Central and Peripheral Neuroendocrine Factors in Cancer-Associated Anorexia and Cachexia

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To my family, friends, and colleagues

ABSTRACT

Cancer anorexia-cachexia syndrome (CACS) develops in response to tumor-host biochemical interactions. A loss of appetite (anorexia) and increased metabolism results in a progressive wasting of adipose and skeletal muscle tissues (cachexia). This syndrome is linked to a reduced tolerance to anti-cancer treatments, lower quality of life, and poor prognosis. The specific mechanisms that cause CACS are still unknown, and there exists no curative treatment for this syndrome. In this thesis, rodent models for induced anorexia, MCG101 tumor-induced CACS, and acute inflammation paradigms were used.

The first stages of this thesis project were aimed at identifying central mechanisms by which cancer-associated anorexia could be overriding homeostatic feeding controls. Our initial investigations involved cocaine- and amphetamine-regulated transcript peptides (CARTp) and the thyrotropin receptor (TSHr). Centrally acting CARTp are known to potently inhibit feeding. Similarly, infusions of TSH into the lateral ventricles have been shown to reduce food intake in rats. The precise mechanisms through which CARTp elicits its effects, and the distribution of functional TSHr have been unknown.

A previous *in vitro* study showed CARTp activity was antagonized by PACAP6-38. We built upon previous findings by showing that PACAP6-38 could block CARTp-induced anorexia *in vivo* in rats; thus, we provided further support that PACAP6-38 is a useful tool for elucidating endogenous CARTp effects. In addition, we report that TSHr proteins are present in nuclei of the hypothalamus and brainstem with relevance for feeding. Indeed, putative stimulation of TSHr in the nucleus of the solitary tract reduced solid food intake in healthy rats.

Using mouse models for acute inflammation and CACS, we investigated gene expression changes in areas of the brain with relevance for feeding, namely the hypothalamic paraventricular nucleus (PVN), arcuate nucleus (ARC), and the dorsal vagal complex of the brainstem. We investigated mRNA for compounds expressed in brain regions involved in feeding behavior under healthy conditions: CART, TSHr, TSH, thyrostimulin, nesfatin-1, and corticotropin-releasing hormone (CRH). Acute inflammation was associated with reduced gene expression for TSHr and CART in the ARC and increased expression of CART mRNA in the pituitary. CACS also resulted in a decrease in CART gene expression in the PVN, which was a response secondary to reduced food intake as shown by pair-fed controls. Interestingly, we saw a tumor-specific effect on nesfatin-1 gene expression in the PVN. Therefore, it appears that CARTp is not inducing anorexia in CACS, but seems to participate in an adaptive response. In addition, hypothalamic nesfatin-1 may be a likely candidate for mediating a CACS response.

Acute inflammation induced a prostanoid-independent increase of plasma CARTp, which correlated positively with degree of inflammation. Tumor-bearing mice similarly had elevated plasma CARTp concentrations. Putative antagonism of circulating CARTp by PACAP6-38 in tumor-bearing animals protected against loss of fat mass, but did not improve food intake or any other metrics. These findings highlight plasma CARTp as a potential mediator of lipolysis in CACS.

Keywords: cancer anorexia-cachexia syndrome; MCG101; inflammation; CART; TSH receptor; CRH; nesfatin-1; hypothalamus; brainstem
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SAMMANFATTNING PÅ SVENSKA

Cancer-orsakad anorexi-kakexi (CACS) är ett tillstånd som drabbar ett stort antal patienter med olika typer av cancersjukdom. CACS kännetecknas av aptitlöshet (anorexi) samt förlust av fettväv och skelettmuskelmassa (kakexi). Både vid CACS och vid svält är energiintaget minskat. En viktig skillnad mellan dessa tillstånd är dock att vid CACS är energiintaget lågt på grund av att aptiten är kraftigt minskad, samtidigt som metabolismen och energibehovet är ökat. Syndromet beror på att tumören utsöndrar faktorer som påverkar värden med förlust av aptit och avmagring som följd, men de exakta mekanismer som orsakar CACS är ännu inte kända i detalj. Forskning om detta tillstånd har hittills främst varit inriktad på att förstå perifera mekanismer för CACS. De kunskaper som finns som rör aptitreglering i allmänhet rör framförallt hur aptit regleras under normala, hälsosamma förhållanden eller vid obesitas. Den övergripande målsättningen med denna avhandling har varit att undersöka hur en tumör som leder till CACS påverkar några centralnervösa mekanismer för aptitreglering, samt att studera hur sjukdom som beror på inflammation kan påverka dessa mekanismer.

I avhandlingen har en experimentell modell för CACS hos mus använts. Då tumörer även medför en inflammatorisk reaktion hos värden, har vi undersökt om och hur akut inflammation kan leda till förändrade nivåer av genuttryck för cocaine- and amphetamine-regulated transcript peptide (CARTp), TSH receptorn och dess kroppsegna agonister i mättnadscentra i hypotalamus, hjärnstam och hypofys. Vidare har vi undersökt om peptiden PACAP6-38 kan fungera som en antagonist till en av de CARTp och förhindra dess effekter på födointag. Eftersom att TSH injicerat i laterala hjärnventrikeln hos råtta liksom CARTp leder till minskat födointag, har vi även undersökt om receptorer för TSHr finns lokaliserade till nervkärnor i hypotalamus och hjärnstam som har betydelse för aptitreglering, metabolism och autonoma funktioner.

I delstudie I var syftet att försöka finna en antagonist till den födointagshämmande peptiden CARTp. PACAP6-38 och CARTp injicerades i fjärde hjärnventrikeln hos råtta. PACAP6-38 hade ingen egen effekt på födointag, men blockerade den aptithämmande effekten av CARTp. Däremot föreföll PACAP6-38 inte påverka tidigare beskrivna motoriska effekter av CARTp. Slutsatsen av denna studie är att PACAP6-38 är en funktionell antagonist till CARTp avseende anorexi. Då vissa motoriska effekter av CARTp fortfarande förelåg trots PACAP6-38 behandling, existerar det sannolikt fler än en CARTp-receptor subtyp. En betydelse av resultatet är att man med hjälp av en funktionell antagonist lättare kommer att kunna undersöka effekter och funktioner av kroppseget CARTp.

Delstudie II syftade till att med hjälp av immunohistokemi kartlägga förekomst av receptorproteiner för TSH anatomiskt och på cellulär nivå i områden i hypotalamus och hjärnstam som styr aptit, autonoma och gastrointestinala funktioner. En för TSH-receptorn selektiv och monoklonal antikropp användes, och vi fann att TSH-receptor-liknande immunoreaktivitet i cytoplasman i ett stort antal nervceller i hypotalamus och hjärnstam. I hypotalamus sågs färgning vara positiv ibland annat i N. arcuatus (ARC), N. paraventricularis (PVN) parvocellulära del, Mediana eminencen och venteromediala hypotalamiska (VMH) kärnan. I hjärnstammen sågs positiv färgning i Nukleus tractus solitarii (NTS), vagus dorsala motorkärna, area postrema (AP) och N. Hypoglossus. Western blot visade att medan TSH-receptorer i thyroidea främst verkar utgöras av kluvna receptorprotein, förekommer TSH-receptorprotein i

CNS huvudsakligen i form av den aktiva s.k. holoreceptorn. För att testa hypotesen att receptortypen är aktiv och fungerande, injicerades TSH lokalt in i NTS, där celler som färgats positivt för receptorproteinförekomst hade noterats. Vi fann då att TSH medförde hämmat födointag. Sammantaget påvisar studien att funktionella TSH-receptorer uttrycks i många områden i hypotalamus av betydelse för aptitreglering, receptorerna förefaller vara aktiva holoreceptorer och aktivering av dessa i NTS medför minskat matintag.

I delstudierna III och IV studerades hur sjukdom påverkar genuttryck av födointagshämmande signaler i aptitcenter i CNS. I delstudie III behandlades möss med LPS, ett ämne som orsakar en akut inflammatorisk reaktion i kroppen. Vi fann att efter LPS minskade uttryck av mRNA för CART och TSH receptorn i ARC och PVN, men ökade i hypofysen, hos mus. I delstudie IV inokulerades möss med MCG101, en tumör som medför CACS och som även medför inflammation och ökade plasmanivåer av prostaglandin E2 hos värden. Liksom efter LPS sågs vid CACS ett minskat uttryck av CART i ARC, dock påverkades inte genuttryck av mRNA för TSH receptorn i hypotalamus. Vid CACS sågs även ett ökat genuttryck för nesfatin-1, en peptid som ger minskat födointag. Med hjälp av en kontrollgrupp som parmatades mot de tumörbärande djuren, fann vi vidare att de förändrade genuttrycket av CART i hypotalamus förefaller utgöra en sekundär adaptation till det minskade kaloriintaget, kanske för att försöka skydda organismen mot svält. Våra fynd talar också för att det ökade genuttrycket av nesfatin-1 kan innebära att denna peptid kan vara en orsaksmekanism bakom uppkomst av CACS. Våra data tyder också sammantaget på att TSHr är reducerade vid akut sjukdom, men inte efter kronisk sjukdom, såsom vid cancerorsakad anorexi/CACS.

I delstudierna III och IV fann även vi att vid akut inflammation och CACS ökade CARTp-koncentrationen i plasma. Vi fann vidare att medan plasmanivåer av CARTp tycks vara kopplade till graden av inflammation, så var effekten inte beroende av prostanoider, eftersom att behandling med COX-hämmaren indomethacin inte påverkade CARTp-ökningen i plasma. Eftersom att genuttryck för CART saknades hos tumören var ökningen av CART i plasma helt beroende på en frisättning från värden.

Mot bakgrund av den kraftigt ökade plasmakoncentrationen av CARTp som sågs hos tumörbärande djur, prövades i delarbete V hypotesen att peptiden kunde bidra till det förändrade födointag eller kroppsammansättning som ses vid CACS. CARTp har tidigare visats medföra ökad lipolys i samband med betaadrenerg aktivering, vilket även förekommer vid CACS. I delstudie V undersöktes därför om den funktionella CARTp-antagonisten som vi påvisat i delstudie I, kunde motverka någon eller några CACS-effekter. MCG101-bärande möss tumörer som behandlades med PACAP6-38 en gång om dagen uppvisade en ökad andel kroppsfett jämfört med tumörbärande djur som fick kontrollinjektion. Behandling med PACAP6-38 påverkade inte i sig total kroppsvikt eller fettfri vikt. Vi fann inte heller att PACAP6-38 i sig påverkade tumörstorlek och noterade inga negativa bi-effekter. Våra resultat antyder att en inflammatorisk ökning av CARTp i plasma skulle kunna vara en möjlig orsaksmekanism för fettvävsförlust vid CACS. Med dessa fynd har vi också påvisat att PACAP6-38 kan motverka sådana fettvävsförluster vid CACS, och skulle kunna vara en ny möjlig farmakologisk behandlingsväg vid detta tillstånd.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Burgos JR, Iresjö B-M, Smedh U.**
Pituitary adenylate cyclase-activating polypeptide 6-38 blocks cocaine- and amphetamine-regulated transcript peptide-induced hypophagia in rats.
PLoS ONE, 2013; 8(8): e72347.

II. **Burgos JR, Iresjö B-M, Wärnåker S, Smedh U.**
Presence of TSH receptors in discrete areas of the hypothalamus and brainstem with relevance for feeding controls—support for functional significance.
Manuscript, *submitted.*

III. **Burgos JR, Iresjö B-M, Olsson L, Smedh U.**
Lipopolysaccharide immune challenge reduces TSHR and CART mRNA in the Arcuate nucleus and elevates circulating CART peptides in mice.
Manuscript, *submitted.*

IV. **Burgos JR, Iresjö B-M, Smedh U.**
MCG101-induced cancer anorexia-cachexia features altered expression of hypothalamic *Nucb2* and *Cartpt* and increased plasma levels of cocaine- and amphetamine-regulated transcript peptides.
Oncology Reports, *Accepted for publication* 9 November 2015.

V. **Burgos JR, Iresjö B-M, Smedh U.**
PACAP6-38, a functional CARTp antagonist, counteracts loss of adipose tissue in MCG101 tumor-bearing mice with elevated host plasma CART and anorexia-cachexia syndrome.
Manuscript.

ABBREVIATIONS

3V	Third ventricle
4V	Fourth ventricle
AgRP	Agouti-related peptide
AP	Area postrema
ARC	Arcuate nucleus of the hypothalamus
BBB	Blood-brain barrier
CACS	Cancer anorexia-cachexia syndrome
CART	Cocaine- and amphetamine-regulated transcript
CARTp	CART-associated peptides
CCK	Cholecystokinin
CDNA	Complementary DNA
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Ct	Threshold cycle
DMH	Dorsomedial nucleus of the hypothalamus
DMX	Dorsal motor nucleus of the vagus
DVC	Dorsal vagal complex
ELISA	Enzyme-linked immunosorbent assay
i.c.v.	Intracerebroventricular; administered into the ventricle
i.p.	Intraperitoneal; administered into the peritoneum
i.v.	Intravenous; administered into the vein
IgG	Immunoglobulin type G antibody
IHC	Immunohistochemistry
-ir	-immunoreactivity/-immunoreactive
LHA	Lateral hypothalamic area
LPS	Lipopolysaccharide
ME	Median eminence

mRNA	Messenger RNA
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
p.o.	*per os*; administered into the mouth
PACAP6-38	Pituitary adenylate cyclase-activating polypeptide fragment 6-38
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
SAP	Serum albumin P component
T3	Triiodothyronine
T4	Thyroxine
TNFα	Tumor necrosis factor alpha
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin (Thyroid stimulating hormone)
TSHr	Thyrotropin receptor
VMH	Ventromedial nucleus of the hypothalamus
WB	Western blot

Gene Name	**Corresponding protein/peptide**
Crh	Corticotropin-releasing hormone
Nucb2	Nesfatin peptides
Cartpt	Cocaine- and amphetamine-regulated transcript
Tshb	Thyrotropin β subunit
Tshr	Thyrotropin receptor
Gphb5	Thyrostimulin β subunit

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1. INTRODUCTION

Cancer anorexia-cachexia syndrome (CACS) is the simultaneous development of a loss of appetite (anorexia) and wasting of adipose and skeletal muscle tissues (cachexia) in patients with progressive cancers (FIGURE 1) [1, 2]. Patients with CACS suffer not only from their primary malignancies but also have diminished physical function [3], are less tolerant to anti-cancer therapies [4, 5], and ultimately have a worse prognosis [4, 6, 7] due to the peripheral tissue wasting. Living with the syndrome reduces quality of life for patients and their caregivers [8–10].

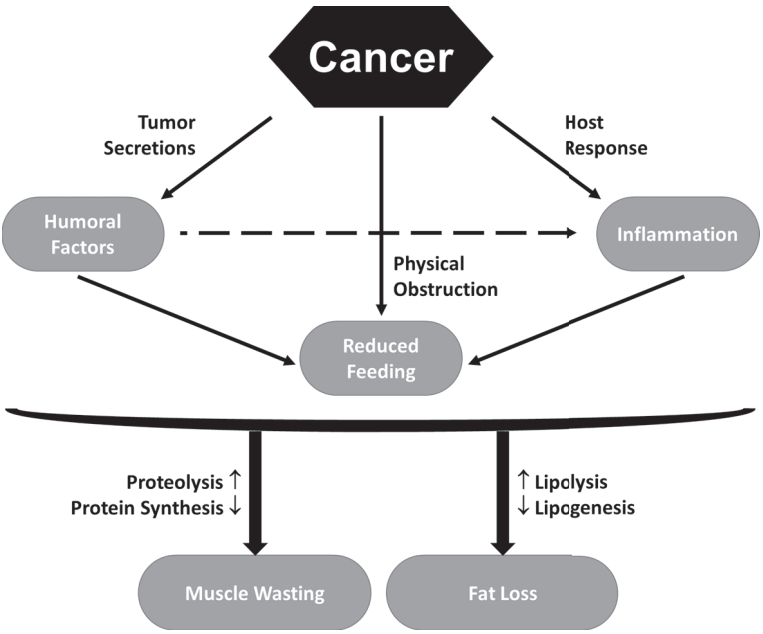

FIGURE 1 Schematic of processes occurring in the cancer anorexia-cachexia syndrome. The cancer can directly or indirectly (secretion of humoral factors or eliciting a host inflammatory response) reduce food intake. The anorexia, inflammation, and other tumor-associated humoral factors can each contribute the catabolic state featured in CACS. In some cases, tumors can obstruct the GI tract preventing food intake leading to starvation, which in combination with CACS will further escalate deterioration.

Prior to 2011, when an international consensus was reached clinically defining CACS [1], the reported prevalence for the syndrome

varied depending on the criteria used [11–13]. Some types of cancer—for example, pancreatic and gastrointestinal—are at higher risk for developing CACS [7]. Chronic malnourishment and pro-catabolic states are the causes of death in an estimated 20% of patients with CACS [14]. The causal mechanism(s) underlying the development cancer-associated anorexia and cachexia remains elusive to researchers. Due to the complex physiological interactions between the tumor and the host, it is agreed that CACS is a multifaceted problem that requires a multimodal treatment approach [15–20].

Restoration of appetite among patients with CACS is one such treatment option. Patients given nutritional support or appetite stimulants do show improvements in body weight and survival [16, 19, 21]. Increasing nutrient intake, to some extent, does help to offset the deficit in energy balance; this treatment approach alleviates a symptom rather than truly reversing cancer anorexia and cachexia. Thus, energy balance cannot truly be attained. However, a causal removal of the anorexia component from CACS may reduce the severity of tissue wasting and improve the ability to treat patients with progressive cancers. In this thesis, the potential involvement of select neuroendocrine mediators known to be involved in feeding inhibition and metabolism in the healthy state are explored in CACS.

1.1 Central controls for food intake and metabolism

Feeding is a regulated behavior that is centrally controlled by an integrated network of nuclei in the hypothalamus and brainstem (FIGURE 2). Under normal conditions, food intake is modulated in order to maintain homeostatic energy balance over the long term. Energy balance is achieved when energy consumed equals the energy expended. Feeding bouts commence when relevant brain centers integrate signals for nutrient status (e.g. leptin, amino acids) with transient depressions in blood glucose that fit a defined, time-dependent pattern [22]. Meal size is determined by gastric volume, and gastric filling will activate vago-vagal reflexes that are integrated by the dorsal vagal complex. Thus, meal size

is controlled at the brainstem level. A variety of gastrointestinal hormones are released into the circulation when nutrients are introduced into the lumen of GI tract. Such satiety signals will convey to the brain information about the meal ingested. One such example is CCK (cholecystokinin), which signals to the CNS about meal size and induces satiation [23–25] as well as causes an inhibition of gastric emptying. In addition, CCK causes satiety via vagal afferent signaling. It is through this interplay of central and peripheral signaling that feeding behavior is regulated to maintain homeostasis.

1.1.1 The hypothalamus

The hypothalamus is regarded as the primary control center for energy balance including feeding behavior and thermoregulation. Early lesion experiments showed that damage to the ventromedial (VMH) or lateral hypothalamic (LHA) areas resulted in voracious overeating or complete abolishment of food intake, respectively [26–28]. Later neuroanatomical studies further characterized these hypothalamic nuclei by their neuropeptide products as well as their functional aspects. Other areas, such as the dorsomedial hypothalamic nucleus (DMH) are implicated in regulating circadian and feeding behaviors [29–32]. Studies included within this thesis focus primarily on the arcuate (ARC) and paraventricular (PVN) nuclei of the hypothalamus, which together regulate satiety and endocrine function. In Paper II, we included the DMH and VMH in our immunohistochemistry investigations.

The ARC nucleus has a key role in feeding controls, and it is situated laterally around and inferior to the third ventricle (3V). The ARC contains neuron populations that are important for appetite regulation. Neurons containing cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC), both of which suppress feeding, are present within the lateral ARC [33]. Another subpopulation of neurons located in the medial ARC co-express the orexigenic peptides neuropeptide Y (NPY) and Agouti-related peptide (AgRP) [34]. Gold thioglucose administration produces chemical lesioning to the ARC indicating glucose sensitivity [35]. Receptors for leptin and cortisol have

also been reported within the ARC [36–38]. While the ARC—like much of the brain—is sequestered from the circulation by the blood-brain barrier (BBB), the ARC's proximity to the 3V makes it susceptible to some signals that are present in the cerebrospinal fluid (CSF). The ARC also has efferent projections to other hypothalamic nuclei allowing signals to be relayed and integrated.

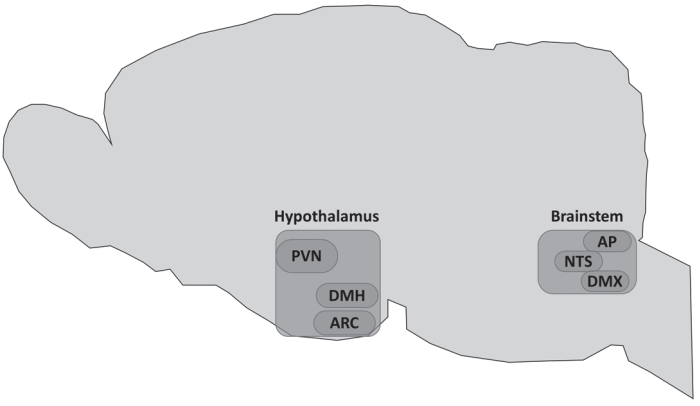

FIGURE 2 Representative diagram of the rodent brain with labels to indicate hypothalamic and brainstem nuclei with relevance for feeding. **AP**, area postrema; **NTS**, nucleus of the solitary tract; **DMX**, dorsal motor nucleus of the vagus; **PVN**, paraventricular nucleus of the hypothalamus; **DMH**, dorsomedial nucleus; **ARC**, arcuate nucleus.

The PVN surrounds the 3V in the anterior hypothalamus, and it consists of two main sub-regions—the magnocellular and the parvocellular nerve cell populations. The magnocellular neurons contain vasopressin and oxytocin, which are important for water retention and milk letdown, respectively. The parvocellular region, which is a focus in the present thesis, contains corticotropin-releasing hormone- (CRH), thyrotropin-releasing hormone- (TRH), and CART-expressing neurons [39, 40]. As such, the PVN is the hypothalamic root of the hypothalamic-pituitary-adrenal and -thyroid axes. Secretion of releasing-hormones from the PVN provides downstream stimulation of adrenal and thyroid function, and thus govern the respective stress response and metabolic rate. Parvocellular neurons of the PVN project fibers to the median

eminence (ME) and to the brainstem, including the dorsal vagal complex (DVC) [41]. It also receives afferent projections from the nucleus of the solitary tract (NTS) [42]. The neuroanatomical organization of the PVN allows for the receipt and modulation of gastrointestinal function and food intake.

1.1.2 The brainstem

While the hypothalamus has a key role in regulating feeding and metabolism, the dorsal hindbrain also contributes to feeding controls directly as well as by integrating and relaying viscerosensory information to hypothalamic nuclei [43–45]. Direct evidence for the dorsal hindbrain's role in regulating feeding behavior is demonstrated in chronic decerebrate animals, in which hindbrain-forebrain connections have been severed. These decerebrate animals are able to initiate and cease feeding bouts when sustenance is provided intraorally, but they cannot initiate food-seeking behaviors. These observed behaviors highlight the role of the hindbrain in meal size determination [44, 46–48]. There are several subsets of hindbrain nuclei that contribute to the feeding response. In this thesis, particular focus is placed on the DVC given its direct control over gastrointestinal function as well as meal size determination.

The DVC consists of the area postrema (AP), the dorsal motor nucleus of the vagus nerve (DMX), and the nucleus of the solitary tracts (NTS; FIGURE 2). The AP is located in direct proximity to the NTS and the DMX, and the permeable BBB of the AP is important for emetic responses [49, 50]. The NTS receives vagal afferent fibers via the nodose ganglion, and also projects afferent fibers to hypothalamic structures that regulate feeding including the ARC and PVN [42, 51]. The DMX contains vagal motor neurons which innervate the gastrointestinal tract via efferent nerve fibers to control gastrointestinal physiology [52]. The afferent vagal fibers contain receptors for a variety of peptides and cytokines including CCK, leptin, and interleukin-1β [53–56]. Immunohistochemistry studies have further shown CARTp expression in vagal fibers and CART mRNA in the nodose ganglion [57] suggesting CARTp as a vagal afferent signaling factor.

1.2 Neuroendocrine mechanisms in feeding and metabolism

This thesis explores select neuroendocrine factors that, under normal conditions, inhibit food intake. These factors are the thyrotropin receptor (TSHr) and its agonists, CARTp, and the recently discovered nesfatin-1 with respect to illness-associated anorectic conditions. These select neuroendocrine factors are shown to be inflammation-responsive in brain regions with relevance for feeding [58–61].

1.2.1 CART

Shortly after its discovery [62], the protein translated from CART mRNA was described as an endogenous satiety molecule in the hypothalamus [33]. The CART pro-peptide is post-translationally cleaved to produce two endogenous functional peptides: CARTp 55-102 and CARTp 62-102 [63–65]. CART is widely distributed throughout the brain [66, 67] as well as in peripheral tissues [68–71]. In contrast to centrally acting CARTp, the role for CARTp in the periphery is less well known; although a role in pancreatic function has been proposed [70, 72, 73]. Central injections of CARTp induce an acute inhibition of food intake in healthy and fasted rats [33, 74–77] as well as changes in motor behavior [33, 78]. CARTp has been described to induce a conditioned taste aversion after intracerebroventricular (i.c.v.) infusion [79]. In addition, centrally acting CARTp acts to inhibit gastric emptying and gastric acid secretion [76, 80].

The precise sites of action for CARTp remain unclear, though data from intraparenchymal-injection studies report region-specific effects [77, 81]. Attempts to anatomically identify CARTp binding sites with autoradiography have been unsuccessful due to a high degree of unspecific background [82], and the identity of the putative CARTp receptor protein structure has yet to be demonstrated. There is, however, evidence to support that CARTp acts on G-protein coupled receptors [65, 83–85], and CARTp binding can be antagonized by the PACAP6-38 fragment [85, 86].

1.2.2 Nesfatin-1

Nesfatin-1 is a polypeptide in the CNS and periphery that is encoded by the *Nucb2* gene in mice. Within the CNS, nesfatin-1 is present in hypothalamic nuclei including the PVN, NTS, and DMX [87, 88]. In the PVN, nesfatin-1 has been demonstrated to interact with TRH and CRH neurons and alter feeding behavior [89], and 3V infusions of nesfatin-1 suppress food intake and reduce body weight in the rat [90]. Intracerebroventricular injections were further shown to inhibit gastric emptying in a dose-dependent manner [91]. Food deprivation and refeeding alters expression of *Nucb2* mRNA in the PVN [87]. Together, this is suggestive of a role for nesfatin-1 in central feeding controls in the healthy state. In the present thesis, we tested the hypothesis that hypothalamic *Nucb2* expression may be altered as part of a host response to a murine model for CACS (Paper IV).

1.2.3 Brain-derived thyrotropin receptor

Thyrotropin-releasing hormone, produced in the PVN, promotes the release of thyrotropin (TSH) from the anterior pituitary, which acts on the TSH receptor (TSHr) in the thyroid. Activation of TSHr results in the release of thyroid hormones (thyroxine, T4; and the active triiodothyronine, T3) into the circulation. Thyroid hormones control basal metabolic rate, and this will indirectly impact feeding behavior [92, 93]. High concentrations of circulating thyroid hormones initiate energy-taxing molecular processes, which increase appetite as a secondary effect to meet the higher energy requirements and maintain energy balance. Conversely, periods of low caloric intake reduce thyroid activity to spare energy [93–95]. The relative concentration of thyroid hormones is regulated by a series of feedback mechanisms along the hypothalamus-pituitary-thyroid axis.

While interactions between TSH and its receptor are traditionally thought to occur exclusively in the periphery, there is evidence supporting the presence of TSH and TSHr in the CNS as well [96–102]. The endogenous actions of central TSH signaling are not well described. Although TSHr have been shown in hypothalamic extracts [60, 101, 102],

the neuroanatomical locations of TSHr proteins in the hypothalamus and brainstem have yet to be demonstrated. Lateral i.c.v. infusion of TSH resulted in a reduction in food intake among rats [100], thus suggesting a role for central TSHr in the regulation of feeding.

Based on previous findings, the hypothesis that functionally relevant TSHr protein is present in distinct areas of the hypothalamus and brainstem of importance for feeding behavior is tested (Paper II). In addition, the central mRNA expression coding for TSH, TSHr, and the newly proposed endogenous agonist thyrostimulin [60, 103–109] are investigated in hypothalamic and brainstem feeding centers in response to acute inflammation (Paper III) and a CACS-promoting tumor (Paper IV). Furthermore, the effects these pro-inflammatory states had on pituitary mRNA expression and circulating levels for CARTp, TSH, and of serum albumin P component—an acute phase protein marker among mice [110]—were evaluated.

1.3 Inflammation, cancer, and anorexia

Celsus originally characterized inflammation by its cardinal features: *calor* (heat), *dolor* (pain), *rubor* (redness), and *tumor* (swelling). Galen later appended with *functio laesa* (loss of function). The systemic inflammatory response of the host is aimed at defending against foreign infiltrations and to promote wound healing. A common feature of the host inflammation response is loss of appetite, increased temperature, and fatigue, which will lead to reduced physical activity. Appetite can be suppressed during illness usually due to pain, nausea, and/or the enhancement of satiety signaling. Pro-inflammatory mediators—including tumor necrosis factor α (TNFα), some interleukins, and prostaglandin E_2—reduce food intake by actions at a central level in acute as well as in chronic illnesses [111–115]. A systemic inflammatory response produces an activation of the hindbrain and hypothalamic feeding-associated structures in both acute lipopolysaccharide-induced inflammation [116] and in chronic tumor-associated illnesses [117, 118].

In a parabiotic animal study (where two animals share a circulatory system), CACS was shown to develop in the tumor-free rat, thus suggesting that CACS is a result of a humoral factor transferred from the tumor-bearing animal to the healthy rat [119]. However, the specific humoral factor(s) underlying this effect has yet to be isolated. Since a specific mechanism underlying CACS remains to be identified, there exists no curative treatment for the affliction. In the lack of specific mechanistic treatments, current clinical care is aimed mainly at reducing the severity of the symptoms, and delaying the progression of the condition. The provision of supplemental nutrition to patients with CACS prolongs survival but does not restore lean muscle mass [19, 120, 121]. Similarly, daily long-term provision of the gut-hormone ghrelin was shown to improve perceived appetite in unselected cancer patients with CACS and improved body weight [122].

Little is known about how the regulatory networks for food intake are affected in CACS and in response to inflammation. Importantly, CACS differs from starvation, wherein reduced caloric intake is met with a compensatory reduction in metabolic rate. In healthy organisms, starvation/nutrient deprivation results in a reduced physical activity and body temperature in an attempt to conserve energy. Patients and experimental models with CACS exhibit an increase in energy expenditure even though physical activity and nutrient consumption have decreased. The hallmark feature of CACS is the lack of compensatory feeding or metabolic changes to maintain energy balance. Thus, the mechanistic conclusions derived from studies of feeding regulation in the normal or the obese conditions cannot be directly transferred to, or assumed to apply for, CACS.

1.3.1 The MCG101 tumor model for CACS

The MCG101 tumor mouse model was used to investigate the impact of CACS on central and peripheral host responses. The MCG101 tumor was originally derived from methylcholanthrene-induced sarcoma in mice [123]. This model is well-investigated and it produces a phenotype that resembles CACS in human cancer patients (TABLE 1) [124]. Although

originally classified as a sarcoma, MCG101 now appears as an undifferentiated epithelial-like solid tumor [125]. This tumor is a non-metastasizing, transplantable tumor, with a doubling time of approximately 57 hours, and produces high amounts of the pro-inflammatory mediator prostaglandin E_2 [125].

Transplanted MCG101 tumors become palpable usually about the seventh post-implantation day with anorexia developing shortly thereafter. Mice bearing the MCG101 tumor present with decreased *ad libitum* feeding and loss of adipose and skeletal muscle masses [126–128].

TABLE 1 Features shared by the MCG101 experimental mouse model and clinical cancer anorexia-cachexia syndromes. Studies expressing these features are indicated by reference number in brackets.

Feature	MCG101	Clinical CACS
Anorexia	Reduced food intake [125, 126, 129]	Self-reported loss of appetite [130, 131]
Tissue wasting	Less whole-body lipid content, fat-free dry carcass weights, increased edema [127, 129, 132]	Lowered computed fat and skeletal muscle masses [133–135]
Ghrelin	Elevated circulating ghrelin [129]	Reduced sensitivity to ghrelin [136, 137]
Energy Expenditure	Increased metabolism [132]	Elevated resting energy expenditure [16, 138, 139]
Thyroid Function	Reduced plasma T3 [127]	Normal to reduced T3 [94, 135, 140, 141]
Inflammation	Elevated plasma SAP and cytokine concentrations [115, 142]	Elevated concentrations of CRP and cytokines in circulation [135, 138, 143]

2. SPECIFIC AIMS

I. To investigate if PACAP6-38 is a functional antagonist to CARTp 55-102 *in vivo* with respect to food intake.

II. To identify and localize TSH receptors in areas of the brain with relevance for feeding and gastrointestinal controls, and to verify the functionality of such putative receptors.

III. To explore responses to acute inflammation of *Cartpt*, *Tshb*, *Tshr*, and *Gphb5* gene expression in areas of the brain with relevance for feeding controls. Further, to examine possible inflammation-induced changes in plasma CARTp concentrations under similar conditions, and if such changed levels may be prostanoid-dependent.

IV. To explore effects of the CACS-inducing MCG101 tumor and/or food restriction on gene expression changes in mouse brain/pituitary extracts for *Crh*, *Cartpt*, *Nucb2*, *Tshr*, and *Tshb* in areas of relevance for feeding controls. Second, to determine whether plasma CARTp levels may be affected by tumor and/or reduced caloric intake.

V. To test whether putative antagonism of CARTp with PACAP6-38 can improve feeding behavior or body composition of MCG101-bearing mice.

3. METHODOLOGY

3.1 Animals

Adult male Sprague-Dawley rats (purchased from Charles River, Germany; Scanbur, Sweden; or Taconic Farms, Denmark) were used to investigate the central effects of CARTp and TSH *in vivo* (Papers I and II). Female mice of the strain C57BL/6JBomTac (Charles River, Germany) were used to model acute inflammation (Paper III) and cancer anorexia-cachexia (Papers IV and V). Animals were kept at the Laboratory for Experimental Biomedicine under controlled ambient temperature, lighting, and relative humidity. Animals were group-housed in polysulfone cages supplied with woodchip bedding, nesting material, and gnawing sticks (rats) before experiments began. During experiments, mice were housed in groups of 2-4 animals per cage, whereas rats were individually housed due to cannulation for central injections. Ethical permissions were obtained from the Gothenburg Regional Animal Ethics Committee.

3.2 Central injections

In Papers I and II, we addressed specific central effects of feeding-modulatory substances. Rats were anesthetized and affixed to a stereotaxic instrument for chronic guide cannulas aimed at the fourth ventricle (4V; Paper I) or the nucleus of the solitary tract (NTS; Paper II).

The coordinates for the 4V and NTS were determined from a rat brain atlas and have been used previously [77, 144, 145]. The 4V guide was situated 2.5 mm anterior to the occipital crest. The NTS was targeted by unilaterally aiming the guide 1.6 mm anterior to the occipital crest and 0.6 mm lateral of the midsagittal line. Injection sites were verified functionally, in Paper I, by administering 5-thio-D-glucose into the 4V, which induces a robust increase in blood glucose concentration [146]. Animals in Paper I were included when blood glucose had at least doubled in response to 4V injections. Alternatively, in Paper II, injection

sites were verified by including rat urocortin I as a positive control peptide [147] in addition to using ink traces at the end of the study.

Injections were given over a period of 30-60 seconds just before lights-off using a syringe affixed to an infusion pump. Injectors were left in place for an additional 30-60 seconds to reduce the risk of back flush (i.e. withdrawing test substance along with the injector), and there was a two-day washout period between sessions to minimize possible carry-over effects. The doses used in Papers I and II were selected based on prior experience (CARTp 55-102; [76]) and an earlier pilot study (TSH).

3.3 Modelling acute inflammation

Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria, and it is a tool used to produce a robust acute inflammation response in experimental settings. LPS activates the immune system, which recognizes the cell wall component and initiates a general inflammatory response. The resulting inflammatory reaction resembles that of a septic response, but without the bacterial load. Group-housed mice were injected with LPS (7.5-10 mg/kg mouse; *S.enterica* serotype enteritidis) or saline vehicle i.p. 24 h prior to tissue collection. These LPS doses are similar that used in a previous study [60], and produced a strong inflammatory response within 24 h. The anti-inflammatory drug indomethacin was provided to some mice to the drinking water to yield an effective dose of 1 mg/kg mouse p.o. Indomethacin inhibits the enzyme that synthesizes prostanoids, including prostaglandins. This indomethacin dose was determined to be effective against tumor-associated inflammation in previous studies [115, 125, 142, 148, 149].

3.4 MCG101-induced CACS

Tumor-bearing mice in Papers IV and V underwent bilateral subscapular implantation of MCG101 tumor fragments taken from donor mice under aseptic conditions. In brief, donor mice were anesthetized (3.5% isoflurane inhalation, 350 mL/min flow rate). The MCG101 tumors were

removed from the donor mice, and non-necrotic tissue was placed in McCoy's 5A medium and cut to 1-3 mm^3 pieces with surgical scissors. Healthy mice were similarly anesthetized and MCG101 pieces were subcutaneously placed using Tuohy needles. The entire procedure was performed such that the mice were anesthetized for no more than 10 min. Control mice underwent sham implantations with empty Tuohy needles. All mice were killed on post-implantation day 14.

The PACAP6-38 dose selected for Paper V was determined to be sufficient for putative CARTp antagonism *in vivo* given plasma CARTp levels obtained in Paper IV, the estimated blood volume of an adult mouse, and the inhibitory constant (K_i) reported by Lin *et al.* [85]. In Paper V, mice were given either PACAP6-38 (1 μg/mouse or approximately 0.25 nmol) or saline vehicle as control. These injections were given i.p. one daily, and administration began on post-implantation day 10 when anorexia was evident among tumor-bearing mice. The PACAP6-38 treatment regimen lasted for four days. This treatment schedule parallels the clinical setting where treatment begins after CACS has become evident.

3.5 Food Intake

Rodents are nocturnal animals, and as such have their feeding bouts predominately during the dark phase. In acute feeding experiments (Papers I and II), rats were provided with pre-weighed portions of food at lights-off and consumption was recorded at regular intervals (FIGURE 3). The schedule for drug administration and food intake measurements was adjusted to accommodate the animals' natural nocturnal feeding patterns. Therefore, food intake was measured from the onset of the dark period when the motivation to eat is most pronounced. During the experimental observation periods, animals were placed in wire-floored cages so that food intake could be corrected for spillage.

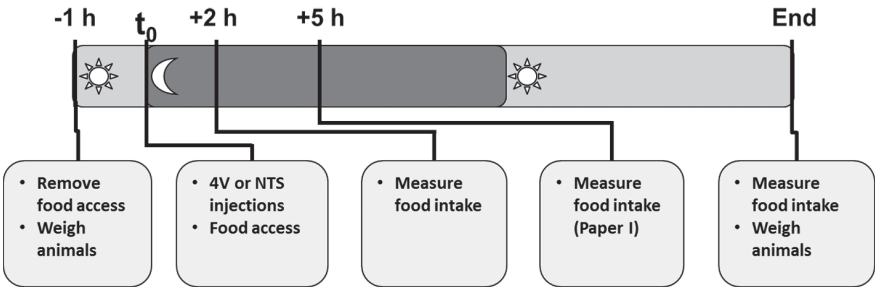

FIGURE 3 Representative schematic of the study design for functional feeding experiments performed in the rat (Papers I and II).

Mice were acclimated to wire-floor cages several days prior to tumor or sham implantations. Food intake and body weights were measured once daily (FIGURE 4). Anorexia develops progressively in MCG101 tumor-bearing mice (Papers IV and V) with reductions in *ad libitum* feeding beginning approximately 7-10 days after tumor implantation. Tumor-specific anorexic responses were differentiated from caloric intake *per se* by including pair-fed controls (Paper IV). Pair-fed mice were provided a portion of food equal to the amount eaten *ad libitum* by the tumor-bearing mice. Pair-fed mice began to eat shortly after they received access to food early in the light period.

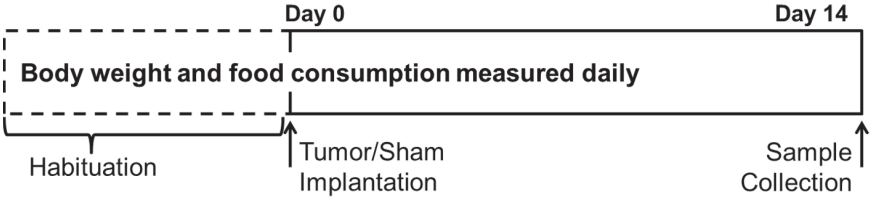

FIGURE 4 Representative schematic of the study design used for chronic exposure experiments performed in MCG101-bearing mice (Papers IV and V).

3.6 Gene expression analysis

In order to minimize the risk of RNA degradation, samples were placed in RNAlater solution immediately after sacrifice. The hypothalamus blocks were defined as the tissue sounding the 3V bounded by the optic

chiasm and mammillary bodies (Papers II-IV). In mice, the hypothalamus was further dissected using 2 mm biopsy punches into the PVN (dorsal two-thirds of 3V) and ARC (ventral third of 3V) in a standardized way.

Tissues were mechanically disrupted using a rotor/stator homogenizer in QIAzol lysis solution. Soluble total RNA was isolated using RNeasy microcentrifuge spin columns and genomic DNA was digested with enzyme treatment. Total RNA was eluted with nuclease free water and evaluated in a Nanodrop ND-1000 instrument for quantification. The quality/integrity of RNA was determined using an RNA 6000 Nano kit in a Bioanalyzer 2100 instrument. RNA integrity numbers—the index for sample quality ranging from 0 to 10—greater than 7.0 units were considered suitable for use. Complementary DNA (cDNA) was synthesized from 0.2-1.0 μg extracted total RNA using reverse transcriptase kits. These commercially available kits use reverse transcriptase enzymes and oligo-dT primers to selectively convert messenger RNA (mRNA) in the sample to cDNA.

Commercially available primers probe the cDNA sample for gene-specific sequences. These primers, as well as a fluorescent reporter dye (SYBR Green I), are mixed with the cDNA sample. The dye emits a signal when these specific primers align with corresponding sequences of cDNA forming double-stranded DNA segments. The polymerase chain reaction (PCR) consists of a series of melting (separation of DNA strands), annealing (alignment of primer with the DNA strand), and elongation (production of a new strand of DNA). With each successive PCR cycle, the amount of cDNA for a given transcript is doubled. The fluorescence intensity is proportional to the concentration of gene-specific cDNA.

The cycle number in which the signal intensity crosses a set threshold (Ct) is used to quantify the gene expression levels. Gene expression can be quantified by either the relative standard curve method (Papers II and III) or the $2^{-\Delta\Delta Ct}$ method (Paper IV). In the former quantification method, Ct values are compared to an external standard

curve yielding expression levels relative to that standard. The latter $2^{-\Delta\Delta Ct}$ method is calculated relating Ct values obtained from the control condition and reference gene(s); the output for this method is the relative fold-change in gene expression rather than a concentration.

3.7 Protein detection and quantification

Protein detection methodologies take advantage of the ability for the adaptive immune system to produce highly specific antibodies. Immunoglobulins (often of the G-subtype; IgG) are able to recognize specific peptide sequences (epitopes) in the antigen. The immunoglobulin subtype most often used is the IgG. These immunoglobulins are classified as either monoclonal (recognize one peptide sequence of antigen) or polyclonal (recognizes multiple epitopes of an antigen). Antibody-antigen interactions are used in a number of methodologies in order to isolate, identify, and/or quantify proteins.

While only one antibody is necessary to recognize the protein of interest, antibody-based methods regularly use two or more antibodies. The use of multiple antibodies improves the signal-to-noise ratio, thus allowing for reliable detection of even low-abundant proteins. The first—or primary—antibody is used to recognize and bind to the antigen of interest. Secondary antibodies are often conjugated to a reporter protein, which are used to visualize successful immunoreactivity.

3.7.1 Western blot

Western blots (WB) characterize proteins by combining the specificity of immunodetection with the size discrimination of gel electrophoresis. In Paper II, WB analysis was used in a qualitative manner to confirm that TSHr proteins are present in brain tissues of the adult rat. Two primary antibodies raised against the human TSH receptor protein were used to improve confidence of detection: a mouse monoclonal, directed at the extracellular receptor domain; and a rabbit polyclonal selective for the intracellular portion of the receptor protein. Protein sequences for rat and human TSH receptors were compared using the standard protein

BLAST tool (National Library of Medicine, National Center for Biotechnology Information) to ensure species cross-reactivity.

Rat tissues were homogenized and denatured proteins were resolved using gradient acrylamide gels under reducing conditions. The size-separated proteins were then transferred to polyvinyl difluoride (PVDF) membranes and blocked for unspecific binding sites. PVDF-bound proteins were subsequently incubated with primary and respective secondary antibodies. Immunoreactivity was visualized via enhanced chemiluminescence, which was recorded using a CCD camera (Paper II, Fig. 5).

3.7.2 Immunohistochemistry

Immunohistochemistry (IHC) relies on similar principles as WB. Whereas WB can inform on physical characteristics (such as size or multimerization) of the immunoreactive protein, IHC informs on the anatomical location of a protein. Therefore, WB in conjunction with IHC provides evidence for *which* protein is detected and *where* it natively resides. IHC can be further extended to probe for multiple proteins of interest in order to determine co-localization. Such co-localization experiments provide an anatomical basis for possible protein-protein interactions. In IHC the protein of interest is in its native, folded—tertiary or quaternary—structure in the cells where it is presumably produced. The same anti-TSHr mouse monoclonal antibody used in WB analysis was also used for IHC.

In Paper II, rats were perfusion-fixed with a solution of 4% paraformaldehyde in phosphate buffer followed by overnight post-fixation of dissected tissue samples. Brains, thyroid, liver, and pituitaries were collected. The samples were dehydrated by incubation with increasing concentrations of ethyl or isopropyl alcohols. Tissues were then cleared in xylene and embed with paraffin wax. Brains were subsequently sectioned to levels that displayed the paraventricular hypothalamic nucleus (PVN; coordinated Bregma -1.5 mm), the arcuate nucleus (ARC; Bregma -3.24 mm), and the caudal brainstem (Bregma -

13.76 mm) approximated from the rat brain atlas [144]. Paraffin-embedded tissues were sectioned to a thickness of 5 μm on a sliding microtome and fixed to positively charged glass slides.

IHC was performed on the de-waxed, rehydrated tissue sections. The slides then underwent heat-induced epitope retrieval under acidic conditions followed by quenching of endogenous peroxidase activity. Unspecific binding sites were blocked prior to incubation with the primary antibody. Immunoreactivity was primed for visualization using a biotinylated secondary antibody and the Vectastain Elite ABC kit. Cells containing TSHr-immunoreactive (TSHr-ir) proteins produced a brown precipitate after a brief incubation with a 3,3'-diaminobenzidine (DAB) chromogen. Finally, the sections were hematoxylin counterstained, cover-slipped, and mounted using a permanent mounting medium.

3.7.3 ELISA

Enzyme-linked immunosorbent assays (ELISA) are routinely used to quantify the amount of a protein present in biological samples. ELISA-based methods take place in two stages: first, the protein of interest is captured onto a solid phase; second, the protein concentration is quantified. In standard ELISAs, microwell plates are coated with capture antibodies. Multiplex ELISA methods are also available such as the Luminex xMAP system (Paper V). In the multiplex assay, the solid phase is a bead coated with specific capture antibodies. Several types of coated beads can be used in tandem on a single sample to capture multiple proteins to be quantified. Quantitative ELISAs may be "sandwich" or competitive assay types. In a sandwich ELISA, the captured protein is flanked between the capture antibody and a second enzyme-conjugated reporter antibody. Competitive ELISAs use an enzyme-conjugated protein of a known concentration, which displaces the captured protein of interest. Sample concentrations are quantified by relating the signal intensity to a calibration curve of known concentrations.

ELISAs were used to quantify plasma concentrations for CARTp (Papers III-V), serum albumin P component (SAP; Papers III and IV),

TSH (Papers III and V), and thyroid hormones (Paper V). SAP is a mouse acute phase protein and behaves similarly to the human c-reactive protein (CRP) [110]. Both SAP and CRP belong to the pentraxin-family of proteins, and they are useful markers for degree of inflammation in the mouse and man, respectively. Whole blood was collected from anesthetized mice by cardiac puncture with EDTA added as an anticoagulant. Blood samples were centrifuged and plasma was collected and stored at -80 °C until use. All ELISA procedures were performed in accordance with the manufacture-supplied protocols.

3.8 Body composition

One of the aims of Paper V was to determine whether peripheral treatments of PACAP6-38 could reduce the severity of tissue wasting in MCG101-bearing mice. Tumor-free carcasses were dried at 80 °C to constant mass, thus providing a measure for whole body fluid content. Whole-body lipid content was extracted from the dried mouse carcasses by a series of solvent extractions (1:1 chloroform:methanol; 1:1 acetone:ethanol; and pure diethyl ether) [129, 132]. The solvents were pooled into a pre-weighed beaker and evaporated until only lipid mass remained. The remaining fat-free dry weight functions as a surrogate measure for the lean tissue compartment [127, 128].

4. RESULTS AND DISCUSSION

There is a vast body of literature on normal feeding controls; however, less is known about the specific mechanisms for feeding that are altered during conditions of disease. Feeding behavior during states of illness is a multifaceted problem, which is likely due to several mechanisms working in tandem to decrease food intake. While anorexia is a common feature to both acute inflammation and chronic tumor exposure, the central mechanisms underlying acute-illness anorexia may differ from those governing chronic, progressive illnesses. Due to the complex biochemical interactions that arise during states of disease, conclusions on how cancer-anorexia occurs cannot be directly drawn from how feeding signals operate in a healthy state. Instead, the mechanisms behind illness-associated anorexia must be considered in the context of its own physiological situation and not simply as a deviation from the normal state. In this thesis, our focus was on CART, the TSH receptor and its ligands, CRH, and nesfatin-1 after exposure to LPS or MCG101 tumor. These neuroendocrine factors all shown to impact feeding under normal conditions [33, 76, 100, 150] and to be sensitive to inflammation [58–61].

4.1 Central manipulations on feeding in healthy animals

4.1.1 CARTp anorexia is antagonized by PACAP6-38 *in vivo*

In Paper I, we tested whether PACAP6-38 was able to act as a functional antagonist to CARTp-induced inhibition of food intake *in vivo* [86]. This study built on previous data reporting *in vitro* antagonism of CARTp binding and ERK-phosphorylation [85]. Since CARTp potently inhibits feeding from a dorsal hindbrain site [78], we decided to test the hypothesis that 4V pretreatment with PACAP6-38 could antagonize CARTp-induced anorexia.

Injections of CARTp 55-102 into the 4V without the putative antagonist reduced food intake by up to 67% in 2 h, which was sustained throughout the 22 h observation period. PACAP6-38 pretreatments into

the 4V blocked CARTp-induced inhibition of feeding for 2-5 h (FIGURE 5) at lower doses; however, the highest dose appeared to have a longer-lasting antagonism. PACAP6-38, when administered alone, did not significantly influence feeding or body weight after being injected either 4V or s.c. at doses corresponding to those used to antagonize CARTp (0.3-6.0 nmol PACAP6-38). The lack of effect of PACAP6-38 *per se* on feeding and body weight further suggests that energy expenditure was similarly unaffected in the short term. Together, the findings that PACAP6-38 alone did not affect body weight but did counteract CARTp-associated weight loss provide further support for CARTp antagonism.

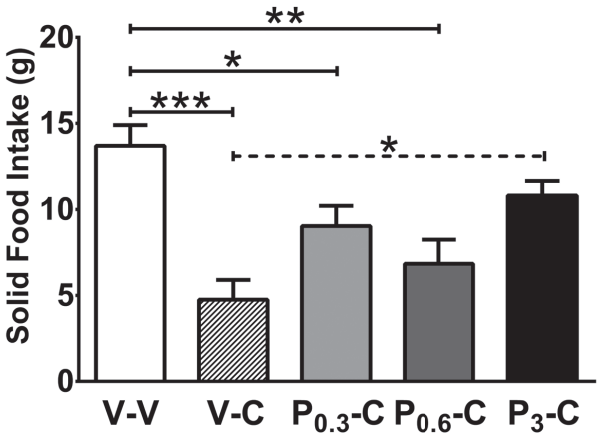

FIGURE 5 The average (±SEM) five-hour cumulative food intake following 4V peptide injections in adult male Sprague-Dawley rats (n = 8). Definitions: **V**, saline vehicle; **C**, CARTp 55-102 at 0.3nmol; **P**, PACAP6-38 at 0.3, 0.6, or 3.0 nmol. Statistical significance: * P<0.05; ** P<0.01; *** P<0.001. This figure is reproduced from Burgos *et al.* 2013 (Paper I; see ref. [86]).

While the molecular environments *in vitro* and *in vivo* are vastly different, our feeding data were consistent with Lin *et al.* in showing functional CARTp inhibition by PACAP6-38 [85, 86]. Binding studies performed by Lin *et al.* [85] showed that active CART peptides could be displaced by PACAP1-38 and PACAP6-38. They further showed that the CARTp binding was not displaced by maxadilan or vasoactive intestinal

peptide, thus indicating that CARTp was not interacting with any known PACAP-sensitive receptors. Conclusions drawn from the *in vitro* experiments were that PC12 cells in fact express a receptor structure for which CARTp has a high agonistic affinity. In our *in vivo* 4V i.c.v. paradigm, the lack of any observable PACAP6-38 effects indicates that there was a low background of endogenous agonist activity on a PACAP6-38- or CARTp-sensitive receptor structure. This interpretation is consistent with studies showing endogenous CARTp activity is low early in the dark period, when feeding motivation is high [33, 151].

We further noted that while PACAP6-38 pre-treatment mitigated CARTp-induced anorexia and body weight change, there was no apparent attenuation of stereotypical motor behavior (e.g. wobbly gait and flat posture). The inhibition of some but not all i.c.v. CARTp-associated behaviors is indicative of separate mechanisms by which CARTp elicits its feeding and motoric effects. One such possibility would be the existence of at least two CARTp-sensitive receptor subtypes.

Having access to putative CARTp receptor ligands may help to facilitate the future identification of CARTp receptor structures. CARTp antagonists are also useful for exploring functional actions of endogenous CARTp. In Paper V, we investigated how peripheral administration of PACAP6-38 would influence feeding and body composition in mice with elevated plasma CARTp levels. Our works support the notion of PACAP6-38 as a potential tool for identifying yet-unknown CARTp binding sites and physiological mechanisms of action. Biochemical modification of PACAP6-38 may improve its affinity and selectivity for the putative CARTp receptor(s), thus further increasing its pharmacological utility.

4.1.2 Functional TSHr are present in hypothalamic and hindbrain centers for feeding controls

In Paper II, we investigated the distribution and some functional aspects of neuronal TSHr proteins in centers with relevance for feeding in the

rat. While there is previous evidence for TSHr in the hypothalamus and other forebrain structures [101, 102], the specific localization within the hypothalamus and brainstem are still largely unknown. Our rationale for exploring TSHr anatomically was from the standpoint of understanding anorexia in disease given: first, that acute inflammation and experimental CACS activate neurons in the hypothalamus and DVC [116, 117] of the rat; and secondly, that central TSH inhibits feeding [100].

We first set out to test the hypothesis that the *Tshr* gene is expressed in the adult rat brain. We found that *Tshr* transcript was present in the hypothalamus and brainstem at readily detectable, but far lower than thyroid, concentrations. In separate WB analyses, proteins extracted from hypothalamus and brainstem tissues indicated TSHr-ir proteins were indeed present (Paper II, Fig. 5). Bands for these brain-derived TSHr-ir proteins were at molecular weights consistent with reports of the uncleaved TSH holoreceptor [152, 153]. We found hindbrain TSHr-ir localized to the dorsal vagal complex (including AP, NTS, and DMX) and the hypoglossal nucleus using IHC (Paper II, Fig. 2). Hypothalamic IHC preparations additionally showed TSHr-ir cells and fibers in the PVN, DMH, VMH, ARC and ME (Paper II, Fig. 3-4). Together, these findings provide support that hypothalamic and hindbrain TSHr may be of significance in feeding controls given the neuroanatomical distribution.

In order to confirm the functionality of the receptor and to address whether the receptor subpopulation of the NTS may be involved in feeding, local nano-injections of TSH were administered. TSH significantly reduced solid food consumption with no signs of refractory compensation under the 14 h observation period to a degree comparable to the urocortin control. Taken together, we are able to provide support for functional TSH holoreceptors in the dorsal hindbrain of the rat with relevance to feeding behaviors. In addition, 4V infusions of TSH were associated with an increase in average core temperature (Smedh *et al.*, unpublished results), while TSH via lateral i.c.v. decreased body temperature [100]. We also reported TSHr proteins are distributed not

only in the DVC but also in forebrain structures that receive NTS afferent projections [42]. The endogenous role—or multiple roles—for central TSHr and its ligands is still unclear, but present evidence suggests it may involve energy balance and thermoregulation among other yet unknown effects.

In this thesis, by showing the presence and location of TSHr in hypothalamic and hindbrain centers, we provide a basis for the possible involvement of TSHr in feeding and autonomic controls. Although further identification of neurotransmitter identity of TSHr-expressing neurons is important, the presence of TSHr in specific subregions—for example, the parvocellular PVN—of the hypothalamus provides some suggestive support of the possible physiological implications. We further present two novel manipulations on feeding behavior in the rat: antagonism of CARTp-sensitive receptor structures and ligand activation of brain-derived TSHr in the dorsal hindbrain. These experimental manipulations were performed in order to influence feeding behavior in the healthy state. Whereas CARTp is considered to participate in normal feeding controls in the healthy condition [33], the endogenous role for neuronal TSHr warrants further investigation. Based on these observations, the investigations were widened to address the possible roles for CARTp or brain-derived TSH receptors in the context of acute illness or cancer-associated anorexia.

4.2 Responses in central gene expression associated with acute inflammation and CACS

We hypothesized that mRNA coding for select anorexigenic compounds may be altered in CNS centers for feeding in response to illness. Mice were used to model acute LPS inflammation (Paper III) and exposure to MCG101-induced CACS (Paper IV).

4.2.1 CART is altered in CACS as an adaptation to nutrition status

CART and its associated peptides in the CNS have been implicated as endogenous satiety signals [33, 154]. This proposed role in satiety signaling is supported by the fact that fasting reduces hypothalamic CART expression in sheep [155], rats [33, 156], mice [157], and non-human primates [75]. Conversely, inhibition of endogenous CARTp with a specific anti-sera increases food intake in the rat [33]. The potent anorectic effect of central CARTp highlights the peptide as a possible cause of anorexia during inflammation and such a role has been proposed [58, 158–160].

We initially hypothesized that in states of prolonged illness *Cartpt* gene expression would be one candidate contributor to the development of the anorexia in CACS in MCG101-bearing mice. Instead, we observed decreases of *Cartpt* gene expression in the ARC and PVN of LPS- and MCG101-bearing mice, respectively (TABLE 2). Our own findings in addition to other CACS models [161–163] suggested that CART is responsive to the caloric consumption of the host during illness. These previous studies, performed in rats, raised the hypothesis that CART is adaptive to rather than the cause of reduced feeding in CACS. In order to conclusively address whether *Cartpt* expression is directly responsive to tumor factors or altered as a consequence of reduced caloric intake, a pair-fed condition was included in our investigation. We found decreases in PVN *Cartpt* expression could be largely explained by decreased food intake (Paper IV). This observation supports the suggestion that the tumor is not strictly influencing appetite by exploiting normal feeding controls in general, as would have been the case had *Cartpt* expression been elevated. We thusly conclude that hypothalamic *Cartpt* expression is an adaptive measure presumably to defend body weight rather than causal to reduced feeding in CACS.

Other research groups have shown that central injections of CARTp induce further feeding inhibition in fasted [33, 64, 74, 164] but not in tumor-bearing [162] animals. These findings could implicate

central CARTp resistance as a long-term adaptation to negative energy balance. The notion of decreased CARTp sensitivity among tumor-bearing animals would suggest reduced expression of putative CARTp receptors within the CNS. Consistent with this speculation, Ihnatko *et al.* found expression of the dynamin 1 protein to be up-regulated in hypothalami of MCG101-bearing mice [165]. Dynamin 1 facilitates endocytosis, and it may presumably be utilized for receptor turnover in MCG101-bearing mice. However, specific knowledge of a CARTp receptor is needed in order to validate such a claim of CARTp resistance.

4.2.2 TSHr in the CNS is down-regulated in acute inflammation but not CACS

A previous study in mice reported that LPS inflammation down-regulated *Tshr* in the hypothalamus and pituitary [60]. We have shown that TSHr proteins are expressed in the hypothalamus and hindbrains of healthy rats (Paper II). Our first aim was to investigate the effects of acute LPS inflammation on *Tshr, Tshb, Gphb5* gene expressions in the brainstem in addition to the hypothalamus and pituitary (Paper III). Tumors also induce inflammation, and the MCG101 tumor typically produces high plasma concentrations of prostaglandin E_2 [125, 148]. Our next aim was to evaluate the central response of *Tshr* and *Tshb* in MCG101-bearing mice (Paper IV) in addition to the central *Cartpt*, *Crh*, and *Nucb2* effects.

Similar to *Cartpt,* we found that LPS reduced hypothalamic *Tshr* expression in the ARC (TABLE 2). We detected no transcripts for either of the known TSHr agonists (TSH and thyrostimulin) in brain extracts, though other researchers have provided evidence for the presence of neuronal TSH protein and thyrostimulin gene transcript [60, 96–99]. The finding that *Tshr* were in fact down-regulated in the ARC implies a role for the TSHr in response to acute inflammatory illness modulate thyroid function. We did not investigate effects on food intake with regard to LPS; however, it is well known that LPS induces an anorexic response [58, 61]. Consistent with this, we did note an acute reduction in body weight in LPS-treated mice, which can be used as a proxy indicator of

overnight food intake. Our inability to detect TSH or thyrostimulin transcript in the CNS is likely due to the quantities being below limit of detection, though the possible existence of other as yet unknown TSHr agonists cannot be excluded.

Contrary to the LPS condition (Paper III), *Tshr* expression was not influenced by either MCG101 burden or by pair feeding in the hypothalamus and pituitary extracts (TABLEs 2 and 3). The difference in expression patterns may well be attributed to the differences in inflammatory profiles induced by LPS or MCG101, where the tumor-associated inflammation is more progressive in nature. Another possible explanation is that in MCG101-bearing mice there was a long-term exposure to inflammation and tumor-derived factors in addition to other CACS features. It cannot be ruled out that expression patterns would be more similar between MCG101 and LPS had the acute condition been sustained for longer than 24 hours.

4.2.3 Nesfatin-1 is increased in MCG101-bearing mice

As stated previously, Paper IV included in its scope the gene coding for the nesfatin-1 peptide, *Nucb2*. An interesting finding in Paper IV was that MCG101 CACS was associated with an elevation in *Nucb2* expression while pair-fed mice exhibited a tendency towards reduced expression relative to freely fed control mice (TABLE 2). While neither *Nucb2* expressions differed significantly from freely fed mice, the difference between tumor-bearing and pair-fed mice was significant. This finding indicates that decreased feeding is associated with the down-regulation of the gene coding for nesfatin-1. This down-regulation of *Nucb2* is consistent with a state of negative energy balance. MCG101-induced CACS appears to be promoting an overexpression of *Nucb2*, which if processed into peptide nesfatin-1 in the PVN could be causally contributing to cancer-anorexia.

Central and peripheral administration of nesfatin-1 peptide reduce food intake in healthy rodents [150, 166]. It is tempting to speculate that during CACS, central *Nucb2* expression reduces feeding via

a leptin-independent pathway [166]. Under normal conditions, circulating leptin is proportional to fat stores acts centrally to inhibit food intake. Although plasma concentrations of leptin were not measured here, both clinical and experimental CACS are well documented as having low circulating leptin concentrations [135, 137, 167, 168]. Regardless, in contrast to *Cartpt* and *Tshr* expressions in the hypothalamus in response to acute and tumor-illness, *Nucb2* expression stands out as primary response to tumor rather than adaptive to caloric intake. In addition, since *Nucb2* codes for the anorexigen nesfatin-1, we propose this peptide as a candidate for centrally elicited anorexia during CACS. Central provision of nesfatin-1 antagonists in CACS may conclusively address this hypothesis.

TABLE 2 Summarized changes in hypothalamic relative gene expression compared to respective controls.

Hypothalamus	Acute Inflammation (Paper III)	MCG101 (Paper IV)	Pair-Fed (Paper IV)
Cartpt	↓ ARC	↓ PVN	↓ PVN
Tshr	↓ ARC	↔	↔
Tshb	nd	nd	nd
Gphb5	nd	np	np
Crh	np	↔	↔
Nucb2	np	↑ PVN[a]	↔

nd, below limit of detection
np, evaluation not performed
[a], Significant difference compared to Pair-Fed condition

4.2.4 Hindbrain *Cartpt* and *Tshr* expression and inflammation

Gene expression levels for *Cartpt* and *Tshr* in the brainstem were unaffected in both the LPS and the MCG101 paradigms. The brainstem is an essential integrator of afferent signals from the periphery and yet both models for disease preferentially altered hypothalamic gene expression. The lack of gene expression differences reported within this thesis may be due to methodological limitations. In Papers III and IV, mRNA was extracted from a block of hindbrain tissue rather than from microdissected nuclei—as done in the hypothalamus. We cannot entirely exclude that significant regional effects could have been cancelled out in the *en bloc* analysis.

We showed in Papers I and II that exogenous CARTp and TSH, respectively, could reduce feeding; however, hindbrain *Cartpt* and *Tshr* expression levels remained unaffected by acute (Paper III) as well as by tumor-induced CACS (Paper IV). Further, the *Tshb* and *Gphb5* genes expression were detectable only in the pituitary tissue preparations. These negative findings do not exclude the possibility of changed gene expressions for a yet-to-be-identified CARTp receptor or TSHr agonists, which were below limit of detection in the brainstem extracts. Whereas we did not detect differences in diseased states where inflammation is present, CARTp, and possibly TSHr (Paper II), may still be involved in normal feeding controls and gastrointestinal function. Our data provide rationale for the further evaluation of the central role of TSHr and its involvement in the regulation of food intake and autonomic functions.

4.3 Peripheral responses to illness

The impact of acute LPS inflammation as well as MCG101 CACS on pituitary expression for *Cart, Tshr, Tshb,* and *Gphb5* were examined. The pituitary is subdivided into its neuronal (posterior) and glandular (anterior) tissues. TSH and CART [40, 66] are known to be produced and excreted from the anterior pituitary lobe. Since our analysis did not separate the pituitary into its neuronal and glandular constituents, the gene expression results of the pituitary are discussed as peripheral responses.

4.3.1 LPS and MCG101 enhances *Cartpt* expression in the pituitary and plasma CARTp concentrations

We showed in Papers III and IV that pituitary *Cartpt* expression was elevated (TABLE 3). Expression of the *Cartpt* gene and associated peptides are not restricted solely to the CNS, but rather they is widely distributed throughout the body [66, 69–71]. Circulating CARTp has also been reported to have diurnal rhythmicity, and other studies have shown CARTp to influence pituitary hormone release [169–174]. The physiological relevance for CARTp in the periphery, much like its specific site of action, remains unknown. Several control experiments

show no effects on feeding or gastric motor function when CARTp is administered peripherally as a single bolus [76, 80, 175–177]. During states of inflammatory illness, circulating thyroid hormones are reduced. We raised the hypothesis that peripheral *Cartpt* expression and/or plasma CARTp could be affected similarly in our paradigms.

In the pituitary, acute inflammation (Paper III)—but not MCG101 or pair-feeding (Paper IV)—caused a significant decrease in *Tshr* expression and tendency towards decreased *Tshb* expression (TABLE 3). We also reported evidence for increased *Gphb5* (thyrostimulin) expression among LPS-treated mice. This pattern was consistent with a previous report [60], though levels were below the limit of quantification in our study. With regard to the MCG101 study, *Tshr* expression was unaffected, while *Tshb* expression was in fact increased among both tumor-bearing and pair-fed groups. These data from the LPS and MCG101 studies suggest that thyroid activity is acutely down-regulated, but chronic exposures result in no observable effect. Tumor-bearing mice did show elevated plasma TSH though no change in T3 (Paper V), an observation that is consistent with the previously described non-thyroidal illness/secondary hypopituitarism that attends CACS [127, 140].

Cartpt expression was elevated in the pituitaries of MCG101-bearing, LPS-treated, and pair-fed mice compared to respective control conditions (TABLE 3). Interestingly, a proportional increase in plasma CARTp was also measured (Papers III-V) in response to these conditions. Reduced caloric intake could only partially explain the elevations in *Cartpt* expression and plasma CARTp, as there seemed to be a tendency towards a more pronounced response among tumor-bearing CACS mice.

TABLE 3 Summarized changes in pituitary relative gene expression compared to respective controls. Specific measures and statistical evaluations are reported in respective papers.

Pituitary	Acute Inflammation (Paper III)	MCG101 (Paper IV)	Pair-Fed (Paper IV)
Cartpt	↑↑	↑↑	↑
Tshr	↓	↔	↔
Tshb	↔[b]	↑↑	↑
Gphb5	↑[c]	np	np
Crh	np	↔	↔
Nucb2	np	↔	↔

nd, below limit of detection
np, evaluation not performed
[b], trend for decrease
[c], evidence for increase based on Ct values

The exact role for CARTp actions in the periphery is not yet fully described. Circulating CARTp levels have, however, been associated to worsened outcomes and duration of disease [178–181]. It has been suggested that CARTp may be a useful biomarker for neuroendocrine tumors (NET). However, our works (and others [180]) provide evidence to the contrary, since the MCG101 tumor *per se* did not express *Cartpt* (Paper IV). Plasma CARTp as a host response is further supported by the fact that acute LPS inflammation resulted in similarly high CARTp concentrations in the absence of a tumor (Paper III). Progressive cancer disease typically features increased inflammation, which as such is also associated a worsened prognosis. In Papers III and IV, CARTp plasma levels were positively correlated with SAP levels. Thus, it is plausible that CARTp may be an indirect proxy measure for inflammatory response or physiological stress seen in progressive cancer disease.

The observation that hypothalamic *Cartpt* was suppressed in animals that also displayed increased plasma CARTp (Papers III-V), could be assumed to be indicative of an inhibitory feedback loop on central expression. However, *Cartpt* was suppressed in the sham-implanted, pair-fed group as well (Paper IV) despite having plasma CARTp concentrations that were significantly lower than the tumor-

bearing condition. This, together with observations that peripherally administered CARTp does not cause any behavioral or physiological effects typically observed after CNS application, would argue against such a peripheral-central CARTp feedback mechanism. Whether there is a functional interaction between CARTp and TSH within the pituitary is not clear and not addressed in this thesis. However, the possibility of such an interplay between TSH and CARTp is illustrated by the latter peptide's ability to affect pituitary hormone release [40, 171–173, 182–188]. Unlike CARTp, plasma TSH concentrations were not affected by the acute inflammation induced with LPS (Paper III). Chronic MCG101 exposure was associated with a doubling in plasma TSH (Papers IV and V)[127], as well as a robust increase in plasma CARTp concentrations. Our findings cannot exclude the possibility of CARTp-TSH interactions in tumor-bearing animals, but the results obtained from the chronic tumor condition may motivate further investigations.

4.3.2 Plasma CARTp correlates with degree of inflammation

In both the LPS and tumor paradigms, the animals were shown to have inflammatory responses, as indexed by their plasma SAP concentrations, and CARTp levels (Paper III and IV). We thus hypothesized that the CARTp response was prostanoid-mediated, given the fact that MCG101 tumors secretes prostaglandin E_2 resulting in high steady-state plasma concentrations [125, 189]. We also reported in these studies that CARTp and SAP had a positive correlation (Paper III, Fig. 3) suggesting that inflammatory processes may induce CARTp. In Paper III, we provided half of the mice with indomethacin as prophylaxis into the drinking water for three days prior to LPS injections. Indomethacin, an inhibitor of prostanoid synthesis, failed to block LPS-induced elevations in plasma CARTp, thus indicating a prostanoid-independent mechanism. We noted, but did not grade, in-cage behavior of LPS-injected mice, and we found that indomethacin reduced apparent sickness behavior. Mice bearing the prostaglandin E_2-producing MCG101 tumor also showed elevated CARTp and SAP, and the positive correlation between CARTp and SAP levels was evident (Paper IV, Fig. 5). Given the failure of indomethacin to inhibit plasma CARTp elevation in acute inflammation

(Paper III), it is less likely that the high plasma CARTp levels seen in MCG101-bearing animals (Paper IV) would be dependent on prostaglandin-E_2 release. MCG101 and LPS may well result in different inflammatory profiles. We, therefore, hesitate to speculate on specific factors that may induce plasma CARTp elevations, but it is reasonable to assume that other inflammatory mediators, such as TNFα or other interleukins, may be of importance.

4.3.3 PACAP6-38 as a possible CARTp antagonist in CACS

Given the effectiveness of PACAP6-38 to antagonize CARTp effects *in vivo* (Paper I; [86]) as well as *in vitro* [85] and our reports of elevated plasma CARTp in response to inflammation (Papers III & IV), we tested the hypothesis that peripheral PACAP6-38 treatments could improve the appetite and/or body composition of MCG101-bearing mice (Paper V).

Ad libitum feeding began to decrease shortly after tumors became palpable approximately 7 days after MCG101 implantation (Paper V, Fig. 1A). Half of the tumor-bearing and control mice were then given once-daily injections of PACAP6-38 i.p. over the last four days of the study, when anorexia had become evident. This treatment schedule was designed to resemble the clinical situation, where treatments to counteract weight loss begin when CACS is manifest. We found that daily PACAP6-38 treatments administered in Paper V appeared sufficient to attenuate the severity of fat mass loss. PACAP6-38 did not, however, show any effect on feeding, tumor burden, or lean tissue loss. Feeding and body composition were unaffected by PACAP6-38 in tumor-free control animals showing no intrinsic effect of the peptide. The adipose tissue-preserving effect of PACAP6-38 could be of potential clinical relevance for CACS treatment.

One concern when introducing new treatments to cancer patients is the risk for the treatment to enhance cancer cell proliferation. From this perspective, it is important to note that PACAP6-38 treatments have in fact been shown to reduce cell growth in prostate and breast cancers *in vitro* [190, 191] and *in vivo* [191]. Once-daily treatments of PACAP6-38—

beginning when tumors became palpable—reduced tumor burden in nude mice [191]. We did not observe any difference in tumor size in the MCG101 model, and we noted no obvious negative effects. The data so far would suggest that PACAP6-38 is a well-tolerated compound that does not seemingly accelerate tumor growth.

From this, we conclude that the preservation in adipose tissue seen in MCG101 animals treated with PACAP6-38 was not secondary to a reduction in tumor burden. Instead, we interpret our findings to mean that PACAP6-38 may have a local effect on adipose tissue. As reported in Papers IV and V, MCG101-bearing mice feature high concentrations of plasma CARTp. In addition, CARTp 55-102 enhanced isoprenaline-induced lipolysis in cultured rat adipocytes *in vitro* [71]. Moreover, clinical and experimental CACS are associated with elevated basal metabolic rate, likely due to elevated sympathetic outflow [192–194]. Taken together, this raises the possibility that PACAP6-38 acts to preserve adipose tissue in MCG101 mice by blocking CARTp-induced lipolysis.

While four PACAP6-38 treatments did reduce the severity of fat loss in tumor-bearing mice, there appeared to be no improvement in fat-free dried carcass weights. It is possible that lean tissue mass would have also benefited had the PACAP6-38 treatments began sooner or the study extended by a few days. Given the present study design, we cannot exclude the possibility that PACAP6-38 treatments were antagonizing endogenous PACAP signaling as well. However, putative CARTp antagonism is perhaps more likely given the high concentration of plasma CARTp and the fact that PACAP6-38 did not alter fat mass in sham-implanted mice (Paper V, Fig. 2C).

PACAP6-38 treatments attenuated the loss of fat mass in MCG101 mice with CACS and elevated plasma CARTp concentration (Paper V). This finding has two implications: first, it further supports that PACAP6-38 could be a competitive CARTp antagonist in the periphery as well as within the CNS; and second, it provides support for circulating CARTp to modulate lipolysis in CACS. As previously mentioned,

PACAP6-38 is not specific for a putative CARTp receptor structure; therefore, we cannot exclude that there may be other additional interactions in these paradigms.

5. SPECIFIC CONCLUSIONS

I. PACAP6-38 is an effective functional CARTp 55-102 antagonist on feeding effects *in vivo*.

II. TSH receptor proteins are functionally active and present—likely as holoreceptors—in the rat dorsal vagal complex and hypothalamus. Activation of these receptors with TSH in the NTS has an inhibitory effect on solid food intake.

III. Acute LPS-inflammation decreases hypothalamic *Cartpt* and *Tshr* mRNA, increases pituitary *Cartpt* mRNA, and elevates plasma CARTp levels. Plasma CARTp levels induced in inflammation are not dependent on cyclooxygenase-associated pro-inflammatory mediators.

IV. In CACS, *Cartpt* gene expression is altered. This phenomenon is secondary to reduced caloric intake rather than a causal, primary effect of the tumor. In contrast, *Nucb2* expression is increased in response to tumor, and could possibly be a causal factor in CACS. A peripheral manifestation of the tumor is an elevation of plasma CARTp which correlates to degree of inflammation.

V. Once-daily PACAP6-38 treatments conserve fat mass in MCG101-bearing mice that display increased plasma CARTp, possibly due to competitive antagonism of putative CARTp-sensitive receptors.

6. PERSPECTIVES

Cancer anorexia-cachexia syndrome continues to be a common and deadly comorbidity among cancer patients today [195]. This thesis aimed to investigate how some central mechanisms of feeding controls respond in models for acute inflammation and CACS. Knowledge of such mechanisms would be conducive to obtaining a causal cure for some or all parts of the syndrome. Our studies focused on genes coding for a few candidate anorexigens in the hypothalamus and the caudal brainstem: CARTp, TSH, TSHr, thyrostimulin, CRH, and nesfatin-1. A schematic summary of some of my main findings are presented as FIGURE 6 below.

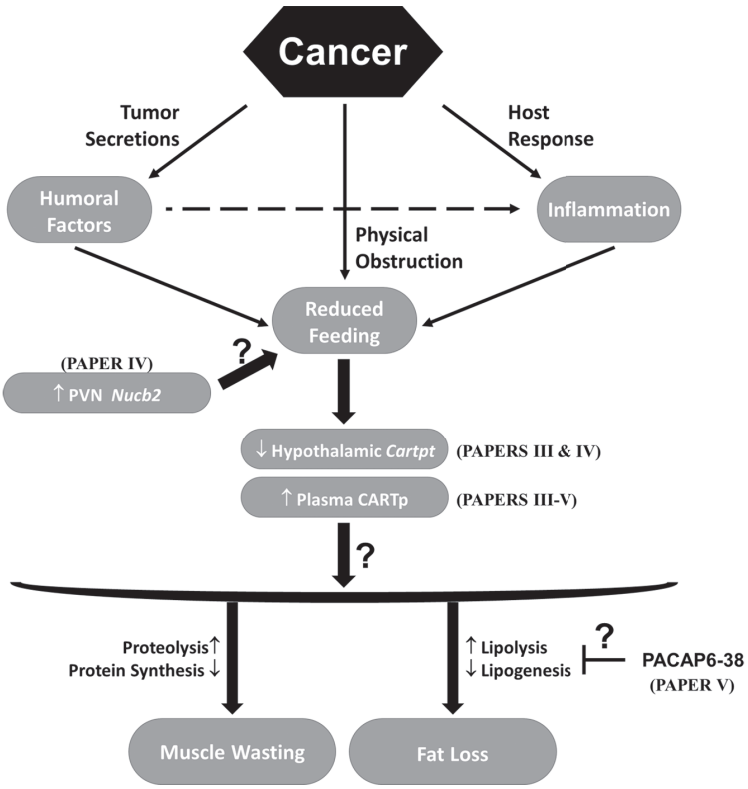

FIGURE 6 Schematic representation of how findings from this thesis may be involved in the cancer anorexia-cachexia syndrome.

While we initially hypothesized that hypothalamic CARTp may mediate cancer-anorexia, we found support for central *Cartpt* expression being adaptive—rather than causal—to the malnourished state. Circulating CARTp in the plasma, however, was associated with the degree of inflammation in both paradigms. CARTp has been hypothesized to be involved with glucose homeostasis and lipid metabolism [71, 72, 196], but the exact mechanisms of central or peripheral CARTp remain unknown. Our results also give support for PACAP6-38 as a tool to investigate endogenous CARTp mechanisms, and possibly as a future treatment option for CACS patients. Future studies in patients with CACS are needed in order to better understand the role of peripheral CARTp during disease. Conversely, knowledge of exact CARTp mechanisms may also be useful in developing a treatment for over-eating and obesity at the other end of the disease spectrum.

There has been evidence supporting neuronal TSH-TSHr signaling for many years [96–102]. We confirmed the presence of functional TSHr proteins in the adult rat brain, which are likely appearing as uncleaved holoreceptors. We also provided an anatomical map showing these receptor proteins are located in brain regions with relevance for feeding and autonomic controls (Paper II). We also provided further support showing that central *Tshr* expression in the hypothalamus is down-regulated in response to acute inflammatory challenge. We presently found no evidence to suggest that hindbrain *Tshr* expression is affected during either acute inflammation or MCG101-induced CACS. Tumor-bearing mice also showed no alterations in hypothalamic *Tshr* expression. The lack of changed gene expression for hypothalamic and brainstem TSHr mRNA in this CACS model does not exclude the TSHr from being involved in cancer-associated anorexia. However, our gene expression findings do suggest that brain-derived TSHr may not be influenced by peripheral thyroid hormone concentrations. TSHr perhaps are not part of the particular circuits affected by tumor, and thus are not functionally involved in CACS. Additionally, the alternative TSHr agonist, thyrostimulin, has only recently been discovered [107] and is acutely inducible predominately in

the pituitary [60, 197]. It is possible that another unknown agonist is interacting with the TSHr to maintain thyroid function in conditions of chronic disease, such as CACS.

Nucb2 expression in the PVN was significantly increased in tumor-bearing, but not pair-fed mice, which suggests a causal role for reduced feeding and weight loss in CACS. Our findings in Paper IV of alterations in gene expression for nesfatin-1 and its suggested contribution to cancer-anorexia warrant further investigations. To our knowledge only one study has investigated the association between circulating nesfatin-1 and weight loss in cancer patients [198]. This study among lung cancer patients reported reduced circulating nesfatin-1 among patients with weight loss compared to weight-stable cancer patients. The possible peripheral response of nesfatin-1 to experimental CACS, as well as other clinical cancers, requires further study.

In conclusion, our findings highlight the notion that the anorexia seen in acute inflammation and tumor-bearing mice is not a result of normal feeding controls favoring satiety. Instead, the role of specific factors for feeding controls should be individually explored in the diseased state. This conclusion is further supported by the fact that tumor-bearing, but not fasted, rats are unresponsive to CARTp [162]. Furthermore, in another rat study normal feedback mechanisms for POMC and the melanocortin receptor appeared intact in rats with cancer-anorexia [199]. Moving forward, research on central mechanisms of cancer-anorexia should not be approached from the classical "under-active feeding" and "over-active satiety" cues, but rather with particular attention on alternative pathways seen only in states of disease.

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