment of obesity [286]. Also the combination of leptin administration with low calorie intake was shown to circumvent some of the negative metabolic side effects of dieting, e.g. reduction of EE, which favor weight regain [256].

Insulin and leptin act in brain areas that control eating and EE. Arc neurons are thought to be the key site of leptin's and insulin's actions because Arc NPY neurons co-express LepR^b and IRS-2. DMH NPY neurons, in contrast, do not, so may be stimulated by leptin and insulin only indirectly [122, 243, 287-289]. Administration of insulin or leptin into the 3rd cv suppresses Arc NPY expression [244, 290]. In line with this, genetically obese rodents that do not produce leptin (ob/ob mice) or LepR^b (db/db mice and fa/fa Zucker rats) have increased hypothalamic NPY levels (reviewed in [100]). Reestablishing leptin signaling, for instance by subcutaneous administration of leptin in ob/ob mice, reduced body weight gain and Arc NPY mRNA levels [291]. Further, leptin's effect to reduce food intake was reduced by administration of NPY directly into the PVN [292]. The importance for NPY in leptin signaling, however, is questioned by a report of no difference in the effect of leptin to reduce body weight in NPY-knockout and control mice; NPY-knockout mice, however, do not have an obese phenotype, suggesting developmental compensation for the lesion [120]. On the other hand, acute knockdown of NPY in ob/ob mice reduced excess body weight by decreasing food intake and increasing EE [293]. Similarly, AgRP, which is co-expressed with NPY in Arc neurons, is inhibited by leptin. Intraperitoneal administration of leptin reduced Arc

AgRP expression in mice [294]. Lateral cv administration of leptin was able to reverse fasting-induced upregulation of Arc AgRP expression. Interestingly, however, insulin administration was not [295].

Insulin and leptin appear to act on distinct populations of Arc POMC expressing neurons [296-298]. Mice lacking the leptin receptor in POMC neurons were obese and had reduced hypothalamic POMC expression [299]. Administration of insulin into the 3rd cv increases Arc POMC expression [296, 300]. Furthermore, insulin's effect to reduce food intake and body weight was inhibited by administration of antagonists to MC3/4R into the 3rd cv [154, 296]. Leptin administered into the lateral cv and 3rd cv increased POMC mRNA levels in the Arc [279, 296, 300, 301]. Leptin was also shown to affect its downstream targets: intraperitoneally injected leptin increased MC4R mRNA on PVN neurons [302]. Furthermore, leptin's effect to reduce food intake, body weight and c-fos expression in the PVN was inhibited by pretreatment with 3rd cv injections of MC3/4R antagonists [154, 296]. Arc POMC mRNA levels was increased in db/db mice, and this was reversed by leptin treatment [166].

Leptin also stimulated the production of CART, which is co-expressed with POMC in Arc neurons [303]. Chronic ip administration of leptin increased Arc CART expression and decreased body weight in db/db mice [169]. Lateral cv administration of leptin, but not of insulin, reversed fasting-induced down-regulation of Arc CART expression [295]. Furthermore, leptin induced c-fos and SOCS-3 expression in CART neurons in the Arc, PVN, DMH and ventral premammillary nucleus, all of which also express LepR^b [168].

The activation of POMC/CART and NPY/AgRP neurons by leptin and insulin leads projections to MCH and orexin neurons in the LHA, to CRH, oxytocin, and other PVN neurons, and to the DMH and VMH. More recently it has been shown that leptin can also act directly on many of these neurons [242, 302, 304].

Study 2 of this thesis includes investigation of PVN CRH signaling. Several data relate the hypothalamic effects of leptin and insulin to PVN CRH: (1) Central administration of leptin increased the expression of CRH mRNA in the PVN, which was associated with a reduction in food intake [244]. (2) Co-administration of leptin and a CRH antagonist attenuated the effects of leptin to reduce food intake and body weight [305]. (3) The effect of insulin to reduce food intake was enhanced by co-administration of CRH [306]. Whether leptin activates CRH expression directly via acting on LepR^b on PVN neurons or indirectly via activation of Arc neurons is not yet clear [147].

Amylin

Amylin, or islet amyloid polypeptide, is secreted from pancreatic beta cells together with insulin. Basal levels of amylin (~3 pM) are much lower than those of insulin (~80 pM) in normal-weight adult humans subjects, and their plasma levels rise in this proportion after an oral glucose load [307]. Amylin's half-life in the blood is 15 min. Amylin has also been proposed to act as adiposity signal [222].

Amylin reflects AT mass. Basal plasma amylin levels increase with adiposity in humans [308-310], rats and mice [311-313], but no correlation analysis has been reported. Plasma amylin levels have not been tested during dynamic regulation of body adiposity.

Chronic amylin administration decreases AT mass. Chronic subcutaneous and central administration of amylin into the 3rd or lateral cv of reduces food intake by decreasing meal size and body weight, especially body fat [314-319]. Chronic administration of amylin did not seem to affect EE when normalized to lean mass, but may do so when normalized to body weight [318-320]. UCP-2 expression also did not change in fat or skeletal muscle tissue after 5 d of subcutaneous amylin administration [318, 319]. However, amylin was shown to prevent compensatory decreases in EE in weight-reduced rats [320]. In obese humans, the amylin analogue pramlintide effectively induced weight loss [321].

There are several studies indicating that administration of amylin antagonists attenuate amylin's eating-inhibitory effect, supporting the physiological role of endogenous amylin in controlling food intake and body weight (reviewed in [237, 322, 323]). Most convincingly, antagonizing brain amylin receptors by chronic 3rd cv administration of the amylin receptor antagonist AC 187 increased food intake and body fat [317].

Amylin acts on central mechanisms involved in eating and EE. Amylin seems to act on neurons in the AP, an area without a BBB. The amylin receptor consists of a calcitonin receptor and receptor activity modifying proteins (RAMP) and is densely expressed in the AP, although it has not yet been shown that it is specifically expressed by amylin-responsive neurons [321]. Amylin no longer inhibited eating in AP-lesioned rats [324, 325]; amylin administration directly into the AP stimulated food intake, and administration of amylin antagonist AC 187 directly into the AP had the opposite effect [326]. The extracellular-signal regulated kinase 1 and 2 (ERK1/2) cascade might be involved in transducing amylin's neural effects [326]. Recent studies suggest that noradrenergic neurons in the AP are necessary for amylin's eating-inhibitory effect (reviewed in [327]). Amylin-induced neural signaling projects from the AP via the NTS and lateral parabrachial nucleus (LPBN) to the Arc, VMH and other forebrain areas (reviewed in [222, 327]). Peripheral administration of amylin or its agonist

salmon calcitonin down-regulated expression of orexin and MCH in the LHA, but had no effect on Arc NPY, AgRP, POMC or CART expression [328].

Ghrelin

Ghrelin, discovered in 1999 [329], is produced by gastric X cells, some CNS neurons. Ghrelin differs from other gut hormones in that its plasma levels increase before meals and decrease after meals and in that ghrelin administration increases food intake, whereas other gut hormones that affect eating decrease food intake [223]. Ghrelin circulates in both as acylated form and as des-acyl ghrelin; although both forms may have physiological roles, only acylated ghrelin stimulates eating (reviewed in [330]).

Ghrelin reflects AT mass. In contrast to insulin, leptin and amylin, basal plasma ghrelin levels are decreased in obesity. Basal ghrelin was negatively correlated with body mass index (BMI) in humans [331, 332] and with body weight and fat pad weight in rats [333]. Ghrelin plasma levels also increased after experimentally induced body weight loss [140, 334, 335] and decreased after weight gain [259, 333, 336, 337]. Ghrelin- and ghrelin receptor-knockout mice do not have an obesity phenotype when maintained on chow, but are protected against obesity when fed a high-fat diet (reviewed in [330]). Ghrelin levels were decreased in mice that are obese due to genetic defects in leptin signaling, so ghrelin apparently was not contribute to their hyperphagia here [335]. Like leptin and insulin, ghrelin was dissociated from AT mass in obese patients who underwent Roux-en-Y gastric bypass surgery, although this has not been seen in all studies (reviewed in [223]). For example, In one study diurnal plasma ghrelin levels were lower in Roux-en Y gastric bypass than in BMI-matched controls who lost body weight by dieting [334], whereas in another, ghrelin levels were reduced after surgery similarly to BMI-matched controls [264].

Chronic ghrelin administration increases AT mass. Chronic ghrelin administration into the 3rd or lateral cv increased food intake and body weight, predominately due to increase in fat, in rats and mice [338-341]. Some [342], but not all [339] studies indicate that ghrelin decreases in EE. 4th cv administration of ghrelin also was reported to increase food intake [343].

GHS-R1a which is a G protein-coupled receptor, is thought to mediate ghrelin's eating effects (reviewed in [330, 344]). Ghrelin-induced eating was blocked by lateral cv administration of the GHS-R antagonist [D-Lys-3]-GHRP-6 [345], and specific inhibition of GHS-R expression in the Arc of the hypothalamus decreased food intake, body weight and

the weight of several fat pads [346]. Finally, acute administration of a ghrelin antibody alone reduced food intake, suggestive of a physiological role for endogenous ghrelin in controlling eating [338].

Ghrelin acts on central mechanisms involved in eating and EE. Where ghrelin acts in the brain to control eating is not clear [223]. The ghrelin receptor is present in the Arc, VMN and PVN as well as other brain areas, including the brainstem (e.g. DVC). Abdominal vagal afferents also express ghrelin receptors, but vagal afferents were shown to be unnecessary for ghrelin's eating-inhibitory effect [347].

GHS-R1a are expressed by Arc NPY/AgRP neurons [348], and ghrelin administration into the lateral cv increased Arc NPY and AgRP expression [340]. That NPY might mediate ghrelin's eating-stimulatory effect is suggested by reports that icv administration of antibodies against NPY or AgRP or Y 1 and Y 5 receptor antagonists blocked ghrelin's eating-stimulatory effect [338]. Administration of α -MSH reduced ghrelin's effect on food intake [338], although ghrelin itself did not affect Arc POMC expression [348].

5. Role of estrogens in the regulation of adipose tissue mass and distribution

Sex is a physiological modulator of body weight and body composition. Differences in the levels of circulating gonadal steroid hormones (estrogens, androgens, progestins) are thought to contribute to differences in AT mass and regional AT distribution [12, 349, 350]. In the following I briefly review: (1) sex differences in AT mass and distribution and their health consequences; (2) relationships between plasma estrogen levels and AT mass and distribution; (3) potential brain mechanisms for sex differences in eating and EE; and (4) potential mechanisms for sex differences in regional AT distribution.

Sex differences in adipose tissue mass and distribution and their health consequences

The regulation of AT is sexually differentiated: women have more AT and less lean tissue than men of similar weight or BMI [34, 251], and young adult female rats and mice have more fat than equal-age males [54, 351]. In addition, AT is differently distributed in men and women: women accumulate more fat in the gluteo-femoral region (gynoid fat), and men in the abdominal region (android fat) [4]. Abdominal fat includes abdominal subcutaneous AT (SAT) and intra-abdominal AT (IAAT), which in turn includes visceral AT, whose circulation drains into the hepatic portal vein (i.e., omental and mesenteric AT), and non-visceral AT, whose circulation enters the vena cava (gonadal and peri-renal AT) [352] [4]. Men tend to have more abdominal SAT as well as more IAAT [34, 251]. The differences are difficult to quantify, however, because of differences in different populations of humans as well as the imprecision of all but whole-body imaging studies. Even computed tomography (CT) and magnetic-resonance imaging (MRI) scans, which are increasingly used in basic research, are usually limited to one or a few levels, are not sufficiently precise to detect physiologically significant changes in the amount and regional distribution of AT [34, 353-358]. The few whole body imaging studies available showed that women have relatively more whole-body SAT, whereas men had more IAAT [34, 251]. In women the percentage of IAAT of total AT increased between 25 and 65 years from 2-8 % and in men from 6-17% of total AT [34].

In rats, the relative and absolute amounts of IAAT compared to SAT are much larger than in humans. Imaging IAAT in male rats by CT or MRI revealed that about 40% of total AT is IAAT [359, 360]. There are no imaging studies comparing SAT and IAAT in male and female

rats. Clegg et al. estimated that male rats have relatively more IAAT than SAT [361], but did not measure AT depots directly; rather, SAT was resected together with the skin and IAAT was estimated as the fat contents of the remaining carcass as measured by chemical analysis. This estimate is very imprecise, however, because in the MRI study referred to above showed that obese male rats deposited substantial amounts of excess fat, about 1/3, outside the AT [360].

Obesity-related health risks depend on the site, as well as the amount, of AT deposition. IAAT is thought to be the most deleterious form of adiposity, and gluteo-femoral SAT, the least deleterious [4-7]. Many epidemiological studies indicate that increased waist circumference or increased waist to hip-circumference ratio is associated with increased disease risk [5, 6, 362, 363]. This is consistent with the fact that in premenopausal women the risk of developing obesity-related metabolic disorders, including diabetes, hyperlipidemia, hypertension, and atherosclerosis, is less than in men (reviewed in [12]).

Relationships between plasma estrogen levels and adipose tissue mass and distribution

Estrogens appear to affect both AT mass. Although the course of menopause is quite variable, there is usually an abrupt and marked decrease in ovarian secretion of estrogens that occurs around menopause [364]. This is associated with an increase in AT mass. The best available estimates of the effect of menopause on total adiposity come from studies of whole-body imaging or dual-energy x-ray absorptiometry (DEXA) scans. We are aware of six cross-sectional studies of this type in which multiple-regression analysis was used to isolate effects of menopause from those of aging *per se* [365-370]. In these studies, menopause increased body fat ~5-10% body mass. This increase did not appear to depend on premenopausal body mass. This is interesting because it suggests that menopause adds to other causes of increased adiposity, in particular to obesity related to the increased availability and consumption of palatable, high energy-dense foods that is thought to be the main impetus for the obesity epidemic (i.e., “dietary obesity”) [8-11]. The effect of menopause on IAAT has been measured with whole-body imaging and analyzed by multiple regression only once. It was found that menopause increased IAAT by ~2 kg, from 4.3% of TAT in premenopausal women to 8.8% in postmenopausal women [369]. Several more limited imaging studies have reported similar results [370-373], although others have not found any selective increase in IAAT [374, 375].

The most common model for studying the role of estrogens in the regulation of AT mass and distribution is ovariectomy (OVX), which has long been known to increase body mass and adiposity in rats, mice and other species. In several studies in which whole-body fat content was analyzed by chemical carcass analysis 4-8 wk after OVX in chow-fed rats, body masses increased by means of ~35-60 g and body fat contents increased ~6-20 g [361, 376-381]. Similar effects have been reported in mice [382, 383]. The effect of OVX on regional AT deposition, however, has not been clearly established in either rats or mice. Several groups have described increases in the mass of one or a few resected AT depots following OVX [384-388], but none has described the total of all depots. Others have reported the total fat content of different body areas, but not of the AT depots *per se*. For example, Ainslie et al. [389], using DEXA, reported that OVX increased “abdominal” and “peripheral” fat gain ~11 g each. The landmarks distinguishing abdominal and peripheral, however, were not given, and, as noted, abdominal DEXA does not distinguish abdominal SAT from IAAT. Clegg et al. [361] estimated that OVX increased IAAT more than SAT, but did not measure AT depots directly, as described above. Therefore, one of the goals of **study 3** of this thesis was to determine the effects of OVX on SAT and IAAT directly with microCT.

Studies of hormone replacement therapy (HRT) in postmenopausal women also support a role for estrogens in the control of AT mass. A meta-analysis [390] of four studies [391-394] in which a total of 129 postmenopausal women were randomly allocated to HRT or to placebo or no treatment revealed that HRT decreased body fat mass by ~7% and increased lean body mass ~3%. In regard to the effects of HRT on abdominal SAT and IAAT in postmenopausal women, however, both positive [373, 394] and negative [375, 395] effects have been reported. Furthermore, in two randomized trials [395, 396] that were not included in the meta-analysis above, together involving 128 women, no effects of HRT on fat or fat-free mass were detected. The different outcomes of these randomized trials [391-396] may be related in part to the form of HRT used. That is, HRT regimens involving larger amounts of estrogens or smaller amounts of progestins [393, 394] tended to produce the larger effects. It is likely that these differences affect AT because the effects of estradiol treatment on body mass in OVX rats was dose-dependent and can be reduced by pharmacological progestin treatment [397]. In addition, none of the studies reviewed in this section included imaging of the entire abdomen, much less the whole body. As reviewed above, this almost certainly introduced substantial errors. Finally, there are also no imaging studies of the effects of estrogens on SAT and IAAT in OVX rats. Therefore, another goal of **study 3** was to provide such information.

Relationships between plasma estradiol and food intake

Plasma levels of estradiol may affect both food intake and EE across the menstrual cycle in women (**Figure 8**). Food intake decreases in the peri-ovulatory phase of the human cycle, when estradiol levels peak. Some studies also indicate that food intake is lower during the follicular phase compared to the luteal phase [398-400]. Sleeping and resting EE increase in the post-ovulatory (luteal) phase and total and sleeping EE decrease after menopause [373]. There are no data on the effects of menopause on eating.

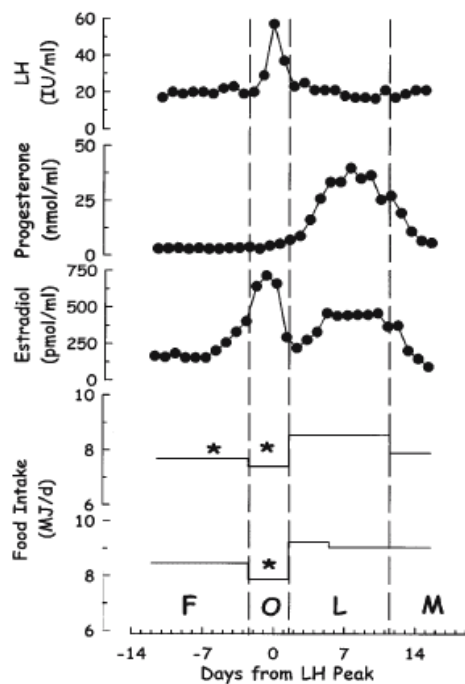


Figure 8. Plasma hormone levels and food intake through the menstrual cycle in women. F = follicular phase, O = peri-ovulatory phase, L = luteal, M = menstrual phase; LH = luteinizing hormone.

Female rats (**Figure 9**) and mice usually have 4 or 5 d ovulatory cycles. During the pre-ovulatory phase, i.e. diestrus and proestrus, plasma estradiol levels increase and estradiol exerts mainly positive feedback on the hypothalamus and pituitary to stimulate further gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion, until follicular stimulating hormone (FSH) and LH reach levels that initiate ovulation, which then occurs 10-12 h later (estradiol is the major estrogen and most commonly measured). Progesterone plasma levels peak 4-6 h after the estradiol surge. Increases in both estrogens and progesterone are necessary for the increase in sexual receptivity (“estrus”) in rats and mice, which begins around ovulation. Behavioral estrus persists for 12-20 h; estradiol levels

are very low through most of estrus. During the night of estrus female mice and rats decrease their food intake by decreasing meal size and increase locomotor activity [400-403]. That the increased plasma estradiol levels do not coincide with the decrease in food intake in rats and mice may be explained by the latency of most of estrogens' genomic effects, i. e. at least 12 h [404].

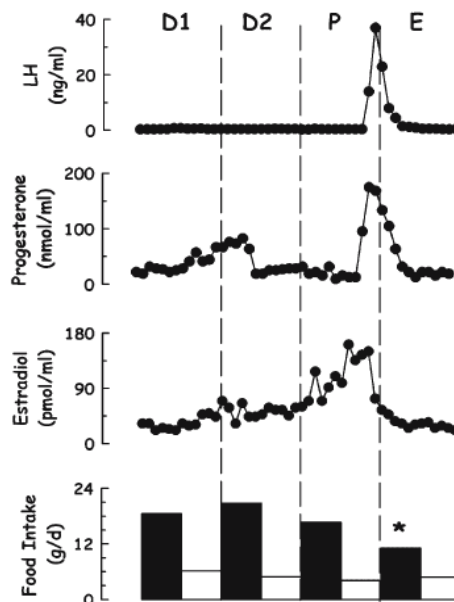


Figure 9. Plasma hormone levels and food intake during the estrous cycle in rats. D1 = diestrus (beginning at dark onset), D2 = diestrus, P = Proestrus, and E = estrus. The black bars indicate food intake in the 12 h dark-phase and the white bars food intake of the 12 h light-phase [400]

In OVX rats and mice, the cyclic eating pattern is replaced by tonic hyperphagia, due to increased meal size. Locomotor activity also decreases [405, 406]. Overall energy balance is clearly positive, however, and body weight and body fat increase [380, 384, 389, 401, 406, 407]. These effects can be reversed by subcutaneous, nearly physiological estradiol treatment in rats [361, 406].

Estrogens act on nuclear estrogen receptors (ER) that have genomic effects (ER α and ER β) and membrane-bound ER that have both genomic and non-genomic effects (ER α , ER β , and various novel ERs). The effects on eating seem to be mediated through ER α and not through ER β . Selective ER α , but not ER β , agonists reduced food intake and body weight in ovariectomized rats [408, 409]. In mice, ER α knockout (ER α KO) results in increased fat pad mass and body weight [410]. That ER α is the mediating receptor is also supported by the absence of any additional effect of OVX in ER α KO mice or of estradiol treatment in OVX

ER α KO mice [411]. In women, several ER α gene polymorphisms have been associated with increased adiposity, and with onset around puberty, when estrogen levels increase [412, 413].

Potential brain mechanisms for sex differences in eating and EE

(a) Direct brain mechanisms

Estrogens' effects on food intake appear to be wholly brain mediated [414], and their effects on EE appear to be at least partially so. The mechanisms, however, are only beginning to be understood. Most hypothalamic nuclei that are involved in energy homeostasis possess estrogen receptors and could therefore mediate effects on eating by estrogens (reviewed in [350]). But few consistent, positive effects have been reported, either for brain areas where estrogens might work or for estrogenic modulation of hypothalamic signaling molecules (reviewed in [400, 404]). For example, Clegg et al. failed to find any sex difference in the effects of the MC3/4R agonist MTII or in gonadally intact rats, and Polidori and Geary failed to see any effect of estradiol treatment on the potency of either MTII or SHU9119 to affect food intake in OVX rats [351, 415].

VMN ER α may be involved in estrogen's effects on EE. A specific knockdown of ER α in the VMN by adeno-associated virus-vectored anti-ER α RNA was associated with increased body weight, increased fat mass, and decreased REE in gonadally intact rats; in addition, estradiol no longer increased locomotor activity after similar VMN ER α -knockdown in OVX mice [416]. Food intake was also increased after VMN ER α knockdown in intact mice, but tests in OVX mice indicated that this did not depend on estrogens; that is, food intake was also increased in VMN ER α -knockdown OVX mice and estradiol administration did not affect this. This latter finding is consistent with studies of direct implantation of estradiol into the hypothalamus, which in general do not support a hypothalamic site for the estrogens' eating-inhibitory action (reviewed in [400, 404]).

The hindbrain may play an important role in estrogens' effects on eating. Local estradiol implants over the caudal NTS was shown to decrease food intake and body weight [417]. The gastrointestinal hormone CCK reduces food intake by acting on receptors on vagal afferents that project to this part of the NTS, and estradiol was shown to increase the satiating potency of both exogenous [418] and endogenous [419] CCK in OVX rats. Furthermore, endogenous CCK's satiating potency increased during estrus in intact rats [420]. Finally, knockdown of ER α in the same region of the NTS with the same siRNA

construct described above prevented both the increase in endogenous CCK satiation and the normalization of body weight by estradiol in OVX rats [421].

With regard to the central signaling molecules mediating the estrogenic control of eating, two promising leads are related to the LHA neuropeptide MCH, as estradiol treatment reduced the eating-stimulatory effect of MCH administered into the lateral cv [422], and to serotonin, for which a variety of evidence exists [404].

(b) Interaction with adiposity signals

Estrogens might interact with adiposity signals to modulate AT regulation. Leptin in particular seems an attractive candidate because increased leptin is required to initiate puberty and normal leptin levels are required to maintain ovarian cycling. In addition, women have higher leptin levels than men when corrected for fat mass, and leptin levels decrease after menopause [251, 423]. This is consistent with the fact that subcutaneously administered estradiol can increase leptin production in the AT [424]. Furthermore, basal plasma leptin levels reflect SAT mass more closely, and thus the gynoid type of AT distribution, than they do IAAT mass [251]. Finally, administration of insulin and leptin into the 3rd cv had different effects on food intake in male and female rats. Whereas male rats reduced food intake and body weight more potently in response to insulin, female rats were more sensitive to leptin, and the effects in females were reduced by OVX and reinstated by central or peripheral estradiol treatment [351, 361]. On the other hand, chronic infusions of estradiol did not affect the potency of chronic subcutaneous infusions of leptin to decrease body fat mass in either intact or OVX mice [425], and a similar effect was observed in rats [380]. In regard to insulin, a recent human study failed to support the idea that insulin's inhibitory effects on food intake might be modulated by estrogens; that is, although intra-nasal insulin administration decreased eating in men but not premenopausal women, as in the rat studies, postmenopausal women, unlike OVX rats, also failed to decrease eating following insulin [426].

Ghrelin is another hypothesized adiposity signal. Female rats were differently sensitive to 3rd cv administered ghrelin across the estrous cycle, i.e. they increased food intake on diestrus day 1 and 2 but not during proestrus and estrus. These effects were apparently estrogen-mediated, as untreated OVX rats were more sensitive to the eating-stimulatory effects of 3rd cv or systemic administration of ghrelin than intact female intact rats or estradiol-treated OVX rats [427]. Further, the period of post-OVX hyperphagia and weight gain corresponded to an increase in plasma ghrelin levels [427], which is different from overfeeding-induced weight gain, which was associated with decreases in plasma ghrelin levels [259].

Finally, two studies of the effect of estradiol treatment on the eating-inhibitory action of amylin have produced apparently conflicting results. In an unpublished study, Asarian and Lutz found that estradiol treatment increased the acute satiating effects of exogenous and endogenous amylin in OVX rats. In contrast, Trevaskis et al. reported that estradiol reduced the weight-reducing effect of chronic amylin treatment in rats rendered obese by diet and OVX (reviewed in [407, 428]).

Potential mechanisms for sex differences in regional AT distribution

Despite the marked sex differences in AT distribution and intensive research efforts, the physiological controls of regional AT distribution are very poorly understood [429]. As an example, a recent very large scale (nearly 200,000 subjects) genome-wide association study of 14 gene loci associated with sex differences in waist-hip ratio independent of BMI accounted for only 1.3% of the variance in waist-hip ratio in women and 0.5% of the variance in men, despite the fact that estimates of the heritability of waist-hip ratio indicate that 22-61% of the variability in the phenotype is heritable [430].

Androgen, estrogen and progesterone receptors are all present in AT, suggesting that effects of gonadal steroid hormones could be direct. ER expression is higher in the SAT than in the IAAT, and the opposite is true for androgen receptors [429]. Because this difference is similar in men and women, however, it is unlikely to explain the sex difference in AT distribution. Further ER α KO results in increased fat pad mass in both male and female mice [410].

6. Thesis Aims

As reviewed in the previous sections, demonstrations of dynamic regulation of AT mass, i.e., observations of dynamic compensatory changes in eating and EE and the return to near normal levels of AT mass following experimentally induced increases or decreases in AT mass are the strongest evidence that AT mass is dynamically regulated [14-19]. Therefore this thesis involved studies using the model of dynamic regulation of AT mass in rats recovering from obesity. The most prevalent model of AT mass regulation posits that adiposity signals such as insulin, leptin, ghrelin and amylin inform the brain about the level of AT mass and, in the case of increased or decreased AT mass they prompt central neuronal networks to adjust eating and EE appropriately. Insulin and leptin are thought to act on neurons in the Arc of the hypothalamus to initiate such regulatory responses. Arc neurons, which are sensitive to insulin and leptin, express NPY and α -MSH, which are considered to be crucial signaling molecules in this neuronal network. Similarly, downstream signaling molecules, including PVN CRH neurons, are thought to be involved [20-25].

AT mass regulation is modulated by the environment, such as the availability of palatable, energy-dense foods, which leads animals and humans to develop “dietary obesity” [11, 25]. Therefore the thesis also included a model of diet-induced obesity. Other influences are less environmental, for instance the effects of menopause in women. The loss of estrogen production that occurs around menopause is thought to contribute to the associated increase in AT mass, in particular more IAAT [369]. OVX in rats leads to increases in body weight and AT mass and is a commonly used model of menopause [404]. For these reasons, this model also was used in the present thesis.

The overall aim of the present thesis was to investigate AT mass regulation in these three models of obesity. In each study we were interested in the regulation of AT mass and the distribution of IAAT and SAT mass, which we were able to measure by micro-computed tomography (microCT), a method that has not been applied previously to any of these models.

Despite the strong evidence for dynamic AT regulation in rats recovering from obesity, there are only few descriptions of the involvement of hypothesized adiposity signals [19] or their downstream brain mechanisms during such dynamic regulation. Therefore in **study 1** we tested whether basal plasma levels of insulin, leptin, amylin and ghrelin encode information about AT mass during dynamic AT mass regulation in rats. Basal plasma levels of these hormones and AT mass were measured during the development of obesity induced by

chronic intragastric (IG) overfeeding and after termination of IG overfeeding, i.e. during dynamic regulation of AT mass. In **study 2**, in situ hybridization for mRNA was used to test whether hypothalamic levels of NPY, α -MSH or CRH might be involved in this regulatory response.

The effects of OVX on AT distribution have not been measured directly in rats. Therefore, in **study 3** we tested whether OVX affects adiposity in rats similarly to the effect of menopause in women; that is, by increasing TAT and the relative deposition of IAAT, and whether a physiological regimen of estradiol treatment is sufficient to prevent these effects. We also included intact and OVX groups that were offered palatable, energy-dense foods (i.e., Ensure in addition to chow) to test whether dietary-obesity changes the effects of OVX.

7. Studies

Basal plasma levels of insulin, leptin, ghrelin and amylin do not signal adiposity in rats recovering from forced overweight

This study is published:

Gloy VL, Lutz TA, Langhans W, Geary N, Hillebrand JJ: Basal plasma levels of insulin, leptin, ghrelin and amylin do not signal adiposity in rats recovering from forced overweight. *Endocrinology*. 2010 Sep;151(9):4280-8. Epub 2010 Jul 28.

Abstract

This study examined how adiposity signals are related to adiposity during recovery from forced overweight. Rats were rendered overweight by chronic intragastric overfeeding (overweight, OW). Overfeeding was stopped when OW rats reached 126-129% of saline-infused normal weight (NW) rats. Adipose tissue (AT) mass was estimated by computed tomography and blood samples were drawn from a chronic atrial cannula throughout. Basal levels (i.e., after 2-3 h fast late in the diurnal phase) of the hypothesized adiposity signals insulin, leptin, ghrelin and amylin were assayed. OW rats gained ~130 g more body weight and ~100 g more AT mass during overfeeding. Plasma levels of insulin and leptin increased, whereas those of ghrelin decreased, linearly with AT mass; amylin did not change reliably. During recovery, OW rats' body weight and AT mass decreased, but were still elevated versus NW rats after 39 d. OW rats' insulin returned to NW levels on d 1 of recovery and decreased below NW levels thereafter. Leptin was no longer elevated after d 8 of recovery. Ghrelin and amylin did not change reliably during recovery. Although AT mass decreased in OW rats during each inter-measurement interval between d 0 and d 23 of recovery, insulin and leptin did so during only the first interval (d 0 – 5). Insulin and leptin levels were exponentially related to AT mass during recovery. These data indicate that basal insulin, leptin, ghrelin and amylin do not encode AT mass in rats dynamically regulating weight and adiposity during recovery from overweight.

Introduction

Energy homeostasis has been a subject of physiological investigation for over a century [431, 432]. Since Kennedy's classic 1953 paper [40], the major organizing principle of this work has been the "lipostatic" theory. According to this theory, the brain actively regulates adipose tissue (AT) mass by controlling eating so as to correct deviations between the levels of circulating energy metabolites, including metabolites originating in the AT, and an internal set point. There have been two major adjustments to this theory in the last 50 years. First, it has become apparent that energy expenditure (EE) is controlled as well as eating [14, 15, 56, 258]. Second, basal levels of endocrine "adiposity signals", rather than levels of metabolites, are now hypothesized to be feedback signals that encode AT mass. Indeed, two hormones, insulin and leptin, are now widely accepted to fulfill this role [15, 20, 21, 23, 228]. Amylin [433], ghrelin [223], and peptide YY [224] have also been proposed as adiposity signals, although the evidence for their involvement is less strong.

Earlier studies of energy homeostasis focused mainly on the organism's capacity to adjust energy intake to maintain a steady state of body weight (BW) or AT when challenged with, for example, energy-dilute or energy-dense diets or conditions of increased or decreased energy loss, such as cold or warm ambient temperatures, lactation, etc. [39, 40, 434]. Contemporary authors [15, 31, 37] continue this tradition by noting how small, constant errors in the balance of energy intake and EE would lead to large increases or decreases in AT over time. A natural extension of this approach is to investigate the relationships between levels of hypothesized adiposity signals and BW or body AT in cross-sectional studies under steady-state situations. Nearly all the evidence that basal levels of hormones encode AT is of this type [19]. Numerous cross-sectional studies indicate that basal plasma levels of insulin, leptin, and ghrelin are significantly correlated with BW or estimates of AT, such as BMI, resected AT mass, or AT volume estimated by computed tomography (CT) or magnetic resonance imaging, in stable, steady-state conditions in humans [250, 255, 261, 331, 435] and rats [19, 252, 333, 436]. Similar, but less extensive, correlational data exist for several other hormones and adipokines [437-439]. Basal amylin has been reported to change with adiposity [311], but we know of no correlational analyses.

A stronger test of the hypothesis that AT is actively regulated was introduced by Cohn and Joseph in 1962 [42], who measured dynamic changes in eating while rats were recovering from increases in adiposity induced by intragastric (IG) overfeeding. By now there are many reports indicating that experimentally induced increases in AT lead to coordinate decreases in eating and increases in EE and that experimentally induced decreases in AT lead to the opposite responses [14-17]. Furthermore, the magnitudes of the compensatory responses

often parallel the degree of perturbation. Such data strongly support the hypothesis that AT mass is dynamically regulated by an active, negative-feedback mechanism. In Cohn and Joseph's [42] and many subsequent studies, however, BW often recovers more exactly than AT. This remains unexplained and is usually ignored.

Despite the probative value of tests of compensatory responses after manipulations of AT mass, there are very few descriptions of levels of adiposity signals during such dynamic regulation [19]. Furthermore, in most of these studies, design weaknesses prevent clear interpretations. For example, in several, manipulations were too short, i.e., only 1-3 d, to clearly distinguish the influences of changed AT mass on hormone levels from influences of acute changes in energy metabolite flux, gut contents, etc. In others, AT mass was not measured, the induced changes in AT mass or BW were not sufficient to induce longer-lasting compensatory responses, or the responses were measured when AT mass or BW was no longer significantly changing. For example, White et al. [266] recently reported that plasma concentrations of insulin, leptin and PYY returned to control levels within 2 d after rats that had been rendered 45 g overweight by chronic IG infusions were returned to ad libitum feeding. At the 2 d time point, however, rats were no longer losing BW, and AT was not measured, so it was not clear if any effective compensation was occurring. In two earlier studies insulin was tracked during longer periods of dynamic compensation for substantial increases in AT mass [14, 267], and neither supported the hypothesis that basal plasma insulin functions as an adiposity signal. Rather, insulin returned to control levels during recovery while AT was still significantly increased and while eating was still decreased.

We know of no other reports on changes in basal insulin, leptin, ghrelin or amylin during compensation for overweight. Therefore, we IG overfed rats until they were substantially overweight and tracked changes in eating, AT mass and basal levels of insulin, leptin, ghrelin and amylin during recovery. None of these hormones accurately encoded AT mass during this period of dynamic regulation of AT mass.

Materials and Methods

Animals. Male Long Evans rats (bred from founders from Charles River, Sulzfeld, Germany), were housed individually in wire-mesh cages at 21 ± 1 °C, with a 12:12 h light-dark cycle (lights off, 1200) and water and ground chow available ad libitum. Chow intake and BW were measured between 1000 and 1100 daily. All procedures were approved by the Cantonal Veterinary Office.

Surgeries. Catheters were implanted into the stomach and into the right atrium via the right external jugular vein. Rats were food deprived ~6 h, pretreated with subcutaneous (SC) injections of trimethoprim-sulfadoxine antibiotic and atropine sulfate, and anaesthetized with ip injections of a mixture of 4.8 mg/kg xylazine and 96 mg/kg ketamine. Gastric catheter headsets consisted of 1.5 cm of 16 ga surgical stainless-steel tubing, bent into U-shapes and connected to 20 cm of silastic tubing (id 1.02 mm, od, 2.16 mm), shielded with 1.5 cm pieces of silastic tubing (id 1.47 mm, od 1.95 mm), and sutured to 2 x 3.5 cm pieces of polypropylene surgical mesh. Narrow rings of silicone glue were placed ~7 and 10 mm from the free end of the catheter. Atrial catheters were constructed as described previously (31) and sutured to the same piece of surgical mesh. Two cm dorsal midline cutaneous incisions 2 cm caudal to the interscapular midline, 1.5 cm incisions above the right clavicle, and 3 cm laparotomies were made. The free ends of the catheters were led SC to the appropriate incision. Atrial catheters were implanted as described before [440]. Gastric catheters were led through punctures in the abdominal muscle and gastric corpus so that the gastric wall lay between the silicon rings and were anchored with purse-string sutures. Muscle and skin incisions were closed separately. Carprofen (5 mg/kg) was SC injected after surgery and the next 2 d, and additional antibiotic was injected the day after surgery. Rats were allowed to recover ≥ 10 d. Atrial catheters were filled with heparinized polyvinylpyrrolidone and flushed every second day with sterile saline between 900-1000. The exteriorized ends of the catheters were sealed with plugged pieces of stainless-steel tubing.

Procedure. Rats were IG overfed until substantially overweight, and then tested during recovery. On the first day, subsequently designated d -25, AT mass was measured as described below. On d -23, rats were divided into overweight (OW, n = 9) and normal-weight (NW, n = 8) groups that were matched for BW and total AT mass. Beginning on d -22, OW rats were IG infused with 5 mL liquid diet (**Table 1**) and NW rats with equivolemic loads of 0.9% saline. This was increased gradually over 7 d to four 11 mL infusions / d (~189 kcal total), done at 1100, 1300, 1500, and 1700. Infusions lasted until the mean BW of OW rats reached 125% of the mean BW of NW rats, which occurred on d -4. On d -4 through d 0, infusions were reduced to 141 kcal / d to stabilize the OW rats at ~125% of NW rats' BW. This was done because leptin and insulin levels are known to be affected by the state of energy balance as well as by AT mass [15, 37, 255]. When IG infusions were stopped on d 0, OW rats' BW was 129% of NW rats'. The recovery phase lasted from d 1-39, after which rats were sacrificed with overdoses of pentobarbital.

Table 1. Composition of the liquid diet used for intragastric overfeeding.

| Component | Day -22 to -5 | Day -4 to 0 |
|--------------------------------|---------------|-------------|
| Protein, g / L (% energy) | 100 (10) | 15 (10) |
| Corn oil, g / L (% energy) | 300 (66) | 45 (66) |
| Maltodextrin, g / L (% energy) | 267 (24) | 40 (24) |
| Water (mL / L) | 470 | 550 |
| Energy density (kJ / L) | 18000 | 13400 |

The diet ingredients were a dairy-soy protein product (15 g protein / 17.8 g product, supplemented with vitamins and minerals, PZN 5566143, Biomedica Pharma GmbH, Heinrichsthal, Germany), corn oil, maltodextrin (maltodextrin 19, SHS Society for Clinical Nutrition, Heilbronn, Germany; 16.3 kJ / g), and Tween 80 emulsifier (8 mL / L, Sigma-Aldrich, Saint Louis, MO). Energy equivalents of protein and fat were estimated to be 18.4 and 81.5 kJ / g, respectively [441].

AT mass. AT was estimated by computed tomography (CT, LCT 100, Aloka, Tokyo, Japan) between 1000 and 1100 on d -25, -11, -2, 5, 8, 17, 23, and 39. Rats were briefly anesthetized with isoflurane and scanned from the anterior aspect of lumbar vertebra 1 to the posterior aspect of lumbar vertebra 6 (L1-L6), using a method that we have shown to be sensitive, accurate, and reliable [359]. Intra-abdominal (IA) and subcutaneous (S) AT were distinguished by the abdominal muscle layers, converted to mass by Aloka's software, and extrapolated to whole-body IAAT and SAT masses using formulae based on correlations between CT estimates and resected AT. SAT included subcutaneous white and brown AT, and AT interspersed among the limb muscle fascicles [359]: whole-body IAAT = $1.21 \times [\text{L1-L6 IAAT}] + 1.47 \text{ g}$, whole-body SAT = $3.23 \times [\text{L1-L6 SAT}] + 8.57 \text{ g}$, and total AT (TAT) = IAAT + SAT.

Hormone assays. One mL blood samples were taken on d -24, -12, -4, -1, 1, 5, 8, 17, and 23 between 900 and 1000, i.e., late in the diurnal phase, following 2-3 h of food deprivation, which we define as basal. Blood was mixed with EDTA and a proteinase inhibitor, and plasma was separated, aliquoted and stored at -20 °C. Plasma insulin, leptin, and total ghrelin (i.e., active acylated ghrelin and desacyl-ghrelin; referred to as ghrelin below) were measured by RIA. Plasma amylin was measured by fluorescence immunoassay, except on d -4. Table 2 summarizes assay information and performance.

Table 2. Assay performance characteristics.

| | Insulin | Leptin | Ghrelin | Amylin |
|--------------------|---------|---------|---------|------------------|
| Linear range | 0.2-5 | 1-10 | 0.7-1.5 | 3.9 – 250 |
| Slope (log) | -1.26 | -1.70 | 0.88 | 0.73 - 0.88 |
| y-intercept (log) | 0.86 | 1.3 | 0.52 | 0.27 - 0.74 |
| r ² | 0.99 | 0.99 | 0.95 | 0.98 - 0.99 |
| SEE (log) | 0.06 | 0.04 | 0.10 | 0.07 - 0.12 |
| MSD (log) | 0.05 | 0.03 | 0.08 | 0.07 |
| Intra-assay CV | 10 ± 10 | 14 ± 13 | 6 ± 9 | 10 ± 9 – 16 ± 13 |
| Inter-assay CV | - | - | - | 13 ± 12 |

Plasma levels of insulin, leptin and total ghrelin are ng/mL and were measured by RIA (kits RL-83K, RI-13K, and GHRT-89HK, Millipore, Zug, Switzerland; SI conversions for 1 ng/mL are 172 pM insulin, 63 pM leptin, and 302 pM total ghrelin); plasma levels of amylin are pM and were measured by fluorescence immunoassay (FIA, Milliplex MAP kit, rat endocrine RENDO-85K; 1 pM = 3.96 pg/mL). RIA standard plots were fraction bound versus log concentration (ng/mL); FIA standard plots were log fluorescence versus log concentration. Linear ranges were judged by eye and standard lines fit by least squares using the means of the duplicate standard concentrations. The slopes, y-intercepts, r², and standard errors of the estimate (SEE) of these standard lines are given. MSD is the minimum detectable significant group difference by a t-test, based on the SEE and a sample size of 8. Sample values outside the linear range were not used. The intra-assay CV is the coefficient of variation for standards in the linear range. The inter-assay CV of the same standards is given for amylin, because this assay required several assay plates.

Statistical analyses. The overfeeding and recovery phases were analyzed separately with time x group ANOVA, with data from the last day of overfeeding also used as the initial recovery-phase values. Food intake and BW data were analyzed only for the days on which blood sampling or CT scanning was done. For food intake, 3-d means around the day of blood sampling were used to minimize transient changes associated with the sampling and scanning. Log amylin levels were analyzed to obtain homogenous sample variances. Significant ANOVA interaction effects were followed up with planned Bonferroni-Holm post-hoc comparisons (OW-NW differences on each day analyzed and, during the recovery phase, sequential changes in hormone levels and TAT mass in OW rats). The sequential change of SAT as % TAT in NW and OW rats were compared with t-tests. The forms of the relationships between mean hormone levels and mean TAT masses during each phase were determined by regression analyses. To increase statistical power, data were converted

to standard scores using the median absolute deviate method, and standard scores with absolute values > 1.96 (i.e., $P < 0.05$) were excluded [442, 443]. Minimum level of significance was $P < 0.05$. Data are reported as mean \pm SEM. The standard error of the difference (SED) is given as a measure of residual error.

Results

Overfeeding phase

Energy intake, BW, and AT. IG overfeeding markedly reduced eating (**Figure 1A**) and increased BW and TAT (**Figure 1B-C**). On d 0, at the end of overfeeding, OW rats were ~ 130 g (29%) heavier than NW rats ($P < 0.05$). On d -2, the last CT scan during overfeeding, OW rats had ~ 100 g (250%) more TAT mass ($P < 0.05$). Representative CT images are shown in **Figure 1D**. Regional fat distribution did not change in OW rats during overfeeding (69% SAT at onset and end), but on d -2 percent SAT was only $\sim 62\%$ in NW rats, resulting in a group difference (**Table 3**).

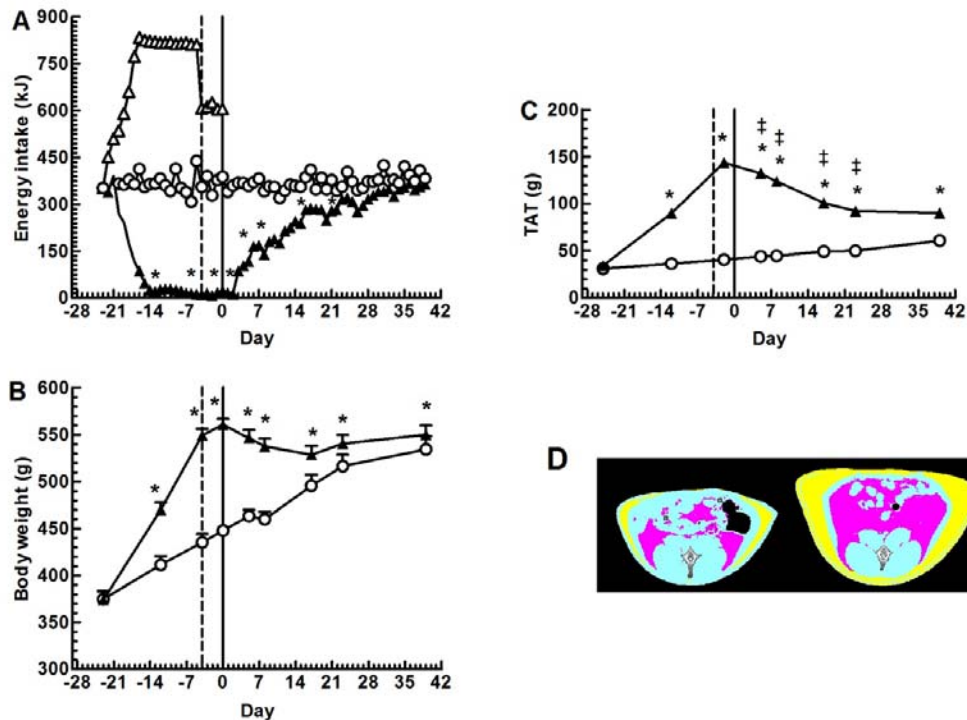


Figure 1. **A.** Energy intake (EI; **A**), body weight (BW; **B**), and total adipose tissue mass (TAT; **C**) during and after intragastric infusions (IG) of liquid diet in overweight (OW) and of saline in normal-weight (NW) rats. The dashed vertical line on d -4 indicates the beginning of the stabilization period, and the solid vertical line on d 0 indicates the beginning of the recovery period. Open circles, NW rats; filled triangles, OW rats (in **A**: filled triangles, oral EI; open triangles, total EI). Representative computed tomography images showing subcutaneous (yellow) and intra-abdominal (magenta) adipose tissue at the level of lumbar vertebra 6 in a NW rat (left; TAT, 40 g) and an OW rat (right; TAT, 147 g) at d -2 are shown in **D** (white / gray, bone; black, air; and blue, remainder). Data are mean \pm SEM. Statistical contrasts were based on significant ANOVA interaction effects; during overfeeding: EI: $F(3, 38) = 154.3$, $SED = 13$ kJ; BW, $F(40, 3) = 568.8$, $SED = 3$ g; and TAT, $F(2, 25) = 749.5$, $SED = 2$ g, all $P < 0.001$; during recovery: EI, $F(6, 75) = 95.43$, $SED = 12$ kJ; BW, $F(6, 76) = 193.3$, $SED = 3$ g; TAT, $F(5, 63) = 143.7$, $SED = 2$ g, all $P < 0.001$. *Different from NW on same day, $P < 0.05$. †Different from OW on preceding measurement day, $P < 0.05$.

Table 3. Effects of overfeeding and recovery on regional adipose tissue distribution.

| | Initial values | | End of Overfeeding | | End of Recovery | |
|---------------------------------------|----------------|--------|--------------------|---------------------|-----------------|---------------------|
| | NW | OW | NW | OW | NW | OW |
| Total Adipose Tissue | | | | | | |
| g | 31 ± 2 | 34 ± 0 | 41 ± 3 | 144 ± 2* | 61 ± 5 | 90 ± 4* |
| Δ g | - | - | 10 ± 2 | 108 ± 3 | 19 ± 2 | -54 ± 4 |
| Intra-abdominal Adipose Tissue | | | | | | |
| g | 9±1 | 11±0* | 16 ± 2 | 44 ± 1* | 22 ± 2 | 30 ± 2* |
| Δ g | - | - | 5 ± 1 | 35 ± 0 | 8 ± 1 | -14 ± 1 |
| Subcutaneous Adipose Tissue | | | | | | |
| g | 22 ± 1 | 24±0 | 25 ± 2 | 99 ± 2* | 36 ± 2 | 59 ± 3* |
| Δ g | - | - | 4 ± 1 | 74 ± 3 | 11 ± 1 | -40 ± 3 |
| g, % TAT | 70 ± 1 | 69 ± 1 | 62 ± 2 | 69 ± 1* | 60 ± 1 | 67 ± 0* |
| Δ g, % TAT | - | - | 40 ± 3 | 69 ± 1 [†] | 57 ± 3 | 75 ± 1 [†] |

Data shown are absolute amounts (g) and changes (Δ g) in TAT, IAAT, and SAT, mean ± SEM in normal weight (NW) and overweight (OW) rats. SAT data are also shown as percentages of TAT (% TAT). *Different from NW on same day, $P < 0.05$, Bonferroni-Holm test after significant ANOVA interaction effects: for overfeeding: TAT: $F(2, 25) = 749.5$, $SED = 2$ g; IAAT, $F(2, 27) = 535.1$, $SED = 1$ g; SAT: $F(2, 26) = 601.0$, $SED = 1$ g; SAT as % TAT, $F(2, 27) = 30.5$, $SED = 1\%$; all $P < 0.001$; for recovery: TAT: $F(5, 63) = 143.7$, $SED = 2$ g; IAAT: $F(5, 64) = 111.4$, $SED = 1$ g; SAT, $F(5, 63) = 115.9$, $SED = 2$ g; SAT as % TAT, $F(5, 59) = 6.4$, $SED = 1\%$; all $P < 0.001$. [†]Different from NW, $P < 0.05$, t-test.

Endocrine profile. No significant differences were detected between OW and NW rats in basal levels of insulin, leptin, ghrelin or amylin on d -24, before overfeeding began. On d -1, the last blood sampling during overfeeding, basal insulin and leptin levels were significantly increased, basal ghrelin level was significantly decreased, and basal amylin level was not significantly affected (**Figure 2A-D**). Unexpectedly, ghrelin levels also tended to decrease in NW rats during overfeeding. Mean basal levels of insulin, leptin and ghrelin changed linearly in relation to TAT mass during overfeeding (**Figure 3A-C**).

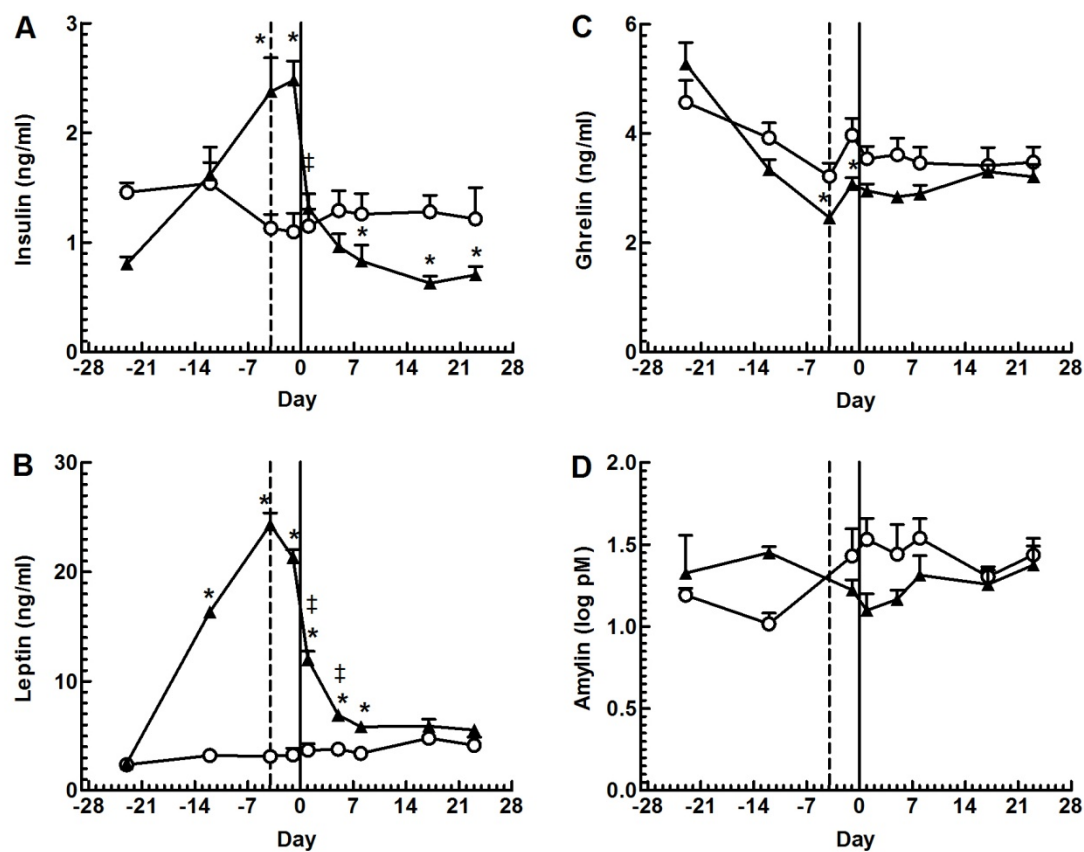


Figure 2. Basal plasma levels of insulin (A), leptin (B), ghrelin (C), and amylin (D) during and after intragastric infusions (IG) of liquid diet in overweight (OW) and of saline in normal-weight (NW) rats. The dashed vertical line on d -4 indicates the beginning of the stabilization period, the solid vertical line on d 0 indicates the beginning of the recovery period. Open circles, NW rats; filled triangles, OW rats. Data are mean \pm SEM. Statistical contrasts were based on significant ANOVA interaction effects; during overfeeding: insulin, $F(3, 33) = 7.58$, $P < 0.001$, $SED = 0.3$ ng/mL; leptin, $F(3, 34) = 117.69$, $P < 0.001$, $SED = 0.8$ ng / mL; ghrelin, $F(3, 34) = 7.18$, $P < 0.001$, $SED = 0.3$ ng / mL; and amylin, $F(2, 16) = 2.91$, $P = 0.08$, $SED = 0.6$ log pM; during recovery: insulin, $F(5, 52) = 22.40$, $P < 0.001$, $SED = 0.2$ ng / mL; leptin, $F(5, 56) = 65.26$, $P < 0.001$, $SED = 0.8$ ng / mL; ghrelin, $F(5, 57) = 1.27$, $P = 0.29$, $SED = 0.2$ ng / mL; amylin, $F(5, 57) = 1.61$, $P = 0.17$, $SED = 0.1$ log pM. *Different from NW on same day, $P < 0.05$. †Different from OW on preceding measurement day, $P < 0.05$.

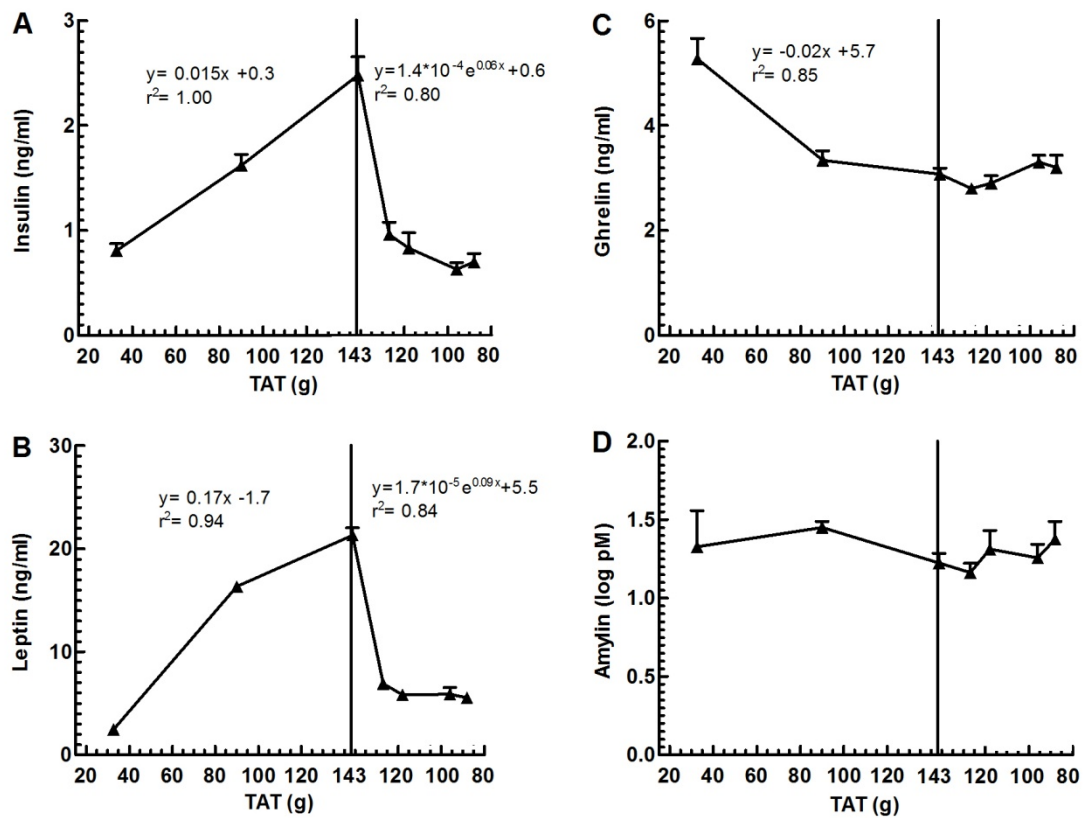


Figure 3. OW rats' basal plasma levels of insulin (**A**), leptin (**B**), ghrelin (**C**), and amylin (**D**) plotted against total adipose tissue (TAT) during and after intragastric infusions (IG) of liquid diet. The solid vertical line indicates the end of IG infusions and beginning of the recovery period. Insets in **A-C, left**, are least squares fits and correlation coefficients for linear trend during overfeeding (amylin did not change significantly during overfeeding); insets in **A** and **B, right**, are least squares fits and correlation coefficients for exponential trend during recovery (amylin and ghrelin did not change significantly during recovery). Data are mean \pm SEM.

Recovery phase

Energy intake, BW, AT. Eating was significantly inhibited in OW rats for ≥ 23 d of recovery (**Figure 1A**). BW, TAT, IAAT and SAT gradually decreased, but remained significantly elevated compared to NW rats on d 39 (**Figure 1BC**, and **Table 3**). The mean differences in BW between OW rats and NW rats on d 23 (24 g) and d 39 (16 g), however, were much smaller than the mean differences in TAT on those days, 42 g and 30 g, respectively. OW rats lost predominately SAT during recovery ($P < 0.05$), so that regional AT distribution remained similar to the initial value (**Table 3**). NW rats again gained similar amounts of SAT and IAAT, so that regional AT distribution still differed between groups on d 39 (**Table 3**).

Endocrine Profile. Basal plasma insulin, which was ~2.3 fold higher in OW rats than NW rats at the end of overfeeding, fell on d 1 of recovery to the NW level, was not different between groups on d 5, and was significantly below the NW on d 8 and all subsequent measurements (**Figure 2A**). Basal plasma leptin, which was ~6.5 fold higher in OW rats than NW rats at the end of overfeeding, also fell markedly on d 1 of recovery, but was still ~3 fold higher in OW rats. The difference became smaller on d 5 and 8 and was no longer significant on d 17 or 23 (**Figure 2B**). Neither basal plasma ghrelin nor basal plasma amylin changed reliably during recovery (**Figures 2C and 2D**).

Analysis of sequential changes in hormone levels revealed that although TAT in OW rats decreased significantly through each inter-measurement interval between d 0 and d 23, basal plasma insulin and leptin each decreased significantly during only the first interval, d 0 to 5 (**Figure 1C, 3A and 3B**). Regression analyses indicated that the relations between TAT and mean basal levels of insulin or leptin during recovery were fit by exponential functions (**Figure 3A and 3B**); 90% of the total decreases in basal insulin and leptin levels took place during between d 0 and 5 of recovery, in comparison to only 25% of the total decrease in AT mass.

Discussion

The brain orchestrates dynamic compensatory changes in eating and EE in response to perturbations in normal BW or AT mass. How the brain senses weight or AT mass, however, remains unclear. Here we tested the hypotheses that basal plasma levels of insulin, leptin, ghrelin and amylin encode information about increased AT mass that enables the brain to produce appropriate compensatory responses. The hypotheses were not supported. Levels of insulin, leptin and ghrelin, but not of amylin, did change in accordance with the hypotheses during the overfeeding phase. During the subsequent ad libitum-feeding phase, however, none of the hormones tracked the compensatory decreases in OW rats' eating and AT mass.

The finding that OW rats' insulin levels were significantly below NW levels on d 8-23 of recovery extends previously reported dissociations between basal plasma insulin and AT mass in similarly designed tests [14, 267]. In addition, we report that the relationships between insulin and AT mass were different during forced AT gain and voluntary, compensatory AT loss. Insulin increased linearly with AT mass during weight gain, which is consistent with the linear relationships reported in cross sectional studies of basal insulin and AT or BW in rodents [19, 252] and humans [250]. In contrast, during compensatory AT

loss, insulin decreased exponentially. Why the relationships between insulin dynamics during AT gain and loss are so different is unknown and warrants investigation. It is not due to changes in regional AT distribution, because this remained remarkably constant in OW rats throughout the experiment. Interestingly, insulin levels also failed to fall linearly with AT loss in chronically food-restricted rats.

The dynamics of leptin changes during recovery were not associated with AT loss. That is, leptin fell significantly only through the first 5 d of recovery, during which rats lost ~13 g AT, it did not change significantly between d 5 and 8 or 8 and 17, despite that AT mass decreased ~30 g in this interval. Daily energy intake was also significantly decreased for more than two weeks after leptin was no longer significantly elevated. In addition, similar to insulin, leptin increased linearly with AT gain, but fell exponentially with compensatory AT loss. Leptin also failed to fall linearly with AT loss in chronically food-restricted rats [444]. Neither ghrelin nor amylin changed significantly, while rats lost AT mass. Our assays for ghrelin and amylin appeared sufficiently sensitive to detect small differences (**Table 2**), so it is unlikely that we failed to detect a meaningful change. It is possible that the assay of active ghrelin, rather than total ghrelin, would reveal a different result, but we are not aware of any evidence that altered AT mass differentially affects the ratio of active to total ghrelin [335].

In sum, we conclude that the dissociations between AT mass and basal plasma levels of insulin, leptin, ghrelin and amylin during recovery from forced overweight indicate that none of these hormones encode AT mass in a way that could be used by the rats' brain to guide dynamic compensatory changes in eating or EE, i.e., they are not signaling adiposity in rats under these dynamic conditions. Furthermore, because dynamic regulation proceeded in the absence of significant differences in basal levels of these hormones, their elevations (insulin, leptin, amylin) or decreases (ghrelin) are not necessary for compensatory decreases in eating or increases in EE. Finally, because compensatory decreases in eating seemed to track BW rather than AT mass, our data question which variable is actually regulated in long-term energy homeostasis. Indeed, beginning with Cohn and Joseph's [42] original study, it has been repeatedly observed that rats return to nearly normal BW but maintain significant increases in AT mass after experimentally induced obesity [14, 48]. Increased AT mass, without an increase in BW indicates that another body compartment must be decreased. Possibly a feedback signal from this compartment opposes further compensation for the increased AT mass, but what this signal might be is unknown.

Although compensatory reductions in BW or AT mass also occur after rats are switched from chronic feeding of diets that they overeat back to standard chow [48], we are aware of only one study of this type in which an adiposity signal was measured during the dynamic phase

of AT reduction. In that study, insulin levels returned to control levels within 3 d after rats were switched from high-fat to chow [258]. Recently, Shi et al. [445] reported that plasma leptin levels returned to control levels within two weeks after mice were switched from a high-fat diet to chow. At that time, however, the mice were no longer hypophagic, although they still had markedly more AT. Similarly, in humans, basal plasma insulin and leptin levels have been measured just at the end of weight changes induced by overfeeding or underfeeding, but not during the dynamic compensation period itself [255]. Finally, basal plasma insulin and leptin levels are also dissociated from adiposity in nursing rats, which have decreased levels of both hormones despite being substantially overweight, perhaps because of chronic negative energy balance during nursing [446].

The endocrine coding criterion that we tested here is only one of several criteria for establishing normal or physiological endocrine function [447]. Two other crucial criteria relate to “physiological dose” and hormone “antagonism”. Data relevant to these criteria yield mixed support for the hypotheses that insulin, leptin, ghrelin and amylin are adiposity signals. The concentrations of these hormones reaching their hypothesized primary central sites of action (described below) have not been measured, and appropriate tests of chronic systemic infusions have been done only for leptin. These tests indicate that plasma leptin levels must be elevated at least 4-fold in mice [448] and 2-fold in rats [449] before any effect on eating or BW occurs. Positive results of chronic antagonist infusions have been reported for insulin [450], leptin [281], and amylin [317], but not ghrelin. Importantly, however, these antagonist tests were done in lean animals, and, as discussed below, hormone signaling may operate differently in obese and normal- or underweight animals. The failure of basal hormone levels to encode AT mass in obesity that we report here suggests that positive results in antagonist tests may reflect only a permissive role for the hormone, unrelated to hormone level, in the control of eating and EE and not a role as a proximal or efficient cause.

Regulation of the hypothalamic melanocortin (MC) system is supposed to contribute to the regulatory effects of leptin, insulin and ghrelin on eating and EE [20, 21, 23, 223, 228]. One study indicates that α -melanocyte stimulating hormone (α -MSH) is involved in compensatory undereating. That is, injections of a MC 3/4 receptor antagonist into the third ventricle selectively reversed compensatory undereating in rats that had been rendered ~45 -70 g overweight by IG overfeeding [163]. These data, together with ours, suggest that the MC system might be activated independent of changed insulin, leptin, or ghrelin plasma levels.

Increased BW usually elicits central leptin and insulin resistance, i.e., decreased responsivity to central injections. Because the levels of these hormones were normal or below normal during dynamic compensation for increased AT mass here, however, it seems unlikely that

resistance phenomena are relevant to insulin or leptin signaling during the compensatory response to overweight.

The failure of basal insulin, leptin, ghrelin and amylin to encode AT mass during dynamic compensation for overweight does not rule out the possibility that these hormones may signal adiposity in other situations, e.g. during compensation for forced losses in AT mass. There are several indications that underweight is more tightly regulated than overweight [70]. Perhaps most convincing is a recent study in humans who were maintained at a 10% reduced BW. In these subjects, reinstatement of normal-weight levels of leptin reversed the compensatory decrease in EE and other metabolic adaptations to underweight and led to further weight loss [256]. The situation in rats, however, may differ. For example, in male rats that had sustained an 11% BW loss induced by chronic food restriction and then returned to ad libitum food access, elevation of plasma leptin level to ~3 times normal did not delay weight recovery [451].

In summary, rats rendered overweight by forced overfeeding reduced BW and AT mass when returned to ad libitum food intake. Most of this dynamic regulatory response occurred in the absence of any significant elevations in basal plasma insulin, leptin, or amylin or significant decreases in basal plasma ghrelin. These data fail to support the hypotheses that these hormones function as adiposity signals in overweight or obese individuals. Whether other hormones, cytokines, or adipokines function as adiposity signals in this situation is unclear [438, 439, 452]. As recently discussed by Woods [30], Kennedy's (1953) hypothesis that levels of circulating metabolites encode information about AT mass to the brain remains a viable alternative.

Hypothalamic CRH mRNA, but not POMC or NPY mRNA, changes during compensatory under-eating in rats recovering from forced overweight

This study is being prepared for publication:

Gloy V, Koss M, Langhans W, Geary N, Hillebrand JJG: Hypothalamic CRH mRNA, but not POMC or NPY mRNA, changes during compensatory under-eating in rats recovering from forced overweight.

Abstract

We tested whether hypothalamic NPY, POMC or CRH mRNA levels are altered in hypophagic rats dynamically regulating adipose tissue levels following forced overweight. Overweight (OW) rats were intragastrically overfed 21 d, resulting in ~141 g (~35%) excess body weight and ~98 g (~245%) excess total adipose tissue (AT), measured by microCT, in comparison to saline-infused normal weight (NW) rats. Overfeeding was then stopped. Blood samples were taken 8 d later and rats were killed on the day after, when rats were undereating by ~40% and still had ~23% (~94 g) excess body weight and ~188% (~75 g) excess AT. In situ hybridization indicated that Arc and DMH NPY mRNA and Arc POMC mRNA were not significantly changed in OW rats, but PVN CRH mRNA was significantly increased by ~25% in OW rats. Plasma corticosterone on d 8 was not significantly different between groups. Basal plasma insulin levels were increased ~2.7 fold at the end of overfeeding and had returned to NW levels by d 8. Basal plasma leptin levels were increased ~6.8 fold by overfeeding and were still increased ~2.4 fold on d 8. Thus leptin may have stimulated the increase in PVN CRH mRNA. These data do not support the canonical view that tonic changes in Arc NPY and α -MSH synaptic secretion mediate weight-regulatory changes in eating or that basal insulin is an endocrine adiposity signal in this situation. In contrast, neurocrine PVN CRH synaptic secretion may contribute. Our negative data challenge the current view on the neurocrine regulation of AT mass.

Introduction

The brain controls eating and EE in order to regulate AT mass, or stored energy. An interconnected group of hypothalamic nuclei, including the Arc, PVN, LHA, VMN and DMH, is thought to form the core of the neural network mediating this regulation [20-25]. Arc NPY and α -MSH neurons project to the PVN, LHA, DMH and other forebrain and hindbrain sites, and levels of Arc NPY and POMC mRNA are changed in underweight and obesity [9, 20]. Administration of NPY in or near several of these hypothalamic sites elicits “anabolic” (i.e., increased eating and decreased EE) responses and increased body weight [102-110], and administration of α -MSH elicits “catabolic” (i.e., decreased eating and increased EE) responses and decreased body weight [105, 146, 148-150]. Furthermore, reports that antagonism of hypothalamic NPY [117] or α -MSH [137, 150-154] leads to the opposite responses as the agonists support the idea that these systems are normally involved in the control of eating and EE. Similar evidence implicates a variety of downstream signaling molecules, including CRH [9, 105, 182, 184, 189-192], in this functional network.

Perhaps the strongest evidence for an active regulation of AT mass is the dynamic compensatory changes in eating and EE that follow experimental manipulations of AT mass [14-19]. However, we are aware of only one study of hypothalamic signaling molecules during such dynamic recovery. This study [163] implicated α -MSH signaling in compensatory hypophagia during recovery from forced overweight. First, Arc POMC mRNA was increased almost 3 fold in rats that had been rendered 5% overweight by chronic intragastric overfeeding. Second, in two trials, 3rd cerebro-ventricular injections of the MC3/4 R antagonist SHU9119 increased eating more in rats tested 1-3 d after ending intragastric overfeeding regimens that rendered them 10 or 16% overweight than in normal-weight control rats.

We [18] previously reported that basal levels of insulin, leptin, amylin and ghrelin did not accurately encode adiposity information during dynamic recovery from overweight induced by chronic intragastric overfeeding. The aim of the present study was to determine whether, despite this, changes in hypothalamic NPY, α -MSH or CRH mRNA occur during dynamic regulation of AT.

Materials and Methods

Animals. Male Long Evans rats, bred from founders from Charles River (Sulzfeld, Germany), were housed individually in wire-mesh cages at 21 ± 1 °C, with a 12:12 h light-dark cycle (lights off, 1200). Ground chow and water were available ad libitum except as described. Chow intake and body weight were measured between 1000 and 1100 daily. All procedures were approved by the Veterinary Office of the Canton of Zurich. Rats were equipped with chronic intragastric catheters, as previously described [18], and allowed to recover ≥ 10 d.

Procedure. Dynamic compensation following forced overweight was tested as previously described [18]. On the first day of the experiment (designated d -21), AT mass was measured by computed tomography (CT, LCT 100, Aloka, Tokyo, Japan), as described below, and rats were divided into overweight (OW, n = 6) and normal-weight (NW, n = 7) groups that were roughly matched for body weight and total adipose tissue (TAT) mass. Beginning on d -19, OW rats were intragastrically infused at 1100, 1300, 1500, and 1700 with liquid diet and NW rats were infused with 0.15 M NaCl. The liquid diet contained 15% energy as protein, 45% as maltodextrin, and 40% as corn oil, with an energy density of 4.3 kcal/ml. Intragastric loads were initially 5 ml and were increased gradually to 11 ml, which resulted in delivery of ~ 189 kcal/d, or ~ 2.5 fold control energy intake¹. On d -17 infusions were reduced to 141 kcal/d to stabilize OW rats' weight until d 0. We did this initially (Gloy 2010) because in similarly designed human studies, leptin and insulin levels were affected by energy flux as well as by AT mass [15, 37, 255]. We saw no such phenomenon [18]. Intragastric infusions were stopped on d 0.

On d -21, -4, -1, and 8, rats were food deprived at 0700, and 600 μ l blood samples were taken from tail vein incisions between 1000 and 1100, i.e., 1-2 h before dark onset, which we define as basal. Blood was mixed with EDTA, and plasma was separated and stored at -20 °C. Immediately following blood sampling, rats were briefly anesthetized with isoflurane, and axial CT scans were made at 2 mm intervals from the anterior aspect of lumbar vertebra 1 to the posterior aspect of lumbar vertebra 6 (L1-L6). Intra-abdominal AT (IAAT) and subcutaneous AT (SAT) were distinguished by the abdominal muscle layers, converted to mass by Aloka's software, and extrapolated to whole-body IAAT and SAT masses using formulae based on correlations between CT estimates and resected AT: whole-body IAAT = $1.2 \times [\text{L1-L6 IAAT}] + 1.5$ g, whole-body SAT = $3.2 \times [\text{L1-L6 SAT}] + 8.6$ g, and TAT = IAAT + SAT. We have shown this method to be sensitive, accurate, and reliable [359].

After completion of 8 d recovery, rats were food deprived for 2-3 h, anaesthetized with intraperitoneal injections of 200 mg/kg pentobarbital about 2 h before dark onset, and transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed and incubated overnight in 4% PFA on an orbital shaker at 250 Hz, then placed in 20% sucrose, which was changed after one day, and stored at -4 °C. Coronal brain sections (20 µm) from 0.80 to 4.08 mm posterior to bregma were cut on a cryostat (Leica Biosystems Nussloch GmbH, CM 1950, Nussloch, Germany), thaw-mounted on RNase-free glass slides (SuperfrostPlus, Gerhard Menzel Glasbearbeitungswerk, Braunschweig, Germany) and stored at -80 °C until *in situ* hybridization.

Hormone assays. Plasma insulin and leptin were measured by fluorescence immunoassay (FIA, Milliplex MAP kit, rat endocrine RENDO-85K, Billerica, MA, USA). Standard lines were fit by the method of least squares using the means of the duplicate standards provided. The insulin standard curve between 0.3-8.7 ng/ml insulin was best fit by a linear function ($\text{ng/mL insulin} = 0.002 \cdot \text{fluorescence} + 0.3$; $r^2 = 1.0$; standard error of the estimate (SEE) = 0.08 pM). The leptin standard curve between 2.7 - 71.4 ng/ml leptin was best fit by an exponential function ($\text{ng/ml leptin} = 1.3 \cdot e^{0.0005 \cdot \text{fluorescence}}$, $r^2 = 1.0$, SEE = 0.09 ln pM). Sample values outside these ranges were not used. The intra-assay CVs for the two plates run were 6 ± 6 and 10 ± 15 for insulin and 11 ± 22 and 12 ± 16 for leptin (mean \pm SD). Corticosterone was measured by competitive ELISA (Assaypro, St. Charles, MO, USA) using plasma samples that were diluted 100 fold. The corticosterone standard curve between 0.391 and 6.25 ng/mL was best fit by exponential function ($\text{ng/mL corticosterone} = 36.08e^{-2.59 \cdot \text{optical density}}$, $r^2 = 1.0$, SEE = 0.05 ln ng/mL). All sample values fell within this range.

Antisense RNA probes. Labeled antisense RNA probes were made by *in vitro* transcription of specific fragments of rat NPY, POMC and CRH cDNA. We used plasmids containing a 288 bp Xba I-Ava I fragment of exon 2 of the rat NPY gene or a 190 bp EcoRI PstI fragment of exon 2 and exon 3 of the rat POMC gene. For the CRH-probe, the cDNA was obtained using total rat RNA as a template (Super Array Bioscience Cooperation, Frederick, MD, USA). The cDNA was amplified by using a primer assay for CRH (QT00183533, QuantiTect Primer Assay, QIAGEN AG, Hombrechtikon, Switzerland), leading to a 88 bp fragment that was subsequently cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). The insertion of positive clones was verified by DNA sequencing. Blast-sequence alignment indicated homology between the inserted sequence and the sequence of CRH mRNA and that the region bound by the primers corresponded to nucleotides 149 -235 of the CRH transcript (GenBank accession no. NM 031019). The plasmids were linearized by digestion with NdeI (Fermentas GmbH, St. Leon-Rot, Germany). *In vitro* transcription was done at 37° C with the appropriate (depending on plasmid and orientation of fragment) RNA polymerase

(T7: P2075 or SP6: P1085, Promega) to obtain antisense probes with radioactive labeled UTP (^{33}P -UTP, Perkin Elmer Inc, Boston MA USA). Incorporation of the ^{33}P label into the transcript was checked using a beta counter.

***In situ* Hybridization.** Slide-mounted sections were fixed in 4% PFA for 10 min, rinsed in phosphate-buffered saline (PBS), pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, rinsed again in PBS, and dehydrated in graded ethanol rinses, followed by 100% chloroform and, finally, 100% ethanol.

The antisense probe solution was added to a hybridization mix containing 65% deionized formamide, 2.6x standard saline citrate (SSC), 1.3x Denhardt's solution, 6.5 mM EDTA and 13 mM phosphate buffer (H_2PO_4 , KP_2O_4) and heated for 5 min at 80 °C. The final probe concentration was adjusted to 6.7×10^6 cpm / ml. Slides were then incubated overnight with 150 μl of the hybridization mix at 72 °C, and on the following day were rinsed briefly in 5x SSC at 72 °C, washed for 2 h in 0.2x SSC at 72 °C to remove aspecific binding, dehydrated with washes in a graded ethanol series, and finally air dried. Slides were exposed to X-ray film (Kodak BioMax MR Sicientific imaging film, Sigma Aldrich GmbH, Steinheim, Germany) for 2 (NPY and CRH) or 3 d (POMC). Films were developed using a film processor (Curix60, Agfa-Gevaert N.V.,Mortsel, Belgium), and data were digitalized using an image scanner (Epson perfection V500 Photo, Meerbusch, Germany). POMC, NPY and CRH mRNA levels were quantified by densitometry (ImageJ, image Processing and Analysis in Java) by an experimenter blind to sample identities. The Paxinos and Watson's rat brain atlas was used to define regions of interest (ROI) in the Arc, PVN, and DMH [453]. Two sections per rat in which NPY was expressed in both the DMH and Arc (i.e., about -2.80 to -3.14 mm posterior to bregma) were analyzed. Neuropeptide mRNA expression was expressed as optical density (arbitrary units) in the ROI minus background measured in the same section, OW rats' data were expressed as percentages of the NW group mean.

Statistical analyses. Amount chow eaten expressed in kcal and body weight were analyzed with separate 2-way (day x group) ANOVA during overfeeding (d -21, -12, -4 and 0) and recovery (d 0, 4 and 8). For amount eaten, 3-d means around the day of blood sampling were used to minimize transient changes associated with the sampling and scanning. Hormone and CT data were also analyzed with 2-way ANOVA. Significant ANOVA interaction effects were followed with the Hochberg variant of planned Bonferroni-Holm comparisons (i.e., OW-NW differences on each day, and OW differences on d 8 vs. d 0 of recovery). Group differences in mRNA expression were tested with independent t-tests. To increase statistical power, data were converted to standard scores using the median absolute deviate method, and standard scores with absolute values > 1.96 (i.e., $P < 0.05$)

were excluded. Minimum level of significance was $P < 0.05$. Data are reported as mean \pm SEM. The standard error of the difference (SED) is given as a measure of residual error.

Results

Food intake and body weight. As shown in **Figure 1**, at the end of the intragastric overfeeding phase (d 0) rats were ~ 141 g ($\sim 35\%$) heavier than NW rats ($F(3, 27) = 495$, $SED = 3$ g, $P < 0.05$). OW rats gradually increased eating during recovery, but still ate $\sim 38\%$ less than NW rats on d 8 ($F(2, 17) = 111$, $SED = 2$ kcal, $P < 0.05$). OW rats lost ~ 47 g weight, but were still ~ 94 g ($\sim 23\%$) heavier than NW rats on d 8 ($F(2, 18) = 154$, $SED = 2$ g, $P < 0.05$).

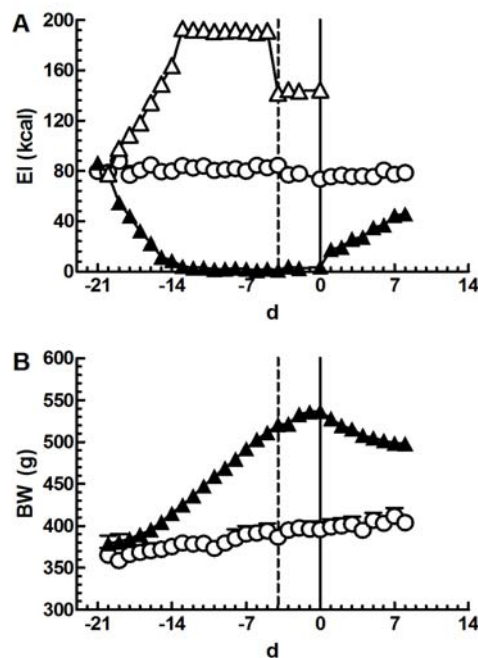


Figure 1. Compensatory undereating and dynamic body weight loss. Energy intake (EI; **A**) and body weight (BW; **B**) during and after intragastric infusions (IG) of liquid diet in overweight (OW) and of saline in normal-weight (NW) rats. The dashed vertical line on d -4 indicates the beginning of the stabilization period, and the solid vertical line on d 0 indicates the beginning of the recovery period. At d 9 rats were killed. Open circles, NW rats; filled triangles, OW rats (in **A**: filled triangles, oral EI; open triangles, total EI). Data are mean \pm SEM.

Adipose tissue. As shown in **Figure 2**, by the end of overfeeding, OW rats had ~ 98 g ($\sim 245\%$) more TAT ($F(2, 20) = 288$, $SED = 3$ g, $P < 0.05$), ~ 68 g ($\sim 280\%$) more SAT ($F(2, 18) = 170$, $SED = 3$ g, $P < 0.05$), and ~ 31 g ($\sim 190\%$) more IAAT ($F(2, 14) = 471$, $SED = 1$ g, $P < 0.05$) than NW rats. During 8 days of recovery OW rats lost only $\sim 15 - 20\%$ of their excess AT, so that TAT, SAT and IAAT were all still significantly elevated (TAT by ~ 75 g,

$F(1, 9) = 154$, $SED = 2$ g, $P < 0.05$; SAT by ~ 54 g, $F(1, 9) = 25$, $SED = 2$ g, $P < 0.05$; and IAAT by ~ 20 g, $F(1, 9) = 408$, $SED = 0$ g, $P < 0.05$). OW rats deposited more excess AT as SAT during overfeeding, so that SAT as a percentage of TAT was greater in OW rats ($66 \pm 1\%$) than NW rats ($60 \pm 1\%$, $F(2, 17) = 18$, $SED = 1\%$, $P < 0.05$) at the end of overfeeding. This difference was maintained through the 8 d of recovery (68 ± 0 vs. $59 \pm 0\%$, $F(1, 7) = 13$, $SED = 1\%$, $P < 0.05$).

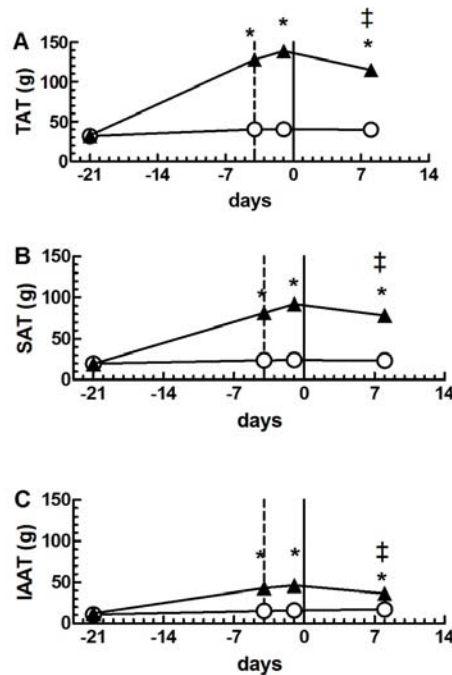


Figure 2. Dynamic adipose tissue (AT) mass loss. Total AT (TAT, A), subcutaneous AT (SAT, B) and intra-abdominal AT (IAAT, C) during and after intragastric infusions (IG) of liquid diet in overweight (OW) and of saline in normal-weight (NW) rats. The dashed vertical line on d -4 indicates the beginning of the stabilization period, and the solid vertical line on d 0 indicates the beginning of the recovery period. At d 9 rats were killed. Open circles, NW rats; filled triangles, OW rats. Data are mean \pm SEM. Statistical contrasts were based on significant ANOVA interaction effects; *Different from NW on same day, $P < 0.05$. ‡Different from OW on preceding measurement day, $P < 0.05$.

Endocrine profile. Basal plasma levels of insulin and leptin are shown in **Figure 3**. No significant differences were detected between OW and NW rats in basal levels of leptin on d -21, before overfeeding began, but insulin levels, for reasons unknown, were significantly higher in OW rats. On d -1, the last blood sampling during overfeeding, basal insulin was almost 3 fold higher in OW rats than NW rats, and basal leptin was over 6 fold higher (insulin: $F(2, 14) = 12$, $SED = 0.25$ ng / ml, $P < 0.05$; leptin: $F(2, 13) = 82$, $SED = 1.2$ ng / ml, $P < 0.05$). By d 8 of recovery, basal plasma insulin levels were no longer significantly

different in NW and OW rats ($F(1, 8) = 18$, $SED = 0.24$ ng / ml, $P < 0.05$). Basal plasma leptin levels also fell markedly during recovery, but were still ~2.4 fold higher in OW rats than NW rats on d 8 ($F(1, 5) = 78$, $SED = 1.3$ ng / ml, $P < 0.05$). Plasma corticosterone levels appeared slightly elevated in OW rats on d 8, (279 ± 14 vs. 313 ± 8 ng / ml in NW rats), but this was not significant ($t(10) = 2.06$, $SED = 16$ ng / mL, $P = 0.07$).

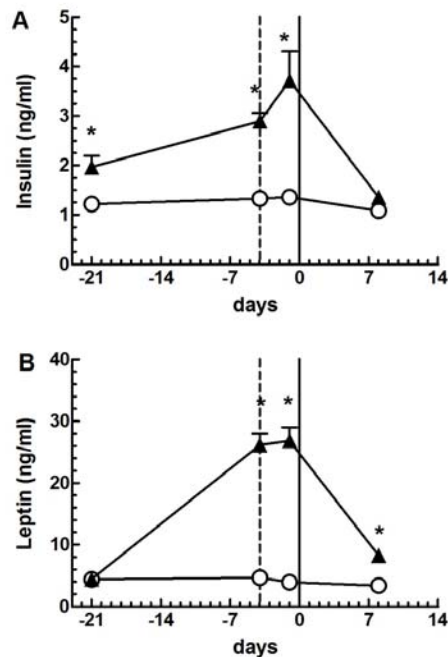


Figure 3. Endocrine profile. Basal insulin (A) and leptin (B) levels during and after intragastric infusions (IG) of liquid diet in overweight (OW) and of saline in normal-weight (NW) rats. The dashed vertical line on d -4 indicates the beginning of the stabilization period, and the solid vertical line on d 0 indicates the beginning of the recovery period. At d 9 rats were killed. Open circles, NW rats; filled triangles, OW rats. Data are mean \pm SEM. Statistical contrasts were based on significant ANOVA interaction effects; *Different from NW on same day, $P < 0.05$. †Different from OW on preceding measurement day, $P < 0.05$.

Hypothalamic gene expression. Gene expression data are shown in **Figure 4**. PVN CRH mRNA levels were increased significantly by ~25% in OW rats on d 9 of recovery ($t(9) = 2.29$, $SED = 8\%$, $P < 0.05$). In contrast, Arc POMC mRNA levels ($t(8) = 0.36$, $SED = 2\%$ AU, $P = 0.73$), Arc NPY mRNA levels ($t(10) = 1.11$, $SED = 12\%$, $P = 0.29$), and DMH NPY mRNA ($t(9) = 0.92$, $SED = 11\%$, $P = 0.38$) did not differ reliably in OW and NW rats.

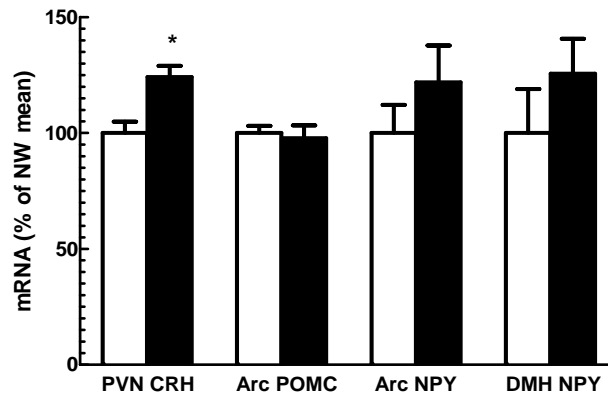


Figure 4. Hypothalamic gene expression. PVN CRH, Arc POMC, Arc NPY, and DMH NPY mRNA levels at d 9 of recovery following intragastric infusions (IG) of liquid diet in overweight (OW) and of saline in normal-weight (NW) rats. Open bars, NW rats; filled bars, OW rats. Data are mean \pm SEM. OW rats' data were expressed as percentages of the NW group mean. Statistical contrasts were based on t-test; *Different from NW, $P < 0.05$.

Discussion

In the present study we tested for the first time whether hypothalamic NPY and POMC mRNA levels are affected in hypophagic rats recovering from forced overweight, i.e., during dynamic regulation of AT mass. NPY and the post-translational product of POMC, α -MSH, are considered to be crucial signaling molecules in the neural network mediating the regulatory control of eating and energy expenditure (EE) [20-25]. Their hypothalamic mRNA levels have been shown to change in, for example, fasting, dietary obesity, genetic obesity, and other steady-state situations. [20, 21, 77, 100, 136]. Therefore, the lack of decreased Arc or DMH NPY levels or increased Arc POMC mRNA levels in a situation in which overweight rats are in marked negative energy balance represents an important challenge to their putative roles in energy homeostasis. At the time of our sample, rats were undereating by ~40% and still had ~23% (~94 g) excess body weight and ~188% (~75 g) excess AT. Furthermore, in our previous, similarly designed study [18], we demonstrated that food intake was decreased through a ~3-4 week recovery period, i.e., ~ 2 weeks past the time of the sample here. Thus, the absence of changed hypothalamic NPY and POMC mRNA levels were unlikely to be because dynamic regulation of AT had already stopped. It is possible, of course, that NPY or POMC mRNA levels may have been changed earlier in recovery, and therefore may play roles in the initial stages of recovery.

Arc POMC mRNA levels have not been measured before during dynamic weight regulation. Hagan et al. [163], however, reported that Arc POMC mRNA was markedly increased at the end of an intragastric overfeeding regimen that increased body weight by only 5%. They also showed that 3rd cerebro-ventricular injections of the MC3/4R antagonist SHU9119 reversed compensatory undereating 1-3 d after ending an intragastric overfeeding regimens that rendered rats ~10 or 16% overweight [163], indicating that α -MSH signaling is involved in dynamic weight regulation. The apparent discrepancy between these data and ours may be because of their earlier sampling time or because the change in α -MSH signaling they detected was due to a change in MC3/4R activity and not a change in α -MSH synthesis and release, which presumably would be reflected in changed POMC mRNA levels. This issue deserves further study.

Seeley et al. [164] measured Arc NPY mRNA levels in rats recovering from 5% overweight induced by intragastric overfeeding. Although Arc NPY mRNA was significantly increased at the end of the overfeeding period, it was not 3 d later. The implication of the latter finding is unclear, however, because the rats were still hypophagic, they were no longer significantly overweight on d 3 of recovery (adiposity was not measured).

CRH neurotransmission is thought to be involved in the control of eating and EE [181-183]. Our finding that PVN CRH mRNA expression was increased during recovery from forced overweight is consistent with the idea that hypothalamic CRH neurotransmission might contribute to the dynamic regulation of AT mass. We measured plasma corticosterone to determine whether a change in PVN CRH mRNA would be more likely to represent neuroendocrine CRH, which stimulates ACTH and subsequently corticosterone release, or neurocrine CRH, which does not. Plasma corticosterone levels were similar to those reported before for this time of the light-dark cycle in rats [454, 455] and did not differ significantly between NW and OW rats, although OW rats tended to have slightly higher levels ($P = 0.07$). Therefore, we conclude that our rats were not chronically stressed at the time of sampling, and thus the increased PVN CRH mRNA in OW rats represents at least in part neurocrine CRH, and that CRH neurotransmission is tonically upregulated in rats dynamically recovering from forced overweight. Our data do not, however, reveal the functional role of CRH in dynamic regulation of AT. For a functional role of CRH in compensatory undereating other criteria have to be fulfilled [24], one of which being reversal of compensatory undereating by antagonism of CRH receptors.

One important question is whether the degree of upregulation of PVN CRH mRNA is quantitatively related to the magnitude of compensatory responding during recovery, which would be consistent with the hypothesis that it is an effective cause of these responses.

Seeley et al.'s [164] finding that PVN CRH mRNA was significantly elevated at the end of overfeeding but not 3 d later provides mixed support for this possibility, as body weight had recovered on d 3 but food intake was still reduced. These investigators also reported that plasma corticosterone levels, measured at light onset, were not increased at the end of overfeeding, but were increased 3 d later. Plasma corticosterone was also increased in normal-weight rats that were pair-fed to the recovering rats. Their interpretation was that the corticosterone increases were linked to negative energy balance independent of weight. Our finding that corticosterone levels were not significantly elevated on d 8 of recovery, when energy intake was reduced by ~40%, does not support this interpretation.

As in our previous, similarly designed study [18] basal plasma leptin levels were slightly, but significantly elevated on d 8 of recovery (previously, leptin was 6.5 fold elevated at the end of over feeding and 1.7 fold elevated on d 8; here they were 6.8 and 2.4 fold elevated, respectively). These decreases in basal plasma leptin were disproportionately large in comparison to the decreases in AT mass. In addition, as is evident in Figure 3, basal leptin levels on d 8 of recovery were much lower than the levels in similarly obese rats during the overfeeding phase. These data suggest that basal plasma leptin does not accurately encode adiposity information and did not serve as an endocrine adiposity signal sufficient to drive dynamic regulatory responses in our test. Nevertheless, leptin was still elevated on d 8 of recovery and, therefore, may have stimulated the increase in PVN CRH mRNA, although it probably did not do so via the canonical Arc NPY/ α -MSH pathway. Finally, others showed that when plasma leptin levels were elevated a similar amount (~2x) by peripheral leptin infusion, Arc NPY mRNA was significantly decreased and PVN CRH mRNA was unchanged [449]. The distinction between these data and ours is another indication that plasma leptin levels alone are not sufficient to explain the changes in hypothalamic signaling molecule expression.

Basal plasma insulin could not have functioned as an endocrine adiposity signal on d 8 of recovery because insulin levels were no longer elevated. We reached the same conclusion previously [18], when basal plasma insulin decreased below those of NW rats by d 8 of recovery and remained below NW levels through 23 d.

Conclusions

The dynamic compensatory changes in eating and EE provoked by forced weight gain or loss are considered the strongest evidence that body weight or adiposity is actively regulated. In recent years, a great deal of attention has been focused on the hypothesis that

the regulation of body weight is mediated by a neural network centered in the hypothalamic Arc, which is sensitive to basal levels of leptin, insulin and perhaps other adiposity signals and in which NPY and α -MSH are principal signaling molecules. Nevertheless, there have been very few studies of the functioning of this neural mechanism during compensatory responding to weight perturbations. The present data (1) fail to support the hypothesis that basal insulin, Arc NPY or Arc α -MSH mediate compensatory hypophagia after forced weight gain, (2) give only mixed support for the involvement of basal leptin, and (3) support the hypothesis the neurocrine CRH in the PVN does participate in this regulatory capacity.

Therefore, future studies are warranted to attempt to understand (1) the role of endogenous CRH in weight regulation, (2) the discrepancies between our data and results on MC3/4 receptor antagonism [163], as well as in different contexts of overweight [163, 164] (3) whether these mechanisms drive compensatory responses to underweight more effectively than those to overweight [70, 456], and (4) whether the regulatory actions of these molecules might occur in other brain areas, such as the caudal brainstem [457].

Our results support the emerging view that regulatory changes in eating and EE cannot be attributed to a small number of integratory neurons (especially, Arc POMC and NPY neurons) and peripheral signals (especially, insulin and leptin). Instead it appears that a variety of metabolic-sensing neurons in several peripheral and central sites activate a widely distributed neural network to achieve energy homeostasis [90].

Ovariectomy and overeating palatable, energy-dense food increase subcutaneous adipose tissue more than intra-abdominal adipose tissue in rats

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Abstract

Background. Menopause is associated with increased adiposity, especially increased deposition of intra-abdominal (IA) adipose tissue (AT). This differs from common or 'dietary' obesity, i.e., obesity apparently due to environmentally stimulated overeating, in which IAAT and subcutaneous (S) AT increase in similar proportions. The effect of menopause on adiposity is thought to be due to the decreased secretion of ovarian estrogens. Ovariectomy in rats and other animals is a commonly used model of menopause. It is well known that ovariectomy increases adiposity and that this can be reversed by estradiol treatment, but whether ovariectomy selectively increases IAAT has not been measured directly. Therefore, we used micro-computed tomography (microCT) to investigate this question in both chow-fed and dietary-obese rats.

Methods. Ovariectomized, ovariectomized and estradiol treated, and sham-operated (intact) rats were fed chow or chow plus Ensure (Abbott Nutrition; n = 7/group). Total (T) AT, IAAT and SAT were measured periodically by microCT. Regional distribution of AT was expressed as IAAT as a percentage of TAT (%IAAT). Excesses in these measures were calculated with respect to chow-fed intact rats to control for normal maturational changes. Chemical analysis of fat was done in chow-fed intact and ovariectomized rats at study end. Data were analyzed by t-tests and planned comparisons.

Results. Body mass, TAT, total fat mass, fat-free body mass, and %IAAT all increased in chow-fed intact rats during the 41 d study. Ovariectomy increased excess body mass, TAT, fat mass, fat-free body mass, and SAT, but had little effect on IAAT, leading to a decrease in %IAAT, in chow-fed rats. Ensure feeding markedly increased SAT, IAAT and TAT and did

not significantly affect %IAAT. Ovariectomy had similar effects in Ensure-fed rats as in chow-fed rats, although less statistically reliable. Estradiol treatment prevented all the effects of ovariectomy.

Conclusions. Both ovariectomy in rats and menopause are associated with increased TAT. After ovariectomy, fat is preferentially deposited as SAT and lean body mass increases, whereas literature shows that after menopause fat is preferentially deposited as IAAT and lean body mass decreases. These opposite effects of ovariectomy and menopause on regional AT distribution and lean body mass indicate that ovariectomy in rats is not a homologous model of menopause-associated changes in body composition and should be used with great caution in investigations of adiposity-related diseases.

Background

Menopause increases the risks of a number of diseases [458-460]. The abrupt and marked decrease in ovarian secretion of estrogens that occurs around menopause [364] is thought to be the main cause of these increases in health risks. In many cases, such as osteoporosis and stroke, increased risk appears to result from losses of direct estrogenic actions on the target tissues [459, 461]. In others, such as type 2 diabetes mellitus and cardiovascular disease, however, decreased estrogen production also appears to increase risk indirectly, by increasing adiposity [362, 459, 462-464].

Body mass index (BMI, mass in kg / height in m²), axial computed tomography (CT) or magnetic-resonance imaging (MRI) scans limited to one or a few levels are commonly used measures of adiposity. A growing literature, however, indicates that these measures are not sufficiently precise to detect physiologically significant changes in the amount and regional distribution of adipose tissue (AT) [34, 353-358]. The best available estimates of the effect of menopause on total adiposity come from studies of whole-body imaging or dual-energy x-ray absorptiometry (DEXA) scans. We are aware of six cross-sectional studies of this type in which multiple-regression analysis was used to isolate effects of menopause from those of aging *per se* [365-370]. In these studies, menopause increased body fat ~5-10% body mass. In terms of physical health, this is a substantial gain, as epidemiological data indicate that in moderately obese women, the risk of diabetes decreases 16% for each kilogram, or ~2% body mass, lost [465].

The effect of menopause on adiposity in the studies above did not appear to depend on premenopausal body mass. This is interesting because it suggests that menopause adds to

other causes of increased adiposity, in particular to obesity related to the increased availability and consumption of palatable, high energy-dense foods that is thought to be the main impetus for the obesity epidemic (i.e., “dietary obesity”) [8-11].

Obesity-related health risks depend on the site, as well as the amount, of AT deposition. Intra-abdominal AT (IAAT) is thought to be the most deleterious form of adiposity, and lower-body (or gluteo-femoral) subcutaneous AT (SAT), the least deleterious [4-7]. Many epidemiological studies indicate that increased waist circumference or increased waist to hip-circumference ratio is associated with increased disease risk [5, 6, 362, 363]. Waist circumference, however, does not distinguish abdominal SAT from IAAT. This distinction requires direct measurements with CT, MRI or other imaging techniques. The effect of menopause on IAAT has been measured with whole-body imaging and analyzed by multiple regression only once. It was found that menopause increased IAAT by ~2 kg, from 4.3% of TAT in premenopausal women to 8.8% in postmenopausal women. In addition, the increase in IAAT was associated with increased signs of cardio-metabolic health risk, including fasting concentrations of plasma insulin, triglycerides and the inflammation mediators C-reactive protein and tissue plasminogen-activator antigen [369]. This is consistent with several other menopause studies using less direct measures of IAAT [371, 460].

The most common model for studying the physiology of menopause is ovariectomy, which has long been known to increase body mass and adiposity in rats and mice. In several studies in which whole-body fat content was analyzed by chemical carcass analysis 4-8 wk after ovariectomy in chow-fed rats, body masses increased by means of ~35-60 g and body fat contents increased ~6-20 g [361, 376-381]. Similar effects have been reported in mice [382, 383]. The effect of ovariectomy on regional AT deposition, however, has not been clearly established in either rats or mice. Several groups have described increases in the mass of one or a few resected AT depots following ovariectomy [384-388], but none has described the total of all depots. Others have reported the total fat content of different body areas, but not of the AT depots *per se*. For example, Ainslie et al. [389], using DEXA, reported that ovariectomy increased “abdominal” and “peripheral” fat gain ~11 g each. The landmarks distinguishing abdominal and peripheral, however, were not given, and, as noted, abdominal DEXA does not distinguish abdominal SAT from IAAT. Clegg et al. [361] estimated that ovariectomy increased IAAT more than SAT, but did not measure AT depots directly; rather, SAT was resected together with the skin and IAAT was estimated as the fat contents of the remaining carcass as measured by chemical analysis. In view of the fragmentary data on the effects of ovariectomy on AT mass and distribution in rats, our goal here was to provide an improved platform for the use of ovariectomy as a rodent model of

menopause in obesity research. We used a combination of microCT and chemical carcass analysis to provide the first direct measures of the effects of ovariectomy on TAT, IAAT, SAT and fat outside the TAT (non-TAT fat) in chow-fed and, except for the chemical analysis, dietary-obese rats. We hypothesized that ovariectomy would affect adiposity in rats similarly to the effect of menopause in women; that is, by increasing TAT and the relative deposition of IAAT.

Materials and Methods

Animals. Female Long-Evans rats (bred from founders from Charles River, Sulzfeld, Germany) were housed individually in cages with wood-chip bedding, in a colony room with a 12:12 h light-dark cycle (lights off 1700 h) and an ambient temperature of 20 – 22 ° C. Water and ground chow were available ad libitum, except as indicated. At study onset (d 0) animals weighed 200 - 270 g and were ~12 weeks old. All procedures were approved by the Veterinary Office of the Canton Zurich.

Measurement of AT mass. SAT, IAAT and TAT masses were measured periodically by microCT (LCT 100, Aloka, Tokyo, Japan). Rats were anesthetized with isoflurane and placed supine in the machine, and serial 2 mm scans were done from the anterior aspect of lumbar vertebra 1 to the posterior aspect of lumbar vertebra 6 (L1–6). Aloka software estimated the volumes of AT, bone, air and the remainder on the basis of their different x-ray densities, and distinguished SAT and IAAT by detecting the abdominal muscle layers. These data were converted to masses and extrapolated to whole-body SAT, IAAT and TAT masses as previously described and validated for male rats [18, 359]. Whole-body scans of 44 female rats weighing 270 – 412 g were done to generate regression formulae to extrapolate L1-6 AT masses to whole-body AT masses in females. The formulae were (data in g): whole-body IAAT mass = 1.1 (L1-6 AT mass) + 1.5, ($r^2 = 0.99$); whole-body SAT mass = 3.0 (L1-6 AT mass) + 7.1 ($r^2 = 0.91$); and TAT mass = SAT mass + IAAT mass. Data below are transformed to whole-body values. Rats were assigned to one of 6 groups (n = 7 each), matched for body and TAT masses on the basis of d 0 data.

Surgery and hormone treatment. On d 1, rats were food deprived ~6 h and pretreated with 5 mg/kg trimethoprim sc and 20 mg/kg sulfadoxine sc for antibiotic prophylaxis, 50 µg/kg atropine sulfate sc, and 80 µg/kg acepromazine ip. About 20 min later, they were anesthetized with 5 mg/kg xylazine and 50 mg/kg ketamine, both ip. Four groups were ovariectomized via a 4 cm midline laparotomy and two groups were sham operated by laparotomizing them and visualizing the ovaries. Immediately after surgery, 5 mg/kg

carprofen was sc injected for analgesia. This was repeated on d 2 and 3, and the antibiotic prophylaxis was repeated on d 2. Rats recovered pre-surgical body masses within 24 h. Hormone treatments began on d 5. Two groups of OVX rats received sc injections of 2 µg 17β-estradiol-3-benzoate (Sigma-Aldrich, Buchs, Switzerland; Cat # E8515) in 100 µl sesame oil (Sigma-Aldrich), and two ovariectomized groups and the two sham operated groups received oil alone. This was repeated every 4th d thereafter. This estradiol regimen has been shown to elicit a near-physiological pattern of plasma estradiol concentration and to be sufficient to maintain normal food intake, spontaneous meal patterns, body mass, and (with progesterone) lordotic reflexes in ovariectomized rats [406].

Procedure. Beginning on d 5, three groups were offered Ensure (chocolate Ensure Plus, Abbott Nutrition, Baar, Switzerland; 1.5 kcal/ml [4.7 kcal/g solids], ~28% energy from soy oil and ~57% from sugar) ad libitum in addition to chow, leading to the final allocation of rats into the six groups listed in **Table 1**. Ensure feeding rapidly leads to marked dietary obesity in male rats [320]. Routine maintenance, injections, body mass measurements, and vaginal cytology sampling [420] were done daily between 0900 and 1000 h. On d 0, 20, 27, 34, and 41, AT mass was measured by microCT between 1300 and 1500 h. Blood samples were mixed with EDTA, and plasma was separated and stored at -20°C. Rats were euthanized by CO₂ inhalation on d 42. Carcasses of CH-Intact and CH-OVX rats were stored at -20 °C.

Table 1 - Group designations

| Endocrine status | Diet | |
|---|-----------|-----------------|
| | Chow | Chow and Ensure |
| Intact | CH-Intact | EN-Intact |
| Ovariectomized | CH-OVX | EN-OVX |
| Ovariectomized with estradiol treatment | CH-OVX+E2 | EN-OVX+E2 |

Chemical carcass analysis. Frozen carcasses of CH-Intact and CH-OVX rats were cut in 3 mm slices, lyophilized (BenchTop 2K Freeze Dryer, VirTis, Gardiner, NY, USA) to a constant mass, and homogenized in a blender. Fat content of the carcass was analyzed in duplicate ~2 g aliquots by automated petroleum ether extraction (Soxtec Avanti 2050, Foss Tecator, Hamburg, Germany). In addition, in order to determine the amount of fat in rat AT, samples of inguinal, epididymal, mesenteric, omental and retroperitoneal AT from 3 male Long Evans rats were resected, combined and subjected to chemical analysis in the same run as the

female samples. The CVs (mean \pm SEM) of rat and AT analyses were 0.06 ± 0.05 and 0.05 ± 0.05 , respectively. The recoveries of $\sim 0.25 - 0.5$ g sesame oil (Sigma) added to rat ($n = 8$) and AT ($n = 2$) samples were $0.96 \pm 0.02\%$ and $0.98 \pm 0.03\%$, respectively, and data were corrected for this. We also compared the chemical analysis data to calculations of fat content from carcass water content, as described by Cox et al. [466], i.e., percent fat = $-1.28 * \text{percent carcass water} + 95.22$. The two methods agreed well: chemical extraction (g) = $0.92 * (\text{Cox method}) - 0.5$, $r^2 = 0.92$, $\text{SEE} = 4$ g, $F(1, 11) = 131.4$, $P < 0.05$).

Data analysis. Our design depended upon regular ovarian cycling in the intact rats and hyperphagia and the development of dietary obesity in the Ensure-fed rats. All intact rats displayed regular 4 or 5 d cycles and were included in the analysis. One Ensure-fed rat gained substantially less body mass (63 g) and TAT (21 g) than all the other Ensure-fed rats (mass gain range, 95 – 244 g; TAT gain range (46 – 107 g); body mass and TAT gains were statistical outliers (z-scores 2.34 and 2.14, $P < 0.01$ and < 0.016 , respectively) as determined by the median-absolute deviate method as described previously [18] and the rat was excluded from the analysis. Data were analyzed only on the days of CT scans. In addition to analyzing and presenting the raw data, we also expressed them as excesses, i.e. the difference between the changes in test groups minus the mean change in the CH-Intact control group. This was done to exploit our longitudinal design, to increase statistical power, and to make the data more comparable to the forms recommended for studies of human obesity [467]. Subtracting the change in the CH-Intact control rats is necessary because chow-fed rats normally gain appreciable amounts of body mass and AT throughout adulthood; i.e., they are a dynamically changing control [360]. To characterize this normal maturation, the d 0 - d 41 differences in CH-Intact rats were analyzed with t-tests. Because our focus in the main analysis was on a small number of comparisons that included complex comparisons, statistical power was maximized by analyzing the data with planned comparisons. ANOVA was done to generate an experiment-wide residual error, which was used to compute standard errors of the difference (SED) and t-tests, the significances of which were determined using the Hochberg variant of the Bonferroni-Holm method [468], with an experiment-wide two-tailed α -level of $P < 0.05$. Five comparisons were tested: CH-OVX vs. CH-Intact, CH-OVX vs. CH-OVX-E2, EN-Intact vs. CH-Intact, EN-OVX vs. EN-Intact, and EN-OVX vs. EN-OVX-E2. Analysis of pilot data indicated that with five comparisons, we would detect as significant differences of ~ 10 g TAT, which we consider biologically meaningful. The variability of data from the Ensure-fed groups increased relatively faster than those of chow-fed groups, necessitating square-root or logarithmic transformation to achieve homogeneity of variance. Chemical analysis data were analyzed

by t-tests. Data are reported as means \pm standard errors of the mean (SEM), and SED are given to indicate experiment-wide residual errors.

Results

Effects of maturation and Ensure feeding. Body composition changed significantly in CH-Intact rats during the 41 d study. Body mass increased by 73 ± 6 g (SED = 11 g, $P < 0.001$), of which TAT, measured by microCT, was 15 ± 3 g (SED = 4 g, $P < 0.01$) (**Table 2**). SAT and IAAT increased in similar amounts (SAT, 7 ± 2 g, SED = 3 g, $P < 0.03$; IAAT, 8 ± 1 g, SED = 2 g, $P < 0.01$) (Table 3), leading to an increase in IAAT as a percentage TAT from $41 \pm 1\%$ to $46 \pm 1\%$ IAAT (SED = 2%, $P < 0.01$) (**Table 3**). These dynamic longitudinal changes in CH-Intact rats were used to calculate changes in other groups in terms of excesses, as described in the **Data Analysis** section.

Table 2 - Body mass and TAT mass at study onset and end

| | BM (g) | | TAT (g) | |
|-----------|-------------|----------------------------|------------|--------------------------|
| | d 0 | d 41 | d 0 | d 41 |
| CH-Intact | 226 \pm 6 | 298 \pm 9 ^{###} | 18 \pm 1 | 33 \pm 4 ^{##} |
| CH-OVX | 226 \pm 6 | 357 \pm 10 ⁺ | 19 \pm 1 | 51 \pm 2 ⁺ |
| CH-OVX+E2 | 226 \pm 7 | 281 \pm 7 | 20 \pm 1 | 34 \pm 3 |
| EN-Intact | 226 \pm 7 | 363 \pm 7 [*] | 19 \pm 1 | 84 \pm 4 [*] |
| EN-OVX | 229 \pm 7 | 399 \pm 19 ^{ϕ+} | 18 \pm 2 | 95 \pm 10 |
| EN-OVX+E2 | 233 \pm 8 | 356 \pm 14 | 20 \pm 1 | 83 \pm 7 |

Initial (d 0) and final (d 41) body mass (BM) and total adipose tissue (TAT) mass, determined with microCT, of chow- and Ensure-fed intact rats (CH-Intact and EN-Intact), ovariectomized rats (CH-OVX and EN-OVX), and estradiol-treated ovariectomized rats (CH-OVX+E2 and EN-OVX+E2), means \pm SEM. Initial and final levels in CH-Intact rats were compared with t-tests to characterize normal maturational changes. Group differences on d 41 were analyzed by planned comparisons, with experiment-wide significance $P < 0.05$, as described in the text. [#]Different from d 0, $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$; ^{*}Different from CH-Intact; ⁺OVX different from OVX+E2, same diet group; ^ϕEN-OVX different from EN-Intact.

Table 3 - SAT mass and IAAT mass at study onset and end

| | SAT (g) | | IAAT (g) | | IAAT (%) | |
|-----------|---------|----------------------|----------|----------------------|----------|----------------------|
| | d 0 | d 41 | d 0 | d 41 | d 0 | d 41 |
| CH-Intact | 11 ± 0 | 18 ± 3 [#] | 8 ± 1 | 15 ± 2 ^{##} | 41 ± 1 | 46 ± 1 ^{##} |
| CH-OVX | 12 ± 0 | 30 ± 1 ^{*‡} | 7 ± 0 | 20 ± 1 | 38 ± 1 | 40 ± 1 [*] |
| CH-OVX+E2 | 12 ± 0 | 19 ± 2 | 8 ± 1 | 16 ± 2 | 40 ± 1 | 45 ± 1 |
| EN-Intact | 12 ± 1 | 46 ± 3 [*] | 7 ± 1 | 38 ± 2 [*] | 38 ± 2 | 45 ± 1 |
| EN-OVX | 11 ± 1 | 54 ± 6 | 7 ± 1 | 41 ± 4 | 39 ± 4 | 43 ± 1 |
| EN-OVX+E2 | 11 ± 1 | 44 ± 4 | 8 ± 1 | 39 ± 2 | 42 ± 2 | 47 ± 1 |

Initial (d 0) and final (d 41) levels (mean ± SEM) of subcutaneous adipose tissue (SAT) and intra-abdominal adipose tissue (IAAT) masses of chow- and Ensure-fed intact rats (CH-Intact and EN-Intact), ovariectomized rats (CH-OVX and EN-OVX), and estradiol-treated ovariectomized rats (CH-OVX+E2 and EN-OVX+E2). Regional adipose tissue distribution is expressed as percentage IAAT/ (SAT + IAAT). Initial and final levels in CH-Intact rats were compared with t-tests to characterize normal maturational changes. Group differences on d 41 were analyzed by planned comparisons, with experiment-wide significance $P < 0.05$, as described in the text. [#]Different from d 0, $P < 0.05$, ^{##} $P < 0.01$; ^{*}Different from CH-Intact; [‡]OVX different from OVX+E2, same diet group.

Ensure-feeding led to marked dietary obesity. Body mass, TAT mass, SAT mass and IAAT mass were significantly increased in EN-Intact rats compared to CH-Intact rats at study end, as shown in Tables 2 and 3 and Figures 1, 2, and 3. The changes were progressive: excess body mass and TAT mass were detected at each measurement point and by d 41 reached levels of 64 ± 9 g (SED = 12 g, $P < 0.05$) excess body mass and 50 ± 5 g (SED = 7 g, $P < 0.05$) excess TAT (Figure 2). Excess SAT and IAAT were increased in EN-Intact rats on each test day as well (e.g. d 41, excess SAT: 28 ± 3 g, SED = 5 g, $P < 0.05$; excess IAAT: 23 ± 2 g, SED = 3 g, $P < 0.05$) (Figure 3). IAAT as a percentage TAT on d 41 was similar in EN-Intact rats and CH-Intact rats (46 ± 1 and $45 \pm 1\%$, respectively, SED = 2%, n.s.), (Table 3).

Ovariectomy and estradiol treatment in chow-fed rats. Ovariectomy significantly increased body mass and TAT mass levels in CH-Intact rats (Table 2). Significant excesses in body mass were detected in CH-OVX rats on each test day and significant excesses in TAT, on d 20 and subsequently (**Figure 1 and Figure 2**). On d 41, CH-OVX rats had 58 ± 5 g excess body mass (SED = 12 g, $P < 0.05$) and 17 ± 2 g excess TAT (SED = 7 g, $P < 0.05$).

CH-OVX rats had significant excesses in SAT on d 21 and subsequently, but a significant amount of excess IAAT only on d 34 (Figure 3, open squares). As a result, IAAT as a percentage of TAT increased more slowly in CH-OVX rats than in CH-Intact rats and was significantly less on d 41 ($40 \pm 1\%$ vs. $46 \pm 1\%$ in CH-OVX and CH-Intact rats, respectively, SED = 2%, $P < 0.05$) (Table 3). Finally, estradiol treatment significantly ameliorated all these effects of ovariectomy, except IAAT as a percentage of TAT still tended to be increased in CH-OVX-E2 rats (SED = 1%, n.s.), (Tables 2 and 3; Figures 2 and 3).

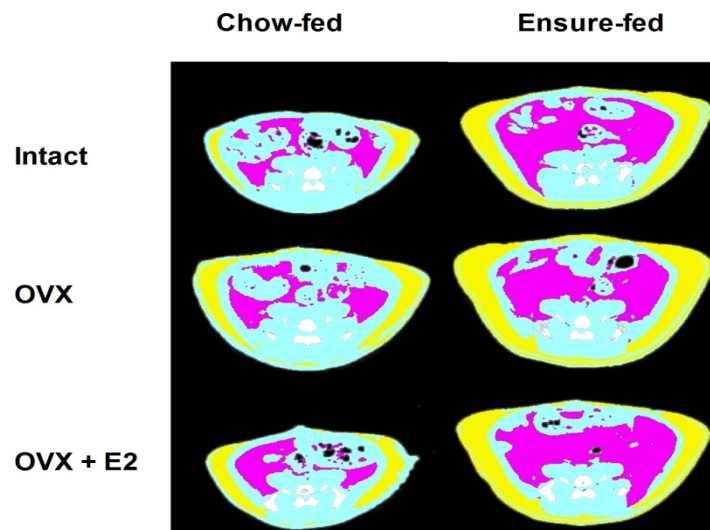


Figure 1 - Representative microCT images. Representative microCT images showing SAT (yellow) and IAAT (magenta) at the level of lumbar vertebra 6 on d 41 in chow-fed and Ensure-fed rats that were sham-operated (Intact), ovariectomized (OVX), or ovariectomized and estradiol-treated (CH-OVX + E2) are shown; *white/gray* is bone, *black* is air, and *blue* is the remainder.

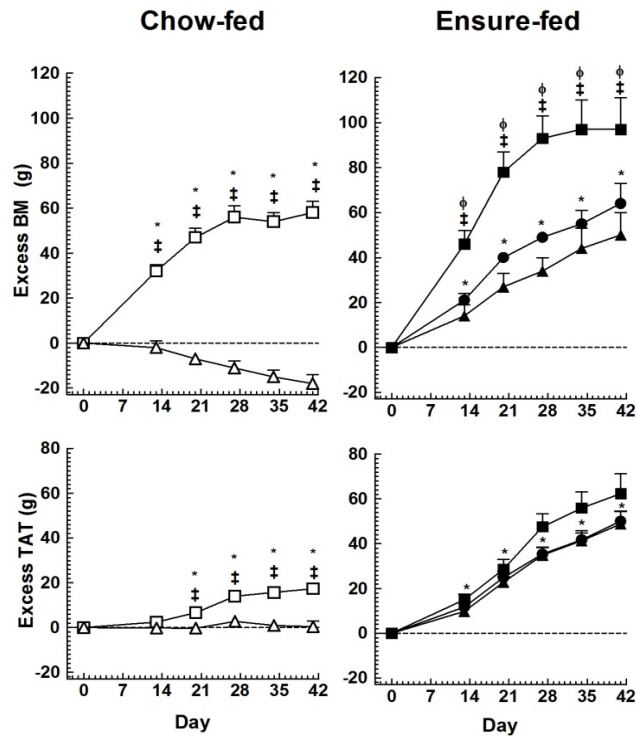


Figure 2 - Effects of ovariectomy and estradiol treatment on excess TAT and body mass. Excess TAT measured by microCT and body mass were calculated with respect to the chow-fed intact group (CH-Intact, y-axis = 0), as described in the text. The left panel shows chow-fed rats that were ovariectomized (CH-OVX; open squares) or ovariectomized and estradiol-treated (CH-OVX+E2; open triangles). The right panel shows Ensure-fed rats that were sham operated (EN-intact, filled circles), ovariectomized (EN-OVX, filled squares), or ovariectomized and estradiol-treated (EN-OVX+E2, filled triangles). Data are means \pm SEM and were analyzed by planned comparisons, with an experiment-wide significance of $P < 0.05$, as described in the text. *Different from CH-Intact; †OVX different from OVX+E2, same diet group; ϕ EN-OVX different from EN-Intact.

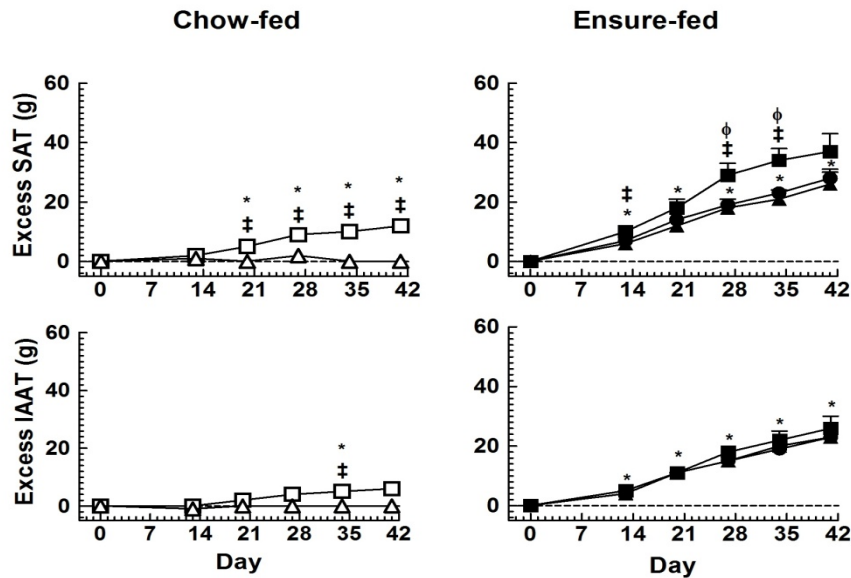


Figure 3 - Effects of ovariectomy and estradiol treatment on excess SAT and IAAT.

Excess SAT and excess IAAT were calculated with respect to the chow-fed intact group (CH-Intact, y-axis = 0), as described in the text. The left panel shows chow-fed rats that were either ovariectomized (CH-OVX; open squares) or ovariectomized and estradiol-treated (CH-OVX+E2; open triangles). The right panel shows Ensure-fed rats that were sham operated (EN-Intact, filled circles), ovariectomized (EN-OVX, filled squares), or ovariectomized and estradiol-treated (EN-OVX+E2, filled triangles). Data are means \pm SEM and were analyzed by planned comparisons, with an experiment-wide significance of $P < 0.05$, as described in the text. *different from CH-Intact; #OVX different from OVX+E2, same diet group; ϕ EN-OVX different from EN-Intact;

Chemical carcass analysis was used to further characterize the effects of ovariectomy in chow-fed rats. At study end, CH-OVX rats had ~ 56 g excess body mass than CH-Intact rats ($t(11) = 3.75$, $SED = 14$ g, $P < 0.01$), of which chemical analysis indicated was ~ 24 g fat ($t(11) = 4.37$, $SED = 5$ g, $P < 0.01$) and, by subtraction, ~ 33 g fat-free body mass ($t(11) = 2.63$, $SED = 12$ g, $P < 0.05$) (**Table 4**; note body mass is not identical to the values above because one rat's sample was lost from the chemical analysis). Chemical analysis of resected AT samples indicated that AT contains $84 \pm 1\%$ fat (of wet mass), which agrees well with published data [469, 470]. This percentage was used to calculate the fat content of AT measured by microCT. CH-OVX rats ~ 14 g more fat stored in the TAT at study end ($t(11) = 3.43$, $SED = 4$ g, $P < 0.05$) and ~ 9 g more fat stored outside the TAT ($t(11) = 2.82$, $SED = 3$ g, $P < 0.05$). Ovariectomy significantly changed body composition: the amounts of body fat, fat in the TAT, and fat outside the TAT normalized to fat-free body mass were all significantly increased in CH-OVX rats ($t(11) = 3.68$, $SED = 0.018$, $P < 0.01$; $t(11) = 2.95$, $SED = 0.013$, $P < 0.05$; and $t(11) = 2.63$, $SED = 0.011$, $P < 0.05$, respectively) (Table 4).

The percentage of total body fat stored in TAT, however, did not differ significantly between CH-OVX and CH-Intact rats ($t(11) = 1.34$, $SED = 4\%$, $P = 0.21$).

Table 4 - Body composition data at study end

| | BM | FFBM | Body fat | | TAT fat | | non-TAT fat | |
|-----------|------------|----------|----------|--------|----------|--------|-------------|-------|
| | G | g | g | /FFBM | g | /FFBM | g | /FFBM |
| Ch-Intact | 301 ± 11 | 266 ± 7 | 37 ± 4 | 0.14 | 28 ± 4 | 0.10 | 9 ± 1 | 0.03 |
| Ch-OVX | 357 ± 10** | 296 ± 9* | 61 ± 3* | 0.21** | 42 ± 2** | 0.14** | 18 ± 3* | 0.06* |

Fat-free body (FFBM) and body-fat masses measured at study end (d 42) by chemical carcass analysis and partitioning of fat between the TAT and non-TAT compartments of chow-fed intact (CH-Intact; $n = 6$) and ovariectomized rats (CH-OVX; $n = 7$). FFBM is body mass – body fat. Fat content of TAT was calculated using the factor of 84% determined in the present study. Data (mean ± SEM) are absolute levels and, for fat data, are also normalized to FFBM. Data were analyzed by t-tests. *Different from CH-Intact, $P < 0.05$; ** $P < 0.01$.

Ovariectomy and estradiol treatment in Ensure-fed rats. Ovariectomy significantly increased body mass in Ensure-fed rats, both in absolute terms (Table 2) and expressed as excesses (Figures 2). TAT mass tended to increase as well, but this was not statistically significant (Figures 2; Table 2). Ovariectomy did, however, increase excess SAT in EN-OVX rats in comparison to EN-Intact rats on d 27 and 34 (9 ± 4 g and 11 ± 4 g, respectively, $SEDs = 3$ g, $Ps < 0.05$, Figure 3). The difference on d 41 was similar, but not significant due to increasing variability (**Table 3; Figure 3**). The difference on d 41 was similar, but not significant due to increasing variability. Ovariectomy did not increase excess IAAT in EN-OVX rats in comparison to EN-Intact rats on any day; the resulting trend for a decrease in %IAAT, however, was not significant (on d 41, $43 \pm 1\%$ in EN-OVX rats and $45 \pm 1\%$ in EN-Intact rats, $SED = 2\%$, n.s.; **Table 3**). Estradiol treatment significantly ameliorated all the effects of ovariectomy that were detected in Ensure-fed rats.

Although we did not include a planned comparison to compare the effects of ovariectomy in the two diet groups (i.e., excess in CH-OVX rats vs. the difference in excesses between EN-OVX and EN-Intact rats), exploratory t-tests suggested that ovariectomy did not differentially affect either excess body mass (d 41, 58 ± 10 vs. 36 ± 19 g, $t(12) = 1.04$, $SED = 22$ g, $P = 0.32$) or excess TAT (d 41, 17 ± 2 vs. 26 ± 10 g, $t(12) = 0.63$, $SED = 10$ g, $P = 0.54$).

Discussion

It has been known for nearly a century that ovariectomy increases adiposity in rats [471], and in recent years ovariectomy has become the most prevalent model of the increase in adiposity precipitated by menopause [12, 349, 404, 472]. Nevertheless, many aspects of ovariectomy-induced obesity have not been well characterized. Therefore, we used microCT to determine directly for the first time the effects of ovariectomy on the development of excess AT and on regional AT distribution in chow-fed and dietary-obese rats. In addition, we further characterized the effects of ovariectomy effects on adiposity in chow-fed rats with chemical analysis of body fat and tested whether a physiological regimen of estradiol treatment was sufficient to prevent the effects of ovariectomy.

Ovariectomy produced ~56 g excess body mass in ~6 wks in the chow-fed rats that were used for chemical analysis. This consisted of ~33 g fat-free body mass and ~2 g fat. These data are similar to several previous reports [361, 377-379], although our effects are larger than most, presumably due to the slightly longer study duration. Combining these data with the microCT data indicated that ovariectomy led to ~17 g excess TAT, which contains ~14 g fat and, consequently, ~9 g fat was deposited outside the AT. We know of only a single other report of the relative amounts of fat inside and outside the AT in rats: Tang et al. [360] found a similar effect in lean and dietary-obese male rats, although this was not emphasized in their report of the data. Ovariectomy increased relative adiposity as well as absolute adiposity: normalized to the increase in fat-free body mass, body fat, fat in the AT, and fat outside the AT all increased significantly. The increases in absolute and relative adiposity in rats appear to parallel studies in normal-weight women revealing that menopause increases absolute and relative adiposity independent of aging [365, 366, 368-370].

Use of microCT also enabled us to provide the first direct measures of regional AT deposition in ovariectomized rats. Based on the limited menopause data available [369], we hypothesized that ovariectomy would increase IAAT relatively more than SAT. This hypothesis was clearly disconfirmed. In chow-fed rats, ovariectomy led to significant excess SAT (~12 g on d 41), but only non-significant excess IAAT (~6 g). This resulted in a significant reduction in the percentage of TAT deposited as IAAT compared to CH-Intact rats (~40 vs. 46%). Similarly, ovariectomy increased excess SAT clearly more than it did excess IAAT in Ensure-fed rats. We previously demonstrated that our microCT technique provides valid and accurate estimates of SAT and IAAT in rats [359]. Therefore, we conclude that ovariectomy leads to the deposition of more SAT than IAAT and, depending on the diet, may not be associated with any significant increases in IAAT. This is different from previous

reports in rats that were based on less complete or indirect measurements, e.g., resection of one or a few AT depots [384-388] or DEXA of the abdominal regions [389], which does not distinguish abdominal SAT from IAAT. Clegg et al. [361] resected SAT and assumed that chemical analysis of the remaining carcass reflected IAAT, i.e., that there is little fat outside the AT. Our data indicate that this is not the case; i.e., we found that ovariectomy increased the fat content of the IAAT ~5 g and the fat content outside IAAT ~10 g. We believe that this, perhaps together with Clegg et al.'s rather small total ovariectomy effect (increases of ~32 g body mass and only ~7 g body fat over 4 wk, vs. our increases of ~54 and ~24 g, respectively) account for the apparent difference in results.

The selective effect of ovariectomy on SAT appears different from the effect of menopause on regional adipose tissue deposition, although the database is surprisingly thin. There has been only one whole-body imaging study with a statistical age control [369]. In this study, menopause increased IAAT about twice as much as it increased SAT. Several more limited imaging studies have reported similar results [370-373], although others have not found any selective increase in IAAT [374, 375]. Given that IAAT, especially truly visceral IAAT, i.e., IAAT that drains into the hepatic-portal vein, poses the more serious challenge to metabolic health [4-6], these data suggest that the metabolic and cardiovascular consequences of ovariectomy in rats may differ importantly from those produced by menopause-induced obesity in women.

We also found that fat-free body mass increased ~33 g in chow-fed ovariectomized rats. We assume that a substantial percent of this increase represent lean body mass, as suggested by several previous reports in which lean body mass was measured directly by chemical analysis [378-381, 473]. This effect is unlike menopause, which is associated with a decrease in lean body mass [365, 367, 368]. The metabolic milieu associated with the marked increases in lean body mass in rats vs. a loss of lean body mass in women is likely to affect many of the same metabolic variables that menopause-induced adiposity does. This seems an important issue to consider in using ovariectomy as a model of menopause, although we are unaware of studies that have done so. These different effects of ovariectomy and menopause on lean body mass may be due to differences in the effects of estrogens on growth hormone and insulin-like growth factor I. This is because ovariectomy increases and estradiol decreases secretion of both hormones in rats [474, 475], whereas menopause decreases and estrogen treatment increases secretion of both [476-478]. It is not clear whether these are species differences or are related to the difference in relative age i.e., the ovariectomy data are from young adult rats, whereas menopause occurs in middle age.

We included groups of Ensure-fed, “dietary-obese” rats as a model of idiopathic human obesity, which is attributed in large part to overconsumption of palatable, high-fat, high-sugar, energy-dense food [8-11]. As expected, feeding intact rats Ensure led to further increases in excess body mass (~65 g on d 41) and TAT (~50 g). Ovariectomy led to further increases in body mass (~35 g) and TAT mass (~12 g, which was not significant). The smaller relative difference between body mass and TAT mass produced by Ensure feeding compared to the effects of ovariectomy in both chow- and Ensure-fed rats suggests that the chronic positive energy balances associated with ovariectomy and with dietary obesity were partitioned into fat and lean tissue in qualitatively different fashions. We assume that dietary obesity more closely mimics human obesity,

The effects of Ensure feeding that we observed may be to a certain extent diet-specific. Lemieux et al. [378] reported that ovariectomy produced much larger effects on body mass and adiposity in rats fed a 45% sucrose, 10% fat diet than in chow-fed rats, whereas here the effects of ovariectomy were similar or smaller in Ensure-fed rats than chow-fed rats. It is important to note that both we and Lemieux et al. [378] began the dietary-obesity regimen only after ovariectomy, whereas women are more often obese before menopause. A better model of dietary obesity and menopause may be afforded by designs like that used by Noel and Fleming [479], who made rats obese by force feeding prior to ovariectomy. In this situation, ovariectomy produced similar amounts of excess body mass, which was the only obesity measure, in obese and control rats (~40 and 45 g, respectively, at d 30).

Estradiol treatment significantly ameliorated the effects of ovariectomy on the SAT, IAAT, TAT, and body mass in both diet groups. The effects on total adiposity and body mass are consistent with many previous studies [404], and the effects on SAT and IAAT are novel. These results indicate that loss of estrogen secretion is the crucial ovariectomy-induced lesion disrupting normal energy homeostasis and causing increased adiposity. That estradiol appeared to reduce body mass below intact levels was unexpected here, because this was not the case in several previous studies using the same, near-physiological estradiol regimen [361, 406, 417]. We have no explanation for this apparent discrepancy.

The effects of estradiol treatment on adiposity that we observed appear to parallel the effects of hormone replacement therapy (HRT) in postmenopausal women. A meta-analysis [390] of four studies [391-394] in which a total of 129 postmenopausal women were randomly allocated to HRT or to placebo or no treatment revealed that HRT decreased abdominal body fat mass by ~7% and increased lean body mass ~3%. In regard to abdominal SAT and IAAT, however, both positive [373, 394] and negative [375, 395] effects of HRT on IAAT have been reported in studies in which part of the abdomen was imaged. Furthermore, in

two randomized trials [395, 396] that were not included in the meta-analysis above, together involving 128 women, no effects of HRT on fat or fat-free mass were detected. The different outcomes of these randomized trials [391-396] may be related in part to the form of HRT used. That is, HRT regimens involving larger amounts of estrogens or smaller amounts of progestins [393, 394] tended to produce the larger effects. Furthermore, progestin treatment alone apparently can increase adiposity in women. For example, in a 30 month longitudinal study of US American women, chronic medroxyprogesterone treatment increased fat mass 23% more than other methods of contraception [480]. This is consistent with rat studies, which indicate that the effects of estradiol treatment on body mass in ovariectomized rats is dose-dependent and can be reduced by pharmacological progestin treatment [397].

Conclusions

Ovariectomy in rodents is a convenient model that mimics the rapid decrease in plasma estrogens and the increase in TAT associated with menopause. Rodent ovariectomy, however, differs from menopause in two ways that are likely to produce important differences in metabolism and obesity-related pathophysiology. First, as we report here, ovariectomy preferentially increases SAT in rats, whereas menopause preferentially increases IAAT, which is metabolically more deleterious. Second, as our data suggest and others [378-381, 473] have documented, ovariectomy induces gain of lean body mass, at least in relatively young rats, whereas menopause induces loss of lean body mass. These two opposite effects indicate that rodent ovariectomy is not a homologous model of menopause-related changes in adiposity. Therefore, ovariectomy should be used with great caution in investigations of adiposity-related cardio-metabolic disease.

Preliminary study: Ovariectomy and estradiol treatment affect subcutaneous more than intra-abdominal adipose tissue in rats.

This initial study was designed to test whether feeding an 'obesifying' diet changes the effects of ovariectomy (OVX) when the development of excess TAT is held constant.

Methods

Intact and OVX rats were offered either chow ad libitum (CH, n = 5, Ch-OVX n = 8) or 3 daily rations of Ensure Plus (En, n = 8, En-OVX n = 8) in amounts that led to excess TAT similar to CH-OVX rats for 30 d (Phase 1). Then En-fed rats were switched to chow ad libitum and OVX rats were treated with estradiol (E2, 2 µg/4 d SC) for 25 d (Phase 2). AT was measured weekly by CT. Statistical comparisons were based on significant ANOVA interactions. Comparisons were CH-Intact vs. CH-OVX, EN-Intact, or EN-OVX, and EN-Intact vs. EN-OVX; for TAT in phase 1, comparisons were CH-Intact vs. CH-OVX, EN-Intact, or EN-OVX. Raw data were also expressed as excesses, i.e. the difference between the in test groups minus the mean change in the CH-Intact control group.

Results

Phase 1: Matching of TAT in Phase 1 was relatively successful. After 30 d, CH-OVX rats had 9 g, EN-intact 6 g, and EN-OVX 10 g excess TAT compared to CH-intact ($F(3, 25) = 4.2$, $SED = 0.1 \log g$).

Ensure feeding alone. Phase 1: In EN-Intact rats, excess TAT was ~5 g SAT and ~1 g excess IAAT, neither of which was significant (Figure 1, left). The percentage of TAT deposited as IAAT (%IAAT) was less in EN-intact compared to CH-Intact (37 ± 1 vs. $41 \pm 1\%$, $F(3, 25) = 12$, $SED = 1\%$). EN-Intact rats had no have any excess BW (Figure 2, left). Plasma levels of insulin were 1.4 fold and those of leptin 1.3 fold increased, which were not significant vs. CH-Intact rats (Figure 3, left). Phase 2: At the end of recovery EN-Intact did not have any excess in TAT, SAT, IAAT or BW (**Figure 1 and 2, right**). AT distribution was not different. Plasma levels of insulin and leptin were not different from CH-Intact (**Figure 3, right**).

Ovariectomy and estradiol treatment in chow-fed rats. Phase 1: CH-OVX rats had 9 g excess TAT was 6 g SAT ($F(3, 25) = 5.8$, $SED = 2 g$, $P < 0.05$) and 3 g IAAT (**Figure 1, left**), which did not change AT distribution. CH-OVX rats had 44 g excess BW ($F(3, 25) = 7.8$, $SED = 0.04 \ln g$, $P < 0.05$) (**Figure 2, left**). Plasma levels of insulin were 1.4 fold (n.s.) and

leptin 2 fold increased ($F(3, 25) = 7.8$, $SED = 1$ ng/ml, $P < 0.05$) in CH-OVX compared to CH-Intact rats (**Figure 3, left**). Phase 2: At the end of the recovery phase, estradiol treated CH-OVX rats had 28 g excess BW (**Figure 2, right**), which was not significant, but still had significant amounts of excess TAT (10 g, $F(3, 25) = 4.9$, $SED = 3$ g, $P < 0.05$), which was 5 g excess SAT ($F(3,25) = 4.2$, $SED = 2$ g, $P < 0.05$) and 5 g excess IAAT ($F(3,25) = 4.9$, $SED = 2$ g, $P < 0.05$) (**Figure 1, right**). AT distribution did not change. Plasma levels of insulin were 1.5 fold (n.s.) and leptin 1.8 fold increased ($F(3, 25) = 6.2$, $SED = 1$ ng/ml, $P < 0.05$) compared to CH-Intact (**Figure 3, right**).

Ovariectomy and estradiol treatment in Ensure-fed rats. Phase 1: Compared to EN-intact rats EN-OVX did not significantly increase excess BW (1 g), TAT, SAT (4 g) or IAAT (0 g) (**Figure 1 and 2, left**). %IAAT, however, was increased in EN-Intact vs. EN-OVX (37 ± 1 vs. 33 ± 0 , $F(3, 25) = 12$, $SED = 1\%$) and leptin levels were significantly elevated (1.7 fold, $F(3,25) = 7.8$, $SED = 1$ ng/ml). Insulin levels were not increased (1.1 fold) (**Figure 3, left**). Phase 2: 25 d after switching rats to chow and starting estradiol treatment, there were no significant differences in any of the excesses measured in EN-OVX compared to EN-Intact rats (**Figure 1-3, right**).

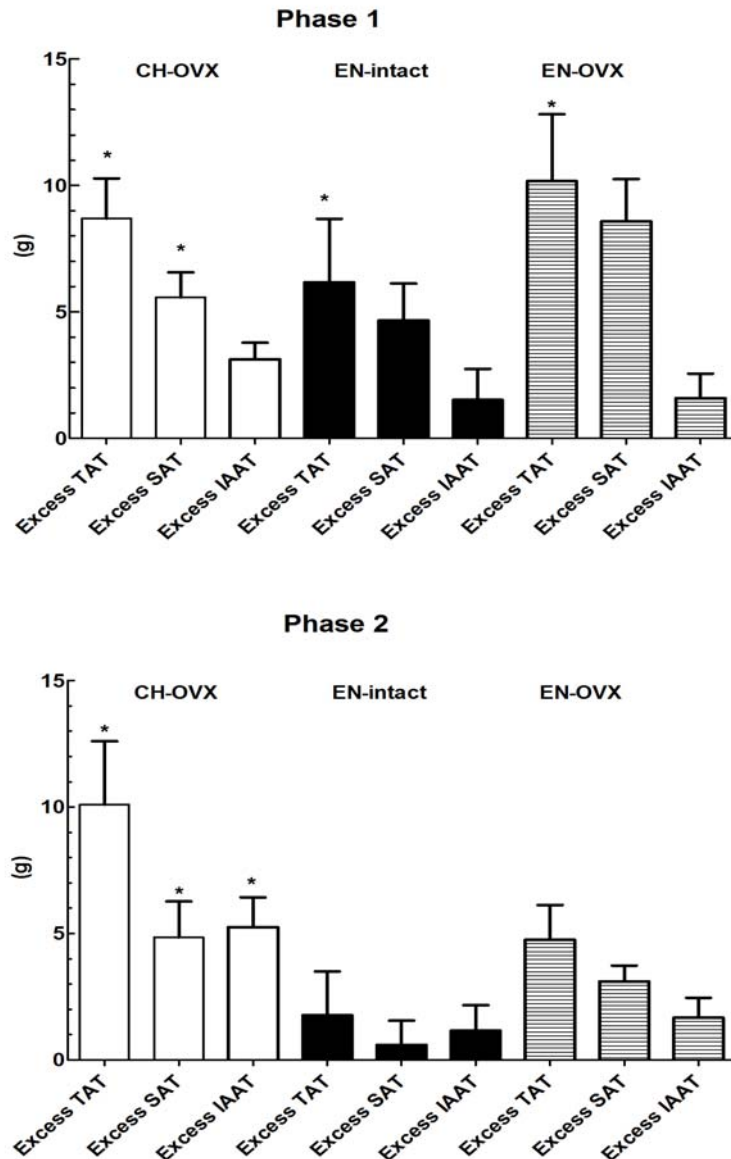


Figure 1. Excess total (T), subcutaneous (S) and intra-abdominal (IA) adipose tissue (AT) mass at the end of phase 1 (top) and 2 (bottom). Intact and ovariectomized (OVX) rats were offered either chow ad libitum (Ch n=5, Ch-OVX n=8) or 3 daily rations of Ensure Plus (En n=8, En-OVX n=8) in amounts that led to excess (T) AT similar to Ch-OVX rats (Phase 1). Then En-fed rats were switched to chow ad libitum and OVX rats were treated with estradiol (E2, 2 μ g/4 d SC) for 25 d (Phase 2). * Different from CH-Intact; ϕ EN-OVX different from EN-Intact, P < 0.05.

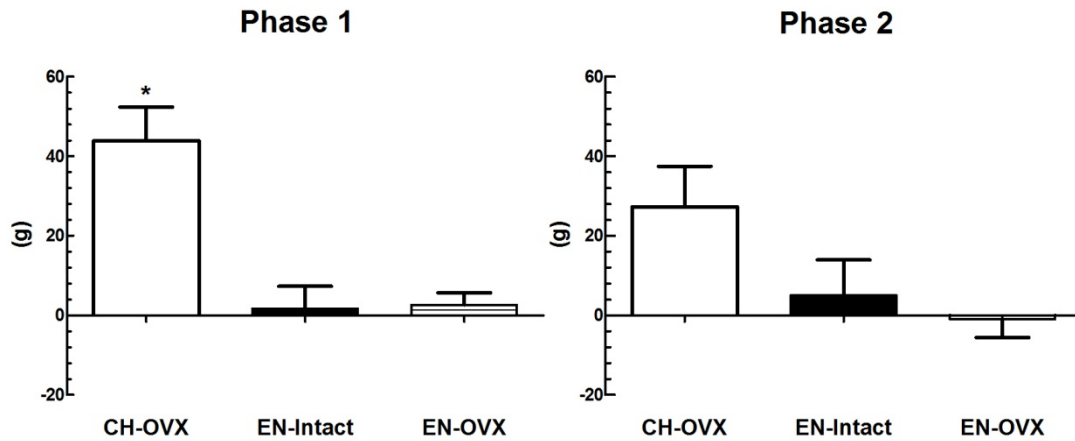


Figure 2. Excess body weight (BW) at the end of phase 1 (left) and 2 (right). Intact and ovariectomized (OVX) rats were offered either chow ad libitum (Ch n=5, Ch-OVX n=8) or 3 daily rations of Ensure Plus (En n=8, En-OVX n=8) in amounts that led to excess (T) AT similar to Ch-OVX rats (Phase 1). Then En-fed rats were switched to chow ad libitum and OVX rats were treated with estradiol (E2, 2 μ g/4 d SC) for 25 d (Phase 2). *Different from CH-Intact; ϕ EN-OVX different from EN-Intact, P < 0.05.

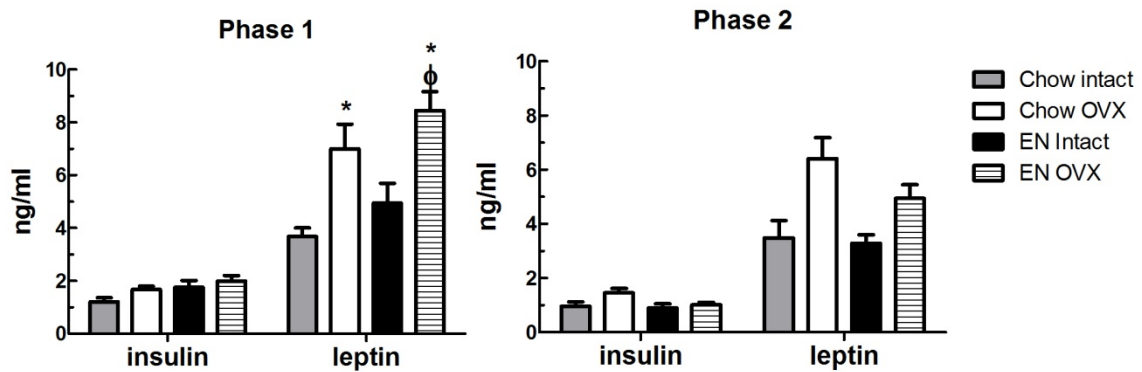


Figure 3. Basal insulin and leptin plasma levels at the end of phase 1 (left) and 2 (right). Intact and ovariectomized (OVX) rats were offered either chow ad libitum (Ch n=5, Ch-OVX n=8) or 3 daily rations of Ensure Plus (En n=8, En-OVX n=8) in amounts that led to excess (T) AT similar to Ch-OVX rats (Phase 1). Then En-fed rats were switched to chow ad libitum and OVX rats were treated with estradiol (E2, 2 μ g/4 d SC) for 25 d (Phase 2). *Different from CH-Intact; ϕ EN-OVX different from EN-Intact, P < 0.05.

Discussion

The feeding regimen in this experiment produced unexpected results. Most unexpected was that, although all test groups gained excess TAT compared to CH-intact, only CH-OVX rats increased excess BW (44 g). Excess BW in CH-OVX was ~4 times higher than excess TAT, which suggests that lean mass increased. This is consistent with studies measuring lean mass in OVX rats directly [378-381, 473]. In contrast, neither EN-intact nor EN-OVX increased excess BW, which, together with the increase in TAT, suggests a loss of lean mass. The results do not identify why Ensure feeding did not lead to significant increases in excess BW. We suspect that the Ensure feeding regimen used in this study might have been the reason. In particular, casual observation indicated that the Ensure-fed rats typically consumed most of each of the three daily rations very soon after it was offered. Such 'gluttony' has long been known to increase relative adiposity [260]. Because we did not have the capacity to prevent this unusual meal pattern, i.e., by imposing a yoked-feeding regimen, we abandoned this approach and tested the effects of Ensure feeding in ad libitum-fed intact and OVX rats in study 3 of the thesis.

Switching from feeding intact rats Ensure to feeding them chow ad libitum in phase 2 reversed excess TAT, and had no significant effect on excess BW, which suggests a regain of lean mass. This appears to be another example of how the amount consumed and the nutrient composition of the food interact to affect body composition, with Ensure containing relatively more energy derived from fat compared to chow. The absence of a change in BW also may have been due to the Ensure feeding regimen.

In contrast to the change in excess TAT with no significant effects on excess BW in intact rats switched from Ensure to chow, estradiol treatment in CH-OVX rats did not reverse excess TAT, but partially reversed excess BW. That is, during the estradiol treatment in phase 2, CH-OVX maintained, but did not reduce, the amount of excess TAT gained during phase 1. In addition, estradiol treatment reduced excess BW from 44 g to 27 g, which, although no longer significant, is not a complete reversal. Thus, starting estradiol treatment 30 d after OVX might be sufficient to stop further increases in excess TAT and BW, but was not sufficient to reverse OVX-induced increases in excess TAT and BW. A similar effect on BW was reported by Noel and Fleming [479]. In contrast, starting the same estradiol treatment shortly after OVX was shown to completely prevent excess fat and BW gain [361, 406]. Thus the timing of the estradiol treatment after OVX seems to be crucial for the effects on TAT on BW.

As already mentioned, although Ensure feeding in intact rats during Phase 1 did not increase excess SAT or IAAT significantly, it did decrease the percentage of TAT deposited as IAAT (%IAAT). EN-Intact rats still had no excess SAT or IAAT at the end of Phase 2, but the decrease in %IAAT was reversed. Therefore, the change in diet and feeding schedule that we used affected regional AT distribution independent of the amount of TAT. To our knowledge, this has not been described before. Because this analysis of this phenomenon was not part of the goals of this thesis, however, we did not pursue it and did not include a switch from feeding a palatable energy-dense food to chow in study 3.

In chow-fed rats OVX increased excess SAT during phase 1, but did not significantly increase excess IAAT or changed %IAAT. OVX in Ensure-fed rats also increased excess SAT selectively. These data are different from the apparent increase in IAAT in women after menopause [369]. Starting estradiol treatment in CH-OVX rats did not reverse excess SAT, and unexpectedly resulted in a significant increase in excess IAAT, although %IAAT did not change significantly. In contrast, EN-OVX rats treated with estradiol did not have any significant excess SAT or IAAT at the end of phase 2. Again, our data do not permit distinction between the relative contributions of Ensure feeding per se and the feeding schedule to these effects, and we did not further pursue them.

Plasma leptin levels (2 and 1.7 fold), but not plasma insulin levels (1.4 and 1.1 fold), were significantly increased in both CH-OVX and EN-OVX rats compared to CH-intact rats at the end of phase 1. Furthermore, at the end of Phase 2, the continued increase in excess TAT in CH-OVX rats was associated with increased plasma leptin levels, but not plasma insulin levels. We have no explanation for these unexpected dissociations between insulin and leptin, but it is consistent with a study by Chen & Heiman [380]. In this study plasma leptin levels, but not insulin plasma levels, were significantly increased 35 d after OVX. At this point in time OVX rats had 50 g excess BW and 13 g excess fat mass [380]. Finally, EN-OVX had significantly higher plasma leptin levels compared to EN-Intact (1.7 fold), which might be due to the apparent difference in the amount of excess TAT (10 vs. 6 g). In EN-OVX treated with estradiol, leptin and insulin plasma levels were not significantly different from CH-Intact at the end of Phase 2, which is consistent with their lack of excess TAT.

8. Summary and Discussion

Background and approach

The increasing prevalence of obesity poses a major health risk for the development of metabolic disorders, diabetes, heart disease, etc., and is associated with increased mortality [2, 481]. Increased IAAT is most strongly associated with increased risk for obesity-related diseases [4-7]. Increased AT mass arises from a chronic imbalance between energy intake and energy expenditure (EE). The main cause is seen as the increasing availability and consumption of palatable, high energy-density foods (i.e., “dietary obesity”) [8-11]. Although the prevalence of dietary obesity seems to cast doubt on the idea that AT mass is actively regulated, there are good reasons to think that it is. First, AT mass during adulthood is kept remarkably constant in most individuals. Second, and more impressive, are demonstrations of dynamic regulation of AT mass; i.e., the dynamic compensatory changes in eating and EE and the return to near normal levels of AT mass following experimentally induced increases or decreases in AT mass, which have been observed in both animals and humans [14-19]. Because little is known about the mechanisms of such dynamic regulation, **studies 1 & 2** of this thesis used the model of dynamic regulation following increases in AT mass induced by chronic IG overfeeding.

The brain is thought to regulate of AT mass by detecting feedback signals related to AT mass and initiating appropriate compensatory responses when the feedback indicates deviations beyond some threshold. The compensatory responses are changes in eating or EE. The most prevalent model of AT mass regulation posits that the feedback signals are basal levels of various endocrine ‘adiposity signals,’ especially insulin and leptin [20]. The Arc of the hypothalamus is generally considered to be the core integratory site where these feedback signals are detected and initiate compensatory responses. Arc neurons are sensitive to insulin and leptin, express NPY and α -MSH, which are considered to be crucial signaling molecules in the neural network mediating the regulatory control of eating and EE, and project to the PVN, LHA, DMH and other forebrain and hindbrain areas. A variety of downstream signaling molecules, including PVN CRH neurons, have been implicated in this functional network [20-25].

There are few descriptions of the involvement of adiposity signals [19] or brain mechanisms during dynamic AT regulation in rats recovering from forced overweight. Therefore, in **study 1** I tested whether basal plasma levels of insulin, leptin, amylin and ghrelin encode

information about AT mass during dynamic AT mass regulation in rats. Basal plasma levels of these hormones were assayed and AT mass was measured by microCT during the development of obesity induced by chronic IG overfeeding and after termination of overfeeding. In **study 2**, in situ hybridization for mRNA was used to test whether hypothalamic levels of NPY, α -MSH or CRH might be involved in compensatory undereating during recovery from forced overweight.

AT mass regulation is modulated by the environment in which an individual lives. Most familiar is the dysregulatory effect of access to palatable, energy-dense foods; i.e., “dietary obesity”, as described above [11, 25]. Other influences are less environmental, for instance the effects of menopause in women. Influences of the reproductive axis on AT mass is especially prominent in women, who gain AT mass at puberty, during pregnancy, and after menopause [349]. The loss of estrogen production that occurs around menopause is thought to contribute to the associated increase in AT mass, in particular more IAAT [369]. OVX in rats and many other species leads to increases in BW and AT mass [404] and is the most commonly used model of menopause. The effects of OVX on AT distribution have not been measured directly. Therefore, in **study 3** I tested whether OVX affects adiposity in rats similarly to the effect of menopause in women, that is, by increasing AT mass and the relative deposition of IAAT, and whether a physiological regimen of estradiol treatment is sufficient to prevent these effects. I also included intact and OVX groups, which were offered palatable, energy-dense foods (i.e., Ensure in addition to chow) to test whether dietary-obesity changes the effects of OVX.

In the following sections I summarize and discuss the implications of each individual study, after which I draw some across-study conclusions. Some across-study discussion is justified because (1) all three Studies used the same strain of rats, held under identical conditions, and (2) the main planned outcome variables, i.e., microCT scans of AT mass and basal plasma insulin and leptin levels, were identical. Across-study conclusions are limited by the facts that Study 3 used female rats and did not include a recovery phase, i.e., the dynamic regulation of AT mass following the induction of obesity by access to palatable energy-dense diet. In addition, unfortunately, some of the hormone data were lost.

Study 1. OW rats were IG overfed for 23 d, resulting in ~130 g excess BW and ~100 g excess TAT, measured by microCT, in comparison to saline-infused NW rats. When the IG overfeeding regimen was terminated, OW rats underate for at least 23 d, and lost excess AT mass and BW. At the end of the study, after 39 d of recovery, rats had still 30 g excess AT mass and 15 g excess BW. Basal levels of insulin, leptin and ghrelin, but not of amylin, changed linearly with AT mass during the IG overfeeding phase. During the subsequent

phase of dynamic AT regulation, none of the four hormones accurately reflected AT mass. Plasma levels of insulin returned to control levels on d 1 of recovery and decreased below control levels thereafter. Leptin also decreased disproportionately to AT mass, but fell more slowly, i.e., was still elevated on d 1, 5 and 8 of recovery, but not thereafter. Furthermore, although AT mass decreased in OW rats during each inter-measurement interval between d 0 and d 23 of recovery, insulin and leptin did so during only the first interval (d 0 – 5). Finally, neither ghrelin nor amylin changed reliably during recovery.

These data disconfirm the hypotheses that these hormones encode AT mass during dynamic recovery from forced overweight. The dissociations between basal plasma insulin and AT mass are consistent with similarly designed tests [14, 267]. The finding that insulin levels were significantly below control levels on d 8-23 of recovery is, however, new. The dynamics of leptin changes during recovery were not associated with AT loss. Although leptin was elevated throughout d 8, leptin fell significantly only through the first 5 d of recovery. This is different from AT mass, which decreased until d 23. Daily food intake was also significantly decreased for more than two weeks after leptin was no longer significantly elevated. It is possible, however, that during the early phase (d 1 to d 8) of dynamic regulation, leptin plasma levels encoded information related to AT mass. Although ghrelin decreased during AT mass gain, it did not change during dynamic AT mass loss. Thus, similarly obese rats with similar amounts of AT mass during the initial phase and the recovery phase had very different ghrelin levels. Amylin did not change reliably during the study. The lack of an increase of amylin with AT mass during IG overfeeding is inconsistent with cross-sectional studies in which increases in plasma amylin levels were associated with increased body weight [313]. The assays for amylin appeared sufficiently sensitive to detect small differences, so it is unlikely that a meaningful change went undetected.

In sum, I conclude that the dissociations between AT mass and basal plasma levels of insulin, leptin, ghrelin and amylin during recovery from forced overweight indicate that none of these hormones encode AT mass in a way that could be used by the rats' brains to guide dynamic compensatory changes in eating or EE, i.e., they are not signaling adiposity in rats under these dynamic conditions. Furthermore, because dynamic regulation proceeded in the absence of significant differences in basal levels of these hormones, their elevations (insulin, leptin, amylin) or decreases (ghrelin) are not necessary for compensatory decreases in eating or increases in EE.

Study 2. OW rats were IG overfed for 21 d, resulting in ~141 g excess BW and ~98 g excess TAT in comparison to saline-infused NW rats. IG overfeeding was terminated and on d 8 blood samples were taken and on the day after rats were killed. At the time of our

sample, on d 9 of recovery, OW rats were undereating by ~40%, had ~94 g excess body weight and ~75 g excess TAT mass. In situ hybridization indicated that Arc and DMH NPY mRNA and Arc POMC mRNA in OW rats were not significantly different from NW rats. In contrast PVN CRH mRNA was significantly increased by ~35% in OW rats. Plasma corticosterone on d 8 was not significantly different between groups. Insulin and leptin levels were consistent with study 1. That is, during recovery from forced overweight, decreases in basal plasma levels of insulin and leptin were disproportionately large in relation to the decreases in AT mass, basal plasma insulin was back to control levels on d 8 of recovery, and basal plasma leptin levels were still elevated on d 8 of recovery, but were ~70% lower than at the end of IG overfeeding, whereas AT mass had decreased only ~20%.

I conclude from these data that the lack of decreased Arc or DMH NPY levels or increased Arc POMC mRNA levels in a situation in which OW rats are in marked negative energy balance represents an important challenge to their putative roles in energy homeostasis. I further conclude that our rats were not chronically stressed at the time of sampling and therefore, that the increased PVN CRH mRNA represents at least in part neurocrine CRH. One important question is whether the degree of upregulation of PVN CRH mRNA is important for synaptic function. That is, other criteria for an endogenous neurocrine role of CRH in compensatory undereating have to be fulfilled [24]. One important criterion is to establish whether antagonizing CRH would reverse compensatory undereating.

Consistent with study 1, study 2 suggests that basal plasma insulin and leptin do not accurately encode adiposity information sufficient to drive dynamic regulatory responses under the conditions tested. In contrast, leptin was still elevated on d 8 of recovery and, therefore, may have stimulated the increase in PVN CRH mRNA, although it apparently did not do so via the canonical Arc NPY/ α -MSH pathway.

Arc POMC mRNA levels have not been measured before during dynamic regulation of AT mass. Hagan et al. reported that Arc POMC mRNA was markedly increased at the end of an IG overfeeding regimen that increased body weight only 5%. They also showed that 3rd cv injections of the MC3/4R antagonist SHU9119 reversed compensatory undereating 1-3 d after ending an IG overfeeding regimen that rendered rats ~10 or 16% overweight [163], indicating that α -MSH signaling is involved in dynamic weight regulation. The apparent discrepancy between their and present data may be because of their earlier sampling time or because the change in α -MSH signaling they detected was due to a change in MC3/4R function and not a change α -MSH synthesis and release. These issues deserve further study.

Seeley et al. [164] measured Arc NPY and PVN CRH mRNA levels in rats recovering from 5% overweight caused by IG overfeeding. Arc NPY mRNA was significantly decreased and PVN CRH mRNA was significantly increased at the end of the overfeeding period, but not 3 d later. The implications of these findings are unclear, because although the rats were still hypophagic, they were no longer significantly overweight on d 3 of recovery (adiposity was not measured).

In summary, study 2 (1) fails to support the hypothesis that basal insulin, Arc NPY or Arc α -MSH mediate compensatory hypophagia after forced weight gain, (2) give only weak support for the involvement of basal leptin, and (3) support the hypothesis that neurocrine CRH in the PVN does participate in this regulatory capacity.

Study 3. In the final study of the thesis I examined two additional models of obesity, OVX and dietary obesity, which was induced by feeding palatable energy-dense food (Ensure). OVX, OVX and estradiol treated, and sham-operated (intact) rats were fed chow or chow plus Ensure (Abbott Nutrition; n = 7/group) for 41 d. TAT, IAAT and SAT were measured periodically by microCT. Regional distribution of AT was expressed as IAAT as a percentage of TAT (%IAAT). Chemical analysis of fat was done in chow-fed intact and OVX rats at study end. Chemical carcass analysis in chow-fed rats revealed that the 56 g excess body weight produced by OVX consisted of 24 g fat mass and 33 g fat-free body mass, which is in line with previous reports in OVX rats [361, 377-379]. OVX increased absolute and relative adiposity (fat mass normalized to fat-free body mass), which parallels the effects of menopause in normal-weight women [365, 366, 368-370]; the increase in lean body mass, however, is in contrast to menopause, which is associated with a decrease in lean body mass. In addition, as measured by microCT, OVX increased TAT 17 g, indicating that a substantial amount of fat, about 7 g, was deposited outside the AT. Whether this occurs in menopause is unclear. Finally, OVX increased SAT (12 g) more than IAAT (5 g, which was non-significant), leading to a decrease in %IAAT (~40 vs. 46%). This is the first direct measure of AT distribution in OVX rats and clearly disconfirms the hypothesis that OVX would, like menopause, shift regional AT distribution toward more IAAT (in fact, there is also only one whole-body imaging study in women, which indicated that menopause increased IAAT about twice as much as it increased SAT [369]).

As expected, feeding Ensure ad libitum markedly increased excess body weight (65 g), TAT (53 g), SAT (28 g), and IAAT (23 g) and did not change AT distribution. This is similar to human obesity, in which there is little or no gain of lean body mass. OVX led to further increases in body weight (~35 g) and TAT mass (~12 g, which was not significant).

Unfortunately, the study did not have sufficient statistical power to detect whether the effects of OVX were additive or slightly less in rats simultaneously developing dietary obesity.

Estradiol treatment prevented all the effects of OVX in both chow- and Ensure-fed rats.

For reasons that are unclear, the hormone assays in the main study failed. A preliminary study, however, indicated that plasma levels of insulin were not changed, but those of leptin were 2-fold increased after 30 d in CH-OVX rats that had somewhat smaller increases in excess TAT (9 g), SAT (6 g) and IAAT (3 g). The preliminary study also included a group of Ensure-fed intact and OVX rats. The feeding pattern of Ensure was different compared to Study 3 (main study) in that Ensure was fed in three daily rations instead of ad libitum, in order to match EN-Intact and EN-OVX to CH-OVX for excess TAT. Plasma levels of insulin were not changed in either En-OVX or CH-OVX rats compared to CH-intact rats, but plasma levels of leptin were 1.7 and 2-fold increased. Similar to Study 3, OVX increased SAT more than IAAT, in both chow-fed and Ensure-fed rats in the Preliminary study. Thus, the essential finding that OVX selectively increased SAT was consistent between the preliminary and the main study. Furthermore, it seems that leptin plasma levels were associated with changes in TAT, whereas insulin was not. This is consistent with a study by Chen & Heiman [380]. In this study leptin but not insulin plasma levels were significantly increased 35 d after OVX, when OVX rats had 50 g excess BW and 13 g excess fat mass [380].

An unexpected result in the preliminary study was that although all test groups gained excess TAT compared to CH-intact rats, only CH-OVX rats increased excess BW (44 g). Excess BW in CH-OVX was ~4 times higher than excess TAT, which is similar to Study 3 and suggests that lean mass increased. This is consistent with studies measuring lean mass in OVX rats directly [378-381, 473]. In contrast, neither EN-intact nor EN-OVX increased excess BW, which suggests a loss of lean mass. The results do not identify why Ensure feeding did not lead to significant increases in excess BW. We suspect that the Ensure feeding regimen (three daily rations) used in this study might have been the reason. And, indeed, ad libitum feeding of Ensure in the main study led to excess BW. Nevertheless, more direct measurements of body composition, as were done for CH-OVX in Study 3, would provide important additional information about the effects of diet and feeding schedule on regional body composition.

Another point of comparison between the main and the preliminary study regards the importance of the timing of beginning estradiol treatment after OVX. In the preliminary study, 25 d of estradiol treatment in CH-OVX, begun 30 days after OVX, did not reverse either excess TAT nor excess SAT, although excess BW was reduced from 44 to 28 g, after 25 d.

In contrast, starting the same estradiol treatment shortly after OVX completely prevented the development of excess TAT and excess SAT (excess fat and BW gain were also prevented, similar to previous reports [361, 406]). Thus, the timing of estradiol treatment after OVX seems to be crucial for its effects on body composition and regional AT distribution and needs further testing.

Overall, study 3 indicates that OVX in rats is not homologous to the effects of menopause in regard to regional AT distribution and change in lean body mass. The degree of excess ectopic fat deposition may also differ. Thus, OVX should be used with great caution as a model of menopause in investigations of adiposity-related diseases our data. In addition, the data suggest that physiological estradiol treatment can prevent the effects of OVX on body composition, but may not be able to reverse them. These finding emphasize the need for future studies investigating the importance of the duration between OVX and onset of estradiol treatment.

General discussion

In this thesis I investigated regulation of AT mass in three models of obesity. The first model involved dynamic AT mass regulation in rats recovering from forced overweight (**Studies 1 + 2**), the second was “dietary obesity” obesity, and the third was OVX-induced obesity (**Study 3**). These models are meant to be relevant to situations in humans: (a) Humans, like rats, dynamically lose AT mass after a period of experimentally overeating [45, 46]; (b) Common or idiopathic human obesity is thought to be dietary obesity, i.e., to be attributable in large part to overconsumption of palatable, high-fat, high-sugar, energy-dense food [8-11]; (c) OVX is the most common model for studying postmenopausal obesity in women [349].

There were common aspects to the approaches of the three studies. First, in each study I was interested in the effects on AT mass and AT distribution, which I was able to measure by microCT, a method that has been validated to be sensitive and accurate [359]. This method has not been applied previously to any of these models, and this is perhaps the most important novel aspect of the experiments. Second, I was interested in endocrine controls of obesity, in particular basal plasma levels of the putative adiposity signals insulin, leptin, amylin and ghrelin. The measures of the latter three hormones are the first descriptions of their behavior during dynamic weight regulation, and thus represent a second important novel contribution of this work. Third, I planned to include tests of dynamic regulation of adiposity in each model. Unfortunately, this became impractical in study 3, so I have only some preliminary data related to this point. There were, of course, important differences among the studies: Only in **study 2** did I investigate brain mechanisms, and in **study 3** I used female rats and, as mentioned, did not include a recovery phase in the main study. The discussion below focuses on four main questions about AT regulation that were addressed in the studies.

Do adiposity signals encode adipose tissue?

The regulation of AT mass is assumed to be based on detection of feedback signals related to AT mass by the brain and initiation of appropriate compensatory responses in eating or EE when the feedback indicates deviations beyond some threshold. The most prevalent model of AT mass regulation posits that the feedback signals are basal levels of various endocrine ‘adiposity signals’ primarily insulin and leptin [20, 21, 78].

Most of the evidence that insulin and leptin encode information about the level of AT mass comes from cross-sectional studies demonstrating correlations between their plasma levels and AT mass under steady-state conditions [19]. Plasma levels also change appropriately in situations of experimentally induced increases or decreases of AT mass in humans [46, 255-257] and rats [140, 232, 258-261]. The present data were consistent with this. That is, increased AT mass due to IG overfeeding (**Studies 1 & 2**) was associated with increased basal plasma levels of insulin and leptin. Increased AT mass due to OVX (**Study 3**, preliminary study) was also associated with increased basal plasma levels of leptin, but not insulin. This is consistent with a study by Chen & Heiman [380]. Unfortunately hormone data in the model of dietary-induced obesity did not have enough statistical power to permit clear interpretations (**Study 3**). **Studies 1 & 2** included a phase of dynamic AT mass regulation, which was initiated by termination of IG overfeeding. In both, the decreases in basal plasma insulin and leptin levels were quantitatively dissociated from decreases in AT mass. Furthermore, in **study 1**, amylin and ghrelin levels were dissociated from dynamic AT mass loss; rather, levels of these hormones did not change. These data clearly indicate that plasma levels of these hormones do not accurately encode information about AT mass. Rather, rats underate and dynamically regulated AT mass in the absence of any significant changes in any of them.

The dissociations between basal plasma insulin and AT mass are consistent with two previous, similarly designed tests by Harris and her colleagues in which insulin returned to control levels while body fat mass was still significantly increased and while eating was still decreased [14, 267]. Other studies on recovery from forced overweight appear consistent with the present results on insulin and also leptin; these, however were limited by the facts that in each BW, and not AT mass, was measured [258, 266] or that insulin and leptin were measured when rats were no longer losing body weight [164, 266], so it was not clear if any effective compensation was occurring. One of these studies, however, is interesting because it appears to be the only one in which EE was measured during dynamic body weight loss. In this study, insulin returned to control levels within 3 days, although rats still were undereating and had increased EE [258].

Compensatory reductions in AT mass also occur after rats are switched from chronic feeding of diets that they overeat back to standard chow [48]. In one study, insulin was measured during compensatory undereating and dynamic BW loss (not AT mass) after rats had been switched from high-fat diet to chow; insulin returned to control levels within 3 days, although the rats were still overweight [258]. Shi et al. reported that plasma leptin levels returned to control levels within two weeks after mice were switched from a high-fat diet to chow. At that time, however, the mice were no longer hypophagic, although they still had markedly more

AT [445]. Similar failures of insulin and leptin to encode AT mass during dynamic recovery from forced overweight occurred during recovery from forced underweight in rats. In three studies, insulin and leptin were shown to be back to control levels despite reduced AT mass and compensatory overeating [14, 19, 451].

Leptin also has been dissociated from AT mass in other situations. During chronic food restriction in humans, leptin levels were lower than after chronic maintenance of the reduced body weight [255]. Further, obese patients who underwent RYGB had significantly reduced leptin and insulin levels compared to BMI-matched controls and comparable levels to lean subjects [264]. In one recent study, leptin fell by about 50% within the first week after RYGB, although weight loss was only 4% [265]. All these data further indicate that the answer to question (1) is no. Rather, there is little reason to believe that leptin or insulin provides sufficient feedback about adiposity to drive dynamic AT-regulatory responding.

What does control the levels of these hormones? In all the situations reviewed above, whether the animals or people were in positive or negative energy balance appeared to associate with insulin and leptin levels more closely than did their AT mass. Furthermore, it is known that leptin levels change dramatically in response to short term energy surfeits or deficits [234, 284], which do not affect AT mass at all, and of course, eating leads to acute increases in insulin levels. Thus, it seems that momentary energy balance status affects levels of these hormones more than AT mass. Nevertheless, this seems unlikely to be the sole factor because levels of insulin and leptin were not below control levels (except for insulin in **study 1**), as one would expect and which occurs, for example, in rats pair-fed to the reduced food intake of rats recovering from forced overweight [164] or other situations of negative energy balance in rats that were not overfed before.

Are Arc POMC and NPY neurons involved in adipose tissue mass regulation?

The most prevalent model of AT mass regulation posits that adiposity signals signal the state of AT mass by acting on receptors on Arc α -MSH- and NPY-producing neurons, from which information spreads into other brain areas [20]. The measurements in **study 2** of POMC (the precursor of α -MSH) and NPY mRNA in the Arc in rats recovering from forced overweight did not support this view because mRNA levels did not change despite the ongoing compensatory hypophagia and dynamic AT mass loss. To the extent that mRNA levels reflect tonic activity of the neurons, these data indicate that these neurons were not effective causes of the regulation.

In contrast, further downstream in the neural network, in the PVN, mRNA levels of CRH were found to be elevated. Because plasma corticosterone was not significantly elevated, we concluded that the CRH mRNA reflected neurocrine CRH and that these neurons were likely to be involved in dynamic AT mass loss. Together, the data suggest that PVN CRH mRNA was not increased via the canonical Arc NPY/POMC pathway, which raises the important question, what signals did affect PVN CRH mRNA? Consistent with the view of Levin and Strack [90], I suggest that a variety of different types of signals, rather than just “adiposity signals,” contribute Levin and Strack emphasize that a widely distributed network of “metabolic-sensing” neurons in the Arc, several other hypothalamic areas, the NTS, ventrolateral medulla, some of the raphe nuclei, and other brain areas sense a variety of signals (metabolites, hormones, peptides, sensory nerves, vagal afferents) related to the energy status of the body (**Figure 6**). They further emphasize that non-metabolic information from gastrointestinal satiety signals, orosensory (and learned) reward signals, etc., converges onto this network of metabolic-sensing neurons to result in adaptations in eating and EE.

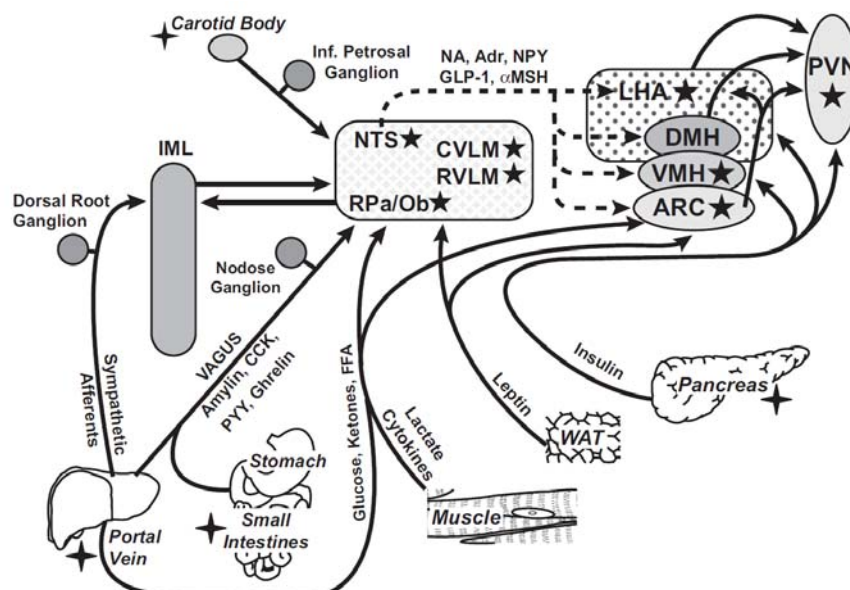


Figure 6. Model of convergence of a variety of peripheral signals (neuronal, energy metabolites, hormones) related to energy metabolism by neurons in the hypothalamus and hindbrain [90]. Metabolic-sensing neurons are in the caudal (CVLM) and rostral ventrolateral medulla (RVLM), contain noradrenaline, adrenaline, neuropeptide Y (NPY), glucagon-like peptide (GLP-1), and/or (α -melanocyte stimulating hormone (α -MSH)), and project to the arcuate nucleus (ARC), lateral hypothalamic area (LHA), ventromedial nucleus (VMN) and dorsomedial nucleus (DMN) of the hypothalamus. Metabolic-sensing serotonin neurons in the raphé pallidus and obscurus (RPa/Ob) project to sympathetic preganglionic neurons in the intermediolateral (IML) cell column of the spinal cord.

Is adipose tissue mass regulated?

Observations of dynamic compensatory changes in eating and EE and the return to near-normal levels of AT mass following experimentally induced increases or decreases in AT mass have long been considered the strongest evidence for active AT regulation of AT mass [14-19]. In this context, it is usually overlooked that body weight and AT mass often do not change in parallel. Beginning with Cohn and Joseph's [42] seminal study on dynamic regulation, it has been often observed that rats return to nearly normal body weight, but maintain significant increases in AT mass, after experimentally induced obesity [48]. The present results confirm this observation. When rats stopped undereating during recovering from forced overweight, AT mass was still increased, whereas body weight was almost back to the control level (**Study 1**). Thus compensatory decreases in eating seemed to track BW rather than AT mass, questioning which variable is actually regulated in long-term energy homeostasis. In another, similarly designed study, Harris et al. (1986) showed that body weight and AT mass both remained elevated, although protein content, which is a more accurate indicator of lean body mass, returned to control levels [14]. Such observations indicate that AT mass is not the sole variable driving the regulation of energy homeostasis.

In **study 3**, rats offered a palatable, energy-dense diet in addition to chow developed excess body weight, which was mainly due to an increase in excess AT mass. This supports the notions that the nature of the food has an enormous influence on AT mass regulation and that the regulatory system might operate less strongly in preventing excess AT mass than in preventing underweight (**Study 3**). The present work also reflects the importance of estrogens in females for AT mass regulation. Estradiol treatment, when started shortly after OVX, precisely prevented OVX-induced increases in AT mass in chow-fed, lean rats and in Ensure-fed, obese rats (**Study 3**). Estradiol also prevented the OVX-induced increases in body weight, and, in fact, tended to reduce body weight below the control level, suggesting that estradiol treated OVX rats lost lean mass. This result was unexpected because the estradiol treatment used was reported to reinstate near-physiological estradiol levels and body weight [406]. Estradiol treatment seems to have different effects on body composition, when started later after OVX. Estradiol treatment started 30 d after OVX (**Study 3**, preliminary study) was not able to reverse excess TAT, and only partially reversed excess BW after 25 d. It is possible of course that prolonged treatment would eventually reduce excess TAT.

Furthermore, it is important to note that whereas AT mass was primarily affected in the models of dynamic AT mass regulation and "dietary" obesity, this was different in the OVX model. OVX rats increased both AT mass and lean mass, as indicated by the large

difference between excess BW and excess AT mass. Furthermore, estradiol treatment seemed to affect non-AT mass more than AT mass, both when begun at OVX (**Study 3**) or 30 d later (**Preliminary study**). Thus I conclude that the regulation of AT mass is integrated with, and affected by, physiological control of other variables that affect body weight.

Lean body mass may also contribute to the regulatory signals, shown in Figure 6. Increased body weight induced experimentally or in obese people is due mainly to increased fat mass (about 70-80%), with some increase in lean mass (about 20-30%) [255, 256, 482, 483]. As demonstrated by **study 3** this is consistent with the model of dietary-induced obesity in rats: Ensure feeding led to excess body weight, about 75% of which was explained by AT mass. Menopause is associated with increases in absolute or relative adiposity [365, 366, 368-370] whereas lean body mass decreases [365, 367, 368]. The increases in AT mass in OVX rats appear to parallel the effects of menopause. Unlike menopause, fat-free body mass increased in OVX rats, which may represent lean body mass, as suggested by several previous reports in which lean body mass was measured directly by chemical analysis [378-381, 473]. It is not clear whether these are species differences or are related to the difference in relative age i.e., the OVX data are from young adult rats, whereas menopause occurs in middle age. Thus, I conclude that OVX is not a homologous model of menopause with regard to lean body mass may not apply to older rats.

Ectopic fat is another factor that may affect the precision of regulation of AT mass. Obesity in humans leads to deposition of ectopic fat, i.e., fat inside liver and muscle cells, in the blood vessels, around the heart, etc. Such ectopic fat is thought to contribute to metabolic disorders and malfunctions of the organs [484]. Combining data of chemical carcass analysis with microCT data (**Study 3**) in intact female rats indicated that ~25% of total body fat was deposited outside the AT. Note that this fat did not seem to include brown AT or intermuscular AT in the legs, because our extrapolation from L1-6 SAT to whole body SAT included these in the SAT depot. The amount of fat outside the AT increased to 30% in OVX rats. An even larger amount of non-AT fat was detected in a study of obese male rats [360]. This appears different from humans. Whether this fat is regulated and its impact on AT regulation and metabolism in rats are unclear. It appears to be another dimension in which the rat model differs from the human model.

How similar is AT distribution in rats and humans?

Regional AT distribution depends on sex and age [34, 369]. The few whole-body imaging studies show that women have relatively more whole-body SAT, whereas men have more

IAAT [34, 251]. In women the percentage of IAAT of total AT increases with age from 2-8% and in men from 6-17% of total AT [34]. During maturation in rats, female (**Study 3**) and male rats (**Studies 1 & 2**) increased the relative distribution of TAT as IAAT (females: 40 to 46% in study 3, 30-41% in the preliminary study; males: 30 to 40% in study 1 and 36 to 41% in study 2). Note, however, that in humans, the relative and absolute amounts of IAAT compared to SAT are much less in rats. Furthermore, the size of the intermuscular AT depot appears relatively smaller in rats (unpublished observations) than in humans [485]. These are potentially important differences between rat and human obesity.

In addition, as discussed above, the relatively abrupt and marked decrease in ovarian secretion of estrogens that occurs around menopause [364] appears to be associated with an increase in IAAT and percentage of AT mass deposited as IAAT [369], which was not the case in OVX rats, where SAT increased selectively in both chow- and Ensure-fed rats (**Study 3**). Thus, the effect of estrogens on regional AT distribution differs between female rats and humans.

Conclusions

In summary, the present work investigated the regulation of AT mass in 3 different models of human obesity. I conclude from the model of dynamic AT regulation, that (1) basal plasma levels of insulin, leptin, ghrelin, and amylin do not accurately reflect AT mass and are unlikely to be feedback signals related to the levels of AT mass; (2) compensatory undereating and AT mass loss do not require changes in Arc and DMH NPY mRNA and Arc POMC mRNA, whereas increases in PVN CRH mRNA might be involved.

I conclude from the model of diet-induced obesity that dietary-obesity in female rats parallels the dysregulatory effect of palatable, energy-dense diet on AT mass in humans. Finally, I conclude from the model of OVX-induced obesity that OVX is not a homologous model of the effects of menopause on AT distribution and lean body mass in women. Furthermore, the duration between OVX and onset of estradiol treatment seems to be crucial for its effects on TAT and AT distribution.

These experiments should contribute to a better understanding of the regulation of AT mass, which could in the future be used for the development of more effective, physiologically based, non-surgical therapies for obesity.

Limitations and future directions

There are certain limitations to this work. The studies using the model of dynamic regulation of AT mass were associational, so no mechanisms were identified. Future studies are warranted to attempt to understand whether manipulations of insulin, leptin, amylin or ghrelin plasma levels by systemic administration of the hormones has an impact on dynamic regulation of AT mass or whether the regulation continues independent of them. For instance, in rats recovering from forced underweight, increases in plasma leptin levels by systemic administration failed to affect BW regain, which was similar to non-treated rats [451]. Also, administration of leptin receptor antagonists into the lateral cerebral ventricle, which was shown to increase food intake and BW [281], could be used to test whether endogenous leptin on d 8 of recovery from forced overweight affects compensatory undereating.

Manipulations of endogenous NPY, α -MSH and CRH in dynamic regulation of AT should be investigated. Especially important is to determine whether antagonizing their function would affect, or in the case of CRH, reverse, compensatory undereating or dynamic AT mass loss. Perhaps most interesting would be to determine whether administration of CRH2R antagonists into the lateral cv, which was shown to increase food intake and BW [186], could affect compensatory hypophagia and AT mass loss. Similarly, administration of NPY antibodies into the 3rd cv [114], which was shown to decrease food intake, could be used. This was tested for α -MSH: antagonizing MC3/4R by administration of SHU9119 decreased compensatory undereating in rats recovering from forced overweight [163]. Why this should be if, as study 2 indicated, α -MSH is not up-regulated in dynamic recovery from obesity needs further study.

Another limitation of this work is that EE was not determined. EE measures may have led to different insights. For example, in **study 1**, plasma leptin levels were elevated through about 8 d of recovery, and it is possible that, although the compensatory decrease in eating lasted much longer, a compensatory increase in EE occurred only during this interval.

The model of diet-induced obesity as well as the OVX model did not include a recovery phase, i.e., termination of Ensure feeding and starting estradiol treatment after already established OVX-induced obesity. It is not clear how plasma levels of adiposity signals are related to AT mass in such situations, in particular whether recovery from diet-induced obesity is different from the model using IG overfeeding. In the case of estradiol it is also not clear whether OVX-induced obesity can be reversed. In the preliminary study estradiol treatment was begun 3 weeks after OVX and did not reverse the increased AT mass, but

reduced excess BW. This apparent discrepancy between this result and the effects of estradiol treatment begun shortly after OVX should be further investigated. In addition, although the combination of Ensure feeding to OVX rats was meant to mimic menopause in obese women, rats in **study 3** were not obese when ovariectomized. Thus, inducing dietary obesity in female rats prior to OVX would be a potentially more valuable model.

In addition, as mentioned above, age might have an influence on the effects of OVX on fat-free mass and AT distribution. The experiment could be done with older rats, for example, rats that have already reached the growth plateau, which begins around 5 month of age.

Finally, in only one study chemical carcass analysis was combined with microCT, and doing so led to important results (i.e., that OVX increased the amount of fat that was deposited outside the AT). It seems likely that combining the two techniques in studies of dynamic regulation or diet-induced obesity would also lead to additional important information that neither method alone yields.

9. References

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| September 2004 - March 2005 | Erasmus Program Scholarship, <i>Ecole Nationale Supérieure d'Agronomie de Montpellier, France</i> |
| October 2001 - September 2004 | Bachelor of Science (German final grade 2.2), <i>Christian-Albrechts-University of Kiel, Germany</i> Nutrition and Food Science, "Oecotrophologie", |
| September 1992 - June 2001 | Abitur (qualification studies for university entrance) (German final grade 2.7), <i>Katharineum Lübeck, Germany</i> |

Courses during doctoral studies

ETH Zurich: Physiology of Eating, Theory and Practice in Nutritional Science, Presentation Skills, Laboratory Animal Usage, Project Management, French; *University of Zurich*: Functional Anatomy of the Rodent Brain; *Clinical Trial Unit, University of Basel*: Clinical Investigator Course

Research Methodologies

Animal behaviour methodology, computerized monitoring of eating and drinking, radio- and fluorescence-immunoassay, in situ hybridization, micro (rodent) computed tomography, surgical techniques in rats: intra-gastric and jugular vein catheter implantation, ovariectomy

Awards

July 2010 New investigator Travel Award, *Society for the Study of Ingestive Behavior*

Conference Presentations

- August 2010 Symposium of the Zurich Centre for Integrative Human Physiology, *Zurich, Switzerland*: "Hypothalamic CRH mRNA, but not POMC or NPY mRNA, changes during compensatory undereating in rats recovering from forced overweight" (poster presentation)
- July 2010 Annual Meeting of the Society for the Study of Ingestive Behavior, *Pittsburgh, PA, USA*: "Ovariectomy and estradiol treatment affect subcutaneous more than intra-abdominal adipose tissue in rats" (oral presentation)
- June 2010 Workshop: The Intestinal Wall - The Regulatory Interface in Energy Homeostasis, *Ascona, Switzerland*: "Lack of change in hypothalamic POMC and NPY mRNA during compensatory undereating in rats recovering from forced overweight" (poster presentation)
- August 2009 Symposium of the Zurich Centre for Integrative Human Physiology, *Zurich, Switzerland*: "Leptin and insulin plasma levels do not reflect adiposity in rats recovering from forced overweight" (oral presentation)
- February 2009 Swiss Winter Conference on Ingestive Behaviour, *St. Moritz, Switzerland*: "Leptin and insulin plasma levels do not reflect fat pad mass in rats recovering from forced overweight" (oral presentation)

Publications

Gloy VL, Lutz TA, Langhans W, Geary N, Hillebrand JJ. Basal plasma levels of insulin, leptin, ghrelin, and amylin do not signal adiposity in rats recovering from forced overweight. *Endocrinology*. 2010 Sep; 151(9):4280-8.

Asarian L, Gloy V, Geary N. Homeostasis. In: *Encyclopedia of Human Psychology, 2nd Edition*, edited by V.S. Ramachandran. San Diego: Academic Press, in press.

Gloy V, Langhans W, Hillebrand JJ, Geary N, Asarian L. Ovariectomy and overeating palatable, energy-dense food increase subcutaneous adipose tissue more than intra-abdominal adipose tissue in rats. *Biology of Sex Differences*, submitted.

Gloy V, Koss M, Langhans W, Geary N, Hillebrand JJ. Hypothalamic CRH mRNA, but not POMC or NPY mRNA, changes during compensatory under-eating in rats recovering from forced overweight. In preparation.

Internships

August 18 - September 12, 2003 Clinical Nutrition Team, Hannover Medical School, Germany, Department of Gastroenterology, Hepatology and Endocrinology

July - August 2001 Neuropaediatric Nursing, *University Clinic, Lübeck, Germany*

Student Jobs

October 2003 - September 2004 Student assistant, *Christian-Albrechts-University of Kiel, Germany*, Institute of Human Nutrition and Food Science

2006 - 2007 Conference assistant, *German Nutrition Society, section Baden-Wuerttemberg*

Professional Memberships

2008 - present Society for the Study of Ingestive Behavior

2007 - present Verband der Oecotrophologen, *Germany*

Language and Computer Skills

English (very good) and French (good)

SAS, SPSS, Graph Pad Prism, Reference Manger, Endnote, Microsoft Office

References

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Dr.ir. J.J.G. Hillebrand, Tel. 074-2904487 j.hillebrand@zgt.nl, Ziekenhuisgroep Twente, Clinical Chemistry, Geerdinksweg 141, Postbus 546, 7550 AM, Hengelo, The Netherlands

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