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

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
AUTONOMIC NERVOUS CONTROL OF WHITE ADIPOSE TISSUE

Felix Kreier

STUDIES ON THE ROLE OF THE BRAIN IN BODY FAT DISTRIBUTION
STELLINGEN

behorende bij het proefschrift

AUTONOMIC NERVOUS CONTROL OF WHITE ADIPOSE TISSUE

1. The vagus nerve stimulates the growth of fat tissue. *current thesis*

2. The brain (including the biological clock) controls intra-abdominal and subcutaneous fat via separate nerves. *current thesis*

3. The brain controls intra-abdominal fat, pancreas and liver via the same vagal nerve fibers. *current thesis*

4. The dysfunction of intra-abdominal fat, pancreas and liver might not be the cause of disturbed brain function in the metabolic syndrome, but the disturbed brain might be the cause of these symptoms; thus, diabetes type 2 might be a brain disease. *current thesis*

5. Today, neurologists handle the brain like pulmonologists handle the lungs: they treat infection, infarction, intoxication, trauma and tumors, neglecting the ultimate purpose of the brain, which is the coordination of vital body functions.

6. Today, endocrinologists work with a decapitated model.

7. Diabetes got divorced from the brain after attractive insulin came up in 1921. Hopefully, their re-unification in neuroendocrinology will last longer.

8. Science is the record of dead religions. *Oscar Wilde*

9. It is not enough to say I made a mistake. You must explain how. *Claude Bernard*

10. Medicine is a science of uncertainty and an art of probability. *William Osler*

11. Art is I, science is we. *Claude Bernard*

12. De beste stuurlui staan op de Kloveniersburgwal. *David de Wied*
AUTONOMIC NERVOUS CONTROL OF WHITE ADIPOSE TISSUE

Studies on the role of the brain in body fat distribution

ACADEMISCH PROEFSCHRIFT
ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam,
op gezag van de Rector Magnificus
prof. mr. P.F. van der Heijden
ten overstaan van een door het college
voor promoties ingestelde commissie,
in het openbaar te verdedigen
in de Aula der Universiteit
op woensdag 7 september 2005, te 14:00 uur
door

Felix Kreier

geboren te Neurenberg, Duitsland
PROMOTIECOMMISSIE

Promotores: prof. dr. R.M. Buijs
            prof. dr. E. Fliers

Co-promotores: prof. dr. J.A. Romijn
                prof. dr. H.P. Sauerwein

Overige leden: prof. dr. R.A. Adan
               prof. dr. M.F. Dallman
               prof. dr. H.A. Delemarre-van de Waal
               prof. dr. J.B. Hoekstra
               prof. dr. D.F. Swaab

Faculteit der Geneeskunde

Cover illustration from:
Andreas Vesalius (1514-1564)
De corporis humani fabrica libri septem
(Basel: Johannes Oporinus, 1543)

Book design: Henk Stoffels
Print: Febodruk bv, Enschede
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER 1</th>
<th>Introduction</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>PART A</td>
<td>FINDINGS</td>
<td></td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>Selective parasympathetic innervation of subcutaneous and intra-abdominal fat – functional implications</td>
<td>17</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>Neuronal tracing from metabolic organs: An autonomic (anatomical) basis for type 2 diabetes</td>
<td>33</td>
</tr>
<tr>
<td>CHAPTER 4</td>
<td>Dual sympathetic and parasympathetic hypothalamic output to white adipose tissue</td>
<td>47</td>
</tr>
<tr>
<td>CHAPTER 5</td>
<td>Estrogen receptor alpha and glucocorticoid receptor expression in (pre-)parasympathetic neurons that project to white adipose tissue</td>
<td>57</td>
</tr>
<tr>
<td>COLOR SECTION</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td>PART B</td>
<td>DISCUSSION AND PERSPECTIVES</td>
<td></td>
</tr>
<tr>
<td>CHAPTER 6</td>
<td>Hypothesis: Shifting the equilibrium from activity to food leads to autonomic unbalance and the metabolic syndrome</td>
<td>81</td>
</tr>
<tr>
<td>CHAPTER 7</td>
<td>Hypothesis: HIV-associated adipose redistribution syndrome as a selective autonomic neuropathy</td>
<td>91</td>
</tr>
<tr>
<td>CHAPTER 8</td>
<td>Perspectives for follow-up studies</td>
<td>97</td>
</tr>
<tr>
<td>SUMMARY</td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>SAMENVATTING</td>
<td></td>
<td>115</td>
</tr>
<tr>
<td>AUTHORS</td>
<td></td>
<td>119</td>
</tr>
<tr>
<td>PUBLICATIONS</td>
<td></td>
<td>121</td>
</tr>
<tr>
<td>THANKS</td>
<td></td>
<td>123</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td></td>
<td>127</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

Based on:
Central nervous determination of food storage-a daily switch from conservation to expenditure: implications for the metabolic syndrome.

Felix Kreier, Andries Kalsbeek, Marieke Ruiter, Ajda Yilmaz, Johannes A. Romijn, Hans P. Sauerwein, Eric Fliers, Ruud M. Buijs


It is important to look at the integrated organism at different times [...] and to examine the events in different tissues, because these events might not be similar or comparable

Per Björntorp, 1983

'NEW' CONCEPTS

Claude Bernard, a pioneer in scientific theory and practice of modern medicine, proposed in the 1840s that nerves are either sensory or motor, that vasomotor nerves regulate blood supply by modulation of arterial tone, that the liver stores glucose and that the pancreas secretes digestive enzymes. He assumed -at that time a groundbreaking concept- that these important processes should be coordinated by the best-protected organ of the body: the brain. To test his hypothesis, Claude Bernard punctured the fourth ventricle of rabbits and found them to turn diabetic. His famous conclusion from these observations was that these mechanisms all serve one common objective: keeping the internal environment stable.

When Charles Darwin observed in the 1870s that "a hungry man, if tempting food is placed in front of him, may not show his hunger by any outward gesture, but cannot control the secretion of glands (like saliva)", he assumed that the brain controls the body by will-dependent and will-independend nerves.

When Harvey Cushing observed in the 1920s that a fatty liver could be induced within 9 hours after peripheral injection of hypophysin (an extract of the posterior lobe of the pituitary), he hypothesized a role for the brain and the autonomic nervous system in
this effect. Indeed, when he injected hypophysin intracerebroventricularly, the fatty liver developed already within 4 hours after injection. Moreover, he could abolish this effect by lesions to either the central nervous or the peripheral autonomic nervous system.

Interestingly, these three scientific pioneers shared the same concept to comprehend metabolism in mammals: they presumed a central role for the brain in the coordination of energy homeostasis. However, the interpretation of these experiments was extremely complex. After the discovery of various powerful hormones in the first half of the 20th century, concepts changed. The interpretation of experiments with hormones seemed less complex. In addition, the directly measurable effects were much stronger, such as the effects of intravenous injection of insulin. One could conduct an experiment easily by injecting a hormone or removing a gland. This experimental reduction was less feasible in the nervous system in vivo; it seemed a major obstacle to govern the experiment without inducing uncontrolled counterregulatory mechanisms or major side effects.

During this period a paradigm shift occurred: endocrinology grew out to an independent area of research and specialization of medicine, focused on hormones and becoming dissociated from neuroscience. Extensive endocrinological research of the 20th century revealed an amazing amount of knowledge about the role of hormones in energy homeostasis. However, the open question of what coordinates body homeostasis has concerned more and more researchers over the past decades. Today, a re-unification of neurosciences and endocrinology is starting to develop which may eventually allow an insight into the "big picture" of energy homeostasis. In this chapter, we will focus on the role of the brain as a coordinator of metabolism, using both neurons and hormones to communicate with the body. We will describe the coordinating function of the brain and provide mechanisms that maybe causing the metabolic syndrome and type 2 diabetes.

THE BIOLOGICAL CLOCK MODULATES HYPOTHALAMIC INTEGRATION

One basic principle of central nervous integration is the active filtering of noise. The brain decides which stimuli are relevant. E.g., the time of the day defines how a certain stimulus will reach the hypothalamus and even when it reaches the hypothalamic integration sites, the time will determine the level of response. The blood-brain-barrier (BBB) actively selects humoral factors that can pass to the hypothalamus. E.g. leptin and TNF-alpha get access to the brain depending on the time of the day. This circadian filter can help us to order and direct the flood of information.

Evolution forced us to develop adaptive body functions to survive a hostile world. The daily switch between light and dark and the outside temperature forced mam-
INTRODUCTION

Animals to develop a circadian rhythm generator that saves energy and avoids predation by specialization to an active and an inactive period\(^8\). The central biological clock is situated in the suprachiasmatic nucleus of the hypothalamus (SCN), adjacent to the optic chiasm, from where the SCN receives light information from the eyes through the retinohypothalamic tract\(^8\). Our biological clock oscillates the body between an inactive phase for regeneration and preparation and an active phase, in which energy is invested in physical activity (e.g. hunting).

Here, we will focus on the modulation of the hypothalamic output by the SCN. The central clock receives information from the external environment about the time of the day e.g. by light. Humoral input from the internal environment enables the SCN to read the internal synchronization message of the body, such as melatonin. In return, the SCN transports its time-of-the-day message throughout the body via various hormones, such as corticosterone and melatonin\(^9\). The SCN communicates its state of the day selectively through the sympathetic and parasympathetic branches of the autonomic nervous system\(^10\). In addition, the SCN modulates the activity of neurons within the hypothalamus itself.

SCN-lesions in animals result in the disruption of circadian behavioral and metabolic rhythms, leading to flattened hormonal rhythms and an equal distribution of locomotor activity during the day and the night. Since SCN transplants in a semi-permeable membrane, preventing neuronal sprouting, could restore locomotor activity in SCN-lesioned hamsters, one could reason that its message is broad and unspecific\(^11\)-\(^12\). However, later experiments also revealed that transplants were unable to restore hormonal rhythmicity in cortisol and gonadal function, illustrating the complexity of the regulation of hormones by the SCN. Neuroanatomical and functional studies revealed that the SCN uses different sets of hypothalamic neurons to deliver selective messages to other brain regions. The target areas can be divided into four functional groups of neurons.

1. Hypothalamic neurons projecting to the pituitary axes involved in the hormonal control of the body.
2. Hypothalamic neurons projecting to the autonomic nervous system involved in the neuronal control of the body. Note that the SCN is able to affect the sympathetic and parasympathetic branch selectively via separate projections\(^10\).
3. Hypothalamic neurons of integrative centers involved in e.g., energy homeostasis and temperature regulation, such as the dorsomedial nucleus and the medial preoptic area, putatively building an intermediate step between the SCN and the hormonal or neuronal output signal via the neurons of 1. and 2.
4. Thalamic neurons in the lateral geniculate nucleus and the paraventricular nucleus, synchronizing hypothalamic-induced behavior with locomotor activity.
INTRODUCTION

CIRCADIAN OUTPUT: THE BRAIN AFFECTS BODY FUNCTIONS

The adrenal - a model for the dual control of an organ

The central clock uses hormones as an output signal to the adrenal - sufficient to predict corticosterone levels?

Corticosterone and glucose peak in the beginning of the active phase, just one or two hours before the central clock has planned to awaken us\(^{(13,14)}\). This so-called dawn phenomenon prepares us for the new day\(^{(15)}\). How is this rise in corticosterone accomplished? The SCN modulates the cascade of corticotrophin-releasing hormone-containing neurons that stimulate the secretion of adrenocorticotropic hormone from the pituitary, which in turn results in corticosterone secretion from the adrenals, well-known as the hypothalamus-pituitary-adrenal axis\(^{(11)}\). However, activity of the HPA-axis cannot explain the pattern of corticosterone secretion as a whole: 24-hour plotting of ACTH against corticosterone levels in plasma reveals that the sensitivity of the adrenal for ACTH is restricted to the active period\(^{(16)}\).

The parallel output signal of the central clock: neuronal projections to the adrenal

The SCN talks to the body not only via the pituitary, but also via the autonomic nervous system. With the aid of neuroanatomical tools such as retrograde tracers, central neurons controlling a particular organ can be visualized. The retrograde transneuronal tracer Pseudorabies virus travels against the direction of the neuronal signal and crosses synapses, which enables it to identify a chain of neurons in control of a particular organ.

Using this technique, for the first time, a multisynaptic projection from the SCN to the adrenal by the autonomic nervous system was demonstrated\(^{(17)}\). Moreover, physiological experiments revealed that the function of this multisynaptic pathway from the SCN to the adrenal cortex is to modulate its sensitivity for ACTH\(^{(17)}\).

We propose that fat tissue is also controlled by hormones and neurons. In chapters 2 and 4 we describe the projections from the biological clock via the sympathetic and parasympathetic nervous system to fat tissue. In chapter 2 we study the physiological impact of vagal input to fat tissue.

The SCN and the oscillating body

Potentially, all cells of the body have clock genes forming an intracellular clock. Here, we review data indicating that the central clock sets the peripheral clocks on time by means of neurons and hormones\(^{(8)}\).

The neuroanatomical substrate for neuronal control was shown recently by experiments using Pseudorabies virus. The SCN has specialized neurons affecting the sympathetic or the parasympathetic branch\(^{(10)}\). Neuroanatomical tracer studies revealed a multisynaptic pathway from the SCN to various organs, e.g. heart, pancreas, liver, thyroid and pineal\(^{(18-21)}\).
INTRODUCTION

The best-studied peripheral circadian oscillator is the pineal; here the SCN uses the autonomic nervous system to induce melatonin secretion. Recently, the sympathetic nervous system has been demonstrated to affect clock gene expression in the liver. Physiological studies on liver and pancreas revealed a circadian rhythm in glucose, insulin and glucagon secretion, induced by the SCN and modulated by food intake. Also daily levels of the fat-derived hormone leptin are driven by the SCN. Within the cardiovascular system, heart rate, blood pressure, QT-interval length and k+ channels show a circadian rhythm.

It is attractive to assume that the biological clock might be the basis for the observation that body performance during exercise is best at the time of the day where training was regularly performed. In order to test the hypothesis that peripheral metabolic information is communicated to the clock by neural pathways, in addition to humoral factors, we investigated the neuronal feedback from fat tissue to the brain in chapter 3.

Another group of peripheral non-circadian oscillators is illustrative of the neuronal induction of organ rhythmicity. The sympathetic nervous system has been shown to induce 10 rapid oscillations per hour in lipolysis. In a series of studies on the endocrine pancreas, precise analysis of insulin and glucagon levels in the portal vein also revealed a pulsatile secretion pattern.

The hypothalamus-autonomic nervous system-body-axis: simply top-down?

The central role of the hypothalamus in the control of energy homeostasis was deduced from lesion studies. Stereotactic lesions in the region of the ventral medial hypothalamic nucleus cause overfeeding and obesity, whereas lesions in the lateral hypothalamic area result in an anorexia-cachexia syndrome. These data suggest a simple one-way top-down control of the body by the hypothalamus in the control of energy homeostasis.

However, as the brain senses the body on all its integrative layers via humoral factors and afferent nerves, it also does so on the level of the autonomic motor neurons in brainstem and spinal cord. As reviewed by Grill and Kaplan, the decerebrate animal model with a hypothalamus disconnected from the brainstem sheds light on the autonomic integration of autonomic afferents and efferents. The isolated autonomic nervous system is capable of coordinating oral movements and digestion, resulting in similar weight with intra-orally placed meals as compared to intact rats. Interestingly, the isolated autonomic nervous system is not capable to compensate for food deprivation, as demonstrated by intact rats increasing their meal size but not the decerebrate rats. Thus, while the autonomic nervous system can function autonomously in the short-term control of food intake, the hypothalamus is needed for long-term coordination.
INTRODUCTION

We propose that the hypothalamic-brainstem interaction is a system in control of energy homeostasis, built up of nested autonomous units that are modulated by higher integrative layers. In chapters 2, 3 and 4 we discuss the hypothalamic network projecting to fat tissue.

The autonomic nervous system differentiates between functional body compartments

The purpose of the SCN is to anticipate fluctuations in the external environment in order to keep the internal environment stable. However, the central clock needs to activate or silence tissues, depending on their function at different time points of the day. For example, muscles work in the active phase, when the digestive tract slows down. Therefore, opposite autonomic tone on vasculature redirects blood away from the abdominal compartment towards the movement compartment, whereas cerebral blood flow is kept constant. In contrast, at night the SCN slows down heart function, resulting in a dip in blood pressure.²¹

How can different regions of the body be controlled selectively? The brain has two avenues of communication: hormones and neurons. Hormones are present throughout the body and obtain their specificity by acting on receptors with a tissue-specific, fluctuating expression, whereas neurons deliver their message to a precisely targeted tissue in the body.

We propose that the body can be divided into different functional autonomic compartments and that at least a thoracic and movement compartment and a visceral compartment should exist. In this setting, a balanced and flexible autonomic nervous system can oscillate the activities of the organs within the compartments according to the actual needs of the body.

In chapters 2 and 3 we demonstrate that the autonomic nervous system differentiates between intra-abdominal and subcutaneous fat tissue.

In chapter 3 we reveal that the abdominal organs share autonomic control.

In chapter 5 we investigate the role of the brain in body fat distribution. We study whether estrogen and glucocorticoid receptors are expressed by pre-autonomic neurons that project to fat tissue. This might provide an anatomical basis for the effect of these hormones on fat tissue metabolism and body fat distribution.

In chapter 6 we propose that the metabolic syndrome is caused by a disturbed central clock.

In chapter 7 we speculate whether HIV-related lipodystrophy might be caused by toxicity of HIV or HIV-therapy on central (pre)autonomic neurons projecting to fat tissue.

In chapter 8 we suggest follow-up studies for the present thesis.
REFERENCES

5. Cushing, H. Pituitary body, hypothalamus and parasympathetic nervous system (Charles C Thomas, Baltimore, 1932).
Part A

Findings
CHAPTER 2

Selective parasympathetic innervation of subcutaneous and intra-abdominal fat — functional implications

The Journal of Clinical Investigation, November 2002, Volume 110, Number 9, p. 1243-1250

Felix Kreier, Eric Fliers, Peter J. Voshol, Corbert G. Van Eden, Louis M. Havekes, Andries Kalsbeek, Caroline L. Van Heijningen, Arja A. Sluiter, Thomas C. Mettenleiter, Johannes A. Romijn, Hans P. Sauerwein, and Ruud M. Buijs

The wealth of clinical epidemiological data on the association between intra-abdominal fat accumulation and morbidity sharply contrasts with the paucity of knowledge about the determinants of fat distribution, which cannot be explained merely in terms of humoral factors. If it comes to neuronal control, until now, adipose tissue was reported to be innervated by the sympathetic nervous system only, known for its catabolic effect. We hypothesized the presence of a parasympathetic input stimulating anabolic processes in adipose tissue. Intra-abdominal fat pads in rats were first sympathetically denervated and then injected with the retrograde transneuronal tracer pseudorabies virus (PRV). The resulting labeling of PRV in the vagal motor nuclei of the brain stem reveals that adipose tissue receives vagal input. Next, we assessed the physiological impact of these findings by combining a fat pad–specific vagotomy with a hyperinsulinemic euglycemic clamp and RT-PCR analysis. Insulin-mediated glucose and FFA uptake were reduced by 33% and 36%, respectively, whereas the activity of the catabolic enzyme hormone-sensitive lipase increased by 51%. Moreover, expression of resistin and leptin mRNA decreased, whereas adiponectin mRNA did not change. All these data indicate an anabolic role for the vagal input to adipose tissue. Finally, we demonstrate somatotopy within the central part of the autonomic nervous system, as intra-abdominal and subcutaneous fat pads appeared to be innervated by separate sympathetic and parasympathetic motor neurons. In conclusion, parasympathetic input to adipose tissue clearly modulates its insulin sensitivity and glucose and FFA metabolism in an anabolic way. The implications of these findings for the (patho)physiology of fat distribution are discussed.
INTRODUCTION

The dissimilar distribution of white adipose tissue over the intra-abdominal and subcutaneous fat compartments depends on factors like gender, age, and nutritional condition. Local effects of humoral factors cannot readily explain such distribution, because both compartments are subject to the same endocrine environment, and regional differences in receptor expression are not sufficient to explain the differences (1). Only recently, the biological clock in the hypothalamus was shown to regulate diurnal changes in adipose tissue leptin production (2). This and other evidence indicates that the autonomic nervous system exerts direct control at the cellular and molecular levels in adipose tissue (3). This principle of regulation of adipose tissue is unlikely to be limited to control of leptin secretion only, since the autonomic nervous system plays an important role in the control of energy homeostasis (4–8).

Neuroanatomical and physiological evidence for sympathetic innervation of adipose tissue was presented earlier, suggesting a role for this branch of the autonomic nervous system in lipolysis (9, 10). Parasympathetic innervation, however, was reported to be absent. In the energy-spending, catabolic state of the body, the sympathetic nervous system is predominant (11), whereas in the energy-saving anabolic state, the parasympathetic branch prevails (12, 13). Therefore, we hypothesized the presence of parasympathetic innervation in order to explain the buildup of adipose tissue.

To investigate the innervation of adipose tissue, we used two neuronal retrograde tracers. We injected a retrograde tracer, FluoroGold (Fluorochrome, Englewood, Colorado, USA), and a transsynaptic retrograde tracer, pseudorabies virus (PRV), which is taken up exclusively by neuronal terminals and transported toward the cell body (14–20) into different fat pads in rats. Here, we show that all these fat pads receive parasympathetic input.

In order to assess the physiological impact of the parasympathetic innervation, we applied a hyperinsulinemic euglycemic clamp to determine glucose and FFA uptake in intact as well as in vagotomized retroperitoneal fat pads in the same animal. Sham operated animals were used as a control. Furthermore, we established the activity of hormone-sensitive lipase (HSL), a marker of lipolysis, and the expression of leptin, resistin, and adiponectin mRNA after fat pad–specific vagotomy.

Finally, we hypothesized a selective control of fat compartments by the autonomic nervous system as a neuroanatomical basis for body fat distribution. First, we investigated the organization of parasympathetic motor neurons projecting into the intra-abdominal or subcutaneous compartments. Two retrograde tracers were simultaneously injected into two different fat pads in a single animal: FluoroGold into subcutaneous inguinal fat and PRV into sympathetically denervated retroperitoneal fat. The latter has been reported to be metabolically comparable to omental fat tissue (21). The combination of tracers was used because FluoroGold rapidly and retrogradely fills all
neurons that innervate a particular organ, and it is not transported transsynaptically like PRV is. By combining PRV with selective sympathetic denervation, its transport was restricted to the parasympathetic branch of the autonomic nervous system. Thus, we used neuroanatomical, physiological, and molecular biological methods to elucidate the presence and functionality of parasympathetic input to adipose tissue. Moreover we demonstrated a specialization of autonomic motor neurons projecting into one fat compartment only.

**METHODS**

**PRV tracing**

Retrograde transneuronal labeling of PRV, a swine neurotrophic α herpes virus, was applied in this study. Uptake, but not replication, by glial cells prevents diffusion of the virus to other neurons (14–20).

Intravenous deposition of 5 μl PRV suspension containing 5 × 10⁶ plaque-forming units [a generous gift of C.E. Jacobs (Institute for Animal Science and Health, Lelystad, The Netherlands)] in the abdominal cavity or on top of a fat pad did not result in labeling of the CNS in six male Wistar rats. Similarly, complete denervation of a fat pad followed by PRV injection in five animals did not result in infection of the CNS. Thus, labeling via blood capillaries in the fat pad did not occur.

Fifteen animals received a single injection of 5 μl of a suspension of the Bartha strain of PRV (PRV-Bartha) containing 5 × 10⁶ plaque-forming units. The injection was performed using a 30-gauge needle connected to a Hamilton syringe at a single spot 1 cm below the most rostral tip of the left retroperitoneal fat pad. By carefully controlling the amount and site of injection, we obtained a reproducible infection rate. In our experience, multiple injections can result in uncontrolled superinfection, probably due to more rapid proliferation of neuronal PRV, resulting in faster lysis of neurons.

Since the level of PRV labeling progresses with time (15), we denote the first neurons in the CNS that were shown to contain PRV as the “first-order neurons.” These were all sympathetic and parasympathetic motor neurons. Sacrificing the animals on several time points (3–4 days after injection of PRV), we followed the progress of infection retrogradely from the autonomic motor neurons to the preautonomic neurons (the “second-order neurons”) and further upstream. We analyzed five intact animals with first-and second-order infection.

**Sympathetic denervation**

Twenty-eight animals were used to develop the procedure of sympathetic denervation of the retroperitoneal fat pad. In a series of experiments, we dissected different nerves entering the left retroperitoneal fat pad and then injected PRV. Successful denervation
was achieved when the PRV injection resulted in staining of the vagal motor nuclei of the brain stem, but not staining in the spinal cord (see Figure 2-1). With this approach, the vagal input could be identified and a reproducible denervation technique was developed. The autonomic input to the retroperitoneal fat pad is characterized by diffuse sympathetic nerve fibers, running mostly along blood vessels from the lateral and dorsal directions, and one focused vagal input nerve traveling along blood vessels from the diaphragm into the fat pad.

For histological analysis, sympathetic nerves were removed from a fat pad in 15 animals just prior to PRV injection. The left retroperitoneal fat pad was dissected completely from the connecting tissues except for the nerve bundle traveling along a blood vessel from its rostral tip to the diaphragm. The fat pad was lifted up and inspected for residual nerve bundles. Then PRV was injected in the same way as described above.

*Parasympathetic denervation*

For the physiological experiments, we reversed the sympathetic denervation procedure. Instead of removing all diffuse input (which has been shown to be sympathetic) and leaving the vagal input along the blood vessel from the rostral tip to the diaphragm intact, we dissected the vagal input only. Histological control experiments were conducted to examine the reproducibility of this procedure. We showed in eight of eight animals the absence of any labeling in the vagal motor nuclei after 4 days of survival.

*Histological techniques*

After 3 days and 4 days, the animals were perfused with saline and then a solution of 4% paraformaldehyde and 0.15% glutaraldehyde in PBS (pH 7.4). They were postfixed overnight and cryoprotected by immersion with 30% sucrose in 0.2 M PBS (pH 7.4) for a further 24 hours. Brain and spinal cord were frozen and coronal sections (40 µm) were cut. After rinsing in 0.05 M Tris-buffered saline (pH 7.4), brain sections were incubated overnight at 4°C with a polyclonal rabbit anti-PRV (anti-α-Aujeszky) antibody (1:10,000; a generous donation of C.E. Jacobs, Institute for Animal Science and Health), then incubated for 60 minutes in the secondary antibody, biotinylated goat anti-rabbit (Vector Laboratories Inc., Burlingame, California, USA), followed by incubation in ABC complex (Vector Laboratories Inc.). Finally, the sections were reacted with 0.025% 3,3-diaminobenzidine tetrahydrochloride in Tris-buffered saline containing 0.5% H2O2.

The light microscopy color figures were imported using a Zeiss axioplan 2 microscope (Zeiss, Jena, Germany) fitted with a Progress Camera 3012 (Jenoptik, Jena, Germany). The figures were of 1,488 x 1,120 pixel size in RGB 24-bit true color. Contrast and color were adapted using Adobe Photoshop (Adobe Systems Inc., Mountain View, California, USA) without any other image manipulation.
Glucose and FFA uptake and HSL activity

Animals were either parasympathetically denervated on one side \( n = 6 \), see section on parasympathetic denervation, above) or sham operated \( n = 6 \). Permanent catheters were placed in the jugular vein for infusion and in the inner carotid artery for sampling \( (22, 23) \). After 7 days, two pumps were started, one for input of insulin (Actrapid; Novo Nordisk, Chartres, France) at a constant rate of 3.5 mU/kg/min and another for D-glucose \( (25\% \text{ solution}; \text{Sigma-Aldrich, St. Louis, Missouri, USA}) \). Insulin levels increased to \( 450 \pm 160 \) versus \( 333 \pm 170 \text{ pmol/l} \), sham versus vagotomy, respectively, while the D-glucose pump was adjusted to maintain blood glucose around \( 6.0 \text{ mM} \) \( (6.5 \pm 1.0 \) versus \( 6.6 \pm 0.7 \text{, sham versus vagotomy, respectively}) \). After glucose reached steady-state levels (ca. 1 hour), a bolus of \( 3\text{H}-2\text{-deoxy}-D\text{-glucose} \) \( (20 \mu\text{Ci; Amersham International, Little Chalfont, United Kingdom}) \) was given to trace tissuespecific glucose uptake. Forty-five minutes later, a bolus of \( 14\text{C}-\text{palmitate} \) \( (10 \mu\text{Ci; Amersham International}) \) was given to trace tissuespecific FFA uptake. One minute later the animals were killed.

To determine adipose glucose uptake, fat pads were homogenated in water and boiled for 10 minutes. After centrifugation, \( 3\text{H}-2\text{-deoxy}-D\text{-glucose-phosphate} \) was separated from free \( 3\text{H}-2\text{-deoxy}-D\text{-glucose} \) \( \text{present in plasma} \) by ion-exchange chromatography \( (\text{Dowex-column X-100; Sigma-Aldrich}) \) to measure tissue glucose uptake. \( 3\text{H}-2\text{-deoxy}-D\text{-glucose} \) is taken up by the tissue, converted into \( 3\text{H}-2\text{-deoxy}-D\text{-glucose-phosphate} \), and not metabolized further \( (24) \).

Adipose FFA uptake was determined in homogenized fat pads after lipid extraction \( (25) \). We confirmed by TLC that no \( 14\text{C} \) FFAs were incorporated in the lipid fractions (triglycerides and cholesteryl esters).

HSL activity was determined by homogenization of 200 mg of fat pad in buffer containing protease inhibitors \( (26) \). The homogenates were centrifuged and the supernatant was used to determine HSL activity with cholesteryl-14C-oleate \( \text{(Amersham International)} \) as a substrate. All determinations were done in duplicate.

Leptin, resistin, adiponectin, and reference gene mRNA expression

The left retroperitoneal fat pad of nine animals was parasympathetically denervated and was compared with the intact right pad. Seven days later, the retroperitoneal fat pads were removed and directly frozen in liquid nitrogen. RNA extraction was performed in Trizol \( \text{(Life Technologies Inc., Gaithersburg, Maryland, USA)} \) according to the instructions of the manufacturer. Total RNA was reverse transcribed using 2 \( \mu\text{g} \) of RNA, 500 ng of oligo-dT, and 200 U reverse transcriptase \( \text{(SuperScript II RT; Life Technologies Inc.)} \) for 1 hour at 37°C. Quantitative assessment of mRNA levels was performed using a GeneAmp 5700 sequence detection system \( \text{(PE Biosystems, Foster City, California, USA)} \). RT-PCR was performed using the SYBR Green core reagents kit \( \text{(PE Biosystems)} \). Primer pairs were designed using Primer Express software \( \text{(Ap-} \)
Somatotopy: FluoroGold/PRV tracing

**Brain stem.** Seven animals received a 2-μl injection of 2% FluoroGold solution, a retrograde neuronal tracer, in the left subcutaneous inguinal fat pad. Simultaneously, a 5-μl injection of PRV solution was applied to the sympathetically denervated left retroperitoneal fat pad to show the somatotopic organization of the dorsal motor nucleus of the vagus (DMV). Spinal cord sections were controlled for the absence of PRV as described above. Five animals with only first-order neuronal labeling were included for analysis. In the control group, both tracers were injected into the left sympathetically denervated retroperitoneal fat pad (three of five animals with first-order infection were included for analysis). Brain stem sections were incubated overnight at 4°C with a polyclonal rabbit anti-FluoroGold antibody (1:15,000; Sigma-Aldrich) and polyclonal mouse anti-PRV (a generous donation of C.E. Jacobs, Institute for Animal Science and Health), and then incubated for 60 minutes with the FITC-conjugated secondary antibody to detect PRV and the CY3-conjugated secondary antibody to detect FluoroGold as described above. **Spinal cord.** Sixteen animals received, at the same time, two different strains of PRV: PRV-Bartha β-galactosidase B80 (PRV β-gal) and PRV green fluorescent protein (PRV-GFP). A total of 5 × 10^7 plaque-forming units were given of each PRV. PRV β-gal was injected into subcutaneous inguinal fat and PRV-GFP into mesenteric fat without denervation. Animals were sacrificed after 3 days. Both PRV strains showed the same infection rate. As a control, 11 animals (four with first-order labeling included for analysis) received injections of both viruses into the same fat pad. Sections of thoracic segments of the spinal cord were incubated overnight at 4°C with polyclonal rabbit anti–PRV-GFP (Molecular Probes Inc., Eugene, Oregon, USA) and polyclonal mouse anti–PRV β-gal (Sigma-Aldrich) and then incubated for 60 minutes with the FITC-conjugated secondary antibody to detect PRV β-gal and the CY3-conjugated secondary antibody to detect PRV-GFP.
CHAPTER 2 VAGAL CONTROL OF FAT TISSUE

RESULTS

Parasympathetic innervation of adipose tissue

Neuroanatomy. In the control experiments, intravenous deposition of PRV in the abdominal cavity, on top of a fat pad or into a completely denervated fat pad, never resulted in labeling of the CNS. In contrast, after injection of PRV into the intact retroperitoneal fat pad, the sympathetic preganglionic motor neurons in the intermediolateral column of the spinal cord (IML) were rapidly labeled by PRV. In a later stage of infection (4 days survival), the DMV and nucleus ambiguus (AMB) plus multiple sites in the brain stem and hypothalamus became visible (Figure 2.1).

Microsurgical denervation of all sympathetic fibers entering the retroperitoneal fat pad combined with injection of PRV selectively infected the antagonistic vagal branch alone without labeling in the spinal cord, where the sympathetic motor nuclei are situated. Six animals were allowed to survive for 3 days and five for 4 days. All animals denervated according to this procedure showed complete sympathetic denervation as evidenced by the absence of sympathetic labeling in the spinal cord. Six animals showed infection beyond the DMV and revealed second-order labeling, while five animals showed first-order labeling only. Among the six animals killed after 3 days, five showed first-order labeling only and one had no staining.

PRV labeling appeared in parasympathetic motor nuclei (DMV and AMB). Subsequently, areas that project into the vagal motor neurons became infected in the brain stem (rostroventrolateral medulla, nucleus of the solitary tract [NTS]), and the hypothalamus (paraventricular nucleus, lateral hypothalamic area).

Physiology. Plasma levels of glucose, insulin, FFAs, cholesterol, glycerol, and triglycerides did not differ between the animals that had the left retroperitoneal fat pad locally vagotomized and the sham-operated animals. Analyzing the ratio between intact and parasympathetically denervated fat pads revealed a 33% (by Mann-Whitney U test, \( P = 0.02 \)) reduction in insulin-mediated glucose uptake and a 36% (by Mann-Whitney U test, \( P = 0.02 \)) reduction in insulin-mediated FFA uptake in vagotomized fat pads (Figure 2.2). Interestingly, concurring with the reduced FFA uptake in the vagotomized fat pad, the activity of the catabolic enzyme HSL, the most important enzyme involved in hydrolyzing triglyceride in adipose tissue, increased by 51% (Mann-Whitney U test, \( P = 0.03 \)).

Endocrine function. Compared with the intact right retroperitoneal fat pad, resistin and leptin mRNA expression after fat pad–specific vagotomy on the left side decreased by 71% (Mann-Whitney U test, \( P = 0.001 \)) and 45% (Mann-Whitney U test, \( P = 0.004 \)) respectively, whereas adiponectin and reference gene mRNA did not change significantly (Figure 2.3). In the control group, mRNA expression of leptin, resistin, adiponectin, and reference gene did not change after sham operations. Somatotopy After injection of FluoroGold into the subcutaneous inguinal fat pad and PRV into the sympathetic-
PART A FINDINGS

Figure 2.1  Transverse section of the spinal cord (at Th7) and the rat brain stem at the level of the obex shows spinal cord and brain stem neurons projecting into adipose tissue. Transneuronal retrograde tracing by PRV injection into retroperitoneal fat in rats before (a and b) and after (c and d) sympathetic denervation of adipose tissue. In a and b (PRV tracing from adipose tissue before denervation), since both sympathetic and parasympathetic fibers are intact, PRV is seen to spread via the vagus and the sympathetic nerves. Interestingly, the route via the IML is favored in intact animals such that second-order neurons in the brain stem are already evident when the first-order parasympathetic motor neurons appear in the DMV (arrow). In b, the Ai region, the raphe nucleus (R), and the nucleus of the solitary tract (NTS) project into the sympathetic motor neurons. In c (with d, showing PRV tracing after sympathetic denervation of the left retroperitoneal fat pad), there is no labeling of PRV in the IML. In the brain stem shown in d, neurons are clearly visible in the parasympathetic motor nuclei: DMV and caudal part of the AMB. CC, central canal. Bar in a and c = 0.5 mm. Bar in b and d = 0.4 mm. See color section.

cally denervated retroperitoneal fat pad in the same animal, both retrograde tracers were demonstrated within cell bodies of the parasympathetic motor nuclei of the vagus nerve. In all five animals that showed first-order labeling only, the tracers were localized in the same nuclei but in different neurons, which demonstrates a separation of autonomic control at the level of the parasympathetic motor neuron (Figure 2.4).
Vagal motor neurons in the DMV projecting into intra-abdominal fat pads tended to be localized medially to the neurons projecting into subcutaneous fat. As a control, we injected both Fluoro-Gold and PRV into the same sympathetically denervated fat pad.
PART A FINDINGS

Figure 2.3  Hormone mRNA expression in adipose tissue after parasympathetic denervation. The left retroperitoneal fat pad was parasympathetically denervated \((n = 9)\) and compared with the right intact pad for the expression of mRNA of resistin, leptin, adiponectin, and elongation factor-1α (as a reference gene) by means of real-time RT-PCR. Shamoperated animals were used as control \((n = 5)\). While resistin and leptin mRNA expression was reduced \((-71\%, \text{ Mann-Whitney } U \text{ test, } ^*P = 0.001; -45\%, \text{ Mann-Whitney } U \text{ test, } ^{**}P = 0.004, \text{ respectively})\), adiponectin and reference mRNA did not change significantly. Thus, parasympathetic denervation of adipose tissue specifically changes mRNA expression of fat-derived hormones. One relative unit is the equivalent cDNA corresponding with 0.1 μg per well of the pooled cDNA of the control fat pads. Values are expressed as mean ± SEM. See color section.

This resulted in colocalization of the tracers, confirming the specificity of the method. Next, the organization of sympathetic motor neurons was investigated by injecting two different strains of PRV: one carrying β-gal into mesenteric fat and the other, with GFP as a marker, into subcutaneous fat. After 3 days, the first neurons appeared in the IML (thoracic segments Th5–Th10). Four animals displaying only first order labeling of both PRV strains in the sympathetic motor nuclei in the IML were included for analysis. The other animals showed either no infection (two animals), infection with only one PRV strain (eight animals), or a massive infection (two animals). The IML also exhibited staining of the two PRVs, but again in separate neurons (Figure 2.5). In contrast, if injected into the same fat pad (mesenteric or subcutaneous inguinal) as a control, both strains of PRV were found in the same neurons.

26
CHAPTER 2 VAGAL CONTROL OF FAT TISSUE

Figure 2.4 Somatotopic organization of the parasympathetic nervous system. Laser scanning photomicrograph of transverse sections of the brain stem. The central canal is on the right side. Vagal motor neurons project into one fat compartment only (subcutaneous or intraabdominal). PRV (stained green) was injected into the intraabdominal fat compartment after sympathetic denervation. At the same time, FluoroGold (stained red) was injected into the subcutaneous fat compartment. Both tracers were transported back to the dorsal motor nucleus DMV and AMB in different neuron populations. Somatotopic segregation can be observed within the DMV. Bar = 50 μm. See color section.

DISCUSSION

Adipose tissue receives sympathetic and parasympathetic control. PRV injected into the adipose tissue of intact animals resulted in a more rapid labeling of sympathetic motor neurons than occurred in vagal motor neurons. Recent studies have shown that neuronal tracing can be modulated by neuronal activity (27, 28). Also in our study the activity of the vagus nerve modulated the velocity of PRV replication and transport. In the next stage of infection, the transneuronal tracer PRV labeled preautonomic neurons (second order neurons) in brain stem and hypothalamus projecting into the sympathetic motor neurons. In that stage neurons also became visible in the dorsal vagal complex, in which sympathetically-labeled NTS neurons were in proximity to the vagal first-order neurons, making their classification infeasible. This probably explains why parasympathetic innervation of adipose tissue was not noticed in the experiments of Bamshad et al., although their schematic figures showed labeling throughout the dorsal vagal complex (including the DMV) (9).

To distinguish between vagal motor neurons and preautonomic neurons projecting into sympathetic motor neurons, we used two different methods. First, we applied FluoroGold, a nontransneuronal retrograde tracer that reaches the CNS only via pre-ganglionic parasympathetic motor neurons. We found vagal input to various differ-
Figure 2.5  Somatotopic organization of the sympathetic nervous system. Sympathetic motor neurons project into one fat compartment only (subcutaneous or intra-abdominal). Two strains of PRV were injected simultaneously into the intra-abdominal fat compartment (mesenterial fat) and the subcutaneous fat compartment (subcutaneous inguinal fat). Confocal laser scanning photomicrograph of transverse thoracic spinal cord sections (Th5–Th10). Both tracers were transported back to the IML and show clear separation of the different tracers (red/green). Insert (control): Injection of both tracers into the same mesenterial fat pad resulted in colocalization of the two tracers (yellow). Specific laser analysis of the indicated neuron (arrow) also showed colocalization of the tracers with a strong signal of FITC (green) and a much weaker signal of CY3 (red). Bar = 50 μm. See color section.
ent fat pads (retroperitoneal, mesenteric, epididymal, and subcutaneous inguinal fat). Second, a combination of sympathetic denervation of retroperitoneal fat followed by injection of the transneuronal retrograde tracer PRV prevented infection of the spinal cord, but showed infection of the DMV and AMB. Cutting the readily labeled sympa-
thetic branch forces the virus through the residual branch. As infection continues in an upstream direction, neurons projecting into the vagal motor neurons (i.e., the NTS and rostroventrolateral medulla) become infected. The projection of the same brain areas into vagal motor neurons has been reported earlier from studies of the innervation of the pancreas (20).

At present we cannot answer the question of which transmitters are used by the vagal system to affect adipose tissue. The transmitters used by the vagal system may be (a) acetylcholine, (b) acetylcholine in combination with nitric oxide and/or vasoactive intestinal peptide, or (c) nitric oxide with vasoactive intestinal peptide without acetylcholine (29, 30).

The parasympathetic input to adipose tissue demonstrated in this study illustrates that white adipose tissue receives a dual autonomic control like other (endocrine) organs (4).

Parasympathetic input to adipose tissue modulates insulinmediated glucose uptake and FFA metabolism in an anabolic way and can selectively modulate its endocrine function. Selective denervation of an intra-abdominal fat pad was chosen instead of subdiaphragmatic vagotomy because the latter changes the neuronal communication between the brain and the whole intra-abdominal compartment (e.g., liver, pancreas, stomach, and intestines). The local vagotomy of one retroperitoneal fat pad allows its comparison with the intact pad on the other side. In addition, because of the surgical complications associated with removing the diffuse sympathetic input, we chose to investigate vagotomy only. Insulin-dependent uptake of glucose and FFAs in adipose tissue was strongly reduced after fat pad--specific vagotomy, while the activity of the catabolic enzyme HSL was increased. The opposite directions of the observed changes indicate that they do not merely reflect a gross change in metabolism or circulation but indeed reflect specific catabolic changes in the adipose tissue after vagotomy. The results show that the anabolic effect of the parasympathetic nervous system on adipose tissue antagonizes the wellknown catabolic effect of the sympathetic nervous system (31). Moreover, the endocrine function of adipose tissue is selectively modulated by parasympathetic input. Leptin and resistin mRNA synthesis is decreased in vagotomized fat pads, while adiponectin mRNA synthesis does not change. Thus, the data show a stimulation of the release of resistin and leptin by the vagus nerve (32, 33).

Our results clearly show the physiological impact of parasympathetic innervation on intra-abdominal adipose tissue, indicating its potential to stimulate glucose and FFA uptake, i.e., growth of adipose tissue. The parasympathetic input might mediate in the etiology of obesity by directly influencing the metabolic state of adipose tissue.

Body fat distribution reflects central somatotopic organization. The present study revealed the capacity of the CNS to directly control adipose tissue by means of two different principles: a balance of the sympathetic and parasympathetic output and a
selective control of the output with respect to the site of the fat compartment. In other terms, individual central autonomic neurons are specialized to control one fat compartment. Earlier studies of selective peripheral sympathetic control of adipose tissue support our findings (34).

This viscerotopic or rather somatotopic organization reveals the potential of the autonomic motor centers of both branches to selectively affect the anabolism and/or catabolism of either subcutaneous or intra-abdominal fat. Future studies will have to determine whether this somatotopic organization of the autonomic nervous system forms the anatomical basis for the dissociation of intra-abdominal and subcutaneous fat accumulation, i.e., body fat distribution (e.g., in the metabolic syndrome, Cushing syndrome, or AIDS lipodystrophy) (35–40). It is possible that a misbalanced autonomic outflow to the intra-abdominal compartment, including liver, pancreas, and intra-abdominal fat, is an important factor in the pathogenesis of prevalent diseases related to intra-abdominal obesity.

In summary, we show that adipose tissue receives vagal input, modulates its metabolism in an anabolic way, and can selectively stimulate endocrine function. In addition, we demonstrate that parasympathetic innervation differentially modulates the endocrine function of adipose tissue. Finally, we demonstrate a somatotopic organization with respect to the selective innervation of subcutaneous versus intra-abdominal fat by both the sympathetic and parasympathetic nervous systems.

ACKNOWLEDGEMENTS

We would like to thank Joke Wortel and Marieke Ruiter for technical assistance and Michel Hofman for statistical advice.

REFERENCES


CHAPTER 3

Neuronal tracing from metabolic organs: an autonomic (anatomical) basis for type 2 diabetes

submitted

Felix Kreier, Yolanda S. Kap, Thomas C. Mettenleiter, Caroline van Heijningen, Jan van der Vliet, Andries Kalsbeek, Hans P. Sauerwein, Eric Fliers, Johannes A. Romijn, Ruud M. Buijs

The hypothalamus uses hormones and the autonomic nervous system to balance energy fluxes in the body. Here, we show that the autonomic nervous system has a distinct organization in different body compartments. The same neurons control intra-abdominal organs (intra-abdominal fat, liver, and pancreas), whereas subcutaneous adipose tissue located outside the abdominal compartment receives input from another set of autonomic neurons. This differentiation persists up to preautonomic neurons in the hypothalamus, including the biological clock, that have a distinct organization depending on the body compartment they command. Moreover, we demonstrate a neuronal feedback from adipose tissue that reaches the brainstem. We propose that this compartment-specific organization offers a neuroanatomical perspective for the regional malfunction of organs in type 2 diabetes (T2 diabetes), where increased insulin secretion by the pancreas and disturbed glucose metabolism in the liver coincide with an augmented metabolic activity of visceral compared to subcutaneous adipose tissue.

INTRODUCTION

To balance energy fluxes, the brain needs a precise and clear view on the metabolic state of the body. Circulating humoral factors are in fact averaged whole body signals, while sensory neurons add detailed information from specific regions of the body to this global view. Hence, the first part of this study addressed the question, whether adipose tissue might act as any other organ and provides neuronal feedback to the brain as well. We investigated the presence of sensory feedback to the central nervous system by an injection of the neuronal tracer Cholera Toxin B (CTB) into intra-abdominal adipose tissue.

The hypothalamus integrates peripheral signals delivered by the blood (fatty acids, glucose, and hormones) and by neuronal input from peripheral organs. This hy-
pothalamic integration of peripheral information results in a modulated metabolic state. For instance, fatty acids and insulin are sensed in the hypothalamus and inhibit endogenous glucose production by the liver, stimulate glycogen synthesis in muscle and reduce food intake. The biological clock, located in the suprachiasmatic nucleus (SCN), modulates the metabolic set point of the body according to a diurnal pattern. The SCN induces diurnal metabolic variations such as the rise of glucose and glucocorticoids in the early morning, known as the “dawn-phenomenon”.

In general, in a chain of four events, the brain receives input, integrates it, and generates a hormonal and autonomic output that finally affects the peripheral organs. Two steps in this chain are much less established than the others: That is the direct neuronal sensory information from the organs and the neuronal output of the hypothalamus to the organs. The exact mechanism by which the hypothalamus directs the organs into the desired metabolic state is far from evident.

In order to clarify the output of the brain to the abdominal cavity we analyzed the vagal output to the abdomen by means of CTB using different fluorescent labels into intra-abdominal fat, liver and pancreas. Injecting the retrograde neuronal tracer Pseudorabies Virus (PRV) into retroperitoneal and subcutaneous fat allowed us to determine the projections from hypothalamus and brainstem to these fat compartments. Thus, we uncovered from the biological clock complete separate sets of neurons controlling subcutaneous and visceral adipose tissues. In contrast, located in the same body region, visceral adipose tissue, liver and pancreas share the same vagal motor neurons. We propose that this region-specific organization contributes to the concord in malfunction of organs in type 2 diabetes mellitus (T2 diabetes), in which condition an increased insulin secretion by the pancreas and hepatic insulin resistance coincides with an augmented metabolic activity of visceral compared to subcutaneous adipose tissue.

MATERIALS
All experiments were performed in adult male Wistar rats (250–350 g Harlan, Zeist, The Netherlands) according to the NIH guidelines for animal experiments and with approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

Fat denervation
The sympathetic or parasympathetic fibers entering the retroperitoneal fat pad were cut, as described earlier by our group. Please refer for a detailed description including perioperative photos to the supplemental information online.
Liver denervation

The liver was sympathetically denervated by a technique described earlier by Buijs et al. The bile duct was isolated from the portal vein complex. At the level of the hepatic portal vein the hepatic artery, a branch of the celiac artery, branches into the hepatic artery proper and the gastroduodenal artery. This division occurs on the ventral surface of the portal vein. At this point the arteries were separated via blunt dissection from the portal vein. The nerve bundles running along the hepatic artery proper were removed using microsurgical techniques. Then that part of the hepatic artery was closed by surgical thread on two sides and cut in between the knots in such a way that all sympathetic nerves were sectioned.

CTB tracing

2μl of CTB (A, 2%, Sigma-Aldrich, #C167) or CTB-alexa fluor 488/555/647 (Φ1%, Molecular Probes, #C22841/C22843/C22844) were injected in retroperitoneal fat, liver or pancreas using a 30-gauge needle connected to a Hamilton syringe at a single spot. As controls, CTB was applied on top of the intact or the totally denervated organ. 3, 4 or 5 days after tracer injection, the animals were perfused with saline and then a solution of 4% paraformaldehyde and 0.15% glutaraldehyde in PBS (pH 7.4). They were postfixed overnight and cryoprotected by immersion with 30% sucrose in 0.2 M PBS (pH 7.4) for a further 24 hours. Brains were frozen and coronal sections (40 μm) were cut. After rinsing in 0.05 M Tris-buffered saline (pH 7.4), brain with CTB sections were incubated overnight at 4°C with polyclonal rabbit anti-CTB (Sigma-Aldrich, #C362), then incubated for 60 minutes in the secondary antibody, biotinylated goat anti-rabbit (Vector Laboratories Inc., Burlingame, California, USA), followed by incubation in ABC complex (Vector Laboratories Inc.). Finally, the sections were reacted with 0.025% 3,3-diaminobenzidine tetrahydrochloride-nickel in Tris-buffered saline containing 0.5% H2O2. The light microscopy color figures were imported using a Zeiss axioplan 2 microscope (Zeiss, Jena, Germany) fitted with a Progress Camera 3012 (Jenoptik, Jena, Germany). The figures were of 1,488 x 1,120 pixel size in RGB 24-bit true color. Contrast and color were adapted using Adobe Photoshop (Adobe Systems Inc., Mountain View, California, USA) without any other image manipulation. Brain sections with CTB-alexa fluor were rinsed extensively in phosphate-buffered saline (PBS) pH 7.2, and coverslipped in 50% PBS glycerol for examination under a Philips (Eindhoven, The Netherlands) confocal laser-scanning microscope (LSM410/510). Digital images of the fluorescent sections were obtained using filters that prevented cross-talk of the fluorophores. Figures were contrast-enhanced but not otherwise manipulated in Adobe Photoshop.
PRV tracing

5μl PRV-Bartha ($5 \times 10^6$ plaque-forming units, a generous gift of C.E. Jacobs from the Institute for Animal Science and Health, Lelystad, The Netherlands) or PRV B80 ($5 \times 10^7$ plaque-forming units PRV β-galactosidase B80, Institute for Molecular Biology, Insel Riems, Germany), or PRV GFP ($5 \times 10^7$ plaque-forming units PRV green fluorescent protein, Institute for Molecular Biology, Insel Riems, Germany) were injected into liver, subcutaneous inguinal or retroperitoneal fat using a 30-gauge needle connected to a Hamilton syringe at a single spot. As controls, PRV was applied on top of the intact or the totally denervated organ.

3, 4 or 5 days after tracer injection, the animals were perfused with saline and then a solution of 4% paraformaldehyde and 0.15% glutaraldehyde in PBS (pH 7.4). They were postfixed overnight and cryoprotected by immersion with 30% sucrose in 0.2 M PBS (pH 7.4) for a further 24 hours. Brains were frozen and coronal sections (40 μm) were cut. Sections were incubated overnight at 4°C with a polyclonal mouse anti-PRV Bartha (a generous donation of C.E. Jacobs, Institute for Animal Science and Health), rabbit-anti GFP (Molecular Probes Inc., Eugene, Oregon, USA) or mouse-anti galactosidase (Sigma-Aldrich) depending of the tracers used and then with a secondary antibody for 60 min for analysis under a confocal laser-scanning microscope (see above).

RESULTS

Adipose tissue feeds back to nociception-related central structures

2μl of 2% CTB solution was either injected into (n=5) or as control applied onto retroperitoneal fat in rats (n=5). The absence of CTB-label in the central nervous system of rats that received an injection into a completely denervated fat pad or a topical application of CTB onto fat tissue served as a control and excluded false positive results due to leakage. In six animals neurons in the dorsal motor nucleus of the vagus (DMV) and nerve endings in the gracile nucleus were positive, whereas nine did not show any central CTB (figure 3.1). The gracile nucleus is known as a part of sensory systems, reporting nociceptive stimuli from the whole body$^{17-21}$.

Liver, pancreas and intra-abdominal fat share one set of vagal motor neurons, intra-abdominal or subcutaneous fat do not

2μl of 1% CTB-488 (fluor-conjugate), CTB-555 or CTB-647 was injected alternately into liver and pancreas (n=8). In the control group, rats received CTB-fluor-conjugate into the vagal denervated liver or pancreas (n=6). Since the fluorescence signal in the CNS was absent in the control groups, false positive results due to leakage could be excluded. In the DMV labeled vagal motor neurons contained both fluorescent labels from liver and pancreas. In a second set of experiments, the liver was sympathetically
CHAPTER 3 ANS CONTROL OF METABOLIC ORGANS

Figure 3.1 Neuronal feedback from fat tissue to the gracile nucleus of the brainstem. After injection of 2μl of 2% Cholera Toxin B into retroperitoneal fat the gracile nucleus of the brainstem is labeled with nerve endings (1a+b). In 1a, retrograde labeling of a vagal motor neuron in the DMV is visible. This result demonstrates that direct sensory fibers run from fat tissue to the brain. See color section.

denervated as described earlier and injected with 5μl of the retrograde tracer PRV and 2μl 2% CTB was injected into retroperitoneal fat (n=12). In the control groups, injection of PRV or CTB into the denervated liver or fat pad or application onto liver or fat pad did not result in labeling of the CNS (n=7). In the intervention group all vagal motor neurons projecting to retroperitoneal fat labeled with CTB contained also PRV from the liver (figure 3.2, five animals with tracing of CTB and PRV in the DMV). Some vagal motor neurons were filled with PRV only, indicating that more neurons might control the liver than retroperitoneal adipose tissue (figure 3.2). Earlier, we reported that subcutaneous and intra-abdominal fat pads are controlled by separate sets of vagal motor neurons.

Distinct sets of hypothalamic and amygdalar neurons project to either intra-abdominal or subcutaneous adipose tissue

5μl of PRV B80 or PRV GFP was injected into intra-abdominal or subcutaneous adi-
Liver, pancreas and intra-abdominal fat share one set of vagal motor neurons, but not intra-abdominal and subcutaneous fat, demonstrated by laser scanning microscopy. 

- **a:** 2μl of 1% CTB-488 (fluor-conjugate), CTB-555 or CTB-647 was injected alternately into liver and pancreas: Colocalization in the DMV demonstrates a shared autonomic control.

- **b:** The liver was sympathetically denervated and injected with 2μl of the retrograde tracer PRV and 2μl 2% CTB was injected into retroperitoneal fat: As in 2a, Colocalization in the DMV demonstrates a shared autonomic control of liver and intra-abdominal fat.

- **c:** We reported earlier that subcutaneous and intra-abdominal fat do not share their neuronal control. These experiments show that the brain controls the intra-abdominal compartment with the same autonomic neurons, in contrast to a different set of neurons to control the subcutaneous compartment. See color section.

Figure 3.2 Liver, pancreas and intra-abdominal fat share one set of vagal motor neurons, but not intra-abdominal and subcutaneous fat, demonstrated by laser scanning microscopy. a: 2μl of 1% CTB-488 (fluor-conjugate), CTB-555 or CTB-647 was injected alternately into liver and pancreas: Colocalization in the DMV demonstrates a shared autonomic control. b: The liver was sympathetically denervated and injected with 2μl of the retrograde tracer PRV and 2μl 2% CTB was injected into retroperitoneal fat: As in 2a, Colocalization in the DMV demonstrates a shared autonomic control of liver and intra-abdominal fat. c: We reported earlier that subcutaneous and intra-abdominal fat do not share their neuronal control. These experiments show that the brain controls the intra-abdominal compartment with the same autonomic neurons, in contrast to a different set of neurons to control the subcutaneous compartment. See color section.

Injecting PRV into completely denervated intra-abdominal fat or applying the tracer on top of intra-abdominal or subcutaneous adipose tissue did not result in labeling of the CNS (n=6). After parasym pathetic denervation of both the right and left retroperitoneal fat pad, PRV B80 and PRV GFP were injected alternately (n=32). In animals with comparable infection rate of both tracers (n=9), neuronal colocalization of both tracers exceeded 95%, both in second order infection of PRV in PVN and MPO (n=5) as well as in third order infection in SCN and amygdala (n=4). This demonstrates that the used viruses have the capacity to infect simultaneously or shortly
CHAPTER 3  ANS CONTROL OF METABOLIC ORGANS

Figure 3.3  CNS specialization in body compartments from spinal cord up to the biological clock of the hypothalamus and the amygdala. a: 5μl of PRV B80 and PRV GFP was injected into parasympathetically denervated intra-abdominal adipose tissue and in subcutaneous adipose tissue. As we reported earlier, the IML of the spinal cord shows a separate control of the compartments, therefore the survival time of the animals was chosen such that second or third order neurons were labeled. b: In an upstream direction, the PVN of the hypothalamus shows specialized sets of neurons per compartment. c: MPO. d: Central biological clock of the hypothalamus (SCN). e: Amygdala. See color section.

after each other the same neuron; in addition it indicates a shared control of both intra-abdominal fat pads. Parasympathetic (n=37) denervation of the left retroperitoneal fat pad and alternately injection of PRVB80 or GFP in this fat pad and subcutaneous fat, forced the virus to infect the brain via the sympathetic motor neurons only and allowed us to investigate whether these different sympathetic neurons receive input from different or the same preautonomic neurons. Now instead of major overlap as found with infection via functionally the same fat pads, none or only sparse overlap of both tracers (maximal one neuron per section) could be observed in animals with comparable infection (figure 3.3) (PVN/MPO: n=6, SCN/amygdala n=5). Earlier we showed that sympathetic motor neurons are specialized in intra-abdominal or subcutaneous fat pads13. Thus, the projections of the PVN, MPO, SCN and amygdala are specialized by body region.
DISCUSSION

Neuronal feedback from adipose tissue
Several fat-derived humoral factors have been demonstrated to affect the brain. For instance, the hormone leptin acts on the hypothalamus and other brain regions and inhibits food intake and stimulates sympathetic nerve activity. Other studies have shown that FFAs inhibit endogenous glucose production by the liver via the hypothalamus. The presence of primary afferent projections from adipose tissue to the gracile nucleus of the brainstem not only presents evidence for neuronal feedback of fat tissue but also opens the question of the functional role of this feedback. The gracile nucleus receives afferent signals from the whole body and has a role in nociception. In view of this pain-related feedback, the anatomical position of adipose tissue within the body suggests a function in monitoring the metabolism of skin and visceral organs. A role of subcutaneous fat tissue in the perception of pain has been suggested earlier by dermatologists.

The afferents could sense mechanical, temperature or hormonal stimuli such as cytokines not only under the skin, but also from the viscera. Few studies addressed nociception in brown and white fat tissue. It has been shown that capsaicin sensitive fibers are present in brown adipose tissue. The nociceptive function of the afferents is supported by experiments where capsaicin was injected into white subcutaneous fat tissue on the back. As a consequence skin lesions appeared 10 days later on the back but also in the neck, suggesting a reaction mediated by the ANS. Fat pads in the knee joint and around spine ligaments contains nociceptive substance P fibers. Recently, a study demonstrated the induction of local and referred pain by injection of saline into the infrapatellar knee fat pad.

Thus, nociceptive fibers from adipose tissue to the gracile nucleus might sense mechanical stress or paracrine factors. Consequently the present study shows that adipose tissue has equal hormonal and neuronal access to the brain as any other organ.

Shared (pre-) autonomic output links intra-abdominal obesity to diabetes
Recently, several studies reported early dysfunction of the ANS in the development of type 2 diabetes. Other publications demonstrate a link between cardiovascular disease or insulin resistance in muscle and a sympathetic overweight, contrariwise hyperinsulinemia, obesity and fatty liver are connected to parasympathetic overweight. Consequently, when the status of the ANS in a certain area of the body is understood as an indicator of autonomic balance of the whole body, the picture becomes confusing: different authors suggest a high sympathetic or high parasympathetic or low sympathetic and parasympathetic tone on whole body level as a cause for T2 diabetes.

Our experiments show that the ANS controls the body by compartment. These observations make it clear that the central nervous system may affect different organs in
a different manner, while at the other hand different organs may also share the same neurons that control them. We reveal a shared parasympathetic control of the abdomen, that connects single neuronal stimulation to visceral fat growth, hyperinsulinemia and a fatty liver by a parasympathetic overweight\textsuperscript{46}. Using the first order tracer CTB, we demonstrate that liver, pancreas and intra-abdominal fat indeed share the same vagal motor neurons. In contrast, distinct sets of vagal motor neurons project to intra-abdominal and subcutaneous fat \textsuperscript{15}.

Moreover, we describe the output of hypothalamus and limbic system to the intra-abdominal and subcutaneous compartment using two different labels of the transneuronal retrograde tracer PRV. Tracing from subcutaneous fat tissue results in a strong (pre-) sympathetic picture, with much slower development of parasympathetic labeling, and in the controls with PRV tracers injected into subcutaneous and sympathetically denervated retroperitoneal fat no colocalization was found\textsuperscript{51}. In consecutive groups with increasing survival times, we analyzed sequentially first order neurons in the sympathetic motor nuclei, then upstream second order neurons in the hypothalamus and third order neurons in the hypothalamus and amygdala, and found them separated on all levels.

These findings are in agreement with earlier studies that suggest that higher brain regions such as the hypothalamus affect body fat distribution. Lesions rostral from the autonomic motor neurons in the midbrain and LH lead to a different body fat distribution than lesions in the VMH\textsuperscript{47}. As to the function of such differentiation, it has been proposed that the hypothalamic temperature center, the MPO, might selectively activate the projections to intra-abdominal compartment to mobilize energy in times of low food and low temperature by burning specifically visceral fat. Then the isolation layer of the body, the subcutaneous fat, can be spared\textsuperscript{48,52}.

In the amygdala, we found separated groups of neurons projecting either to the intra-abdominal or to the subcutaneous body compartment. Amygdala lesions lead to a change in body composition in favor of fat, hyperinsulinemia and impaired skin conduction\textsuperscript{53,54}. The direct connections to brain regions that process smell and taste suggest that the amygdala prepare the body for upcoming food. After detection of food by the nose, a specific parasympathetic activation of the intra-abdominal compartment by the amygdala might induce secretion of insulin and enhance the uptake of substrate in fat tissue and liver.

Earlier we proposed a role for the biological clock in the metabolic syndrome, where disturbed circadian rhythms play a prominent role, such as hormonal rhythms, a less pulsatile insulin secretion or a reduced dipping of the heart rate at night\textsuperscript{37,48,55-57}. We show that indeed a somatotopic organization exists up to the biological clock (SCN) in the hypothalamus. This neuronal network might coordinate the “Dawn-phenomenon”, where enhanced glucose production by the liver and high insulin levels coincide with
enhanced glucose uptake of the target organs in the beginning of the active phase of the day. Inappropriate timing and protracted activation of the shared vagal input to intra-abdominal compartment (intra-abdominal fat, pancreas and liver) might lead to intra-abdominal obesity, hyperinsulinemia and a fatty liver. At the same time, an enhanced sympathetic activation in the thorax compartment and to the vasculature of the muscles might induce cardiovascular disease and insulin resistance. In the presented model, the cause of the failure of the hypothalamus and ANS might be induced by an endogenous error due to a genetic or developmental defect. However, probably in a majority of obese patients, the main cause of the system failure is a huge "environmental mutation" of our life style. Overeating without compensation by physical activity might induce confusing feedback directly in the brain or via neuronal feedback from e.g. the portal system.

Since energy homeostasis is warranted by countless mechanisms in our body, it is unlikely that a single cause of T2 diabetes will be identified. Unbalanced food intake is not an endocrinological disease that can be cured by replacement therapy of one single hormone. The step from a physiological buildup of energy stores to a metabolic derailment and T2 diabetes might occur at many points of the system. Future experiments that address the physiological relevance of the neuroanatomical network established in the present paper will incorporate the cross talk between blood born factors and neurons in their experimental design.

REFERENCES


CHAPTER 4

Dual sympathetic and parasympathetic hypothalamic output to white adipose tissue

submitted

Felix Kreier, Laura Veder, Andries Kalsbeek, Hans P. Sauerwein, Eric Fliers, Johannes A. Romijn, Thomas C. Mettenleiter, Ruud M. Buijs

The balance between lipogenesis and lipolysis in fat tissue is regulated by blood born factors and by the autonomic nervous system. The sympathetic branch stimulates lipolysis, whereas, the parasympathetic branch, induces lipogenesis. Here, we describe a hypothalamic network behind the two branches of the ANS, by applying two different strains of the retrograde transneuronal tracer Pseudorabies Virus (PRV). After an injection of PRV-B80 into the right, sympathetically denervated, retroperitoneal fat pad and a simultaneous injection of PRV-GFP into the left, parasympathetically denervated, retroperitoneal fat pad we could trace both branches up to the hypothalamus within the same animal. We found that preautonomic neurons in the suprachiasmatic nucleus (SCN), the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus (LH) are specialized to project either to sympathetic or to parasympathetic motor neurons. This dual hypothalamic pathway enables hypothalamic centers such as the biological clock together with temperature and feeding centers to coordinate adipose tissue physiology.

INTRODUCTION

The balance between lipogenesis and lipolysis in fat tissue is regulated by blood born factors and by the autonomic nervous system (ANS). Endocrine factors such as insulin and cortisol promote accumulation of fat, whereas sex hormones and growth hormone exert lipolysis. With respect to the ANS, denervation studies show that the sympathetic nervous system stimulates mobilization of fat tissue. Conversely, the vagus nerve increases uptake of glucose and FFA and reduces the activity of Hormone Sensitive Lipase, in keeping with an anabolic role for parasympathetic input to fat tissue.
PART A FINDINGS

The hypothalamus is the central regulator of energy balance, as shown by many studies involving the application of hormones and substrates, while electrical stimulation or lesion of hypothalamic regions can result in profound changes in energy reserves. Integrating body information received both via the circulation and by neurons, the hypothalamus warrants a secure level of energy to the body.

The present study addresses the way in which the hypothalamic will is being translated to the fat tissue via the ANS. At present, it is unknown how hypothalamic areas such as the Suprachiasmatic Nucleus (SCN) (the biological clock), the Media Preoptic area (MPO) (the temperature regulation region) or feeding centers such as LH, PVN and the limbic system inform the ANS how to change metabolic organ function to fulfill their needs. Specifically, it is not known if the hypothalamic nuclei send a general message to the ANS that is subsequently divided in lower neural centers into separate sympathetic and parasympathetic signals. Alternatively, particular hypothalamic regions may have direct access to each branch by parallel pre-sympathetic and pre-parasympathetic projections to the ANS. These two alternatives of hypothalamic-ANS integration have profound impact on our view of the role of the CNS on metabolic regulation. If the first hypothesis is true, a functional unit should be present that transforms a general message from hypothalamic centers into distinct sympathetic or parasympathetic tones. By contrast, the second hypothesis is true i.e. if parallel pathways exist within the hypothalamus, the hypothalamic-ANS communication should be organized by the same antagonistic principle as the ANS itself. Consequently, hypothalamic regions would have the potential to modulate physiological processes in the body, controlled by sympathetic and/or parasympathetic innervation, by either stimulation or inhibition.

During the last years, Pseudorabies virus (PRV) has become a valuable tool to explore multisynaptic neuronal chains back from a specific organ upstream to higher brain regions. After injected into an organ of interest, PRV invades the synaptic endings of autonomic nerves, is retrogradely transported, gets replicated within the neuronal cell body and is taken up by synapses that are connected to the infected neuron, and so on. Tracing studies with PRV from adipose tissue revealed fibers that run from hypothalamic centers such as the SCN and PVN via the brain stem to the sympathetic motor neurons in the spinal cord and finally to fat tissue. An earlier study with tracing from the adrenal gland (sympathetic labeling) and the sympathetically denervated liver (parasympathetic labeling) with two different strains of PRV demonstrated separate pre-sympathetic or pre-parasympathetic neurons in PVN and SCN and other hypothalamic centers. In order to test the two hypotheses on the interaction between the hypothalamus and both branches of the autonomic nervous system, we used bilateral retroperitoneal fat pads as a model by sympathetic denervation of one fat pad and parasympathetic denervation of the contralateral fat pad. This allowed us to follow
the tracer from both retroperitoneal fat pads separately via the two different branches of the ANS. As a control, we denervated both fat pads parasympathetically, leading to double labeling of hypothalamic neurons, on both sides due to crossover. We found separate parallel pre-sympathetic or pre-parasympathetic neurons in SCN, PVN and LH, giving these centers direct access to stimulate or inhibit fat tissue metabolism.

**METHODS**

All experiments were performed in adult male Wistar rats (250–350 g Harlan, Zeist, The Netherlands) according to the NIH guidelines for animal experiments and with approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

**Sympathetic fat denervation**

The techniques for denervation are described extensively in [1]. The autonomic supply of the retroperitoneal fat pad in male wistar rats is characterized by a) diffuse sympathetic nerve fibers, from lateral and dorsal directions, and b) one single vagal nerve branch traveling along blood vessels from the diaphragm to the superior tip of the fat pad.

The abdominal cavity is opened such that retroperitoneal fat pad, kidney and part of the diaphragm are visible. The vagal fibers run along an artery and vein from the diaphragm to the tip of the fat pad. Multiple small sympathetic fibers arise from medial and dorsal directions, three large sympathetic fibers enter the fat pad medially [4]. The retroperitoneal fat pad is cleaved at the level of the kidney and carefully removed from the kidney, dorsal muscles, abdominal wall and connective tissues, working from caudal to rostral. For this step, microsurgery is obligatory to warrant the integrity of the sectioned fat pad, which is moved laterally to expose the vagal and superior sympathetic fibers. Finally, the superior sympathetic fibers are cut such that only a bundle of vagal fibers and adjacent blood vessels remain as a connection between the fat pad and the body of the animal. The fat pad is lifted up and inspected for residual nerve bundles [7]. After the denervation the tracers is injected into the fat pad. This procedure demands an intact, undamaged fat pad, since placement of one bolus of tracer in the superior part of the fat pad without leakage is necessary to achieve successful tracing.

**Parasympathetic fat denervation** [21]

The vagal denervation procedure is the opposite of the sympathetic denervation: in this procedure all adjacent fibers of the blood vessels entering the superior tip of the retroperitoneal fat pad are removed.

For a detailed description, including preoperative photos, please refer to the supplemental information online.
PART A FINDINGS

PRV tracing
5µl PRV B80 (5x10^7 plaque-forming units PRV β-galactosidase B80, Institute for Molecular Biology, Insel Riems, Germany), or PRV GFP (5x10^7 plaque-forming units PRV green fluorescent protein, Institute for Molecular Biology, Insel Riems, Germany) were injected into the right or left retroperitoneal fat using a 30-gauge needle connected to a Hamilton syringe at a single spot. In a control group, we denervated both retroperitoneal fat pads sympathetically. As tracer controls, PRV was applied on top of the intact or the totally denervated organ. 3, 4 or 5 days after tracer injection, the animals were perfused with saline and then a solution of 4% paraformaldehyde and 0.15% glutaraldehyde in PBS (pH 7.4). They were postfixed overnight and cryoprotected by immersion with 30% sucrose in 0.2 M PBS (pH 7.4) for a further 24 hours. Brains were frozen and coronal sections (40 µm) were cut. PRV B80/GFP: Sections were incubated overnight at 4°C with a polyclonal mouse anti-PRV Bartha (a generous donation of C.E. Jacobs, Institute for Animal Science and Health), rabbit-anti GFP (Molecular Probes Inc., Eugene, Oregon, USA), rabbit-anti GFP (Molecular Probes Inc., Eugene, Oregon, USA) or mouse-anti galactosidase (Sigma-Aldrich) depending of the tracers used and then with a secondary antibody for 60 min and coverslipped in 50% PBS glycerol for examination under a Philips (Eindhoven, The Netherlands) confocal laser-scanning microscope (LSM410/510). Digital images of the fluorescent sections were obtained using filters that prevented cross-talk of the fluorophores. Figures were contrast-enhanced but not otherwise manipulated in Adobe Photoshop.

RESULTS

Hypothalamic neurons are either pre-sympathetic or pre-parasympathetic
Injection of PRV into completely denervated retroperitoneal fat or applying the tracer on top of intra-abdominal or subcutaneous adipose tissue did not result in any labeling of the CNS (n=6).

Parasympathetic denervation of both fat pads
After parasympathetic denervation of both the right and left retroperitoneal fat pads, PRV B80 and PRV GFP were injected in the left and right side (n=32). In animals with comparable infection rate of both tracers that did not exceed third order (n=9), neuronal colocalization of both tracers exceeded 95%, both in second order infection of PRV in PVN, MPO and LH (n=5) as well as in third order infection in SCN and amygdala (n=4) (figure 4.1). The fact that e.g. in SCN and amygdala just a few cells are visible and yet show colocalization show that indeed these neurons share output to both fat tissues. Furthermore this demonstrates that the applied viruses have the capacity to infect simultaneously, or shortly after each other, the same neurons. In addition, this observation indicates a shared sympathetic control of both intra-abdominal fat pads.
Parasympathetic denervation of one fat pad and sympathetic denervation of the contralateral fat pad

The parasympathetic denervation of the right retroperitoneal fat pad in combination with a sympathetic denervation of the left fat pad ($n=32$) in the same animal with alternate injection of PRV B8o or GFP forced the two different virus tracers to infect the brain either via the sympathetic or the parasympathetic motor neurons only. Instead of major overlap as found with infection via fat pads with the same autonomic innervation, none, or only sparse, overlap of both tracers (maximal one neuron per section) could be observed in animals with comparable infection (PVN: $n=7$, SCN: $n=5$)(figure 4.2).
**DISCUSSION**

The hypothalamus has direct parallel access to both branches of the ANS. In the present study, we show that the biological clock, the PVN and the lateral hypothalamus (LH) contain pre-autonomic neurons that are specialized to project either to the sympathetic or the parasympathetic branch innervating fat tissue. Interestingly, the neurons projecting to these separate autonomic systems share the same brain nuclei in spite of opposite functionality and the different location of sympathetic and parasympathetic motor neurons\(^{22-23}\). Thus, the antagonistic principle of the ANS extends into higher brain regions like the hypothalamus and is even present in neighboring neurons within the same nucleus. Our results are in agreement with earlier data obtained in the adrenal gland and in the liver, where also specialized pre-autonomic neurons appeared to be present SCN, PVN and LH\(^\text{20}\).
The PVN contains a whole range of different neuroendocrine and pre-autonomic neurons. Analogous to the neuroendocrine outflow from the PVN to the median eminence and posterior pituitary, the present results demonstrate that the autonomic parasympathetic and sympathetic output of the PVN has a specialization in different neurons. Our findings support the notion of the PVN as a central output center of the hypothalamus, coordinating hormonal and autonomic body controls. Similarly, the LH utilizes its connections to the cerebral cortex, periaqueductal gray, and autonomic centers to play an integrated role in metabolism and associated behavior. This neuronal network enables the PVN and the LH to fulfill their roles as modulators of fat tissue metabolism.

The biological clock possesses neurons to modulate fat tissue metabolism in two directions by a lipolytic (sympathetic) and a lipogenic (parasympathetic) branch. Interestingly, lipoprotein lipase (LPL), a key enzyme in lipogenesis and hormone sensitive lipase (HSL), a key enzyme in lipolysis, have been shown to follow a circadian rhythm, which would support such a bimodal action of the SCN. After lesioning of the SCN, the circadian rhythm in leptin production was abolished. Moreover, during hibernation, fat tissue metabolism changes profoundly, which cannot be completely understood by the action of hormones, such as melatonin only. Our finding of selective neurons within the biological clock offers a new perspective on the way the SCN imposes its rhythms on fat tissue, i.e., in addition to hormones, the ANS might be employed in the generation of diurnal variations in fat tissue function.

Previously, we proposed a role for the biological clock in the development of the metabolic syndrome or type 2 diabetes. We suggest that the flattening of natural circadian rhythms by a westernized life style, mainly by irregular abundant eating in combination with reduced need for physical examination lead to autonomic failure. In this context, a high parasympathetic tone might induce hyperinsulinemia, visceral obesity and a fatty liver. In contrast, the neuroanatomically separated thoracic and subcutaneous compartments exhibit high sympathetic tone with cardiovascular disease as a consequence.

In summary, we describe an antagonistic organization of the pre-autonomic neurons in the hypothalamus, empowering the biological clock to modulate fat tissue metabolism according to needs as calculated by the hypothalamus.

REFERENCES
22. Sawchenko, P. E. & Swanson, L. W. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. J Comp Neurol 205, 260-72 (1982).
Estrogen and glucocorticoids have strong effects on fat tissue metabolism and body fat distribution. So far, the differential effects of these hormones on the abdominal versus subcutaneous fat compartment have not been explained satisfactorily. Recently, the autonomic innervation of fat tissue has been described, with separate sets of neurons innervating the abdominal and subcutaneous fat compartments. The aim of the present study was to investigate glucocorticoid and estrogen alpha receptor (GR and ER alpha) expression by the (pre)parasympathetic neurons projecting to intra-abdominal fat tissue by combined Pseudorabies virus (PRV) tracing and ER alpha and GR immunocytochemistry. A large proportion of neurons in the brainstem (DMV) and hypothalamus (PVN, LH and amygdala) projecting to abdominal fat expressed GR. In contrast, less neurons were double stained for PRV and ER alpha. These findings show for the first time that glucocorticoids and estrogens might affect adipose tissue metabolism and distribution indirectly via its autonomic innervation.

INTRODUCTION

Estrogen and cortisol have profound local effects on fat tissue metabolism. Cortisol is lipogenic, while estrogen stimulates lipolysis. Interestingly, both cortisol and estrogen also change body fat distribution: cortisol promotes central obesity and peripheral lipoatrophy (like in Cushing syndrome), in contrast to estrogen, that causes subcutaneous fat accumulation and reduces visceral fat. The heterogenic distribution of estrogen receptor alpha (ER alpha) and glucocorticoids receptor (GR) throughout central and peripheral fat depots has been proposed to play a role in these observations. However, local application of stress- and sex hormones on fat tissue does not completely explain their effect of fat tissue in vivo.
PART A FINDINGS

The balance of lipogenesis and lipolysis in fat tissue is regulated by humoral factors and by the autonomic nervous system. Denervation studies show that the sympathetic nervous system stimulates mobilization of fat tissue\textsuperscript{6,8}. Conversely, the vagus nerve induces increased uptake of glucose and FA and a reduction of hormone sensitive lipase activity\textsuperscript{9}. Both ER alpha and GR are present in the same brain regions as (pre-)autonomic neurons that project to fat tissue, but a direct connection between ER alpha and GR and these neurons has never been studied. We hypothesized that estrogen alpha and glucocorticoids might modulate fat tissue metabolism via differential ER and GR expression in (pre)autonomic neurons innervating adipose tissue\textsuperscript{10,11}.

During the last years, the retrograde transneuronal tracer PRV has become a valuable tool to explore multisynaptic neuronal chains from a specific organ upstream to higher brain regions. Injected into an organ, PRV invades the synaptic endings of autonomic nerves, is retrogradely transported, gets replicated in the cell body and is taken up by synapses that are connected to the infected neuron, followed by another cycle\textsuperscript{12,14}.

By a combination of retrograde neuronal PRV tracing and ER alpha and GR staining, we could test the hypothesis that (pre)autonomic neurons projecting to adipose tissue express ER alpha and GR, thereby offering a neuroanatomical substrate for central effects of estrogen and glucocorticoids on adipose tissue metabolism and distribution. In a first step, we investigated the first and second order (pre)parasympathetic neurons in DMV, PVN, LH and amygdala, since they have a short connection to fat tissue and can influence lipogenesis by the vagus nerve\textsuperscript{9}(chapter 2, 3 and 4).

METHODS

All experiments were performed in 29 adult male Wistar rats (250–350 g Harlan, Zeist, The Netherlands) according to the Netherlands Institute for Brain Research guidelines for animal experiments and with approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

PRV tracing

5\textmu l PRV (a generous donation of C.E. Jacobs, Institute for Animal Science and Health) were injected into the sympathectomized retroperitoneal fat using a 30-gauge needle connected to a Hamilton syringe at a single spot. For a detailed description of the denervation technique, see chapter 3. Three (n=8), four (n=11) or five (n=11) days after tracer injection, the animals were perfused with saline and then a solution of 4\% paraformaldehyde and 0.15\% glutaraldehyde in PBS (pH 7.4). They were postfixied overnight and cryoprotected by immersion with 30\% sucrose in 0.2 M PBS (pH 7.4) for a further 24 hours.
CHAPTER 5 GR & ER AND FAT PROJECTING NEURONS

Histology
Brains were frozen and coronal sections (40 μm) were cut. The brainstem sections were incubated overnight at 4°C in the presence of polyclonal rabbit anti-ERa 1:2000 (Santa-Cruz Biotechnology Inc., Santa Cruz, CA) or polyclonal rabbit anti-GR 1:100,000 (a generous gift from Dr. Kawata (Kyoto Prefectural University of Medicine, Japan)). The next day the sections were incubated 60 min. in presence of the second antibody, biotinylated goat anti-rabbit (1:400), followed by a 60 min incubation in presence of mixture of Vecta stain ABC kit reagents A and B (1:800) (Vector, Burlingame, CA, USA). After each incubation the sections were rinsed with TBS for 30 minutes. The peroxidase antibody complex was visualized by incubating the sections in substrate 0.025% 3,3’-diaminobenzidine tetrahydrochloride (DAB) in TBS with 0.5% ammonium nickel sulphate supplemented with 0.05% H2O2. This precipitate covers the underlying antibody, making it inaccessible for the following antibodies. The sections were again incubated overnight at 4°C with polyclonal rabbit anti-PRV (anti alpha-Aujerszky) antibody 1:10,000, rabbit-anti GFP (Molecular Probes Inc., Eugene, Oregon, USA) antibody 1:2000 or mouse-anti galactosidase (Sigma-Aldrich) antibody 1:2000, followed by the same procedure, except no ammonium nickel sulphate was added to the DAB-solution. The sections were mounted on gelatin-coated slides, dehydrated in serial alcohol solutions and coverslipped using Entallan.

The light microscopy color figures were imported using a Zeiss axioplan 2 microscope (Zeiss, Jena, Germany) fitted with a Progress Camera 3012 (Jenoptik, Jena, Germany). The figures were of 1,488 x 1,120 pixel size in RGB 24-bit true color. Contrast and color were adapted using Adobe Photoshop (Adobe Systems Inc., Mountain View, California, USA) without any other image manipulation.

RESULTS
(Pre-)parasympathetic neurons contain ER alpha and GR
Three types of staining were visible in brainstem and hypothalamus: neurons labeled with PRV alone, neurons labeled with ER alpha or GR receptor and neurons labeled with both PRV and ER alpha or GR receptor.

Glucocorticoid receptor
GR was present in a substantial number (approximately 25-50%) of the pre-parasympathetic fat projecting neurons in brainstem (DMV), hypothalamus (PVN and LH) and amygdala (figures 5.1-5.4).

Estrogen receptor alpha
In contrast to the expression of GR, we detected less neurons expressing both ER alpha and PRV in brainstem and hypothalamus, since most neurons were labeled exclusively either with PRV or ER alpha (figures 5.5-5.8).
Figure 5.1 GR expression on parasympathetic motor neurons in the DMV projecting to retroperitoneal fat. a. overview, b. detail: brown: PRV from sympathectomized RP fat, black: GR. Arrows indicate examples of colocalization. Figure 5.2: GR on pre-parasympathetic PVN neurons. a. overview, b. detail. Figure 5.3: GR on pre-sympathetic LH neurons. Figure 5.4: GR on pre-parasympathetic neurons in the central amygdala. a. overview, b. detail. See color section.
Figure 5.5 ER alpha expression on parasympathetic motor neurons in the DMV projecting to retroperitoneal fat. a. overview, b. detail: brown: PRV from sympathectomized RP fat, black: ER alpha. Arrows indicate examples of colocalization. Figure 5.6: ER alpha on pre-parasympathetic PVN neurons. a. overview, b. detail. Figure 5.7: ER alpha on pre-sympathetic LH neurons Figure 5.8: ER alpha on pre-parasympathetic neurons in the central amygdala. a. overview, b. detail.
DISCUSSION

In this study, we found a substantial expression of GR in fat projecting neurons in brainstem and hypothalamus. In contrast, ER alpha was present on a smaller number of fat projecting neurons. The colocalization of PRV, GR and ER alpha indicates that glucocorticoids and estrogen could influence metabolic effects via central mechanisms. Glucocorticoids have been reported to exert both anabolic and catabolic effects if administered into the CNS. Here, we labeled neurons that project to intra-abdominal fat tissue via the parasympathetic branch with a lipogenic effect. Consequently, glucocorticoids on these neurons potentially can influence intra-abdominal fat accumulation, which would be in agreement with the notion of visceral obesity in Cushing disease due to hypercortisolism. The expression of GR on (pre)sympathetic neurons projecting to intra-abdominal fat tissue and on pre-autonomic neurons projecting to subcutaneous compartments should be studied to get deeper insight on the central effect of glucocorticoids in body fat distribution.

Estrogens are well-known for their effect on body shape. Transsexuals that receive estradiol during gender transition experience a change in body shape towards the gynoid (peer) form. In ER alpha knock out mice, the brain has a reduced sensitivity for leptin and fat mass is increased, indicating that estrogen has a central effect on energy homeostasis. Moreover, sympathectomized fat pads are relatively insensitive (23%) to the lipolytic effects of systemic estradiol compared to intact pads. We suggest that ER alpha and beta colocalization studies in female rats and androgen receptor staining in male rats should be performed to answer the question if ER alpha inhibits visceral (pre)parasympathetic neurons or whether ER alpha is more expressed on neurons that are connected with subcutaneous fat. In female rats, ER expression varies through the estrous cycle.

In conclusion, we show for the first time that estrogen and glucocorticoid receptors are present on fat projecting neurons indicating that the effect of sex and stress hormone on fat tissue is (partly) mediated by the brain.

ACKNOWLEDGEMENT

We thank Mitsuhiro Kawata from the Kyoto Prefectural University of Medicine (Japan) for the generous gift of glucocorticoid antibodies.

REFERENCES


Figure 2.1 Transverse section of the spinal cord (at Th7) and the rat brain stem at the level of the obex shows spinal cord and brain stem neurons projecting into adipose tissue. Transneuronal retrograde tracing by PRV injection into retroperitoneal fat in rats before (a and b) and after (c and d) sympathetic denervation of adipose tissue. In a and b (PRV tracing from adipose tissue before denervation), since both sympathetic and parasympathetic fibers are intact, PRV is seen to spread via the vagus and the sympathetic nerves. Interestingly, the route via the IML is favored in intact animals such that second-order neurons in the brain stem are already evident when the first-order parasympathetic motor neurons appear in the DMV (arrow). In b, the A1 region, the raphe nucleus (R), and the nucleus of the solitary tract (NTS) project into the sympathetic motor neurons. In c (with d, showing PRV tracing after sympathetic denervation of the left retroperitoneal fat pad), there is no labeling of PRV in the IML. In the brain stem shown in d, neurons are clearly visible in the parasympathetic motor nuclei: DMV and caudal part of the AMB. CC, central canal. Bar in a and c = 0.5 mm. Bar in b and d = 0.4 mm.
Figure 2.4  Somatotopic organization of the parasympathetic nervous system. Laser scanning photomicrograph of transverse sections of the brain stem. The central canal is on the right side. Vagal motor neurons project into one fat compartment only (subcutaneous or intraabdominal). PRV (stained green) was injected into the intraabdominal fat compartment after sympathetic denervation. At the same time, FluoroGold (stained red) was injected into the subcutaneous fat compartment. Both tracers were transported back to the dorsal motor nucleus DMV and AMB in different neuron populations. Somatotopic segregation can be observed within the DMV. Bar = 50 μm.
Figure 2.5  Somatotopic organization of the sympathetic nervous system. Sympathetic motor neurons project into one fat compartment only (subcutaneous or intra-abdominal). Two strains of PRV were injected simultaneously into the intra-abdominal fat compartment (mesenterial fat) and the subcutaneous fat compartment (subcutaneous inguinal fat). Confocal laser scanning photomicrograph of transverse thoracic spinal cord sections (Th5–Th10). Both tracers were transported back to the IML and show clear separation of the different tracers (red/green). Insert (control): Injection of both tracers into the same mesenterial fat pad resulted in colocalization of the two tracers (yellow). Specific laser analysis of the indicated neuron (arrow) also showed colocalization of the tracers with a strong signal of FITC (green) and a much weaker signal of CY3 (red). Bar = 50 μm.
Figure 3.1 Neuronal feedback from fat tissue to the gracile nucleus of the brainstem. After injection of 2μl of 2% Cholera Toxin B into retroperitoneal fat the gracile nucleus of the brainstem is labeled with nerve endings (1a+b). In 1a, retrograde labeling of a vagal motor neuron in the DMV is visible. This result demonstrates that direct sensory fibers run from fat tissue to the brain.
Liver, pancreas and intra-abdominal fat share one set of vagal motor neurons, but not intra-abdominal and subcutaneous fat, demonstrated by laser scanning microscopy. 

**a:** 2μl of 1% CTB-488 (fluor-conjugate), CTB-555 or CTB-647 was injected alternately into liver and pancreas: Colocalization in the DMV demonstrates a shared autonomic control.

**b:** The liver was sympathetically denervated and injected with 2μl of the retrograde tracer PRV and 2μl 2% CTB was injected into retroperitoneal fat: As in 2a, Colocalization in the DMV demonstrates a shared autonomic control of liver and intra-abdominal fat.

**c:** We reported earlier that subcutaneous and intra-abdominal fat do not share their neuronal control. These experiments show that the brain controls the intra-abdominal compartment with the same autonomic neurons, in contrast to a different set of neurons to control the subcutaneous compartment.
Figure 3.3  CNS specialization in body compartments from spinal cord up to the biological clock of the hypothalamus and the amygdala. a: 5μl of PRV B80 and PRV GFP was injected into parasympathetically denervated intra-abdominal adipose tissue and in subcutaneous adipose tissue. As we reported earlier, the IML of the spinal cord shows a separate control of the compartments, therefore the survival time of the animals was chosen such that second or third order neurons were labeled. b: In an upstream direction, the PVN of the hypothalamus shows specialized sets of neurons per compartment. c: MPO. d: Central biological clock of the hypothalamus (SCN). e: Amygdala.
Figure 4.1 *Intra-abdominal fat pads share pre-autonomic control:* Injection of PRV GFP and PRV B80 into vagotomized right and left retroperitoneal fat pad. In PVN, LH and SCN, both tracers label the same neurons indicating the same neurons project to both fat pads.
Figure 4.2 Subcutaneous and intra-abdominal do not share pre-autonomic control. Injection of PRV GFP / PRV B8o into the right vagotomized and the left sympathectomized retroperitoneal fat pad. In PVN, LH and SCN, tracers label separate neurons indicating their specialization as pre-sympathetic or pre-parasympathetic.
Unbalanced autonomic nervous system

Metabolic syndrome

Figure 6.1 Model of the metabolic syndrome caused by a central nervous deregulation. The disturbed output of the biological clock affects the selective balance of the ANS in different parts of the body. In the intra-abdominal compartment, the ANS is shifted in favor of the parasympathetic branch (blue), resulting in increased insulin secretion and growth of intra-abdominal fat tissue compared with normal values (grey). Contrarily, in the thorax and movement compartment, the sympathetic branch (red) prevails, leading to high blood pressure and impaired glucose uptake by the muscle compared with normal values. In this model, the symptoms of the metabolic syndrome are the result and not the cause of the disease.
Figure 5.1  GR expression on parasympathetic motor neurons in the DMV projecting to retroperitoneal fat. a. overview, b. detail: brown: PRV from sympathectomized RP fat, black: GR. Arrows indicate examples of colocalization. Figure 5.2: GR on pre-parasympathetic PVN neurons. a. overview, b. detail. Figure 5.3: GR on pre-sympathetic LH neurons. Figure 5.4: GR on pre-parasympathetic neurons in the central amygdala. a. overview, b. detail.
Figure 5.5 ER alpha expression on parasympathetic motor neurons in the DMV projecting to retroperitoneal fat. a. overview, b. detail: brown: PRV from sympathectomized RP fat, black: ER alpha. Arrows indicate examples of colocalization.

Figure 5.6: ER alpha on pre-parasympathetic PVN neurons. a. overview, b. detail.

Figure 5.7: ER alpha on pre-sympathetic LH neurons.

Figure 5.8: ER alpha on pre-parasympathetic neurons in the central amygdala. a. overview, b. detail.
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.
Part B

Discussion and perspectives
CHAPTER 6

Hypothesis: shifting the equilibrium from activity to food leads to autonomic unbalance and the metabolic syndrome

Perspectives in Diabetes

Diabetes 52, 2652-6 (2003)

Felix Kreier, Ajda Yilmaz, Andries Kalsbeek, Johannes A. Romijn, Hans P. Sauerwein, Eric Fliers, and Ruud M. Buijs

'The stability of the internal environment is the condition that life should be free and independent ... So, far from the higher animal being indifferent to the external world, it is on the contrary in a precise and informed relation with it, in such a way that its equilibrium results from a continuous and delicate compensation, established as by the most sensitive of balances.'

Claude Bernard (1865)

The incidence of the metabolic syndrome, the most threatening epidemic in industrialized countries, is rapidly rising. Nonetheless, the mechanisms causing visceral obesity and its associated comorbidity of type 2 diabetes, cardiovascular disease, and dyslipidemia are incompletely understood. Extensive endocrine research has identified important players in the metabolic syndrome but has failed to present a unifying hypothesis regarding its pathogenesis. Evolution created powerful tools to keep our internal environment stable, mainly by forecasting the conditions of the external environment by synchronizing activity and rest to the day/night cycle by means of biological clock mechanisms. During the last century, life has changed dramatically in industrialized countries. Food has become abundant, snacking frequency increased and shifted toward the end of the day, and simultaneously, the necessity for physical effort became considerably reduced (1-6). Moreover, physical activity does not need to coincide with the light period anymore. As a result, the environment sensed by the brain has become metabolically flattened and arrhythmic. From the perspective of a longstanding evolutionary development, this has been an abrupt "environmental mutation." We hypothesize that in such conditions the susceptible brain loses its feeling for internal and external rhythm. Since the brain uses the autonomic nervous system to implement the internal rhythmicity, we propose an unbalanced and arrhythmic autonomic nervous system as a major cause of the metabolic syndrome.
The autonomic nervous system and neuroendocrine circuits maintain harmony between internal and external environment

To maintain homeostasis, the brain has two avenues of communication: hormones and neurons. Hormones present themselves broadly throughout the body and obtain their specificity by acting on their receptors expressed in specific tissues, whereas neurons deliver their message to a precisely targeted tissue in the body.

This communication network coordinates the transition of the body from the inactive to the active period and vice versa. For instance, in the preparation for an upcoming active period, cortisol and glucose blood levels rise just before awakening, known as the "dawn phenomenon." The autonomic nervous system (ANS) coordinates the "dawn phenomenon," by modulating the adrenocorticotrophic hormone sensitivity of the adrenal glands and the glucose output of the liver (7-11). The autonomic nervous system commands the organs through two antagonistic branches: the sympathetic nervous system, predominant in the active period ("fight, fright, and flight"), whereas the parasympathetic nervous system rules the body in the inactive period ("rest and digest"). For instance, in the active period the sympathetic tone to the heart is enhanced, in contrast, in the inactive period the parasympathetic input prevails and heart rate and blood pressure decrease.

Still, the brain needs to translate this general message to different parts of the body in a selective manner. The sympathetic nervous system directs blood to certain parts of the body by selectively constricting blood vessels. With physical activity in the active period, the movement apparatus requires blood, while the digestive apparatus slows down; the opposite holds for the inactive period (12). Thus, blood vessels in these different regions must receive different autonomic signals depending on the time of the day.

A neuroanatomical network for distinct regional control has been described recently. Both branches of the ANS were shown to discriminate between different fat compartments throughout the body. Within the motor nuclei of both the sympathetic and the parasympathetic nervous system the intra-abdominal and the subcutaneous fat compartment is represented by specific neurons. This compartmentalization of autonomic motor neurons provides the neuroanatomical basis for selective changes of the sympato-parasympathetic balance in different compartments of the body (13).

Supported by these anatomical data, we propose that the body can be divided into different functional autonomic compartments and that at least a thoracic and movement compartment and a visceral compartment should exist. In this setting, a balanced and flexible autonomic nervous system can oscillate the activities of the organs within the compartments according to the actual needs of the body.

The brain anticipates the diurnal rhythm of the internal and external environment

The central biological clock in the hypothalamus (suprachiasmatic nucleus [SCN])
uses both this differentiated autonomic network and hormonal signals to generate and organize metabolic rhythms (10,14). The crucial role of the SCN has been demonstrated by lesion studies. Without a functioning SCN, cortisol and glucose do not rise before the beginning of the active period and blood pressure does not dip anymore in the inactive period.

The central clock requires information from the environment to keep running on time. Light information from the eyes reaches directly into the SCN via the retinohypothalamic tract (15). The sensory organs inform the brain about the external environment (16). The state of the internal environment is reported to spinal cord and brain stem through feedback from virtually all organs (16,17). In addition, the brain integrates information about circulating hormone and substrate availability through receptors located in areas where the blood-brain barrier allows this information to be passed to the brain (18-20).

In the active period, the movement compartment uses glucose and free fatty acids. As a reaction, the brain facilitates liberation of energy substrate from storage organs, such as liver and fat tissue. If physical activity is being repeated on a daily basis, the SCN will be programmed to facilitate performance at the entrained time point (10,21,22). In contrast, in the inactive period the brain shifts the body toward an anabolic state of recovery. In summary, information from the internal and external environment sets the central clock to run on time and prepares the body for the upcoming (inner and outer) tasks.

A metabolically shifted environment

We hypothesize that the brain needs repeated metabolic clues from both the external and the internal environment to maintain endogenous physiological rhythms in autonomic output. The western lifestyle is characterized by increased energy intake and decreased energy expenditure; in fact, evolution prepared us to use this anabolic state very efficiently, referred to as the “thrifty genotype” hypothesis (1-6,23,24). The precisely timed seasonal development of obesity in animals might be an expression of the thrifty genotype induced by the biological clock (24). The effects of lifestyle on a population can develop very rapidly, as shown by the increase of the BMI in children within 10 years in Eastern Germany after reunification and westernization (25). Due to our current sedentary lifestyle, the brain no longer senses the urge to oscillate the body between the anabolic and catabolic states. The fact that such information is crucial can be inferred from the effect of absent hepatic feedback to the brain: after hepatic vagotomy, food intake in the inactive phase of rats increases, leading to weight gain, while insulin resistance develops in muscle (26,27).

The central clock might also be affected by the changed environment (24). In humans, the circadian rhythm in insulin secretion and sensitivity is disturbed and flattened in
diabetic patients, and their nondiabetic offspring has reduced diurnal blood pressure variation (28,29). An impaired functioning of the SCN would explain early changes in the metabolic syndrome, such as the absence of a physiological dip in blood pressure at night caused by an impaired circadian rhythm in sympatho-vagal balance (30–35). In hypertensive patients, postmortem neuroanatomical assessment revealed indeed a disturbed SCN structure (36). The impairment of the central clock function in the elderly might relate to the high incidence of the metabolic syndrome in this population (37). Epidemiological analysis of a 6-year survey among 2,000 workers revealed a correlation between irregular eating and snacking habits and insufficient hours of sleep, while smoking or alcohol drinking did not affect sleep (38). Nocturnal eating leads to an abnormal endocrine response (39). Interestingly, other studies showed that overeaters tend to consume a larger part of their daily energy in the evening (40). In OLETF rats (a model of type 2 diabetes and selective visceral obesity), induced by a lack of the cholecystokinin-α receptor-mediated vagal feedback to the brainstem, spontaneous activity, sleep, and temperature rhythms are also disturbed (41). In general, it should be noted that although a certain metabolic value is physiological at one time point, it might be pathophysiological at a different time point of the day.

Hypothesis: the unbalanced autonomic nervous system causes the symptoms of the metabolic syndrome

The metabolic syndrome consists of visceral obesity, hyperglycemia, hyperinsulinemia, dyslipidemia, and cardiovascular diseases. A common pathophysiological denominator underlying these epidemiological correlations has not been identified. However, the autonomic nervous system was shown to play a role in the metabolic syndrome. Recently, a prospective cohort study in 8,000 patients from 1987–1998 revealed a high relative risk to develop type 2 diabetes if autonomic dysfunction is present in healthy subjects independent from other risk factors, such as body weight (42). However, if the status of the autonomic nervous system in a certain compartment is understood as an indicator of autonomic balance of the whole body, the picture becomes confusing. As a result, the finding of reduced plasma catecholamines, increased heart rate, decreased parasympathetic activity measured by R-R interval after α-adrenergic blockade, and increased pupil latency period before but not after muscarinic blockade is summarized as an overall decreased sympathetic and parasympathetic tone in the development of obesity (43). In rat models of hypothalamic obesity, a reduction in sympathetic tonus to the pancreas, white and brown fat tissue was shown to play a role in fat growth (44). Others show evidence for a high parasympathetic activity in obesity, leading to high insulin and fat storage (45,46). Contrarily, an overactive sympathetic nervous system is described in type 2 diabetes, resulting in increased heart rate, vascular resistance, and sodium retention (47). Consequently, we propose that the picture has become confus-
Figure 6.1 Model of the metabolic syndrome caused by a central nervous deregulation. The disturbed output of the biological clock effects the selective balance of the ANS in different parts of the body. In the intra-abdominal compartment, the ANS is shifted in favor of the parasympathetic branch (blue), resulting in increased insulin secretion and growth of intra-abdominal fat tissue compared with normal values (grey). Contrarily, in the thorax and movement compartment the sympathetic branch (red) prevails, leading to high blood pressure and impaired glucose uptake by the muscle compared with normal values. In this model, the symptoms of the metabolic syndrome are the result and not the cause of the disease. See color section.

The metabolic syndrome is associated with enhanced insulin secretion and fat accumulation in the abdomen. Interestingly, parasympathetic input to fat tissue has been shown to enhance insulin sensitivity and fat accumulation (13). Moreover, insulin secretion by the pancreas is parasympathetically driven, and parasympathetic input to the liver has been shown to increase insulin sensitivity and glucose uptake (26,48). Less pulsatile insulin secretion in type 2 diabetic patients indicates a more rigid autonomic tonus to the abdomen and might be used as a marker, since profound defects of pulsatile secretion are already present in glucose-intolerant individuals (49,50).
These data indicate that all organs receiving an enhanced parasympathetic tone are situated in the visceral compartment. Interestingly, in apparent contrast at the same time, in the metabolic syndrome the balance is shifted to a sympathetic overweight to the heart, resulting in increased blood pressure and insulin-resistant muscles (51,52). Thus, organs in the thoracic and movement compartment act metabolically opposite to the visceral compartment (53). While in healthy subjects the autonomic balance of the compartments oscillates, these findings indicate that an unbalanced autonomic output develops in the metabolic syndrome with increased parasympathetic dominance in the visceral compartment and increased sympathetic tone in the thoracic and movement compartment.

Can this vicious cycle be broken?
If our hypothesis is correct, interventions on the level of feedback to the autonomic centers or to the central clock should be beneficial in the metabolic syndrome.

During exercise, energy is consumed, which is sensed by the brain. As a reflex, the autonomic input to the visceral compartment shifts to sympathetic dominance and visceral fat decreases (54–57), and at the same time the sympathetic outflow to the heart and arteries decrease in order to facilitate blood flow to the muscles, resulting in lower blood pressure and an improvement of insulin sensitivity of the muscle (58–60). Consequently, daily exercise and weight loss reestablishes the counteracting metabolic balance between the anabolic and catabolic state such that the autonomic outflow becomes rhythmic again (60,61).

Another possible intervention at the level of the SCN is its reentrainment by melatonin, which is expressed in the pineal gland in a circadian fashion as the signal of the night. Diabetic patients with autonomic disturbances and patients with coronary artery disease have a flattened melatonin rhythm (62,63). Interestingly, melatonin supplementation reentranges the SCN and restores the diurnal variation in blood pressure in hypertensive patients and allows blood pressure to fall at night (64). In rats, administration of melatonin at night induces visceral fat loss and improves the metabolic syndrome (65).

In conclusion, the reversal of the metabolic syndrome by these entrainment procedures of the SCN argues for a possible treatment aimed at restoring a physiological daily rhythm in energy balance.

ACKNOWLEDGMENTS
This project was financially supported by the Dutch Diabetes Foundation (grant 03.11007) and the Dutch Heart Foundation (grant 00B180). We thank Henk Stoffels for the artwork.
REFERENCES

PART B DISCUSSION AND PERSPECTIVES

CHAPTER 6 HYPOTHESIS ON DIABETES TYPE 2


44. Bray GA: Obesity—a state of reduced sympathetic activity and normal or high adrenal activity (the autonomic and adrenal hypothesis revisited). Int J Obes 14 (Suppl. 3):77–91 (see discussion 91–72), 1990


CHAPTER 7

Hypothesis: HIV-associated adipose redistribution syndrome as a selective autonomic neuropathy


Eric Fliers, Hans P. Sauerwein, Johannes A. Romijn, Peter Reiss, Mark van der Valk, Andries Kalsbeek, Felix Kreier, Ruud M. Buijs

Abnormal body-fat distribution in HIV-1-associated adipose redistribution syndrome (HARS) remains unexplained at present. White adipose tissue is controlled by humoral factors and by neural regulation. Sympathetic innervation stimulates lipolysis, whereas parasympathetic innervation has an anabolic influence on white adipose tissue. Results of neuroanatomical studies showed a clear somatotopy with respect to autonomic control of white adipose tissue by both the sympathetic and parasympathetic branch, with separate sets of autonomic neurons innervating either the subcutaneous or the visceral fat compartment. Thus, the CNS is likely to be a key player in regulation of body-fat distribution. We propose that HARS is mediated by effects of antiretroviral treatment on the CNS and could indicate a change in autonomic balance resulting in redistribution of adipose tissue.

Potent combination antiretroviral therapy for HIV-1 infection has resulted in striking reductions in associated morbidity and mortality. However, soon after the widespread introduction of such treatment, a novel lipodystrophy syndrome was reported in HIV-1-infected patients, which was characterised by insulin resistance, hypertriglyceridaemia, and abnormal distribution of adipose tissue. Many studies have since addressed the pathogenesis of this disorder, mainly focusing on pharmacologically induced mitochondrial toxic effects and altered adipocyte differentiation. However, the abnormal distribution of adipose tissue has remained unexplained. Here, we propose that this HIV-1-associated adipose redistribution syndrome (HARS) is mediated via the CNS and represents a selective neuropathy.
Background

Body-fat redistribution in HARS entails disproportional deposition of intra-abdominal adipose tissue and wasting of subcutaneous fat, especially in the extremities, buttocks, and face. Initially, the syndrome was solely linked to use of protease inhibitors, but the present idea is that both protease inhibitors and nucleoside reverse transcriptase inhibitors contribute to the syndrome, although in-vitro evidence is sparse for nucleoside reverse transcriptase inhibitors. HARS was initially reported to arise in more than half of HIV-1-positive patients treated with protease inhibitors with an average delay of 10 months, although a lower prevalence (17% after 18 months) has been reported.

Since visceral accumulation of white adipose tissue with peripheral fat wasting is a prominent feature of Cushing’s syndrome, several studies have looked at the hypothalamus-pituitary-adrenal (HPA) axis in HIV-1-infected patients with antiretroviral-associated lipodystrophy. Yanovski and colleagues investigated this axis in patients with HARS, and reported that they had lower urine excretion of free cortisol and normal glucocorticoid receptor number and affinity compared with controls or patients with Cushing’s syndrome. Therefore, hypercortisolism is unlikely to account for the noted phenotype, confirming initial reports of normal urine cortisol excretion and dexamethasone suppression in HARS. On the other hand, the serum cortisol/dehydroepiandrosterone ratio has been reported to be associated with clinical progression and atherogenic lipid alterations in HARS. However, the relevance of this ratio for fat redistribution is unclear at present. Alteration of tumour necrosis factor alpha homeostasis induced by combination antiretroviral therapy has been proposed as a risk factor for HARS development, but again is unlikely to account for fat redistribution. Alternative pathophysiological mechanisms have been the subject of intense research. Findings of in-vitro studies have shown protease inhibitors to affect fat-cell differentiation, and these observations have been extended to altered adipocyte differentiation markers in subcutaneous fat tissue of patients with HARS. Furthermore, results of several studies have shown nucleoside reverse transcriptase inhibitors to induce mitochondrial toxic effects, and dysfunction induced by these inhibitors has been proposed to be important in the metabolic changes in adipose tissue. However, these mechanisms offer no explanation whatsoever for adipose tissue being affected differently in distinct regions of the body—i.e., for the redistribution of fat tissue characteristic of HARS.

Our group has shown novel neural pathways in rats between the CNS and white adipose tissue compartments with the retrograde transneuronal tracer pseudorabies virus. There seemed to be clear separation of central autonomic neurons projecting to either the subcutaneous or the intra-abdominal fat compartment. This pronounced somatotopy represents a plausible model to account for effects of hormones and phar-
macological agents on body fat distribution. White adipose tissue appeared to be innervated by parasympathetic input from the dorsal motor nucleus of the vagal nerve in the brainstem. Vagal motor neurons in this nucleus projecting to intra-abdominal fat pads were localised medially to neurons projecting to subcutaneous fat. Selective surgical lesions of this parasympathetic input resulted in striking local insulin resistance, with a 30% fall in glucose and fatty-acid uptake and a more than 50% rise in hormone-sensitive lipase activity. Thus, parasympathetic innervation of white adipose tissue enhances insulin sensitivity congruent with an anabolic function—i.e., fat accumulation. Conversely, sympathetic stimulation of subcutaneous adipose tissue induces lipolysis and free fatty-acid mobilisation.

The sympathetic motor neurons in the spinal cord, receiving input from sympathetic brainstem areas such as the A1 region, show a clear somatotopic organisation similar to parasympathetic outflow to the visceral and subcutaneous white adipose tissue compartments. Parasympathetic neurons in the dorsal motor nucleus of the vagal nerve receive innervation from at least four regions where the blood–brain barrier is absent for various hormones and nutrients, such as the hypothalamic arcuate nucleus and the area postrema. If antiretroviral drugs could reach these regions and have differential adverse effects on autonomic neurons in the CNS that project either to the visceral or to the subcutaneous white adipose tissue compartment, an imbalance in the autonomic control of subcutaneous and visceral fat tissue might ensue, and hence an aberrant distribution of adipose tissue, as seen in people with HARS.

We noted raised plasma norepinephrine concentrations in patients with HARS, providing circumstantial evidence for altered sympathetic input in this disorder. Indeed, diffuse autonomic dysfunction has been noted in HIV-1-infected patients, with more frequent and severe autonomic involvement in patients with AIDS. These findings, however, cannot account for the localised adipose tissue changes seen in HARS. Data suggest that a substantial number of antiretroviral agents, including protease inhibitors, penetrate the CNS, as shown by detectable concentrations in cerebrospinal fluid. Indinavir is easily detected in cerebrospinal fluid, and there have been reports of limited, but detectable, concentrations of amprenavir, saquinavir, and ritonavir. However, at present, the relation between concentrations of drugs in cerebrospinal fluid and tissue in the brain areas involved in our hypothesis (area postrema and arcuate nucleus) is unclear. The arcuate nucleus, in which the blood–brain barrier is absent for various compounds, is a potential target; it is essential in neuroendocrine feedback of leptin and insulin on the brain because it expresses leptin and insulin receptors and responds via intrahypothalamic neuropeptidergic pathways to the endocrine milieu. Selective damage to the arcuate nucleus of rats by means of peripherally injected monosodium glutamate in the neonatal period induces adiposity in the presence of hypophagia and signs of reduced sympathetic outflow from the hypothalamus. Moreover, leptin
administration to rats treated with monosodium glutamate fails to reduce the size of intra-abdominal fat depots compared with a pronounced reduction in intact rats.\(^9\)

This finding might be explained in this model via damage to autonomic neural connections originating in the arcuate nucleus and innervating visceral white adipose tissue via multisynaptic pathways. Glutamate is assumed to have an important role in the pathogenesis of HIV-1-related central neurotoxic effects.\(^2\)

Quantitative differences in limb fat loss between different treatment regimens, even within one particular class of antiretroviral drugs, might be associated with differences between the various drugs in blood-brain barrier passage, neurotoxic effects, or both.

Hypothesis

HARS is a disorder resulting from selective damage by antiretroviral drugs to autonomic pathways in the CNS, innervating either the subcutaneous or visceral fat depots. Specifically, increased sympathetic over parasympathetic tone of subcutaneous fat innervation induces selective loss of subcutaneous fat, and decreased sympathetic over parasympathetic tone of visceral fat innervation induces accumulation of intra-abdominal fat. The damage might be reversible (altered neuronal function) or irreversible (neuronal cell death).

Testing the hypothesis

For antiretroviral agents to affect autonomic neurons within the CNS they must enter the brain. Experiments in rats exposed to different combinations of antiretroviral drugs including protease inhibitors and nucleoside reverse transcriptase inhibitors should assess their penetration in dissected and homogenised hypothalamus and brainstem areas containing, respectively, the preautonomic and autonomic motor neurons. Furthermore, stereotaxic local administration of antiretroviral agents in the hypothalamus and brainstem should be followed by assessment of body fat distribution and metabolic variables, to confirm that these treatments induce the expected changes. Imaging techniques (MRI) and hyperinsulinaemic clamp studies will be needed to monitor body fat distribution and insulin sensitivity. Moreover, conventional morphometric techniques (Nissl staining in combination with computer-assisted image analysis) should indicate selective neuron loss or altered neuronal activity in the nuclei involved after systemic treatment and local administration. A combination of retrograde neuronal tracing (immunocytochemical detection of pseudorabies virus) from the subcutaneous and visceral fat depots, selective surgical lesioning of the vagal input, and morphometry of hypothalamic and brainstem nuclei should indicate if indeed depot-specific damage takes place during chronic antiretroviral treatment. Furthermore, the mechanism of the postulated selective damage by antiretroviral agents to either visceral or subcutaneous autonomic output should be assessed, focusing on differential receptor expression or
enzyme activities in these neuron sets. Predominant damage to autonomic innervation of only one adipose tissue compartment is a possibility and might accord with clinical observations of dissociations between subcutaneous fat wasting and abdominal fat gain.\(^2\) Finally, differential changes in autonomic innervation of lipoatrophic and lipogenetic compartments should be investigated in HIV-1-infected patients with HARS. One way to compare these compartments is by microdialysis of neurotransmitters and their metabolites in phenotypically distinct fat compartments. Comparison of subcutaneous thigh fat as a lipoatrophic compartment with visceral fat could, however, be hampered by ethical considerations about obtaining intra-abdominal fat biopsy specimens for research purposes. Dorsocervical fat accumulation might serve as an alternative for visceral fat, although the source of the autonomic innervation of this fat depot has not been established.

REFERENCES


'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 state. 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 lipogenesis. 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 lipolysis. However, humoral factors cannot completely explain fat tissue metabolism in vivo.

Cantu demonstrated in 1967 that sympathetic denervation of the retroperitoneal fat pad results in impaired lipolysis after fasting. In the 1980ies, Stock and Rothwell discovered that sympathetic tone induces mitochondrial oxidation in brown fat tissue. In the same decade, Trayhurn and Dalziel refined the theoretical frame on the autonomic innervation of fat tissue. 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\textsuperscript{5,15}. 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\textsuperscript{16-25}. Except the adrenal gland, these organs appeared to receive both sympathetic and parasym pathetic innervation, originating in the biological clock and other hypothalamic nuclei.

We injected PRV into intact and locally sympathectomized fat pads (chapter 2)\textsuperscript{26}. 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 where cut, the sympathetic motor neurons in the spinal cord where 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\textsuperscript{17}. 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:
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 antiphase 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 pear 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?
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, hyperinsulinemia and a fatty liver as a consequence.
CHAPTER 8 PERSPECTIVES FOR FOLLOW-UP STUDIES

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\textsuperscript{53,54}. Saporin conjugated to a retrograde tracer, such as CTB, has been used in to retrogradely kill autonomic motor neurons in rodents\textsuperscript{52,53-57}. 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-gII is a swine neurotrophic a 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-gII has not the ability to enter a neighboring neuron and spread upstream through the CNS\textsuperscript{58-60}. Therefore, PRV-gII is a neuroanatomically a marker and physiologically a neurotoxin at the same time. In a similar approach to CTB-SAP, we injected PRV-gII 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-gII in the CNS.

Transport of CTB-SAP and PRV-gII has been described after dipping in cut nerve endings or intra-neuronal injections\textsuperscript{52,56-58}. 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.

\textit{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 a strong association between disturbed sleep, ANS function and the development of the metabolic syndrome\textsuperscript{36,48,61-66}.

\textit{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\textsuperscript{67}.  

106
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


SUMMARY

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

The hypothalamus controls 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 chapters 6-8 we discuss the implications of our findings and propose follow-up studies.

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 (chapters 2 and 4).

Finding #2
The brain distinguishes between organs from different body compartments and combines the organs of one body compartment (chapters 2 and 3).

Finding #3
Sex and stress hormones can access central neurons projecting to fat tissue (chapter 5).

Perspective #1
The metabolic syndrome might be a brain disease (chapter 6).

Perspective #2
HIV-associated body fat redistribution syndrome might be a brain disease (chapter 7).

FINDINGS
Chapter 2
Selective parasympathetic innervation of subcutaneous and intra-abdominal fat – functional implications
Fat tissue was reported earlier to be innervated by the sympathetic nervous system only, known for its catabolic effect. For the first time, we demonstrate parasympathetic input from the vagal motor nucleus to fat tissue, clearly modulating its insulin sensitivity and glucose and free fatty acid metabolism in an anabolic way. Moreover, we demonstrate that sympathetic motor neurons in the spinal cord and parasympathetic neurons in the DMV are specialized to project either to intra-abdominal or subcutaneous body compartment only.
Chapter 3
Neuronal tracing from metabolic organs: an autonomic (anatomical) basis for type 2 diabetes
In this chapter we extend the studies of chapter 2 on the specialization of autonomic motor neurons. We show that the same neurons control intra-abdominal organs (intra-abdominal fat, liver, and pancreas), whereas subcutaneous adipose tissue located outside the abdominal compartment receives input from another set of autonomic neurons. This differentiation persists up to pre-autonomic neurons in the hypothalamus, including the biological clock. Thus, pre-autonomic neurons have a distinct organization depending on the body compartment they command. Moreover, we demonstrate a neuronal feedback from adipose tissue that reaches the brainstem.

Chapter 4
Dual sympathetic and parasympathetic hypothalamic output to white adipose tissue
We describe a hypothalamic network behind the sympathetic and parasympathetic branches of the ANS projecting to fat tissue as described in chapter 2. Preautonomic neurons in the suprachiasmatic nucleus (SCN), the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus (LH) are specialized to project either to sympathetic or to parasympathetic motor neurons. This dual hypothalamic pathway enables hypothalamic centers such as the biological clock, together with temperature and feeding centers, to coordinate adipose tissue physiology.

Chapter 5
Estrogen receptor alpha and glucocorticoid receptor expression in (pre-)parasympathetic neurons that project to fat tissue
Estrogen and glucocorticoids have strong effects on fat tissue metabolism and body fat distribution. So far, the differential effects of these hormones on the abdominal versus subcutaneous fat compartment have not been explained satisfactorily. We describe that a large proportion of neurons in the brainstem (DMV) and hypothalamus (PVN, LH and amygdala) projecting to abdominal fat express the glucocorticoid receptor (GR). In addition, some neurons were double stained for PRV and estrogen receptor (ER) alpha. These findings show, for the first time, that glucocorticoids and estrogens might affect adipose tissue metabolism and distribution indirectly via its autonomic innervation.
DISCUSSION AND PERSPECTIVES FOR FOLLOW-UP STUDIES

Chapter 6
Hypothesis: shifting the equilibrium from activity to food leads to autonomic unbalance and the metabolic syndrome
During the last century, life has changed dramatically in industrialized countries. Food has become abundant, snacking frequency increased and shifted toward the end of the day, and simultaneously, the necessity for physical effort became considerably reduced. Moreover, physical activity no longer needs to coincide with the light period anymore. As a result, the environment sensed by the brain has become metabolically flattened and arrhythmic. From the perspective of a longstanding evolutionary development, this has been an abrupt “environmental mutation.” We hypothesize that in such conditions the susceptible brain loses its feeling for internal and external rhythm. Since the brain uses the autonomic nervous system to implement internal rhythmicity, we propose an unbalanced and arrhythmic autonomic nervous system as a major cause of the metabolic syndrome.

Chapter 7
Hypothesis: HIV-associated adipose redistribution syndrome as a selective autonomic neuropathy
Abnormal body-fat distribution in HIV-1-associated adipose redistribution syndrome (HARS) remains unexplained at present. White adipose tissue is controlled by humoral factors and by neural regulation. Sympathetic innervation stimulates lipolysis, whereas parasympathetic innervation has an anabolic influence on white adipose tissue. Results of neuroanatomical studies showed a clear somatotopy with respect to autonomic control of white adipose tissue by both the sympathetic and parasympathetic branch, with separate sets of autonomic neurons innervating either the subcutaneous or the visceral fat compartment. Thus, the CNS is likely to be a key player in the regulation of body-fat distribution. We propose that HARS is mediated by effects of antiretroviral treatment on the CNS and could indicate a change in autonomic balance resulting in redistribution of adipose tissue.

Chapter 8 contains suggestions for follow-up studies.
SAMENVATTING

De hypothese van dit proefschrift luidt: de hersenen spelen een rol bij de distributie van lichaamsvet en de aan vetverdeling gerelateerde stofwisselingsziekten, zoals diabetes.

De achtergrond van dit proefschrift is dat de hypothalamus door middel van hormonen uit de hypofyse lichaamsfuncties reguleert. Pas onlangs zijn we de bijdrage van het autonome zenuwstelsel op waarde gaan schatten.

De hoofdstukken 2 tot 5 beschrijven de interactie tussen hypothalamus en metabole organen. In de hoofdstukken 6 tot 8 bespreken we de implicaties van onze bevindingen doen we voorstellen voor vervolgonderzoek.

Het proefschrift is opgebouwd uit twee onderdelen: conclusies en vooruitzichten.

Conclusie # 1
De hersenen gebruiken twee neuronale systemen die een antagonistisch effect op metabole organen hebben (hoofdstuk 2 en 4).

Conclusie # 2
De hersenen zijn in staat organen uit verschillende regionen van het lichaam van elkaar te onderscheiden, en combineren de organen afkomstig uit dezelfde regio van het lichaam (hoofdstuk 2 en 3).

Conclusie # 3
Geslachts- en stresshormonen hebben toegang tot centrale neuronen die naar het vetweefsel projecteren (hoofdstuk 5).

Vooruitzicht # 1
Het metabool syndroom is mogelijk een hersenaandoening (hoofdstuk 6).

Vooruitzicht # 2
Het herdistributie-van-HIV-geassocieerd-lichaamsvet syndroom is mogelijk een hersenaandoening (hoofdstuk 7).

CONCLUSIES

Hoofdstuk 2
Selectieve parasympathische innervatie van subcutaan en intra-abdominaal vet - functionele implicaties

Over vetweefsel is in eerder onderzoek gemeld dat het uitsluitend geïnnerveerd wordt door het sympathisch zenuwstelsel, dat bekend staat om zijn catabolisch effect. Wij laten voor het eerst de parasympathische input van de vagus motor kern (DMV) in
vetweefsel zien: insulinegevoeligheid, glucose en vrije vetzuren metabolisme worden duidelijk anabool gedomineerd.

Verder laten we zien dat sympathische motorneuronen in het ruggenmerg en para-sympathische neuronen in de DMV een specifieke taak hebben, namelijk uitsluitend projecteren naar de intra-abdominale of subcutane lichaams compartimenten.

Hoofdstuk 3
Neuronale tracing van metabole organen: Een autonome (anatomische) basis voor diabetes type 2.
In dit hoofdstuk breiden we het onderzoek over de specialisatie van autonome motorneuronen zoals beschreven in hoofdstuk 2 uit. De organen in de buikholte (intra-abdominaal vet, lever, alvleesklier) blijken door dezelfde neuronen bestuurd te worden, terwijl subcutaan vetweefsel input ontvangt van een heel andere set autonome neuronen. Deze differentiatie wordt doorgevoerd tot in de pre-autonome neuronen in de hypothalamus, inclusief de biologische klok. De pre-autonome neuronen zijn strikt georganiseerd naar het lichaams compartiment dat ze besturen. Verder laten we een neuronale feedback zien van vetweefsel dat de hersenstam bereikt.

Hoofdstuk 4
Tweeledige sympathische en parasympathische hypothalamische output naar vetweefsel
Hier beschrijven we een hypothalamisch netwerk achter de sympathische en parasympathische takken van het autonoom zenuwstelsel die naar vetweefsel projecteren, zoals beschreven in hoofdstuk 2. Pre-autonome neuronen in de suprachiasmatische kern, de paraventriculaire kern van de hypothalamus en de laterale hypothalamus zijn gespecialiseerd in het projecteren naar ofwel de sympathische ofwel de parasympathische motorneuronen.

Dit tweeledige hypothalamische pad stelt de hypothalamische centra, zoals de biologische klok en temperatuur- en voedselcentra, in staat om de vetweefselfysiologie te coördineren.

Hoofdstuk 5
Oestrogeen-receptor alpha en glucocorticoid-receptor expressie in (pre)para-sympathische neuronen die naar vetweefsel projecteren
Oestrogenen en glucocorticoiden hebben beide een hevige effect op vetweefselmetabolisme en lichaamsvetdistributie. Tot nu toe is er geen bevredigende verklaring gevonden voor de gedifferentieerde effecten van deze hormonen op buikholtevet- en subcutaan vetcompartimenten. Een groot deel van de neuronen in de hersenstam en in de hypothalamus (paraventriculaire kern, laterale hypothalamus en amygdala) die naar buikholtevet projecteren beschikken over glucocorticoid receptoren. Bovendien
waren een aantal neuronen dubbelgekleurd voor PRV en oestrogeen receptor alpha. Het is hiermee voor het eerst aangetoond dat glucocorticoiden en oestrogenen via de autonome innervatie invloed zouden kunnen uitoefenen op vetweefselmetabolisme en -distributie.

**DISCUSSIE EN VOORUITZICHTEN OP VERVOLGONDERZOEK**

**Hoofdstuk 6**

Hypothese: verschuiving van de balans van lichaamsbeweging naar voedsel leidt tot verstoring van het evenwicht en tot het metabool syndroom

In de afgelopen eeuw is het leven in de geïndustrialiseerde landen ingrijpend veranderd. Er kwam voedsel in overvloed, er werd meer gesnackt, de eetmomenten verplaatsten zich naar een later tijdstip, terwijl tegelijkertijd de noodzaak fysiek in actie te komen beduidend afnam. Bovendien was lichamelijke beweging ineens niet meer iets wat zich per se overdag afspeelde. Het gevolg hiervan was dat het brein voor wat betreft de stofwisseling de omgeving als aritmisch en afgevlakt begon te beschouwen. In termen van evolutionaire ontwikkeling kunnen we hier dus wel spreken van een abrupte mutatie van de omgeving. We stellen dat in deze omstandigheden het ontvankelijke brein zijn gevoel voor interne en externe ritmes kwijtraakt. Aangezien het brein het autonome zenuwstelsel gebruikt om het interne ritme te kunnen opleggen, wijzen we het autonome zenuwstelsel - uit balans geraakt en aritmisch - aan als de hoofdoorzaak van het metabool syndroom.

**Hoofdstuk 7**

Hypothese: Herdistributie van met HIV-geassocieerd vetweefsel syndroom als selectieve autonome neuropathie

Er is nog altijd geen verklaring voor de abnormale distributie van lichaamsvet in het HAR (HIV-associated adipose redistribution) syndroom. Vetweefsel wordt gestuurd door humorale factoren en door neurale regulatie. Sympathische innervatie stimuleert de lipolyse, terwijl parasympathische innervatie een anabole werking op vetweefsel heeft. Uit neuroanatomische studies is gebleken dat er een duidelijke somatotopie is wat betreft autonome sturing van vetweefsel door zowel de sympathische als parasympathische tak, met aparte sets autonome neuronen die ofwel het subcutane-vetcompartiment ofwel het buikholtevetcompartiment innerveren. Het centraal zenuwstelsel speelt dus naar alle waarschijnlijkheid een hoofdrol in de regulatie van de distributie van lichaamsvet. We stellen voor dat de antiretrovirale behandelingen veranderingen in het autonome evenwicht induceren, hetgeen resulteert in een herdistributie van vetweefsel.

Hoofdstuk 8 bevat suggesties voor vervolgonderzoek.
AUTHORS

Maurice Bizino
Netherlands Institute for Brain Research, Amsterdam, The Netherlands
m.bizino@nih.knaw.nl

prof.dr. Ruud M. Buijs
Netherlands Institute for Brain Research, Amsterdam, The Netherlands
r.buijs@nih.knaw.nl

dr. Corbert G. Van Eden
Netherlands Institute for Brain Research, Amsterdam, The Netherlands
c.van.eden@nih.knaw.nl

prof.dr. Eric Fliers.
Departement of Endocrinology and Metabolism, Academic Medical Center of
the University of Amsterdam, Amsterdam, The Netherlands
e.fliers@amc.uva.nl

prof.dr. Louis M. Havekes
Netherlands Organization for Applied Scientific Research — Prevention and
Health, Gaubius Laboratory, Leiden, The Netherlands
Department of Internal Medicine and Cardiology, Leiden University Medical
Center, Leiden, The Netherlands
lm.havekes@pg.tno.nl

Caroline L. Van Heijningen-Pirovano
Netherlands Institute for Brain Research, Amsterdam, The Netherlands
c.pirovano@nih.knaw.nl

dr. Andries Kalsbeek
Netherlands Institute for Brain Research, Amsterdam, The Netherlands
a.kalsbeek@nih.knaw.nl

Yolanda Kap
Netherlands Institute for Brain Research, Amsterdam, The Netherlands
y.kap@nih.knaw.nl

prof.dr. Thomas C. Mettenleiter
Friedrich-Loeffler-Institute
Federal Research Institute for Animal Health, Insel Riems, Germany
thomas.c.mettenleiter@rie.bfav.de
dr. Peter Reiss  
Department of Infectious Diseases, Tropical Medicine, and AIDS, Academic Medical Centre of the University of Amsterdam, Amsterdam, The Netherlands  
p.reiss@amc.uva.nl

prof.dr. Johannes A. Romijn  
Department of Endocrinology and Metabolism, Leiden University Medical Center, Leiden, The Netherlands  
j.a.romijn@lumc.nl

dr. Marieke Ruiter  
Netherlands Institute for Brain Research, Amsterdam, The Netherlands  
m.ruiter@nih.knaw.nl

prof.dr. Hans P. Sauerwein  
Department of Endocrinology and Metabolism, Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands  
h.p.sauerwein@amc.uva.nl

Arja A. Sluiter  
Netherlands Institute for Brain Research, Amsterdam, The Netherlands  
a.sluiter@nih.knaw.nl

Cara Snel  
Netherlands Institute for Brain Research, Amsterdam, The Netherlands  
c.snel@nih.knaw.nl

dr. Marc van der Valk  
International Antiviral Therapy Evaluation Centre, Academic Medical Centre of the University of Amsterdam, Amsterdam, The Netherlands  
m.vandervalk@amc.uva.nl

Laura Veder  
Netherlands Institute for Brain Research, Amsterdam, The Netherlands  
l.veder@nih.knaw.nl

dr. Peter J. Voshol  
Department of Internal Medicine and Cardiology, Leiden University Medical Center, Leiden, The Netherlands  
p.j.voshol@lumc.nl

Ajda Yılmaz  
Netherlands Institute for Brain Research, Amsterdam, The Netherlands  
a.yilmaz@nih.knaw.nl

120


THANKS

In January 2000 I entered the (surprisingly small) office of Ruud Buijs, professor at the Netherlands Institute for Brain Research. I was an undergraduate student and came for an interview concerning my scientific internship from medical school. I was nervous, because I feared that it would not be long before Ruud discovered how ridiculously meagre my knowledge of the nervous system was.

To my surprise the interview went okay, and after we had made all the formal arrangements, I asked him: "You wouldn't happen to have some basic literature for me, to get to know the brain somewhat better? I did my best at the neurology courses, but I still feel I don't understand all that much of it."

Ruud smiled, stood up, extended his hand and said: "Welcome to the club, Felix - that is exactly the reason why I come to the institute every morning, the same as quite a few other people in this building."

His reaction characterizes the open and innovative atmosphere of Ruud Buijs' group "Hypothalamic Integration" at the NIBR. I am grateful for the way Ruud made me feel at home in the institute and for the way he stimulated me to develop my own approach to science. He always supported me in times of scientific despair, and when experiments or manuscripts ground to a complete halt he would come up with a refreshing, sometimes breathtaking, new idea. Ruud gave true scientific training, not just orders to conduct experiments.

Eric Fliers, professor at the AMC and promoter of this thesis, suggested the study of the innervation of fat tissue, an idea he developed together with Prof. Hans Sauerwein. Eric's enthusiasm, even at the very beginning, when we had no results at all, helped to overcome all kinds of unforeseen problems. His ideas and his critical clinical view have been a major contribution to the project. I am very grateful for the way Eric supported me in the last month of the project to finish this thesis.

Hans Sauerwein, co-promotor and professor at the AMC, would run all the way down from the outpatient clinics in the AMC - in between two consultations - to the NIBR to see the first microscope slides showing that different fat compartments receive separate innervation. With his broad knowledge and longstanding experience in the field of endocrinology and metabolism he helped us discover the direction to take. As an experienced senior scientist he often cut down my planning to a realistic time schedule, thereby maximizing the results of the project.

Hans Romijn, professor at Leiden University Medical Center and co-promotor, put a great deal of energy in the studies, both intellectually and practically. Meeting with Hans Romijn meant getting new ideas and motivation. Moreover, Hans put me in touch with his collaborators Prof. Louis Havekes and Dr. Peter Voshol, who helped us
to perform the clamp studies. Dr. Andries Kalsbeek, senior scientist in the group “Hypothalamic Integration”, was involved in every meeting and contributed his experience to the project. He always warned us when our plans became too ambitious.

Prof. Thomas Mettenleiter from the Friedrich-Loeffler-Institut (Insel Reims, Germany) provided us with the labeled PRVs for the double-labeling studies.

I thank Professors Roger Adan (Rudolf Magnus Institute, Utrecht), Mary Dallman (UCSF, San Francisco), Henriette Delemarre-van de Waal (VUMC medical center, Amsterdam), Joost Hoekstra (AMC medical center, Amsterdam) and Dick Swaab (NIBR, Amsterdam) for taking part in the reading committee of this thesis.

I met Rixt Riemersma at the institute. She has been a wonderful colleague and friend. Almost every day she would listen patiently to all my complaints, annoyances, problems and - even worse - to all kinds of so-called ‘success stories’.

Martin zur Nedden, physicist and friend, taught me to perceive the beauty of science. He believed in me and gave me substantial practical support at a time when everybody thought I was mad to go to medical school at the age of 29. Without Martin, this thesis would not have been written.

I was lucky with the undergraduate students: Cara Snel (pharmacy), Yolanda Kap (biomedical techniques), Laura Veder (medicine) and Maurice Bizino (medicine). They carried out a substantial part of the work for this thesis. They were truly interested in the subject and nice people to work with.

Susanne la Fleur and Joke Wortel had already developed the PRV tracing technique on the liver, which meant I could start my research of the vagus from the word go. Technicians Jan van der Vliet and Caroline van Heyningen-Pirovano worked with me on several experiments, providing ideas and practical help.

It was a pleasure to share a room in the brain institute with Marieke Ruiter, Stephanie Perreau, Cathy Cailloto, Frank Scheer, Gerben Meynen, Ajda Yilmaz and Jun Lei, discussing science and eating chocolate. Corbert van Eden, Inge Huizinga, Nico Bos, Marie-Laure Garidou, Chunxia Yi and Lars Klieverik from the group “Hypothalamic Integration” in the NIBR were great colleagues, both scientifically and socially. Chris Pool, thanks for the patient reactions to my DEC protocols. Anita van den Hoek and Annemieke Heijboer from Leiden helped with the experiments in Leiden and have been challenging scientific discussion partners. Jenneke Kruisbrink managed to get hold of every article I asked for, no matter whether it was from 1921 or only available in China. Jilles Timmer took care of the rats and made sure that they did not get ill by cleaning the whole animal department every week with great accuracy. Joop van Heerikhuize explained to me (more than once…) how to make photographs from microscopic slides. Bart Fisser was an important figure for the foreigners in the brain institute, helping everybody out and explaining the core aspects of Dutch culture, with a great sense of humor. Henk Stoffels made our figures clear to people outside our
group and made this book. Tini Eikelboom en Wilma Verweij helped me through the bureaucracy of the AMC, UvA and KNAW.

Finally, my special thanks go to Dick Swaab. As a first year medical student I read an interview with Dick Swaab in a students' journal. It was inspiring; he formulated questions that stuck in my head for weeks afterwards. I don't remember these questions now, except this quote: Student's question: "Will you donate your brain to your Brain Bank when you're dead?" Swaab: "Of course, at least then I can be sure that in the end society benefits from my brains." Reading this, I decided to wanted to be at that place where he worked. Dick Swaab is an outstanding neuroscientist. As director of the NIBR he made the institute into a non-hierarchical organization, a safe environment for everybody with significant ideas and willing to try to practice science seriously. His style is humane and relaxed and his door is always open.

Next, I want to thank my friends and especially Sabine Plamper, Rogier Alleblas, Teo van Kooten, Katrin Hammer, Ralf Bohlsen, Hartmut Janssen, Matthias Janssen, Jens Voortman, Julius and Anna Riemersma for their help and patience, when I, once again, had to cancel an appointment because I hadn't finished an experiment or manuscript on time.

My sisters Kathrin Kreier and Birgit Simon always calmed me down when I was getting just a bit overexcited telling them about all the ins and outs of my experiments, either by just yawning directly in my face or by making a cynical joke. Cooling down can be helpful to get back to earth and they always made sure my touch down was smooth. And when I reported a scientific disappointment, they never yawned, but listened with empathy. I would like to thank my nephew Robin Maron, who always helped me when I asked him; he is the only person I know able to enter 400 articles into Endnote in just six hours. Finally: Thanks, Mom!
CURRICULUM VITAE

Felix Kreier was born on 1 April 1967 in Nurnberg, Germany. In 1988 he finished high school at the Gymnasium Eversten (Oldenburg, Germany) and started two years of social services, working with handicapped children. He then began a six-year search for his profession: In 1990, he started the study of philosophy at the University of Hamburg (Germany). He moved to Amsterdam in 1992.

In Amsterdam, Felix Kreier worked for a computer company and became team leader in 1996 (and received two "company achievement awards", in 1995 and 1996), but discovered that neither philosophy nor business suited him when it came to a fulfilling career.

In 1996 he therefore started the study of medicine at the University of Amsterdam, and joined the group "Hypothalamic Integration" at the Netherlands Institute for Brain Research of Prof. Ruud Buijs, working on the project: "Vagal Innervation of Fat Tissue" in collaboration with Prof. Eric Fliers, Prof. Hans Sauerwein (Academic Medical Center, Amsterdam) and Prof. Johannes Romijn (Leiden University Medical Center) in January 2000. In August 2000 this scientific internship as part of his medical training became a PhD project: "Autonomic Innervation of Fat Tissue", which resulted in the present thesis.

Felix Kreier received the "Best Endocrinology Article 2002" award of the Dutch Endocrine Society, the "Best AMC Publication 2002" award of the Academic Medical Center of the University of Amsterdam and the "Hippocrates Study Price 2002". In 2004, Felix Kreier started his medical internships. He plans to specialize as a pediatrician after receiving his MD. He aims to combine working in the clinic and doing research in the field of pediatric neuroendocrinology.