Autonomic nervous control of white adipose tissue: studies on the role of the brain in body fat distribution
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Neuronal tracing from metabolic organs: an autonomic (anatomical) basis for type 2 diabetes

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
2 μl of CTB (A, 2%, Sigma-Aldrich, #C167) or CTB-alexa fluor 488/555/647 (B, 1%, 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, #C3062), 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.
PART A FINDINGS

PRV tracing

5μl PRV-Bartha (5 x 10⁶ plaque-forming units, a generous gift of C.E. Jacobs from the Institute for Animal Science and Health, Lelystad, The Netherlands) or PRV B80 (5x10⁷ plaque-forming units PRV β-galactosidase B80, Institute for Molecular Biology, Insel Riems, Germany), or PRV GFP (5x10⁷ 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=15) 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 body¹⁷-²¹.

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
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 (ia+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-
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.

pose tissue. 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 parasympathetic 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 pads15. 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 contain 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. 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.

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. 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. 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.

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. 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. 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\textsuperscript{(58-60)}. Inappropriate timing and protracted activation of the shared vagal input to the 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\textsuperscript{(6)}. However, probably in a majority of obese patients, the main cause of the system failure is a huge "environmental mutation" of our lifestyle. Overeating without compensation by physical activity might induce confusing feedback directly in the brain or via neuronal feedback from e.g. the portal system\textsuperscript{(62-64)}.

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
