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## GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Adapted from:

- Sympathetic nervous system control of triglyceride metabolism: novel concepts derived from recent studies. *J Lipid Res.* 2014, 55:180-9
- Brown fat as a novel target to attenuate hyperlipidemia and atherosclerosis. *Circ Res.* 2015, *in press*
- Regulation of brown fat by AMP-activated protein kinase. *Trends Mol Med* 2015, *in press*
- Hypothalamic regulation of brown fat activity. *Trends Endocrinol Metab* (2015) *in press*

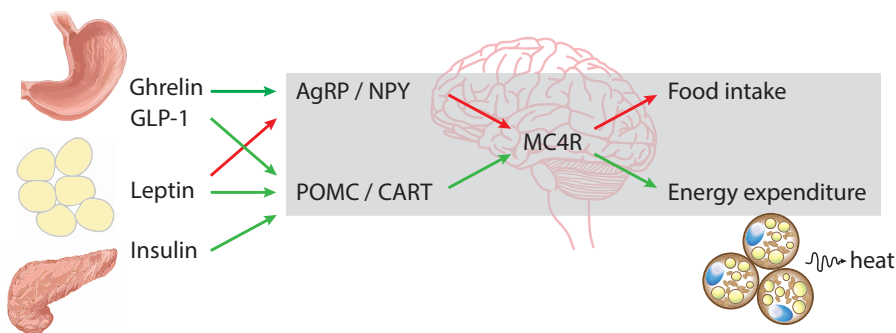


One of the first studies confirming a crucial role of the nervous system in homeostasis dates back to 1942 when Hetherington and Ranson described that hypothalamic lesioning of rats frequently causes obesity by increasing food intake and decreasing energy expenditure (1). In following fascinating experiments, Hervey surgically connected the circulatory system of hypothalamic lesioned rats via subcutaneous tissues to normal rats. In these parabiotic experiments, the normal rats that were connected to the obese rats decreased their food intake and lost weight (2). About 35 years later, leptin was isolated and identified as the satiety signal (3) that was overproduced in obesity but to which hypothalamic lesioned rats could not respond.

Today, the hypothalamus and autonomic nervous system (ANS) are increasingly recognized as the regulators of body homeostasis and as possible treatment target in obesity and related disorders including type II diabetes (T2D) and cardiovascular disease (CVD). This thesis further revealed the role of the ANS in the control of lipid metabolism and inflammation, and identified pathological consequences of disturbed regulation. We found that mainly the parasympathetic nervous system (PNS) is required for maintaining anti-inflammatory reflexes, while the sympathetic nervous system (SNS) is highly important for energy expenditure and lipid metabolism, largely via regulating brown adipose tissue (BAT) activity.

### Hypothalamic multi-tasking to maintain energy balance

Leptin was initially discovered as the satiety signal lacking in genetically obese *ob/ob* mice (3). More recent studies specified the central melanocortin system as main target of leptin (4). Leptin and other anorexigenic peptides including insulin, glucagon-like peptide-1 (GLP-1), peptide YY (PYY) and cholecystokinin (CCK) act by activation of cocaine- and amphetamine-regulated transcript (CART)/pro-opiomelanocortin (POMC)-expressing neurons and suppression of neuropeptide Y (NPY)/Agouti-related protein (AgRP)-expressing neurons, resulting in activation of melanocortin 4 receptor (MC4R) signalling (**Figure 1**). Probably because of MC4R's integral role, MC4R deficiency is the most common form of monogenic obesity (5) and associated with increased risk of cardiovascular disease. In addition to increased food intake in MC4R deficient individuals, energy expenditure is lower and nutrient usage shifts from fatty acid oxidation towards carbohydrate oxidation (6). Similarly, we (**Chapter 2**) and others (6) have shown that inhibition of MC4R signalling in mice by intracerebroventricular (ICV) administration of the synthetic antagonist SHU9119 increases food intake and weight gain, and also reduces fat oxidation. Interestingly, we observed that the increase in adiposity was also present in mice receiving ICV SHU9119 that were limited in food intake to the amount consumed by the control group, indicating that the reduction in fat oxidation contributes to the increase in fat mass. Apparently, the central melanocortin system exerts a dual-role in maintaining energy balance, namely by regulating energy intake as well as by regulating energy



**Figure 1 – The melanocortin system as central regulator of food intake and energy expenditure.** See text for details. AgRP, agouti-related protein; CART, cocaine- and amphetamine-regulated transcript; GLP-1, glucagon-like peptide-1; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; POMC, pro-opiomelanocortin. Red arrow, inhibition; green arrow, stimulation.

expenditure. Indeed, ICV administration of leptin (7), insulin (8) and GLP-1 (**Chapter 3**) result in both reduced food intake and an increase in energy expenditure, while ghrelin that is produced by the stomach upon fasting exerts opposing effects (9). By performing dedicated mechanistic experiments as described in this thesis, we now identified regulation of BAT activity as mechanism underlying the changes in energy expenditure (**Chapters 2 and 3**).

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Considering the importance of energy balance for survival, in addition to the response to satiety hormones, the brain also directly monitors energy status of the body. High energy availability drives *de novo* lipogenesis not only in metabolic organs but also in the hypothalamus, yielding increased levels of malonyl coenzyme A (CoA) and long-chain fatty acyl-CoAs that signal the need to reduce food intake. Correspondingly, ICV administration of long-chain fatty acids, *e.g.* oleic acid, reduces food intake (10). Conversely, ICV administration of inhibitors of fatty acid synthase (FAS) has a profound orexigenic effect (11). Interestingly, fatty acid synthesis is tightly regulated/inhibited by 5' adenosine monophosphate-activated protein kinase (AMPK), that is a cellular energy sensor by itself being active upon low energy availability (high AMP/ATP). This mechanism also mediates at least part of the GLP-1-mediated reduction in food intake. GLP-1 signalling lowers hypothalamic AMPK activity thereby increasing neuronal fatty acid synthesis resulting in decreased expression of orexigenic peptides and increased expression of anorexigenic peptides and finally reduced food intake. Consistent with this pathway, both ICV administration of AICAR [potent AMPK activator] and an adenoviral vector overexpressing constitutively active AMPK diminish weight loss induced by the GLP-1 analogue liraglutide (12).

So far, we have discussed that the hypothalamus monitors the body's energy status directly (e.g. by fatty acid synthesis and AMPK activity) and indirectly (e.g. signaling induced by anorexigenic and orexigenic peptides) and responds by regulating both food intake and energy expenditure. This dual mode of action makes the hypothalamus and more specifically the melanocortin system an attractive target in the treatment of obesity and related disorders. In fact, GLP-1 analogues (e.g. exenatide, liraglutide and dulaglutide) and DPP-4 inhibitors (e.g. sitagliptin, saxagliptin and alogliptin) that prevent the breakdown of GLP-1, are already implemented in the treatment of T2D. The potential of these drugs to prevent CVD remains to be determined and future studies may focus on combinations of drugs targeting multiple aspects of the central melanocortin system thereby enhancing efficacy.

### Hypothalamic (dys)regulation of circadian rhythms

Via several pathways, feeding provides negative feedback to the hypothalamus in order to enhance satiety thereby reducing food intake. In addition, feeding behavior is a so-called Zeitgeber for the central clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and for peripheral clocks that drive circadian rhythms. In general, disturbed circadian rhythmicity leads to adiposity. For example, dim light at night [13] shifts circadian rhythmicity and increases body fat mass. When we exposed mice to more hours of light per day (*i.e.* 16 h or 24 h compared to regular 12 h), we observed increased adiposity (**Chapter 4**). Genetic mouse models of circadian dysfunction, among others *Clock<sup>mt/mt</sup>* mice [14] and *Per2<sup>-/-</sup>* mice [15], are also prone to develop obesity. Lesioning of the central circadian clock in the SCN in mice results in disturbed rhythmicity and an acute induction of weight gain [16]. The underlying mechanism may include timing of feeding that is disturbed in these animal models. This is reflected by mouse studies showing that feeding restricted to the light phase (*i.e.* the inactive period) leads to increased body weight gain compared to dark phase restricted feeding [17,18]. Conversely, restricting food intake to the active period of mice was shown to prevent diet-induced obesity [19,20].

In humans, plasma levels of lipids display diurnal variations independent of food intake [21], suggesting that the circadian clock is an important determinant of lipid levels. Consequently, disturbed rhythmicity may lead to dyslipidemia and eventually CVD. In fact, already in 1949 a Scandinavian observational study among factory workers reported an association between shift-work and cardiovascular mortality [22]. The underlying mechanism remained elusive. Meanwhile, the behavioral patterns of human activity, especially in industrialized countries, have undergone dramatic changes with respect to adherence to day and night rhythms. For example, 1) electrical light has uncoupled behavioral active period from the natural occurring day, 2) aircraft travel has resulted in jet-lag phenomenon, and 3) the 24-hour economy necessitates working at night and social activities are

shifted, simultaneously affecting sleep duration. Notably, the increasing prevalence of obesity is associated with disrupted sleep-wake pattern (23) and coincides with the availability of artificial light (24,25). However, for these association studies, it is rather difficult to identify causality. For example, shift-work leads to changes in locomotor activity, food intake and light exposure that all potentially affect circadian rhythmicity, adiposity and the development of CVD. Randomized clinical trials and animal studies are needed to determine the contribution of the different factors to metabolic health and to design specific intervention strategies.

Circadian rhythms are not limited to energy metabolism but are also present in other factors involved in the development of CVD, including blood pressure (26), thrombotic factors (27) and the immune system (28). Maintenance of circadian rhythmicity may be an additional strategy to prevent and treat human dyslipidemia and CVD. This could be achieved by for example providing advice on life style changes regarding sleep and food intake behavior. Currently, the feasibility of extending sleep duration by giving sleep advice is being explored in randomized controlled trials. Pilot data show that sleep duration of obese individuals may be extended by from less than 6 hours to more than 7 hours for more than 20 nights out of 28 nights, resulting in body weight loss, appetite loss and improvement of sleep quality (29).

## Sympathetic activation of brown fat to protect against dyslipidemia and CVD

Retrograde tracing in Syberian hamsters identified neuroanatomical connections between the SCN and BAT (30), indicating possible involvement of BAT in the associations between disturbed circadian rhythm and obesity. Indeed, we now showed that prolonging day light exposure not only changes circadian rhythmicity but also reduces sympathetic outflow towards BAT accompanied by decreased BAT activity and increased body fat mass in mice (**Chapter 4**). Recent evidence suggests that in humans, BAT activity is also physiologically regulated by the circadian clock. The detectability of BAT by [<sup>18</sup>F]fluorodeoxyglucose (FDG)-PET-CT imaging at room temperature follows a circannual cycle (31,32), with low detectability of BAT in summer (*i.e.* short day) as compared to winter (*i.e.* long day). Although differences in outside temperature over the year would be a likely explanation for this phenomenon, the detectability of BAT showed a stronger correlation with day length than with outside temperature (31). Likewise, we demonstrated that exposure of mice to short day length of 8 h enhances BAT activity reflected by the total daily uptake of lipids from the circulation when compared to 12 and 16 h of light (**Chapter 5**). It is tempting to speculate that this seasonal adaptation of BAT activity to day length precedes changes in temperature and thereby prepares the body for upcoming changes in ambient temperature. Future studies should reveal the contribution of prolonged

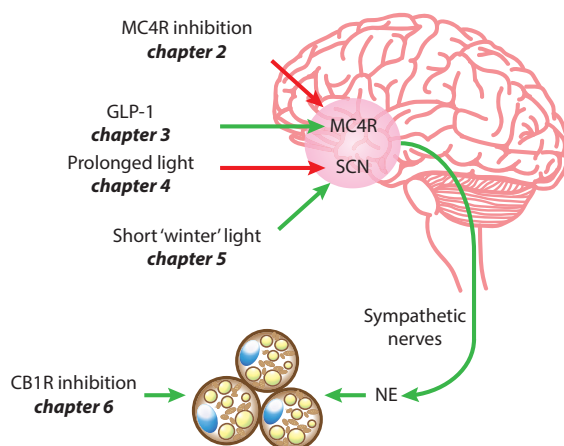
light exposure, shift-work and other circadian disturbances on human BAT function and metabolism.

The importance of the SNS for BAT function is reflected by the number of nerve endings in the tissue. Each brown adipocyte is in close proximity to a nerve ending that releases norepinephrine upon sympathetic activity initiated by for example cold exposure. In addition to its crucial role in non-shivering thermogenesis, BAT is probably required for maintaining energy balance and is activated upon overeating, a process called diet-induced thermogenesis (DIT). Although the existence of DIT is under debate (33), we and others have identified the central melanocortin system, crucial in the regulation of food intake and energy expenditure as described above, as an important determinant of BAT activity. Inhibition of MC4R receptor signaling reduces BAT activity (**Chapter 2**), while activation of the melanocortin system by ICV administration of GLP-1 (**Chapter 3**), insulin (34) and leptin (35) results in enhanced sympathetic outflow and activation of BAT. These studies suggest that reduced BAT activity may underlie at least part of the obese phenotype in MC4R deficient individuals, and underscore the potency of targeting the melanocortin system as therapeutic target for obesity and related disorders.

In humans, BAT activity, as quantified by [<sup>18</sup>F]FDG PET-CT scan, inversely correlates with body mass index and body fat mass (36-39). BAT activation by cold exposure alleviates hypertriglyceridemia through its large potential to take up and combust TG-derived fatty acids, at least in mice (40,41) and likely also in humans (37). Other potent BAT activators that act via the SNS are thyroid hormone (42), estradiol (43), nicotine (44) and bone morphogenetic protein-8 (45). Alternatively, BAT can be activated directly by for example metformin (46), irisin (47), salsalate (48) or CB1R inhibitors (**Chapter 6**), the latter being dependent on sympathetic signaling. Central and peripheral contributors to BAT activity as described within this thesis are summarized in **Figure 2**. SNS-mediated activation of BAT and subsequent lowering of plasma TG can be pharmacologically mimicked by  $\beta$ 3-adrenergic receptor ( $\beta$ 3-AR) agonists in mice (49,50). Interestingly, a recent study showed that the  $\beta$ 3-AR agonist mirabregon acutely increases BAT activity in human volunteers. Future chronic studies are evidently needed to illuminate the role of  $\beta$ 3-AR agonism in human obesity and hyperlipidemia.

The primacy of BAT activation on hypertriglyceridemia is clear, however, the effect on hypercholesterolemia, which especially underlies development of atherosclerosis, is less well known. Experimental studies report either reduced (46,51) or increased (52) levels of plasma cholesterol after BAT activation. Dong *et al.* (52) were the first to document an effect BAT activation on atherosclerosis development, and unexpectedly found that cold BAT activation via exposure aggravates atherosclerosis development in *Ldlr*<sup>-/-</sup> and *ApoE*<sup>-/-</sup> mice. However, it should be noted that BAT activation increases lipolytic processing of (V)LDL particles (41),





**Figure 2 – Regulation of brown adipose tissue by central systems and peripheral pharmaceutical compounds.** See text for details. CB1R, cannabinoid 1 receptor; GLP-1, glucagon-like peptide-1; MC4R, melanocortin 4 receptor; NE, norepinephrine; SCN, suprachiasmatic nucleus.

leading to enhanced formation of cholesterol-rich remnants that are normally taken up by the liver, mainly through ApoE-LDLR interactions, both of which obviously do not occur in *Ldlr<sup>-/-</sup>* and *ApoE<sup>-/-</sup>* mice. Strikingly, we recently showed that B3-AR agonism in *APOE\*3-Leiden.CETP* mice, with functional ApoE-LDLR interactions, not only alleviates hypercholesterolemia but also reduces atherosclerotic lesion area and lesion severity [49].

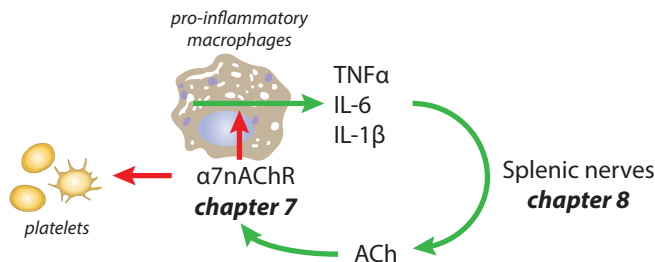
The effect of BAT activation on hypercholesterolemia and atherosclerosis development in humans is still largely unknown as only few studies investigated the effect of BAT activation on plasma cholesterol levels. One study reported that subjects with detectable BAT have lower plasma total cholesterol and LDL-cholesterol levels as compared to subjects without detectable BAT [53]. De Lorenzo *et al.* [54] showed that daily cold exposure of 20 min for 90 days reduced total cholesterol, LDL-cholesterol and body mass in hypercholesterolemic patients. These data show that SNS-mediated BAT activation may also improve hypercholesterolemia in humans and underscore its potential as anti-atherogenic treatment as well.

## Modulation of the immune response by the autonomic nervous system

Metabolic regulation and inflammatory responses are highly integrated. Salicylate and its derivative salicylate not only activate BAT, but also reduce NF- $\kappa$ B activity thereby lowering inflammation [48] and atherosclerosis [55]. Anti-inflammatory processes

allow skewing of macrophages from M1 macrophages to norepinephrine-producing M2 macrophages in BAT, thereby inducing similar responses as via SNS-mediated BAT activation (56). The ANS itself exerts a complex control on inflammation, which depends on bidirectional communication between the brain and the immune system. Lymphoid organs such as bone marrow and the spleen are mainly innervated by sympathetic noradrenergic neurons. Primary afferent visceral neurons are often not labelled parasympathetic or sympathetic but do form reflex circuits with the autonomic pathways. Neuroanatomical evidence is limited for the involvement of the parasympathetic nerves in immune organs and its functional existence is highly under debate (57). However, electrical stimulation of the vagus nerve does inhibit pro-inflammatory responses (58) and recent studies indicate evidence for presence and functional activity of parasympathetic branches in the spleen (59,60). We now observed that surgical dissection of these splenic parasympathetic nerves enhanced inflammation in mice (**Chapter 8**), consistent with disruption of the cholinergic anti-inflammatory reflex as proposed by Tracey *et al.* (61) and the pro-inflammatory effects of hematopoietic  $\alpha 7nAChR$  deficiency (**Chapter 7**). In addition, hematopoietic  $\alpha 7nAChR$  deficiency resulted in a higher activation status of platelets. Regulation of the immune response by autonomic reflexes as described within this thesis are summarized in **Figure 3**.

As the ANS mainly responds to pro-inflammatory cytokines, pharmaceutical targeting of these systems to reduce inflammation does not seem feasible. Surprisingly, training of the ANS by meditation, breathing techniques, and exposure to cold (*i.e.* immersions in ice cold water) results in increased plasma epinephrine levels and reduced inflammation upon endotoxemia (62). However, it remains to be determined whether such an intervention can be used to prevent or treat chronic inflammation as is often the case in obesity or during atherosclerosis development. At least, one may question the autonomy of the ANS.



**Figure 3 – Regulation of the immune response by autonomic reflexes and pharmaceutical compounds.** See text for details.  $\alpha 7nAChR$ ,  $\alpha 7$  nicotinic acetylcholine receptor; ACh, acetylcholine; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-6, interleukin-6; TNF $\alpha$ , tumor necrosis factor  $\alpha$ .

Currently large-scale phase III trials with cardiovascular events as end-point are underway for drug agents that reduce IL-6 and C-reactive protein (e.g. canakinumab and low dose methotrexate). Pre-clinical studies suggest that at least part of the anti-inflammatory and atheroprotective effects of methotrexate in mouse models result from increased adenosine release and subsequent activation of the  $A_{2A}$ -receptor leading to enhanced reverse-cholesterol transport and reduced foam-cell formation (63). Combined with the observation that  $A_{2A}$ -receptor agonists activate BAT (64), these studies indicate that  $A_{2A}$ -receptor agonism is a potential treatment target for CVD. Unfortunately, many  $A_{2A}$ -receptor ligands are known to alter cardiac function.

### Concluding remarks

The central nervous system is an important regulator of lipid metabolism and inflammation. The fact that many of the systems involved, including the melanocortin system, exert multiple modes of action, makes these systems attractive and powerful targets for obesity and related disorders. Although pharmaceutical targeting of the brain appears to be difficult in humans, one may circumvent this by improving circadian rhythmicity, training the ANS by cold exposure, or the use of sympathomimetic compounds. Additionally, several unique approaches have been developed including combinatorial compounds (65) and nanoparticles (66) to specifically target hypothalamic sites.

Since its (re-)discovery in 2009, BAT became a hot research topic in the field of metabolism. Pre-clinical studies, including those described in this thesis, have shown that BAT activation protects against obesity, dyslipidemia and even atherosclerosis. So far only a couple of studies explored the metabolic benefits of BAT activation in humans. Future studies should elucidate to what extent the promising results obtained in animal studies can be extrapolated to the treatment of human obesity and related disorders.

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