Autonomic nervous control of white adipose tissue: studies on the role of the brain in body fat distribution
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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.
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\(^8\). Integrating body information received both via the circulation and by neurons, the hypothalamus warrants a secure level of energy to the body\(^15\).

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\(^15\). 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\(^16\)–\(^18\). 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\(^19\). 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\(^20\). 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 21. 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 medially4. 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 bundles7. 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 B8o (5x10⁷ plaque-forming units PRV β-galactosidase B8o, 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 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 B8o 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 B80 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).
Figure 4.2   Subcutaneous and intra-abdominal do not share pre-autonomic control. Injection of PRV GFP / PRV B80 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. See color section.

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\textsuperscript{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\textsuperscript{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, periaquaductal 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 the 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