Autonomic nervous control of white adipose tissue: studies on the role of the brain in body fat distribution
Kreier, F.H.K.

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CHAPTER 8

Perspectives for follow-up studies

'The brain has a role in body fat distribution and its associated metabolic diseases' is the general hypothesis of this thesis.

An increasing number of researchers have been studying the role of the hypothalamus as the central region that controls energy homeostasis; they initially showed that manipulations of the hypothalamus can induce major changes in energy homeostasis. But what really launched the research on the hypothalamic role in metabolism was the discovery of leptin in 1995, when it became clear to what extent the hypothalamus integrates information of the body's state4. The hypothalamus translates its drive into the body by means of pituitary hormones, and only recently we have started to appreciate the contribution of the autonomic nervous system (ANS). In chapters 2-5 we describe a part of the interaction between the hypothalamus and metabolic organs. In this chapter we aim to add some comments with respect to the developed hypothesis and the essential experiments that still need to be done for investigating this hypothesis.

The present thesis consists of two main parts: findings and perspectives.

FINDING #1 The brain uses two neuronal systems with an antagonistic effect on metabolic organs (chapter 2 and 4)

We show that fat tissue is innervated by (pre-)sympathetic and (pre-)parasympathetic neurons, running from the biological clock (SCN), via the PVN and LH through the ANS to fat tissue. Bartness and co-workers proved that sympathetic tone stimulates lipolysis; we demonstrated that parasympathetic tone induces lipogenesis5. Until now, lipogenesis was believed to be solely under hormonal control. Insulin and cortisol have been shown to stimulate lipogenesis, whereas estrogen and growth hormone induce lipolysis6,7. However, humoral factors cannot completely explain fat tissue metabolism in vivo8.

Cantu demonstrated in 1967 that sympathetic denervation of the retroperitoneal fat pad results in impaired lipolysis after fasting9. In the 1980ies, Stock and Rothwell discovered that sympathetic tone induces mitochondrial oxidation in brown fat tissue10,11. In the same decade, Trayhurn and Dalziel refined the theoretical frame on the autonomic innervation of fat tissue12,13. In the 1990ies Bartness and coworkers substantiated in a series of experiments that the hypothalamus is connected to white adipose tissue...
by the sympathetic nervous system exerting a lipolytic effect. However, lipogenesis was believed not to be controlled by the ANS.

Earlier, our group applied neuroanatomical and physiological techniques to study the hypothalamic autonomic control of heart, liver, pancreas, thyroid and adrenal. Except the adrenal gland, these organs appeared to receive both sympathetic and parasympathetic innervation, originating in the biological clock and other hypothalamic nuclei.

We injected PRV into intact and locally sympathectomized fat pads (chapter 2). Transneuronal tracing from intact fat pads revealed a dominant sympathetic labeling as compared to the number of parasympathetic neurons. However, if prior to the tracer injection the sympathetic nerves were cut, the sympathetic motor neurons in the spinal cord were empty, while the dorsal motor nucleus of vagus and the nucleus ambiguous where strongly labeled. Finally, we conducted a series of control experiments to exclude false positive labeling due to leakage into the abdominal cavity and blood stream. (See supplemental material chapter 3 for a detailed description.) A point of discussion remains whether PRV transport and replication is partly depending on neuronal activity. We speculate that the dominant sympathetic labeling in our non-denervated animals the consequence of a higher sympathetic activity.

After the neuroanatomical evidence for parasympathetic innervation was established, we tested the hypothesis whether the vagal input would be antagonistic to the lipolytic action of the sympathetic arm. In a study with a hyperinsulinemic euglycemic clamp we compared the insulin dependent uptake of glucose and fatty acids (FA) between vagotomized and intact fat within the same animal. The vagotomized fat pads took up 33% less glucose and 36% less FA, moreover the lipolytic hormone sensitive lipase (HSL) was increased by 51%. In other words, the vagus plays an anabolic role in fat tissue by inducing lipogenesis.

In chapter 4, we aimed to reveal neural connections between the hypothalamus and the autonomic motor neurons projecting to fat tissue. We used the bilateral retroperitoneal fat pads as a model by ipsilateral sympathetic and contralateral parasympathetic denervations. Injection of two differently labeled PRV strains into the fat pads allowed us to follow both branches upstream through the brain at the same time. We found two parallel chains of pre-sympathetic and pre-parasympathetic neurons that appeared neighbored but separated in the PVN, LH and SCN.

This finding implies that the biological clock, the neuroendocrine and autonomic nucleus of the hypothalamus (i.e., the PVN) and the feeding center (LH) all have direct access to both antagonistic branches and therefore the potency to drive fat tissue metabolism into an anabolic or catabolic direction.
Follow-up studies

**What is the proportional contribution of hormones versus neurons in the regulation of fat tissue metabolism?**

This remains a fundamental question in neuroendocrine research. While it is crucial to find the answer in order to estimate the impact on clinical patient care, it depends strongly on the experimental setting. Our studies on the vagal impact on fat tissue—not to forget the first—were done after a 24 hours fasting and 2 hours refeeding by intravenous glucose in combination with hyperinsulinemia. This situation is not a perfect image of normal physiology. Future experiments should aim to develop methods that imitate normal physiology, e.g. study the non-fasted state and the reaction of the body on a standard meal.

**Does the ANS control circulation in addition to adipocyte metabolism?**

Histological studies have shown that nerve endings are present on fat cells. Active fat cells need increased blood flow to exchange metabolites, both during lipogenesis and lipolysis. We know that parasympathetic tone stimulates lipogenesis and sympathetic tone lipolysis, but also vasoconstriction. Future research needs to investigate how the ANS arranges the control of both parenchymal tissue and its blood vessels.

**What are the neurotransmitters of the vagal input to fat tissue?**

Histology studies on fat tissue have not been successful yet due to the cell structure of the white adipocytes. Traditionally, acetylcholine is assumed to be present in vagal neurons. Alternately, vasoactive intestinal peptide (VIP) or nitric oxide (NO) might be present alone or in combination with acetylcholine. Histological analysis of content in brown fat tissue—feasible due to relatively less intracellular lipid drops and a higher proportion of mitochondria—revealed acetylcholine as vagal transmitter. In the liver, acetylcholine, VIP and NO are known to be present.

**Surgical sympathetic denervation of subcutaneous fat tissue**

We did not succeed to develop a surgical method to denervate subcutaneous fat tissue. Investment in the development of a local surgical or chemical denervation will be needed to study the functional role of subcutaneous fat tissue metabolism in the future.

**Could the activity dependent transport of PRV be used as a marker of neuronal activity?**

Future experiments might address whether the infection rate is indeed depending on neuronal activity and if yes, whether this finding could be used as a tool to assess neuronal activity in vivo. We assume however that due to the many variables that may
determine virus uptake that it will be difficult to absolutely ascertain that variation in uptake and transport are due to the activity of the neurons.

**FINDING #2  The brain distinguishes between organs from different body compartments and combines the organs within one body compartment (chapter 2 and 3)**

We show that intra-abdominal fat and liver, as well as liver and pancreas share input from the same vagal motor neurons (chapter 3). In addition we show that subcutaneous and intra-abdominal fat pads are innervated by two parallel chains of neurons, running from the biological clock (SCN), the PVN and MPO through the sympathetic nervous system and from vagal motor neurons to fat tissue.

Assessment of ANS function is of central interest in studies on the role of the brain in the control of vital functions. Autonomic tone on the heart has been well-studied by the analysis of electrocardiograms. Skin, eye and muscle are organs that are easy to approach and therefore they are often used in ANS studies as well.

Most of those studies assume that local autonomic tone in a certain organ is equal to the general autonomic tone in the whole body. This is surprising because the advantage of nerves in contrast to hormones (in fact integrated whole body signals) is their ability to point to a specific target in the body. As an example, blood distribution is depending on the local constriction of blood vessels. Blood flow is low in the abdomen and high in muscle during exercise, while it is opposite during rest due to local differences in autonomic tone.

Therefore, we hypothesized that organs from different body compartments are controlled by different neurons. We used retrograde tracers to label the central origin of autonomic input to intra-abdominal and subcutaneous fat pads. In chapter 2, we studied the sympathetic motor control by injection of two PRV strains injected into intact retroperitoneal and subcutaneous fat and the assumed lesser activity of the parasympathetic neurons. We found that sympathetic motor neurons in the intermediolateral column of the spinal cord are specialized to project either to the intra-abdominal or the subcutaneous compartment.

Also in chapter 2 we aimed at the vagal motor neurons, here we used the transneuronal PRV in sympathectomized retroperitoneal fat and the non-transneuronal tracer fluorogold in subcutaneous fat pads. Since fluorogold cannot cross a synapse and the peripheral parasympathetic ganglia are located in the organs itself, the only central labeling to be expected are the vagal motor neurons. We found distinct groups of vagal neurons projecting to either intra-abdominal or subcutaneous fat tissue.

In chapter 3 we followed the neuronal pathways upstream to the hypothalamus. We injected two PRV strains into vagotomized retroperitoneal and intact subcutaneous
fat pads. We found that in addition to the IML and brain stem, also in the SCN, PVN, MPO and amygdala the neurons are divided by body region. In the control experiment, we sympathectomized both retroperitoneal fat pads and injected two PRV strains, resulting in double labeled neurons in SCN, PVN, MPO and amygdala, demonstrating that both intra-abdominal fat pads share neuronal control.

In chapter 3, we tested whether the abdominal organs are innervated separately. We hypothesized a shared autonomic control of functional groups of organs. Organs with an anabolic function in the intra-abdominal cavity might share input, such as visceral fat, pancreas and liver. In the first experiment, we injected PRV into the sympathectomized liver and Cholera Toxin B (CTB), a non-transneuronal tracer, into intra-abdominal fat tissue. We found an overlapping group of neurons in the vagal motor nucleus. In the second experiment, we injected two differently labeled CTB’s into the pancreas and the liver and found double labeled neurons in the vagal motor neurons.

In conclusion, we demonstrated that intra-abdominal fat, pancreas and liver share control in the vagal motor nucleus and that intra-abdominal and subcutaneous fat are separated through the sympathetic nervous system up to the biological clock.

Follow-up studies

What is the relationship between the autonomic control of the thorax compartment and the intra-abdominal cavity?
Since circulation and heart are central parts of our metabolic system we need to know whether the thorax is controlled by different neurons than the intra-abdominal and movement compartment. Moreover, we need this information because the heart is the most studied organ of the body to measure autonomic tone. We draw the conclusion that the body is controlled by body compartments. However, future research has to investigate the neuronal control in more detail to test whether the system is in any detail different from what we found in the first approach.

How could we develop tests that measure autonomic tone simultaneously in multiple body compartments?
An autonomic test battery should be cheap, fast and as non-invasive as possible. The finding of autonomic functions test especially for the abdominal region in human would be of significant clinical impact. The autonomic test battery is mandatory for fundamental and clinical research to understand the mechanisms of ANS function and evaluate future therapies. To measure autonomic tone in the thorax compartment, electrocardiography might be used. Sympathetic tone in muscle can be measured by postganglionic muscle sympathetic nerve activity (microneurography). For the intra-abdominal compartment, no validated method is available yet. Six parameters that are under autonomic control might be promising to validate:
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a. Ultrasound of gall bladder contraction after oral mineral water.
b. Doppler ultrasound of brachial versus splanchnic blood vessels.
c. Standard meal and cephalic insulin response.
d. Electrogastroentergraphy.
e. Pulsatility of insulin secretion and.
f. Radiological assessment of gastrointestinal transition time.

What is the physiological relevance of hypothalamic compartmental body control?
If we combine the results of our experiments, we might assume that the hypothalamus can control the body compartments separately. However, the physiological impact of hypothalamic control has to be tested. E.g., SCN lesions combined with assessment of autonomic tone in the abdominal, thorax and movement compartment (for method see above) should demonstrate if the biological clock indeed oscillates the compartments in an antiphasic circadian rhythm.

FINDING #3  Sex and stress hormones can access central neurons projecting to fat tissue (chapter 5)
We demonstrate that estrogen alpha and glucocorticoid receptors are expressed on neurons in hypothalamus and brainstem that are connected to fat tissue.

Body fat distribution has clear sex differences: visceral fat causes the android apple form and subcutaneous fat the gynoid peer form. In addition, patients suffering from Cushing syndrome (hypercortisolism) show the remarkable combination of visceral obesity and peripheral (subcutaneous) lipoatrophy. As a first step to test whether the hormonal effect on fat tissue might be centrally mediated, we stained brainstem and hypothalamus for PRV and for either estrogen receptor alpha (ER alpha) or glucocorticoid receptor (GR). In DMV, PVN, LH and amygdala we found a strong colocalization with GR, indicating that glucocorticoids might affect fat tissue metabolism via (pre)-autonomic neurons in the CNS. These findings might give a new view on body fat distribution in Cushing syndrome: we show that a neuroanatomical network is present that might mediate the effect of stress hormone on fat tissue. In contrast, in male rats, we only found less colocalization with ER alpha (see future research).

Follow-up studies
Are ER alpha/beta/testosterone receptors expressed on fat projecting neurons in females?
The sparse presents of ER alpha on fat projecting neurons suggest a minor effect of estrogen on fat metabolism in male rats. How is this organized in female rats, and what is the effect of testosterone in males?
CHAPTER 8 PERSPECTIVES FOR FOLLOW-UP STUDIES

What is the distribution of ER alpha/beta and GR on fat projecting neurons depending on their compartment?
To draw any conclusion about the effect of sex- and stress hormones on fat tissue metabolism, a comparison of the receptors on neurons projecting to visceral versus subcutaneous fat tissue is mandatory. Currently experiments in our group try to answer these questions.

What is the physiological impact of ER alpha and GR on fat projecting neurons?
To assess this, in a first step, intracerebral estrogen or cortisol versus peripheral hormone suppletion might be applied. The results could be measured as the uptake of substrate or the body fat distribution as detected by MRI.

PERSPECTIVE #1 The metabolic syndrome might be a brain disease (chapter 6).
The biological clock can oscillate the activity of individual body compartments according to the day night rhythm. Disturbed circadian rhythms are a prominent feature of the metabolic syndrome. We speculate that a metabolically flattened environment induces flattened endogenous rhythms with high sympathetic tone in the thorax and movement compartment, leading to hypertension and insulin resistance in muscle and high parasympathetic tone in the intra-abdominal compartment leading to visceral obesity, hyperinsulinemia and fatty liver disease.

Recently, new insights in the field support our hypothesis. Epidemiological analysis of a large cohort study on sleep behavior revealed a positive relationship between to short or to long sleep with obesity and cardiovascular disease. In mutant mice, the metabolic syndrome develops if the key transcription factor of the biological rhythms CLOCK is missing. New clinical studies revealed that obese patients suffer from significant circadian dysfunction, especially the non-dipping of blood pressure at night, while weight loss restores the normal circadian blood pressure rhythm.

Follow-up studies
How can we test the hypothesis of an arrhythmic biological clock and an unbalanced ANS as the cause of the metabolic syndrome in animals?
Such a study should aim to resolve whether the biological clock is involved, whether the autonomic tone has a parasympathetic overweight in the abdominal compartment and a sympathetic overweight in the thorax compartment.
During the course of this thesis, we conducted experiments with retrograde neurotoxins in rats to test the hypothesis (see figure 6.1-6.5). We aimed to create this autonomic shift by a deactivation of sympathetic motor neurons by neurotoxins. After a surgical
parasympathetic denervation of an intra-abdominal fat pad, we injected retrograde neurotoxin. Due to the vagotomy, the neurotoxins forced to enter the sympathetic motor neurons and will partly kill them. Since sympathetic control is shared by intra-abdominal fat, pancreas and liver, the expected result of this manipulation is a relatively high parasympathetic tone to these organs, with excessive visceral fat, hyperinsulinenia and a fatty liver as a consequence.
Figure 8.1 The abdominal compartment receives both sympathetic and parasympathetic shared input via the sympathetic and parasympathetic nervous system (chapter 2, 3 and 4). We assume that the thorax compartment receives sympathetic and parasympathetic input from a separate group of neurons (chapter 6).

Figure 8.2 The retroperitoneal fat pad is vagotomized (see for technique supplemental material in chapter 3).

Figure 8.3 Neurotoxin conjugated to a non-transneuronal retrograde tracer (e.g. saporin – CTB) is injected into the vagotomized fat pad.

Figure 8.4 Since the fat pad is vagotomized and the neurotoxin has access to the sympathetic input only, the motor neurons in the peripheral sympathetic ganglion projecting to the abdomen will be killed.

Figure 8.5 Since the abdominal organs share neuronal control, the sympathetic input to the abdomen will be decreased and the net effect will be a parasympathetic overweight in the abdominal cavity, but not in the thorax compartment. If our hypothesis is right, the animal should develop visceral obesity in the intact abdominal fat pads, hyperglycemia and hyperinsulinemia. See color section.

Two types of retrograde neurotoxins have been studied: Cholera toxin B / saporin conjugate (CTB-SAP) and Pseudorabies Virus gII (PRV-gII). In a first step we aimed to demonstrate the presents of retrograde neurotoxins in the CNS with sympathetic or parasympathetic and without surgical denervation.

CTB is a neuronal tracer that fills autonomic motor neurons after injection into an organ and can be visualized in the CNS by immunohistochemistry; saporin is a ri-
bosome inactivating neurotoxin. Saporin conjugated to a retrograde tracer, such as CTB, has been used in to retrogradely kill autonomic motor neurons in rodents. We applied several types of CTB-SAP that were conjugated in our lab and by commercial suppliers. We could not detect CTB in the CNS with immunohistochemical techniques, nor did we find changes in glucose tolerance test after CTB-SAP treatment.

In a next step, we tested a different retrograde neurotoxin. PRV-\(g\)II is a swine neurotrophic herpes virus that infects the synapse, is being retrogradely transported and replicates in the cell body until the neuron disintegrates. In contrast to PRV-bartha, a transneuronal tracer, PRV-\(g\)II has not the ability to enter a neighboring neuron and spread upstream through the CNS. Therefore, PRV-\(g\)II is a neuroanatomically a marker and physiologically a neurotoxin at the same time. In a similar approach to CTB-SAP, we injected PRV-\(g\)II into abdominal organs. Here, sympathetic denervation was chosen because it forces the virus to the DMV where the presence or neuronal damage is easier to establish than in the celiac ganglion. Using immunohistochemistry staining against PRV, we could not detect PRV-\(g\)II in the CNS.

Transport of CTB-SAP and PRV-\(g\)II has been described after dipping in cut nerve endings or intra-neuronal injections. To develop successful retrograde transport from intra-abdominal fat tissue, the percentage of neurotoxin as well as the medium in the dilution might be changed. Alternatively, a more aggressive variant of PRV could be used, with other insertions and deletions.

In summary, further experiments with different doses or compounds are necessary to test the physiological impact of the shared neuronal control of the intra-abdominal cavity. Since we did not succeed to have central labeling of a retrograde neurotoxin, we cannot verify or reject the hypothesis about the involvement of the ANS in type 2 diabetes.

**How can we test the hypothesis of an arrhythmic biological clock and an unbalanced ANS as the cause of the metabolic syndrome in humans?**

In patients, the circadian rhythms and ANS function should be assessed in prospective cohort studies. If circadian and autonomic malfunctioning is present before the symptoms of the metabolic syndrome develop, our hypothesis would be supported. Until now, prospective cohort studies show an strong association between disturbed sleep, ANS function and the development of the metabolic syndrome.

**How could we treat the metabolic syndrome based on this hypothesis?**

The reintroduction of physiological circadian rhythmicity with high maximum energy intake and utilization in the morning and sufficient sleeping time should ameliorate the metabolic syndrome, as discussed in chapter 6. Treatment with drugs that emphasize the natural rhythmicity such as melatonin might have important additional value.
CHAPTER 8 PERSPECTIVES FOR FOLLOW-UP STUDIES

PERSPECTIVE #2 HIV-related lipodystrophy might be a brain disease (chapter 7)

Patients with the HIV-1-associated adipose redistribution syndrome (HARS) suffer from visceral fat accumulation, peripheral fat atrophy and features of the metabolic syndrome. We speculate that this unbalance might be a result of a high parasympathetic tone to the visceral fat tissue and high sympathetic tone to peripheral fat tissue due to selective neurotoxicity of the HIV-medication, possibly in combination with the HIV virus itself.

Follow-up studies

Is autonomic function disturbed in HARS?

An autonomic test battery is needed to evaluate the autonomic status of these patients (see finding #2).

Does HIV medication has an neurotoxic effect on (pre-)sympathetic or (pre-)parasympathetic neurons projecting to fat tissue?

In an animal model, the effect of HIV medication on the brain could be tested by comparing peripheral and intra-cranial administration, followed by measurement of body fat distribution by MRI, assessment of autonomic tone, and histological analysis of (pre)autonomic neurons projecting to fat tissue. These experiments will show if the drugs indeed have a central effect on body fat distribution that is mediated by the ANS.

REFERENCES


PART B DISCUSSION AND PERSPECTIVES


