MCH AND OREXIN GENE EXPRESSION IN A MURINE MODEL OF CANCER ANOREXIA CACHEXIA SYNDROME

by © Caleb Christopher Morden

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Abstract

Cancer anorexia/cachexia syndrome (CACS) is defined as a severe loss of appetite and body weight that results in a significant increase in cancer-related mortality. CACS is thought to involve the hypothalamus, the brain region that controls energy homeostasis. Melanin-concentrating hormone (MCH) and orexin neurons of the lateral hypothalamus are involved in regulating appetite and body weight. However, their role in CACS is unclear. Here, the Lewis-Lung Carcinoma (LLC) mouse model was used to characterize CACS. Additionally, using quantitative RT-PCR we investigated the levels of MCH and orexin gene expression in the hypothalami of LLC tumour-bearing mice. We found that injection of $0.5x10^6$ LLC cells results in the most robust CACS with consistent anorexia and cachexia. In these mice, orexin mRNA expression was significantly reduced which correlated with food intake. Alternatively, in mice displaying cachexia only, MCH and orexin gene expression were positively correlated with body weight gain. Specifically, tumour-bearing mice with upregulated MCH or orexin mRNA expression did not lose weight, while those that failed to increase these factors lost weight. These results indicate that MCH and orexin may play a protective role against tumour-induced weight loss, while decreased orexin gene expression may contribute to anorexia in tumour-bearing mice. Overall, these findings support the idea that the MCH and orexin system may provide a potential therapeutic target for CACS.

General Summary

Cancer anorexia/cachexia syndrome (CACS) is the combination of a severe loss of appetite and body weight that can result in death in many cancer patients. Importantly, it is thought that the centers in the brain that are responsible for keeping a healthy body weight are disrupted during cancer. Specifically, melanin-concentrating hormone (MCH) and orexin (ORX) neurons, which are two cell types that are responsible for controlling food intake, metabolism and physical exercise, were of interest for their roles in CACS. To investigate this, we used mice with a rapidly growing tumour which produced both a reduction in food intake and body weight to accurately represent human CACS. In these mice, we saw changes in our cell groups that lined up with changes in both body weight and food intake. Overall, we show that MCH and ORX neurons are possible targets for future treatment against CACS, which could potentially increase cancer survivability.

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Co-Authorship Statement

I, Caleb Morden, designed the experiments with my supervisor Dr. Michiru Hirasawa. I performed all data analysis presented within this thesis. Dr. Maria Licursi completed the LLC cell preparations and qPCR analysis of brain samples. In addition, Dr. Licursi and members of Dr Kensuke Hirasawa's lab, in particular Dr. Vipin Shankar, assisted with training and performing s.c. injections for many of the mice cohorts that appear in this study. Lisa Fang and Nick Newhook assisted with animal sacrifice and tissue collection. Lastly, Haley Briggs, an honours student, started data collection on the LLC(low) cohort.

Dedicated to my amazing friend Ashley for her unending support and for sticking with me through this incredible journey. I would not have been able to do this without you and I will never forget what you have done for me.

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List of Abbreviations

5-HT: Serotonin

ACSF: Artificial cerebrospinal fluid

AgRP: Agouti-Related Peptide

AP: Area Postrema

ARC: Arcuate Nucleus

BAT: Brown Adipose Tissue

BBB: Blood-Brain Barrier

BMR: Basal Metabolic Rate

CACS: Cancer Anorexia Cachexia Syndrome

CART: Cocaine-Amphetamine Related Transcript

CCL2: Chemokine (C-C Motif) Ligand 2

CGRP: Calcitonin-Gene Related Peptide

CNS: Central Nervous System

CVO: Circumventricular Organ

DA: Dopamine

DIO: Diet Induced Obesity

IFN-γ: Interferon gamma

IL-1: Interleukin-1

IL-6: Interleukin-6

I.C.V. : Intracerebroventricular

I.P. : Intraperitoneal

I.V. : Intravascular

KO: Knock-out

LH: Lateral Hypothalamus

LLC: Lewis Lung Carcinoma

LPS: Lipopolysaccharide

MCH: Melanin Concentrating Hormone

MCHR1: Melanin Concentrating Hormone Receptor 1

MCHR2: Melanin Concentrating Hormone Receptor 2

MSH: Melanocyte-Stimulating Hormone

NEI: Neuropeptide Glutamine (E)-Isoleusine-(I)

NGE: Neuropeptide G-E

NPY: Neuropeptide Y

NTS: Nucleus Tractus Solitarus

ORX: Orexin

PBN: Parabrachial Nucleus

PBS: Phosphate Buffer Solution

POMC: Pre-Opiomelanocortin

PVN: Paraventricular Nucleus

REM: Rapid Eye Movement

RQ: Relative Quantification value

RT-qPCR: Quantitative Reverse Transcription Polymerase Chain Reaction

TNFα: Tumour Necrosis Factor alpha

VEH: Vehicle

WAT: White Adipose Tissue

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1. INTRODUCTION

1-1 General Overview

Cancer is the most prevalent disease worldwide. In Canada, it is estimated that approximately 50% of Canadians will develop cancer at some point in their life (Poirier et al., 2019). Currently, cancer is the leading cause of death in Canada and a major contributor of mortality and economic burden worldwide (Pilleron et al., 2019). Furthermore, the incidence of cancer is rapidly increasing, where it is expected to reach 24 million new cancer cases globally in 2035 (Pilleron et al., 2019). This is indicative of the increasing population of people aged 65 years or older and to a smaller degree, the increase in total population.

It is well known that cancer has a wide array of negative effects on the human body. It has been shown that pre-treatment patients can present with increased fatigue (Bower, 2017), reduction in sleep quality (Soucise et al., 2018), neuropathic pain (Davis, 2018), psychological distress (Bortolato et al., 2017; Cordova et al., 2017), and anorexia or cachexia (Laviano et al., 2017). These effects often present independently from treatment and are generally associated with an inflammatory response derived from the tumour environment (Gupta et al., 2018). Importantly, all cancer-induced symptoms contribute to a decrease in patients' quality of life. Furthermore, some symptoms such as cancer anorexia/cachexia syndrome (CACS) greatly increase patient mortality. Therefore, investigation into the mechanisms of CACS will provide valuable insights into patient survivability and quality of life during cancer.

1-2 Cancer Anorexia-Cachexia Syndrome (CACS)

Approximately 60 percent of all cancer patients present with a significant decrease in appetite referred to as cancer anorexia (Laviano et al., 2017). In addition, many patients show cachexia, characterized as a significant loss of adipose tissue and skeletal muscle mass, which is collectively termed cancer anorexia-cachexia syndrome (CACS). CACS becomes even more prevalent in late stage cancers where it is estimated to occur in up to 80% of patients (Bruera, 1992). CACS is associated with a reduction in overall physical functioning, tolerance to anticancer therapy and quality of life of cancer patients, and has been regarded as one of the leading causes of cancer-associated mortality (Argilés et al., 2010). However, it still suffers a lack of sufficient research attention.

Defining CACS in cancer patients has taken many years, and the definition has often changed. Many early researchers and clinicians separated CACS into the individual components of anorexia and cachexia. However, it has since been noted that CACS can be regarded as a continuum, where patients with apparent cachexia have a poorer prognosis than patients only displaying anorexia (Fearon et al., 2011). In 2011, a panel of experts classified CACS into 3 notable stages: precachexia, cachexia, and refractory cachexia (Fearon et al., 2011). Precachexia consists of early clinical signs such as anorexia and metabolic changes without a significant loss of body weight (<5%). The cachectic stage is when body weight loss is greater than 5% or with the presence of significant sarcopenia (skeletal muscle wasting). Lastly, in refractory cachexia, active catabolism occurs, and the use of typical weight-loss management is no longer effective.

This stage is usually associated with late stage or rapidly progressive cancers and has a life expectancy below 3 months.

Treatment for CACS is often targeted at the early stages and attempts to stimulate appetite or directly increase nutritional food intake (Mattox, 2017). Most nutritional supplementation has been shown to be ineffective at reducing weight loss, while other types of nutritional support (high in fat or through i.v. or directly into the gastrointestinal tract) has been associated with increased mortality (Laviano et al., 2003; Mattox, 2017). Additionally, broad appetite stimulants such as progestins, corticosteroids, and cannabinoids have been used with limited success at increasing patients' appetite (Jatoi et al., 2002; Mattox, 2017; Yennurajalingam & Bruera, 2014). More promising effects have been seen using the synthetic steroid megestrol, which has a significant impact on patients' appetite (Ruiz Garcia et al., 2013). However, as early CACS is associated with metabolic changes which are partially independent from anorexia, all current appetite stimulants have shown limited success in reducing CACS-related mortality. Therefore, it is important to increase our knowledge of the mechanisms of CACS to develop targeted therapies that will improve both appetite and reduce the metabolic changes in early and late stages of CACS.

1-3 CACS and Energy Homeostasis

When investigating CACS, an understanding of energy homeostasis circuitry and the effect of tumour load on these circuits is a necessity. Energy homeostasis is the coordinated regulation of body weight through the balance of energy intake (caloric intake) and energy expenditure (physical activity, metabolism). Briefly, energy homeostasis requires sensing of peripheral energy balance by the brain, and coordinating energy intake and expenditure through behavioural/metabolic changes (Waterson & Horvath, 2015). Firstly, peripheral signals fluctuate during periods of hunger and satiety. These signals are conveyed through direct neuronal communication from critical organs (i.e. stomach/liver), and through circulating nutrients (e.g. blood glucose) and appetite hormones produced by adipose tissue and the stomach (e.g. leptin/ghrelin). This information is coordinated and integrated in the major energy homeostasis centers in the brain. While not exclusive to these areas, two key brain regions are the brainstem and the hypothalamus, with connections between the two systems. Altogether, these areas project to a wide array of brain regions that assist in the balance of energy intake and expenditure to allow for the regulation of a healthy body weight (Waterson & Horvath, 2015). In the presence of a peripheral tumour, energy homeostasis can be disrupted at many levels (Figure 1).

Firstly, peripheral hormones fluctuate in different energy states. The satiety hormone leptin is produced by adipocytes at the level proportional to the degree of adiposity, where it inhibits hunger and increases nutrient release from adipocytes (Zhang et al., 2005). In the presence of elevated tumour necrosis factor-alpha (TNFα), which is

common during tumour growth, leptin levels are increased (Zumbach et al., 1997). In addition, in human cancer patients, leptin and its receptors are upregulated compared to healthy adults (Ishikawa et al., 2004), suggesting that the effect of leptin is enhanced in the presence of cancer, likely contributing to CACS. In contrast, the hunger hormone ghrelin is mainly produced by enteroendocrine cells of the stomach during the state of hunger and promotes appetite and caloric intake (Kojima & Kangawa, 2005). In the presence of a peripheral tumour, ghrelin insufficiency is reported (Fujitsuka et al., 2011). However, application of ghrelin in tumour-bearing animal models has shown limited success in increasing appetite, which may be due to cancer-induced ghrelin resistance (Wang et al., 2006). Furthermore, as ghrelin has a role in the production of growth hormone, it is likely that tumour growth will be enhanced by ghrelin (Northrup et al., 2013), which indicates that ghrelin has critical confounding effects on tumour growth and is not an ideal therapeutic candidate for CACS. Altogether, these studies together suggest that leptin and ghrelin are involved in CACS.

Secondly, nutrient and stretch receptors in the stomach send signals about gastric capacity/contents through the vagus nerve to the nucleus tractus solitarus (NTS) (Chen et al., 2020). The NTS projects to other brainstem areas such as the area postrema (AP) (Shapiro & Miselis, 1985) and the parabrachial nucleus (PBN) (Roman et al., 2016), which both affect acute feeding behaviours. Moreover, it is known that these brainstem nuclei contribute to CACS (Borner et al., 2018). For example, calcitonin-gene related peptide (CGRP) neurons in the PBN are known for triggering anorexia when exposed to noxious stimuli. In the presence of a peripheral tumour, CGRP neurons become activated

(Campos et al., 2017). In fact, anorexia and lethargy can be abolished using chemogenetic inactivation of these neurons (Campos et al., 2017). Furthermore, brainstem-derived monoamines such as serotonin (5-HT) and dopamine (DA) can potently decrease food intake and increase food seeking behaviours, respectively (Galen et al., 2021; Roitman et al., 2004). The presence of a tumour increases 5-HT and decreases DA production, which is thought to contribute to CACS (Meguid et al., 2004). Additionally, the production of 5-HT and DA are returned to sham-treated control levels upon tumour removal, suggesting that these changes are contingent on the presence of the tumour itself (Meguid et al., 2004). Thus, the brainstem plays critical roles in CACS.

Finally, the hypothalamus is also essential for the energy homeostasis mechanism. The arcuate nucleus (ARC) of the hypothalamus is the main detector for peripheral appetite signals in the central energy balance circuitry. This nucleus contains orexigenic neurons expressing agouti-related peptide (AgRP)/neuropeptide Y (NPY) and anorexigenic neurons expressing pre-opiomelanocortin (POMC)/cocaine-amphetamine related transcript (CART). During cancer, NPY gene expression in the ARC is significantly increased; presumably a homeostatic response to nutrient deficiency (Chance et al., 1998; McCarthy et al., 1993). Additionally, application of NPY would be detrimental during cancer, as tumour cells express the NPY receptor, which promotes tumour cell growth and proliferation (Körner & Reubi, 2007; Tilan & Kitlinska, 2016). On the other hand, POMC-derived neuropeptides, the melanocyte-stimulating hormones (MSH), are over-expressed during some cancers and genetic knockdown of melanocortin receptors markedly decreases the anorectic phenotype (Marks et al., 2003; Wisse et al.,

2001). However, it has been noted that the POMC neuron activity does not change in all cancer anorexia models tested (Hitoshi Suzuki et al., 2011). Thus, while AgRP and POMC neurons may be involved in CACS, the underlying mechanisms remain obscure.

Additionally, there is a plethora of research on hypothalamic energy homeostasis mechanisms in the context of diet-induced obesity. Interestingly, many of the alterations associated with CACS listed above occur inversely during states of obesity. Therefore, there is much to be gathered from investigating obesity related changes of the energy homeostasis systems and relating this to changes during CACS. While this topic is intriguing enough to mention, it is futile to attempt to explain all of the intricacies of diet induced obesity within this work.

The ARC neuronal populations project to various effector regions that collectively regulate energy intake and expenditure. These regions include the ARC itself, the paraventricular nucleus (PVN), and the lateral hypothalamus (LH) (Elias et al., 1998). The LH is a critical effector region for energy homeostasis. It has been shown that the cells of the LH can detect peripheral signals as well as integrate neuronal inputs from critical motivation and cognition based centers of the brain (Petrovich, 2018). Furthermore, the LH has widespread projections to areas that are critical for behavioural and internal energy state changes. These areas include, but are not limited to, the ARC (Luan et al., 2017), the nucleus accumbens (Maldonado-lrizarry et al., 1995; Stratford & Kelley, 1999), the ventral tegmental area (Barbano et al., 2016), and the PVN (Wu et al., 2015). Reviewed by Bonnavion et al. in 2016, the LH contains diverse cell types which are functionally and genetically distinct. Two major cell types of interest are those

containing melanin-concentrating hormone (MCH) and orexin/hypocretin (ORX), which both play a role in the control of food intake and body weight. Due to their role in energy balance and their reciprocal connections with the brain stem and the ARC, the LH is likely to be involved in CACS. However, very little is known about the LH in the presence of a peripheral tumour.

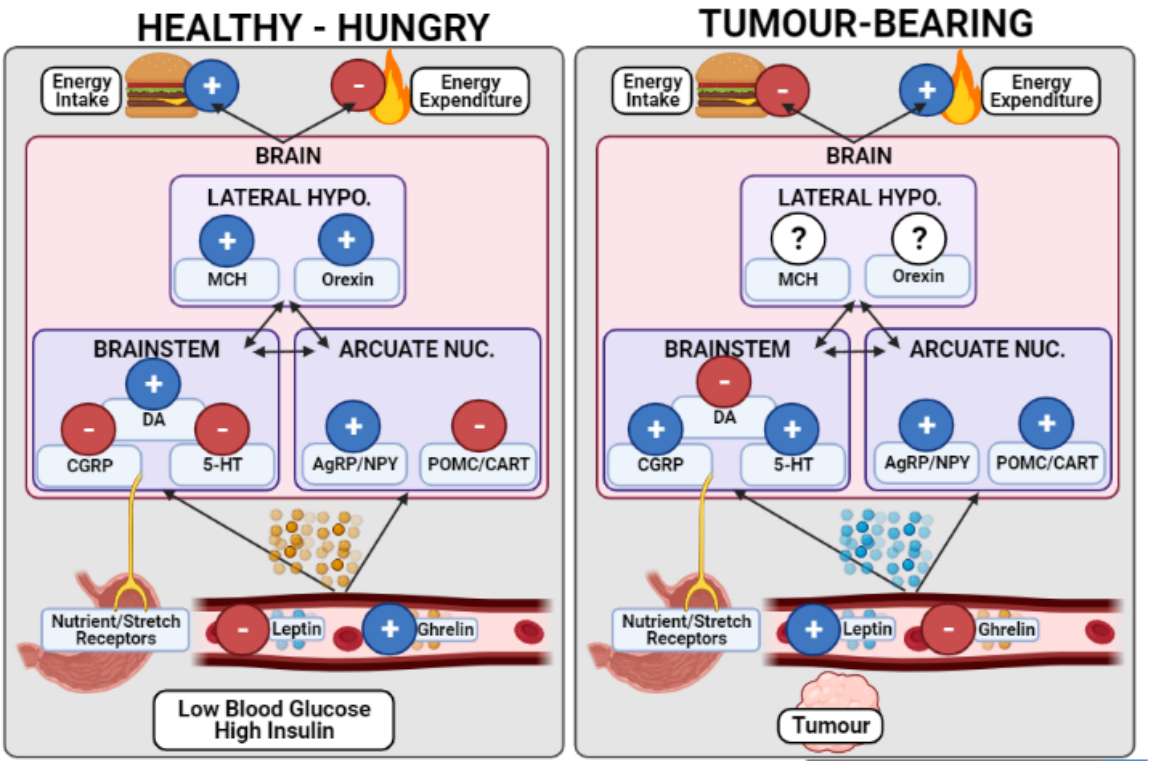

Figure 1. Comparison of critical energy homeostasis mechanisms during a state of hunger and in the presence of a peripheral tumour.

(LEFT) Changes in peripheral and central appetite-related hormones during fasting in order to promote food intake. **(RIGHT)** Changes in appetite-related hormones in the presence of a peripheral tumour and the implications in inducing anorexia and weight loss. **+/-** indicates the directional change in neurotransmitter expression, **?** indicates an unknown change.

CGRP= Calcitonin gene-related peptide; 5-HT= Serotonin; DA= Dopamine; AgRP= Agouti-related peptide; NPY= Neuropeptide-Y; POMC= pro-opiomelanocortin; CART= Cocaine and amphetamine regulated transcript; MCH= Melanin-concentrating hormone.

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1-4 Melanin-Concentrating Hormone (MCH) Neurons

Melanin-concentrating hormone is a 19-amino acid, cyclic neuropeptide that was originally identified in the skin of teleost fish (Kawauchi et al., 1981). It has since been identified in neurons of the LH in humans and rodents (Bittencourt et al., 1992). MCH neurons primarily express the MCH peptide, but often co-express other neuropeptides or transmitters such as CART, neuropeptide glutamine-isoleucine (NEI), neuropeptide G-E (NGE) or glutamate (Schneeberger et al., 2018). Generally, MCH neurons are regarded as regulators of energy balance, however they have additional roles in promoting sleep (Konadhode et al., 2013), memory (Izawa et al., 2019; Monzon et al., 1999), and depression (García-Fuster et al., 2012).

Importantly, MCH neurons have widespread inputs and projections to areas that play key roles in maintaining energy homeostasis (Bittencourt et al., 1992). Firstly, MCH neurons receive innervations from AgRP/NPY and POMC neurons of the arcuate nucleus (Elias et al., 1998). This was thought to be a critical link by which the neurons of the lateral hypothalamus detect peripheral energy balance. However, MCH neurons have since been shown to have the capacity to respond to peripheral signals directly. For example, MCH neurons express the insulin receptor, and insulin directly increases the activity of these neurons (Hausen et al., 2016). Additionally, MCH neurons respond directly to glucose (Kong et al., 2010). Moreover, leptin influences MCH function, despite the lack of leptin receptors on MCH neurons (Kokkotou et al., 2001; Leinninger et al., 2009). Furthermore, the role of MCH neurons in energy homeostasis is exemplified

by their projections, where MCH neurons project to critical appetite-influencing brain regions, including the ARC and the NTS (Kokkotou et al., 2001).

The MCH peptide promotes positive energy balance. In rodents, when the peptide is administered centrally, food intake increases (Rossi et al., 1997). Additionally, when MCH is overexpressed in neurons, rodents present with increased weight gain that leads to obesity (Ludwig et al., 2001). MCH acts on two G-protein coupled receptors; MCHR1 which is found in humans and rodents and MCHR2 which is exclusive to humans (Qu et al., 1996). When MCHR1 is knocked-out (KO) it produces a phenotype of leanness, hyperactivity, and altered metabolism (Chen et al., 2002). This effect is similar to MCH-KO models where animals become lean and are resistant to diet-induced-obesity (DIO) (Whiddon & Palmiter, 2013). Chemogenetic activation of MCH neurons increases lethargy and the initiation of REM sleep (Hausen et al., 2016). In terms of energy homeostasis, MCH neuron activation is causing an overall reduction in energy expenditure that can also contribute to body weight gain. Generally, activity of MCH neurons results in overall body weight gain. In summary, due to the role of MCH neurons in food intake and body weight gain, it is possible that MCH neurons are involved in CACS. However, not much is known about MCH neurons during cancer.

1-5 Orexin Neurons

Orexin-A and B, collectively termed orexins or hypocretins, are neuropeptides originally discovered within the LH of rats. Orexin-A is a highly conserved 33-amino acid neuropeptide, while orexin-B is a 28-amino acid neuropeptide with variants found between species (Sakurai et al., 1999). Both neuropeptides are formed from a single precursor polypeptide, prepro-orexin. Orexin neurons play a crucial role in the regulation of energy homeostasis, sleep-wake cycle, emotion, stress, and reward (Berridge et al., 2010; Harris et al., 2005; Kiyashchenko et al., 2002; Nollet & Leman, 2013).

Orexin neurons have many inputs related to energy homeostasis. Orexin neurons receive inputs from the ARC, where NPY and POMC neurons inhibit orexin neuron activity (Fu et al., 2004; M. López et al., 2007). Moreover, brainstem derived factors such as 5-HT and DA also modulate orexin activity (Yamanaka et al., 2003). In addition, orexin neurons are sensitive to circulating levels of leptin and glucose (Cai et al., 1999; López et al., 2000). More specifically, it has been demonstrated that orexin neurons are responsible for glucosensing through detection of the metabolic byproduct, lactate (Parsons & Hirasawa, 2010). Furthermore, orexin neurons have widespread projections to appetite-related brain regions, including strong projections to the ARC nucleus, the ventral tegmental area, and the nucleus accumbens (Baimel et al., 2017; Marcus & Elmquist, 2006). Moreover, orexin neurons play a crucial role in the regulation of other lateral hypothalamic neurons (Burt et al., 2011). Overall, orexin neurons have inputs and outputs that allow them to be a critical integrator of the energy homeostasis pathway.

When orexin neurons are ablated, animals become obese even with a reduction in overall food intake (Hara et al., 2001). This finding highlights the dual role of orexin neurons in energy homeostasis. Firstly, orexin neurons promote energy expenditure. Orexin neurons project to brainstem wakefulness promoting regions (i.e. locus coeruleus), and cause hyperactivity in animals when stimulated (Hagan et al., 1999; Horvath et al., 1999). Furthermore, orexin neurons promote energy expenditure through the stimulation of metabolism. Orexin neurons are required for proper thermogenesis in rodents (Madden et al., 2012), are thermosensitive (Parsons et al., 2012), and regulate glucose metabolism in muscle tissue (Shiuchi et al., 2009). On the other hand, injection of orexin-A during the dark phase induces food intake (Yamanaka et al., 1999). Moreover, orexin neurons become more active during periods of fasting (Burdakov & Alexopoulos, 2005). While these findings argue that orexin neurons stimulate appetite, it is thought that this is through increases in food-seeking behaviour that is a result of increased activity. In fact, when orexin function is lost, the latency of a rodent to approach food is decreased (Hagar et al., 2017). Overall, this indicates that orexin neurons may promote food seeking, however their role in increasing physical activity and metabolism will typically overshadow this effect.

Orexin neurons have been investigated in some tumour-bearing models. In a sarcoma tumour-bearing model, orexin cell numbers in the perifornical area of the hypothalamus are decreased, which correlates with a decrease in locomotor activity (Grossberg et al., 2011). In addition, an increase in orexin neuron activity was shown to underly a disruption in the sleep-wake cycle in a breast cancer tumour-bearing model

(Borniger et al., 2019). These studies suggest that orexin neurons are affected in the presence of a peripheral tumour. In summary, orexin neurons play a role in the regulation of appetite and metabolism and may be involved in CACS. However, orexin expression during cancer in relation to food intake has not been investigated.

1-6 Rationale and Hypothesis

Peripheral tumours cause anorexia and weight loss in humans, which is critically tied to patient mortality. As the brain plays a major role in regulating body weight, it is important to understand the mechanisms behind the changes in the brain underlying CACS. Tumours are known to influence neuronal activity, specifically in some brainstem and hypothalamic regions responsible for the control of energy balance. However, little is known about the changes that occur in the LH, which contains MCH and orexin neurons that comprise the central energy balance circuitry. These peptidergic systems may be involved in CACS, which remains to be investigated. The above information led to the following hypothesis, visually presented in *Figure 2:*

The presence of a tumour alters MCH and Orexin expression, which contributes to cancer anorexia cachexia syndrome.

To address this hypothesis, a tumour-bearing mouse model was used to test the effect of a tumour on genes expressed in the LH. This work will further our understanding of the impacts of cancer on the energy balance circuitry in the brain. This may ultimately lead to identifying potential therapeutic targets for CACS which may improve patient quality of life and reduce overall cancer-related mortality.

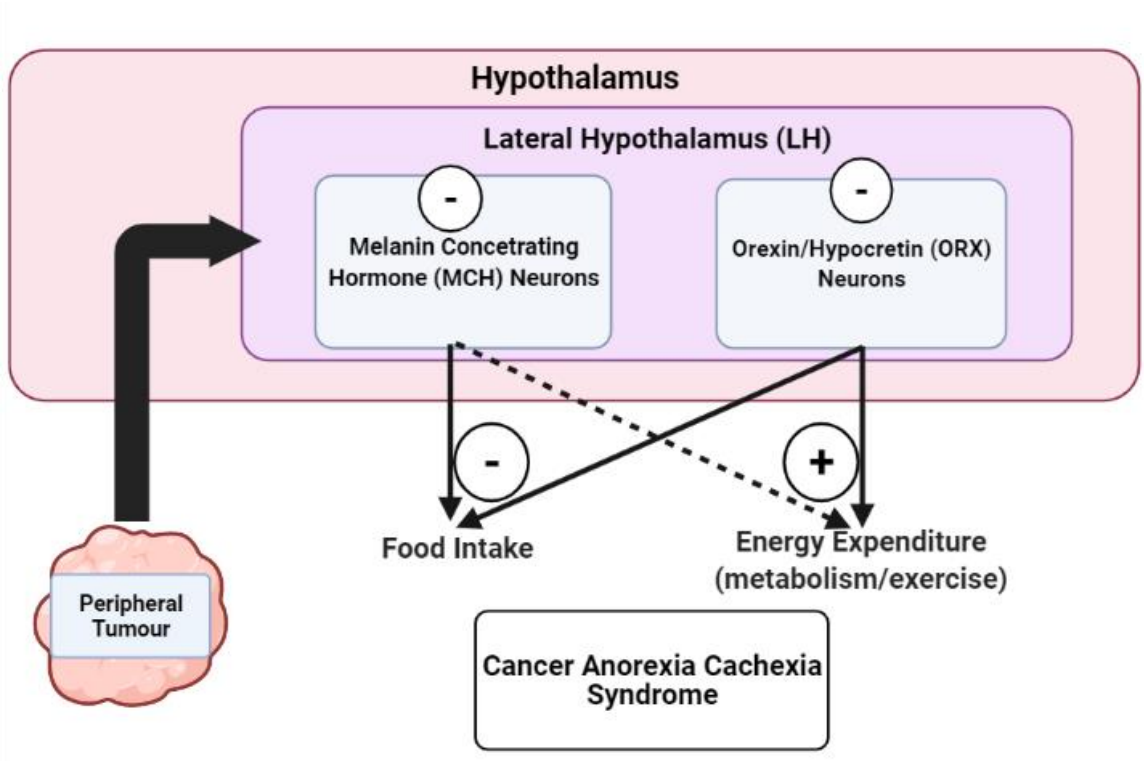

Figure 2. Hypothesis: Cancer anorexia cachexia and the lateral hypothalamus

This hypothesis connects the lateral hypothalamus to CACS. Unknown variables are highlighted using circles, where + indicates a predicted elevation of that factor and – indicates a predicted reduction in that factor. Solid and dashed black arrows indicate an increase or decrease in food intake/energy expenditure, respectively.

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2. METHODS

2-1 Animals

All procedures were conducted in accordance with the Canadian Council on Animal Care and were approved by the Memorial University Institutional Animal Care Committee. Male mice of two age groups were used in this study. *Young mice:* 6-week-old, male C57BL/6 mice, were obtained from Charles River Canada (St. Constant, QC, Canada). *Aged mice:* 10-20 month-old male mice with C57BL/6 background from in-house breeding colony were used.

All mice were held in the animal care facility in the Memorial University, Health Science Centre where they were single housed during all experimental procedures under a 12h light/12h dark cycle (lights on at 08:00 AM). Mice had access to standard rodent chow (Teklad 2018 rodent diet, Envigo) and water *ad libitum*. Body weight and food intake were measured at 12:00 p.m. (Monday, Wednesday, and Friday) for 1-2 weeks during a pre-treatment period (baseline) and following treatment until sacrifice. Food intake was calculated using the difference in pellet weight between measurement periods. In addition, the floor of the cage was investigated for any spillover for the accurate measurement of food intake. Furthermore, the criteria for anorexia were defined based on food intake changes seen in vehicle control mice. More specifically, 95% of young control mice (N=20/21) were found to have food intake above 90% of baseline in the final week before sacrifice. This indicates that a 10% fluctuation in food intake falls within the normal range, and anything beyond this (<90% of baseline) would be considered a loss of appetite or anorexia.

2-2 Lewis Lung Carcinoma (LLC) Inoculation

Mouse Lewis lung carcinoma (LLC) cells used in this study were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained with high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Ontario, Canada), supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic mixture (Invitrogen; 100 units/mL penicillin G sodium). Nearing the day of injection, cells were prepared in 10-cm culture dishes. Cells were then harvested, washed, and counted. Furthermore, the cell number was adjusted to $1x10^6$ cells or $0.5x10^6$ cells in 100 μL of phosphate buffer solution (PBS).

After a 1-2 week pre-treatment baseline period, mice were shaved on the hind left flank to expose the skin. Mice were separated into groups based on body weight and age, so that these parameters were similar among the groups upon injection. The young groups were 8 weeks of age at the time of injections, whereas the aged mice groups had the following averages (Mean +/- SEM: Old LLC(high) = 11.9 +/- 0.68 months, Old VEH(high) = 12.1 +/- 0.78 months, LLC(low) = 13.6 +/- 1.12 months, VEH(low) = 16.0 +/- 1.53 months). Groups were randomly assigned to one of three injection solutions: **(1)** Vehicle (PBS 100 μL), **(2)** LLC High ($1x10^6$ LLC cells/100 μL PBS), or **(3)** LLC Low ($0.5x10^6$ LLC cells/100 μL PBS). The following day, LLC cells and control vehicle (PBS) were prepared and brought to the animal care facility on ice for immediate use. Subcutaneous injections occurred at 12:00 p.m. by lifting the skin with fingers or forceps, inserting a sterile 26-gauge needle, and slowly injecting 100 μL of solution until a bolus below the skin was formed.

Mice were excluded from the study if infection or premature open wounds developed around the injection site, or LLC injection did not result in a subcutaneous solid tumour. During the post-injection period, body weight and food intake continued to be measured Mondays, Wednesdays, and Fridays.

Entire cohorts were sacrificed once the criteria for euthanasia was met for multiple tumour-bearing mice. Specifically, the criteria for euthanasia were defined as the tumour reaching a critical diameter of 17 mm or the presence of open wounds persisting for more than 2 days. If a single mouse displayed prolonged open wounds in the absence of a large tumour, they were removed from analysis without sacrificing the entire cohort. Additionally, all vehicle control mice were sacrificed at time points matching LLC tumour-bearing mice. Mice were deeply anaesthetized with isoflurane, decapitated and tissue samples were extracted and weighed immediately. Tissues collected included the hypothalamus, brown adipose tissue (BAT), white adipose tissue (WAT; retroperitoneal, epididymal, and inguinal fat pads), lean muscle (gastrocnemius and soleus muscle) and tumour. Additionally, body weight change was calculated by subtracting the tumour mass from the total body weight gained at sacrifice (represented as Body Weight Change (g)). Moreover, to assess the change in food intake, the average food intake of the final week (over 3 time points) was normalized to the average of baseline food intake for each mouse (represented as Food Intake (%Baseline)).

2-3 Reverse Transcriptase Quantitative PCR

During tissue sampling, mouse brains were quickly removed and coronal sections (1.25 mm thick) of the hypothalamus were collected. Brains were cut using a vibratome while immersed in ice cold artificial cerebrospinal fluid (ACSF) containing: 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 18 mM $NaHCO_3$, 2.5 mM glucose, 2 mM $CaCl_2$. ACSF was continuously bubbled with 95% O_2 and 5% CO_2. Next, coronal sections were trimmed under a stereoscope to isolate the hypothalami, which were placed in RNAse/DNAse free tubes and stored at -80°C until further use.

RNA was isolated from brain samples using TRIzol (Invitrogen) following manufacturer's instructions. 0.5 μg of RNA was reverse transcribed to cDNA from random hexamers using a first-strand cDNA synthesis kit (Amersham Biosciences). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed in triplicate on StepOnePlus (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Cycling conditions consisted of 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, followed by melt-curve analysis. The absence of nonspecific amplification was confirmed by observing a single peak in the melt-curve and the absence of amplification in the no-template control wells. Primers used for this experiment were: *Pro-Melanin-concentrating hormone* forward primer (*qmusPMCH F1*; GCAGAAAGATCCGTTGTCGC), *Pro-Melanin-concentrating hormone* reverse primer (*qmusPMCH R1*; GTTTGGAGCCTGTGTTCTTTGA), *Orexin* forward primer (*qmusOREX F3*; GGCCTCAGACTTCTTGGGTATT), *Orexin* reverse primer (*qmusOREX R3*; CAGGGAACCTTTGTAGAAGGAAAGT), *GAPDH* forward

primer (*mus GAPDH F Q*; ATGTGTCCGTCGTGGATCTGA), and *GAPDH* reverse primer (*mus GAPDH R Q*; TGCCTGCTTCACCACCTTCTT) (Integrated DNA Technology). GAPDH was used as a housekeeping gene, which all cycle threshold (CT) values were normalized against to control for variations in tissue size. Lastly, all qPCR data are represented as a relative quantification value (RQ value), where MCH/orexin mRNA levels of individual mice are compared against the average of time-matched controls.

2-5 Data Analysis

Prism 8 was used for statistical analysis (GraphPad Software, La Jolla, CA). Outliers were identified for each variable and removed if values were larger than the mean +/- 2SD of each experimental group. Note, outliers in body weight or food intake were removed from all analysis, as they were not displaying signs of CACS (YOUNG/HIGH = 3; YOUNG/LOW = 2; OLD/HIGH = 1; OLD/LOW = 0). Outliers in qPCR results were excluded from the gene expression analysis but included in characterizing the body weight/food intake of the LLC-model (qPCR outliers: YOUNG/HIGH = 0; YOUNG/LOW = 2; OLD/HIGH = 1 MCH only, 1 MCH&ORX; OLD/LOW = 1). Comparison between groups utilized two-tailed unpaired t tests or two-way ANOVA as appropriate. Linear regression analysis was performed to assess correlation. The data is represented as mean +/- SEM. For all analysis, P values <0.05 were considered statistically significant. The N-value indicates the number of mice used in each experiment.

3-0 RESULTS

3-1 The LLC Tumour-Bearing Mouse Model Displays Body Weight Change Without Significant Anorexia

Characterization of the LLC tumour-bearing mouse model was required to begin the investigation into the effect of tumour burden on energy balance-related neuropeptides. First, after a 1–2-week baseline, 8-week-old mice were injected subcutaneously with a high number of LLC cells ($1x10^6$ cells; Figure 3), as this is the most commonly used tumour load in the current literature. As seen in *Figure 3A*, vehicle and tumour-bearing mice continued to increase in body weight after the LLC-cell injection. This was expected as mice continue to gain significant weight during young adulthood. Many mice reached the humane endpoint at 14-days post injection, when all animals were sacrificed collectively. Upon sacrifice, tumour-bearing mice gained significantly less body weight than their vehicle counterparts when tumour weight was subtracted from final body weight (VEH n = 13 vs. LLC n = 13, unpaired t-test: $t(24) = 2.14$, $p = 0.0427$, *Figure 3B*).

Interestingly, food intake during the final week post-injection did not differ significantly from the baseline in both LLC group and vehicle controls (VEH n = 13 vs. LLC n = 13, unpaired t-test: $t(24) = 1.301$, $p = 0.2055$, *Figure 3C*). This indicates that overall, LLC-tumour bearing mice were not displaying an anorexic phenotype in our hands. Nonetheless, a greater proportion of tumour-bearing mice displayed anorexia compared to controls, where anorexia is defined as <90% baseline food intake (Fischer's

exact test: p = 0.0302, *Figure 3D)*. Furthermore, body weight changes in LLC tumour-bearing mice were positively correlated to changes in food intake, while vehicle mice did not correlate in these factors (standard linear regression; VEH: p = 0.5128, R^2 = 0.0399, n = 13; *Figure 3E;* LLC: p = 0.0111, R^2 = 0.4581, n = 13; *Figure 3F*). These results suggest that anorexia may contribute to reduced weight gain due to tumour load.

Some tissue weights were also measured to identify if cachexia was present in this model. Tumour-bearing mice had significantly less white adipose tissue (WAT) (VEH n = 13 vs. LLC n = 13, unpaired t-test: t(24) = 2.084, p = 0.0480, *Figure 3G*) and brown adipose tissue mass (BAT) (VEH n = 13 vs. LLC n = 13, unpaired t-test: t(24) = 2.481, p = 0.0205, *Figure 3H*), which may be indicative of alterations in thermogenesis and metabolism. However, lean mass (gastrocnemius & soleus muscles) in tumour-bearing mice were not significantly altered compared to vehicle controls (VEH n = 13 vs. LLC n = 13, unpaired t-test: t(24) = 1.417, p = 0.1692, *Figure 3I*).

Together, these results indicate that in our hands, the LLC tumour-bearing model displays heterogeneous response to tumour load, where significant anorexia is accompanied by a significant reduction in body weight gain and fat accrual in some mice.

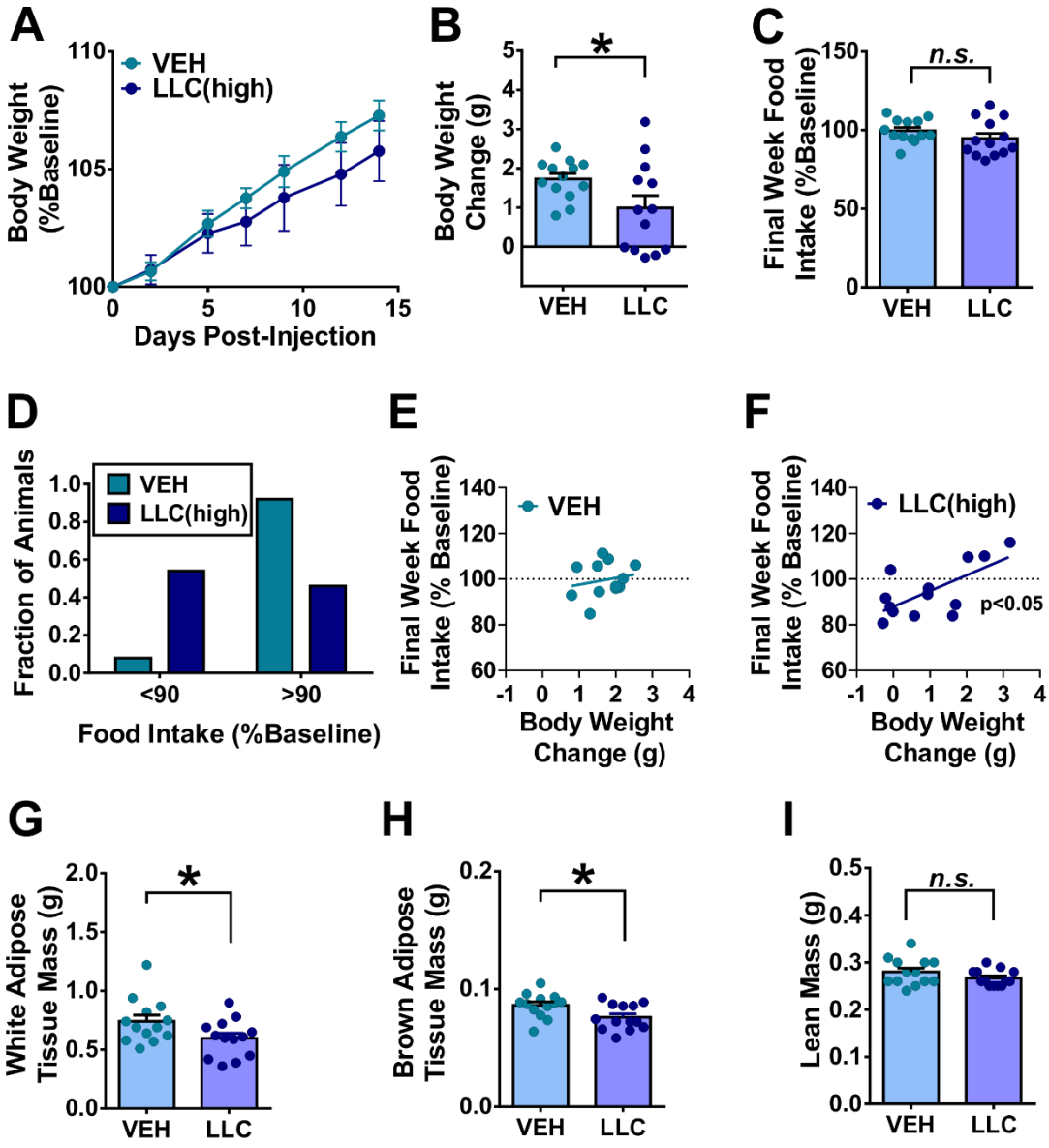


Figure 3. Characteristics of LLC(high) tumour-bearing mice

(A) Body weight during the post injection period; vehicle (VEH) and high number LLC-injected mice (1x10^{6}cells; LLC(high)). **(B)** Change in body weight post injection corrected for the tumour weight. **(C)** Food intake during the final week prior to sacrifice, normalized to the average baseline food intake. **(D)** Frequency of animals that display anorexia (<90% baseline) and normal food intake (>90% baseline) **(E&F)** Correlation between the change in total body weight and food intake during the final week **(G-I)** Tissue weight upon sacrifice as indicated. *p<0.05, n.s. not significant.

3-2 LLC Tumour-Bearing Mice Have Varied MCH/ORX Gene Expression Levels that Correlate with Body Weight Change

MCH and orexin (ORX) neurons play a role in regulating food intake, metabolism, and physical activity and may be involved in the reduction in body weight under tumour-bearing conditions. As stated above (Section 3-1), injections of high number of LLC cells resulted in a reduction of body weight gain with variable presence of anorexia. Therefore, it was hypothesized that MCH and ORX gene expression may be dysregulated in these mice.

We found that LLC tumour-bearing mice displayed a trend towards an increase in the expression of MCH and ORX genes compared to controls, although it did not reach statistical significance (MCH: VEH n = 13 vs. LLC n = 10, unpaired t-test: $t(21) = 1.762$, $p = 0.0926$; *Figure 4A*; ORX:VEH n = 11 vs. LLC n = 10, unpaired t-test: $t(19) = 2.007$, $p = 0.0592$; *Figure4E*). It was noted that the variation in gene expression was larger within the LLC tumour-bearing group compared to controls (F-test; MCH: $p=0.0013$; ORX: $p=0.0262$; *Figure 4A&E*). While some LLC tumour-bearing mice expressed MCH/ORX mRNA levels similar to vehicle controls, some showed higher expression up to 2-3 times that of vehicle controls.

Further analysis was used to determine if the variation in MCH/ORX gene expression was correlated to any aspects of the phenotype (*Figure 4B-D & F-H*). In vehicle control mice, no correlations were seen between MCH/ORX mRNA and changes in body weight (*Figure 4B&F*) or food intake (*Figure 4C&G*). In contrast, in LLC-tumour bearing mice, a significant positive correlation was seen between body weight

change during post-injection period and MCH (*Figure 4B)* or ORX (*Figure 4F*) mRNA expression. Interestingly, the expression of these genes did not correlate with food intake during the final week post-injection (*Figure 4C&G*). Furthermore, the relationship between tumour mass and gene expression was investigated. This was important as all mice within a cohort were sacrificed once some mice reached their endpoint, meaning that some mice had much smaller tumours upon sacrifice. However, final tumour mass did not correlate with MCH or ORX mRNA expression (*Figure 4D&H*), suggesting that the size of the tumour was not a factor in differential gene expression. Overall, these results suggest that LLC mice with a higher MCH/ORX gene expression maintained normal body weight gain while those without gene upregulation presented with smaller weight gain or weight loss.

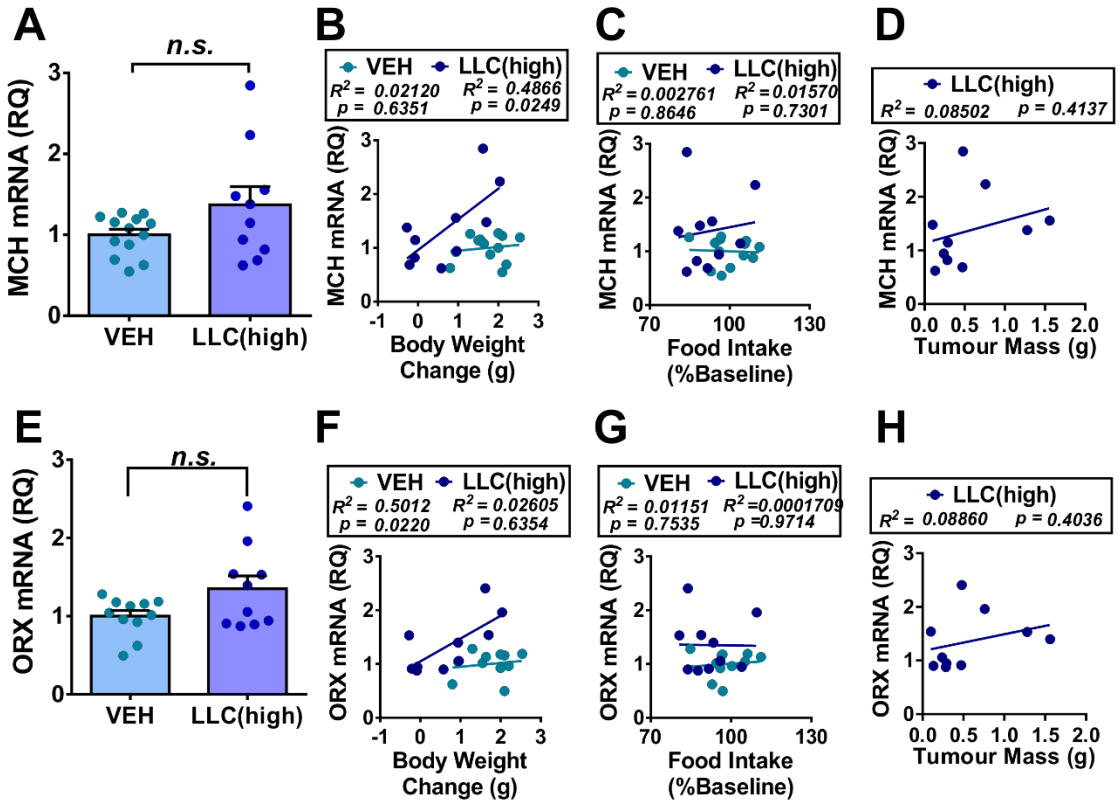


Figure 4. MCH and orexin gene expression correlates with body weight change in LLC(high) tumour-bearing mice

(A&E) MCH (A) and orexin (ORX; E) mRNA expression in the hypothalamus of mice injected with vehicle or LLC cells ($1x10^6$cells). **(B-D & F-H)** Correlation analysis between MCH/Orexin gene expression and body weight change **(B & F),** food intake during the final week post-injection **(C & G)**, and final tumour mass upon sacrifice **(D & H)**.

3-3 Effect of Injecting Low Number of LLC Cells on Body Weight and Food Intake

When investigating cancer anorexia-cachexia syndrome, it is ideal for robust, consistent anorexia to occur in the animal model. As seen previously (Section 3-1), our LLC(high) model was inconsistent, as some animals displayed anorexia while many did not. Overall as a group, the LLC-tumour bearing group did not significantly differ from the vehicle controls in food intake. This could be because many of our mice reached the endpoint within approximately 2 weeks, which may be too short to display an anorexic phenotype (Bennani-Baiti & Walsh, 2011). Therefore, we aimed to develop a model where the tumour grows slowly and prolongs the post-injection period.

To do this, 8 week old mice were injected with half the cell number ($0.5x10^6$ cells, termed LLC(low)). This strategy prolonged the time to reach the humane endpoint with some variation (20 days; n= 5, 22 days; n=2, 25 days; n=1, 30 days; n=2). Time-matched controls were always sacrificed for tissue sampling at the same point as LLC tumour-bearing mice for comparison. In both vehicle and LLC(low) mice, body weight continued to increase throughout the post injection period (*Figure 5A*). However, subtracting tumour weight from final body weight revealed that LLC(low) tumour-bearing gained significantly less body weight compared to vehicle controls (VEH n = 8 vs. LLC n = 10, unpaired t-test: $t(16) = 5.646$, $p < 0.0001$, *Figure 5B*). In fact, many mice did not gain weight or lost weight during the post-injection period. Furthermore, LLC(low) tumour-bearing mice displayed significantly less food intake during the final week post-injection compared to control mice (VEH n = 8 vs. LLC n = 10, unpaired t-test: $t(16) = 3.042$, $p = 0.0078$, *Figure 5C*). It is apparent that a few mice within the vehicle injected group had

significantly elevated food intake compared to baseline, which further differentiated this group from the LLC(low) mice. This may be due to their young age, where body weight and food intake are expected to increase as the mice grow. Moreover, a significantly higher proportion of LLC(low) tumour-bearing mice displayed anorexia than controls (Fischer's exact test; $p = 0.0359$, *Figure5D*). However, food intake during the last week post-injection did not correlate to body weight change (standard linear regression; VEH: $p = 0.1514$, $R^2 = 0.3140$, $n = 8$; *Figure 5E;* LLC: $p = 0.1845$, $R^2 = 0.2086$, $n = 10$; *Figure 5F*). This may indicate that caloric intake is not the sole factor influencing body weight loss and there may be an alteration in metabolism or physical activity in this model. Additionally, LLC(low) tumour-bearing mice displayed a significant decrease in WAT, likely contributing to some of the body weight loss during the post-injection period (VEH $n = 8$ vs. LLC $n = 10$, unpaired t-test: $t(16) = 2.670$, $p = 0.0168$, *Figure 5G*). Lastly, BAT (VEH $n = 8$ vs. LLC $n = 10$, unpaired t-test: $t(16) = 0.2118$, $p = 0.8349$, *Figure 5H*) and lean muscle mass (VEH $n = 8$ vs. LLC $n = 10$, unpaired t-test: $t(16) = 1.380$, $p = 0.1864$, *Figure 5I*) did not change in LLC(low) tumour-bearing animals compared to controls. This indicates that lowering the LLC cell number injected prolonged the tumour-bearing period, resulting in consistent anorexia, fat loss and decreases in body weight gain without lean mass loss.

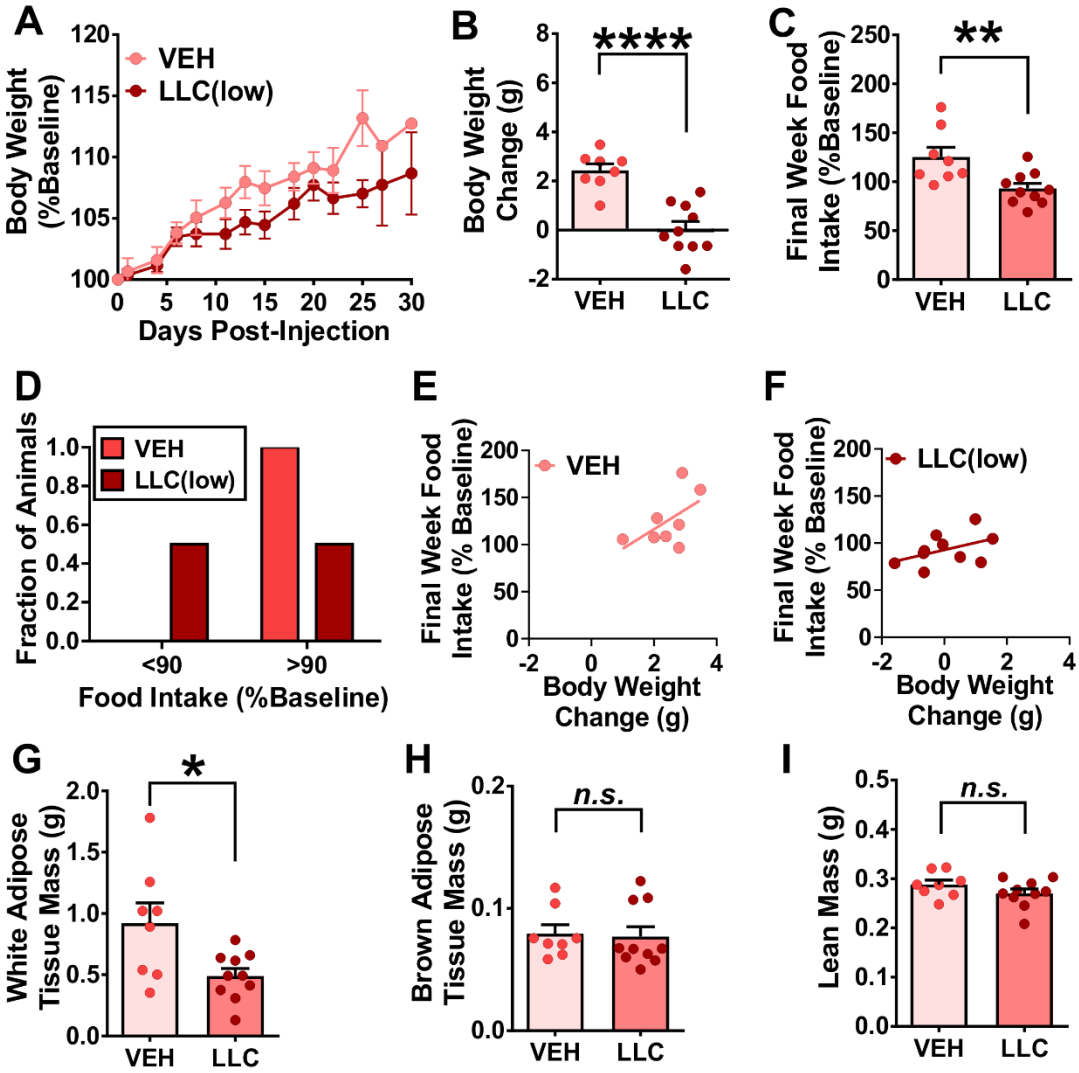


Figure 5. Prolonged tumour load in young mice reduces body weight gain and food intake

(A) Body weight during the post injection period; vehicle (VEH) and low number LLC-injected mice ($0.5x10^6$cells; LLC(low)). **(B)** Change in body weight post-injection, corrected for tumour weight. **(C)** Food intake during the final week post-injection normalized to individual baseline. **(D)** Frequency of animals that display anorexia (<90% baseline) or normal food intake (>90% baseline). **(E&F)** Correlational analysis between body weight and food intake. **(G-I)** Tissue weight upon sacrifice as indicated. *p<0.05, **p<0.01, ****p<0.0001, n.s. not significant.

3-4 LLC(low) Tumour-Bearing Mice have Decreased Orexin Gene Expression

MCH and ORX gene expression was measured in the hypothalamus of LLC(low) tumour-bearing mice and vehicle controls. MCH gene expression was not different between the two groups (VEH n = 6 vs. LLC n = 8, unpaired t-test: $t(12) = 0.3028$, $p = 0.7672$, *Figure 6A*). In addition, levels of MCH mRNA were not correlated with changes in body weight, food intake, or tumour mass in controls or LLC(low) tumour-bearing mice (*Figure 6B-D)*. Interestingly, ORX mRNA expression was significantly decreased in LLC(low) tumour-bearing mice compared to vehicle controls (VEH n = 6 vs. LLC n = 8, unpaired t-test: $t(12) = 2.512$, $p = 0.0273$, *Figure 6E*). However, ORX expression in LLC(low) mice was not correlated to body weight change, food intake or tumour mass (*Figure 6F-H*). This may be due to the tight clustering of data within the tumour-bearing animal group. In fact, when LLC(low) and vehicle control groups were combined, there was a positive correlation between orexin mRNA expression and food intake (*Figure 6F&G bottom)*. This indicates that mice with the lowest expression of orexin mRNA were tumour-bearing mice that consumed the least calories, suggesting that the decrease in orexin mRNA expression may be contributing to the cancer-induced anorexia phenotype.

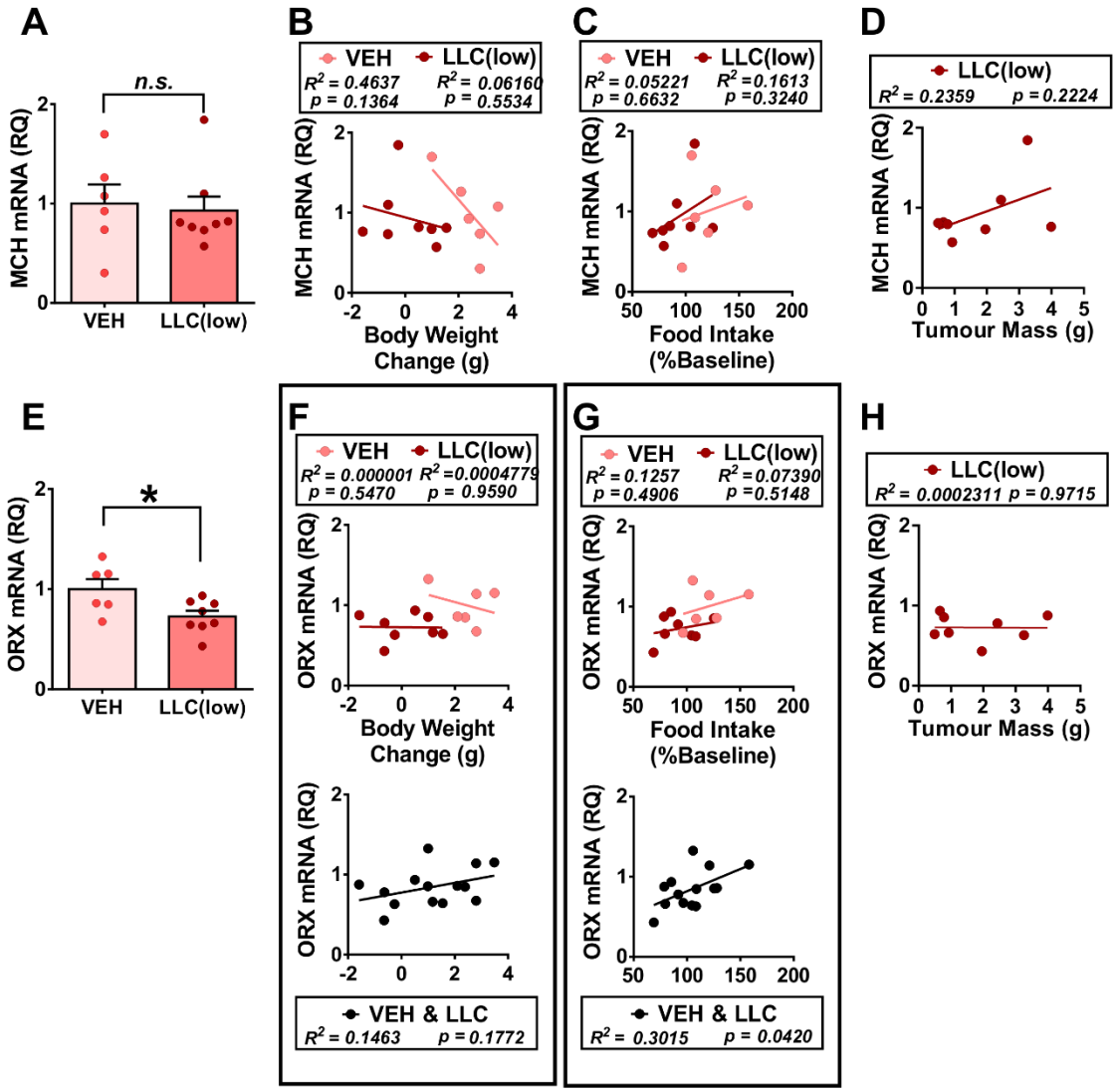


Figure 6. LLC(low) injections result in a decrease in orexin gene expression within the hypothalamus

(A&E) MCH (A) and orexin (E) mRNA expression in the hypothalamus of mice injected with vehicle or LLC cells ($0.5x10^6$cells). **(B-D & F-H)** Correlation analysis between MCH/Orexin gene expression and body weight change **(B & F),** food intake during the final week post-injection **(C & G)**, and final tumour mass upon sacrifice **(D & H).** Bottom panels of F & G represent combined data of vehicle and LLC tumour-bearing animals. *p<0.05, n.s. not significant.

3-5 Aged Mice Show a Loss of Body Weight Without Fat Loss or Changes in Food Intake in Response to High Tumour Load

As cancer incidence rates rise exponentially in older populations (DePinho, 2018) and CACS is most detrimental for aged cancer patients (Skipworth et al., 2007), it was of interest to investigate the LLC tumour-bearing model in aged mice. In order to make direct comparisons of age groups, both LLC(high) and LLC(low) were tested in aged mice.

We found that aged LLC(high) tumour-bearing mice took longer to reach their humane endpoint than younger mice injected with the same number of cells (19 days for aged mice vs. 14 days in young mice; *Figure 7A*). There was no significant difference in total body weight between groups during the post injection period (Two-way ANOVA; Time: $p<0.0001$, LLC vs VEH: $p=0.2465$; *Figure 7A*). However, subtraction of tumour mass from final body weight revealed a significant weight loss in tumour-bearing mice during the post-injection period, while vehicle controls were unchanged (VEH n = 8 vs. LLC n = 10, unpaired t-test: $t(16) = 2.598$, $p = 0.0194$, *Figure 7B*). Moreover, food intake during the final week post-injection was not significantly different in tumour-bearing animals and vehicle controls (VEH n = 8 vs. LLC n = 10, unpaired t-test: $t(16) = 1.406$, $p = 0.1789$, *Figure 7C*). However, vehicle injection alone rarely produced significant individual anorexia (<90% food intake; *Figure* 7D), while more than half of the LLC tumour-bearing mice displayed anorexia, yet this was not significantly different (Fischer's exact test; $p = 0.1880$, *Figure* 7D). Nevertheless, food intake was positively correlated with body weight change in tumour-bearing mice but not controls (standard

linear regression; VEH: p = 0.7025, R^2 = 0.0261, n = 8; *Figure 7E;* LLC: p = 0.0425, R^2 = 0.4208, n = 10; *Figure 7F*). This indicated that in tumour-bearing mice, the decrease in food intake contributed to weight loss. Surprisingly, white adipose tissue (VEH n = 8 vs. LLC n = 10, unpaired t-test: t(16) = 1.141, p = 0.2707, *Figure 7G*), BAT (VEH n = 8 vs. LLC n = 10, unpaired t-test: t(16) = 0.5892, p = 0.5639, *Figure 7H*), and lean mass (VEH n = 8 vs. LLC n = 10, unpaired t-test: t(16) = 0.7358, p = 0.4725, *Figure 7I*) were not different in tumour-bearing aged mice compared to controls. Perhaps weight loss is derived from changes in other tissues or bone density, however this was not investigated in this study.

Overall, these results suggest that aged LLC(high) tumour-bearing mice display body weight loss correlated with anorexia, which is similar to young animals.

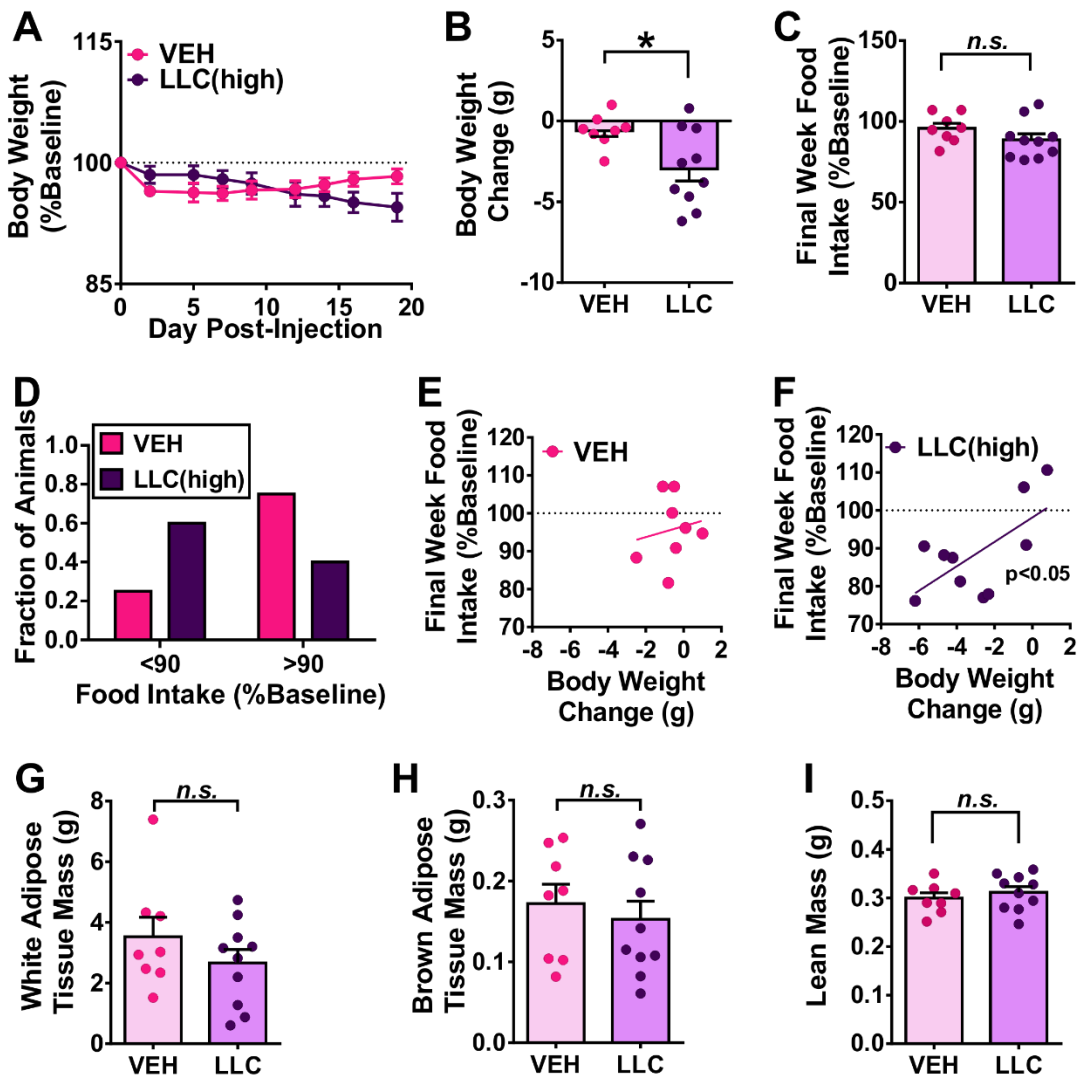


Figure 7. LLC(high) injections in aged mice result in body weight loss

(A) Body weight of mice injected with vehicle (VEH) or LLC cells ($1x10^6$cells; LLC(high)). **(B)** Total change in body weight corrected for tumour weight. **(C)** Food intake during the final week post-injection normalized to individual baseline. **(D)** Frequency of mice that display anorexia (<90%) and normal food intake (>90%) **(E&F)** Correlational analysis between food intake and body weight **(G-I)** Tissue weight upon sacrifice as indicated. *p<0.05, n.s. not significant.

3-6 Aged LLC(high) Mice have Elevated Orexin Gene Expression

MCH mRNA expression in the hypothalamus in LLC(high) mice was not significantly different from that of vehicle controls (VEH n = 7 vs. LLC n = 9, unpaired t-test: $t(14) = 0.8749$, $p = 0.3964$, *Figure 8A*). However, there was increased variation in MCH expression, similar to that seen in young LLC(high) mice (F test: $p=0.0154$; *Figure 8A*). Therefore, a correlational analysis was completed to determine if this variation correlated with the anorexic/cachexic phenotype (*Figure 8B-C*). Unlike in young mice, MCH expression did not correlate with body weight change (*Figure 8B*), food intake *(Figure 8C)* or tumour mass (*Figure 8D)*.

In contrast, orexin gene expression was significantly increased compared to vehicle controls (VEH n = 7 vs. LLC n = 10, unpaired t-test: $t(15) = 2.365$, $p = 0.0320$, *Figure 8E*). Some variation was seen in the orexin expression of tumour-bearing animals, although it was not significantly different from controls (F test; $p= 0.0951$; *Figure 8E*). Furthermore, no correlation was present between orexin expression and body weight change, food intake, or tumour mass (*Figure 8F-H*).

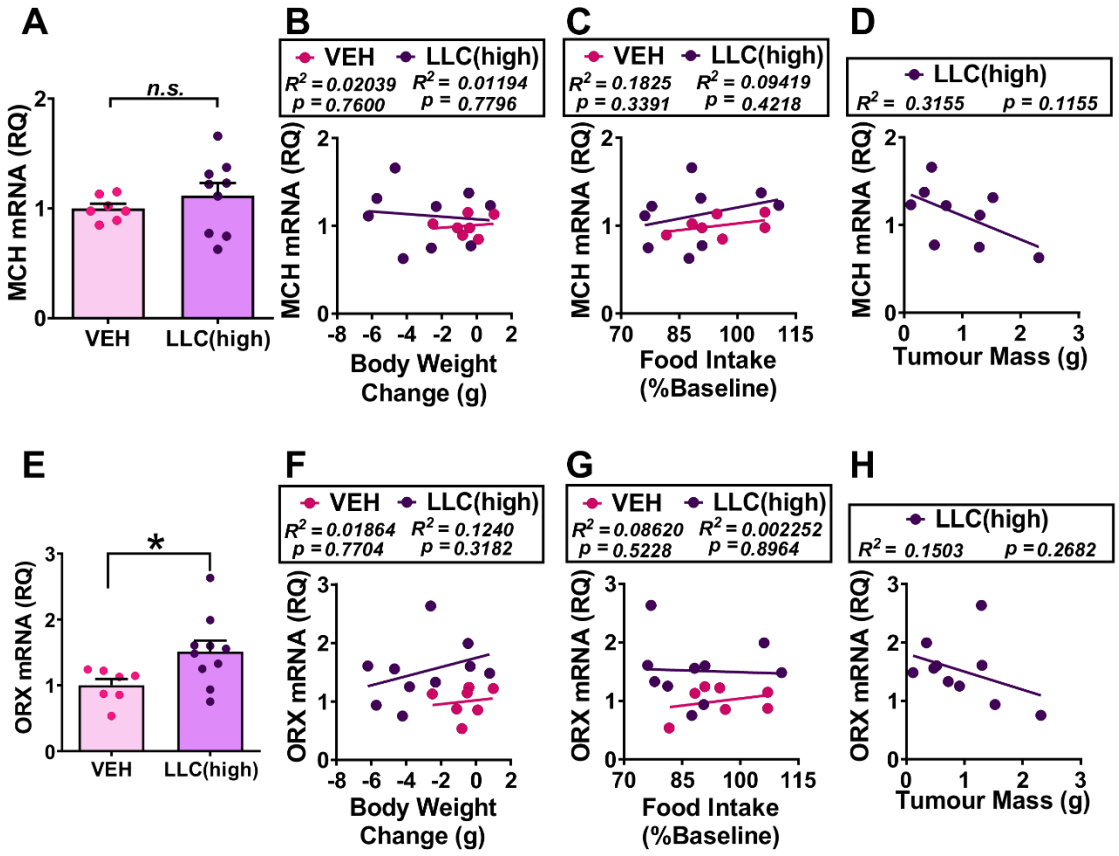


Figure 8. High LLC cell number in aged mice results in increased orexin expression in the hypothalamus without a correlation to body weight

(A&E) MCH (A) and orexin (E) mRNA expression in the hypothalamus of mice injected with vehicle or LLC cells ($1x10^6$cells). **(B-D & F-H)** Correlation analysis between MCH/Orexin gene expression and body weight change **(B & F)**, food intake during the final week post-injection **(C & G)**, and final tumour mass upon sacrifice **(D & H)**. *p<0.05, n.s. not significant.

3-7 Aged LLC(low) Mice Display Significant Anorexia and Body Weight Loss

As reducing the number of LLC cells injected resulted in some critical differences in young mice, we wanted to investigate if this also occurred among aged mice. Aged mice treated with a low number of LLC cells ($0.5x10^6$) reached their endpoint after 23 days post-injection (*Figure 9A*), similar to the average of the young LLC(low) mice. As seen in Figure 9A, vehicle controls maintained their body weight following injection. However, LLC(low) mice continued to lose weight over the entire post injection period, leading to a significant reduction in body weight after 20 days post-injection (VEH n = 3 vs. LLC(low) n = 5; Two-way ANOVA; Time: p=0.0015, LLC vs VEH: 0.2148, Time x LLC vs VEH: p<0.0001; Sidak's multiple comparisons test: $p < 0.05$ at 21 and 23 days; *Figure 9A*). Furthermore, when final tumour weight was subtracted from body weight, tumour-bearing animals lost a significant amount of weight compared to vehicle controls (VEH n = 3 vs. LLC n = 5, unpaired t-test: $t(6) = 2.983$, $p = 0.0245$, *Figure 9B*).

Additionally, aged LLC(low) mice displayed a significant anorexia compared to vehicle controls (VEH n = 3 vs. LLC n = 5, unpaired t-test: $t(6) = 3.626$, $p = 0.0110$, *Figure 9C*). Moreover, the majority of LLC(low) mice displayed anorexia (<90% baseline food intake), while no controls were anorexic (*Figure 9D).* However, this change in food intake was not correlated to body weight change in LLC(low) mice (standard linear regression; VEH: $p = 0.9536$, $R^2 = 0.0053$, n = 3; LLC: $p = 0.2306$, $R^2 = 0.4286$, n = 5; *Figure 9E*). This suggests that caloric intake is not the only factor influencing body weight loss. However, this may also be due to low n-value, and a tight clustering of data, which makes it difficult to find a correlation. In fact, when vehicle controls and LLC(low)

mice were combined, food intake and body weight are positively correlated (standard linear regression; $p = 0.0122$, $R^2 = 0.6761$, $n = 8$), where the higher the food intake the greater the body weight change.

Tissue weights were also investigated in old LLC(low) mice. No significant change was seen in either WAT (VEH n = 3 vs. LLC n = 5, unpaired t-test: $t(6) = 0.7935$, $p = 0.4577$, *Figure 9F*) or lean mass (VEH n = 3 vs. LLC n = 5, unpaired t-test: $t(6) = 2.002$, $p = 0.0922$, *Figure 9H*) compared to vehicle controls. However, BAT was significantly reduced in tumour-bearing mice compared to vehicle controls, indicating potential metabolic disruption (VEH n = 3 vs. LLC n = 5, unpaired t-test: $t(6) = 2.931$, $p = 0.0263$, *Figure 9G*).

In these mice, no significant differences were seen in gene expression of either MCH (VEH n = 2 vs. LLC n = 4, unpaired t-test: $t(4) = 1.482$, $p = 0.2124$, *Figure 9I*) or orexin (VEH n = 2 vs. LLC n = 4, unpaired t-test: $t(4) = 0.3534$, $p = 0.7416$, *Figure 9J*). Correlational analysis was not completed due to low N-value. In fact, these data would be greatly strengthened with additional cohorts, however this was not completed due to time limitations.

Overall, these experiments showed that aged mice injected with a low number of LLC cells present with significant anorexia, loss of body weight and BAT independent of changes in lean mass, WAT or MCH/ORX mRNA expression.

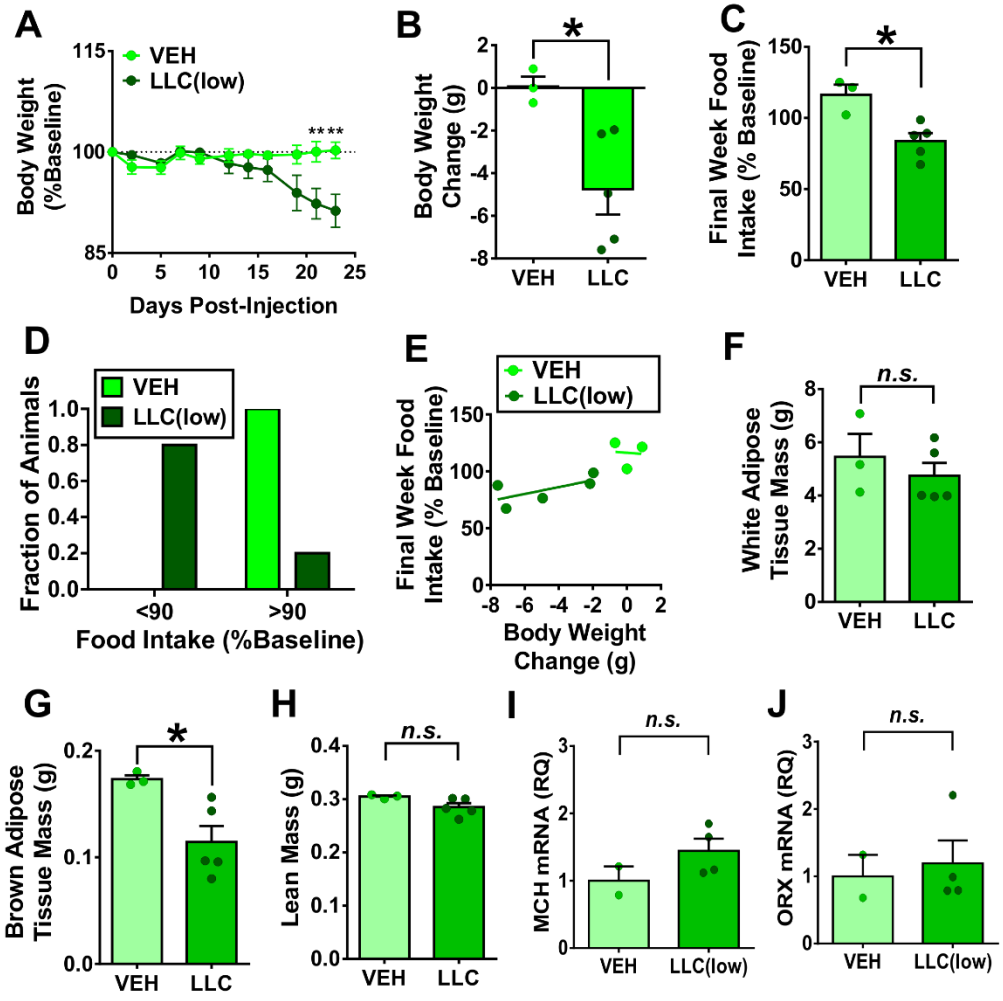


Figure 9. Low cell number LLC injections in old mice results in body weight loss and anorexia

(A) Body weight during the post-injection period; vehicle (VEH) or LLC cells ($0.5x10^6$cells; LLC(low)). **(B)** Change in body weight from baseline to the end point. **(C)** food intake during the final week post-injection normalized to individual baselines. **(D)** Frequency of animals that display anorexia (<90%) and normal food intake (>90%) **(E)** Correlational analysis between food intake and body weight **(F-H)** Tissue weight upon sacrifice; **(I & J)** MCH/Orexin mRNA expression in the hypothalamus. *p<0.05, **p<0.01, n.s. not significant.

3-8 Effects of Age and Tumour Load on Anorexia, Weight Loss and Appetite-related Peptide mRNA Expression

In order to further elucidate the effects of age or tumour load on CACS, the results from the four experiments described above were compared directly. In Figure 10, all data from the four LLC groups (two age groups that received a low or high number of LLC cells) are represented relative to time-matched vehicle controls to account for differences intrinsic to the treatment or age.

Mice in the LLC groups had a significantly less body weight change compared to controls (*Figure* 10A). Relative to the change in young mice, old mice lost more weight. Furthermore, mice injected with the low cell number lost more weight than those injected with a high cell number (Two-way ANOVA; Cell #: $p = 0.0024$, Age: $p = 0.0046$, Interaction: $p = 0.5264$; *Figure 10A*).

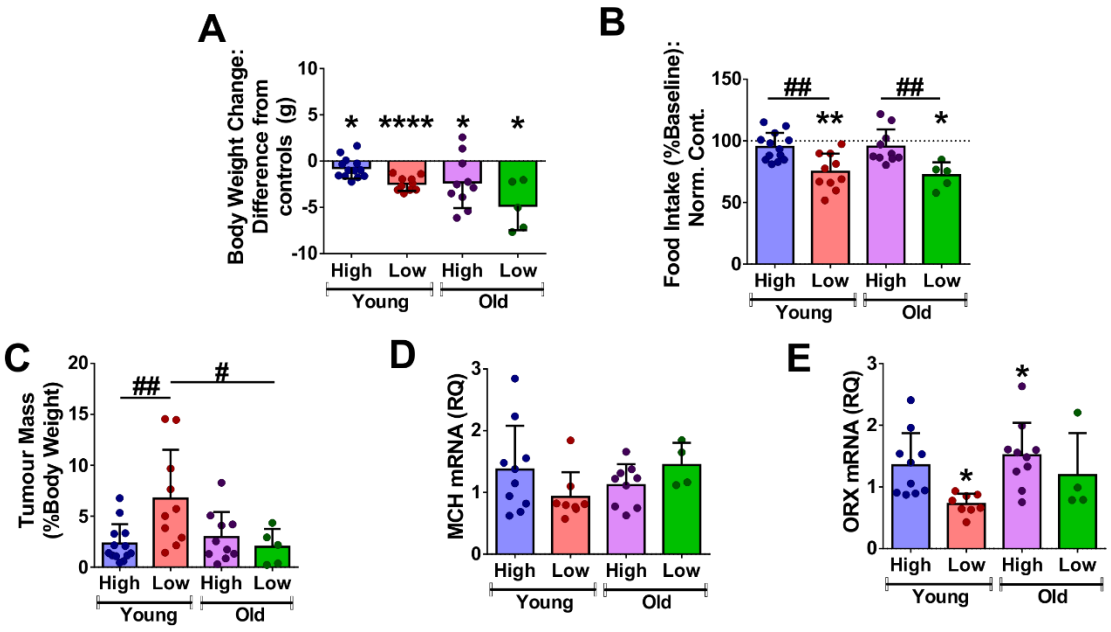
Next, food intake was first normalized to individual baselines, and then to the mean of time-matched controls. A group comparison indicates that anorexia was only significantly present in the LLC(low) tumour-bearing groups irrespective of age (Two-way ANOVA; Cell #: $p < 0.0001$, Age: $p = 0.7798$, Interaction: $p = 0.7350$; *Figure 10B*). This indicates that a greater number of cancer cells at the beginning may not necessarily predict a greater degree of anorexia.

Aged mice had much larger body weight than young mice. However, the tumour mass as a percentage of final body weight was similar regardless of age. Neither age nor cell number significantly altered final tumour mass, yet there was a significant interaction

effect between the two variables (Two-way ANOVA; Cell #: p = 0.1096, Age: p = 0.0615, Interaction: p = 0.0154; *Figure 10C)*. In fact, it should be noted LLC(low) mice had significantly larger tumours as a percent body weight compared with young LLC(high) mice and old LLC(low) mice.

The expression levels of MCH and orexin mRNA in tumour-bearing mice were also compared to see if age or injected cell number affected their expression differently. MCH mRNA expression in tumour-bearing mice was not different between groups based on age and injected cell number (Two-way ANOVA; Cell #: p = 0.7719, Age: p=0.5052, Interaction: p = 0.0581; *Figure 10D*). In contrast, orexin mRNA expression was significantly lower in mice treated with low number LLC regardless of age (Two-way ANOVA; Cell #: p = 0.0155, Age: p=0.0966, Interaction: p = 0.4127; *Figure 10E*).

Overall, these results suggest that the initial tumour load (injected cell number) has a significant impact on body weight change, food intake, and orexin gene expression, where LLC(low) injections resulted in a decrease in all factors. The age of mice only affects the degree of body weight loss, where aged mice are more prone to a greater degree of body weight loss than younger mice.



F

Two-Way ANOVA	BW change	Food Intake	Tumour Mass	MCH	ORX
Cell Number	*0.0024*	*<0.0001*	*0.1096*	*0.7719*	*0.0155*
Age	*0.0046*	*0.7798*	*0.0615*	*0.5052*	*0.0966*
Interaction	*0.5264*	*0.7350*	*0.0154*	*0.0581*	*0.4127*

Figure 10. Effects of age and injected cell number in LLC tumour-bearing mice

Comparison of all LLC-tumour bearing animals in each age group (Young = 8 weeks old, Old >10 months old) and each cell number injected (High = $1x10^6$ cells, Low = $0.5x10^6$ cells). **(A)** Body weight is represented as the total body weight change over the post-injection period compared to the average of time-matched vehicle controls, where 0 indicates no difference in body weight gain compared to controls. **(B)** Food intake during the final week normalized to the mean intake of vehicle control mice, where 100% indicates no difference compared to controls **(C)** Final tumour mass represented as the a percent of final body weight **(D,E)** MCH and orexin mRNA expression in the hypothalamus of LLC tumour bearing animals. **(F)** Two-Way ANOVA statistical analysis. Tukey's post test #p< 0.05, ##p< 0.01, ####p< 0.0001 vs other conditions. Unpaired t-test *p< 0.05, **p< 0.01, ****p< 0.0001 vs time matched vehicle controls.

4-0 DISCUSSION

In the present study, we have shown that the LLC-tumour bearing mouse model displays anorexia and cachexia, which depend on both age and cancer cell number injected. In addition, some changes in both MCH and orexin gene expression were associated with CACS induced by LLC tumours. The following discussion will investigate the LLC-tumour bearing model and dive into the possible implications of appetite-neuropeptide alterations regarding CACS.

4-1 Lewis Lung Carcinoma Model as a Model of CACS

There are many tumour-bearing models utilized to investigate anorexia and cachexia in animals. As reviewed thoroughly by Bennatti & Walsh in 2011, each model varies slightly in the degree of anorexia and cachexia, and other factors such as inflammation. Typically, in animal models, anorexia is measured directly as a reduction in cumulative food intake during the post-injection period or in daily food intake in the final days before the endpoint (Busquets et al., 2004; Campos et al., 2017; Liu et al., 2020). Additionally, cachexia is commonly defined as a significant loss of carcass weight compared to control animals (Busquets et al., 2004; Choi et al., 2013; Kir et al., 2014; Llovera et al., 1998). Moreover, cachexia can be assessed by body composition analysis, observed as significant decreases in either WAT or skeletal muscle mass (Campos et al., 2017; Lee et al., 2017; Liu et al., 2020; Puppa et al., 2014; G. Zhang et al., 2017). Models that produce both significant anorexia and cachexia are useful when investigating CACS.

The LLC tumour-bearing mouse model has been a widely used model of cancer cachexia and anorexia, although several studies showed limited CACS phenotype. Some studies have demonstrated that the LLC tumour-bearing model has the capacity to present with anorexia, cachexia, lethargy and inflammation, all while maintaining an intact immune system (Bennani-Baiti & Walsh, 2011; Zhu et al., 2019). Furthermore, the LLC tumour-bearing mouse model has been used to show anorexia-related changes in neuronal function, namely CGRP neurons of the parabrachial nucleus (Campos et al., 2017). In this study, significant anorexia was present after only 5 days post-injection (Campos et al., 2017). However, other studies using the same LLC tumour-bearing model did not see anorexia before 14 days post-injection or until tumour mass was above 6% body weight (Busquets et al., 2004; Kir et al., 2014; López-Soriano et al., 1997)(Kir et al., 2014). Furthermore, while body weight loss is usually consistently observed in LLC tumour-bearing mice (Busquets et al., 2004; Campos et al., 2017; Llovera et al., 1998; Puppa et al., 2014; G. Zhang et al., 2017), some studies failed to detect body weight loss in the presence of the tumour (Brown et al., 2017; Lim et al., 2020).

Importantly, there are a wide variety of initial tumour loads used in the LLC model. In fact, LLC tumour-bearing models have used as low as 50 cells (G. Zhang et al., 2017) to upwards of $5x10^6$ cells (Kir et al., 2014) to induce cachexia. Commonly used cell loads are $0.5x10^6$ (Busquets et al., 2004; Dwarkasing et al., 2015; Llovera et al., 1998; Zhu et al., 2019) and $1x10^6$ cells (Brown et al., 2017; Campos et al., 2017; Lim et al., 2020; Liu et al., 2020; Puppa et al., 2014; Van Leeuwen et al., 2003). However, previous studies have not directly compared the effect of different initial tumour loads. According

to our results, different initial tumour loads may result in different effects including post-injection duration and the CACS phenotype. Overall, these variabilities in the LLC tumour-bearing model call for a standardization in the model between studies.

4-2 Initial Tumour Load and CACS

Previous studies that utilized $1x10^6$ LLC cells to investigate anorexia, which is the same as our LLC(high) group, are variable and difficult to interpret. In fact, one report showed a significant reduction in daily food intake after 5-9 days post-injection in one cohort, while another cohort took 17 days to display a significant reduction in daily food intake (Campos et al., 2017). Additionally, another group saw a significant loss of cumulative food intake at the end of a 21 day post-injection period (Liu et al., 2020). However, it should be noted that in many of these studies, LLC(high) tumour-bearing mice were kept for much longer than our 14 day post-injection period, which may explain some of the discrepancies in the anorexic response. In our hands, using the commonly utilized number of tumour cells (LLC(high); $1x10^6$) did not result in consistent anorexia. Even so, our LLC(high) tumour-bearing mice lost a significant amount of weight compared to vehicle controls, alongside a significant loss in WAT mass. This suggests that even in the absence of anorexia, which often precedes other symptoms of CACS, these tumour-bearing mice can be labelled as cachexic. This is consistent with another LLC(high) tumour-bearing study, where body weight loss was seen to occur before anorexia (Campos et al., 2017). Nevertheless, we found that food intake and body weight

in tumour-bearing mice were positively correlated, indicating that a subtle reduction in food intake may have contributed to the observed cachexia. Additionally, many studies report a decrease in fat mass using the LLC(high) model (Campos et al., 2017; Lim et al., 2020; Puppa et al., 2014), which further aligns with our study. Overall, this indicates that in our hands, the LLC(high) model displays cachexia without significant overall anorexia.

When attempting to establish an animal model, it is important that its phenotype contains the majority of the symptoms of the modeled syndrome. In CACS this includes both anorexia and cachexia. Exclusively using the LLC(high) tumour-bearing model would have made it difficult to draw conclusions surrounding anorexia. It has been implicated that a longer tumour exposure time produces more profound cachexia (Brown et al., 2017; Dwarkasing et al., 2015), which may also apply to anorexia. Therefore, in an attempt to increase the tumour exposure time, we halved the cell number injected.

In these LLC(low) mice, the period to reach the critical endpoint was extended in both age groups examined. These mice showed significant anorexia nearing the experimental endpoint and a greater body weight loss compared with the LLC(high) tumour-bearing mice. This aligns with previous suggestions that anorexia will only become pervasive after 14 days post-injection (Busquets et al., 2004), although another study showed that anorexia in LLC(low) mice display severe anorexia before this 14 day time-point (Dwarkasing et al., 2015). Moreover, the reduction in food intake was correlated with body weight change, where the animals with the most prominent anorexia also had the most significant body weight loss, suggesting that anorexia contributed to the weight loss.

The result showing that lower initial tumour load produces more profound anorexia brings up the question: why did this difference occur? It is possible that prolonged exposure to tumour-derived factors contributes to the anorexic response to the tumour. Future experiments could investigate whether extended presence of tumours would result in higher levels of tumour-derived factors such as inflammatory cytokines. Additionally, final tumour mass may influence the severity of the CACS phenotype. Indeed, mice with larger tumours showed a greater body weight loss and anorexia. A larger tumour would have a more demanding nutritional load and monopolize much of the caloric availability in the body, which may explain the differences in body weight change (Van Leeuwen et al., 2003). However, our aged LLC(low) mice displayed significant anorexia, despite having tumours grow to a similar percent body weight as those of aged LLC(high) mice that showed inconsistent anorexia. This indicates that final tumour size may not be the sole factor influencing the severity of the anorexic response.

Lastly, initial tumour load may have contributed to CACS, regardless of changes in tumour exposure time and tumour size. Perhaps, this may be due to initial tumour cell survivability, or differences in the initial tumour microenvironment. In future studies a more expanded range of injected cell numbers could further our understanding of the influence of cell number on CACS. Regardless, if initial cell number is influencing the CACS phenotype, it may be critical to characterize and standardize the cell numbers used in LLC tumour bearing models for future use.

In conclusion, while the LLC(high) model is most commonly used in the literature, the LLC(low) model results in more significant anorexia and cachexia that may

be more suitable for studying CACS. However, final tumour mass may impact the expression of CACS and should be controlled more rigorously in future studies.

4-3 Mechanisms of Cancer-Induced Cachexia

Body weight change was significantly lower in all LLC tumour bearing mice compared to controls, indicating a pervasiveness of cachexia in all LLC models. this may be explained by the presence of anorexia in many mice, as food intake and body weight were positively correlated. However, it is known that cachexia can precede anorexia in LLC tumour-bearing mice (Campos et al., 2017), and anorexia was not always present in our mice displaying WAT and general weight loss. Besides the reduction in caloric intake, there are a number of possible factors that may contribute to weight loss.

Increases in energy expenditure through increased physical activity or metabolism can contribute to the cachectic response to cancer (Murphy et al., 2012; Porporato, 2016). For example, a breast cancer mouse model shows dysregulation of the sleep-wake cycle and hyperactivity (Borniger et al., 2019). However, LLC injections typically result in increased lethargy (Campos et al., 2017; Zhu et al., 2019). Increased lethargy aligns strongly with common sickness behaviours, which are typical in tumour-bearing mouse models and human cancer patients (Watanabe et al., 1996). Inactivity may contribute to decreased food-seeking behaviour, leading to weight loss. However, it is more likely that a reduction in activity would contribute to conserving energy.

Alternatively, metabolism can be altered in human cancer patients and tumour-bearing animal models. In cachectic cancer patients, metabolic alterations can range from insulin resistance to mitochondrial uncoupling, which can lead to an increased basal metabolic rate (BMR) (Porporato, 2016). In the LLC tumour-bearing model, many metabolic disruptions have been characterized, such as alterations in calcium, lipid, and glucose metabolism, with an overall increase in BMR (Bing et al., 2006; Kir et al., 2014; López-Soriano et al., 1997). Furthermore, LLC tumours are known to produce factors which may influence metabolism, such as prostaglandins and cachexins (Chiabrando et al., 1985; Zhang et al., 2017). Therefore, increased metabolism is at least partially contributing to the body weight loss seen in cancer. However, in our study, mice had significant loss of brown adipose tissue, which would suggest a downregulation of thermogenesis and BMR. Nonetheless, significant adipose tissue browning can occur in LLC tumour-bearing mice (Hu et al., 2018). This is a clinically relevant process, as human cancer patients undergo white adipose browning, (Porporato, 2016), which strongly contributes to their increased energy expenditure (Kir et al., 2014). Thus, BMR could still be elevated without increased mass of the brown adipose tissue. Furthermore, LLC tumours are known to shrink adipocytes (Bing et al., 2006) and LLC tumour-derived factors can directly cause lipolysis of WAT in mice (Hu et al., 2018). This may partially explain the loss of body weight, even in the absence of significant anorexia.

In conclusion, multiple factors can account for weight loss in the presence of tumours, including changes in food intake, physical activity, metabolism, and WAT

shrinkage. It is important to characterize each of these factors in tumour-bearing animal models to distinguish overlapping and distinct neuronal mechanisms involved in CACS.

4-4 Implications of Age in CACS

CACS is most prevalent and detrimental in aged human cancer patients, and using aged mice would more accurately represent the typical CACS seen in the human population (Skipworth et al., 2007). Accordingly, our aged LLC tumour-bearing mice typically lost more weight compared to young mice regardless of the cell number injected. There are several factors that could account for this age-dependent difference. Various tumours are known to grow quicker in young cancer patients (Förnvik et al., 2016). This was reproduced in our mouse model, as the tumour grew slower in aged compared to young mice and prolonged the post-injection period. In addition, the WAT is known to undergo age-related changes, where adipocyte size increases and responsiveness to caloric restriction and metabolic distress decreases with age (Shen et al., 2020). Moreover, differences in total body composition, starting body weight, caloric intake, BMR, and inflammation can all influence tumour growth and weight loss which may underlie the differences in CACS in aged and young mice (Van Leeuwen et al., 2003).

Metabolism is altered due to aging. It is known that aging results in impaired glucose, phospholipid, and polyamine metabolism that may contribute to sarcopenia and general weight loss associated with age (Uchitomi et al., 2019). Up to 44% of pre-

treatment cancer patients show a significant elevation in their BMR compared to healthy controls (Broeder et al., 2001). In animal models, tumours have a high metabolic demand, which may act in concert with age-related metabolic impairment. Therefore, this may contribute to the greater body weight loss observed in aged tumour-bearing mice. In future studies, the use of a calorimeter on young and old tumour-bearing mice may provide further insight into changes in BMR during tumour-growth.

Additionally, a major difference between old and young mice may be the increased level of baseline systemic inflammation associated with aging, termed "inflammaging" (Franceschi & Campisi, 2014). To summarize, inflammaging is the phenomenon seen where the levels of systemic immune activation increase as age progresses. This can include the age-dependent gradual increase in activated CD8+ T-cells (Mogilenko et al., 2021) and cytokines like interleukin-6 (IL-6) and TNFα (de Gonzalo-Calvo et al., 2010; Varadhan et al., 2014). Recently, inflammaging has garnered attention as one of the leading exacerbatory factors for diseases in late life (Fülöp et alc., 2019) . Moreover, the presence of a peripheral tumour can further upregulate the same immune factors (Hajime Suzuki et al., 2013). Together, this means that aged individuals with cancer will likely have elevated inflammation from an additive effect of cancer and age, which may result in greater catabolism of body mass.

4-5 MCH/Orexin during Cancer Cachexia

MCH is a prominent effector for increasing body weight (Ludwig et al., 2001). Furthermore, MCH neurons are likely to be affected directly and indirectly in the presence of cancer, as they are sensitive to inflammatory factors (Le Thuc et al., 2016) and receive inputs from the arcuate nucleus (Elias et al., 1998), which is known to be affected during by tumours (Marcus & Elmquist, 2006; McCarthy et al., 1993). Furthermore, orexin neurons play a critical role in energy balance (Inutsuka et al., 2014), are innervated by the arcuate nucleus, and are inhibited by hypothalamic inflammation (Grossberg et al., 2011). By contrast, a study investigating sleep disruption during cancer demonstrated that orexin neurons are more active in the presence of a peripheral tumour (Borniger et al., 2019). This study suggested that an increase in orexin activity would result in overactivity and reduction in sleep length and quality, leading to insomnia during cancer. Notably, the mammary tumour model that was used displayed hyperphagia, indicating that orexin may be influencing feeding in the opposite direction of CACS in those mice. Overall, both MCH and orexin are potential regulators of anorexia and cachexia that we see during LLC tumour-growth.

Importantly, both MCH and orexin mRNA levels in LLC(high) tumour bearing mice showed a positive correlation with body weight gain. This suggests that MCH/orexin gene overexpression may be a counterregulatory response to oppose decreases in food intake and body weight, and the ability to mount this response is variable among mice. Interestingly, this correlation was not seen in aged LLC(high) tumour-bearing mice. This means that while the mechanism that is causing the elevation

of MCH/orexin gene expression is consistent between the age groups, the downstream, weight-protecting effects of this elevation are not present in aged mice. Conversely, mechanisms that suppress food intake or body weight in aged mice may be silencing the beneficial effects of elevated MCH/orexin on CACS.

The fact that LLC(high) mice with increases in MCH mRNA showed healthy body weight gain seems to be consistent with the role of MCH in promoting weight gain in healthy animals (Ludwig et al., 2001). However, food intake was not correlated with MCH mRNA expression, which was surprising, since MCH is known to promote appetite in rodents (Rossi et al., 1997). However, MCH neurons suppress energy expenditure which may play a more prominent role in maintaining body weight in these animals. MCH or MCHR1 deficient mice are hyperactive (Zhou et al., 2005) and have higher basal metabolic rate (Marsh et al., 2002). Moreover, chemogenetic activation of MCH neurons results in the initiation of REM sleep and increases in lethargic behaviour (Hausen et al., 2016). Therefore, increased MCH expression could result in a lower caloric expenditure, which may contribute to weight gain/maintenance. This counterregulatory effect of MCH may not occur in aged tumour-bearing mice because the increase in gene expression was not as large (2 fold increase in aged vs 3-fold increase in young mice). Alternatively, MCH expression is naturally elevated in aged mice (Kappeler et al., 2003), which is partially responsible for age-related changes in locomotion (Jeon et al., 2006). In fact, in aged MCH knockout mice, locomotion remains consistent to young animals (Jeon et al., 2006). Therefore, in aged tumour-bearing mice, any elevation of MCH mRNA may not have a significant effect on locomotion and may not be protective against weight loss. In

the present study, neither physical activity nor metabolic rate were investigated but would be interesting targets for future studies to elucidate the functional role of elevated MCH gene expression in tumour-bearing animals.

On the other hand, the fact that elevated orexin mRNA correlated with body weight gain in our LLC(high) mice is perplexing. Similarly to MCH expression, orexin mRNA lacked a correlation to food intake. This suggests that orexin neurons, while known as promotors of food seeking behaviour (Hagar et al., 2017), may play a role in body weight gain without altering appetite in our animals. However, the implications of orexin on both physical activity and basal metabolism are counterintuitive to the body weight outcome that we see in our LLC(high) mice. It is known that overactivity of orexin neurons can result in an increase in spontaneous physical activity as well as an elevation of metabolic rate, which would both lead to weight loss (Inutsuka et al., 2014; Kotz et al., 2012). Therefore, it is possible that orexin neuron activity may be influencing CACS in LLC tumour-bearing mice through a mechanism other than direct energy homeostasis. For example, orexin neurons are known to have reciprocal connections to the ARC and the LH (Burt et al., 2011; Luan et al., 2017; Marcus & Elmquist, 2006). Elevated orexin neuron activity may cause a change in the energy homeostasis pathway to encourage weight gain (Jain et al., 2000). Additionally, it has been suggested that elevated orexin expression can suppress tumour growth (Blais et al., 2017), however hypothalamic orexin mRNA does not correlate with tumour mass in these mice, and likely does not play a role in body weight gain in this direction. Overall, MCH and orexin

gene expression is elevated in some tumour-bearing mice, which may protect them from immediate, rapid weight loss.

Young mice injected with LLC(low) had a longer tumour-exposure time and displayed anorexia alongside cachexia. The variation on MCH and orexin gene expression discussed previously, was not apparent in these tumour-bearing mice. Moreover, the mice did not show any significant change in MCH gene expression, while orexin mRNA expression was decreased compared to vehicle controls. These findings may indicate that the previously discussed protective mechanism is time-dependant and only occurs in during initial tumour growth. More specifically, the lack of change in MCH/orexin gene expression at this time point may represent a failure to mount a counterregulatory response which may contribute to CACS. Alternatively, at this point, MCH may not be involved in any aspect of CACS, while lack of orexin may contribute to the anorexic response in the presence of a tumour. In fact, the reduction in orexin mRNA aligns with previous microarray analysis that indicates a reduction in orexin mRNA levels after LLC(low) injections (Dwarkasing et al., 2015). However the stark difference in orexin mRNA between LLC(high) and LLC(low) mice remains a novel finding, which provides an insight into the delayed anorexic response in LLC tumour-bearing mice.

Interestingly, reduced orexin mRNA expression in LLC(low) mice was correlated with food intake. This indicates that the loss of orexin gene expression is related to anorexia, which agrees with orexins role in appetite. In fact, injection of orexin-A stimulates food intake (Yamanaka et al., 1999). However, more recently this finding has been attributed to food seeking behaviour, where orexin activation does not stimulate

food intake but rather appetite-driven, motivated locomotion (Gao & Horvath, 2014). Nonetheless, a reduction in orexin expression during tumour growth would cause a decrease in overall food intake, resulting in the anorexic phenotype. However, it should be noted that the reduction in orexin gene expression was not correlated with body weight loss in these mice, indicating that the effects of orexin reduction tie more into the anorexic response in LLC(low) mice. This is possible, as the use of multiple animal models of CACS have emphasized that anorexia and cachexia can work independently during cancer (Bennani-Baiti & Walsh, 2011).

Overall, orexin gene expression in the hypothalamus can be reduced during tumour growth, which correlates with anorexic behaviour. Further investigation into injections of the orexin peptide, or chemogenetic activation of orexin neurons would be useful in determining the efficacy of targeting orexin neurons to combat CACS.

4-6 Mechanisms for Altered MCH/Orexin during Cancer

Our results support the idea that MCH and orexin neurons play a role in anorexia and/or cachexia through a counterregulatory response or loss of function. However, it remains unclear what is causing the change in MCH or orexin gene expression in tumour-bearing mice. Peripheral and central inflammation, fluctuations in nutrient availability, and alterations in upstream energy balance signals may all play a role in the changes in MCH and orexin gene expression described in this thesis.

Inflammatory signaling would likely cause a change in neural activity and gene products in the brain. In fact, peripheral tumours are known to significantly alter CNS neuron activity through the upregulation of the inflammatory response (Van Norren et al., 2017). Peripherally, the tumour-induced inflammatory response involves a large increase in activated immune cells and the production of inflammatory cytokines such as interleukin-1 (IL-1), IL-6, TNFα, and interferon gamma (IFN-γ) (Hajime Suzuki et al., 2013). Even though the CNS is widely protected against many circulating molecules by the blood-brain barrier (BBB), systemic inflammation is known disrupt, circumvent or signal through the BBB (Varatharaj & Galea, 2017). For example, endothelial cells comprising the BBB can release cytokines into the brain in response to systemic inflammation (Hunt & Jurd, 1998). Furthermore, the BBB can become "leaky" and allow the infiltration of peripheral lymphocytes (Salimi & Klein, 2019). Additionally, circumventricular organs (CVOs) that lack the BBB allow direct access of peripheral signals such as cytokines to the brain parenchyma. More specifically, the median eminence is a CVO located adjacent to the hypothalamus where neurons may be able to directly detect peripheral cytokines (Burfeind et al., 2020). Moreover, vagal afferents can mediate peripheral inflammatory signals to the CNS (Steinberg et al., 2016).

In response to these peripherally-derived inflammatory signals, the hypothalamus has been shown to increase activation of resident glial cells (microglia and astrocytes; Burfeind et al., 2020) and upregulate locally produced cytokines (Dwarkasing et al., 2016). Furthermore, neuropeptides involved in homeostatic mechanisms are modulated by systemic inflammation. Specifically, systemic injection of lipopolysaccharide (LPS),

which increases peripheral inflammation, decreases both MCH mRNA and peptide expression in the LH (Le Thuc et al., 2016). Moreover, MCH neurons contain receptors for inflammatory factors such as CCL2, a known product of tumours (Qian et al., 2011), which inhibit MCH gene expression (Le Thuc et al., 2016). These studies suggest that if central inflammation was occurring in the LLC tumour-bearing model, MCH gene expression can be expected to be downregulated. However, we found that MCH gene expression was not changed or upregulated in the LLC mice. This means that there may not be significant inflammation in our mice. Alternatively, there may be other factors opposing the inhibitory effects of inflammation on MCH gene expression. In contrast, inflammatory mediators are known to suppress the orexin system. i.p. or i.c.v. injection of IL-1beta downregulates orexin-A concentration, orexin neuron activity, and orexin mRNA levels (Grossberg et al., 2011). Furthermore, systemic injection of LPS causes a reduction in orexin activity and reduction in food seeking behaviour that is typically associated with acute sickness behaviour (Becskei et al., 2008; Gaykema & Goehler, 2009). In fact, TNFα downregulates orexin activity by degrading unprocessed orexin mRNA (Zhan et al., 2011). These reports suggest that hypothalamic inflammation is a major mechanism in decreasing orexin gene expression. However, hypothalamic inflammation was not measured directly in this study. It would be important to characterize inflammation in these LLC models in the future including cytokine expression and the activation of resident microglia using immunohistochemistry. This may specify the degree of hypothalamic inflammation and could be focused on the lateral hypothalamus as well.

On the other hand, other known changes due to peripheral tumours may underly the changes of MCH/orexin we see in some tumour-bearing mice. It is known that MCH and orexin gene expression is increased during periods of negative energy balance, such as periods of fasting (Kokkotou et al., 2001; López et al., 2000; Qu et al., 1996). The growth of a peripheral tumour monopolizes nutrients in the body, which may mimic fasting conditions. In fact, tumours significantly reduce blood glucose levels (Tisdale et al., 1987). Furthermore, orexin neurons become hyperactive during periods of hypoglycemia (Cai et al., 1999), which may explain the temporary increase in orexin gene expression in some tumour-bearing mice.

Other changes in the upstream energy balance circuitry seen during tumour growth would have variable outcomes on MCH and orexin activity. Specifically, NPY in the arcuate nucleus has been shown to be upregulated in anorectic tumour-bearing rats, albeit in a different tumour-bearing model (Chance et al., 1998). Moreover, NPY neurons inhibit MCH neurons in the lateral hypothalamus (Elias et al., 1998), which may account for some changes in our tumour-bearing mice. Additionally, appetite-inhibiting factors like leptin or αMSH are upregulated in response to tumour growth (Ishikawa et al., 2004; Marks et al., 2003), and orexin neuron respond directly to both (López et al., 2007; López et al., 2000). Moreover, serotonin is thought to be responsible for many appetite related changes during LLC tumour-growth (Dwarkasing et al., 2015). In fact, serotonin receptors are present on orexin neurons, and serotonin inhibits orexin neurons (Muraki et al., 2004) and MCH neurons (Van Den Pol et al., 2004). Any of these factors may be influencing the orexin and MCH systems in LLC tumour-bearing mice.

4-7 Limitations of the Current Study and Future Directions

Previous studies using the LLC tumour-bearing model commonly report significant anorexia. However, in our hands this model required the reduction of the injected cell number in order to significantly show anorexia in both young and old mice. This may be due to using stricter endpoint criteria, resulting in some mice sacrificed before significant anorexia. Additionally, the presence of cachexia may be the sole factor of interest in other studies, in which case this model would be suitable. Future studies investigating CACS should use the low LLC cell number $0.5x10^6$ cells, which produces a more convincing correlate to CACS. For example, for those interested in investigating neuronal mechanisms of CACS, the LLC(low) model may show these symptoms more consistently. However, the LLC(high) mice may provide a valid model for cancer cachexia in the absence of anorexia. Furthermore, reducing the cell number even further may increase the viable tumour growth period in the case of studies requiring a longer treatment period. In fact, a study investigating the impact of LLC cell number on CACS would provide further insight into this model and could determine the optimal cell number to produce consistent anorexia and cachexia.

Other limitations of this model include no apparent lean muscle mass loss, variable tumour growth rate, and common breakthrough of the skin which may lead to infection or inflammation. In the future, more detailed muscle wasting analysis may prove useful to view cachexic muscle loss. Additionally, to address the variability in tumour growth, mice could be sacrificed when they reach their individual endpoint, rather than collective sacrifice. This may control for tumour size, although consistency in tumour-

exposure time would be lost. Moreover, altering the location of the tumour-injection site may provide protection against external damage. In fact, some previous studies inject LLC cells into the musculature of the hind flank, which may reduce the possibility of outside damage (Busquets et al., 2004; Dwarkasing et al., 2015; Llovera et al., 1998). However, it is questionable whether this would have a negative effect on locomotion and would likely make tumour size measurements more difficult.

Furthermore, the low sample size for the old LLC(low) mouse groups makes it hard to make definite conclusions with the utmost confidence. More data should be added to increase our confidence in the findings of the current study. Additionally, confirming our findings with another model of CACS would prove extremely valuable to determine whether our results are more generally applicable.

While our LLC(low) mice show replicable anorexia and cachexia, there is some missing information in order to fully characterize this model. Firstly, since it is known that LLC tumour-bearing animals have significantly upregulated immune responses (Bennani-Baiti & Walsh, 2011), it would be interesting to view these changes in correlate with body weight change, food intake, and appetite neuropeptide gene regulation. Peripheral and central measurements of immune factors such as IL-6 and TNF-alpha or using immunohistochemistry to identify levels of activated microglia in the hypothalamus would help to further characterize this CACS model. Secondly, since food intake is not the sole factor involved in cachexia, direct measurements of physical activity and metabolism would be useful when attempting to determine the severity of CACS in this

model. Using a calorimeter to measure basal metabolic rate and behavioural analysis to measure lethargy could strengthen the role of MCH and orexin neurons in CACS.

Males typically display more severe cachexia during cancer (Baracos et al., 2010), suggesting that there may be sex differences underlying CACS (Geary, 2001; Zhong & Zimmers, 2020). However, there are relatively few publications available on this topic (Montalvo et al., 2018). For example, the onset of anorexia has been shown to be different in male and female mice injected with methylcholanthrene sarcomas (Geary, 2001) and some cancer anorexia has been shown to be estradiol dependent (Mordes et al., 1984). The building evidence of sex differences points to the need for the use of female animals in the LLC tumour-bearing model in the future. In fact, comparing the two sexes would provide extremely valuable insight into CACS as a whole.

In the present study, we assessed the expression of MCH and orexin mRNA during CACS. While this provides an important insight into the role of these peptidergic neurons in CACS, there are a few critical caveats with this approach. Firstly, MCH and orexin mRNA levels may not directly correspond to neuropeptide levels or neuronal activity. Using ELISA or western blot could prove useful in measuring the levels of MCH and orexin and other neuropeptides involved in energy homeostasis. Neuronal activity can be assessed by electrophysiology or immunohistochemistry using cFos as a marker of neuronal activity. Secondly, both MCH and orexin neurons have co- neurotransmitters that can have entirely separate or additive effects. For example, MCH neurons contain GABA and glutamate, alongside other neuropeptides like neurotensin (Whiddon & Palmiter, 2013). By only measuring the primary neuropeptide produced by these cells, we

are missing secondary influences of these neurons. Moreover, measuring mRNA levels in the entire hypothalamus may not specify whether changes are due to changes in individual cells or cell number. For example, the decrease in orexin mRNA expression in LLC(low) mice could be due to a loss of orexin neurons or a decrease in orexin gene expression in individual orexin neurons. This question could be answered by counting cells that are immunoreactive to these neuropeptides.

In the future it would be interesting to investigate orexin neurons as therapeutic targets for CACS utilizing the LLC(low) model. Elevation of orexin was associated with stable food intake and body weight gain in young and old LLC(high) mice, and loss of orexin was associated with in significant anorexia in young LLC(low) mice. Furthering the potential of orexin neurons as a therapeutic target, changes orexin activity may be related to other negative implications of cancer. This includes the regulation of the sleep/wake cycle (Borniger et al., 2019), changes in mood or depression (Nollet & Leman, 2013), changes in hippocampal-dependent memory (Mavanji et al., 2017), or peripheral tumour growth rate (Blais et al., 2017). Thus, application of an orexin receptor agonist or orexin itself during CACS could provide beneficial in order to combat body weight loss and anorexia.

4-8 Conclusions and Implications

In summary, the results of this thesis suggest that LLC tumour-bearing mice are a good model for studying CACS under certain conditions. We found that injecting a lower number of LLC cells than that commonly used in other studies results in a consistent, robust anorexia and weight loss. These tumour-bearing mice displayed changes in the expression of MCH and orexin mRNA that encode neuropeptides known to be critical for the regulation of energy balance. The direction of the changes suggests that this is a counterregulatory response opposing the weight loss induced by tumour load. Therefore, both MCH and orexin may play a role in the initial protection against cancer-induced weight loss, and the failure to mount this homeostatic response may contribute to CACS. This implies that both the MCH and orexin systems may be therapeutic candidates for combatting CACS. Furthermore, their potential as a therapeutic target is exemplified due to their functions in not only food intake, metabolism, and physical activity, but also sleep regulation, mood, and general wellbeing.

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APPENDIX A: Animal Ethics Approval

3/3/2021

Animal Care Committee (ACC)
St. John's, NL, Canada A1C 5S7
Tel: 709 777-6620 acs@mun.ca
https://www.mun.ca/research/about/acs/acc/

Dear: Dr. Michiru Hirasawa, Faculty of Medicine\Division of BioMedical Sciences

Researcher Portal File No.: 20211267
Animal Care File: 18-01-MH
Entitled: (18-01-MH) Role of MCH neurons in cancer anorexia
Status: Active
Related Awards:

Awards File No	Title	Status	
20182137	Material Transfer Agreement - Not Publishable	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192205	Mechanisms of neuronal response to homeostatic challenges	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20201622	Role of hypothalamic neuropeptide in cancer anorexia and cachexia	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20201649	Role of hypothalamic neuropeptides in metabolic effects of cancer	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

Ethics Clearance Terminates: January 27, 2024

Your Animal Use Protocol has been renewed for a three-year term. The Animal Care ID [[18-01-MH]] remains the same, note the new file ID (if required) when referring to this protocol.

This ethics clearance includes the following Team Members: Dr. Michiru Hirasawa (Principal Investigator)
Dr. Kensuke Hirasawa (Co-Investigator)

This ethics clearance includes the following related awards:

Awards File No	Title	Status	
20182137	Material Transfer Agreement - Not Publishable	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20192205	Mechanisms of neuronal response to homeostatic challenges	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

20201622	Role of hypothalamic neuropeptide in cancer anorexia and cachexia	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20201649	Role of hypothalamic neuropeptides in metabolic effects of cancer	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

An Event [Annual Report] will be required following each year of protocol activity.

Should you encounter an unexpected incident that negatively affects animal welfare or the research project relating to animal use, please submit an Event [Incident Report].

Any alterations to the protocol requires prior submission and approval of an Event [Amendment].