Tracing from Fat Tissue, Liver, and Pancreas: A Neuroanatomical Framework for the Role of the Brain in Type 2 Diabetes

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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 intraabdominal organs (intraabdominal fat, liver, and pancreas), whereas sc 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, where increased insulin secretion by the pancreas and disturbed glucose metabolism in the liver coincide with an augmented metabolic activity of visceral compared with sc adipose tissue. (*Endocrinology* 147: 1140–1147, 2006)

T O BALANCE ENERGY fluxes, the brain needs a precise and clear view on the metabolic state of the body. Circulating humoral factors, in fact, are averaged whole body signals, whereas sensory neurons add detailed information from specific regions of the body to this global view. An early report indicates that information from sc fat tissue to the brain is being transported by the sympathetic nervous system (1). Hence, the first part of this study addressed the question of to which extent adipose tissue acts as any other organ and provides neuronal feedback to the brain. We investigated the presence of sensory feedback to the central nervous system by injection of the neuronal tracer cholera toxin B (CTB) into intraabdominal adipose tissue.

The hypothalamus integrates peripheral signals delivered by the blood (fatty acids, glucose, and hormones) and by neuronal input from peripheral organs (2). This hypothalamic 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 (3–10). The biological

clock, located in the suprachiasmatic nucleus (SCN), modulates the set point of the hypothalamus by oscillating its sensitivity to metabolic signals (11–13). The SCN induces diurnal metabolic variations such as the rise of glucose and glucocorticoids in the early morning, known as the "dawn-phenomenon" (8, 9).

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 far less established than the others, namely 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. Adipose tissue, liver, and pancreas have been shown to receive sympathetic and parasympathetic innervation, but the organization of this autonomic outflow from the hypothalamus to metabolic organs has not been investigated (14–18).

To clarify the output of the brain to the abdominal cavity we analyzed the vagal output to the abdomen by means of injecting CTB with different fluorescent labels in intraabdominal fat, liver, and pancreas. Injecting the retrograde neuronal tracer pseudorabies virus (PRV) into retroperitoneal and sc fat allowed us to determine the projections from hypothalamus and brainstem to these fat compartments. Thus, we uncovered from the biological clock separate sets of neurons controlling sc 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 with sc adipose tissue (19–22).

Materials and Methods

Materials

All experiments were performed in 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.

Fat denervation

The sympathetic or parasympathetic fibers entering the retroperitoneal fat pad were cut, as described earlier by our group (14). For a detailed description including perioperative photos, please refer to the supplemental data published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Liver denervation

The liver was sympathetically denervated by a technique described earlier by Buijs *et al.* (23). 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 from the portal vein via blunt dissection. 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

Two microliters of CTB (2%, Sigma-Aldrich, St. Louis, MO; no. C167) or CTB-alexa fluor 488/555/647 (1%, Molecular Probes, Eugene, OR; no. 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. In a control experiment, CTB was applied on top of the intact or totally denervated organ.

Three, 4, or 5 d after tracer injection, the animals were first perfused with saline and then with 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 h. Brains were frozen and coronal sections (40 μm) were cut. After rinsing in 0.05 M Tris-buffered saline (pH 7.4), brains with CTB sections were incubated overnight at 4 C with polyclonal rabbit anti-CTB (Sigma-Aldrich; no. C3062), then incubated for 60 min in the secondary antibody, biotinylated goat antirabbit (Vector Laboratories Inc., Burlingame, CA), 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% H_2O_2. 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 1488 × 1120 pixel size in RGB 24-bit true color. Contrast and color were adapted using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) without any other image manipulation. Brain sections with CTB-alexa fluor were rinsed extensively in 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

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

Three, 4, or 5 d after tracer injection, the animals were first perfused with saline and then with a solution of 4% paraformaldehyde and 0.15% glutaraldehyde in PBS (pH 7.4). [For a discussion on survival times, see Buijs and colleagues (16)]. They were postfixed overnight and cryoprotected by immersion with 30% sucrose in 0.2 M PBS (pH 7.4) for a further 24 h. 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), rabbit-anti GFP (Molecular Probes), or mouse-anti galactosidase (Sigma-Aldrich), depending on the tracers used, and then with a secondary antibody for 60 min for analysis under a confocal laser-scanning microscope (see *CTB tracing*).

Results

Adipose tissue feeds back to nociception-related central structures

Two microliters of 2% CTB solution was either injected into (n = 15) or, as control, applied onto retroperitoneal fat in rats (n = 5). Consequently, spinal cord, brainstem, and hypothalamus were stained for CTB. 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, the neurons in vagal motor nuclei and nerve endings in the gracile nucleus were positive, but no other areas, whereas nine did not show any central CTB (Fig. 1).
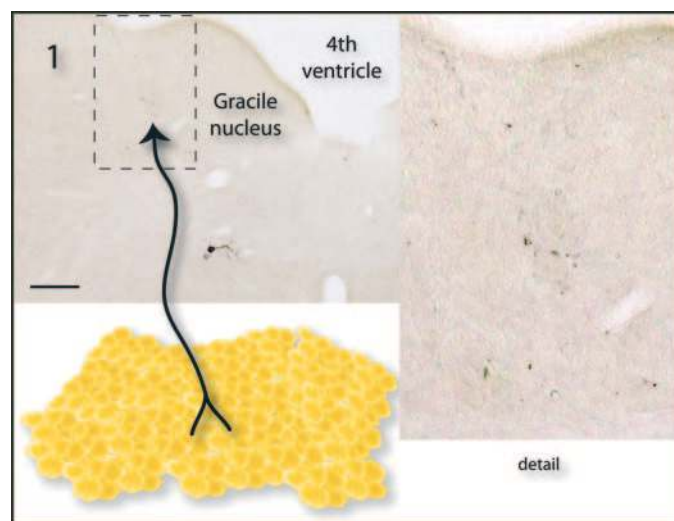

FIG. 1. Neuronal feedback from fat tissue to the gracile nucleus of the brainstem. After injection of 2 μl of 2% CTB into retroperitoneal fat, the gracile nucleus of the brainstem is labeled with nerve endings. Retrograde labeling of a vagal motor neuron in the DMV is visible. This result demonstrates that sensory fibers run from fat tissue to the brain. *Bar*, 200 μm; *detail*, 50 μm.

Liver, pancreas, and intraabdominal fat share one set of vagal motor neurons, intraabdominal or sc fat do not

Two microliters 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). Because the fluorescence signal in the CNS was absent in the control groups, false-positive results due to leakage could be excluded. In the dorsal motor nucleus of vagus (DMV), labeled vagal motor neurons contained both fluorescent labels from liver and pancreas. In a second set of experiments, the liver was sympathetically denervated as described earlier and injected with 5 μl of the retrograde tracer PRV, and 2 μl of 2% CTB was injected into retroperitoneal fat (n = 12) (23). 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 (Fig. 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 (Fig. 2). Earlier, we reported that sc and intraabdominal fat pads are controlled by separate sets of vagal motor neurons (14).

Distinct sets of hypothalamic and amygdalar neurons project to either intraabdominal or sc adipose tissue

Five microliters of PRV B80 or PRV GFP was injected into intraabdominal or sc adipose tissue. Injecting PRV into completely denervated intraabdominal fat or applying the tracer on top of intraabdominal or sc 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 a comparable infection rate of both tracers (n = 9), neuronal colocalization of both tracers exceeded 95%, both in second order infection of PRV in paraventricular nucleus (PVN) and medial preoptic area (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 after each other, the same neuron; in addition, it indicates a shared control of both intraabdominal fat pads. Parasympathetic (n = 37) denervation of the left retroperitoneal fat pad and alternate injection of PRVB80 or GFP in this fat pad and sc 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 (a maximum of one neuron per section) could be observed in animals with comparable infection (Fig. 3) (PVN/MPO, n = 6; SCN/amygdala, n = 5). Earlier we showed that sympathetic motor neurons are specialized in intraabdominal or sc fat pads (14). 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, inhibits food intake, and stimulates sympathetic nerve activity (24). Other studies have shown that free fatty acids inhibit endogenous glucose production by the liver via the hypothalamus (5–7). Sympathetic feedback from sc fat tissue has been demonstrated earlier by labeling of a retrograde tracer in the dorsal root ganglia. However, in our tracing study from intraabdominal fat tissue, no nerve endings were found in the dorsal horn of the spinal cord (1). The presence of primary afferent projections from adipose tissue to the gracile nucleus of the brainstem not only presents evidence of 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 (25–31). Earlier studies demonstrated primary afferents from various organs to the gracile nucleus, for instance from sc tissue of primates, as well as from the hind limb, splanchnic nerve, pelvic nerve, and pudendal nerve in rats (27, 30–33). In view of this pain-related feedback, the anatomical position of adipose tissue within the body suggests a function in monitoring skin and visceral organs. Earlier, dermatologists had suggested a role of sc fat tissue in the perception of pain (34).

The afferents could sense mechanical, temperature, or hormonal stimuli such as cytokines not only under the skin, but also from the viscera (35). Few studies have addressed nociception in brown and white fat tissue. It has been shown that capsaicin-sensitive fibers are present in brown adipose tissue (36). The nociceptive function of the afferents is supported by experiments in rats where capsaicin was injected into white sc fat tissue on the back. As a consequence, skin lesions appeared 10 d later on the back but also in the neck, suggesting a reaction mediated by the autonomic nervous system (ANS) (37). Fat pads in the knee joint and around spine ligaments contain nociceptive substance P fibers (38–40). Recently, a study demonstrated the induction of local and referred pain by injection of saline into the infrapatellar knee fat pad (41).

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 just as other metabolic organs.

Shared (pre-) autonomic output links intraabdominal obesity to diabetes

Recently, several studies reported early dysfunction of the ANS in the development of T2 diabetes (42–46). Other publications demonstrate a link between cardiovascular disease or insulin resistance in muscle and sympathetic overweight (47, 48). In contrast, hyperinsulinemia, obesity, and fatty liver are connected to parasympathetic overweight (49–52). Thus, the ANS might have a different tone in different parts of the body at the same time. However, when the local status of the ANS in a certain region is understood as an indicator of
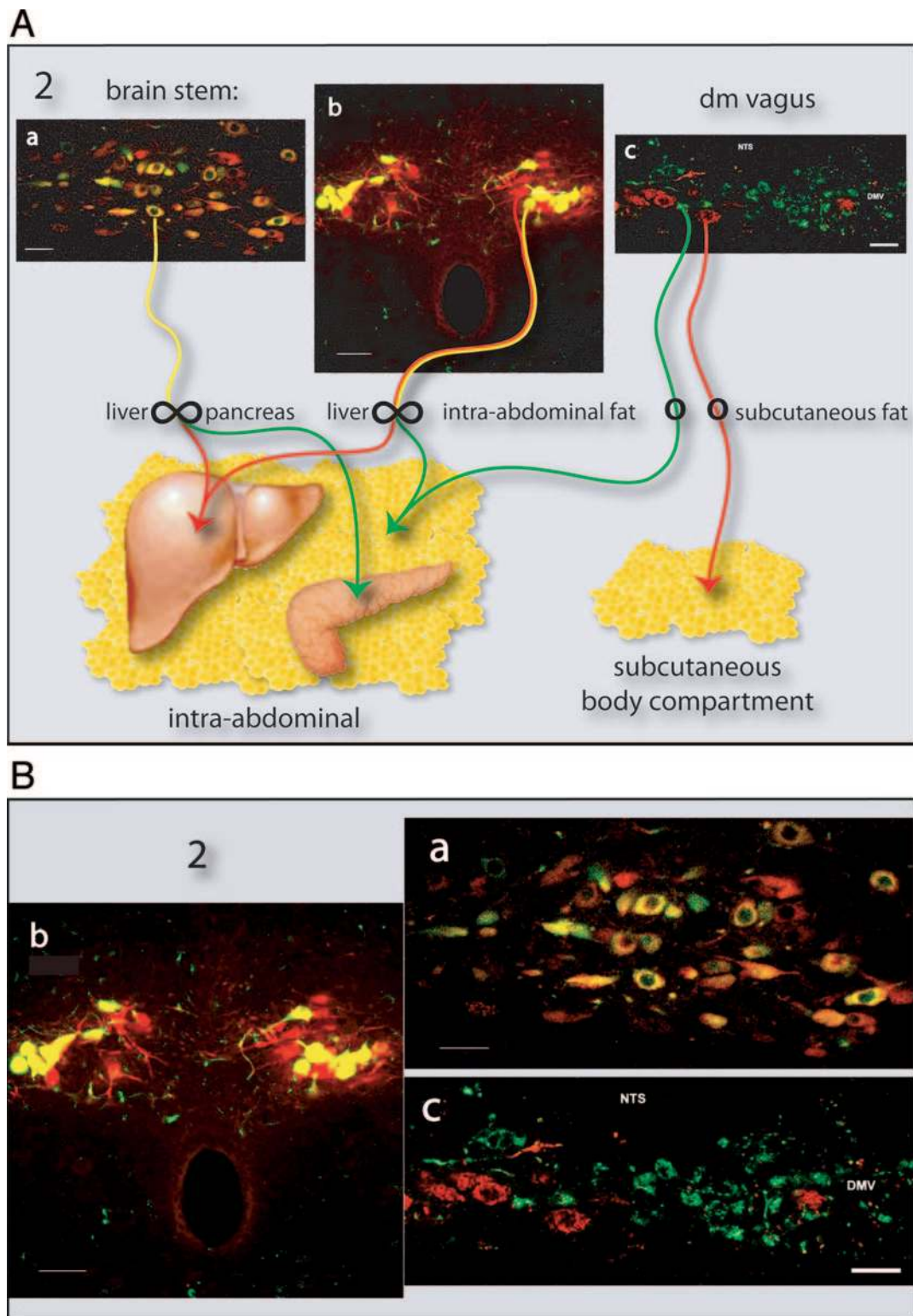
FIG. 2. Liver, pancreas, and intraabdominal fat share one set of vagal motor neurons, but intraabdominal and sc fat have distinct sets of neurons, demonstrated by laser scanning microscopy. A, Two microliters of 1% CTB-488 (fluor-conjugate CTB), CTB-555, or CTB-647 was injected alternately into liver and pancreas. Colocalization (*yellow neurons*) in the DMV demonstrates a shared autonomic control. B, The liver was sympathetically denervated and injected with 2 μl of the retrograde tracer PRV; simultaneously, 2 μl of 2% CTB was injected into retroperitoneal fat. As in A, colocalization in the DMV demonstrates a shared autonomic control of liver and intraabdominal fat. C, We reported earlier that sc and intraabdominal fat do not share their neuronal input. These experiments demonstrate that the brain controls the intraabdominal compartment with the same autonomic neurons, in contrast to a different set of neurons that project the sc compartment. *Bar middle*, 100 μm; *right* and *left*, 50 μm.
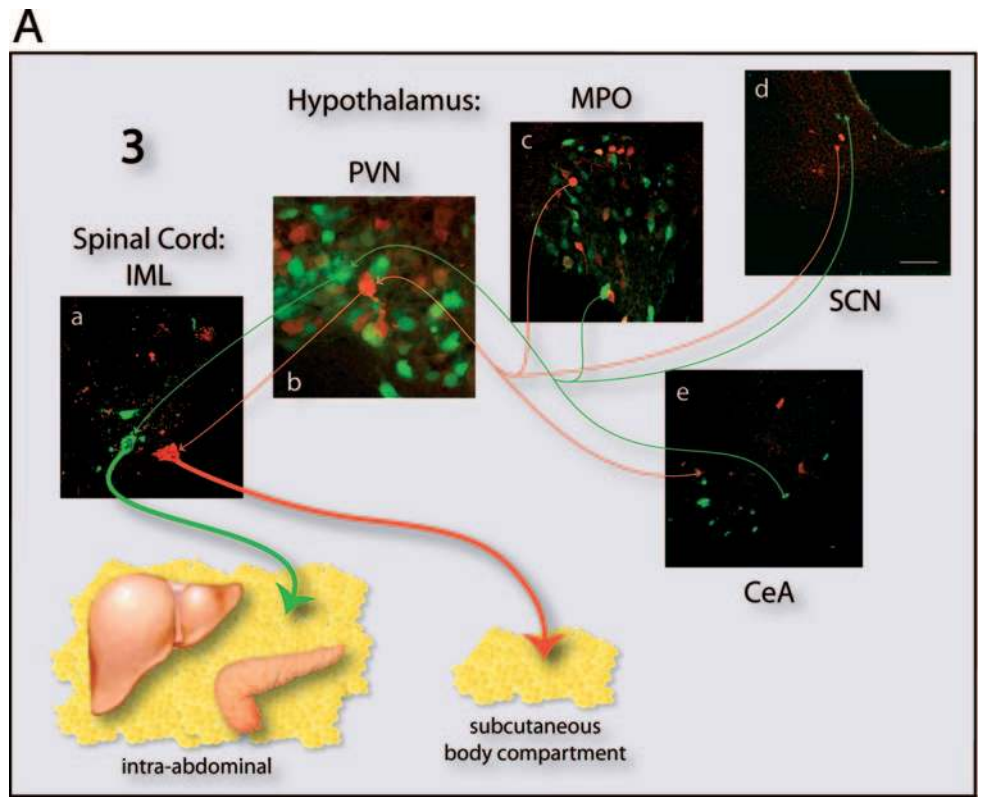
FIG. 3. Selective neurons in hypothalamus, amygdala, and spinal cord project to different body compartments. A, Five microliters of PRV B80 and PRV GFP were injected into parasympathetically denervated intraabdominal adipose tissue and in sc adipose tissue. As we reported earlier, the intermediolateral (IML) cell column of the spinal cord shows a separate control of the compartments; therefore, the survival time of the animals was chosen such that either only second order or third order neurons were labeled. B, In an upstream direction, the PVN of the hypothalamus shows specialized sets of neurons projecting to only one compartment. The same specialization can be seen in the MPO (C), the central biological clock of the hypothalamus (SCN, D), and the amygdala (E). (*Bar* in SCN: IML/PVN = 50 μm; MPO/CeA/SCN, 100 μm)
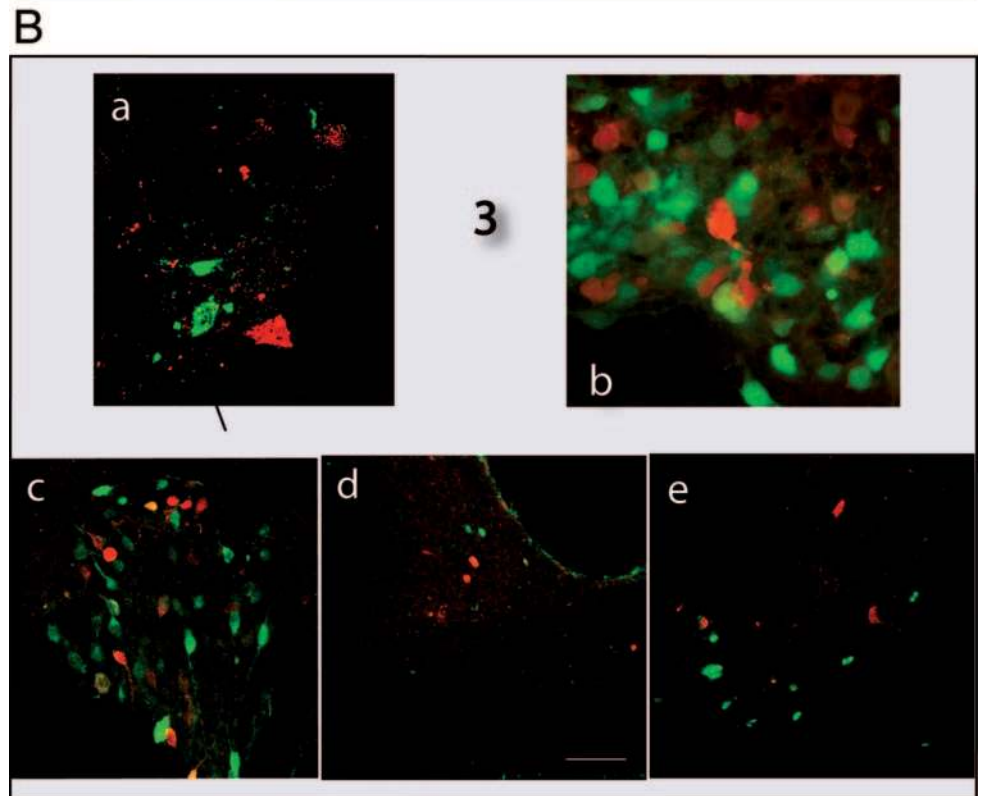
autonomic balance of the whole body, the picture becomes confusing; some authors find a high sympathetic tone, others find a high parasympathetic tone, and a third group finds low sympathetic and parasympathetic tone in patients with T2 diabetes (53–56).

Our experiments show a neuronal network that might

control the body per compartment. These observations indicate that the brain may group the organs by anatomic location. We reveal a shared parasympathetic control of the abdomen that might connect one single neuron to visceral fat growth, hyperinsulinemia, and a fatty liver by a parasympathetic overweight (57). Using the first order tracer CTB, we demonstrate that liver, pancreas, and intraabdominal fat indeed share the same vagal motor neurons. In contrast, distinct sets of vagal motor neurons project either to intraabdominal or to sc fat (14).

Moreover, we describe the output of hypothalamus and limbic system to the intraabdominal and sc compartment using two different labels of the transneuronal retrograde tracer PRV. Tracing from sc 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 sc and sympathetically denervated retroperitoneal fat, no colocalization was found (14). 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 ventromedial hypothalamus (58). As to the function of such differentiation, it has been proposed that the hypothalamic temperature center, the MPO, might selectively activate the projections to the intraabdominal compartment to mobilize energy in times of low food and low temperature by burning specifically visceral fat which means that the isolation layer of the body, the sc fat, can then be spared (59–63).

In the amygdala, we revealed separated groups of neurons projecting either to the intraabdominal or to the sc body compartment. Earlier studies showed that amygdala lesions lead to a change in body composition in favor of fat, hyperinsulinemia, and impaired skin conduction (64, 65). The direct connections to brain regions that process smell and taste suggest that the amygdala prepare the body for upcoming food. When food is detected by the nose, a specific parasympathetic activation of the intraabdominal compartment by the amygdala might induce secretion of insulin and enhance the uptake of substrate in fat tissue and liver.

Our group 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 (49, 57, 66–68). Here, we show that indeed a somatotopic organization exists up to the biological clock (SCN) in the hypothalamus. Possibly, 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 at the beginning of the active phase of the day (69–71). Inap-

propriate timing and protracted activation of the shared vagal input to intraabdominal compartment (intraabdominal fat, pancreas, and liver) might lead to intraabdominal 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 current thinking, the proposed failure of the brain in T2 diabetes might be caused by a genetic or developmental defect (72). 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 compensating for this by physical activity might induce confusing feedback to the brain (73–75).

Because energy homeostasis is warranted by countless mechanisms in our body, it is unlikely that one single cause of T2 diabetes will be identified. Unbalanced food intake is not an endocrinological disease that could be cured by a 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. In our opinion, future experiments that address the physiological relevance of the neuroanatomical network established in the present paper should incorporate the cross talk between blood-borne factors and neurons in their experimental design.

Acknowledgments

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