Hypothalamic neural networks in control of glucose homeostasis
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General discussion

Metabolic feedback to the SCN

The dramatic metabolic phenotypes of some mice with clock gene mutations \(^{254}\) and the association of other clock gene mutations with type 2 diabetes in humans \(^{651}\) have snowballed the interest for the circadian control of energy metabolism in recent years. The circadian timing information is coming from the central biological clock, which is presented in the suprachiasmatic nuclei (SCN) located in the anterior hypothalamus, and synchronizes the daily patterns in locomotor activity, feeding behavior and energy homeostasis. The zeitgeber properties of photic and behavioral inputs to the clock are well established, but the renewed interest has evoked the question whether the central biological clock also receives (feedback) information about the bodily energy homeostasis and fuel supply. This question is all the more relevant as other evidence has emerged recently showing that diet itself affects circadian rhythms and clock gene expression \(^{426, 652-657}\). Thus, in spite of all the evidence that the light-entrainable oscillator and the feeding-entrainable oscillator are separate entities, for instance, because SCN lesions do not eliminate food anticipatory behavior \(^{437, 658}\), it is also evident that energy metabolism and nutrition influence SCN activity. For instance, SCN neurons express receptors for metabolic hormones, such as insulin \(^{319, 659}\), leptin \(^{660}\) and ghrelin \(^{321}\), and they are also sensitive to glucose \(^{661}\). Therefore, a direct input of metabolic information to the SCN seems possible, but little information is available and it is not clear how well this metabolic information can pass the blood-brain-barrier. Leptin informs the brain about the body’s nutritional status and its secretion can be modulated by feeding, glucocorticoids and insulin, but its daily rhythm is timed by the SCN \(^{662}\). In vitro studies have shown that leptin can phase advance the circadian rhythm of SCN neurons at most circadian times, except at the late subjective night \(^{663, 664}\). Ghrelin, the natural antagonist of leptin, with its daily changes in plasma concentrations also being timed by the SCN \(^{665, 666}\), can phase advance the circadian rhythm of SCN neurons, when applied in vitro at circadian time 6 \(^{399}\). The limited evidence for direct and effective metabolic inputs onto the SCN might implicate that this is not a well-studied mechanism or that this is not the major metabolic feedback mechanism. A plausible explanation for the latter might be that in order to reach the SCN an active transport system should be present for these hormones in order to be able to pass the blood-brain-barrier and reach the SCN. Therefore, we hypothesized that the metabolic feedback to the SCN would depend on indirect pathways, involving the circumventricular organs. For instance, in the hypothalamic arcuate nucleus (ARC), circulating metabolic information is already highly integrated, as insulin, leptin, ghrelin, glucose and glucocorticoids all have their specific effects on the ARC neurons. The data pre-
sent in Chapters 3 and 4 show that indeed the ARC-SCN pathway may be important for relaying metabolic information to the SCN. The results of Chapter 5 show that next to the circumventricular organs, metabolic information might also reach the SCN through the dorsomedial hypothalamus (DMH). Clearly the metabolic information from the DMH is different from that provided by the ARC as it probably consists of the integrated inputs from circumventricular organs, the brainstem and sleep centers. DMH-NPFF neurons are mainly sensitive to feeding signals such as food anticipation and fasting-refeeding. We hypothesize that the NPFF-SCN projection onto the light-sensitive VIP neurons is necessary to lift the inhibitory effect of the SCN on daytime vigilance and feeding behavior.

Clock perspectives
We propose that through the ARC the SCN has the ability to selectively sense circulating metabolic information representing the peripheral energy status. In addition, the SCN keeps itself informed about the energetic status of the body in a broader sense via the DMH. In this way the SCN guarantees to time the whole body physiology and behavior in the most efficient way.

Hypothalamic neuropeptides and the control of glucose homeostasis
Our present studies, but also recent studies from others, have evidenced the involvement of several hypothalamic neuropeptides in the control of hepatic insulin sensitivity and glucose production. Each of these peptides has its own divergent physiological function in the control of food intake and energy homeostasis, but in addition they seem to share common signaling pathways and to target similar brain areas and brain output pathways.

Neuropeptide Y (NPY)
Best known among these hypothalamic neuropeptideergic networks are the NPY-containing neurons in the ARC with their projections to several hypothalamic brain areas including the PVN. The first report on the glucoregulatory effects of the hypothalamic NPY system appeared in the mid nineties when it was shown that i.c.v. administration of NPY increases endogenous glucose production in rats, probably by decreasing hepatic insulin sensitivity. Later on these results were confirmed in mice. In view of the inhibitory effects of hypothalamic insulin receptors on hepatic glucose production, the abundant expression of insulin receptors in the ARC, the inhibitory effect of insulin on NPY neuronal activity, and the effects of i.c.v. NPY on sympathetic activity, we decided to test whether NPY could be the hypothalamic intermediate between the insulin receptors in the ARC and the pre-autonomic neurons in the PVN. Hereto we combined the euglycemic hyperinsulinemic clamp
technique with the i.c.v. administration of NPY, and performed these experiments in hepatic sympathetic-, hepatic parasympathetic- and hepatic sham-denervated rats. Our results confirmed that i.c.v. NPY is able to block (partially) the inhibitory effects of peripheral hyperinsulinemia on hepatic glucose production, but they also showed that a specific denervation of hepatic sympathetic nerves blocks the effect on hepatic insulin sensitivity of NPY. Therefore, the brain-mediated inhibitory effect of insulin on hepatic glucose production is probably effectuated via an inhibition of NPY neuronal activity in the ARC. Subsequently, the resulting diminished release of NPY will decrease the stimulatory input to the sympathetic pre-autonomic neurons in the PVN and thus reduce the sympathetic stimulation of hepatic glucose production. The results of Pocai et al. 157, however, show that also the parasympathetic innervation of the liver is involved in the inhibitory effect of insulin on hepatic glucose production. This means that in addition to the effect of NPY on the sympathetic pre-autonomic neurons there is probably another neurotransmitter that is responsible for the transmission of insulin's effects in the ARC to the parasympathetic pre-autonomic neurons in the PVN. Moreover, the effects of NPY also seem to be specific for glucose production as in none of the above experiments there was a significant effect on whole body glucose disposal.

Pro-opiomelanocortin (POMC)

Next to the orexigenic NPY/AGRP neurons, the ARC also contains a population of anorexigenic POMC/CART-containing neurons. The most important POMC-derived peptide with respect to feeding and metabolism is alpha-MSH. The antagonistic function of the NPY/AGRP and POMC/CART cell populations is most clearly illustrated by the fact that AGRP acts as an endogenous antagonist of the melanocortin receptors 3 and 4. The antagonizing mechanism of these neuropeptides are extremely important to adapt the hypothalamic–pituitary–thyroid (HPT) axis to the prevailing food/energy status, i.e., the fasting-induced suppression of TRH mRNA in the PVN needs the reduction in alpha-MSH and the increase in AGRP. Surprisingly, this antagonistic cell population does not seem to be involved in the inhibitory effect of hypothalamic insulin on EGP, as co-administration of a melanocortin antagonist failed to block the decrease in EGP induced by central insulin 210. Blocking alpha-MSH signaling via i.c.v. infusion of the melanocortin 3/4 receptor (MC3R/MC4R) antagonist SHU9119 has no effects on glucose metabolism, but i.c.v. infusion of alpha-MSH itself has a clear stimulatory effect on EGP via gluconeogenesis (GNG) which can be antagonized by SHU9119. In the liver, the stimulation of GNG is confirmed by the increased expression of G6Pase and PEPCK. These central manipulations had no effect on peripheral glucose uptake. It has been proposed that the hypothalamic MC3R/MC4R signaling pathway mediates the effect of systemic leptin on EGP. Central administration of leptin has been proven to be involved in the autoregulation
of hepatic glucose output, i.e. an increase in GNG with a concomitant decrease in glycogenolysis without changing total glucose production. Recently, it was nicely shown that the adenoviral-induced expression of leptin receptors in the ARC of leptin receptor knock-out animals improves glucose tolerance via enhanced suppression of EGP. The ARC-induced expression of the leptin receptor was associated with a reduced hepatic expression of G6Pase and PEPCK, but again, no significant changes in the insulin-stimulated whole body glucose utilization were apparent. Moreover, the effects of hypothalamic leptin signaling on hepatic insulin sensitivity could be blocked by a selective hepatic vagotomy, providing further supportive evidence for the idea that ARC projections to pre-autonomic neurons (in the PVN) are important for the transmission of the effect of leptin on EGP.

**Orexin**

The neuropeptides orexin-A and orexin-B (also known as hypocretin-1 and hypocretin-2) were initially identified as the endogenous ligands for orphan receptors involved in the pathogenesis of narcolepsy. They were recognized as regulators of feeding behavior and energy metabolism because of the exclusive localization of their cell bodies in the lateral hypothalamus (LH), the induction of feeding upon their i.c.v. administration, their responsiveness to peripheral metabolic cues such as leptin and glucose, and the metabolic phenotype of the knock-out animals. More recent studies suggest that the orexin system is particularly important for the maintenance of wakefulness. However, the experiments described in Chapter 6 clearly revitalize the concept of the metabolic control function of the orexin system. Our data show that an increased availability of orexin in the central nervous system, either by i.c.v. infusion, or by local activation via removal of GABA inhibition, increases plasma glucose concentrations through an increase in hepatic glucose production. As with NPY also the stimulatory effect of orexin on EGP could be blocked by a hepatic sympathetic but not parasympathetic denervation. From the results described in Chapter 6 it is not entirely clear yet where in the brain orexin is acting to stimulate EGP. The i.c.v. infusion experiments and the presence of a pronounced orexin-containing fiber network in the PVN suggest that its main action is again at the level of the sympathetic pre-autonomic neurons in the PVN, but in view of the electrophysiological data of Van Den Top et al, a direct effect of orexin at the level of the sympathetic pre-ganglionic neurons in the intermediolateral column of the spinal cord can also not be excluded. Unfortunately, the selective liver denervations do not allow for a distinction between these 2 options. In addition, it is not exactly clear yet what the endogenous triggers are for the stimulatory effect of orexin on EGP, but we propose at least 2 possible pathways: 1. The orexin neurons could be an alternative pathway for the ARC to affect EGP, i.e., in addition to a direct projection to the pre-autonomic neurons in the PVN.
Indeed viral tracing studies have shown second order labeling in orexin neurons after tracer injections in the liver, i.e., orexin neurons are also pre-autonomic neurons 512. In addition, the orexin neurons might also integrate circadian information. Our and other studies clearly showed that the activity of the orexin neurons is under tight control of a GABAergic input that is probably derived from the circadian system 492, 680. These data indicate that the circadian rhythm in orexin release 681 might be implicated in the genesis of the circadian rhythm in plasma glucose concentrations. In order to test this hypothesis, we administered the orexin antagonist SB-408124, either i.c.v. or i.v., during the final 8 hours of the light period and simultaneously measured glucose appearance (Ra) from ZT3-ZT15 with the isotope dilution technique. The i.c.v. administration of the orexin-antagonist completely blocked the endogenous increase in Ra until the start of the dark period. Once the animals start eating, in the dark period, Ra also increases in the i.c.v. orexin-antagonist treated animals. This i.c.v. administration of the orexin-antagonist did not inhibit food intake. Together these data strongly suggest that the perifornical orexin neurons are an important link in the circadian control of the daily peripheral glucose rhythm. Hereby the orexin system provides an example of hypothalamic integration, as the increased activity of the orexin system at the end of the light period not only initiates the wake state but at the same time also ensures a sufficient supply of energy. This may also explain the recently discovered correlation between sleep duration and type 2 diabetes 682-684. We hypothesize that short sleep may cause an over activation of the orexin system, and thereby a disproportionate increase in EGP (Chapter 6).

**Melanin-concentrating hormone (MCH)**

MCH is a cyclic 19-amino-acid polypeptide the expression of which is limited to the lateral hypothalamus, zona incerta and perifornical area, very similar to orexin. However, despite the almost complete overlap in their distribution, the two peptides do not co-localize. The MCH neurons have been implicated as an additional important regulator of food intake, because the central administration of MCH promotes feeding, MCH mRNA levels rise as a result of starvation and leptin deficiency, knock-out animals are hypophagic and lean 506, 685, 686, and MCH neurons are essential for the leptin-deficient phenotype 687. Also over expression of MCH results in hyperglycemia 688. Despite these earlier observations, we found no effect on glucose metabolism of either i.c.v. administered MCH in wild type rats (Chapter 6) or of the MCH knock-out in the MCH knock-out rat (Chapter 7). In fact, the reduced metabolic rate we found in the MCH knock-out rats was perfectly adapted to the leaner body composition of these animals. Of course, these data do not exclude a role for MCH in glucose metabolism; however, apparently at present we have not been able to find the right stimulus to reveal its function in glucose homeostasis.
Pituitary adenylate cyclase activating peptide (PACAP)

PACAP is a 38-amino acid, C-terminally α-amidated neuropeptide, that was originally isolated from the ovine hypothalamus on the basis of its ability to stimulate adenyl cyclase activity in rat anterior pituitary cells. Studies conducted in rodents have shown that PACAP exerts a wide array of biological activities both in the CNS and in peripheral organs. Again the results from knock-out studies indicated the involvement of PACAP in glucose metabolism. However, these studies did not reveal which part of the metabolic phenotype could be attributed to central signaling pathways of PACAP, although some evidence for central effects on energy metabolism was available. Amongst others it has been shown that in the brain PACAP decreases food intake and increases plasma glucose. The data presented in Chapter 8 clearly show that i.c.v. administered PACAP causes a strong increase of EGP. The additional experiments described in Chapter 8 provide strong evidence that also the effects of PACAP are mediated through the pre-autonomic neurons in the hypothalamus. Contrary to the neuropeptidergic systems discussed above, PACAP-producing neurons do not show a restricted localization, but are widespread throughout the CNS. Prominent populations of PACAP neurons can be found in the ARC and the VMH, but the PACAP innervation in the PVN is also derived from other sources such as the brainstem and the BNST. Since, at present only little is known about the stimuli that modulate PACAP release, it is not clear yet what the physiological function of PACAP could be. In Chapter 8 we speculate that it could be involved in the counter-regulatory response to hypoglycemia, but it could also be involved in the hyperglycemic stress response or in the effects of estrogen on glucose metabolism.

Glucagon-like peptide 1 (GLP-1)

GLP-1 is preproglucagon-derived hormone that is secreted from the L-cells in the distal gut in response to meals and is known to decrease food intake in rodents and humans. In addition, GLP-1 also serves as an incretin as it potently augments the release of insulin during food intake. The anorectic effects of GLP-1 are probably mediated through both peripheral and central mechanisms, as a population of GLP-1 positive neurons is located in the brainstem and projects to hypothalamic and brainstem areas important in the control of energy homeostasis. GLP-1 is also involved in glucose metabolism and may lower plasma glucose levels through multiple mechanisms, including central mechanisms. First, Knauf et al demonstrated that during hyperglycemia i.c.v. administered GLP-1 decreases non-insulin-dependent muscle glucose uptake. Although the hypothalamic GLP-1 projections from the brainstem target both the PVN and the ARC, it was shown that direct administration of GLP-1 in the ARC, but not in the PVN, reduces EGP. On the other hand, GLP-1 administration in the PVN causes a decrease in food intake. Interestingly, GLP-1 receptor mRNA is...
also expressed in ~70% of the ARC-POMC neurons. These data seem to suggest that both central and peripheral GLP-1 can work synergistically to regulate food intake and glucose homeostasis. Moreover, they provide a nice example of how the same molecule can work in the periphery as a hormone, and in the CNS as a neurotransmitter.

Interestingly, the results of the dexamethasone infusions described in Chapter 9 show a similar differentiation of the function of PVN and ARC in glucose metabolism. Dexamethasone administration in the ARC, but not in the PVN, decreases hepatic insulin sensitivity. One obvious difference between NPY, orexin and PACAP at the one hand, and GLP-1 and dexamethasone at the other hand, is of course that the first three are (hypothalamic) neurotransmitters that serve as an afferent input to the PVN from other brain areas (amongst which probably the ARC), whereas the latter two are (agonists of) circulating factors acting on the ARC via the systemic circulation. In fact, the PACAP, NPY or orexin might be involved in signaling the effects of peripheral changes in GLP-1 or glucocorticoids via the ARC to the PVN.

**Liver perspectives**

Most of the neuropeptides just discussed regarding their role in the control of EGP share a common effective hypothalamic area, the PVN, although not for all of them it has been proven yet that they regulate glucose metabolism specifically via the PVN. But, in our studies and studies of others, central administration of orexin-A (Chapter 6), PACAP-38 (Chapter 8), NPY \(^{697}\) and synthetic MC3R and MC4R agonist \(^{698}\) are all associated with Fos immunoreactivity in this nucleus. Moreover, for the PACAP-38 induced Fos-ir neurons in the PVN we have shown that they project to the sympathetic pre-ganglionic neurons in the spinal cord. Since some of these peptides are orexigenic (orexin, NPY, MCH), while others (POMC, PACAP) are anorexigenic, this suggests that the mechanism of feeding regulation is separated from that of glucoregulation. Secondly, sympathetic and parasympathetic pre-autonomic neurons in the PVN are separated \(^{699}\); this brings about the question, whether the neuropeptideergic effects we just described can be categorized into two groups, depending on their specific effects on either sympathetic or parasympathetic output pathways. In our studies, the orexin-A and PACAP-38 induced hyperglycemia can only be blocked by hepatic sympathetic but not parasympathetic denervation. Also in the case of NPY, although it does not influence basal glucose turnover, the suppressive effect on hepatic insulin sensitivity is only blocked by hepatic sympathetic denervation. On the other hand, it has been shown that insulin and leptin signaling in ARC influence hepatic insulin sensitivity also via the vagal nerves, supposedly via another type of neurotransmission from the ARC (to the PVN) to influence pre-autonomic neurons in the hypothalamus. Clearly, further studies combining neuroanatomy and physiology are necessary to reveal this “parasympathetic pathway”.

Clinical implications
Obesity, insulin resistance and type 2 diabetes represent a primary health and economic threat for modern societies. How obesity interferes with glucose metabolism is still matter of debate, but it is clear from the above that disorders in autonomic nervous activity or hypothalamic neuropeptidergic signaling may contribute in a major way to hepatic insulin resistance. Future pharmacologic treatments therefore could aim to restore the neuropeptidergic milieu of the hypothalamus or the balance of the hypothalamic outputs to the sympathetic and parasympathetic branch of the autonomic nervous system.