Hypothalamic regulation of food intake during cancer

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Hypothalamic regulation of food intake during cancer

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Summary

Appetite is often reduced in patients with chronic illness, including cancer. Cancer anorexia, loss of appetite, frequently co-exists with cachexia, and the combined clinical picture is known as anorexia-cachexia syndrome. In patients suffering from this syndrome, anorexia considerably contributes to the progression of cachexia, and strongly impinges on quality of life. Inflammatory processes in the hypothalamus are considered to play a crucial role in the development of disease-related anorexia.

The main aim of this thesis was to further elucidate crucial processes involved in the pathogenesis of anorexia in cancer. To investigate mechanisms specifically involved in cancer anorexia, we used two tumour mouse models with opposing food intake behaviours: a C26-colon adenocarcinoma model with increased food intake and a Lewis lung carcinoma model with decreased food intake. In both models, tumour-induced cachexia (body wasting) was strongly present. The contrast in food intake behaviour between tumour-bearing (TB) mice in response to growth of the two different tumours was used to distinguish processes involved in cachexia from those specifically involved in anorexia.

The hypothalamus was used for transcriptomic analysis (Affymetrix chips). We found expression of genes involved in serotonin signalling in the hypothalamus to be differentially regulated between the two tumour models. Furthermore, transcriptional activity of genes involved in serotonin signalling were inversely associated with food intake behaviour. Surprisingly, we also found a strong increase in gene expression of NPY and AgRP, potent orexigenic neuropeptides, in both models, meaning that their expression did not reflect food intake behaviour. However, NPY has also been described to regulate energy storage. Therefore, we hypothesized that this upregulation of NPY/AgRP corresponded to weight loss, which was severe in both tumour models.

Using hypothalamic cell lines we further explored how serotonin might act on food intake regulatory pathways. We showed that serotonin was able to inhibit neuronal NPY secretion, while not affecting gene expression. Inflammatory markers IL-6 and TNFα were also measured in plasma and it was found that C26 TB mice had a lower inflammatory response than LL TB mice. These differences in inflammatory response could be implicated in the differences in feeding behaviour and serotonin signalling between C26 and LL TB mice. We therefore investigated the direct influence of inflammation on hypothalamic serotonin turnover and its contribution to the development of anorexia. To this end,
different doses of TNF and IL-6 were administered by injection to healthy mice, inducing an acute inflammatory response. The injected cytokine doses were estimated from their corresponding plasma levels measured in tumour bearing (TB) mice. Also in this cytokine induced-anorexia model, where anorexia was exclusively induced by an inflammatory response, serotonin metabolism in the hypothalamus was affected. Both TNF and IL-6 increased hypothalamic serotonin turnover while also inducing anorectic behaviour. Furthermore, the effect of cytokines on increasing serotonin turnover was supported by in vitro experiments with hypothalamic neuronal cell lines.

In conclusion, we identified hypothalamic serotonin signalling to play a major role in the decrease in food intake during cancer. Serotonin signalling itself is modulated by inflammatory mediators. Therefore, hypothalamic inflammation is an important trigger in the failure of hypothalamic food-intake regulation, probably by affecting serotonergic signalling, which acts as an upstream modulator of various orexigenic and anorexigenic systems.
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General introduction
Cancer anorexia

Anorexia, literally meaning loss of appetite, in general refers to a condition in which an organism decreases food-intake or even refuses food. Although most people will initially link the term anorexia to the psychological eating disorder anorexia nervosa, it is also considered a common process connected to illness. Anorexia as a consequence of disease is often occurring in patients with cancer.

Different studies indicate an incidence of anorexia in cancer patients from 60-80%, depending on the type and stage of cancer. In these patients, anorexia considerably contributes to the progression of the disease and strongly impinges on quality of life [1-4]. It is still regarded an unmet medical need and current treatments, typically involving corticosteroids and progestagenic steroids, generally improve appetite in less than 30% of the cases [5]. In cancer anorexia, the loss of desire to eat is caused by pathophysiological changes in response to tumour growth and thus also common in patients not receiving chemotherapy [6]. Patients with incurable tumours and near the end of their life, but also newly diagnosed cancer patients often report to experience problems with reduced appetite and involuntary weight loss [3, 7]. Furthermore, anorexia is a strong prognostic factor for reduced survival of cancer patients, even in patients who do not suffer from weight loss [8]. Reports show that cancer patients with a healthy appetite and higher caloric intake have higher survival rates than patients who suffer from anorexia and have a lower energy intake [9, 10]. Anorexia also affects the psychological well-being of the patient. Cancer patients mention anorexia as one of the most prevalent symptoms during disease and often psychologically associate the lack of appetite with disease progression and suffering [11]. On top of that, not eating well and the rejection of food prepared by caring family is a burden for both the patient and his/her family, strongly affecting mental health of both patients as well as their caregivers [12-14].

Anorexia and cachexia: two pathologies, one syndrome?

In a majority of cancer patients, anorexia leads to a reduced food intake [15] and significantly contributes to progression of cachexia [16]. Cachexia is a complex metabolic syndrome associated with underlying illness and is characterized by progressive loss of muscle (muscle wasting) with or without loss of fat mass. This results in weight loss, a reduced quality of life and a shortened survival time [17, 18]. Cachexia is observed in
80% of patients with advanced-stage cancer, and it is estimated to be directly responsible for more than 20% of deaths in cancer [19, 20]. Unfortunately, counteracting body wasting by merely providing nutritional support often fails [21]. At the same time nutritional support can still improve quality of life [22, 23], meaning that nutrition can be a valuable intervention in cancer patients. Cachexia represents an important target in disease management of cancer patients, because it is associated with reduced effectiveness of anti-neoplastic surgery and other anti-cancer treatments, increased risk of therapy-induced side effects, reduced muscle function and physical performance, extended hospital stay, increased postoperative complications and increased infection rates. Most of these complications can be directly correlated to increased morbidity and a reduced quality of life [24-32]. In 2006, anorexia was included as a crucial feature in the framework of factors defining the clinical features of cachexia [33], due to the close entanglement of cachexia and anorexia clinically. Although anorexia and cachexia are likely to be initiated by similar pathologies (underlying illness), it is unclear to what extent anorexia and the metabolic alterations of cachexia affect each other, or to what extent these are distinct entities with their own pathology. Furthermore, food intake is an outcome of various physiological and behavioural processes controlling hunger, satiety and reward systems, making it hard to pinpoint specific causes, as these are highly dependent on the type and stage of the associated disease.

Inflammation is considered to play a pivotal role in the manifestation of both cachexia and anorexia. High concentrations of the prominent pro-inflammatory cytokines IL-1β, IL-6 and TNFα are reported to decrease food intake and to induce features of cachexia [34, 35]. Therefore, the presence of anorexia is not only limited to cancer patients, but also commonly manifested in patients with other chronic illnesses, including acquired immune deficiency syndrome (AIDS) [36], chronic obstructive pulmonary disease (COPD) [37] and chronic heart failure [38].

**Role of the hypothalamus in food intake**

The hypothalamus is considered to be essential in the integration of metabolic and neuronal signals on energy balance. In cancer, an ongoing elevated inflammatory tone in the hypothalamus is suggested to underlie the onset of anorexia during disease. Here, the hypothalamus appears to be unable to respond adequately to pivotal peripheral signals.
involved in appetite regulation [4, 6, 39]. This is particularly relevant when cachexia is also present, which generally demand for an increased energy intake to compensate for increased body weight loss. The hypothalamus displays the highest density of various cytokines receptors in the brain [40], underlying its role in mediating effects of pro-inflammatory cytokines. The hypothalamus contains specific areas that control feeding behaviour with the arcuate nucleus (ARC) being one of the best-characterized regions related to feeding behaviour. This is largely due to its ability to sense peripheral input from its adjacent median eminence. The ARC comprises two neuronal populations with opposing functions on food intake: orexigenic (food intake stimulating) NPY/AgRP, and anorexigenic (food intake inhibiting) POMC/CART neurons [41]. Inflammatory mediators, including TNF and IL-6, cytokines that are commonly elevated in chronic illness, have been shown to interact with neurons dominating these nuclei. Furthermore hypothalamic neurons release various neurotransmitters, including dopamine and serotonin, which are considered to play a role in reward and satiation respectively. Several studies indicate that elevation of hypothalamic serotonin plays a role in the onset of disease-associated anorexia [42-44].

Serotonin in the brain

Serotonin is a common and important mono-amine neurotransmitter and hormone. The body's largest pool resides in the gut, where it plays a role in regulating gastrointestinal tract functioning. In the CNS, serotonin acts as a neurotransmitter and is involved in several behavioural and physiological processes, including food intake regulation [45]. Serotonin is synthesized from the amino acid tryptophan (TRP). Tryptophan has to compete with other branched-chain amino acids (BCAA) at the blood brain barrier (BBB) in order to enter the hypothalamus. Therefore, the ratio of TRP/BCAA is assumed to reflect brain TRP and ultimately serotonin brain status [46, 47]. In cancer patients, limiting TRP transportation to the brain by supplementation of competing brain chained amino-acids (BCAAs) [48] or inhibiting the activity of serotonin at the 5HT3 receptor has shown to be beneficial for enjoyment of food in anorectic cancer patients [49]. Recent findings on cytokine actions on NPY/AgRP and POMC/CART neurons and their effects on food intake are discussed in chapter 2.
Outline of thesis

The main aim of this research presented in this thesis was to further elucidate crucial processes involved in the pathogenesis of anorexia in cancer. The presence of an elevated inflammatory tone reaching the hypothalamus as an underlying cause for the development of anorexia during cancer was taken as an important starting point.

In chapter 2, we discussed the changes that occur on hypothalamic food-intake regulators and serotonin in chronic inflammation. Cancer has also other consequences besides alteration in food intake, including the development of cachexia. In chapter 3 and 4 we used two different tumour models to investigate mechanisms involved in cancer anorexia. In these models, tumour-induced cachexia (body wasting) was strongly present, but different responses in food intake occurred. This contrast in food intake behaviour was used to distinguish between processes involved in cachexia and mechanisms that might be important in food intake regulation. Using transcriptomics, many genes related to food intake behaviour could be studied. In chapter 2, using transcriptomics analysis, we found serotonin signalling to be among the most significantly altered canonical signalling pathways in the hypothalamus. Therefore, we focused on the role of serotonin in food intake. We reported that differences in food intake are inversely associated with hypothalamic serotonin. We also found that the potent orexigenic regulators NPY and AgRP were among the highest altered genes in both tumour models. However, this expression of NPY and AgRP did not reflect food intake status. In conditions of severe cachexia, it is likely that gene expression of NPY and AgRP responds to cachetic processes that ultimately result in severe body wasting. Furthermore, in chapter 4, we showed that serotonin has an inhibiting action on neuronal NPY release, supporting serotonin's modulatory role on food intake regulation. To exclude the influences from cachetic processes (e.g. in the muscle and fat tissue) in cancer on the development of anorexia and changes on hypothalamic serotonin and NPY/AgRP regulation, we focused on the specific influence of inflammatory mediators.

In chapter 5, we investigated the anorexigenic effects of inflammatory mediators TNF and IL-6 and their effects on serotonin metabolism in the hypothalamus. We showed that systemic inflammation leads to elevated serotonin turnover in the hypothalamus. Finally, chapter 6 presents the general discussion and overall conclusions.
References


Hypothalamic inflammation and food intake regulation during chronic illness

J. T. Dwarkasing, D. L. Marks, R. F. Witkamp, K. van Norren
Chapter 2  |  Hypothalamic inflammation and food intake regulation

Abstract

Anorexia is a common symptom in chronic illness. It contributes to malnutrition and strongly affects survival and quality of life. A common denominator of many chronic diseases is an elevated inflammatory status, which is considered to play a pivotal role in the failure of food-intake regulating systems in the hypothalamus. In this review, we summarize findings on the role of hypothalamic inflammation on food intake regulation involving hypothalamic neuropeptide Y (NPY) and pro-opiomelanocortin (POMC). Furthermore, we outline the role of serotonin in the inability of these peptide based food-intake regulating systems to respond and adapt to changes in energy metabolism during chronic disease.
Introduction

In many chronic illnesses including cancer, chronic obstructive pulmonary disease (COPD) and acquired immune deficiency syndrome (AIDS), an ongoing elevated systemic inflammatory status plays a pivotal role in both increased energy expenditure as well as a dysregulation of food intake. As a consequence, increased loss of lean body mass is often accompanied by decreased food intake, ultimately leading to severe malnutrition.

The hypothalamus is important for several metabolic processes including energy homeostasis. It acts as an integrator of metabolic and neuronal signals on energy balance, and regulates the balance between energy expenditure and energy intake. In conditions of increased energy requirements, hypothalamic adaptation generally results in increased food intake, which is for example seen in athletes or persons on incremental exercise training [1, 2] or in individuals residing in a cold environment [1, 3, 4]. However, in conditions where increased energy expenditure is accompanied by the presence of chronic inflammation the hypothalamus is not able to respond adequately to changes in energy balance [5]. Here, this elevated inflammatory status causes loss of body weight, attributed to muscle wasting and increased white adipose tissue lipolysis. On top of that, a loss of appetite beyond a level needed to compensate for increased energy demands is commonly seen, resulting in reduced food intake. This apparent failure of orexigenic (food intake-stimulating) systems of the hypothalamus to respond to peripheral triggers suggests the presence of some form of resistance [5]. This hypothalamic resistance to peripheral neuro-endocrine starvation signals is believed to be directly caused by an increased inflammatory status [5]. Increased plasma levels of pro-inflammatory cytokines are associated with disease progression in a variety of cachectic conditions including cancer [6-8], HIV [9], heart failure [10, 11] and COPD [12].

In experimental models, administration of IL-6, TNFα and IL-1β [13, 14] directly reduces food intake by affecting pivotal food-intake regulating systems in the hypothalamus. At the same time, blocking the action of these cytokines in the presence of underlying disease such as cancer cachexia-anorexia [15-18] or HIV [19] only results in a partial, though significant, reversal of anorexia-cachexia. These studies underline that even though these cytokines are crucial in the pathogenesis of anorexia, their actions and also their interplay with other factors such as anti-inflammatory cytokines [20] are still dependent on the underlying illness via other mechanisms.
In this review we will focus on current insights on the role of hypothalamic inflammation in the reduced food intake during inflammatory conditions. In particular, changes which inflammatory mediators can have on two pivotal food intake regulating systems: 1) Neuropeptide Y (NPY)/Agouti-related peptide (AgRP) and 2) pro-opiomelanocortin (POMC)/Cocaine and amphetamine regulated transcript (CART) will be discussed. It is still unknown how cytokines are able to induce alterations in these systems. The role of serotonin will also be studied in this respect, since serotonin is able to modulate food-intake regulatory systems in chronic inflammatory conditions and able to directly influence food intake. In this review, we explored recent literature to investigate the hypothesis that inflammation alters NPY/AgRP-and POMC/CART-regulated food intake via modulation of serotonergic signalling pathways.

**Hypothalamic inflammation**

Food intake is an outcome of various physiological and behavioural processes controlling hunger, satiety and reward systems of which many originate in the hypothalamus. The hypothalamus consists of a tightly interconnected network of diverse neuronal populations, among them the arcuate nucleus (ARC) and the paraventricular nucleus (PVN). Even though the exact role of the distinct neuronal populations in the complex network of processes is not entirely clear, the observation of dense neuronal projections from the ARC into the PVN [21, 22], provides anatomical substrate for the ARC-PVN axis. This ARC-PVN axis is widely studied in the regulation of food intake behaviour in response to a negative energy balance [23-25]. In relation to this, the ARC-PVN axis is involved in different physiological processes, while interacting with several other neuronal populations. In the present review, we will primarily focus on this ARC-PVN axis in relation to the effect of neural inflammation and food intake. The hypothesis that the hypothalamus plays a crucial role in both reduced food intake and cachectic body wasting is supported by a combination of findings. Firstly, neuroinflammation and disturbed hypothalamic signalling is present in cachectic chronic diseases such as cancer [5], HIV [26, 27], COPD [28] and heart failure [29, 30]. Secondly, the hypothalamus has the highest density of receptors for these pro-inflammatory cytokines in the brain [31]. This inflammatory response in the hypothalamic area can be a consequence of elevated plasma cytokines entering the brain, as several cytokines are able to cross the BBB including TNFα [32], IL-6 [33] and IL-1α [34, 35] and IL-1β [36]. In addition,
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Hypothalamic neurons from the ARC are able to sense systemic circulating factors, including cytokines, and peripheral hormones like leptin and insulin from the adjacent median eminence (ME), which is not protected by the blood-brain barrier. In conditions of food deprivation, permeability and fenestration of microvessels from the ME are increased. This results in an enhanced access of circulating factors entering this region and reaching ARC neurons [37]. In this way ARC neurons might be able to sense peripheral triggers and to project these signals to other neuronal populations including the PVN [38-40]. Subsequently, this response might lead to de novo production of cytokines within the hypothalamus itself. For IL-1β [41], TNFα and IL-6 [42], such de novo synthesis in the hypothalamus has been shown after lipopolysaccharide (LPS) injection in rodents, suggesting that the hypothalamus is both a receiver and an amplifier of the peripheral cytokine signals. Indeed, activation of hypothalamic microglial and astrocyte cells, macrophage-like cells of the central nervous system, appears to be a common phenomenon in chronic inflammatory diseases including myocardial infarction [43], obesity [44] and HIV [45]. Finally, also the vagus nerve is likely to play a role in cytokine signalling to the brain. Vagotony partially attenuates LPS-induced increases in IL-1β expression in murine hypothalamus, while not affecting elevated IL-1β plasma levels [46]. In summary, inflammatory signals reach the hypothalamus by different routes apparently dependent on the type of inflammatory mediator, the hosts’ specifics and the underlying disease.

Hypothalamic inflammation: Orexigenic signalling

The arcuate nucleus (ARC) includes two important populations of neurons: orexigenic NPY/AgRP and anorexigenic POMC/CART neurons. NPY and POMC neuronal populations have opposing effects on food intake. Furthermore, they are oppositely regulated by peripheral triggers including insulin [47], gut hormones including glucagon like peptide-1 (GLP-1) [48] and peptide YY (PYY) [49], and leptin [50]. This is for example indicated by the fall in leptin levels during energy deficit, a condition that generally drives an increase of food intake [51-53]. Subsequently, this drop in leptin levels attenuates the activation of POMC neurons and allows the activation of NPY signalling [47], actions both in favour of stimulation of food intake. This stimulation of food intake during energy deficit is also supported by an increase in gut-derived ghrelin [54, 55]. Ghrelin is a potent stimulator of food intake [56] and acts via activation of
Elevated levels of orexigenic ghrelin have been measured in various chronic cachectic conditions including cancer, COPD and chronic heart failure [58-62]. The ability of ghrelin to initiate and stimulate food intake is contradictory to the high occurrence of anorexia in these diseases, suggesting the occurrence of hypothalamic resistance to ghrelin [63, 64]. This elevation of orexigenic ghrelin might be a compensatory response to negative energy balance in these patients. Furthermore it is likely that this elevation of ghrelin is an attempt to suppress inflammation, as ghrelin has strong anti-inflammatory actions by inhibiting pro-inflammatory cytokines, augmenting anti-inflammatory cytokines [65, 66] and reducing hypothalamic glial activation [67]. Furthermore, administration of ghrelin showed to be beneficial in fasting or high-fat diet induced inflammation [68, 69].

Orexigenic neuropeptide AgRP is exclusively located in the ARC [70, 71], while NPY is abundant throughout the entire mammalian brain, with the highest expression of NPY synthesizing neurons located in the ARC [72]. In contrast, NPY release is found to be highest in the PVN [73], supporting the importance of the ARC-PVN axis in NPY and food intake signalling [74].

NPY is strongly activated during states of negative energy balance. In these conditions, it is at least partly responsible for hyperphagia to compensate energy deficits as reported for food restriction [75], food deprivation [76], exercise [77, 78], lactation [79] and insulin-dependent diabetes [80]. Therefore, NPY is considered a crucial sensor of peripheral signals of energy deficit, and in particular decreases in leptin and insulin plasma levels [52, 53] and increased plasma glucocorticoid levels [81]. Subsequently, NPY can regulate energy balance by altering food intake during energy deficit through its release from ARC projections into the PVN, Dorsomedial hypothalamic nucleus (DMH) and Lateral hypothalamus (LHA) [82] (Figure 2.1).

In experimental models for acute inflammation, such as LPS- or TNFα-induced anorexia, hypothalamic NPY expression is decreased (Figure 2.1), corresponding to the observed decrease in food-intake [15, 83, 84]. More importantly, administration of NPY or blocking the induced inflammatory response in these animal models prevents the development of anorexia [15, 85]. These data suggest that NPY release is necessary to adapt food intake to changes in energy expenditure. Paradoxically, hypothalamic NPY gene expression is reported to be increased in animal models for chronic inflammatory diseases that are characterized by the presence of cachexia, such as cancer cachexia.
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However, this increase in mRNA levels did not correspond to the decrease in food intake [87, 89, 91-93] (Figure 2.1). In contrast, in these models NPY levels and NPY release are either unchanged [87] or decreased [94-96]. This suggests that NPY signalling might be modulated at a post-transcriptional level in these circumstances. Even though increased gene expression of NPY did not correspond to food intake, it was associated with weight loss. Therefore, we hypothesize that gene expression of NPY acts as a sensor to weight loss and that adequate translation and release of NPY is subsequently necessary to adapt food intake to changes in energy expenditure.

Figure 2.1 Hypothesis on the role of serotonin in inflammation-induced reduction of food intake in chronic illness.

Diagram showing how inflammation may alter both orexigenic peptide signalling and anorexigenic signalling in the hypothalamus. Chronic illness is often associated with body wasting (loss of muscle and fat tissue). This negative energy balance usually drives upregulation of orexigenic messenger NPY/AgRP. Serotonin is also elevated in chronic inflammatory illness. This elevation can interfere with NPY and AgRP post-transcriptionally and at the same time can stimulate melanocortin signalling. In this way, serotonin can interfere with orexigenic signalling and stimulate anorexigenic signalling in the hypothalamus and ultimately play a role in disease-induced reduction of food intake.
gene expression of AgRP is increased [97] (Figure 2.1). In addition, hypothalamic food-intake regulation in the anx/anx mouse, a genetic model for anorexia showed that comprised signalling of NPY/AgRP is partly due to dysfunctional axonal transport [98]. Similar to NPY, changes on AgRP are associated with weight loss and failure of AgRP signalling is likely to occur post-transcriptionally. Altogether, these data indicate that inflammatory mediators can affect NPY/AgRP gene expression and also act post-transcriptionally, for example via impaired translation, synthesis, packaging or release of NPY, ultimately altering food intake (Figure 2.1).

Hypothalamic inflammation: Anorexigenic signalling

Two anorexigenic peptides, POMC and CART, are both synthesized in the same ARC neuronal population and are released into the PVN. POMC is a precursor for anorectic alpha-melanocyte stimulating hormone (α-MSH), which is an endogenous agonist of melanocortin receptor 3 and 4 (MC3R and MC4R). Interestingly, AgRP’s orexigenic activity arises from its antagonistic activity on this MC4R receptor [99].

Acute inflammation leads to activation of POMC neurons [100], increased expression of MC4R [101] and increased POMC expression [102, 103] as shown in models of LPS and IL-1β-induced anorexia. Consequently, counteracting the activation of the melanocortin system by MC4R antagonism prevented the development of LPS-induced anorexia [102, 104, 105]. In contrast to an acute immune response, during chronic inflammation such as in cancer, hypothalamic POMC expression is decreased [90, 106]. These opposing differences on POMC expression in acute and chronic inflammation might be explained by changes in leptin plasma levels in these two conditions: LPS administration elevates plasma leptin [107], while in diseases characterized by the presence of cachexia, plasma leptin levels drop [108]. Nonetheless, in chronic inflammatory conditions such as cancer, renal failure and heart failure, the use of AgRP or synthetic MC4R antagonists improved food intake [109-112], but also increased POMC expression and thus possibly αMSH [106]. In contrast, administration of αMSH or synthetic MC4R agonists, have also shown to be beneficial in chronic inflammation including arthritis [113], inflammatory bowel disease [114] and ischemic stroke [115]. This paradox in beneficial effects by both MC4R agonists and antagonists in chronic inflammatory conditions might be explained by another physiological function of the melanocortin systems next to their direct effect on
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Elevation of plasma α-MSH in chronic inflammatory illnesses could suggest an adaptive response in order to control inflammation [116-118]. Both MC4R antagonists [111, 119] and agonists [91, 115, 120, 121] act as anti-inflammatory peptides in experimental models for cancer, rheumatoid arthritis and ischemic stroke either centrally or peripherally [122, 123]. These beneficial suppressive effects of α-MSH on inflammation may occur via increasing circulating anti-inflammatory cytokines [120]. In addition, α-MSH can inhibit pro-inflammatory mediators [91, 115, 121]. In this way α-MSH, via modulation of inflammatory mediators, can attenuate muscle wasting and progression of cachexia [91].

Altogether, it might be that αMSH’s actions on inflammation might be of greater importance on cachexia and anorexia in chronic illness than its direct effects on hypothalamic food-intake regulatory peptides such as POMC and NPY. Of course, the actions of αMSH as a neurotransmitter may be entirely independent of its effects on the immune system. As a consequence, targeting the melanocortin systems is a potential therapeutic approach against cachexia and anorexia induced by inflammation.

Hypothalamic inflammation: serotonergic signalling

Hypothalamic serotonin is an important mediator in the regulation of satiety and hunger and its levels are inversely associated with food intake [124, 125]. Hypothalamic serotonin is suggested to act as an integrator of peripheral triggers modulating energy balance, as it responds to changes in adipokines [126, 127] and gut-derived hormones [128, 129] and subsequently mediates anorexigenic signalling. Therefore, elevated hypothalamic serotonin levels are suggested to play a crucial role in the development of disease-associated anorexia [5, 93, 95] (Figure 2.1). This increase in serotonin can be caused by activation of proinflammatory cytokines, as these are able to directly affect serotonin metabolism. Central administration of LPS, IL-1α, IL-1β or TNFα [130-132] induces a rise of hypothalamic serotonin, serotonin’s metabolite 5-Hydroxyindoleacetic acid (5-HIAA) and serotonin’s precursor tryptophan (TRP). These effects are likely to be at least partly mediated by IL-1 [131]. Furthermore, blockade of 5HT2a and 5HT2c receptors prevents the development of LPS-induced anorexia [133, 134], suggesting that inflammation-induced anorexia is partly mediated by serotonin. Also during anorexia induced by chronic inflammation, such as tumour-driven anorexia, serotonin in the
hypothalamus [95, 135, 136] is elevated, further supporting the importance of serotonin in reduced food intake during both acute and chronic inflammation. In humans, increased levels of TRP, the precursor of serotonin, have been measured in cerebral spinal fluid of anorectic cancer patients [137]. Also, limiting TRP transportation to the brain by supplementation of competing branched chained amino-acids (BCAAs) or antagonizing activity of serotonin at the 5HT3 receptor is beneficial for enjoyment of food in anorectic cancer patients [138]. Serotonergic drugs are extensively investigated for their use in eating disorders in both obesity as well as disease-induced anorexia. Antagonizing serotonin’s activity has been shown to enhance appetite in cancer-cachectic patients [139, 140]. Currently these serotonin antagonists are used to alleviate symptoms of nausea, a symptoms strongly associated with anorexia [141], in cancer patients [142]. At the same time, serotonin agonists are used in treatment of hyperphagia in obesity [124, 140, 141]. Collectively, these observations support the concept that inflammation affects serotonergic activity in the hypothalamus and that serotonin plays an important role in inflammation-induced anorexia.

Serotonin is able to affect food intake via the regulation of several neuronal systems in the ARC. Firstly, serotonin is able to activate the anorexigenic melanocortin system. Serotonergic drugs are used as therapy against obesity. They exert appetite-suppressive effects by increasing the local availability of serotonin or, more specifically, stimulating the 5HT2C receptors [143]. Serotonin’s suppressive effect on food intake is reported to be mediated by 5HT2c and 5HT1b receptors on POMC neurons in the ARC [144–146]. Furthermore, the presence of functional MC4Rs, the downstream target of αMSH, is crucial for this suppressive effect on food intake as well, suggesting a 5HT2c/POMC-MC4R circuit [147]. Secondly, serotonin is able to inhibit the orexigenic activity of ghrelin [129, 148]. This process is likely to be mediated via activation of 5HT2c and 5HT1b receptors on ARC neurons [63, 149]. Vice versa, ghrelin is able to directly inhibit serotonin release [66, 150, 151]. This inhibitory interplay between serotonin and gut-derived ghrelin shows that serotonin may exert its anorectic effects via mechanisms beyond its direct activation of appetite inhibiting neuronal activity [148]. Thirdly, serotonin is able to inhibit orexigenic NPY signalling [95]. Increasing serotonergic activity by the use of serotonin re-uptake inhibitors reduces hypothalamic NPY [152, 153]. In addition, decreasing serotonin concentrations by inhibiting serotonin synthesis results in increased NPY levels [154]. Furthermore, these effects of serotonin on feeding and NPY levels are not apparently accomplished via affecting NPY gene
expression [154, 155]. Also in vitro, serotonin inhibits neuronal NPY secretion, while not affecting NPY gene expression [93]. Therefore, serotonin appears to play an active role in posttranscriptional failure of NPY in anorexia in chronic inflammation.

In summary, several lines of evidence indicate that serotonin mediates food intake by acting as an upstream regulator mediating both anorexigenic and orexigenic signalling. More importantly, serotonin is affected by inflammation and is associated with inflammation-induced anorexia. Consequently, we postulate a neural circuit in which serotonin plays a central role in the failure of food intake regulation taking place during inflammation-induced anorexia (Figure 2.1).

**Conclusion**

Many chronic illnesses are associated with a pro-inflammatory state in the hypothalamic area. This increased hypothalamic inflammatory status plays a crucial factor in reduced food intake in these conditions, ultimately leading to severe malnutrition and acceleration of disease. Pro-inflammatory cytokines affect both orexigenic NPY/AgRP signalling and anorexigenic melanocortin signalling. Hypothalamic serotonin signalling is elevated in disease-induced anorexia. Furthermore, serotonin is able to activate the melanocortin system, and able to inhibit NPY signalling via post-transcriptional regulation. Therefore, we hypothesize that serotonin-regulated mechanisms are playing a key role in the reduction of food-intake during chronic inflammation. Consequently, targeting serotonin signalling is a promising approach in preventing the development of nutritional disorders in disease.

**Acknowledgements**

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**Conflict of interest**

J. Dwarkasing, D.L Marks and R. Witkamp have nothing to declare. K. van Norren is guest employee at Nutricia Research, a medical nutrition company.
References


Chapter 2 | Hypothalamic inflammation and food intake regulation


Hypothalamic inflammation and food intake regulation


Hypothalamic food intake regulation in a cancer-cachectic mouse model


Abstract

Background: Appetite is frequently affected in cancer patients, leading to anorexia and consequently insufficient food intake. In this study, we report on hypothalamic gene expression profile of a cancer cachectic mouse model with increased food intake. In this model, mice bearing C26 tumour have an increased food intake subsequently to the loss of body weight. We hypothesize that in this model, food intake regulating systems in the hypothalamus, which apparently fail in anorexia, are still able to adapt adequately to changes in energy balance. Therefore studying changes that occur on appetite regulators in the hypothalamus might reveal targets for treatment of cancer-induced eating disorders. By applying transcriptomics, many appetite regulating systems in the hypothalamus could be taken into account, providing an overview of changes that occur in the hypothalamus during tumour growth.

Methods: C26-colon adenocarcinoma cells were subcutaneously inoculated in 6 weeks old male CDF1 mice. Body weight and food intake were measured 3 times a week. On day 20, hypothalamus was dissected and used for transcriptomics using Affymetrix chips.

Results: Food intake increased significantly in cachectic tumour-bearing mice (TB), synchronously to the loss of body weight. Hypothalamic gene expression of orexigenic neuropeptides NPY and AgRP was higher, whereas expression of anorexigenic genes CCK and POMC were lower in TB compared to controls. In addition, serotonin and dopamine signalling pathways were found to be significantly altered in TB mice. Serotonin levels in brain showed to be lower in TB mice compared to control mice, while dopamine levels did not change. Moreover, serotonin levels inversely correlated with food intake.

Conclusions: Transcriptomic analysis of the hypothalamus of cachectic TB mice with an increased food intake showed changes in NPY, AgRP and serotonin signalling. Serotonin levels in the brain showed to correlate with changes in food intake. Further research has to reveal whether targeting these systems will be a good strategy to avoid the development of cancer-induced eating disorders.
Introduction

Anorexia affects 60-80% of all patients with cancer and considerably contributes to disease-related malnutrition and cachexia, which in turn strongly affect patient’s morbidity, mortality and quality of life [1].

Anorexia is often linked to cachexia, a complex metabolic syndrome associated with underlying illness which is characterised by progressive loss of muscle (muscle wasting) with or without loss of fat mass resulting in weight loss [2]. Although anorexia and cachexia are likely to be initiated by similar pathologies, several lines of evidence suggest that both conditions progress via distinct mechanisms. However, the presence of cachexia makes it difficult to disentangle the primary underlying mechanisms of cancer anorexia, since this might be due to tumour growth, cachexia progression or other disease related mechanisms.

Cancer anorexia is generally considered to be a multifactorial condition. Contributing to its complexity is the observation that evolution has developed powerful physiological mechanisms favouring food intake. It has been shown that upon shifting the balance to anorexia, pathways can become redundant when they are not functioning properly. This is for example shown by data obtained from studying knock-out animals for well-known appetite regulators, the NPY knock-out mouse [3], the AgRP knock-out mouse [4], or the ghrelin knock-out mouse [5]. These mice display regular food intake and body weight regulation, despite the loss of a significant key modulator in appetite regulation. The difficulties encountered in studying cancer anorexia, inspired us to approach the problem from a different angle. Cancer-induced anorexia is suggested to be predominantly caused by the inability of the hypothalamus to respond adequately to pivotal peripheral signals involved in appetite regulation [6]. This hypothalamic resistance to peripheral neuro-endocrine signals is believed to be due to the increase in pro-inflammatory cytokines resulting from tumour growth [6]. In this study, we report on hypothalamic gene expression profiles in a cancer cachectic model with increased food intake. In this model, appetite regulating systems, which apparently fail in anorexia, are still able to adapt adequately to changes in energy balance. By applying transcriptomics, many appetite regulating systems in the hypothalamus could be taken into account. Here, we provide an overview of changes that occur in the hypothalamus during tumour growth which could be important in the development of cancer-induced eating disorders.
Materials and methods

Tumour model
Male CDF1 (BALB/cx DBA/2) mice aged six-to-seven weeks were obtained from Harlan Nederland (Horst, The Netherlands). Animals were individually housed one week before start of the experiment in a climate-controlled room (12:12 dark-light cycle; 21°C±1°C). Mice were placed on a standard ad libitum diet (AIN93M, research Diet Services, The Netherlands) and had free access to water. Murine C26 adenocarcinoma cells were cultured and suspended as described previously [7]. Under general anaesthesia (isoflurane/N₂O/O₂), tumour cells in 0.2 ml HBSS were inoculated subcutaneously into the right inguinal flank. Controls were sham-injected with 0.2 ml HBSS.

All experimental procedures were approved by the Animal Ethical Committee (DEC, Bilthoven, The Netherlands) and complied with the principles of good laboratory animal care.

Experimental design
On day 0 tumour cells were injected. BW, food intake and tumour size were measured three times a week. Tumour size was determined by measuring the length and width of the tumour with a caliper. On day 20, body composition was determined by DEXA (Lunar, PIXIImus). Subsequently, blood was collected by cardiac puncture. After sacrifice, brain, hypothalamus, organs and lower leg skeletal muscles were weighted and frozen at -80°C. Two studies were performed with similar settings: study A was a pilot study to optimise experimental conditions and was followed by study B. Table 3.1 shows the number of tumour cells used for inoculation in the different groups that were included in the two studies.

Table 3.1 Groups included in study A and study B

<table>
<thead>
<tr>
<th>Study A</th>
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<td>TB-0.5 : Tumor-bearing, 0.5 x 10⁶ C26-cells (n=4)</td>
<td>TB : Tumor-bearing, 1 x 10⁶ C26-cells (n=9)</td>
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<td>TB-1 : Tumor-bearing, 1 x 10⁶ C26-cells (n=3)</td>
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</table>

Pilot study A was performed prior to study B. In study A, mice were injected with different amounts of C26-colon adenocarcinoma cells, while study B comprised of one tumor-bearing group and one control group.
**Blood plasma amino acids and cytokines**

Amino acids were measured by using HPLC with *ortho*-phtaldialdehyde as derivatization reagent and L-norvaline as internal standard (Sigma Aldrich). The method was adapted from van Eijk et al. [8].

Cytokines were measured using a mouse cytokine 10-plex bead immunoassay (Biosource, Etten-Leur, The Netherlands). Prostaglandin E₂ was measured using an enzyme-immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA).

**Serotonin and dopamine levels**

Hypothalamic samples were used for micro array experiments, while remaining brain parts were used to determine serotonin and dopamine levels. Brains were homogenized in 1ml containing 40mM Tris, 1mM EDTA, 5mM EGTA, 0.50% Triton X-100 and PhosSTOP phosphatase inhibitor (Roche Nederland, The Netherlands). Citric acid (1%) was added to prevent serotonin oxidation. Serotonin and dopamine levels were measured using enzyme-immunoassay kits (BAE-5900, BAE-5300, LDN, Nordhorn, Germany).

**Statistics**

Data was analysed by statistical analysis of variance (ANOVA) followed by a *post hoc* Tukey’s multiple comparison/Bonferroni test or by a Student’s *t* test. Differences were considered significant at a two-tailed *P*<0.05. Statistical analyses were performed using Graphpad Prism 5. For statistical analysis of microarray data see microarray section (below).

**Microarray studies**

Total RNA from the hypothalamus was isolated by using RNeasy Lipid tissue kit (Qiagen, Venlo, The Netherlands). RNA concentrations were measured by absorbance at 260 nm (Nanodrop). RNA quality was checked using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technolgies, Amsterdam, The Netherlands) according to the manufacturer’s protocol. For each mouse, total RNA (100 ng) was labelled using the Ambion WT expression kit (Life Technologies, Bleiswijk, The Netherlands). Microarray experiments were performed by using Affymetrix Mouse Gene ST 1.0 (study A) and 1.1 (study B).
For both study A and B, samples were pooled for each group. Also individual samples from study B were included in a subsequent microarray experiment to confirm the findings on food intake regulators and canonical pathways. In this microarray experiment, 4 control samples and 5 samples from tumour-bearing mice were included in this experiment, however 1 control sample gave various spots on the array and was therefore excluded from analysis.

Array data were analysed using an in-house, on-line system [9]. Shortly, probesets were redefined according to Dai et al. [10] using remapped CDF version 15.1 based on the Entrez Gene database. In total these arrays target 21,225 unique genes. Robust multi-array (RMA) analysis was used to obtain expression values [11, 12]. For study B we only took genes into account that had an intensity >20 on at least 2 arrays, had an interquartile range throughout the samples >0.1 and had at least 7 probes per genes. In total 8,763 genes passed the filter. Genes were considered differentially expressed at P<0.05 after intensity-based moderated t-statistics [13]. Further functional interpretation of the data was performed through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Canonical pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Genes from the data set that met the cut-off of 1.3 fold change and p-value cut-off of 0.05 and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. Array data have been submitted to the Gene Expression Omnibus, accession number GSE44082.

**Results**

**Body weight and food intake**

In study A, tumour size and tumour weight did not increase correspondingly to the number of tumour cells injected (Figure 3.1B-3.1C). However, carcass weight, epididymal fat pad weight and skeletal muscle weight decreased proportionally to the number of tumour cells injected, suggesting that body wasting increases with tumour load, despite the weight of the tumour being similar (Supplementary table S3.1). Food intake in all tumour-bearing animals was found to increase after 15 days. At day 19, tumour-bearing (TB) mice in TB-0.5 and TB-1 groups ate approximately 45% more than the controls. An increase of food intake in TB mice was again noticed in subsequent study B (Figure
3.1A and 3.1D). In this study, food intake of TB mice was 40% higher than controls at day 19. On day 13 after tumour inoculation, TB mice started to lose body weight (BW). Synchronously to the decline in body weight, an increase in food intake in TB mice was measured, suggesting compensatory eating by TB mice, in order to cope with loss of BW. The loss of lean mass, fat mass and skeletal muscle weight in TB mice in study B was comparable with that of study A, showing that the level and severity of cachexia developed in TB animals was similar in both studies (Supplementary table S3.1).
**Microarray analysis of the hypothalamus**

The heat map in Figure 3.2 shows fold changes of orexigenic and anorexigenic gene expressions. Orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) expression were found to be significantly higher by 1.9 and 1.6 fold respectively in TB mice. Orexigenic ghrelin expression was comparable between TB mice and controls. However, expression of the growth hormone-secretagogue receptor (GHsR), which mediates ghrelin signalling, showed to be slightly higher by 1.2 fold. In addition, growth hormone (GH) expression, which also acts via GHsR and stimulates food intake, showed to be highly upregulated in TB mice. Expression of anorexigenic somatostatin showed to be 1.2 fold higher in TB mice compared to controls. Somatostatin is a strong negative feedback regulator of GH, suggesting that its upregulation could be a result of increased GH expression. Anorexigenic pro-opiomelanocortin (POMC) and cholecystokinin (CCK) expressions were slightly lower in TB by 1.1 fold and 1.2 fold respectively. PYY, leptin and glucagon expression were not included in the analysis, because absolute expressions were below threshold.

In addition to analysis of food intake regulators, a list of highly-upregulated genes was generated. Genes that were upregulated with a fold change above 1.5 in both study A and B, resulted in a list of 19 genes that were highly upregulated in both studies (Supplementary table S3.2). Lipocalin 2 and leucin-rich α2-glycoprotein 1 are both discussed for their role in tumour progression and for being potential biomarkers for cancer progression [14, 15] and secretoglobin (Scgb3a1) is considered a strong tumour suppressor [16]. Lipocalin 2 expression in hypothalamus has been reported to be strongly elevated upon influenza infection in mice, suggesting that lipocalin 2 in the brain is able to sense inflammatory stressors from the periphery [17]. The strong upregulation of also other inflammatory genes as interleukin 1 receptor and oncostatin M receptor in both studies contribute to the idea of an elevated inflammatory status in the hypothalamic area.

**Pathway analysis: serotonin and dopamine signalling**

Pathway analysis using Ingenuity Systems showed that the serotonin (5-HT) receptor signalling pathway was significantly altered (P<0.05) in the hypothalamic tissues of TB mice (Supplementary figure 3.1). Expression of genes involved in both 5-HT synthesis and 5-HT degradation showed to be lower in TB mice than in controls, pointing towards a compensatory mechanism regulating expression of these enzymes.
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Figure 3.2 Heat map representation of fold-changes of orexigenic- and anorexigenic genes in the hypothalamus in study A and B.

RNA from hypothalamus was used to perform micro-array experiment using Affymetrix chips. Fold changes relative to their control group were calculated and compared between the two studies. Each row represents a gene and each column represents a group of animals. RNA samples from the same group were pooled for analysis in study A. Study A: TB-0.5 = injected with 0.5 x 10^6 tumour cells and TB-1 = injected with 1 x 10^6 tumour cells.

In study B, TB mice were injected with 1 x 10^6 tumour cells and both pooled samples (pools) and individual replicates (mean) were analysed. Pools = RNA from all mice within one group were pooled, mean = calculated mean from replicates.

Red colour indicates genes that were higher expressed as control and green colour indicates genes that were lower expressed as the control. Black indicates genes whose expression was similar to compared to control. Grey indicates genes that were filtered out (NA) because absolute expression values were below the predefined threshold limits (M&M section) ID: Entrez ID; R: receptor, NA: not analysed.
Pathway analysis further showed that besides 5-HT signalling, also dopamine (DA) signalling was altered (Supplementary figure S3.1). Several genes involved in 5-HT signalling are also of importance in dopamine signalling. Changes in these shared genes between the 5-HT and DA pathways are therefore likely to have an effect on both neurotransmitters. Expression of \textit{gch1}, \textit{qdpr} and \textit{ddc}, which are involved in the synthesis of both 5-HT and DA, were strongly downregulated. Also transporter \textit{vmat}, which is important in transporting 5-HT and DA into the neuronal synapse, showed to be 1.7 fold lower in TB mice compared to controls. Tryptophan hydroxylase (\textit{tph}) and tyrosine hydroxylase (\textit{th}), rate-limiting enzymes in the synthesis of 5-HT or DA respectively, were also strongly downregulated. In addition, SERT and DAT, re-uptake transporters of 5-HT and DA respectively in order to terminate activation in the synaptic cleft, showed to be more than 2 fold lower in TB mice. This indicates that besides shared genes between the 5-HT and DA pathways, also genes specifically involved in either DA or 5-HT synthesis, were altered. Figure 3.3A shows an overview of genes involved in 5-HT and DA signalling and their fold changes.

To determine the effects of these changes on gene expression, 5-HT and DA levels were measured. Serotonin levels showed to be significantly lower in the TB mice, whereas DA levels showed not to be different in TB mice compared to control animals (Figure 3.3C-3.3E). Since both DA and 5-HT have been discussed for their role in food intake and feeding behaviour, correlation between these neurotransmitters and food intake were studied. Serotonin levels were found to correlate with food intake in both C and TB mice, while this correlation could not be made for DA and food intake (Figure 3.3D-3.3F).

**Figure 3.3 Serotonin and dopamine signalling in TB mice and correlations with food intake.**
Canonical pathway analysis with IPA (Ingenuity® Systems) revealed serotonin receptor signalling pathway and dopamine receptor signalling pathway as being significantly changed in tumour-bearing mice in both studies. A) Overview of serotonin and dopamine signalling pathway and their overlapping genes (\textit{Gch1}, \textit{Qdpr}, \textit{DDC}, \textit{VMAT} and degrading enzymes \textit{MAO} and \textit{ALDH}). Expression of genes necessary for the synthesis of serotonin/ dopamine as well as genes playing a role in the termination of serotonin/dopamine signalling in the synapse showed to be downregulated. B) Heat map of fold changes of numbered genes. Genes 1-5 represent genes involved in both serotonin and dopamine signalling. C) Serotonin level in brain relative to control mouse. D) Dopamine level in brain relative to control mouse. E) Correlation of serotonin with food intake. F) Correlation of dopamine with food intake. Values are expressed as mean ±SEM. C = sham-injected control, TB = injected with 1 x 10^6 tumour cells * Significantly different from C (P<0.05). DHNTP = 7,8-dihydroneopterin Triphosphate, 6PTS = 6-pyruvoyl-Tetrahydropterin, SHIAA = 5-Hydroxyindole Acetic acid, q-dbtt = q-dihydrobipterin, KYN= kynurenine, TRP = tryptophan, DA = dopamine, SHT = 5-hydroxytryptamine (serotonin), MAO = mono amine oxidase, ALDH = aldehyde dehydrogenase, SULT = sulfotransferase.
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**A**

**B**

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**C, D, E, F**

C: Serotonin (% of control) vs. TB

D: Dopamine (% of control) vs. TB

E: Serotonin (ng/mg protein) vs. Food intake (g)

F: Dopamine (ng/mg protein) vs. Food intake (g)
Plasma amino acid levels and immune parameters

In study B, levels of various amino acids in plasma were measured (Supplementary table S3.3). TRP levels relative to branched-chain amino acids (BCAA) is often used as a predictor for 5-HT status in the brain. Surprisingly, TRP/BCAA ratios showed to be significantly higher in TB animals compared to controls (Figure 3.4A).

To assess tumour-driven inflammatory response, PGE₂, TNFα and IL-6 were measured in blood plasma (Figure 3.4B-3.4D). TNFα levels showed to decrease, while pro-inflammatory mediators IL-6 and PGE₂ showed to be significantly elevated.

![Figure 3.4 Ratio TRP/BCAA and cytokine plasma level in blood plasma. A) Blood plasma aminoacid levels in TB mice. B) TNFα plasma levels. C) IL-6 plasma levels. D) PGE₂ plasma levels. * Significantly different from C (P<0.05). TRP = tryptophan, BCAA = branched chain amino acids, comprising valine, leucine and isoleucine.](image)

**Discussion**

In the present study, we report on the hypothalamic gene expression profile in C26 tumour-bearing. Here, we show gene expressions of important orexigenic genes to be increased, while expression of anorexigenic genes decreased. Remarkable is the downregulation of the complete serotonin signalling cascade in TB mice. To our
knowledge, this is the first study showing that serotonin synthesis, degradation and synaptic release is affected during tumour growth and subsequent changes on serotonin levels are correlated to changes in food intake.

The observed increase in food intake in these C26 TB mice in both experiments has not yet been reported. The C26 cancer-cachexia mouse model as described in 1990 by Tanaka et al. [18] is often referred to as “the standard” for the C26 model. With this set-up, cachexia develops, which is reflected in a decrease in muscle weight as well as adipose tissue depletion. Our findings on cachexia in the present model correspond to the results found by Tanaka et al. A specific characteristic for this model is that in this particular setting, food intake of TB mice does not change and is not different from that of healthy controls. However, in the meantime various research groups have reported a strong decrease in food intake in mice injected with these C26 cancer cells [19, 20], suggesting that changes in morphology of the cell line, variation in the strain of mice and differences in number of tumour cells used for inoculation might led to these discrepancies in findings on food intake. It has already been reported that C26-induced cachexia and anorexia can vary according to the inoculation site [21] and origin of C26 cells [22], as well as the use of solid tumour fragments or cell suspensions for inoculation can cause variation [23].

Also adaptation of C26 cells to \textit{in vitro} culture conditions can cause mutations in the cell line leading to changes in cell characteristics, sensitivity to chemotherapy, metastatic potential and tumour-induced cachexia in mice, suggesting that C26 cells can differentiate to different variants and change tumour characteristics despite being derived from the same source [24]. Subsequently, the extent and type of inflammatory response that is induced by tumour growth might play a role in the severity of cachexia and anorexia. Differences in tumour-driven inflammation, might therefore explain differences between various cancer models. To confirm tumour-induced inflammation in our model, various cytokines and PGE$_2$ levels in blood plasma were measured. IL-6 and PGE$_2$ showed to be elevated in TB mice, which also has been reported previously [25]. However, in contrast to previous results, TNFα levels in blood plasma were not elevated in TB mice compared to control mice.

Elevated concentrations of TNFα are reported to decrease food intake [26], suggesting that the absence of TNFα –mediated inflammation might play a role in compensating feeding behaviour in this model. All together, we would like to propose the hypothesis
that although the “C26 model” is referred to as such, in fact the model is heterogeneous with many varieties. Small differences in experimental settings and spontaneous mutations in the cell line used might lead to great changes in characteristics of the model.

In the present study, pathway analysis indicates serotonin (5-HT) and dopamine (DA) signalling to be altered in TB mice compared to controls. DA and 5-HT are both important neurotransmitters involved in eating behaviour. The signalling pathways of 5-HT, DA and DA-metabolites norepinephrine and epinephrine are closely linked by shared synthesizing enzymes and transporters. Therefore, it is very likely that changes in these shared genes will propose these comprehensive effects. Since both pathways were predicted to be altered, we measured 5-HT and DA levels in whole brain homogenates. Serotonin levels were found to be significantly lower in TB mice compared to control. This might be caused by decreased TPH and SERT expression, which have been directly correlated to lowered 5-HT levels in other studies [27, 28]. However, DA levels in TB mice showed not to be different from levels in controls, suggesting that effects on expression of shared genes are of greater impact on 5-HT synthesis, than on DA synthesis. In addition, in relation to changes in food intake in TB mice, only 5-HT levels showed to inversely correlate with food intake whereas DA levels did not. A limitation of the present set-up is that gene pathway analysis was based on hypothalamic transcripts whereas analysis of 5-HT and DA levels took place in homogenates of remaining brain material. Therefore, levels of these neurotransmitters reflect an indication and local differences in the various regions of the brain in both DA and 5-HT cannot be ruled-out.

Overall, our results suggest that primarily 5-HT is associated with altered food intake regulation caused by tumour growth. This is consistent with findings reported by other research groups. In MCA tumour-bearing rats which showed clear anorexia, 5-HT levels were elevated in the PVN of the hypothalamus [29]. This elevation of 5-HT showed to be clearly tumour-driven, since it did not occur in the pair-fed controls. In addition, 5-HT levels were restored after tumour resection. On the other hand, also DA levels in the hypothalamus were reported to be decreased in that study. However, this decrease in DA level was also found in the pair-fed control group to a similar extent as observed in TB rats. This suggests that the decrease DA in the hypothalamus was a consequence and not a direct cause of decreased food intake. Dopamine has shown to play a role in mechanisms induced during and after feeding, such as rewarding mechanisms [30]. For example DA levels in the hypothalamus have been shown to increase directly after eating
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and the magnitude of DA response is relative to the size of meal ingested [31]. Our results, together with the existing literature, suggest that DA is not a direct causative factor in the development of cancer anorexia, since it is not induced by tumour growth but decreases subsequent to a reduction in food intake. However, DA is likely to play a role in sustaining cancer anorexia, once this has been manifested. Long-term alterations in DA in the hypothalamus are suggested to affect feeding pattern [32] and treatment with L-dopa, precursor of DA, has been shown to be beneficial in restoring appetite in severely anorectic cancer patients [33]. In addition, an increase in hypothalamic expression of several DA receptors (DRD), including DRD2, during tumour growth in anorectic TB rats, might play a role in sustaintment of cancer anorexia [32]. Our results support this finding, as we found a decreased expression of this receptor in TB mice with compensatory feeding behaviour.

In summary, our results suggest that changes in 5-HT signalling and 5-HT levels contribute to compensatory eating during tumour growth. Serotonin is considered an important mediator in the regulation of satiety and hunger [34]. High brain levels induce satiation, whereas lowered levels stimulate food intake. In cancer, elevated brain serotonin has been suggested to play a crucial role in the development of anorexia [6, 29]. On the other hand, lowered serotonin levels and downregulation of SERT are discussed for their role in eating abnormalities and hyperphagia in obesity [35, 36].

Next to changes on 5-HT signalling and 5-HT levels, also tryptophan (TRP) metabolism appeared to change in TB mice. TRP/BCAA ratios in plasma showed to be increased in TB animals. TRP, precursor of 5HT, competes with BCAA at the blood brain barrier. Therefore plasma TRP/BCAA ratio is used as predictor for 5-HT levels in the brain and is often linked to food intake. From this perspective, an elevated TRP/BCAA ratio would result in increased TRP availability for serotonin synthesis in the brain and subsequently higher brain 5-HT levels. However, inconsistencies in this theory have been reported. Several reports show that plasma TRP levels do not predict TRP in brain and consequently brain 5-HT levels [37, 38]. In addition, plasma TRP/BCAA ratio as predictor for changes in food intake [39], appetite [40] and satiety [41] has been reported to fail in several studies. Amino acid profiles in blood reflect skeletal muscle status and total protein metabolism in the body and is dependent on the physical status of the subject [42]. In the case of severe cachexia it could be that large metabolic alterations in muscle [43] and the presence of insulin resistance in the muscle [44] might distort amino acid profiles in blood in order to predict brain 5-HT levels via TRP ratios adequately.
In the present study, various food intake regulators were studied for their role in the observed increased food intake in TB mice. AgRP and NPY expressions were highly upregulated in TB mice. Central infusion of AgRP in cachectic C26 tumour-bearing mice results in an increase in food intake [45], which supports our findings. However, increased expression of NPY and its relation to potentiate feeding in this study is more difficult to interpret, as messenger NPY has been reported to not correlate with NPY levels in the hypothalamus in cancer cachectic conditions [46]. Several studies have shown that in cachectic and anorectic TB mice [47] and rats [46], messenger NPY is also elevated. However, translation of messenger NPY or transport of NPY to NPY terminals showed not to correspond to mRNA changes shown by measurements of NPY levels and immunohistochemistry [46]. Serotonin has been discussed to play a role in this imbalance between messenger NPY and NPY signalling in feeding behaviour in cancer anorexia [29]. Inhibition of 5-HT signalling showed to increase NPY levels [48], while induction of 5-HT signalling reduced NPY levels in rats [49]. All together this suggests that 5-HT signalling can interfere with NPY synthesis or transport. Therefore it could be that in the current study, decreased 5-HT levels and lowered 5-HT signalling might preserve NPY signalling.

In this study, we report on the transcriptomic analysis of a cancer-cachectic model with an increased food intake. In this model, food intake regulating systems, of which failure might contribute to anorexia, are able to adapt properly to changes in energy balance. We showed that alterations in NPY, AgRP and serotonin signalling are likely to explain compensatory eating behaviour of mice bearing C26 tumour. Therefore, targeting these systems might offer promising strategies to avoid the development of cancer-induced anorexia.

**Acknowledgements**

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The authors confirm that they comply with the principles of ethical publishing in the *Journal of Cachexia, Sarcopenia, and Muscle* 2010;1:7–8 (von Haehling S, Morley JE, Coats AJ and Anker SD).
References


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### Supplementary data

**Supplementary table S3.1 Differences in body weight and organ weight at day 20 after tumour inoculation in study A and B**

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C-26 cells were subcutaneously inoculated in CDF1 mice with different numbers of tumour cells. At day 20, organs were dissected and weighted. For skeletal muscles, the average of muscles from both legs was used for calculations. Values are expressed as mean ± SEM. **Study A:** C = sham injected control (n=4), TB-0.5 = injected with 0.5 x 10^6 tumour cells (n=4), TB-1 = injected with 1 x 10^6 tumour cells (n=3). **Study B:** C = sham injected control (n=6), TB = injected with 1 x 10^6 tumour cells (n=9). * Significantly different from C (P<0.05). EDL: *m. extensor digitorum longus*, m. gastroc: *m. gastrocnemius*, m.tib: *m. tibialis anterior.*
### Supplementary table S3.2 List of genes highly upregulated in the hypothalamus in TB groups in both study A and B

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Upregulated genes in TB mice compared to control mice from study A and B that were injected with 1 million C-26 cells were compared. All genes with an induction fold above 1.5 were included. A list of 19 overlapping genes was revealed, showing genes that were highly upregulated in both studies.
### Supplementary table S3.3 Plasma amino acid levels at day 20 in study B

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<th>Plasma amino acid concentration (μmol/L)</th>
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<td>Alanine</td>
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<td>Arginine</td>
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<td>40.9 ± 4.4</td>
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<td>64.4 ± 4.5</td>
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<tr>
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<td>421.3 ± 67.1</td>
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<tr>
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<td>130.5 ± 45.1</td>
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<tr>
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Plasma levels of various amino acids were measured using HPLC. Values are expressed as mean ± SEM. C = sham injected control), TB = injected with 1 x 10⁶ tumour cells. * Significantly different from C (P<0.05).

BCAA: (leucine, isoleucine, valine);
LNAA: (isoleucine, leucine, valine, phenylalanine, tyrosine, histidine, methionine);
EA: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, histidine.

Abbreviations: BCAA = branched-chain amino acids, LNAA = Large neutral amino acids, EA = essential amino acids.
Supplementary figure S3.1 Top 5 Canonical pathways in mice bearing C26-adenocarcinoma. Canonical pathway analysis with IPA (Ingenuity® Systems, www.ingenuity.com) revealed the serotonin receptor signalling pathway and the dopamine receptor signalling pathway as being significantly changed in tumour-bearing mice in both studies. A set of genes involved in serotonin signalling is also of importance in dopamine signalling. These overlapping genes are shown in Figure 3.3. In addition, degradation of these neurotransmitters showed to be altered, since a specific set of genes including aldehyde dehydrogenases was downregulated.
Differences in food intake of tumour-bearing cachectic mice are associated with hypothalamic serotonin signalling


Chapter 4 | Serotonin and neuropeptide Y in cancer cachexia

Abstract

**Background:** Anorexia is a common symptom among cancer patients and contributes to malnutrition and strongly impinges on quality of life. Cancer-induced anorexia is thought to be caused by an inability of food intake-regulating systems in the hypothalamus to respond adequately to negative energy balance during tumour growth. Here, we show that this impaired response of food-intake control is likely to be mediated by altered serotonin signalling and failure in posttranscriptional neuropeptide Y (NPY) regulation.

**Methods:** Two tumour cachectic mouse models with different food intake behaviours were used: a C26-colon adenocarcinoma model with increased food intake and a Lewis lung carcinoma model with decreased food intake. This contrast in food intake behaviour between tumour-bearing (TB) mice in response to growth of the two different tumours was used to distinguish between processes involved in cachexia and mechanisms that might be important in food intake regulation. The hypothalamus was used for transcriptomics (Affymetrix chips).

**Results:** In both models, hypothalamic expression of orexigenic neuropeptide NPY was significantly higher compared to controls, suggesting that this change does not directly reflect food intake status, but might be linked to negative energy balance in cachexia. Expression of genes involved in serotonin signalling showed to be different between C26-TB mice and LLC-TB mice and were inversely associated with food intake. In vitro, using hypothalamic cell lines, serotonin repressed neuronal hypothalamic NPY secretion, while not affecting messenger NPY expression, suggesting that serotonin signalling can interfere with NPY synthesis, transport or secretion.

**Conclusions:** Altered serotonin signalling is associated with changes in food intake behaviour in cachectic tumour-bearing mice. Serotonins’ inhibitory effect on food intake under cancer cachectic conditions is probably via affecting the neuropeptide Y system. Therefore, serotonin regulation might be a therapeutic target to prevent the development of cancer-induced eating disorders.
**Introduction**

Anorexia and consequently a reduced caloric intake [1] affects a majority of all cancer patients [2]. Cancer patients with good appetite and adequate caloric intake have higher survival rates than patients who suffer from anorexia and hence a lower energy intake [3, 4]. Cancer-induced anorexia is considered to be predominantly caused by failure of orexigenic and anorexigenic food intake-regulating systems in the hypothalamus to respond adequately to changes in energy balance [5]. This merits further elucidation of the specific mechanisms involved in this hypothalamic resistance to peripheral neuro-endocrine triggers. Several food intake regulating systems have been mentioned to play a role in the onset of hypothalamic resistance, including the orexigenic triggers neuropeptide Y and ghrelin and anorexigenic melanocortin and CART systems. Cancer anorexia often develops during the progression of cachexia [6], and the combined clinical picture is often called anorexia-cachexia syndrome [7]. Cachexia is a complex metabolic syndrome associated with underlying illness and is characterized by progressive loss of muscle (muscle wasting) with or without loss of fat mass. This results in weight loss, a reduced quality of life and a shortened survival time [6, 8, 9]. Although anorexia is often linked to cachexia and likely to be initiated by similar pathologies (tumour growth), it is unclear to what extent anorexia and the metabolic alterations of cachexia affect each other, or to what extent these are distinct entities with their own pathology. Here, we use two tumour-bearing (TB) mouse models displaying similar development of cachexia. At the same time they show opposite effects on food intake in response to tumour growth. This provides opportunities to disentangle processes involved in anorexia from those causing cachexia. By applying transcriptomics we show that tumour-induced cachexia is associated with several distinct changes in food-intake regulating mediators in the hypothalamus that appear to be independent of food intake status. Furthermore, we found alterations in hypothalamic serotonin signalling and these changes were inversely associated with food intake. In addition, we used hypothalamic cell lines to explore the mechanisms involved in the interaction between serotonin and the NPY-ergic system.
Materials and methods

**Lewis lung tumour model (LLM)**

C57Bl/6 male mice (Harlan, Barcelona, Spain), weighing approximately 20 g, were placed on a standard ad libitum diet (Panlab, Barcelona, Spain) and had free access to water. Lewis Lung carcinoma cells were obtained from exponential tumours. Under general anaesthesia, 0.5x10^6 cells were injected intramuscular in the hind leg. Four groups were included: C, Control mice sacrificed at day 14 (n=6); TB-10, Tumour-bearing mice sacrificed at day 10 (n=6); TB-14 Tumour-bearing mice sacrificed at day 14 (n=8) and TB-17, Tumour-bearing mice sacrificed at day 17 (n=6). Body weight and food intake were measured daily. At the day of sacrifice, mice were anesthetized with a ketamine/xylazine mixture (i.p.; Imalgene* and Rompun* respectively) and blood was collected by cardiac puncture.

**C26 colon adenocarcinoma tumour model (C26)**

Part of the data (Figure 4.1A) from this experimental arm with C26 TB mice have been published previously [10], but are briefly described again here for easy comparison with Lewis lung TB mice.

Male CDF1 (BALB/cx DBA/2) mice aged six-to-seven weeks (Harlan Nederland, Horst, The Netherlands) were placed on a standard ad libitum diet (AIN93M, Research Diet Services, The Netherlands) and had free access to water. Murine C26 adenocarcinoma cells were cultured and suspended as described previously [11]. Under general anaesthesia (isoflurane/ N₂O/O₂), 1 x 10^6 tumour cells in 0.2ml HBSS were inoculated subcutaneously into the right inguinal flank. Controls were sham-injected with 0.2 ml HBSS. Two groups were included: C, Control mice (n=6) and TB, Tumour-bearing (n=9). Body weight and food intake were measured three times a week. At day 19, blood was collected by cardiac puncture under general anaesthesia.

In both tumour models, animals were individually housed one week before start of the experiment in a climate-controlled room (12:12 dark-light cycle; 21°C±1°C).

After sacrifice, brain, hypothalamus, tumour, organs and lower leg skeletal muscles (non-tumour-leg) were weighted and frozen in liquid nitrogen and stored at -80°C. Carcass weight was calculated by body weight at day of section minus tumour weight.
Figure 4.1 Effect of tumour inoculation on food intake, carcass weight and muscle and fat weight in two cachectic models.
A) Time-course of change in food intake in mice bearing the C26 tumour. B) Time course of change in food intake of TB mice baring the Lewis lung carcinoma (LLC). C) Carcass weight of C26 TB mice at day 19. D) Carcass weight of LLC TB mice at day 10, 14 and 17. E) Weight of m. Gastrocnemius (GSN) and epididymal fat pads (WAT) in C26 TB mice at day 19. F) Weight of m. Gastrocnemius (GSN) and epididymal fat pads (WAT) in LLC TB mice at day 10, 14 and 17. * Significantly different from C (P<0.05)

Data is expressed as mean ± S.E.M. C=sham-injected control, TB = C26 injected TB, C-14 = sham-injected control sacrificed at day 14, TB-10 = LLC injected TB sacrificed at day 14, TB-14 = LLC injected TB sacrificed at day 14 and TB-17 = LL injected TB sacrificed at day 17. Carcass weight was calculated by body weight at day of section minus tumour weight.

All experimental procedures were made in accordance with the European Community guidelines for the use of laboratory animals and complied with the principles of good laboratory animal care.
Chapter 4 | Serotonin and neuropeptide Y in cancer cachexia

Cell culture and reagents
Murine derived hypothalamic neuronal cell lines hypoE-46 and hypoA2/12 (CELLutions Biosystems Inc. Canada) were grown and maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 unit/ml penicillin and 100μg/ml streptomycin at 37°C under 5.0% CO₂. Cells were grown in monolayers to 90% confluency. Then medium was replaced by serum-free DMEM containing penicillin and streptomycin. After 4 hours, cells were exposed to serotonin (100 μg/ml, 1 μg/ml, 10 ng/ml or 100 pg/ml) for 24 hours or 60mM KCl for 15 minutes. After exposure, supernatant was collected and used for NPY measurements using an enzyme immunoassay (NPY EIA, Phoenix Pharmaceuticals, CA, USA). Cells were homogenized in 40mM Tris, 1mM EDTA, 5mM EGTA and 0.50% Triton X-100. Homogenates were used to measure protein content (Pierce Bicinchoninic acid Rockford, IL, USA). Serotonin cytotoxicity was determined by measuring LDH leakage and effects on cell viability using an XTT conversion assay (Roche Diagnostics, Mannheim, Germany).

Serotonin levels
Hypothalamic samples were used for micro array experiments, while remaining brain parts were used to determine serotonin levels. Brains were homogenized in 1ml containing in 40mM Tris, 1mM EDTA, 5mM EGTA and 0.50% Triton X-100. Serotonin levels were measured using enzyme-immunoassay kits (BAE-5900, LDN, Nordhorn, Germany) and corrected for the amount of protein (Nanodrop spectrophotometer, Thermo Scientific).

Statistics
Data was analysed by statistical analysis of variance (ANOVA) followed by a post hoc Bonferroni test or by a Student’s t test. Differences were considered significant at a two-tailed P<0.05. Statistical analyses were performed using Graphpad Prism 5. For statistical analysis of microarray data see microarray section (below).

Microarray studies
Total RNA from the hypothalamus was isolated by using RNeasy Lipid tissue kit (Qiagen, Venlo, The Netherlands). RNA concentrations were measured by absorbance at 260 nm (Nanodrop). RNA quality was checked using the RNA 6000 Nano assay on the Agilent
2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) according to the manufacturer’s protocol. For each mouse, total RNA (100ng) was labelled using the Ambion WT expression kit (Life Technologies, Bleiswijk, The Netherlands). Micro-array experiments were performed by using Affymetrix Mouse Gene ST 1.1.

From the C26 model experiment, 4 control samples and 5 samples from tumour-bearing mice were included in this experiment; however 1 control sample gave multiple spots on the array and was therefore excluded from analysis. The Lewis lung experiment included 4 control, 5 TB-10, 7 TB-14 and 6 TB-17 samples.

Array data were analysed using an in-house, on-line system [12]. Briefly, probesets were redefined according to Dai et al. [13] using remapped CDF version 15.1 based on the Entrez Gene database. In total these arrays target 21,225 unique genes. Robust multi-array (RMA) analysis was used to obtain expression values [14, 15]. We only took genes into account that had an intensity >20 on at least 2 arrays, had an interquartile range throughout the samples of >0.1, and at least 7 probes per genes. Genes were considered differentially expressed at P<0.05 after intensity-based moderated t-statistics [16]. Further functional interpretation of the data was performed through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Canonical pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Genes from the data set that met the cut-off of 1.3 fold change and p-value cut-off of 0.05, and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. To list up genes involved in food intake regulation, genes included in GO 0002023, GO 0007631, GO0008343, GO0060259, GO 0042755 (food intake, feeding behaviour and eating behaviour) were analysed (Gene Ontology: tool for the unification of biology. The Gene Ontology Consortium (2000) Nature Genet. 25: 25-29). Also genes POMC (ID 18976), GH ( ID:14599), Sst (ID: 20604) and pro-melanin-concentrating hormone (ID:110312) were additionally looked at, since they also have been described to play a role in food intake behaviour [17]. Array data have been submitted to the Gene Expression Omnibus, accession numbers GSE44082 (C26 tumour) and GSE57190 (Lewis Lung tumour and hypothalamic cell lines). Data set containing the array data on hypothalamic tissues of C26 TB mice has been published previously [10].
Chapter 4  Serotonin and neuropeptide Y in cancer cachexia

Results

Body weight and food intake
In both tumour models, TB mice showed a lower carcass weight, muscle weight and white adipose fat mass (Figure 4.1) compared to their healthy controls, reflecting that cachexia was present in mice bearing either the C26 or the Lewis Lung tumour. Food intake changes in TB animals were found to differ between the two cachectic models. Mice bearing the C26 tumour were found to increase their food intake synchronously to loss of body weight [10], while mice bearing the Lewis lung carcinoma (LLC) showed a lowered food intake from day 7 after tumour inoculation compared to controls (Figure 4.1 A and D). These opposing changes in food intake in TB mice in response to tumour growth of the two different tumours are used to disentangle shared mechanisms of cachexia from those important for food intake regulation.

Microarray analysis of the hypothalamus: comparison C26 and LLM
Expression of genes involved in food intake was analysed to determine if these could explain the opposing changes in food intake between C26 TB and LLC TB mice. A list of genes involved in food intake was generated by using GO categories involved in food intake regulation (M&M). Fold changes of expression of orexigenic and anorexigenic genes were compared between the two tumour models (Figure 4.2a). Interestingly, changes in gene expression of important mediators of food intake regulation, NPY, AgRP and POMC showed to be similar in C26 TB mice and LLC TB mice compared to their controls even though food intake behaviour was different between the two

Figure 4.2 Heat map representation of gene expressions in the hypothalamus in C26 and LLC TB mice.
A) Fold changes of orexigenic and anorexigenic genes relative to their control group were calculated and compared between C26 and LLC TB mice.
B) Top 30 upregulated genes in C26 TB mice compared to their control and top 30 genes upregulated genes in LLC TB mice compared to their control. 12 genes were overlapping between the C26 and LLC tumour model.
Each row represents a gene and each column represents a group of animals.
Red colour indicates genes that were higher expressed as control and green colour indicates genes that were lower expressed as the control. Black indicates genes whose expression was similar to compared to control. ID: Entrez ID. C=sham-injected control, TB = C26 injected TB, C-14 = sham-injected control sacrificed at day 14, TB-10 = LLC injected TB sacrificed at day 10, TB-14 = LLC injected TB sacrificed at day 14 and TB-17 = LLC injected TB sacrificed at day 17.
Figure 4.2a
models. Furthermore, gene expression of oxytocin showed to be altered in TB mice compared to controls and differed between the two models. In addition, analysis of highly upregulated genes (top 30 upregulated genes in TB mice compared to controls) in both tumour models was performed. There was a strong overlap of genes that were highly upregulated in both C26 TB and LLC TB mice compared to their controls, including NPY and AgRP. Furthermore, expression of inflammatory markers lipocalin 2, leucin-rich α2-glycoprotein 1, secretoglobin 3a1 and oncostatin M receptor showed to be highly upregulated in TB mice in both models. Taken together this suggests that major alterations in hypothalamic mechanisms are similar for both tumour models.
Microarray analysis of the hypothalamus: serotonin signalling

We hypothesized serotonin to play a crucial role in the opposing food intake behaviour between the C26 tumour and the LLC tumour model. Therefore, expression levels of genes involved in serotonin signalling were compared between the two tumour models. C26 TB mice expressing compensatory eating behaviour in response to weight loss, showed altered serotonin signalling and lower brain serotonin levels compared to their controls. In LLC TB mice, genes involved in serotonin signalling showed a different expression pattern compared to C26 TB mice. Expression of tryptophan hydroxylase (\textit{tph}) was strongly downregulated in C26 TB mice compared to controls, while expression of this gene was not different from controls in LLC TB mice. Expression of \textit{ddc} and \textit{vmat}, genes involved in serotonin synthesis and serotonin release, respectively showed to be downregulated in C26 TB mice, whereas LLC TB mice showed higher expression of these genes (Figure 4.3B). Serotonin brain levels in LLC TB mice showed to be slightly elevated at day 10, just after the onset of anorexia, while levels were lower in C26 TB mice compared to controls (Figure 4.3C).

Hypothalamic cell lines: Effect of serotonin on messenger NPY and NPY secretion

Murine derived hypothalamic cell lines hypoE-46 and hypoA-2/12 were 24 hours exposed to serotonin (100 pg/ml – 100 μg/ml). Serotonin showed to decrease NPY secretion in the hypoE-46 cells, while it did not have any effect on messenger NPY (Figure 4.4). In HypoA-2/12 cells, serotonin did not have an effect on either NPY secretion or NPY gene expression. Basal expression level of serotonin receptors 5HT1b, 5HT1d, 5HT2a and 5HT2b showed to be significantly different between the two cell lines (Table 4.1). The effect of serotonin on gene expression of 5HT receptors was not different between the two cell lines, except for 5HT2b, which was downregulated in HypoE-46 cells and upregulated in HypoA-2/12 cells after serotonin exposure.

Discussion

Cancer anorexia has been explained by an inability of the hypothalamus to respond adequately to triggers from the periphery during a situation of negative energy balance [18]. Here, we show that this impaired response of food-intake control is associated with
### Chapter 4 | Serotonin and neuropeptide Y in cancer cachexia

**Figure 4.3 Serotonin signalling in C26 and LLC TB mice.**

**A** Schematic view of genes involved in serotonin signalling.  
**B** Serotonin level in brain relative to control mice in C26 and LLC TB mice.  
**C** Heat map of fold changes of expressions of genes involved in serotonin signalling. Values are expressed as mean ± SEM. C = sham-injected control, TB = injected with 1 x 10^6 tumour cells, DNTP = 7,8-dihydroneopterin Triphosphate, 6PTS = 6-pyruvoyl-Tetrahydropterin, q-dbt = q-dihydrobiopterin, TRP = tryptophan, SHT = 5-hydroxytryptamine (serotonin), MAO = monoamine oxidase, ALDH = aldehyde dehydrogenase.

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* The values indicate fold changes in expression levels. Positive values indicate increased expression, and negative values indicate decreased expression. Statistical significance is denoted by * (p < 0.05).
altered serotonin signalling and probably stunted NPY posttranscriptional regulation
in the hypothalamus. In addition this serotonin regulation and brain serotonin levels
were inversely associated with food intake. In *vitro*, serotonin was found to modulate
NPY release by hypothalamic neurons. Taken together, this suggests that serotonin is
likely to play a crucial role in the failure of the NPYergic system during cancer anorexia.

By comparing two cancer models sharing common characteristics of tumour-induced
muscle wasting and fat loss, while displaying opposite effects on food intake behaviour,
we are able to disentangle anorexia from cachectic processes. Peripheral markers for
cachexia reflecting weight loss, muscle loss and fat loss were comparable between
the two tumour models. In addition, the most prominently (top30 based) induced
hypothalamic genes compared to healthy controls, showed to be similar between the two
models. This suggests that there is a common subset of genes that is strongly induced
during tumour growth and progression of cachexia, despite the differences in type of
tumour, inoculation site, strain of mice and independent from effects on food intake
behaviour. These changes might represent markers for cachexia or tumour-induced
inflammation as gene expression of strongly up-regulated inflammatory genes was
overlapping between the two models.

**Figure 4.4** Effect of serotonin on messenger NPY and NPY secretion in hypothalamic cells.
Murine derived hypothalamic cell lines were 24 hours exposed to various concentrations serotonin. KCL
was used to depolarize cells (positive control). A) Effect of serotonin on NPY secretion in HypoE-46 and
Significantly different from C (P<0.05). Data are expressed as mean ± SEM.
Table 4.1 Effect of serotonin on gene expression in hypothalamic cells

<table>
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<th>HypoA-2/12 control</th>
<th>HypoA-2/12 100 μg 5HT</th>
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Murine derived hypothalamic cell lines HypoE-46 were 24 hours exposed to various concentrations serotonin. Gene expression of serotonin receptors (5HTRs) were determined by using microarrays. * Significantly different from control in HypoE-46 cells. ** Significantly different from HypoE-46 cells basal expression. *** Significantly different from control in HypoA-2/12 cells (P>0.01).
Interestingly, also a majority of genes involved in food intake regulation showed to be similar between the models, even though ultimate effects on food intake were different for the two models. Food intake and drive to eat are the outcomes of numerous metabolic, physiological and behavioural cues including satiety, hunger and reward and learning. Therefore, changes on individual genes are difficult to interpret, as the impact on food intake is not similar between these single genes. Furthermore, it might be that, considering the complexity of food intake regulation, by using GO classification on food intake behaviour, some genes are ignored that might be important as well. Finally, a limitation of the current set-up is that we study expression in the hypothalamus only. Although the hypothalamus is a crucial site of food intake regulation, not all processes involved in food intake are regulated in the hypothalamus [19]. Gene expression of NPY, one of the most potent food stimulating neuropeptides in the hypothalamus [20], showed to increase in both C26 TB and LLC TB mice and was among the strongest induced genes in both models. This suggests that expression of NPY in cachectic TB mice is independent of food intake status. NPY is considered to act as a sensor and regulator of energy balance by altering food intake in energy deficits, which is supported by findings that NPY is stimulated in conditions of negative energy balance like food restriction [21], food deprivation [22, 23], and exercise [24, 25]. Several studies have shown that in cachectic TB rodents, NPY mRNA in the hypothalamus is elevated [26-29]. However, this elevation of mRNA NPY does not reflect food intake behaviour [27, 29] and does not correspond to NPY levels in the hypothalamus [27]. In contrast to these elevated messenger NPY levels, anorectic TB rodents have lower PVN hypothalamic NPY levels [30, 31] and lower NPY immunostaining of fibres innervating various hypothalamic nuclei [32]. On the other hand, mice bearing an A375 tumour that do not suffer from cachexia and weight loss, did not show this elevation of NPY mRNA [26], suggesting that cachexia is an important trigger in stimulation of NPY mRNA. This might explain that in both the C26 model and LLC model, messenger NPY was highly upregulated in TB mice compared to controls, despite their difference in food intake behaviour. Consequently, we hypothesize that failure of the NPY-ergic system taking place at a post-transcriptional level, for example by impaired translation, synthesis, transport or secretion, plays a role in the development of tumour-induced anorexia.

In this study, we also found serotonin signalling to be differently regulated between two types of tumour-induced cachexia models, in addition to their opposed effects on food intake behaviour In C26 TB mice, serotonin signalling was strongly down-regulated
and inversely associated with an increased food intake. Anorectic Lewis Lung TB mice displayed less pronounced changes in transcript levels of gene involved in serotonin signalling compared to C26 TB mice. Moreover, transcriptional regulation was different from mice bearing C26 tumour. Furthermore, brain serotonin levels were significantly elevated in TB mice at day 10 compared to controls, shortly after the manifestation of anorexia. This effect was less clear at day 14 and day 17, suggesting that effects on serotonin are more prominent during the initial stages of anorexia development. It has been postulated that cancer anorexia develops by waves of brain activation [33], each of them not necessarily mediated by the same mediators. It could be that serotonin might play a role in the initiation of anorexia. Hypothalamic serotonin is an important mediator in the regulation of satiety and hunger [34]. In cancer-induced anorexia, serotonin is considered a key player in the induction of anorexia [35, 36]. In rodents, tumour-driven elevation of hypothalamic serotonin has been associated with reduced food intake [31, 37, 38]. Furthermore, this elevation of serotonin was not present in the pair-fed control group, meaning that reduced food intake itself does not result in an elevation of hypothalamic serotonin. In humans, a practical and non-invasive method to measure site-specific changes on serotonergic activity in the hypothalamus is not yet available, but increased levels of tryptophan, the precursor of serotonin, have been measured in cerebral spinal fluid of cancer anorectic patients [39].

The exact mechanism on how serotonin is able to regulate food intake is not yet understood. A cross interaction of serotonin with NPY-ergic system has been discussed [31]. Serotonin signalling is negatively associated with NPY activity [40-42]. Active lowering of serotonin levels results in increased NPY levels [43], while induction of 5-HT signalling showed to reduce NPY levels in rat hypothalamus [44]. More importantly, serotonin is able to repress food intake, despite weight loss-induced elevation of messenger NPY [45], suggesting that serotonin might act as an interfering factor between transcription and NPY secretion. Therefore, we hypothesized that serotonin might interfere with NPY signalling at a post transcriptional level. In this way, it could play a role in post-transcriptional failure of the NPY system during certain forms of cancer.

To determine serotonin's ability to repress NPY secretion, we used two murine derived hypothalamic cell lines. We showed that serotonin is able to reduce NPY secretion in HypoE-46 cells, a cell line suggested to originate from the PVN [46], without
affecting NPY gene expression, supporting the hypothesis that serotonin acts post-transcriptionally. Serotonin did not have an effect on NPY secretion in HypoA-2/12 cells a cell line suggested to originate from the arcuate nucleus (ARC) or supraoptic nucleus [47]. In lean and obese Zucker rats, increased levels of serotonin, induced by chronic administration of fluoxetine, resulted in reduced food intake and reduced levels of NPY in the PVN, but not in the ARC and other areas of the hypothalamus [40]. This suggests that NPY release by PVN neurons is more sensitive to serotonin and might explain the difference in response to serotonin between the two cell lines.

The two cell lines did not show differences in their general response to serotonin as reflected by expression of genes involved in serotonin signalling. An exception to this was the effect of serotonin on expression of the 5HT2a receptor, which was down-regulated in response to serotonin in the HypoE-46 and up-regulated in the HypoA-2/12 cell line. Moreover, basal expression of 5HT1b and 5HT2a, 5HT2b and 5HT2c differed between the two cell lines. In the ARC nucleus, serotonin has been reported to inhibit NPY release by hyperpolarization of AgRP/NPY neurons via 5HT1b receptors [48]. The role of 5HT2 receptors might be a target for further research on their role in NPY regulation. However, as NPY secretion is via granule exocytosis [49], also a receptor independent process like serotonylation of GTPases might be implicated. Serotonylation is a process, where intracellular serotonin is able to activate small GTPases via transamination. Dependent on the type of GTPases that are activated, several processes dependent on granule exocytosis can be affected, which has been reported for insulin secretion by pancreatic β cells [50] or platelet aggregation [51].

In this study, we provide evidence that cancer anorexia might be due to post transcriptional failure of the NPY-ergic system in the hypothalamus to respond to increased energy requirement in cancer cachexia. We show that serotonin is able to affect NPY secretion in vitro. In addition, serotonin signalling is found to be altered in TB mice, suggesting that serotonin might play a crucial role in failure of hypothalamic NPY signalling and subsequently the development or sustainment of cancer anorexia.

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that they comply with the principles of ethical publishing in the Journal of Cachexia, Sarcopenia, and Muscle 2010;1:7-8 (von Haehling S, Morley JE, Coats AJ and Anker SD).

**Conflict of interest**

J. Dwarkasing, M. Boekschoten, J. Argilès, S. Busquets, F. Penna, M. Toledo, A. Laviano and R. Witkamp have nothing to declare. M. van Dijk is employee of Nutricia Research, a medical nutrition company. K. van Norren is guest employee of Nutricia Research, a medical nutrition company.

**References**


Increased hypothalamic serotonin turnover in inflammation-induced anorexia

J. T. Dwarkasing, R. F. Witkamp, M. V. Boekschoten, M. C. ter Laak, M. S. Heins, K. van Norren

Submitted for publication.
Abstract

Anorexia can occur as a serious complication of chronic disease. Increasing evidence suggests that inflammation plays a major role, along with a hypothalamic dysregulation characterized by locally elevated serotonin levels. The present study was undertaken to further explore the connections between peripheral inflammation, anorexia and hypothalamic serotonin metabolism and signaling pathways. First, we investigated the response of two hypothalamic neuronal cell lines to TNFα, IL-6 and LPS. Next, we studied transcriptomic changes and serotonergic activity in the hypothalamus of mice after intraperitoneal injection with TNFα, IL-6 or a combination of TNFα and IL-6. In vitro, we showed that hypothalamic neurons responded to inflammatory mediators by releasing cytokines. This inflammatory response was associated with an increased serotonin release. Mice injected with TNFα and IL-6 showed decreased food intake, associated with altered expression of inflammation-related genes in the hypothalamus. In addition, hypothalamic serotonin turnover showed to be elevated in treated mice. Overall, our results underline that peripheral inflammation reaches the hypothalamus where it affects hypothalamic serotonergic metabolism. These hypothalamic changes in serotonin pathways are associated with decreased food intake, providing evidence for a role of serotonin in inflammation-induced anorexia.
Introduction

Loss of appetite (anorexia) leading to insufficient food intake is often seen in chronic illnesses including cancer, HIV and COPD. A chronically elevated increased inflammatory tone is considered one of the major drivers of anorexia in these diseases. Studies suggest that an ongoing elevated inflammatory tone in the hypothalamus, displaying the highest density of various cytokine receptors in the brain [1], is implicated in these disturbances in food intake. Inflammatory mediators affect important orexigenic and anorexigenic regulators including NPY [2] and POMC [3, 4] peptides in the hypothalamus. Cytokines and other pro-inflammatory signalling molecules from the periphery are able to reach the hypothalamus passing the blood brain barrier (BBB) [5-7]. In addition, de novo synthesis of various cytokines in the hypothalamus has been reported [8]. To trigger these processes, sensing of peripheral signals in the adjacent median eminence [9, 10] and activation of hypothalamic microglial and astrocyte cells [11, 12] might be crucial.

We previously described changes in hypothalamic serotonin signalling in rodent tumour models displaying severe body wasting (cachexia). These changes in serotonin formation were inversely associated with food intake [13]. This is in line with findings in a variety of chronic illnesses, where increased hypothalamic serotonin has been implicated for its role in the development of disease-associated anorexia [14-17]. Hypothalamic serotonin plays an important role in food intake, since it is able to respond to peripheral signals on energy status [18-20] and it is able to modulate anorexigenic and orexigenic signalling in the hypothalamus. Serotonin is able to affect food intake via activation of the anorexigenic melanocortin system involving 5HT2c receptors [21] and by inhibition of the orexigenic NPYergic system [15, 22]. Furthermore, reduction of brain serotonin by reducing availability of its precursor tryptophan (TRP) (Figure 5.1) has been shown to be beneficial in the treatment in anorexia during cancer [23].

In the present study, we investigated the anorexigenic effects of TNFα and IL-6, cytokines that are often elevated during chronic illness, on hypothalamic serotonin signalling. To test physiologically relevant concentrations of TNFα and IL-6 in illness, we included two combinations containing both TNFα and IL-6 that reflected plasma levels measured in C26 adenocarcinoma tumour-bearing mice or Lewis Lung tumour-bearing mice respectively [13, 24]. We show that these cytokines when administered intraperitoneally (ip) induce changes in the hypothalamic transcriptome consistent with changes in inflammatory pathways and serotonin signalling. Furthermore, these
cytokines alter hypothalamic levels of serotonin’s main metabolite 5-HIAA (Figure 5.1), indicating that synaptic serotonin release [25, 26] and serotonin turnover [27, 28] is affected by inflammation.

**Materials and methods**

**Cell culture and in vitro studies with IL-6, TNFα and LPS**

Murine derived hypothalamic neuronal cell lines hypoE-46 and hypoA2/12 (CELLutions Biosystems Inc. Canada) were grown and maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and 100μg/ml streptomycin at 37°C under 5.0% CO₂. Cells were grown in monolayers to 90% confluency. Then
medium was replaced by serum-free DMEM containing penicillin and streptomycin. After 4 hours, cells were exposed to LPS (1 μg/ml), TNFα (100 pg/ml), IL-6 (100 pg/ml) for 24 hours, or KCl (60 mM) for 15 minutes. After exposure, supernatant was collected to measure levels of serotonin (BAE-5900, LDN, Nordhorn, Germany), IL-6 (DY406, Abingdon, UK), TNFα (DY410, Abingdon, UK) and MCP-1 (DY479, R&D systems, Abingdon, UK) by enzyme-immuno assay. Cells were homogenized in 40mM Tris, 1mM EDTA, 5mM EGTA and 0.50% Triton X-100. Homogenates were used to measure 5-hydroxyindoleacetic acid (5-HIAA) by ELISA (MBS261481, MyBiosource, Breda, The Netherlands) and corrected for total protein content (Pierce Bicinchoninic acid Rockford, IL, USA). Cytotoxicity was determined by measuring LDH leakage and cell viability using an XTT conversion assay (Roche Diagnostics, Mannheim, Germany). All experiments were performed three times in quadruplicate.

**Animals**

C57BL/6 male mice (Harlan, Horst, The Netherlands), weighing approximately 20 g, were individually housed one week before start of the experiment. Mice were maintained on a 12 hour light:12 hour dark cycle in a climate-controlled room (21°C±1°C). Standard diet was *ad libitum* available during the entire experiment from one hour prior to dark phase until start of the light phase (Arie Blok B.V, Woerden, and The Netherlands). Water was freely available 24 hours a day. Food intake, water intake and body weight were monitored daily from one week prior to the end of the experiment.

All experimental procedures were made in accordance with the European Community guidelines for the use of laboratory animals and complied with the principles of good laboratory animal care.

**Experimental set-up**

Mice were injected intraperitoneal (ip) with 50ul of saline vehicle (G-Biosciences, St.Louis, USA), TNFα (Peprotech, London, UK), IL-6 (Peprotech, London, UK), or both TNFα and IL-6. The study included 6 groups: Control, TNFα, IL-6 Low, IL-6 High, TNFα + IL-6 Low and TNFα + IL-6 High (Table 5.1). The rationale to study different doses and combinations of IL-6 was based on our previous observations in mouse tumour models and the generally recognized central role of IL-6 as link between cancer and inflammation [13, 24]. Combinations TNF + IL-6 Low and TNF + IL-6 High
reflect plasma levels measured in C26 tumour-bearing mice and Lewis Lung tumour-bearing mice respectively. Each group included 12 mice, of which 6 mice were used for determination of hypothalamic metabolites and 6 mice were used for hypothalamic gene expression analysis. Mice were injected one hour prior to the dark phase. Five hours after injection, blood was collected by cardiac puncture under general anaesthesia. After sacrifice, brain, hypothalamus and organs were weighted, frozen in liquid nitrogen and stored at -80°C.

### Table 5.1 Experimental groups

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<tr>
<td>TNF + IL-6 High</td>
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**Hypothalamic metabolites 5-HT, 5-HIAA, DA, DOPAC and TRP**

Hypothalamus tissue was homogenised by sonication in 10 μL of 0.5 M perchloric acid per mg of tissue and stored at -80°C until analysis. Concentrations of DA, 5-HT, DOPAC, 5-HIAA and TRP were determined by HPLC with tandem mass spectrometry (MS/MS) detection, using deuterated internal standards of the analytes. Of each LC-MS sample, an aliquot was injected onto the HPLC column by an automated sample injector (SIL10-20AC-HT, Shimadzu, Japan). Chromatographic separation was performed on a SynergiMax column (100 x 3.0 mm, particle size 3 μm) held at a temperature of 35°C. The mobile phases consisted of A: ultrapurified H₂O + 0.1% formic and B: acetonitrile: ultrapurified H₂O (75:25) + 0.1% formic acid. Elution of the compounds proceeded using a suitable linear gradient at a flow rate of 0.3 mL/min. The MS analyses were performed using an API 4000 MS/MS system consisting of an API 4000 MS/MS detector and a Turbo Ion Spray interface (Applied Biosystems, the Netherlands). The acquisitions on API 4000 were performed in positive ionization mode for 5-HT, DA and TRP and in negative mode for 5-HIAA and DOPAC, with optimized settings for the analytes. The instrument was operated in multiple-reaction-monitoring (MRM) mode.
Data were calibrated and quantified using the Analyst data system (Applied Biosystems, version 1.6.2, the Netherlands). Concentrations in experimental samples were calculated based on the calibration curve in the corresponding matrix.

**Hypothalamic transcriptomics (microarray)**

Total RNA from the hypothalamus was isolated by using RNeasy Lipid tissue kit (Qiagen, Venlo, The Netherlands). RNA concentrations were measured by absorbance at 260 nm (Nanodrop). RNA quality was checked using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) according to the manufacturer’s protocol. For each mouse, total RNA (100 ng) was labelled using the Ambion WT expression kit (Life Technologies, Bleiswijk, The Netherlands). Microarray experiments were performed by using Affymetrix Mouse Gene 1.1 ST arrays. In the TNFα treated group, 1 sample gave multiple spots on the array and was therefore excluded from analysis. Array data were analysed using an in-house, on-line system [29]. Briefly, probesets were redefined according to Dai et al. [30] using remapped CDF version 18.0.1 based on the Entrez Gene database. In total these arrays target 21,266 unique genes. Robust multi-array (RMA) analysis was used to obtain expression values [31, 32]. We only took genes into account that had an intensity >20 on at least 3 arrays and at least 7 probes per genes. Genes were considered differentially expressed at P<0.05 after intensity-based moderated t-statistics [33]. Further functional interpretation of the data was performed through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Genes from the data set that met the cut-off of 1.2 fold change and p-value cut-off of 0.05 were considered for the analysis. Upstream regulators were identified by using cut-off values of z-score>1.96 and z-score<-1.96 combined with P<0.05. Furthermore for this upstream regulators analysis, only endogenous metabolites were considered (chemical drugs and compounds were excluded from analysis). Array data have been submitted to the Gene Expression Omnibus (GEO), accession number GSE69151.

**Plasma cytokines and gut hormones**

Plasma levels of TNF-α, amylin (Active), C-Peptide 2, ghrelin (Active), GIP (Total), GLP-1 (Active), glucagon, IL-6, insulin, leptin, MCP-1, Pancreatic Peptide (PP), PYY and resistin were measured using the 12-plex Mouse Metabolic Hormone Magnetic bead panel (Merck Millipore, Amsterdam, The Netherlands). Serum amyloid was measured
in liver homogenates using SAA mouse ELISA kit (Life technologies, Bleiswijk, The Netherlands).

**Statistics**
Data were analysed by statistical analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test or by a Dunnet test. Differences were considered significant at a two-tailed P<0.05. Statistical analyses were performed using Graphpad Prism 5.

**Results**

**IL-6, TNFα and LPS increase 5HT and 5-HIAA in hypothalamic cell lines**
Exposure of hypothalamic cell lines hypoE-46 and hypoA2/12 to IL-6 (100 pg/ml), TNFα (100 pg/ml) and LPS (1 μg/ml) for 24 hours stimulated serotonin (5HT) release into the medium. Furthermore, levels of intracellular 5-hydroxyindoleacetic acid (5-HIAA) were elevated after exposure to IL-6, TNFα and LPS in both cell lines. Both cell lines produced IL-6 when exposed to IL-6, since levels detected were 4–6 fold higher than exposed levels. In addition, both cell lines produced MCP-1 when exposed to LPS and IL-6. Compared to IL-6 and LPS, TNFα showed to be less potent in inducing the production of MCP-1 and IL-6 (Figure 5.2). No TNFα release was detected (data not shown) after exposure to IL-6, TNFα or LPS.

**IL-6 and TNFα reduce food intake in mice**
Hourly food intake remained constant in control animals during the entire study period. In cytokine treated mice, food intake curves started to deviate from 2 hours after injection, becoming significantly lower 4 hours after injection in the TNFα, IL-6 high, IL-6 Low + TNFα and IL-6 High + TNFα groups, compared to controls (Figure 5.3). These effects did not differ between cytokine treatments. After 5 hours of injection, food intake between all groups was similar again.

Plasma levels of cytokines and serum amyloid A levels in liver were measured 5 hours after injection. There were three groups with significant changes in plasma TNFα, IL-6 or liver SAA levels: the two combination groups and the IL-6 high group. Plasma TNFα was significantly higher in the two combination groups, while IL-6 was increased in the
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IL-6 high and the TNF+ IL-6 Low group. The elevation of the other combination group did not reach significance. Liver SAA was only significantly increased in the IL-6 high group (Figure 5.3). Plasma levels of MCP-1, leptin, resistin, PYY, amylin, GIP, GLP-1 and insulin were not different between groups (Supplemental data Figure S5.1). Ghrelin, pancreatic peptide and glucagon plasma levels were below detection limit of the assay.

**IL-6 and TNFα increase hypothalamic 5-HIAA and TRP**

Total hypothalamic serotonin (5HT) tissue concentrations were not affected by injection with TNF and/or IL-6 five hours after injection. However, serotonin’s
Figure 5.3 Effect of injection with TNFα, IL-6 or both on food intake and plasma cytokines.
A) Time course of food intake after injection with TNFα, IL-6 or both.
B and C) IL-6 and TNFα plasma levels 5 hours after injection.
D) Level of serum amyloid 1 (SAA) in liver homogenates 5 hours after injection.

* significantly different from control group (P<0.05), † significantly different from TNF, IL-6 Low and IL-6 High group (P<0.05). Data is expressed as mean ± SEM (n=12).
metabolite, 5-hydroxyindoleacetic acid (5HIAA), showed to be significantly elevated in hypothalamus homogenates of both the TNF + IL-6 Low and TNF + IL-6 High groups compared to controls (Figure 5.4). These increases were more prominent than those following injection with TNF, IL-6 Low and IL-6 High alone. Tryptophan (TRP) showed to be significantly higher in mice injected with TNF + IL-6 High compared to controls. Hypothalamic levels of dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) showed no differences between groups (Figure 5.4).

![Figure 5.4](image_url)

**Figure 5.4** Levels of tryptophan, serotonin and dopamine and their metabolites in the hypothalamus. Effect of ip injection with TNFα, IL-6 or both on A) 5-hydroxyindoleacetic acid (5HIAA), B) Serotonin (5HT), C) 3,4-Dihydroxyphenylacetic acid (DOPAC), D) Dopamine (DA) and E) Tryptophan (TRP).

* significantly different from control group (P<0.05), † significantly different from TNF, IL-6 Low and IL-6 High group (P<0.05). Data is expressed as mean ± SEM (n=6).
Hypothalamic transcriptome analysis: IL-6 and TNFα have similar effects on serotonin signalling and inflammatory pathways

Expression of genes that were changed with a fold change greater than 1.5 compared to controls were compared among the different groups, resulting in a list of 118 genes (Figure 5.5). From these genes, 87 genes showed to be altered in a similar direction (either up or down compared to controls) in at least 4 out of 5 treatment groups. Furthermore, 96 out of 118 genes were overlapping between animals from the TNF and IL-6 Low or IL-6 High groups. Altogether this shows that induced changes on gene expression were overall similar for treatment with TNF, IL-6 or the combination. More importantly, gene expression of rate-limiting enzymes involved in the synthesis of 5HT, including Tph2 and expression of the serotonin-reuptake transporter, Slc6a4, were found to be among these highly upregulated genes. Strongly down-regulated genes included those of two important orexigenic regulators NPY and AgRP. Treatment effects showed to be similar for NPY and AgRP and these were most prominent in the IL-6 Low and TNFα+ IL-6 Low groups (Figure 5.5). Using Ingenuity, upstream regulators that were present in at least 3 treatment groups were listed (Table 5.2). Overall, this revealed an inflammatory profile of upstream regulators, which included cytokines IFNγ, TGFβ and IL-6 and the enzyme IKBKG which is an encoded protein of the IκB complex, and crucial for activating NFκB. The Ingenuity database used included 75 IFNγ target genes of which 19 out of 27 had an overlap with IL-6 target genes, which might explain the mutual presence of both these cytokines.

Discussion

Decreased food intake (anorexia) often occurs during conditions characterized by an elevated inflammatory response. In this study we investigated the role of serotonin in this decreased food intake during inflammation. Here, we show that both in vitro and
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Serotonin in inflammation-induced anorexia

Overall supporting the viewpoint that changes in hypothalamic 5HT signaling are involved in anorexia resulting from inflammation.

Interestingly, we found that the two hypothalamic cell lines used here (HypoE-46 and HypoA-2/12), which are derived from different neuronal populations of the hypothalamus, the PVN [34] and the ARC [35] respectively, are able to produce inflammatory mediators when exposed to LPS, TNFα or IL-6. So far, studies on hypothalamic inflammation have primarily focussed on the role of microglial and astrocyte activation [11,
However, it has been shown before that neurons also express high levels of various cytokines [38] and that synthesis of cytokines occurs in response to blood-borne inflammatory mediators [39]. This suggests that neurons may play a significant role in development and sustainment of hypothalamic inflammation. Furthermore, we showed that this inflammatory response in hypothalamic neurons coincided with elevated serotonin secretion and increased intracellular 5-HIAA levels, reflecting an increase in serotonin turnover. In the mice, injection with combinations of TNFα and IL-6 resulted in increased hypothalamic 5-HIAA levels, while tissue serotonin levels did not differ from controls. A possible explanation for this apparent difference could be that in cell experiments, secreted serotonin was measured. However, in total tissue homogenates, intracellular serotonin cannot be distinguished from serotonin released into the synaptic cleft. Therefore, total tissue homogenate levels of serotonin do not properly reflect its release, which has also been reported for other monoamines and catecholamines [40]. However, 5-HIAA, the stable metabolic endproduct of 5HT is considered to be a good reflection for serotonin release [41-43] and therefore widely acknowledged as marker for serotonergic activity [25, 26].

Levels of 5-HIAA are measured in different matrices, models and diseases, including various forms of depression and aggressive behaviour disorders, where measurement of 5-HIAA gives better results than that of serotonin. Alterations on 5-HIAA are more pronounced and long-lasting, while changes on serotonin can be rapidly diminished [27, 44-46].

In the current experimental setting, 5 HT and metabolites were measured 5 hours after cytokine injection and it could be that alterations of serotonin levels had already diminished by then. A similar reasoning might explain why elevated levels of serotonin's precursor, tryptophan (TRP), were only prominent in mice injected with the combination of the highest dose of IL-6 and TNFα. Tryptophan levels have been reported to rise upon injection with cytokines depending on the type of inflammatory stimulus used. However at the same time, this inflammatory response is associated with increased TRP breakdown by indoleamine 2,3-dioxygenase m (IDO), to synthesize kynurenine [47, 48].

In plasma, levels of TNFα and IL-6 were not elevated in the groups treated with a single cytokine. However, when these cytokines were given in combination, high levels of IL-6 and TNFα were measured. It is likely that clearance of the administered cytokines already occurred within five hours following injection. For both TNFα and IL-6, complete clear-
ance from the circulation has been reported to occur within 6 hours after ip injection [49, 50]. Furthermore levels of these cytokines have different temporal profiles, with TNFα plasma being fast responsive and also more rapidly cleared than IL-6 from the blood, but at the same time being more persistent in hypothalamus than IL-6 [51, 52].

Food intake showed to decrease in all treated groups and there was no difference between the treatments. It appears that the lowest dose of IL-6 was sufficient to induce an anorexigenic effect and that higher doses produced no additional anorexigenic activity. This is in line with other reports showing that in contrast to other IL-6 effects, there is no clear dose-dependency of IL-6 when it comes to its activity on food-intake [53, 54].

Whole genome gene expression profiles from the hypothalamus and the predicted inflammatory transcriptional regulators showed a high overlap between TNFα, IL-6 and combination groups, indicating that responses of the different treatments were similar. This might be explained by a production of similar cytokines by the host in response to injection with IL-6 or TNFα. In hypothalamic tissue homogenates, increased levels of IL-6 have been reported after injection with TNFα [55], which suggests that injection with TNFα also leads to activation of IL-6 signalling pathways. The strong down-regulation of expression of two important orexigenic neuropeptide regulators NPY and AgRP also showed to be similar between groups and corresponded to lower food intake in treated groups. This decrease in expression induced by TNFα or IL-6 supports findings that have been reported in a variety of experimental models including hypothalamic injection with TNFα and genetic overexpression of hypothalamic IL-6 [56, 57].

Gene expression of two genes involved in serotonin signalling, showed to be upregulated in mice treated with TNFα and IL-6. Serotonin has been reported to have an inhibiting action on NPY [13, 58, 59], suggesting that the increase in serotonergic activity measured in treated mice, might be responsible for the effects on lower NPY expression in these mice.

In summary, we show that TNFα and IL-6 induce similar inflammatory responses in the hypothalamus and similar effects on food intake. This anorexigenic effect of TNFα and IL-6 showed to coincidence with increased hypothalamic serotonergic activity, providing further evidence for a role for serotonin in inflammation-induced anorexia.
Acknowledgements

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References


Supplemental data

Supplemental Figure S5.1 Plasma levels of gut hormones.
Effect of ip injection with TNFα, IL-6 or both on A) Resistin, B) Peptide YY, C) Glucagon-like peptide (GLP-1), D) MCP-1, E) Insulin, F) C-peptide, G) Gastric inhibitory protein (GIP), H) Amylin, I) Leptin.
General discussion
The main aim of this thesis was to further unravel crucial processes involved in the pathogenesis of anorexia during cancer. First, two different mouse models were used to investigate the general processes involved in cancer cachexia. In both models, tumour-induced cachexia (body wasting) was indeed strongly present, but opposing responses in food intake occurred. Therefore, these models were taken as a starting point to compare and disentangle processes primarily underlying cachexia and those specifically involved in anorexia. The differences between the models are discussed below (section Tumour mouse models to study human cancer). Furthermore, to determine the contribution of inflammation to the development of anorexia, we used a cytokine-induced anorexia model. Different concentrations of TNFα and IL-6 were injected in healthy mice, thus inducing an acute inflammatory response. The injected cytokine doses were estimated from plasma levels measured in tumour bearing (TB) mice. In this model, cachexia was absent and the acute inflammatory response exclusively induced anorexia. The usefulness and limitations of this acute inflammatory anorectic model to study food intake regulation during chronic illness are also discussed below (section Acute versus chronic inflammation). We identified serotonin signalling to be important in food intake regulation during cancer and showed that serotonin turnover was also modulated by inflammatory mediators. Finally, we used hypothalamic neuronal cell lines to mechanistically study the interactions between inflammation and the serotonin system. Altogether, we show that hypothalamic inflammation as a result of chronic illness is an important trigger in the failure of hypothalamic food intake regulation. Next, we provide evidence for the involvement of serotonergic pathways, acting as an upstream modulator of various orexigenic and anorexigenic systems.

Tumour mouse models to study cancer and their translation to humans

In chapter 3 and 4, we used two tumour mouse models to study cancer-induced changes in hypothalamic processes involved in food intake. We showed that differences in food intake behaviour were inversely associated with changes in expression of genes involved in serotonin signalling. When using animal tumour models to investigate pathological processes in human diseases it is important to realise that at one hand animal models provide an intermediate step between in vitro and human experiments and that at the other hand their translational value towards humans remains a topic of considerable
controversy. There are several factors that limit the usefulness of animal models in this respect, including the great variety of animal models in use and their lack of predictive value for the highly heterogeneous group of cancers as they occur in human patients. In the following sessions, therefore, specific parts involved in the ability to translate our obtained results to the human situation are discussed.

**Heterogeneity of patient population**

The population of patients suffering from cancer and the disease itself are highly heterogeneous. Validation or verification of biomarkers identified in smaller patients groups often fails in larger cancer population studies. As a result less than 3% of identified potential cancer biomarkers have found their way to the clinic [1]. Even within a patient group that suffers from a similar cancer type, there are numerous differences, including age, gender, disease state, nutritional status, environmental factors and immune status, that all contribute to course of the disease. Consequently, investigational treatments that show promising effects in smaller subpopulations are often producing disappointing results in larger study groups [2]. At the same time, specific genetic profiling of tumours appear to be a promising approach for treatment of various cancer types [3, 4], underlining the tumour-diversity within specific cancer types.

Another concern is that many intervention treatments have often been performed in patients that suffer from final stage cancer, which often co-occurs with irreversible tissue damage, limiting effectivity of treatment and successful outcomes [2, 5]. Fortunately, nowadays more trials are focused on intervention strategies already at diagnosis [6], increasing chances of success in disease progression.

**Heterogeneity of tumour models**

In this thesis, two different tumour mouse models, C26 and Lewis Lung (LL) model were used. These models differed in their experimental settings, including nature and origin of tumour cells, number of cells inoculated, duration of tumour growth, and strains of mice. This underlines the differences between the models found in study outcomes, including food intake behaviour and progression of cachexia. Heterogeneity in progression of cachexia and anorexia is a common phenomenon seen with different
mice models, and even occurs between labs that use the same mouse model. Differences in experimental settings and animal handling, including differences in inoculation site [7], method of administration [8] and strain of mice [9, 10] as well as spontaneous mutations in the cell line used [11] lead to changes in characteristics of the models. These variations can also result in differences between the hosts’ immune response to the tumour, which in turn affects disease progression.

Mice models as representative for human carcinogenesis

Another aspect of the discussion on the use of mice cancer models is their translational value towards humans. Only 8% of cancer treatments showing positive test results in animal models are finally successfully translated to human trials, based on their treatment effectiveness and safety. This generates considerable doubt regarding the usefulness of animal cancer models as suitable models for humans [12]. It appears that this failure in translation from mice to humans is mainly due to disappointing treatment efficacy and safety. Factors underlying this discrepancy include the type of animal model used, differences in size, lifespan [13], drug metabolism [14], functional genes [15] and transcription factor binding sites [16].

Furthermore, psychological changes that affect behaviour in response to tumour growth are complex in humans and difficult to measure in animal models. This is likely to occur in food intake behaviour, since in humans awareness of the disease and its possible consequences will affect mental health and social interactions, which subsequently influences food intake [17-19]. However, even though rodent models possess translational limitations towards humans, they have greatly contributed to our current understanding of molecular and cellular mechanisms in carcinogenesis and their consequences. Furthermore, by using genetic mice models, novel cancer genes have been identified [20, 21]. More importantly, changes in biomarkers that are commonly occurring in cancer patients, including elevated levels of cytokines [22-25], C-reactive protein [26-29], cortisol [30] and muscle-specific markers for cachexia [31] are also found in various animal models used in cancer research. Therefore, similar effects and findings in multiple animal models are likely to be more promising for their efficacy in humans. *Vice versa*, processes involved in migration of tumour cells through the body and specific characteristics of tumour environment in humans can be mimicked in mice.
[32], creating additional research opportunities that are limited in humans. Studies on pathophysiological processes in specific organs in mice are of importance, because of the practical and ethical restrictions of human experiments.

In summary, tumour models in animals are of significant importance to human research, since they provide an intermediate step between in vitro and human experiments. However, limitations on translation from mice to humans, as well as heterogeneity of the cancer patient population should be taken into account.

**Chronic inflammation: low grade and high grade?**

An important aspect of anorexia in cancer is chronic inflammation. A chronically elevated inflammatory tone is common to various illnesses, including cancer, COPD, and HIV infection. In LL TB mice we showed that the presence of anorexia during an increased inflammatory status was associated with increased hypothalamic serotonin signalling. In C26 TB mice, displaying an increased food intake, we found that an increased inflammatory status was associated with decreased hypothalamic serotonin signalling. Taken together, this suggests that tumour-induced chronic inflammation can be associated with both elevated and reduced serotonin in the hypothalamus. Contributing to this paradox is the general observation on lowered hypothalamic serotonin concentration or release in obesity [33-37]. Obesity is also considered to be a state characterized by a chronically increased inflammatory tone. Furthermore, targeting the serotonin system using agonists for serotonin receptors 5HT2c, 5HT1b and 5HT6 in the brain is a current treatment in the fight against overeating in obesity [38, 39]. So, an important question that arises is *what causes chronic inflammation to be associated with both elevated serotonin and reduced serotonin in the hypothalamus?*

Inflammation has different manifestations due to its diversity in type, duration, level and origin [40]. In obesity, a low-grade inflammatory process is ongoing, which is characterized by slightly increased cytokine levels in blood. In contrast, in severe illnesses, including progressive cancer and rheumatoid arthritis, the immune system is highly activated, which is reflected by much higher levels of cytokines compared to those seen in obesity [41-47].
In our experiments, mice bearing C26 tumours showed lower levels of cytokines IL-6 and TNFα in their blood, compared to anorectic LL bearing mice (Figure 6.1), suggesting a more pronounced inflammatory process in LL TB mice compared to C26 TB mice. Plasma levels of TNFα in C26 TB mice showed not to be elevated in our study, while TNFα is commonly reported to be elevated in tumour mouse models, including the C26 model [48]. In contrast, we did find elevated TNFα plasma levels in the LL TB mice. *In vitro*, both C26 and LL cells do not produce TNFα (Figure 6.2), suggesting that the hosts’ immune response differs between the two different tumours. This difference in TNFα

![Figure 6.1](image.png)

**Figure 6.1** Plasma cytokines in C26 and LL TB mice. C26 colon adenocarcinoma (C26) or Lewis Lung (LL) cells were inoculated in mice subcutaneously or intramuscularly, respectively. After sacrifice, blood was collected and cytokines were measured with ELISA (M&M chapter 3 and 4). **A and B** Plasma levels of IL-6 in C26 and LL tumour bearing mice respectively. **C and D** Plasma levels of TNFα in IL-6 and LL tumour bearing mice respectively. Data is presented as means ±SEM. *Significantly different from control (P<0.05).*
production might be implicated in the opposing food intake behaviours in the two cancer models. Both in hyperphagia during obesity as well as in anorexia during cancer, TNFα is reported to play an important role [49, 50]. Blocking TNF activity has been shown to be both beneficial in restoring food intake in cancer and in improving hyperphagia during obesity [51, 52], indicating that TNF has opposing effects on food intake. Also other effects of TNFα, including its actions on proliferation and differentiation of glial cells and on hypothalamic insulin and leptin signalling are reported to be bi-directional, dependent on the dose [53, 54]. Furthermore, the primary site of inflammation appears also to be important in determining inflammation-induced changes of food intake rather than cytokine plasma levels alone [55]. C26 cells are subcutaneously inoculated and C26 tumours rarely have metastases, whereas LL cells injected intramuscularly can show metastases already 14 days after inoculation [56]. Finally, we can speculate on what would happen on food intake and plasma cytokines if cachexia in C26 TB mice would progress. It could be that eventually also in these mice, food intake compensation would fail, leading to development of anorexia. Time-course of tumour and disease development differ between the C26 and LLM model with LL tumours being more progressive in inducing cachexia compared to C26 in our experimental settings. Taken

**Figure 6.2 Production of TNF, IL-6 and MCP-1 in C26 and LL cells in vitro.**

C26 colon adenocarcinoma (C26) or Lewis Lung (LL) cells were seeded in DMEM supplemented with 10% FCS and 1% P/S with a density of 1.10⁶ cells/ml (M&M chapter 4 & 5). After 24 hours, secretion of cytokines in medium was measured with ELISA (M&M chapter 5) N.D not detected (<6.25 pg/ml). Data is presented as means ± SEM.
together, it might be that the nature and degree of inflammation induced by the different tumour models underlies their apparently opposing effects on food intake behaviour.

In summary, a variety of chronic inflammatory diseases are accompanied by food intake disturbances. These disturbances have been reported to be associated with altered serotonin signalling. However, whereas food intake appears to be inextricably inversely linked to serotonin status in the hypothalamus, the link between inflammation and alterations in serotonin signalling during disease remains unclear. It appears that the underlying cause of inflammation (e.g. illness) and subsequently differences in degree (e.g. high/low), duration (acute/chronic), origin (tumour/tissue necrosis) and site of origin (e.g. brain, fat) of inflammation play a role in these different findings.

**Acute versus chronic inflammation**

The differences in plasma levels of TNF and IL-6 measured in C26 and LL TB mice (Figure 6.2), served as basis to estimate the doses of the cytokines administered to mice described in chapter 5. We found that the different doses of IL-6 injected and the combinations of IL-6 with TNFα had similar effects on food intake and hypothalamic gene expression profiles. This might be due to the fact that these interventions induced secondary inflammatory mediators in a comparable fashion. This experimental set-up induces an acute and short-term inflammatory response, which is in contrast to the chronic elevation of cytokine plasma levels induced by the presence of a tumour. Differences in inflammatory responses between the models used in this thesis and their effect on food intake regulation will be discussed below.

**Effects of short and chronic anorexia**

In chapter 3 and 4 tumour models were used, which are characterized by a chronically elevated inflammatory tone. The resulting elevated cytokine plasma concentrations were considered to be important for the development of anorexia in mice bearing LL tumours. In chapter 5, mice were injected with cytokines aiming to achieve plasma levels comparable to those measured in C26 and LL TB mice. Here, the acute effects of cytokines on food intake were investigated. Table 6.1 summarizes the effects found on food intake and the changes in the hypothalamus of C26 TB, LLM TB, and cytokine-
treated mice used in this thesis. As shown, changes in hypothalamic serotonin levels were inversely associated with changes of food intake in all three models. However, alterations in NPY and AgRP gene expression did not reflect food intake behaviour, as NPY was upregulated in TB mice, but downregulated in cytokine treated mice. Hence, both reduced and increased NPY and AgRP gene expression can apparently coincide with decreased food intake and increased hypothalamic serotonin concentrations (Table 6.1), depending on the experimental model used. We hypothesize that gene expression of NPY and AgRP in TB mice is likely to act as a sensor for weight loss. NPY has a dual role in energy homeostasis by modulating both food intake as well as energy storage/expenditure. Regarding the latter, leptin signalling has shown to be crucial [57]. It is likely that cachectic processes promoting lipolysis and muscle wasting indeed act as a trigger for this upregulation of NPY, while this is lacking in acute short-term inflammation as occurring in cytokine-treated mice. This further implies that the dual actions of NPY in food intake and energy expenditure might include acute actions of NPY and long term actions of NPY, being food intake and energy storage respectively. Furthermore, the comparison of cytokine-treated mice with LL TB mice suggests failure of NPY/AgRP signalling in anorectic LL TB mice, which does not occur in cytokine-induced anorexia. This might be due to the differences in type of inflammatory responses in TB mice and cytokine-treated mice.

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<td>Age</td>
<td>6w</td>
<td>6w</td>
</tr>
<tr>
<td>Site</td>
<td>Subcutaneous</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Cachexia</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Duration</td>
<td>20 days</td>
<td>5 hours</td>
</tr>
<tr>
<td>Food intake</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Body weight</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Gene expression NPY/AgRP</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Serotonin</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>

Gene expression of NPY/AgRP was measured via microarray, while indication for changes on serotonin included gene expression of more than 1 gene included in serotonin signalling pathway and/or measurement of serotonin's metabolite 5-HIAA in total tissue homogenate.
In both acute and chronic inflammatory diseases, including infections, injury, and cancer, anorexia is commonly occurring. However, the deleterious effects of anorexia in acute and in chronic inflammation are very different, because effects of decreased food intake largely depend on the duration. In chronic inflammatory diseases including cancer, prolonged anorexia contributes to severe malnutrition and body wasting, increasing mortality and impinging on quality of life in these patients. In contrast, short-term anorexia in acute inflammation can be beneficial, since restriction in the intake of macro- and micronutrients will inhibit bacterial growth [58]. Furthermore, anorexia is considered to facilitate fever response, as anorexia in acute inflammation is almost invariably accompanied by fever [59]. During acute illness due to infection, not being hungry would reduce motivation to find food and interaction with others. Subsequently, this would reduce body heat loss (facilitation fever), reduce activity and mobility and reduce exchange of pathogens with others [60]. It has been shown that food deprivation increases survival during bacterial infection [61]. *Vice versa*, forced-feeding during acute infection increases morbidity and mortality [62]. Together these phenomena suggest a physiological and supportive role of anorexia during acute inflammation. Therefore, the differences in outcome of anorexia between chronic inflammation and acute inflammation might have a functional and evolutionary basis, which in turn underlies that specific processes involved in the development and sustainment of anorexia could be different between the two types of inflammation-induced anorexia. Laviano *et al.* postulated that anorexia in chronic illness such as cancer anorexia develops by waves of brain activation, each of them not necessarily being mediated by the same mediators [63]. This is supported by the development of tolerance in response to continuous cytokine infusion [64]. However, repetitive administration of cytokines, when the anorexic effect of a previous injection is subsided, induces enhanced development of anorexia [65]. Overall, these data suggests that long-lasting anorexia induced by inflammatory mediators occurs in cycles, while this might not be the case in acute inflammation. Furthermore, compensatory mechanisms in response to chronic malnutrition can occur, which might contribute to the sustainment of anorexia in chronic inflammation, developing a vicious cycle where anorexia and its consequences enhance one another.
Future directions

Severe body wasting due to underlying illnesses like cancer has already been known for centuries and was already described by Hippocrates 400 BC [66]. As of today, many targets have been identified as potentially important for the development and outcome of cachexia. Unfortunately, current treatment options to improve appetite and food intake in patients are still limited. Only 20–30% of patients actually report an improvement in food intake upon medical treatment, which usually involves corticosteroids or progestational agents. Furthermore, these conservative treatments can have severe side-effects, including thrombophlebitis and muscle degradation [67, 68], both deleterious conditions in patients who are often less mobile due to their illness and may already suffer from loss of muscle mass.

The knowledge on the regulatory role of serotonin in food intake regulation, together with indications that serotonin brain levels may be elevated due to an underlying chronic illness and may cause anorexia, triggers the question why still no therapeutics have been developed that alter serotonergic activity in order to improve appetite. This is in particular in contrast to the field of drug development for weight management. Here, targeting the serotonin system is considered an effective strategy to reduce food intake in overweight and obese persons [38]. Several current drugs for weight management possess agonistic activity towards 5HT$_{2C}$ or 5HT$_{1B}$ receptors. Consequently, antagonists for 5HT$_{2C}$ or 5HT$_{1B}$ receptors might be interesting for improving appetite, since the satiating effect of serotonin is reported to occur by concomitant activation of POMC/CART neurons via 2HT$_{2C}$, and inhibition of NPY/AgRP neurons via 5HT$_{1B}$ receptors [69-71]. It would be interesting to use both C26 and LL TB mouse models to study the effect of serotonin antagonists on food intake.

Another area that demands for further research is the involvement of other organs in food intake regulation, such as stomach and intestines. It appears that stimulation of gastric emptying can be promising in cancer patients. However, mechanisms involved in delayed gastric emptying during disease are not yet entirely clarified, as various factors, including serotonin, influence gastric emptying [72].

Furthermore, since we showed that inflammation directly leads to an increase in serotonin turnover, molecular processes responsible for this increase still need further research, for example by the use of 5HTR-blockade or the use of knock out models.
It is likely that activation of serotonin signalling leading to anorexia is also involved in modulation of the immune system due to its functions in platelet activation and leukocyte attraction, and this interaction needs to be further elucidated [73, 74].

**Final conclusions**

In this thesis we investigated changes in inflammatory markers and pathways involved in food intake regulation and eating behaviour using different mouse models. The main objectives were to further elucidate processes involved in the pathogenesis of disease-related anorexia, and to determine the role of inflammation in this process. Taken together, the results presented in this thesis show that hypothalamic inflammation during chronic illness is an important underlying mechanism in the failure of hypothalamic food intake regulation. This is likely to be mediated via effects on serotonergic metabolic and (or) signalling pathways, which act as upstream modulators of hypothalamic orexigenic and anorexigenic neuropeptide systems. The degree (high vs low), duration (acute vs chronic), origin and site of inflammation are important factors influencing hypothalamic inflammation, serotonergic and neuronal NPY/AgRP and POMC/CART systems, and ultimately food intake. The serotonergic regulation of food intake during disease merits further investigation as it may deliver novel therapeutic targets for improving food intake during chronic illness.

**References**


Nederlandse samenvatting
Bij een groot deel van de kankerpatiënten (60–80%) komt anorexie, oftewel een verlies van eetlust, voor. Deze vorm van anorexie wordt veroorzaakt door de ziekte zelf en staat los van de problemen met eten die veel patiënten ondervinden als gevolg van chemotherapie. Anorexie komt niet alleen voor bij kanker, maar komt ook veelvuldig voor bij andere chronische ziekten zoals HIV, COPD en chronisch hartfalen. Anorexie gaat vaak gepaard met cachexie, een progressief verlopend verlies van vooral spier- maar ook vetmassa. Deze combinatie wordt algemeen aangeduid met de term anorexie-cachexie syndroom. Dit is een levensbedreigende aandoening, waarbij de patiënt ernstig ondervoed raakt en veel spiermassa verliest. Dit heeft onder andere negatieve gevolgen voor de effectiviteit van chemotherapie en het herstel na operaties. Ook nemen de fysieke conditie van de patiënt en de weerstand tegen infecties af. Deze factoren dragen ertoe bij dat de overlevingskansen en/of de gemiddelde overlevingsduur van de patiënt aanzienlijk afnemen. Belangrijk is daarnaast dat het optreden van anorexie vaak grote psychische gevolgen heeft voor de patiënt en zijn/haar omgeving, omdat verlies aan eetlust in verband wordt gebracht met een slecht ziektebeloop. Helaas zijn er tot op heden nog geen effectieve therapieën ontwikkeld die de ontwikkeling van anorexie tijdens kanker kunnen tegengaan. Dit komt mede doordat er nog niet veel bekend is over de specifieke oorzaken van de ontwikkeling van anorexie tijdens kanker of andere chronische ziekten.

Het doel van het onderzoek beschreven in dit proefschrift was om de mechanismen verder te ontrafelen die betrokken zijn bij de ontwikkeling van anorexie tijdens chronische ziekte. Eerder onderzoek heeft aangetoond dat de hypothalamus, een gebied in de hersenen waar de regulering van voedselname plaatsvindt, hierin een belangrijke rol speelt. Ons onderzoek heeft zich daarom gericht op het bestuderen van de veranderingen in de hypothalamus tijdens kanker. Verder hebben we veel aandacht besteed aan de rol van ontstekingsreacties die sterk verhoogd zijn tijdens kanker en andere ziekten waarbij anorexie veel voorkomt. Om de gevolgen van tumorgroei op de regulatie van processen in het brein te bestuderen hebben we verschillende muismodellen gebruikt als model voor de mens. Muismodellen worden veelvuldig gebruikt in kankeronderzoek omdat de mogelijkheden tot het bestuderen van tumoren en hun interacties met het lichaam in de mens uiteraard beperkt zijn.
Werkwijze

In dit proefschrift hebben we gebruik gemaakt van drie verschillende muismodellen om de processen die een rol spelen bij anorexie als gevolg van kanker te bestuderen. Hiervan waren er twee tumormodellen, waarbij de muizen worden ingespoten met tumorcellen. Bij het derde model was er geen sprake van tumorgroei. Bij het eerste tumormodel (C26) ontwikkelden de muizen cachexie (gewichtsverlies) tijdens de tumorgroei. Ter compensatie van dit gewichtsverlies gingen de muizen meer eten, om zo het gewichtsverlies te beperken. In het tweede muismodel (LL) droegen de muizen een ander type tumor. In deze muizen ontwikkelde er zich naast het gewichtsverlies ook anorexie (minder eten) naarmate de tumor groeide. Door vervolgens deze twee tumormodellen te vergelijken, kon worden onderzocht welke processen specifiek betrokken zijn bij veranderingen in voedselinnname, en welke processen algemeen optreden bij gewichtsafname tijdens tumorgroei.

Het derde muismodel was een model waarbij er geen tumorgroei plaatsvond, maar waarbij de muizen werden ingespoten met bepaalde ontstekingsmediatoren, zogenaamde cytokines. In dit model ontwikkelden de muizen anorexie als gevolg van een ontstekingsreactie zonder dat er sprake was van tumorgroei. Dit werd gedaan om te achterhalen wat de specifieke rol van ontsteking is in de ontwikkeling anorexie. Vervolgens hebben we in deze drie muismodellen de veranderingen die plaatsvinden in de hypothalamus meer in detail bestudeerd en onderling vergeleken. We hebben hiervoor veel gebruikt gemaakt van micro-array-analyse. Bij micro-array-analyse kan de expressie van genen van het gehele genoom tegelijkertijd gemeten worden. Genexpressie is de mate waarmee een gen gekopieerd wordt, zodat het kan worden afgelezen. Dit aflezen is uiteindelijk van belang voor de synthese van het eiwit waarvoor het gen codeert. Omdat het hele genoom geanalyseerd wordt, kunnen veranderingen in veel verschillende biologische processen tegelijk worden opgespoord.

Resultaten en conclusies

Uit onze experimenten is gebleken dat er een aantal sterke veranderingen optreedt in de hypothalamus tijdens de groei van een tumor. Een belangrijke bevinding was het optreden van veranderingen in de vorming van serotonine. Serotonine is een stof die in de hersenen belangrijk is voor de signaaloverdracht tussen neuronen, oftewel de
Nederlandse samenvatting

communicatie tussen zenuwcellen in het brein. Serotonin heeft door deze functie een grote invloed op veel gedragsprocessen, waaronder voedselinnname. Onze resultaten lijken aan te tonen dat tijdens tumorgroei de afgifte van serotonine in de hypothalamus toeneemt. Deze verhoogde afgifte van serotonine leidt er vervolgens toe dat de processen die normaal gesproken de voedselinnname stimuleren worden afgeremd. Hierdoor kan een verhoging in serotonineconcentratie in de hypothalamus leiden tot een afname in eetlust en de ontwikkeling van anorexie. Daarnaast zagen we een verhoging van serotonineconcentratie in de hypothalamus ook in het derde model, waarbij anorexie alleen kon zijn veroorzaakt door ontstekingsreacties en niet door tumorgroei. Dit zou betekenen dat met name de ontstekingsreacties die plaatsvinden tijdens tumorgroei een cruciale rol spelen bij het ontwikkelen van anorexie.

Op basis van dit proefschrift kan worden geconcludeerd dat ontstekingsreacties tijdens chronische ziekten zoals kanker kunnen leiden tot verhoogde afgifte van serotonine in de hypothalamus. Deze verandering is op de lange termijn negatief, omdat ze bijdraagt aan de ontwikkeling van anorexie. Verder onderzoek is nodig om te bestuderen of het verlagen van de serotonineconcentratie of het remmen van de signaaloverdracht via serotonine ook daadwerkelijk effectief is bij de behandeling van anorexie tijdens kanker.
Nederlandse samenvatting
Dankwoord
Serotonine is niet alleen betrokken bij eetgedrag, zoals beschreven in dit proefschrift, maar is ook belangrijk voor het ervaren van geluk en plezier. Het gezelschap en de ondersteuning van een aantal mensen heeft sterk bijgedragen aan het ervaren van dit plezier tijdens mijn AIO-tijd. Deze mensen wil ik hier graag bedanken.

Allereerst wil ik mijn co-promotor bedanken. Klaske, ontzettend bedankt voor je vertrouwen in mij, en voor de gegeven kans om een stage te lopen bij Danone, wat vervolgd werd in een promotietraject. Ik bewonder je gedrevenheid en enthousiasme in alles wat je doet. Door je creativiteit heb je me geleerd om vanuit verschillende invalshoeken het onderzoek te benaderen. Je hebt me ontzettend veel geleerd en ik zal onze samenwerking erg missen. Natuurlijk ook bedankt voor de leuke momenten op onze congresreizen: ik had “de beer of het zwijn?” op het pad in Kobe, de lekkere “extra-noir” thee in Lille, “de spook-hotelkamer” in Boston, de “terror-non” aan de Chiemsee, en het maken van mislukte panoramafoto’s op Mount Real absoluut niet willen missen!

Renger, in 2009 kwam ik bij jou aankloppen voor hulp bij het krijgen van een stage via het RIKILT in Suriname. Helaas brandde het lab daar af vanwege het aansluiten van een apparaat op 220V in plaats van 110V. Achteraf gezien is het allemaal goed gekomen, want ik kon via jou direct voor een andere stage bij Danone Research terecht, onder begeleiding van Klaske. Ik waardeer alle moeite die je voor me hebt gedaan bij het helpen bij al mijn carrièrestappen enorm. Ik vond het erg fijn om in jouw groep te werken en dat je me als promotor begeleidde. Ook al was je altijd erg druk, je wist altijd tijd vrij te maken. Ik moet bekennen dat ik de deadlines voor jou altijd vervroegde met een week, maar steeds weer bleek deze extra week overbodig, want je was er altijd wanneer dat nodig was. Ik heb veel geleerd van al je input op onze artikelen en de discussies op de vrijdagochtend, bedankt!

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About the author
Curriculum vitae

Jvalini Dwarkasing was born on the 1st of February, 1986 in Rotterdam, The Netherlands. In 1994, she temporally moved with her family to Paramaribo, Surinam, to return in 1998 and finish secondary school at the Emmauscollege Gymnasium in Rotterdam. In 2004, she started her studies in Nutrition and Health in Wageningen and continued her studies by following two master programmes: Food Safety and Nutrition and Health. In 2009, she did her first master thesis focused on estrogenic activity of soy flavone genistein at the Division of Toxicology under supervision of prof. dr. I. Rietjens. She continued with a 2nd master thesis at Nutrition and Pharmacology guided by dr. J. Meijerink and prof. dr. R. Witkamp, studying the anti-inflammatory effects of cannabinoids. For an internship she went to Danone Research where she worked on the effects of fish oils on NF-κB-mediated HIV replication under supervision of F. Dijk and K. van Norren. Her work was presented at the ESPEN conference and rewarded with a poster prize. In 2010, Jvalini graduated from the master programmes Nutrition and Health and Food Safety. Then, she started a PhD project at the Pharmacology and Nutrition group under guidance of K. van Norren, R. Witkamp, and M. Boekschoten. During her PhD, she closely collaborated with the Muscle, Protein and Metabolism platform from Nutricia Research. She also joined the Cancer Research Group at the University of Barcelona led by prof. J. Argilès to work on a specific cancer model. In 2012, she received a fellowship award by ESPEN and a poster prize from the Society of Sarcopenia and Muscle Wasting. She finished her PhD in 2015. The results of the PhD project “Hypothalamic regulation of food intake during cancer” are described in this thesis.
**List of publications**


**Other**


Overview of completed training activities

Discipline specific activities

- Pharma-Nutrition Conference (Amsterdam, 2011)
- Advanced visualization, integration and biological interpretation of –omics data (Wageningen, The Netherlands, 2011)
- NUGO week (Wageningen, The Netherlands, 2011)
- Characteristics and implementation Lewis Lung model (Barcelona, Spain, 2012)
- Full4Health Project Meetings (Aberdeen, UK, 2011 and Lille, France, 2013)
- ESPEN (Barcelona, Spain, 2012)
- 1st Cancer Cachexia Conference (Boston, USA, 2012)
- Cachexia Conference (Kobe, Japan, 2013)
- 2nd Cancer Cachexia Conference (Montreal, Canada, 2014)
- Symposium Gut-Nutrient Signalling (Danone Research, Utrecht, The Netherlands, 2013)
- Regulation of energy intake: the role of product properties (Wageningen, The Netherlands, 2012)

General courses

- Implementing and designing experimental models (2011)
- Techniques for Writing and Presenting Scientific Papers (2012)
- VLAG PhD Week (2011)
- PhD Competence test (2011)
- Alisson Douglas Summerschool (2013)
- Efficient Writing Strategies (2014)

Optional activities

- Preparation Research Proposal (2011)
- NMG-Pharma Lab Meetings (weekly, 2011–2015)
- Pharma group meetings (weekly, 2011–2015)
Colophon

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