DISSERTATION

PROOPIOMELANOCORTIN NEURON MANIPULATION IN MOUSE MODELS OF ENERGY BALANCE DISORDERS

Submitted by

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ABSTRACT

PROOPIOMELANOCORTIN NEURON MANIPULATION IN MOUSE MODELS OF ENERGY BALANCE DISORDERS

Proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) of the hypothalamus are critical regulators of energy balance. Highly conserved amongst mammalian species, POMC neurons release peptide transmitters to help an organism maintain appropriate levels of food intake and bodyweight by inhibiting feeding and facilitating metabolism of consumed nutrients. Disruptions in POMC signaling are thought to underlie aspects of energy balance disorders. There are two kinds of energy balance disorders: those of positive energy balance, which includes diseases like obesity, and those of negative energy balance, which includes eating disorders like anorexia nervosa (AN). Given that POMC neurons are believed to be dysregulated in energy balance disorders, treatment strategies for these disorders have focused on POMC neurons or their targets.

The goal of the studies discussed herein was to determine whether manipulation of POMC neurons could improve pathophysiological alterations in bodyweight and food intake in mouse models of energy balance disorders. Mouse models of AN and obesity were used in the current studies. AN was mimicked in the mouse via the well-validated activity-based anorexia (ABA) behavioral paradigm. The results shown in chapters 2 and 3 indicate that POMC neurons are selectively involved in generating food anticipatory activity (FAA) in mice undergoing ABA as disruption of either the POMC

peptide product β-endorphin or inhibition of the entire POMC neuron resulted in decreased FAA. As FAA is the primary output of the food entrainable oscillator (FEO), the circadian clock that allows an organism to anticipate the daily arrival of meals, these results suggest that POMC neurons via the peptide product β-endorphin are possibly involved in the expression of the FEO. As the identity of the FEO has yet to be determined, future studies should further characterize the contribution of β-endorphin and POMC neurons to the FEO.

To determine whether manipulation of POMC neurons is beneficial in a mouse model of obesity, mice fed an obesogenic diet were subjected to chronic POMC neuron stimulation for one month. The unexpected finding that sustained stimulation leads to weight gain as opposed to weight loss indicates that chronic stimulation of POMC neurons may not be a viable option for weight loss, at least under the dosing scheme used in the current study. How POMC neurons adapt to chronic stimulation remains unknown and should be the focus of future work.

ACKNOWLEDGEMENTS

Buckle up folks, it was a long and winding road to get here, and brevity is not exactly my forte…

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than I ever thought possible (though yes, I probably could add more weight to the bar). You must know that you are a very special group of people if I willingly do a workout called “Death Race” multiple times a year with you. I chipped away at this dissertation in much the same way you taught me to approach seemingly impossible WODs: one rep at a time until it’s done, and only then can you collapse on the floor.

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LIST OF ACRONYMS

α-MSH	Alpha-melanocyte stimulating hormone
ABA	Activity-based anorexia
A/P	Anterior/posterior plane
AgRP/NPY	Agouti-related peptide and neuropeptide Y
AN	Anorexia nervosa
ARC	Arcuate nucleus
β-endorphin	Beta-endorphin
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CNO	Clozapine-n-oxide
DMH	Dorsal medial hypothalamus
DREADD	Designer receptors exclusively activated by designer drugs
D/V	Dorsal/ventral plane
FAA	Food anticipatory activity
FDA	Food and Drug Administration
FEO	Food entrainable oscillator
GABA	Gamma-aminobutyric acid
gCaMP6f	Green fluorescent protein-calmodulin fusion protein 6 fast
GIRK	G protein inwardly rectifying potassium channel
GPCR	G-protein coupled receptor
GWAS	Genome-wide association study
HFD	High-fat diet
hM3Dq	Stimulatory DREADD receptor
hM4Di	Inhibitory DREADD receptor
i.p.	Intraperitoneal
LH	Lateral hypothalamus
mRNA	Messenger ribonucleic acid
MCR	Melanocortin receptor

ML	Medial/lateral plane
MOR	Mu opioid receptor
MSH	Melanocyte stimulating hormone
NPY	Neuropeptide Y
PCR	Polymerase chain reaction
PVN	Paraventricular nucleus of the hypothalamus
POMC	Proopiomelanocortin
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCN	Suprachiasmatic nucleus
VMH	Ventromedial hypothalamus

Chapter 1: Introduction

1.1 Overview

The hypothalamus is referred to as the master regulator of homeostatic function in the brain, overseeing control of life-sustaining processes including temperature, sleep/wake cycle, and energy homeostasis (Augustine 2017). How the brain regulates feeding and energy homeostasis has intrigued scientists for over a hundred years. In the early 1900s, the hypothalamus was identified as the part of the brain responsible for overseeing feeding behavior (Castro-Dufourny et al. 2017). Specific hypothalamic sub-regions and cell types have since been discovered that add to our understanding of how the brain controls feeding, yet there remains more to learn, including whether manipulation of these important cell types can correct disturbances in bodyweight and food intake. Specifically, this dissertation will examine whether manipulation of proopiomelanocortin neurons (POMC) in the arcuate nucleus (ARC) of the hypothalamus is beneficial in mouse models of obesity and anorexia nervosa (AN).

POMC neurons in the ARC terminate feeding via the peptide α-melanocyte stimulating hormone (α-MSH) acting at melanocortin receptors (MCRs) in the paraventricular nucleus (Mercer et al. 2013). In addition to α-MSH, POMC neurons also release the peptide β-endorphin, involved in both feeding and reward, as well as the neurotransmitters glutamate and gamma-aminobutyric acid (GABA) (Hentges et al. 2009; Dicken, Tooker, and Hentges 2012). POMC neurons are known to be dysregulated in both humans and animal models of obesity and AN. For instance, a missense mutation in the POMC gene is known to cause obesity in humans, dogs, and

mice (Krude et al. 1998; Raffan et al. 2016; Yaswen et al. 1999), and decreases in either *Pomc* mRNA or POMC peptide products have been associated with exposure to a high-fat diet in rodents (Souza et al. 2016; Çakir et al. 2013). In animal models of AN, transient elevations in *Pomc* mRNA have been observed, as well as increases in the POMC peptide β-endorphin (Hillebrand et al. 2006b; Aravich et al. 1993).

It remains unknown whether manipulation of POMC neurons could improve aberrant bodyweight and food intake in animal models of energy balance disorders. The studies presented here will address whether manipulation of POMC neurons or their peptide products can ameliorate aberrant food intake and bodyweight in animal models of obesity and AN. The results from the work presented here have the potential to inform new therapies for energy balance disorders, a critically important task given that these disorders affect millions of Americans and are responsible for billions of dollars in healthcare spending each year (CDC 2021).

1.2 Hypothalamic control of feeding behavior

> *"Here in this well-concealed spot, almost to be covered with a thumb-nail, lies the very mainspring of primitive existence- vegetative, emotional, reproductive- on which with more or less success, man has come to superimpose a cortex of inhibitions. The symptoms arising from disturbances of this ancestral apparatus are beginning to stand out in their true significance."*
>
> - *Harvey Cushing, 1929*[1]

> *"This bit of brain, 4 grams in weight, integrates almost all higher physiological functions…"*
>
> - *Fred Plum and Robert Van Uitert 1978*[2]

1.2.1 Historical Context

The quotes above refer to the hypothalamus, the brain's primary regulatory center of homeostatic processes. Though the quotes might sound hyperbolic, it is true that the hypothalamus – a small region located just beneath the thalamus in the forebrain (red sections, Figure 1.1) – is responsible for overseeing a myriad of tasks essential to life, including but not limited to regulation of thirst and hunger, blood pressure, body temperature, and sleep/wake cycle (Kalsbeek and Fliers 2016). To execute its many and diverse functions, the hypothalamus sends projections to numerous brain regions. The hypothalamus can be divided into 17 different sub regions referred to as nuclei (Clark 1936). Nuclei are typically named based on anatomic location; the ventromedial nucleus of the hypothalamus, for instance, is located in the ventral and medial section of the hypothalamus (Augustine 2017). Amazingly, an early schematic of the hypothalamic nuclei done by Clark in 1936 is not too different from modern-day depictions (Figure 1.2).

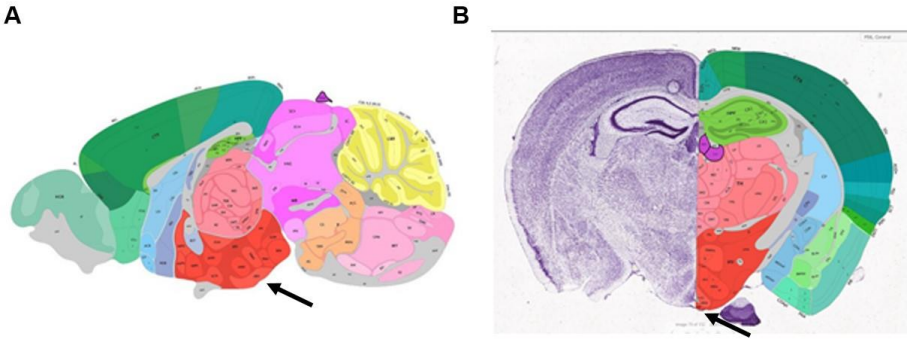

Figure 1.1 Neuroanatomical location of the hypothalamus and the ARC.
Schematic of the mouse brain at post-natal day 56 cut in the sagittal (A) and coronal (B) planes. The hypothalamus is shown in red. The arrow indicates the arcuate nucleus (ARC) of the hypothalamus. Adapted from the Allen Brain Reference Atlas. https://atlas.brain-map.org, accessed 5 July 2021.

The question of how the brain, and in particular, the hypothalamus regulates food intake is one that has intrigued scientists for over a hundred years. The earliest observations leading to the hypothesis that the hypothalamus governs control of food intake were based on case studies of individuals with suspected hypothalamic diseases (Brooks 1988). Alfred Frohlich first described a syndrome that would come to bear his name in 1901 in which a large pituitary tumor disrupted hypothalamic function and led the affected child to have an insatiable appetite, leading to morbid obesity amongst a constellation of endocrine abnormalities. Not everyone agreed that the hypothalamus was responsible for the clinicopathology observed by Frohlich; many thought it was the pituitary gland that was the issue. To solve this puzzle, the first experimental investigations of hypothalamic function were undertaken on a variety of mammalian species, including the dog and the cat. In what will become somewhat of a theme throughout the study of hypothalamic function, investigators like Harvey Cushing (who authored one of the quotes opening this chapter) were led astray in their conclusions based on imprecise methodologies. Cushing's laboratory performed what they believed to be only a hypophysectomy (removal of the pituitary gland), leading to stunted growth, increased fat mass, dull mentation, and underdeveloped secondary sexual characteristics (Cushing 1912), yet it turned out that Cushing's team had accidentally destroyed parts of the hypothalamus located right above the pituitary gland as they performed the surgery. More precise studies by Camus and Roussey would reveal damage to the hypothalamus alone to be the reason for the observed pathologies in the dog model (Castro-Dufourny et al. 2017).

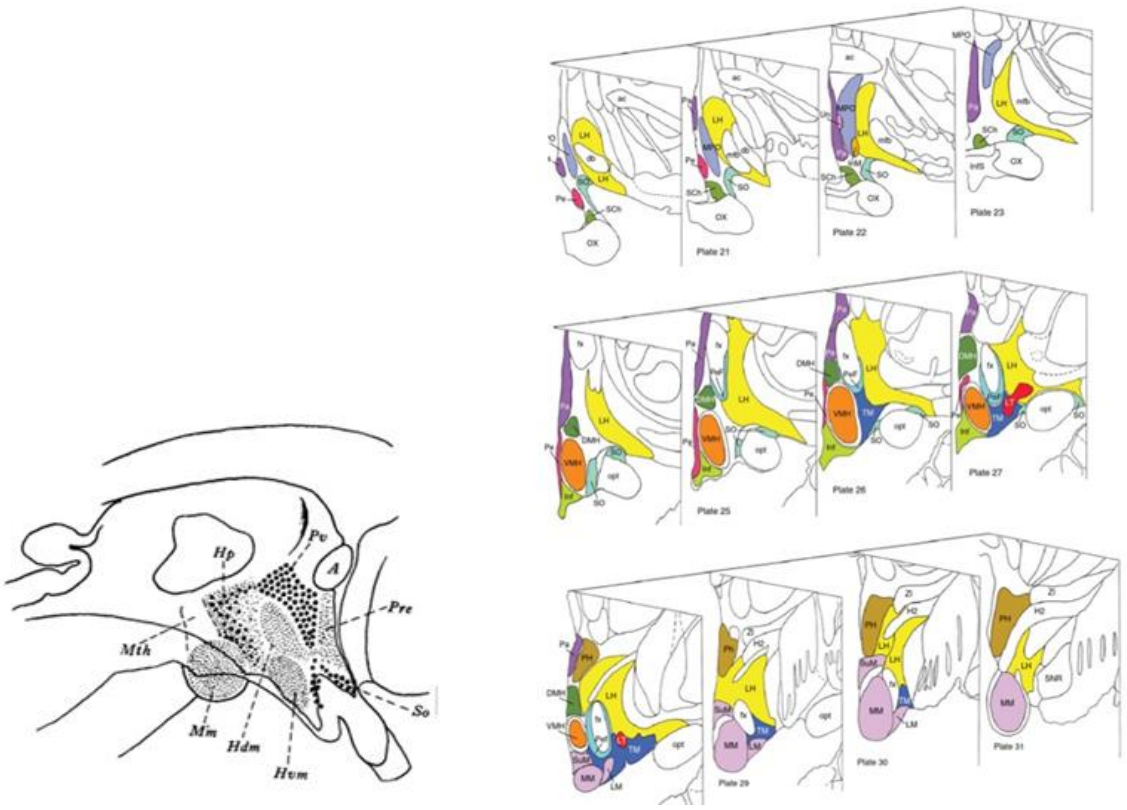

Figure 1.2 The subdivisions of the hypothalamus: then and now. Black and white image (left) is an early schematic of the subdivisions of the hypothalamus. Modified from source: Figure 1, Clark, 1936. Color image (right) is a modern-day schematic of the subdivisions of the hypothalamus presented along the rostral-caudal axis. Modified from Figure 19.5, Augustine, 2017. Hp = VMH, Pv = PVN, Hdm = DMH.

By the 1930s and 40s, the invention of the Horsley-Clarke stereotaxic surgery apparatus would make lesions into specific hypothalamic nuclei possible, allowing scientists to begin addressing more precisely where in the hypothalamus feeding regulation occurs. Early work by Hetherington and Ranson produced severely obese rats, though careful analysis of the lesion area revealed that multiple subnuclei were destroyed in their early experiments, including the dorso- and ventro-medial hypothalamus (VMH), the ARC, the fornix, and some of the lateral hypothalamus (LH), too (Hetherington and Ranson 1940). Refinement of the stereotaxic surgery technique

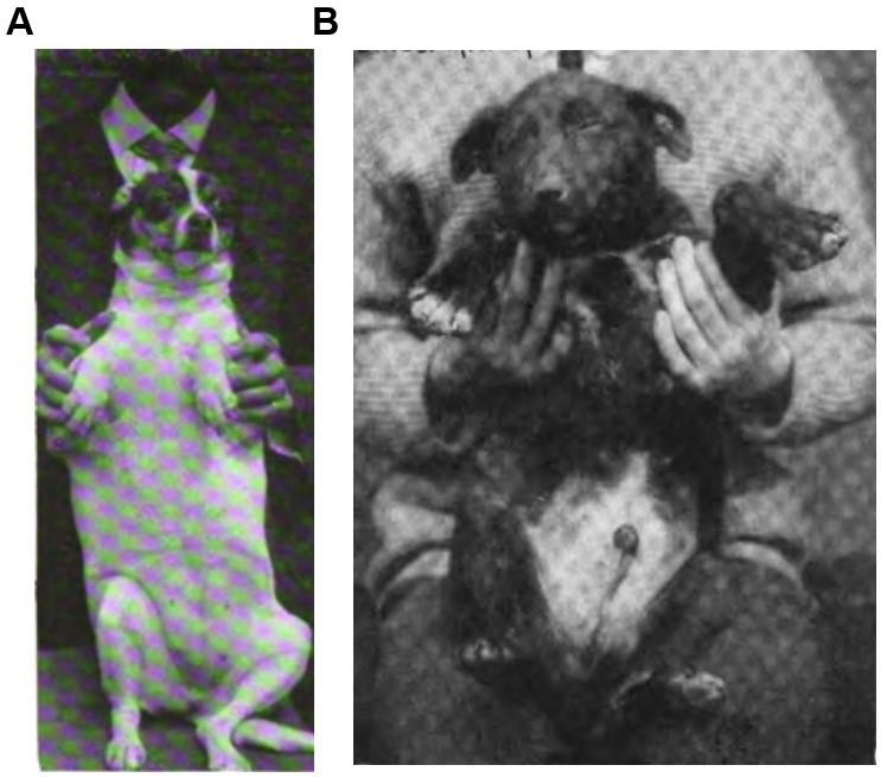

Figure 1.3 Early in vivo experiments of hypothalamic function. Two canine subjects after presumed hypophysectomy (later work would find concurrent hypothalamic damage via the surgical method used to disrupt the pituitary gland). Modified from Figure 9A and Figure 11B from Cushing, 1912 (digitized copy accessed 6 July 2021).

would lead Brobeck and colleagues (1943) to declare that the VMH is the satiety center of the hypothalamus, as they were able to recapitulate the obesity observed by Hetherington and Ranson when the lesion was limited to the VMH (or so they thought) (Brobeck, Tepperman, and Long 1943). Meanwhile, in similar lesion experiments undertaken by Anand and colleagues in the lateral hypothalamus (LH), the opposite was found and the hunger center believed to be identified: "...In four cats...eating was completely and permanently abolished in spite of [food availability]" (Anand, Dua, and Shoenberg 1955). Joliffe synthesized these findings by creating the term "appestat" to

describe the opposing roles of the VMH and LH in turning down or turning up hunger, much like a thermostat is adjusted (Jolliffe 1952).

While concise and seemingly accurate, the dual-center appestat model would be revised over the coming years based on new data. In a report titled, “The myth of the ventromedial nucleus,” Gold detailed his findings that VMH lesions do not cause obesity as the autonomic afferents of the PVN were also damaged in the original Hetherington and Ransom studies (Gold 1973). Similarly, the marked aphagia observed in the studies by Anand and colleagues was due to motor control dysfunction caused by damage to the nigrostriatal bundle just lateral to the LH (Ungerstedt and Arbuthnott 1970). Scientists were left wondering if not the VMH or LH, then where were the satiety and hunger centers? In time, both the VMH and LH would be reinstated as important for regulating feeding, just not in the exact manner that the scientists of the 1940s and 50s initially believed; meanwhile, the ARC would become the focus of intense study in the feeding field.

1.2.2 The arcuate nucleus

The ARC, sometimes referred to as the infundibular nucleus, is in the mediobasal hypothalamus, adjacent to the median eminence and the third ventricle, which gives the ARC privileged access to circulating peripheral signals. In the search for the hypothalamic satiety center, interest in the ARC was initially based on this region’s proximity to circulating hormones (Elmquist, Elias, and Saper 1999), as well as the fact that systemic administration of gold thioglucose (a toxic glucose analog) or monosodium glutamate cause localized destruction of the ARC and the VMH (Debons et al. 1962; Olney 1969). Scientists got to work characterizing the ARC; using Golgi and Nissl

stains, work from the 1980s found neurons within the ARC to be smaller than the neurons of the neighboring VMH, with one to four long, thin dendrites that can span considerable distances (Van Den Pol and Cassidy 1982) (Figure 1.4). Around the same time over 15 neuropeptides and neurotransmitters were identified in ARC neurons based on immunohistological studies, including peptide products of the preprohormone POMC (i.e., α-MSH and β-endorphin), neuropeptide Y (NPY), dopamine, and GABA (summarized in the review by (Chronwall 1985).

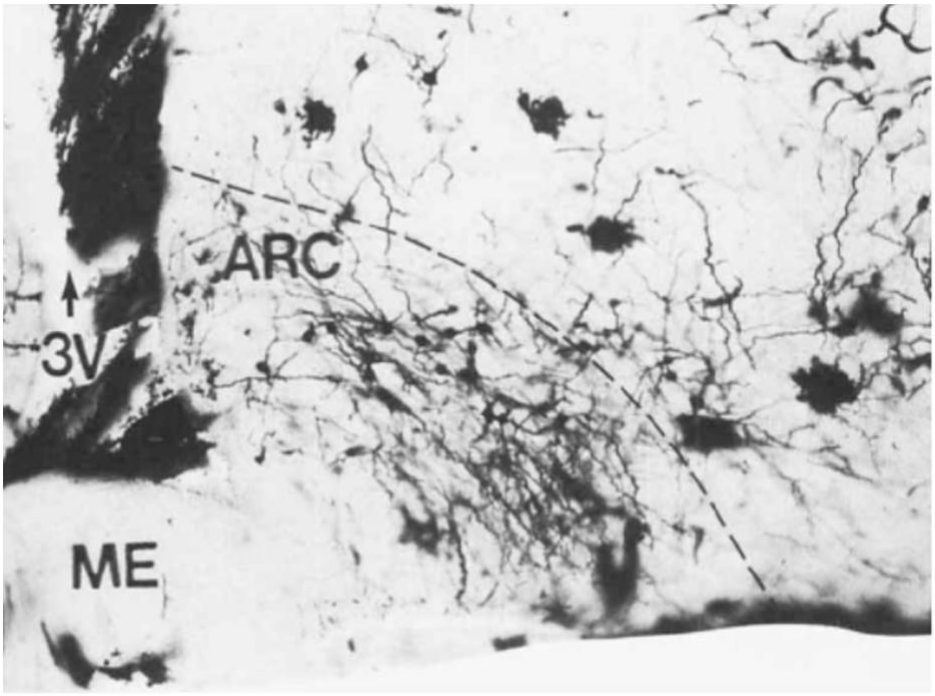

Figure 1.4 Cells within the ARC of the hypothalamus. Coronal section showing neurons in the arcuate nucleus (ARC) visualized with the Golgi stain method. Modified from Figure 3, Van Den Pol and Cassidy, 1982. 3V = 3rd ventricle. ME = median eminence.

Subsequent work would determine that two major cell types within the ARC contain these varied peptides and transmitters and the cell types were named for peptide products they release: POMC neurons and AGRP (agouti-related peptide)

neurons. It was soon realized that these cell types had seemingly opposing actions on feeding behavior: while POMC neurons signal satiety via their peptide product α-MSH acting at MCRs, AGRP neurons promote feeding behavior via its peptide product known by the same name (Fan et al. 1997; Huszar et al. 1997; Ollmann et al. 1997). The canonical view of AGRP and POMC neurons as functionally opposed would become complicated however by the heterogenous nature of POMC neurons, discussed in the subsequent section after a brief introduction to POMC neurons.

1.2.3 Proopiomelanocortin neurons

The vitally important role that POMC neurons play in energy homeostasis was confirmed in much the same way that investigators in the 1800s and 1900s figured out that the hypothalamus was critical for regulating food intake: via case study. While basic laboratory science was finding α-MSH to be a critical signaler of satiety (Fan et al. 1997; Huszar et al. 1997), Krude et al. (1998) published the first report of a genetic mutation in humans leading to a lack of production of multiple POMC peptides including α-MSH which led to morbid obesity very early in life (Krude et al. 1998), Figure 1.5. A similar genetic mutation was later recapitulated in the mouse (Yaswen et al. 1999).

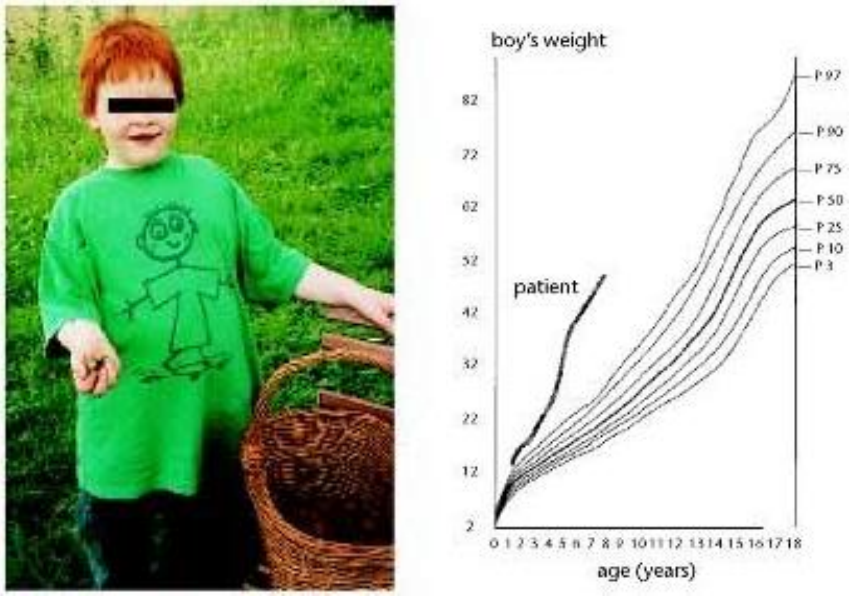

Figure 1.5 Photo and growth curve of a young boy with a POMC missense mutation. A young boy depicted at age 5 (left) with a homozygous mutation in exon 2 of the *POMC* gene, leading to lack of *POMC* translation. The growth curve on the right shows his weight trajectory. He was normal weight at birth but obesity was noted by 5 months of age. Modified from Figure 1, Krude et al., 1998.

There are between 3,000 and 9,000 POMC neurons in the mouse ARC (Cowley et al. 2001; Lemus et al. 2015). POMC neuron cell bodies are either round or fusiform in shape and are roughly 25 µM in diameter (Bugnon, Bloch, and Lenys 1981) (Figure 1.6). POMC neurons receive inputs from diverse brain regions, though most of the inputs come from other hypothalamic nuclei (D. Wang et al. 2015) (Figure 1.7). In addition to diverse inputs, POMC neurons also project to varied brain regions, including other hypothalamic nuclei, the ventral tegmental area, periaqueductal grey, bed nucleus of the stria terminalis, amygdala, and the dorsal vagal complex (King and Hentges 2011).

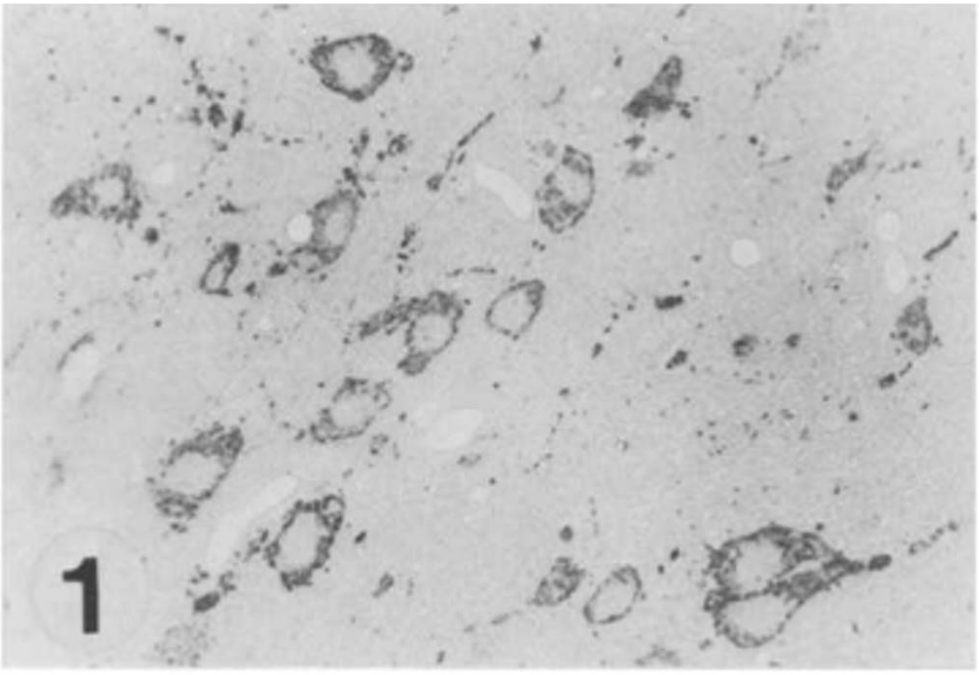

Figure 1.6 POMC neurons in the ARC. Presumptive POMC neurons identified via immunohistochemical labeling of ACTH17-39. Modified from Figure 1, Bugnon, Buch, and Lenys, 1981.

POMC neurons are sensitive to energy state; *Pomc* mRNA synthesis decreases in response to fasting and increases in response to satiety cues such as leptin (Mizuno et al. 1998), though a paradoxical increase in *Pomc* mRNA is transiently observed in the early days of animals undergoing an animal model of AN (Daimon and Hentges 2021). POMC neurons increase firing at the mere sight of food, likely in anticipation of the metabolic response required once eating is initiated (Chen et al. 2015).

Perhaps unsurprisingly given their diversity in afferents and efferents, POMC neurons can respond to a variety of inputs via release of various peptide products or neurotransmitters. Subpopulations of POMC neurons have been found to express receptors for leptin (Cowley et al. 2001), insulin (Qiu et al. 2014), and serotonin (Berglund et al. 2013). In addition to α-MSH, POMC neurons also release β-endorphin,

an endogenous opioid known for its canonical role in reward signaling, but that also affects food intake and energy homeostasis. The role of β-endorphin in feeding behavior is more complex than α-MSH, as both intracerebroventricular administration and genetic knockout of β-endorphin leads to increased food intake and increased bodyweight (Silva et al. 2001; Appleyard et al. 2003). In addition to food intake, POMC neurons also contribute to glucose homeostasis (Berglund et al. 2013; Parton et al. 2007), participate in the regulation of the stress response (Greenman et al. 2013; Qu et al. 2020)and aversion related behaviors (Klawonn et al. 2018).

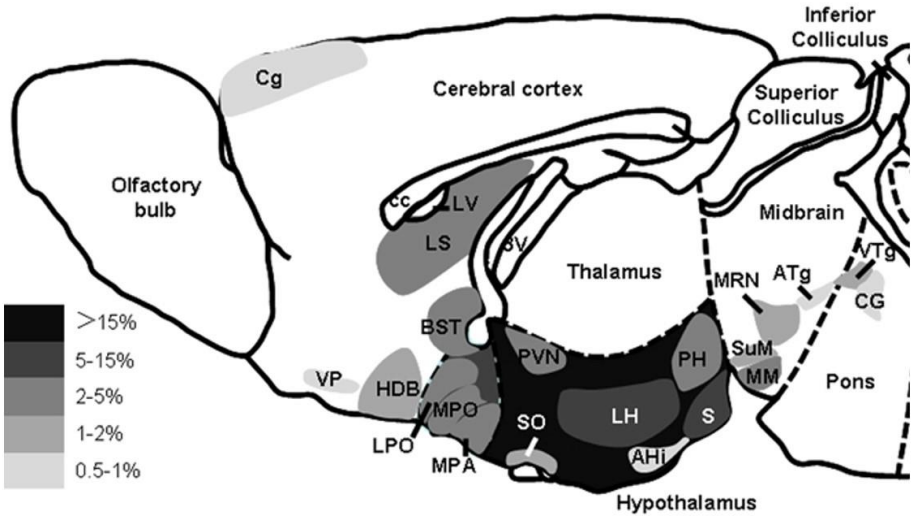

Figure 1.7 Inputs to POMC neurons. Darker shading indicates a higher percentage of inputs to POMC neurons. Modified from Figure 5, Wang et al., 2015.

1.3 When homeostatic mechanisms go awry: Disorders of energy balance

Energy balance disorders such as obesity and eating disorders pose a major health risk to millions of people worldwide. In the US, the prevalence of obesity was 42.4% in 2017, an increase of about 10% since 2000 (CDC 2021). One is considered

overweight if their body mass index (BMI) is between 25 and 30; one is considered obese if their BMI is greater than 30. The CDC also estimates that the economic burden of managing obesity-related healthcare costs amounts to $147 billion dollars a year (CDC 2021).

Obesity is not a uniquely human disease; nearly two-thirds of companion felines and roughly 6 out of 10 canines are overweight or obese. Overweight or obese dogs live 2.5 years less than normal weight dogs (Salt et al. 2019). Veterinarians use a Body Condition Score rather than BMI to determine if a pet is overweight or obese. A 4 or 5 on a 9-point scale indicates that the pet is of a normal weight, while a score of 7 indicates that the pet is overweight and an 8 or a 9 signals obesity (Laflamme 1997).

Figure 1.8 The Body Condition Score system. The Body Condition Score system is used in veterinary medicine over the Body Mass Index system to determine if an animal is overweight or obese. An animal is considered an ideal weight if they rate as a 4 or a 5. Modified from an open access resource provided to veterinarians by the World Small Animal Veterinary Association.

While not as common as overweight and obesity, eating disorders such as AN are also associated with increased risk of death and medical complications. In the United States, the lifetime prevalence of developing AN is 0.69% in women and 0.19% in men (Duncan, Ziobrowski, and Nicol 2017). A diagnosis of AN is warranted if the following criteria are met: 1) the individual restricts food intake to such a degree that significant bodyweight loss is achieved, or, in the case of growing adolescents, expected weight gain commensurate with growth is not achieved; 2) the individual displays an intense fear of weight gain; and 3) the individual's conception of self-worth is tied to bodyweight and/or shape (the individual is disturbed by their body weight/image) (American Psychiatric Association 2013). An individual is considered anorexic if their BMI is less than 17 (severely restricted feeding behaviors in individuals with a BMI greater than 17 have what is referred to as atypical AN). Like obesity, the societal and economic burden of eating disorders is vast: in 2018-2019, 64.7 billion dollars was spent on healthcare to manage eating disorders and the myriad of complications that come from chronic undernutrition (Streatfeild et al. 2021). While companion animals do not (as far as we know) suffer the psychological effects of eating disorders like their human counterparts, many common disease states often lead to inappetence in companion animals, including congestive heart failure, chronic kidney disease, and certain cancers (Freeman 2012).

1.3.1 Obesity and POMC

The POMC null mutations discussed previously in humans and rodents clearly suggested a significant role for POMC in regulating bodyweight and food intake (Krude et al. 1998; Yaswen et al. 1999). Interestingly, this mutation has also been detected in

the Labrador Retriever breed, where it is present at much higher rates than in the human population (Raffan et al. 2016). While the POMC null mutation is rare in humans, POMC dysregulation is one of the earliest changes that can be detected in obesity-prone mice compared to obesity-resistant mice fed a high-fat diet (Souza et al. 2016). Relative to chow fed controls, Souza et al. (2016) found that *Pomc* mRNA levels are increased in obesity-resistant mice, while *Pomc* mRNA levels are decreased in obesity-prone mice. Huang et al. (2003) also found that *Pomc* mRNA decreases following 4 months on a 40% high-fat diet; additionally, the authors show that if mice fed a high-fat diet are then switched to a low-fat diet or a diet high in polyunsaturated fats for 6 weeks, *Pomc* mRNA levels go back up as if the rodents had never received the initial high-fat diet (Huang et al. 2003). However, (Enriori et al. 2007) and (Çakir et al. 2013) have both reported no change to *Pomc* mRNA levels in rodents after 20 or 12 weeks on a high-fat diet, respectively, though Cakir et al. (2013) did find a significant decrease in α-MSH on a high-fat diet (Çakir et al. 2013). In postpubescent obese adolescents, weight loss was associated with increased levels of α-MSH compared to baseline (Dâmaso et al. 2013). The apparent discrepancies in the data are likely due to the complex nature by which POMC peptides both drive and respond to changes in bodyweight and food intake.

1.3.2 Anorexia Nervosa and POMC

Given the lack of data available from prospective studies or studies of individuals early in the disease state, it is not very surprising that there are few generalizations that can be made when it comes to the relationship between AN and POMC. The finding that has held up most strongly across studies is that women diagnosed with AN have lower plasma leptin levels compared to controls (thin women who do not have an eating

disorder); however, Karageorgiou and colleagues as discuss in their meta-analysis, considerable overlap in leptin levels exist between patients and controls and therefore leptin is not helpful as a plasma biomarker (Karageorgiou et al. 2020). A study by Galusca et al. (2015) found plasma α-MSH levels to be significantly lower on average in women with AN compared to controls, though abnormal nocturnal peaks in α-MSH were observed in the AN patients (Galusca et al. 2015). Plasma β-endorphin levels are higher in hospitalized women with AN compared to normal weight controls, and following a meal delivered through a nasogastric tube, plasma β-endorphin levels significantly decreased in women with AN, a response not observed in normal weight controls (Rigaud et al. 2007). Both α-MSH and β-endorphin have been implicated in an animal model of AN referred to as activity-based anorexia (ABA; discussed in detail in subsequent chapters). Administration of α-MSH has been shown to exacerbate ABA, while others have found both increased and decreased *Pomc* mRNA in rodents undergoing ABA, though this discrepancy can be attributed to whether investigators examined mRNA levels during early- or late-stage ABA (de Rijke et al. 2005; Hillebrand et al. 2006a). Whether POMC neurons or their peptide products can be manipulated to improve ABA is unknown, however.

1.4 Aims of the Present Studies

In the chapters that follow, three studies will be described that were undertaken to determine whether manipulation of POMC neurons in animal models of energy balance disorders could ameliorate the negative effects these states have on bodyweight and food intake. Chapters 2 and 3 discuss the effects of manipulating the POMC peptide β-endorphin specifically (Chapter 2) or the entire POMC neuron

(Chapter 3) in an animal model of AN. Chapter 4 discusses the effect of activating POMC neurons in mice fed an obesogenic diet.

Notes

[1] As quoted in the following reference: Augustine, James R. 2017. “Chapter 19: The Hypothalamus.” In *Human Neuroanatomy*, 2nd ed. Hoboken: John Wiley & Sons, Inc.

[2] As quoted in the following reference: Brooks, Chandler Mc C. 1988. “The History of Thought Concerning the Hypothalamus and Its Functions.” *Brain Research Bulletin* 20 (6). https://doi.org/10.1016/0361-9230(88)90075-5.

Chapter 2: β-endorphin differentially contributes to food anticipatory activity in male and female mice undergoing activity-based anorexia

2.1 Overview

While the loss-of-function POMC mutations clearly identified POMC neurons as critical for maintaining a healthy bodyweight, the role of POMC neurons in diseases related to chronic energy deficit is less well-defined. Several factors likely contribute to this relative dearth in knowledge. First, obesity is far more common worldwide than eating disorders of any sort; there are simply more humans with obesity than there are humans with AN. Indeed, two of the first genome-wide association studies (GWAS) on obesity were published back in 2007 (Frayling et al. 2007; Scuteri et al. 2007), while it was only in 2019 that the first adequately powered GWAS study of AN was published (Watson et al. 2019). Second, AN typically goes undiagnosed for years, during which time long-term malnutrition and starvation wreaks havoc on a person's physiology. Comparisons of AN patients to healthy controls become difficult to interpret as it is unclear whether the observed pathology is a cause or consequence of the disease (Hay and Sachdev 2011). Finally, simply selecting inclusion criteria for a human study is difficult, as the researcher must consider factors such as whether the individual was treated in an in-patient versus outpatient treatment setting, previous history of treatment for an eating disorder, presence or absence of a feeding tube, etc.

The intense fear of gaining weight may be a uniquely human experience, but animal models that recapitulate other features of eating disorders are critically important tools for identifying early changes in neurobiology associated with eating disorders. In

the following chapter, the activity-based anorexia (ABA) rodent model of AN is used to investigate the role that β-endorphin potentially plays in the development of ABA. This chapter was first published in the open access journal *Physiological Reports* on March 4th, 2021 in Volume 9, Issue 5 under the same title as this chapter. Slight modifications were made to meet formatting requirements of the dissertation. Note data in chapter 2 are presented as mean±SD to comply with journal standards, while data presented in later chapters are presented as mean±SEM. I designed and performed all the experiments described herein under the supervision of Shane Hentges.

2.2 Summary

Anorexia nervosa (AN) has a lifetime prevalence of up to 4% and a high mortality rate (~5-10%), yet little is known regarding the etiology of this disease. In an attempt to fill the gaps in knowledge, activity-based anorexia (ABA) in rodents has been a widely used model as it mimics several key features of AN including severely restricted food intake and excessive exercise. Using this model, a role for the hypothalamic proopiomelanocortin (POMC) system has been implicated in the development of ABA as *Pomc* mRNA is elevated in female rats undergoing the ABA paradigm. Since the *Pomc* gene product α-MSH potently inhibits food intake, it could be that elevated α-MSH might promote ABA. However, the α-MSH receptor antagonist SHU9119 does not protect against the development of ABA. Interestingly, it has also been shown that female mice lacking the mu opioid receptor (MOR), the primary receptor activated by the *Pomc*-gene-derived opioid β-endorphin, display blunted food anticipatory behavior (FAA), a key feature of ABA. Thus, we hypothesized that the elevation in *Pomc* mRNA

observed during ABA may lead to increased β-endorphin concentrations and MOR activation to promote ABA. Further, given the known sex differences in AN and ABA, we hypothesized that MORs may contribute differentially in male and female mice. Using wild-type and MOR knockout mice of both sexes, a MOR antagonist and careful analysis of food anticipatory behavior and β-endorphin levels, we found 1) increased *Pomc* mRNA levels in both female and male mice that underwent ABA, 2) increased β-endorphin in female mice that underwent ABA, and 3) blunted FAA in both sexes in response to MOR genetic deletion yet blunted FAA only in males in response to MOR antagonism. The results presented provide support for both hypotheses and suggest that it may be the β-endorphin resulting from increased *Pomc* transcription that supports the development of some features of ABA.

2.3 Introduction

Anorexia nervosa (AN) has a lifetime prevalence of up to 4% in women and less than 1% in men (Keski-Rahkonen & Mustelin, 2016; Smink et al., 2012) and has a high mortality rate at roughly 5-10% (Arcelus et al., 2011). Diagnostic criteria for AN include low bodyweight, intense fear of gaining weight, and disturbed body image perception (American Psychiatric Association 2013). While not a formal criterion for diagnosis, excessive exercise is an extremely common feature observed in AN patients, with one study reporting the behavior in over 80% of those surveyed (Casper et al., 2020; Rizk et al., 2020). Unfortunately, no prevention or early intervention strategies for AN currently exist despite an obvious need. Moreover, while current treatment therapies are often initially effective, relapse frequently occurs (Khalsa et al. 2017). Despite the severity of this disease, surprisingly little is known regarding the neurobiological basis of AN (Zipfel

et al. 2015). It is crucial that the gaps in knowledge be addressed to facilitate the development of novel therapeutics for AN.

Studies using animal models have been instrumental in probing the underlying mechanisms of AN; one of the most widely used and well-established animal models is activity-based anorexia (ABA) (Klenotich and Dulawa 2012). ABA closely mimics several key features of AN including severely restricted feeding and excessive exercise (Welch et al., 2018). In ABA, timed feedings restricted to limited hours of the day paired with access to a running wheel results in pronounced reductions in food intake and body weight loss as well as pronounced increases in wheel running activity. Wheel running activity is particularly increased in the hours preceding food presentation during the light cycle in which rodents are typically inactive, a phenomenon referred to food anticipatory activity (FAA; Mistlberger, 1994). Remarkably, animals will continue to lose weight and engage in wheel running to the point of exhaustion and death, as noted in the original reports describing this phenomenon over 50 years ago (Hall & Hanford, 1954; Routtenberg & Kuznesof, 1967). Sex differences have been reported in animals undergoing ABA though this difference remains incompletely understood as both sexes have been identified as more vulnerable to ABA compared to the other (females more vulnerable: Figure 1 in Klenotich & Dulawa, 2012, Pare et al. 1978; males more vulnerable: Achamrah et al 2017; Doerries et al 1991). Despite the evidence suggesting a sex difference is likely albeit incompletely understood, many ABA experiments previously were only conducted in one sex, usually female, given that females are diagnosed with AN in greater numbers than males (Zipfel et al. 2015).

Using the ABA model, a role for the hypothalamic proopiomelanocortin (POMC) system has been implicated in the development of ABA as *Pomc* mRNA is transiently elevated in female rats undergoing ABA (Hillebrand et al., 2006). As a prohormone, POMC is enzymatically cleaved in multiple bioactive peptide products (Cawley et al., 2016; Harno et al., 2018) and previous investigations of POMC involvement in ABA have focused primarily on the cleavage product α-melanocyte stimulating hormone (α-MSH) given its ability to robustly inhibit feeding via activation of the melanocortin-4 receptor (Fan et al., 1997; Huszar et al., 1997). Yet while exogenous administration of α-MSH was found to exacerbate ABA (Hillebrand et al., 2005), subsequent experiments in which the melanocortin receptor antagonist SHU9119 was administered showed the drug incapable of ameliorating ABA (Hillebrand et al., 2006). Interestingly, it has also been shown that female mice lacking the mu opioid receptor (MOR), the primary receptor activated by the *Pomc*-gene-derived opioid β-endorphin, display blunted food anticipatory behavior, a key feature of ABA (Kas et al., 2004).

In the current study, we hypothesized that the elevation in *Pomc* mRNA observed during ABA may lead to increased β-endorphin action and MOR activation to promote ABA. Further, given that AN disproportionately affects women, we hypothesized that MORs may contribute differentially in male and female mice. We first confirm the findings reported by Hillebrand and colleagues in female rats that *Pomc* mRNA is transiently elevated in female mice undergoing ABA as well as report a similar finding in male mice. We then show that circulating levels of β-endorphin increase in response to the ABA paradigm and that inhibiting MOR activation during ABA selectively reduces FAA but does not alter bodyweight or food intake in either male or female mice. Finally,

we found a sex- and behavior-specific difference between the genetic deletion of MORs and pharmacologic inhibition of these receptors. These results indicate a potential sex-specific degree of involvement of the β-endorphin system in ABA and suggest that there could be a need for sex-specific approaches to treatment in patients with AN.

2.4 Materials and Methods

Animals: Mice were initially acquired from the Jackson Laboratory (C57BL/6J, 000664 and B6.129S2-*Oprm*1^{tm1Kff}/J, 007559) and bred at Colorado State University. Standard PCR techniques were used to genotype animals. Male and female mice aged 2-6 months were used in all experiments. Animals were maintained on a 12/12-hour light/dark cycle with *ad libitum* access to food and water unless stated otherwise. In the breeding room where animals were housed prior to entering an experiment, lights turned on at 0600 h. The experimental procedure room was on a modified light/dark cycle with lights turning on at 0200 h. Following transfer to the procedure room, all animals were given a minimum of 10 days to adjust to the altered light/dark cycle. During the acclimation period of ABA (discussed in detail below), wheel running activity was collected and analyzed to verify that proper adjustment to the altered light/dark cycle had occurred. All mice adjusted to the altered light/dark cycle prior to the start of the experiment. Room temperature was kept constant in both the breeding facility as well as the experimental test room at 20-22°C.

Activity-based anorexia model: The activity-based anorexia (ABA) model is a well-validated, commonly used behavioral paradigm in which access to a running wheel paired with restricted feeding results in severe weight loss and reductions in food intake, in addition to increased wheel running activity (Klenotich and Dulawa 2012). At the start

of all experiments, mice were singly housed in clean cages equipped with a running wheel (catalog # 0297; Columbus Instruments, Columbus, OH). Mice were given 3 days to acclimate to their new environment during which Multi Device Interface Software (Columbus Instruments, Columbus, OH) detected the total number of wheel revolutions every 15 minutes. Data were collected during acclimation for two reasons: first, to confirm proper adjustment to the altered light/dark cycle and second, to determine whether the mouse exhibits sufficient baseline wheel running activity to warrant moving forward in the experiment. Mice running less than 1500 revolutions a day were considered non-runners and were not used for ABA. Following the acclimation period, baseline daily bodyweight and food intake values were collected 1 hour prior to lights out for 5 days. Wheel running activity continued to be monitored every 15 minutes. Following baseline data collection, the ABA paradigm or relevant control condition was initiated. Food restricted animals were given access to chow for 2 hours a day, presented at the start of the dark cycle. Males and females were always run separately and cages and wheels were thoroughly cleaned in between cohort runs. Control conditions included mice in cages where running wheels were provided but locked in place to create a food restricted without wheel-running condition (FR only) and *ad libitum* fed animals provided access to a running wheel (WHL only).

Assessment of the temporal dynamics of Pomc mRNA expression or β-endorphin concentration: For determination of *Pomc* mRNA and β-endorphin levels, male and female mice were sacrificed at lights-out after varying lengths of exposure (1 day, 2 days, or 3 days) to the ABA paradigm (FR + WHL) or one of the control conditions. No differences in *Pomc* mRNA expression were detected in either control condition

regardless of day of sacrifice; as such, control data were pooled. At sacrifice, animals were first deeply anesthetized with 200 mg/kg sodium pentobarbital solution (Fatal-Plus, Vortech Pharmaceuticals, Ltd, Dearborn, MI) and lack of deep pain reflex confirmed before transcardial perfusion with 10% sucrose followed with 4% paraformaldehyde in potassium phosphate-buffered saline. Brains were then stored at 4°C in 4% paraformaldehyde until sectioned. Whole blood was collected from the right atrium prior to perfusion and allowed to clot for 30 minutes at room temperature prior to centrifugation at 3,000 rpm x 20 minutes at 4°C. Serum was removed and stored at -80°C.

Fluorescent in situ hybridization: Fluorescent *in situ* hybridization was used to detect *Pomc* mRNA as previously described (Dennison et al., 2016; Jarvie et al., 2017). In brief, brains stored at 4°C in 4% paraformaldehyde were sliced into coronal sections (50 μM) spanning the rostral-caudal axis of the arcuate nucleus. Slices were then incubated at room temperature sequentially in: 6% hydrogen peroxide, Proteinase K (10 μg/ml), glycine (2 mg/ml), post-fixation solution containing 4% paraformaldehyde and 0.2% glutaraldehyde and finally ascending concentrations of ethanol prior to incubation in hybridization solution for 1 h at 60°C (66% (v/v) deionized formamide, 13% (w/v) dextran sulfate, 60 mM NaCl, 1.3x Denhardt's solution, 13 mM Tris-Hcl, pH 8.0, 1.3 mM EDTA, pH 8.0). The *Pomc* probe (0.25 pg/ml, corresponding to bases 531-1000 of GenBank sequence NM_08895.3) was denatured for 5 minutes at 85°C, added to the hybridization solution, and hybridized at 70°C for 18-20 hours. Brain slices were washed in saline sodium citrate buffer post-hybridization before detection of the fluorescein isothiocyanate-labelled *Pomc* probe with a secondary antibody conjugated to Alexa

Fluor 488 (1:400, Invitrogen/Thermo Fisher Scientific, Waltham, MA). Tissue sections were mounted on glass slides, cover-slipped, and stored at 4°C for later image collection and analysis.

Image collection and analysis: All images were collected on a Zeiss 800 confocal microscope at 40x. Imaging parameters were kept consistent between experiments and each experiment contained both control and experimental animals. For each animal, a minimum of 10 tiled z-stack images taken from 10 brain slices at 1 μm intervals were obtained containing both sides of the arcuate nucleus. *Pomc*-expressing cells labeled with AlexaFluor-488 were identified using masks generated in ImageJ. The fluorescent intensity of each *Pomc*-expressing cell was expressed as a percentage of background fluorescence intensity for that given image. An overall average of fluorescent intensity above background was generated for each animal by averaging the values collected from individual z-stack images.

Radioimmunoassay: Peptide extraction and β-endorphin measurement on previously stored serum samples were performed using a commercial radioimmunoassay kit according to the manufacturer's instructions (RK-022-33, Phoenix Pharmaceuticals, Inc., Burlingame, CA). In brief, samples were incubated overnight at 4°C with rabbit anti-β-endorphin antibody, followed by another overnight incubation with ^{125}I-β-endorphin. Samples were then incubated with goat-anti-rabbit IgG serum and normal rabbit serum, centrifuged, and the supernatant discarded prior to detection of bound ^{125}I- β-endorphin in the remaining pellet with a gamma-counter (Perkin-Elmer, Waltham, MA). A standard curve was generated from which the concentration of β-

endorphin present in each sample was extrapolated. The detection range of the kit used is 10-1280 pg/ml.

Disruption of MOR signaling: To determine whether MORs contribute differentially in male and female mice to ABA, MOR function was inhibited in two ways: first by genetic deletion of the MOR using knockout mice discussed above, and second by administration of the MOR antagonist naloxone hydrochloride to wildtype animals (NAL, 5 mg/kg i.p., Sigma-Aldrich, St. Louis, MO). NAL was administered twice daily at 0.5 hours until lights out and again 4.5 hours later. Saline-treated control animals received two injections of 0.9% NaCl sterile saline solution (0.1 mL) at the same time. Animals were first habituated to i.p. injections of saline solution during baseline data collection (one injection per day). Unlike previous ABA experiments in which animals were sacrificed on a predetermined day, animals in these experiments were allowed to proceed through ABA uninterrupted until either 20% of initial bodyweight had been lost or 6 days had passed, at which point all animals were removed from the study. Upon completion of the experiment, animals were humanely euthanized.

Statistical Analyses: Detailed information regarding specific statistical tests used are given in the results section. All data were analyzed using Prism (GraphPad Software Inc., San Diego, CA). Data are presented as mean±SD. Differences were considered significant when $p \leq 0.05$.

2.5 Results

Activity-based anorexia (ABA) can be reliably generated in wildtype mice

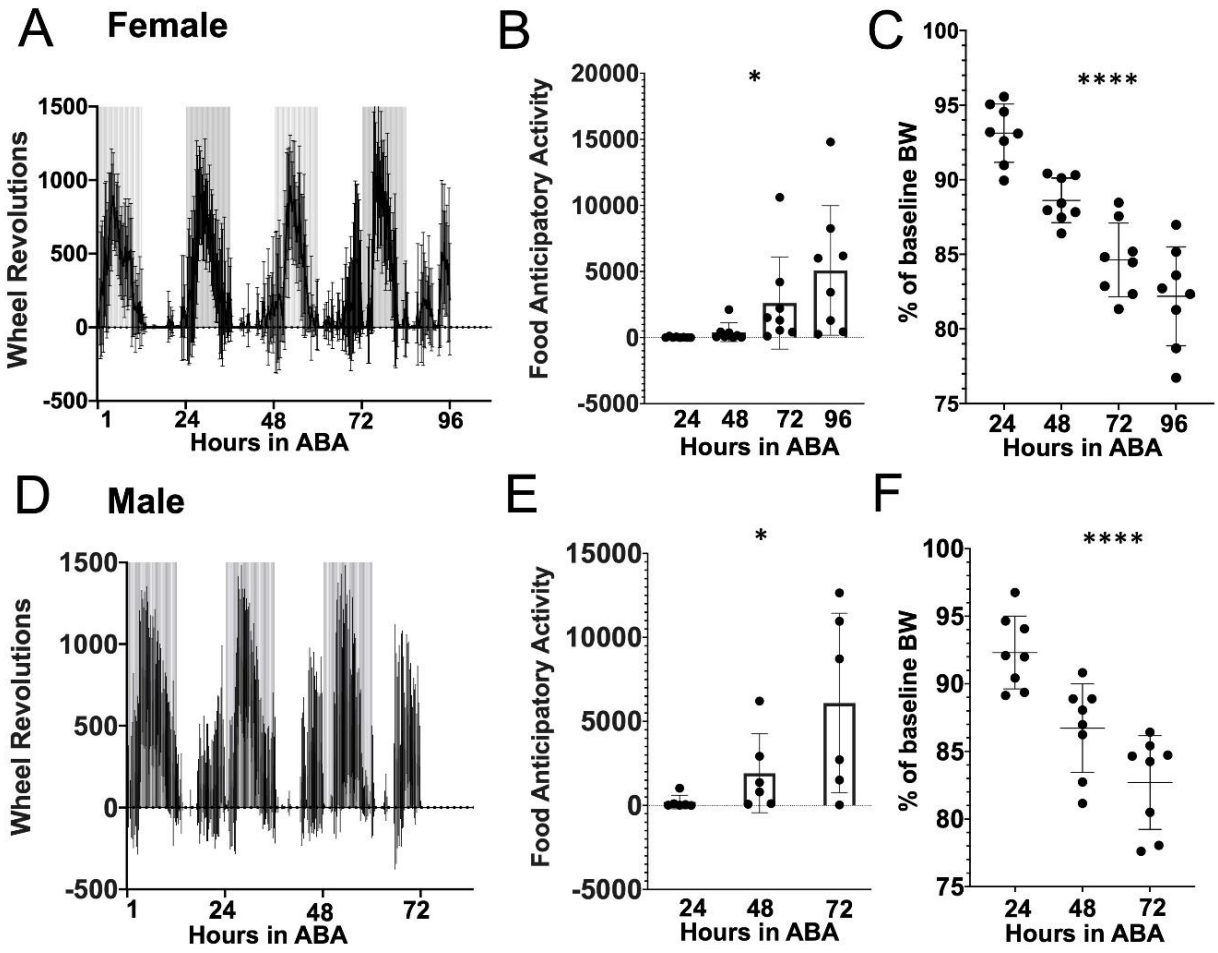

Figure 2.1. Activity-based anorexia (ABA) can be reliably generated in wildtype mice. Wheel revolutions per 15-minute time bin over the course of 3 days in ABA are shown for female (A) and male (D) mice. Gray bars denote the dark cycle. Wheel revolutions during FAA, the four hours preceding food presentation, are shown in panels B (female, n=8; * = p = 0.0224) and E (male, n=7; * = p = 0.0334). Daily bodyweight expressed as a percentage of the animal's baseline average is shown in panels C and F for females and males, respectively. In both panels C and F, **** = p < 0.0001. Summary data are presented as mean±SD. Data were analyzed using repeated measures one-way ANOVA.

One of the most widely used and well-established animal models is activity-based anorexia (ABA) as it closely mimics several key features of AN, including

severely restricted feeding and excessive exercise (Klenotich and Dulawa 2012). We first verified that we were able to reproduce the increased wheel running during the hours preceding food presentation known as food anticipatory activity (FAA) as well as bodyweight loss in response to restricted feeding in female and male wildtype mice (Figure 2.1). Data from female mice are presented in panels A-C. Data from male mice are presented in panels D-F. Wheel revolutions per 15-minute bin over the course of 3 days in ABA are first shown for either sex (Figure 2.1A, 2.1D). The daily cumulative total of wheel revolutions run during FAA, the four hours preceding food presentation, are shown in panels B (female) and E (male). As an ABA experiment progresses the sample size inevitably gets smaller given that animals are removed from an ABA study when they lose 20% of their baseline bodyweight or greater. We have therefore elected to display the data up to the point that removal of animals from the experiment became necessary. For females, this is up to 4 days of ABA; for males, 3 days. In both sexes we were able to reliably observe a significant increase in FAA in response to the ABA paradigm. In females, repeated measures one-way ANOVA revealed a significant overall effect after 4 days of ABA: $F_{(1.784, 12.49)} = 5.45$, $p = 0.0224$ (Figure 2.1B, n=8, 24 h: 29±40.5 mean±SD, 48 h: 403±709, 72 h:2615±3481, 96 h: 5085±4907). Repeated measures one-way ANOVA revealed a significant overall effect in males after 3 days of ABA: $F_{(1.129, 5.646)} = 7.56$, $p = 0.0334$, n=6, 24 h: 202±404, 48 h: 1915±2352, 72 h: 6099±5343 (Figure 2.1E). Bodyweight loss over the course of ABA is shown as the animal's daily bodyweight expressed as a percentage of its baseline average for both females (Figure 2.1C; repeated measures one-way ANOVA; $F_{(1.309, 9.164)} = 71.4$, $p < 0.0001$, n=8, 24 h: 93.1%±1.95, 48 h: 88.6%±1.49, 72 h: 84.6%±2.47, 96 h:

82.2%±3.32) and males (Figure 2.1F; repeated measures one-way ANOVA; $F_{(1.559, 10.92)}$ = 77.6, $p < 0.0001$, n=8, 24 h: 92.3%±2.70, 48 h: 86.7%±3.28, 72 h: 82.7%±3.46). Relative to the night before the initiation of ABA, a significant decrease in food intake was observed when hours of food access were restricted to the first two hours of the dark cycle in both females and males (Table 2.1).

Table 2.1. Food intake data expressed as a percentage of the animal's baseline average food consumption. All data presented as mean±SD. a) One-way repeated measures ANOVA in WT females; $p<0.0001$; $F_{(2.125, 14.88)} = 94.1$. b) Two-way repeated measures ANOVA comparing MOR knockout females to WT females followed by post-hoc analysis via Sidak's multiple comparisons. Sidak's multiple comparisons were not significant (24 hr WT vs. MOR ko: $p = 0.168$; 48 hr WT vs MOR ko: $p = 0.105$, 72 hr WT vs. MOR ko: $p = 0.263$; 96 hr WT vs. MOR ko: $p = 0.892$) despite an overall effect ($p = 0.0149$; $F_{(1,15)} = 7.57$). c) One-way repeated measures mixed effects model to account for missing datapoint due to data collection error in WT males; $p<0.0001$; $F_{(3,26)} = 106$. d) Two-way repeated measures mixed effects model comparing MOR knockout males to WT males followed by post-hoc analysis via Sidak's multiple comparisons. Sidak's multiple comparisons were not significant (24 hr WT vs. MOR ko: $p = 0.555$; 48 hr WT vs MOR ko: $p = 0.126$, 72 hr WT vs. MOR ko: $p = 0.348$) despite an overall effect ($p = 0.003$; $F_{(1,56)} = 9.65$). e) Two-way repeated measures mixed effects model; $p = 0.969$; $F_{(1,42)} = 0.00154$.

Sex	Condition	Food consumed expressed as percentage of baseline average (mean±SD)				
		0 h ABA	24 h ABA	48 h ABA	72 h ABA	96 h ABA
F	WT[a]	n=8, 99.3%±15.3	n=8, 23.4%±8.80	n=8, 38.8%±7.51	n=8, 40.9%±9.23	n=8, 45.3%±6.76
F	MOR ko[b]	---	n=9, 15.5%±4.75	n=9, 29.4%±8.34	n=9, 32.9%±7.74	n=9, 41.9%±10.4
M	WT[c]	n=7, 110%±4.79	n=7, 17.8%±9.30	n=8, 46.7%±13.3	n=8, 39.3%±11.1	---
M	MOR ko[d]	---	n=13, 13.1%±3.65	n=13, 34.0%±11.7	n=13, 31.3%±11.7	---
M	NAL[e]	---	n=9, 18.7%±4.78	n=8, 42.0%±10.7	n=8, 43.5%±10.9	---

Pomc mRNA is transiently increased in both female and male animals undergoing ABA

Pomc mRNA levels change in response to an organism's energy state such that duringtimes of positive energy balance *Pomc* mRNA is increased (Schwartz et al. 1997) and during times of negative energy balance *Pomc* mRNA is decreased (Benoit et al., 2002; Mizuno et al.1998). Paradoxically, Hillebrand and colleagues have previously shown a transient increase in *Pomc* mRNA levels in female rats undergoing ABA despite the animal existing in a state of negative energy balance (Hillebrand et al., 2006). We performed fluorescent *in situ* hybridization to detect changes in *Pomc* mRNA levels in female and male wildtype mice having undergone one, two, or three days of ABA or a control condition (food restriction only or wheel running only). Example images are shown in Figure 2.2A. The fluorescent intensity of each *Pomc*-expressing cell was expressed as a percentage of background fluorescence intensity specific to that image and an overall average of fluorescent intensity above background was determined for each animal for statistical analysis. One-way ANOVA revealed a significant difference in means between treatment groups (Figure 2.2B, $F_{(4,21)} = 3.89$, $p = 0.0162$). After one day of ABA, female mice showed a significant increase in *Pomc* mRNA fluorescent intensity compared to both food restricted controls (Figure 2.2B, Tukey's multiple comparison, * = $p = 0.0500$, FR only: n=6, 223%±29.6, 24 h ABA: n=5, 323%±78.8) and wheel running only controls (Figure 2.2B, Tukey's multiple comparison, # = $p = 0.0177$, WHL only: n=6, 207%±72.0, 24 h ABA: n=5, 323%±78.8). Female mice showed a peak in fluorescent intensity after one day of ABA. By thee days of ABA, fluorescent intensity values had essentially returned to levels observed in either control condition (Figure 2.2B, Tukey's multiple comparison, FR only vs. 72 h ABA: $p = 0.999$, FR only: n=6, 223%±29.8, 72 h

ABA: n=3, 216%±42.1, WHL only vs. 72 h ABA: p = 0.999, WHL only: n=6, 207%±72.0, 72 h ABA: n=3, 216%±42.1).

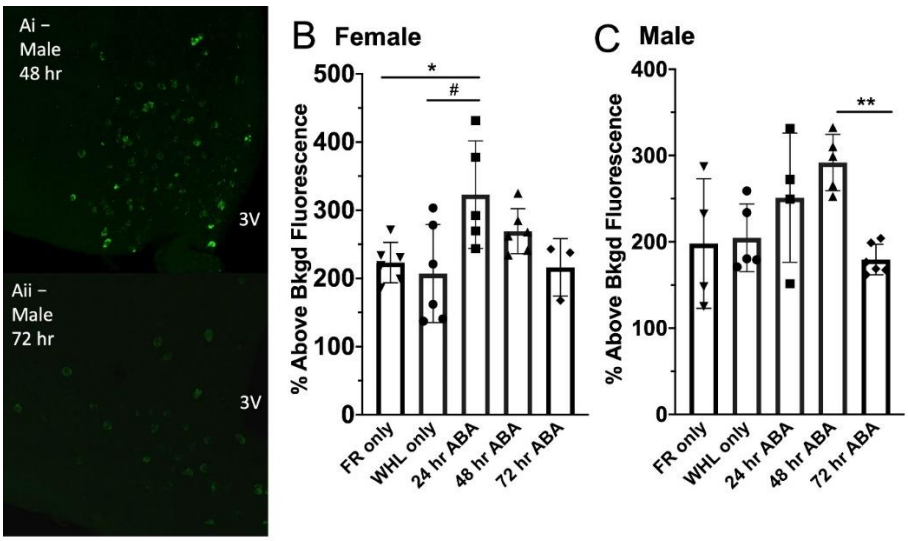

Figure 2.2. *Pomc* mRNA is transiently elevated in both female and male mice undergoing ABA. Representative confocal images taken at 40x of *Pomc* mRNA detected with Alexa488-labeled probe in the arcuate nucleus of the hypothalamus from male animals sacrificed after 48 hours (Ai) or 72 hours (Aii) in ABA. Summary data from females are shown in panel B: * = p = 0.0500, # = p = 0.0177, one-way ANOVA. Summary data from males are shown in panel C: * = p=0.0110, one-way ANOVA. Summary data are presented as mean±SD. Additional details are found in the results section. 3V = third ventricle. FR = food restricted. WHL = wheel. n numbers were as follows: female: FR only: n=6, WHL only: n=6, 24 h ABA: n=5, 48 h ABA: n=6, 72 h ABA: n=3, male: FR only: n=4, WHL only: n=5, 24 h ABA: n=5, 48 h ABA: n=5, 72 h ABA: n=6.

As observed in the female mice, a significant difference in mean fluorescent intensity was observed between treatment groups in male mice (Figure 2.2C, one-way ANOVA, $F_{(4,19)} = 4.398$, * = p=0.0110). Unlike in females where the peak fluorescent intensity was observed after one day of ABA (Figure 2.2B, 24 h ABA: n=5, 323%±78.8), in male animals the peak fluorescent intensity was observed after two days of ABA (Figure 2.2C, 48 h ABA: n=5, 292%±32.7). Fluorescent intensity was significantly decreased by three days of ABA compared to two days of ABA (Figure 2.2C, Tukey's

multiple comparison, 72 h ABA vs. 48 h ABA: ** = p=0.00980, 72 h ABA: n=6, 180%±17.7, 48 h ABA: n=5, 292%±32.7). No significant difference was observed between either control condition and thee days of ABA (Figure 2.2C, Tukey's multiple comparison, FR only vs. 72 h ABA: p = 0.976, FR only: n=4, 198±75.1, 72 h ABA: n=6, 180%±17.7, WHL only vs. 72 h ABA: p=0.913, WHL only: n=5, 205%±39.3, 72 h ABA: n=6, 180%±17.7).

Peripheral levels of β-endorphin increase over the course of ABA

To determine whether the increase in *Pomc* mRNA observed might lead to an increase in circulating levels of β-endorphin we performed radioimmunoassays to detect levels of β-endorphin in serum from animals euthanized after varying days in ABA. One-way ANOVA revealed a statistically significant effect in female mice (Figure 2.3A, $F_{(2,14)} = 4.701$, $p = 0.0274$). Specifically, a significant increase in β-endorphin was observed when female mice completed 3 days of ABA compared to 1 day (Figure 2.3A, Tukey's multiple comparison, p = 0.0223, 24 h ABA: n=5, 71.3±12.8, 72 h ABA: n=7, 176±81.8). No significant difference was found between one and two days in ABA (Figure 2.3A, Tukey's multiple comparison, p = 0.3940, 24 h ABA: n=5, 71.3±12.8, 48 h ABA: n=5, 121±43.8) or two and three days in ABA (Figure 2.3A, Tukey's multiple comparison, p=0.282, 48 h ABA: n=5, 121±43.8, 72 h ABA: n=7, 176±81.8). Mean concentration of β-endorphin from male mice is presented in Figure 3b, though statistical analysis was not performed due to the low number of samples from male mice that met quality standards (Figure 2.3B, 24 h ABA: n=3, 73.2±16.9, 48 h ABA: n=3, 86.9±31.0, 72 h ABA: n=4, 92.1±21.2).

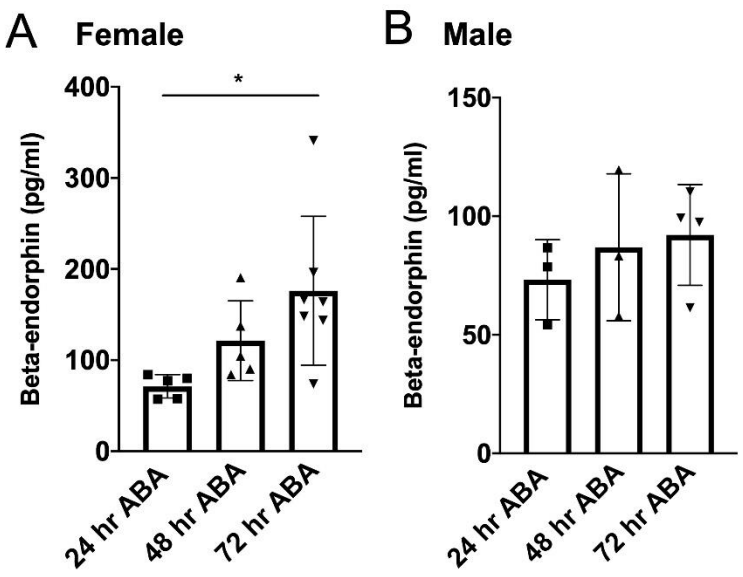

Figure 2.3. Peripheral levels of β-endorphin are elevated in response to ABA. RIA was used to detect circulating levels of beta-endorphin in peripheral blood collected from female (A) and male (B) after varying days of exposure to ABA. Data are presented as mean±SD. * = p = 0.0223. Data were analyzed using one-way ANOVA. Additional details are found in the results section. n numbers were as follows: female: 24 h ABA: n=5, 48 h ABA: n=5, 72 h ABA: n=7, male: 24 h ABA: n=3, 48 h ABA: n=3, 72 h ABA: n=4.

Food anticipatory activity (FAA) is blunted after MOR deletion in both male and female mice

After detecting elevations in *Pomc* mRNA and β-endorphin concentration in response to ABA, we next investigated whether MOR activation contributes to the development of ABA. Previous studies have shown that FAA can be blunted in female MOR knockout mice undergoing ABA (Kas et al., 2004); given the known sex differences in AN and ABA however, we hypothesized that MOR activation may contribute differentially to the development of ABA in males versus females. Data from female and male MOR knockout mice are shown in Figure 2.4. The overall pattern of wheel revolutions per 15-minute bin over the course of 3 days in ABA are shown for

female (Figure 2.4A) and male (Figure 2.4D) mice (wildtype animals in black, MOR knockout animals in blue). There is day-to-day variability in FAA for any given animal and a distribution in days needed to lose 20% of initial bodyweight, thus FAA is presented for each animal at the day that wheel revolutions were highest for the individual. In the majority of instances, the highest level of FAA is displayed on the

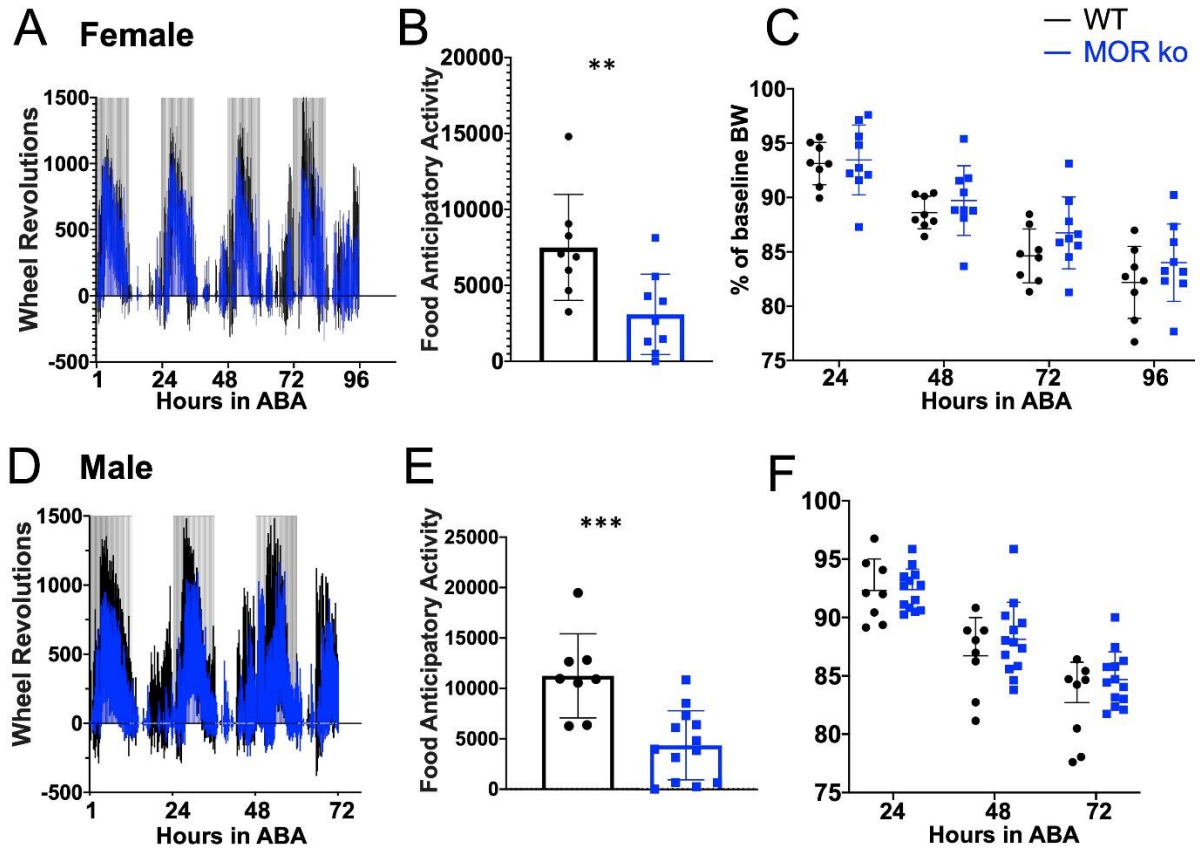

Figure 2.4. Both male and female MOR knockout mice display blunted FAA. Wheel revolutions per 15-minute time bin over the course of 3 days in ABA are shown for female (A) and male (D) mice. Gray bars denote the dark cycle. Highest daily total of FAA for each animal is shown in panels B (female: WT: n=8, MOR ko: n=9, ** = p = 0.0099, unpaired two-tailed T test) and E (male: WT: n=8, MOR ko: n=13, *** = p = 0.0006, unpaired two-tailed T test). Daily bodyweight expressed as a percentage of the animal's baseline average are shown in panels C and F for females and males, respectively. Summary data presented as mean± SD. Data shown in panels C and F were analyzed with repeated measures two-way ANOVA.

same day that the animal reaches the experimental endpoint. In instances where this was not the case, animals appeared to be exhausted and displaying minimal engagement with the running wheel. Both female (Figure 2.4B) and male (Figure 2.4E) MOR knockout mice display significant reductions in FAA compared to wildtype; female: unpaired, two-tailed T test, ** = p = 0.0099, WT: n=8, 7500±3488, MOR knockout: n=9, 3103±2644; male: unpaired, two-tailed T test, *** = p = 0.0006, WT: n=8, 11247±4166, MOR knockout: 4363±3424. No significant differences in bodyweight loss were detected between MOR knockout animals and wildtypes in both females (Figure 2.4C; two-way repeated measures ANOVA; $F_{(1,15)} = 1.12$, p = 0.307, WT: 24 h ABA: 93.1%±1.95, 48 h ABA: 88.6%±1.50, 72 h ABA: 84.6%±2.47, 96 h ABA: 82.2±3.32, MOR knockout: 24 h ABA: 93.5%±3.21, 48 h ABA: 89.7%±3.20, 72 h ABA: 86.8%±3.30, 84.0%±3.56) and males (Figure 2.4F; two-way repeated measures ANOVA; $F_{(1,19)} = 1.014$, p = 0.327, WT: 24 h ABA: 92.3%±2.69, 48 h ABA: 86.7%±3.28, 72 h ABA: 82.7%±3.46, MOR knockout: 24 h ABA: 92.4%±1.74, 48 h ABA: 88.1%±3.18, 72 h ABA: 84.7%±2.37). We did not detect significant differences in food consumption at any specific timepoint in ABA between MOR knockouts and WTs of either sex (Table 1).

MOR antagonism reduces FAA only in male mice

We elected to further investigate the hypothesis that MOR activation contributes to the development of ABA by treating wildtype animals undergoing ABA with the MOR antagonist naloxone hydrochloride (NAL, 5 mg/kg). Here, we observed a sex-specific effect with males but not females show showing a significant reduction in FAA in response to NAL treatment (Figure 2.5A: Female, unpaired T test, two-tailed, p =0.595, WT: n=8, 7500±3488, NAL: n=13, 8695±5588; Figure 2.5B: Male, unpaired T test, two

tailed, * = p = 0.0352, WT: n=8, 11247±4166, NAL: n=9, 6809±3744). Despite the reduction in FAA observed in males administered NAL, there was not a significant change in bodyweight in response to NAL treatment (Figure 2.5C; two-way repeated measures ANOVA; $F_{(1,15)} = 0.5425$, p = 0.473, WT: 24 h ABA: 92.3%±2.70, 48 h ABA: 86.7%±3.28, 72 h ABA: 82.7%±3.46, NAL: 24 h ABA: 91.1%±1.34, 48 h ABA: 86.3%±1.22, 72 h ABA: 82.0%±2.07). There was also no significant difference in food intake in mice receiving NAL compared to WT (Table 1).

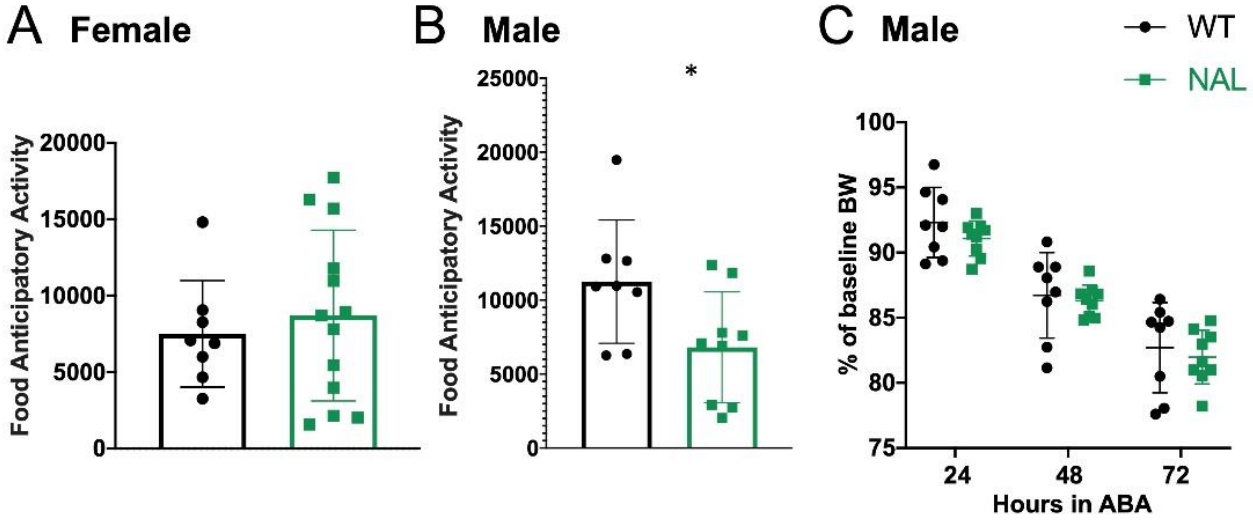

Figure 2.5. Male, but not female, mice display reduced FAA in response to naloxone treatment. Highest daily FAA totals are shown for female (A, n=13) and male (B, n=9) mice undergoing ABA. Daily bodyweight expressed as a percentage of baseline average are shown for male mice (C). Control mice are shown in black; mice treated with naloxone (NAL) are shown in green. Summary data presented as mean±SD. In panel B, * = p = 0.0352. Unpaired two-tailed T tests were used to analyze data shown in panels A and B; repeated measures two-way ANOVA was used to analyze data shown in panel C.

2.5 Discussion

In the current study, we found that *Pomc* mRNA levels were transiently elevated in both female and male mice undergoing ABA and that there was also a concomitant

rise in circulating β-endorphin. A role for β-endorphin action in FAA was indicated in studies in mice lacking MORs and studies where MOR function was pharmacologically antagonized. While previous work had indicated that deletion of MORs decreased FAA in female mice, the present study extends the findings to males as well. Interestingly, administration of the MOR antagonist naloxone hydrochloride (NAL) reduced FAA in male, but not female, mice. We did not observe changes in bodyweight or food intake in response to MOR deletion or NAL administration. The results presented here suggest (1) that increased *Pomc* transcription is transient and observed in both female and male mice, (2) targeted inhibition of β-endorphin function via MOR genetic deletion selectively blunts FAA in female and male animals, yet (3) only male mice show a similar blunting of FAA in response to NAL treatment. These results highlight the potential of sex-specific differences in the mechanisms underlying ABA as well as highlight the potential need for sex-specific treatments in individuals with AN.

Sex differences

Many epidemiological studies have shown eating disorders in general, and especially AN, to be much more common in females compared to males (Keski-Rahkonen and Mustelin 2016; Smink, Van Hoeken, and Hoek 2012). Given the unequal sex distribution observed in the human population, many previous rodent ABA studies were only conducted on female animals (Hillebrand et al., 2005; Hillebrand et al., 2005b; Hillebrand et al., 2006; Kas et al., 2004). However, processes underlying energy homeostasis, and more specifically the physiology of feeding behaviors, are known to be highly sexually dimorphic (Asarian and Geary 2013). Moreover, a considerable degree of evidence suggests that POMC neurons are sexually dimorphic as well:

female mice have significantly more POMC neurons compared to male mice and electrophysiological recordings from POMC neurons of female mice displayed an increase in firing rate as well as a decrease in resting membrane potential (C. Wang et al. 2018); male, but not female, mice from which the vesicular glutamate transporter *Vglut2* is deleted from POMC neurons are unable to maintain normal bodyweight (Dennison et al. 2016); similarly, female mice lacking a G-protein coupled receptor (Gpr17) from POMC neurons better maintained energy homeostasis relative to males (Reilly et al. 2019). We therefore felt it necessary to revisit experiments performed only in female rodents to determine whether increases in *Pomc* mRNA and decreased FAA in MOR knockout mice are also true in males. We show for the first time in male mice a transient increase in *Pomc* mRNA as well as confirm the previously reported observation of a similar transient increase in *Pomc* mRNA in female mice (Hillebrand et al., 2006). Interestingly, we observed a temporal difference in the peak of the increase in *Pomc* transcription, with males showing a peak in fluorescence on day two compared to day one in females.

Wheel running and opioid-mediated reward

Wheel running is considered rewarding in rodents as they display conditioned place preference for a particular side of a chamber where access is given to a running wheel (Lett et al., 2001). Moreover, engagement in voluntary wheel running has been shown to reduce the consumption of other known rewarding stimuli such as a high-fat diet (Liang et al., 2015) as well as various drugs of abuse including heroin (Smith and Pitts 2012) and cocaine (Cosgrove et al., 2002). As an endogenous opioid, β-endorphin is a critical mediator of reward and it has been shown that pharmacological blockade of

this system can reduce wheel running (Rasmussen and Hillman 2011). In ABA, animals will run on the running wheel to the point of exhaustion and death if the investigator does not intervene; we therefore hypothesized that the animals consider wheel running rewarding and that β-endorphin concentrations are increased during ABA to signal this reward. The observed increase in β-endorphin after three days in ABA compared to one day supports this hypothesis and might explain why rodents choose to engage in wheel running despite limited resources (restricted feeding). These findings are in line with previous work that showed that leptin signaling through the ventral tegmental area, a key brain region in reward processing, can reduce hyperactivity observed in ABA (Verhagen et al., 2011). We elected to measure circulating levels of β-endorphin due to being unable to collect an adequate volume of cerebrospinal fluid (CSF) to allow for reliable peptide detection by radioimmunoassay. We acknowledge the pitfalls of using this approach given that positive and negative correlations between central and peripheral levels of β-endorphin have been reported in rodents and humans (Martinez et al., 1993; Vecsei et al., 1992; Yamamoto et al., 2000; Aravich et al., 1993; Kosten et al., 1987; Baker et al., 1997). Despite the caveat that peripheral β-endorphin levels do not always reflect central β-endorphin levels, we nevertheless elected to determine whether our hypothesis might be supported. A second limitation of the current study is the use of a global rather than site-specific MOR knockout. While we are unable in the current study to point towards specific brain sites as likely candidate regions underlying ABA, future studies should address this question using site-specific MOR deletion.

Food intake and bodyweight

In addition to playing an important role in the signaling of rewarding stimuli, β-endorphin has also been shown to influence feeding behavior, albeit in a complex manner given that opposing effects have been observed and given the fact that β-endorphin contributes to both homeostatic and hedonic aspects of food intake (Nogueiras et al. 2012). Exogenous intracerebroventricular administration of β-endorphin has been shown to increase food intake (Silva et al., 2001), yet genetic deletion of β-endorphin also leads to increased food intake and bodyweight in male mice (Appleyard et al., 2003; Low et al., 2003). Given the complex nature of the role of β-endorphin in feeding behavior, perhaps it is not surprising that we did not observe effects on bodyweight and food intake when inhibiting the actions of β-endorphin as the predicted outcome is not clear. Moreover, while we initially hypothesized that the melanocortin system was uniformly involved in all features of ABA, i.e. equal effect on wheel running behavior as well as feeding behavior and resulting weight loss, the results presented in the current study seem to suggest that β-endorphin specifically regulates the wheel running feature of ABA, leaving open the possibility for a second neuronal system to regulate the feeding and bodyweight changes observed in ABA. A potential candidate system could be the agouti-related peptide (AGRP) neurons, residing in the arcuate nucleus of the hypothalamus alongside POMC neurons, that potently stimulate food intake (Aponte et al., 2011); indeed, exogenous administration of AGRP to rodents undergoing ABA show greater food intake relative to controls (Hillebrand et al., 2006; Kas et al., 2003). Exactly how these two neuronal populations with opposing effects on feeding behavior could work in concert to contribute to the development of ABA remains to be determined, however.

Conclusions

Finally, while many findings in the current study were similar between female and male mice (both sexes displaying elevations in *Pomc* transcription, elevated β-endorphin concentration, and blunted FAA in response to MOR deletion), only male mice showed reduced FAA when treated with NAL. This could suggest two things: first is the possibility that there are sex-specific differences in the degree of involvement of the opioidergic system in ABA, highlighting the utility and necessity of using both sexes when conducting ABA studies; and second is the potential need for sex-specific approaches to treatment in individuals with AN. Indeed, while not fully understood, men and women show differential responses to opioidergic drugs (Bartley & Fillingim, 2013) and it is possible that the NAL dose used in the current study, a mid-range dose shown in the literature to be effective in rodent wheel running studies (Sisti & Lewis, 2001) was too low to see the effect observed in males. The potential need for sex-specific approaches to AN treatment has been indicated by emerging evidence suggesting that AN in males has a different clinical presentation when compared to females (Coelho et al. 2018). Eating disorders in males have been described as “under-diagnosed, undertreated, and misunderstood” (Strother et al., 2012). It is therefore imperative that potential sex differences be identified and studied further in both ABA and AN studies.

Chapter 3: Inhibition of proopiomelanocortin neurons in mice undergoing activity-based anorexia selectively blunts food anticipatory activity in male and female mice

3.1 Overview

The previous chapter presented evidence that β-endorphin released by proopiomelanocortin (POMC) neurons is likely an important driver of increased wheel running activity (specifically food anticipatory activity (FAA) in male mice undergoing activity-based anorexia (ABA). Somewhat surprisingly, no changes to bodyweight or food intake were observed with either pharmacological blockade or genetic deletion of mu opioid receptors (MORs) in both males and females. Given that POMC neurons release multiple peptide products, it could be the case that selective inhibition of a single peptide product produces parallel selective inhibition of one aspect of the ABA paradigm; i.e., while it appears that β-endorphin contributes to the development of FAA, perhaps changes to bodyweight and food intake require manipulation of the entire POMC neuron in order to observe changes. In the current chapter, the effect of inhibiting POMC neurons during ABA is evaluated to address this follow-up question. The data presented herein is undergoing final preparation for publication and will be submitted for peer review shortly. As a prelude to this chapter, a brief discussion of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) is first presented to introduce the method by which the studies presented in this chapter inhibited POMC neurons.

DREADDs are an example of a “chemical genetic” or “chemogenetic” tool first developed by Armbruster and colleagues that allows for targeted manipulation of

neurons expressing a designer receptor that is no longer activated by its endogenous ligand (Armbruster et al. 2007). The DREADD used in the current chapter is the hM4Di DREADD, a modified muscarinic (G-protein coupled receptor, GPCR) receptor that no longer binds acetylcholine and is instead activated by an otherwise inert compound, clozapine-n-oxide (CNO) (Roth 2016). Once inserted into the neuronal membrane of interest (POMC neurons, in this case), hM4Di receptors activated by the designer drug CNO couple to G protein inwardly rectifying potassium channels (GIRKS) to dampen synaptic transmission (Urban and Roth 2015). There are several other types of inhibitory DREADDs, though the hM4Di is the most widely used and for which there is the most in vitro and in vivo evidence demonstrating successful attenuation of synaptic signaling. Excitatory DREADDs also exist and can be used to facilitate, rather than attenuate, synaptic transmission. Excitatory DREADDs are further discussed relevant to studies presented in Chapter 4.

3.2 Summary

Anorexia nervosa (AN) is a serious eating disorder that affects millions of individuals each year. Females are more commonly diagnosed with AN than males at a ratio of 8:1. As the etiology of the disease remains unknown, treatment is limited to correcting medical complications associated with starvation and malnutrition. Given the high mortality and relapse rates of the disease, it is imperative that improved treatments specifically targeting the etiology of the disease are developed. Previous research using the ABA model has implicated a role for the hypothalamic POMC system in the development of ABA. POMC neurons are peptidergic neurons that reside in the arcuate nucleus (ARC) of the hypothalamus. More specifically, the POMC peptide β-endorphin

appears to contribute to food anticipatory activity (FAA), a characteristic of ABA, as both deletion and antagonism of the mu opioid receptor (MOR), the β-endorphin receptor, resulted in decreased FAA without altering bodyweight or food intake. In the current study, we set out to determine whether targeted inhibition of POMC neurons would produce similar or perhaps stronger results given that POMC neurons signal through multiple peptides and neurotransmitters, including α-MSH which inhibits feeding. Inhibition of POMC neurons during ABA resulted in reduced FAA in male mice, with no significant changes in bodyweight or food intake. Based on this somewhat surprising finding, we performed an additional chemogenetic experiment in which we concurrently inhibited additional cell types in the hypothalamic ARC known to influence food intake and bodyweight along with inhibition of POMC neurons; if it is true that POMC neuron involvement in ABA is limited to presentation of FAA, then concurrent inhibition of other cell types should not alter the blunted FAA observed. As hypothesized, inhibition of a mixed neuronal ARC population produced the same reduction in FAA as when only POMC neurons were inhibited. Inhibition of POMC neurons during ABA also blunted FAA in female mice but only when the DREADD was delivered virally. The results of the current study indicate that POMC neurons are involved selectively in FAA in male and female mice and that future studies should look to other neurons for the source of bodyweight and food intake modulation during ABA and ultimately AN.

3.3 Introduction

AN is a debilitating eating disorder that affects approximately 1% of the US population (Duncan, Ziobrowski, and Nicol 2017). With an average mortality rate of 5-10%, AN has one of the highest mortality rates of all neuropsychiatric conditions,

second only to substance use disorder as of this writing (Arcelus et al. 2011). Medical complications and relapses are unfortunately commonly associated with AN (Keel & Brown, 2010), yet despite its devastating impact the underlying etiology of this disease remains unknown and treatment options limited (Zipfel et al. 2015). Thus, there is a dire need to better understand the etiology of AN if new treatments and therapies are to be developed.

When studying patients with AN, it is difficult to discern whether observed pathologies are causative or simply a sequelae of the disease (Hay and Sachdev 2011). Prospective studies tracking currently undiagnosed individuals would be incredibly helpful, yet prospective studies to date have focused nearly exclusively on tracking how an individual fares after an initial AN diagnosis is made (Herzog, Schellberg, and Deter 1997; Löwe et al. 2001; Strobel et al. 2019). Because of these limitations in studying the human population, animal models of AN are a critical tool that must be utilized if we are to uncover early neurobiological changes associated with the onset of AN. Activity-based anorexia (ABA) is one of the most widely-used animal models of AN in which limited food access coupled with free access to a running wheel produces profound decreases in bodyweight and food intake (Klenotich and Dulawa 2012). Significant increases in wheel running activity, particularly in the hours preceding food presentation (termed food anticipatory activity; FAA), are also seen in ABA (Mistlberger 1994), and if the investigator does not intervene animals will starve themselves to death (Hall and Hanford, 1954; Routtenberg and Kuznesof 1967).

Using the ABA behavioral paradigm, the hypothalamus, particularly the proopiomelanocortin (POMC) system within the arcuate nucleus (ARC), has been

identified as a key brain region involved in governing ABA (Scharner et al. 2017). POMC is a preprohormone that is enzymatically cleaved into multiple bioactive peptides, including α-MSH, which potently inhibits feeding through its actions at melanocortin receptors (Fan et al. 1997; Huzsar et al. 1997), and β-endorphin, an endogenous opioid that participates in both reward and feeding behaviors (Silva et al. 2001; Appleyard et al. 2003). We and others have shown a transient increase in *Pomc* mRNA at the onset of ABA (Daimon and Hentges 2021; Hillebrand et al. 2006). Additionally, modulation of bioactive peptides produced by cleavage of the preprohormone POMC including α-MSH and β-endorphin can alter the response to ABA: administration of α-MSH exacerbates ABA, while mice lacking the mu opioid receptor (MOR) or those given a MOR antagonist display blunted FAA, one of the hallmarks of ABA (Daimon and Hentges 2021; Hillebrand et al. 2005; Kas et al. 2004). However, it remains unknown whether modulation of POMC neurons themselves, rather than individual bioactive peptide products, would have similar or perhaps even stronger ameliorative effects on ABA presentation.

In the current study, we hypothesized that inhibition of POMC neuron activity via chemogenetic DREADD technology would either prevent the development of or lessen the severity of ABA, i.e., less severe decreases in bodyweight and food intake and less pronounced increases in wheel running activity. We first verify that the inhibitory hM4Di DREADD can be reliably targeted to POMC neurons via two methods and that expression of the DREADD in POMC neurons does not interfere with the establishment of ABA in saline-treated animals. We then show that activation of the inhibitory hM4Di DREADD during ABA produces a selective reduction in FAA in male and female

animals while having no additional impact on bodyweight and food intake. These results indicate that POMC involvement in ABA is limited to the presentation of FAA.

3.4 Materials and Methods

Animals: All animal experiments were performed in accordance with protocols approved by Colorado State University's Institutional Animal Care and Use Committee. The following mouse strains were initially acquired from the Jackson Laboratory and bred and maintained in-house on the C57BL/6J strain: B6.129-G*t(ROSA)26Sor*$^{tm1(CAG-CHRM4*,-mCitrine)Ute}$, stock #026219, *Pomc*$^{cre/+}$, stock #005965, B6N;120-Tg(CAG-CHRM3*,-mCitrine)1Ute/J, stock # 026220. Pomc-cre:ER$^{T2/+}$ mice were originally a gift from Dr. Joel Elmquist, University of Texas Southwestern. Pomc^{dsRED} mice were originally a gift from Dr. Malcolm Low, University of Michigan. Specific breeding schemes of these transgenic mice are subsequently discussed. Standard PCR techniques were used to genotype mice. Male and female mice aged 2-6 months were used in the experiments described. Animals were maintained on a 12/12-hour light/dark cycle. In the breeding colony room, lights turned on at 0600 h; in the experimental procedure room where ABA was performed, lights turned on at 0200 h. All animals were allowed to adjust to the modified light/dark cycle and proper entrainment to the new light/dark cycle confirmed via activity monitoring prior to experiment start. Ad libitum access to food and water was provided unless stated otherwise. Temperature and humidity were kept within an acceptable range in both the breeding facility and experimental procedure room.

Cre-recombinase-dependent DREADD expression in POMC neurons: Not all POMC-expressing progenitor cells in utero develop into mature POMC cells in the post-

natal mouse (Padilla, Carmody, and Zeltser 2010; Wei et al. 2018); in fact, a portion of POMC progenitors develop into agouti related peptide (AGRP)-expressing neurons which, via AGRP's ability to act as an inverse agonist at the same melanocortin receptors that the POMC peptide α-MSH acts at, has directly opposing actions on food intake (Atasoy et al. 2012). To avoid expressing DREADDs in populations of neurons with opposing physiological effects, we either waited until *Pomc*$^{cre/+}$ animals were 8 weeks old to introduce the DREADDs via stereotaxic microinjection or used an inducible Cre-recombinase driven by the *Pomc* promoter (*Pomc*-cre:ER$^{T2/+}$) (Berglund et al. 2013). In the inducible cre-lox system, Cre-recombinase is inactive and fused to mutated hormone binding domains of the estrogen receptor; only after 4-hydroxytamoxifen (75 mg/kg i.p. daily for 5 days, Sigma Aldrich) administration is Cre-recombinase released from the mutated estrogen receptor and able to translocate to the cell nucleus (Feil, Valtcheva, and Feil 2009). In the transgenic approach, an inducible Cre-recombinase driven by the *Pomc* promoter was activated upon systemic administration of 4-hydroxytamoxifen to POMC-cre:ER$^{T2/+}$ animals crossed to a floxed hM4Di sequence tagged with human influenza hemagglutinin (B6.129-G*t(ROSA)26Sor*$^{tm1(CAG-CHRM4*,mCitrine)Ute/J}$). In the viral strategy, animals constitutively expressing Cre-recombinase driven by the *Pomc* promoter (*Pomc*$^{cre/+}$) received bilateral stereotaxic injections with an adenovirus vector containing the inverted floxed hM4Di DREADD sequence at 8 weeks of age (pAAV2-hSyn-DIO-hM4Di-mCherry; 200 nl/hemisphere, Addgene, Watertown, MA). The injections were targeted to the ARC using the following stereotaxic coordinates: from bregma, A/P: -1.63, M/L: +/- 0.32, D/V

-6.00). Animals were given a minimum of 7 days to recover from surgery or 4-hydroxytamoxifen administration prior to initiation of ABA.

Activity-based anorexia model: The activity-based anorexia (ABA) model is a widely used and well-validated behavioral assay in which access to a running wheel paired with limited access to feed leads to decreased bodyweight, decreased food consumption, and increased wheel running activity (Klenotich and Dulawa 2012). Animals are singly housed for the duration of the ABA experiment in clean caging equipped with a running wheel (Columbus Instruments, Columbus, OH). Wheel revolution counts were collected in 15 min bins via Multi Device Interface Software (Columbus Instruments, Columbus, OH). After a 3-day acclimation period, baseline food intake, bodyweight, and wheel running activity was collected 1 hour prior to lights out for 5 days. Mice running less than 1500 revolutions/day were considered non-runners and dropped from the experiment. Following baseline data collection, the ABA paradigm was initiated by limiting access to food to the first 2 hours of the dark cycle. Daily bodyweight measurements collected during the ABA paradigm were compared to the animal's baseline average and animals were removed from the study for ethical reasons if 20% bodyweight loss was achieved. The experiment was terminated after 6 days of restricted feeding regardless of the degree of bodyweight loss. Both control and treatment animals received drug to control for off-target effects of 4-hydroxytamoxifen and/or CNO. CNO was administered twice daily at 0.5 h prior to lights out and again 5 h later.

Tissue collection and processing: Prior to brain tissue collection, animals were first deeply anesthetized with 200 mg/kg sodium pentobarbital (Fatal-Plus, Vortech

Pharmaceuticals, Dearborn, MI) and lack of deep pain reflex confirmed before proceeding with transcranial perfusion with a 10% sucrose solution followed by 4% w/v paraformaldehyde in potassium phosphate-buffered saline. Whole brains were removed and stored at 4C in 4% paraformaldehyde overnight or until tissue processing took place. To confirm DREADD expression in POMC neurons, 50 µM slices were collected using a VT100S Leica vibratome spanning the rostral-caudal axis of the ARC.

Immunohistochemistry: Immunohistochemistry was performed to visualize HA-tagged hM4Di DREADDs in POMC neurons via the following protocol: 50 µM brain slices were washed (3 x 10 min) in potassium phosphate buffer solution, then blocked for 1 h in blocking solution containing 2% goat serum. Slices were next incubated overnight in primary antibody against the HA-tag at 1:1000 dilution (Cell Signaling Technologies). The following day, three additional washes were performed (3 x 15 min) and slices incubated in for 1 h at room temperature in a 1:250 dilution of goat anti-mouse Alexa Fluor 488 antibody (Invitrogen). Slices went through a final series of washes, were mounted on glass slides, and allowed to dry at room temperature overnight prior to imaging. All images were collected on a Zeiss 800 confocal microscope.

Statistical Analyses: The results section lists the specific statistical tests used to analyze the data. All data were analyzed using Prism (GraphPad Software Inc., San Diego, CA). Data are presented as mean ± SEM. Differences were considered significant when $p \leq 0.05$.

3.5 Results

DREADD expression in POMC neurons does not interfere with the development of ABA

It is common practice to use stereotaxic microinjection to deliver viral vectors containing DREADDs to brain regions of interest (Roth 2016). It has previously been shown that ABA can be generated successfully following post-stereotaxic surgery (Miletta et al. 2020; Boekhoudt et al. 2016). Before the use of DREADDs became commonplace, intracerebroventricular injection was used in a number of studies to deliver drugs centrally during ABA and no negative effect on the ABA paradigm was observed (Verhagen, Luijendijk, and Adan 2011; Verhagen et al. 2011; Hillebrand et al. 2006b; Hillebrand, Kas, and Adan 2005). However, there are caveats to using a surgical approach, including inadvertent misses, variations in injection site along the rostral-caudal or dorsal-ventral planes, and potential complications associated with general anesthesia and post-operative recovery. Given that 1) transgenic mouse lines exist to permit targeted DREADD expression in POMC neurons and 2) use of a transgenic breeding strategy is a means to avoid the potential pitfalls associated with surgical DREADD delivery, we decided to use both approaches in the current experiments.

Both methods of DREADD delivery rely on the cre-lox system for site-specific DNA recombination (Akagi et al. 1997). In the transgenic approach, an inducible Cre-recombinase driven by the *Pomc* promoter was activated upon systemic administration of 4-hydroxytamoxifen to POMC-cre:ER$^{T2/+}$ animals crossed to a floxed hM4Di sequence tagged with human influenza hemagglutinin (B6.129-G*t(ROSA)26Sor*$^{tm1(CAG\ CHRM4*,mCitrine)Ute/J}$). In the adenoviral vector strategy, animals constitutively expressing Cre-recombinase driven by the *Pomc* promoter (*Pomc*$^{cre/+}$) received bilateral stereotaxic

injections with an adenovirus vector containing the inverted floxed hM4Di DREADD sequence at 8 weeks of age (pAAV2-hSyn-DIO-hM4Di-mCherry; 200 nl/hemisphere, Addgene, Watertown, MA).

The viral vector microinjection strategy for cell-type specific recombination is the standard method for DREADD delivery to specific brain regions (Roth, 2016) and ABA experiments have been previously performed successfully using mice that received stereotaxic microinjections (Miletta et al., 2020). We confirmed this via a small pilot study in which mice that had previously received stereotaxic surgery were run through ABA without issue (data not shown). The transgenic inducible Cre mouse breeding strategy is much less commonly used and concerns have been raised about the impact of 4-hydroxytamoxifen on behavior (Li et al. 2020); as such, we sought to verify that there were no overt behavioral abnormalities following Cre-recombinase activation via 4-hydroxytamoxifen. Both male and female animals receiving DREADDs via the inducible Cre transgenic breeding strategy were subjected to the ABA behavioral paradigm. 4-hydroxytamoxifen was administered at least 7 days prior to experimental acclimation start. To best mimic the experimental conditions used in the experiments that follow, animals were habituated to i.p. injections during baseline data collection and received twice daily saline injections in place of CNO. Data from males (Figure 3.1A-C) and females (Figure 3.1D-F) receiving the hM4Di DREADD via the transgenic strategy are presented in Figure 3.1. We were able to successfully generate the three hallmark characteristics of ABA in both male and female mice: significant increases in food anticipatory activity, a phenomenon that is not present during ad libitum feeding (Males: Figure 3.1A, * = $p = 0.0210$; Females: Figure 3.1D, * = $p = 0.0149$, paired t tests) as well

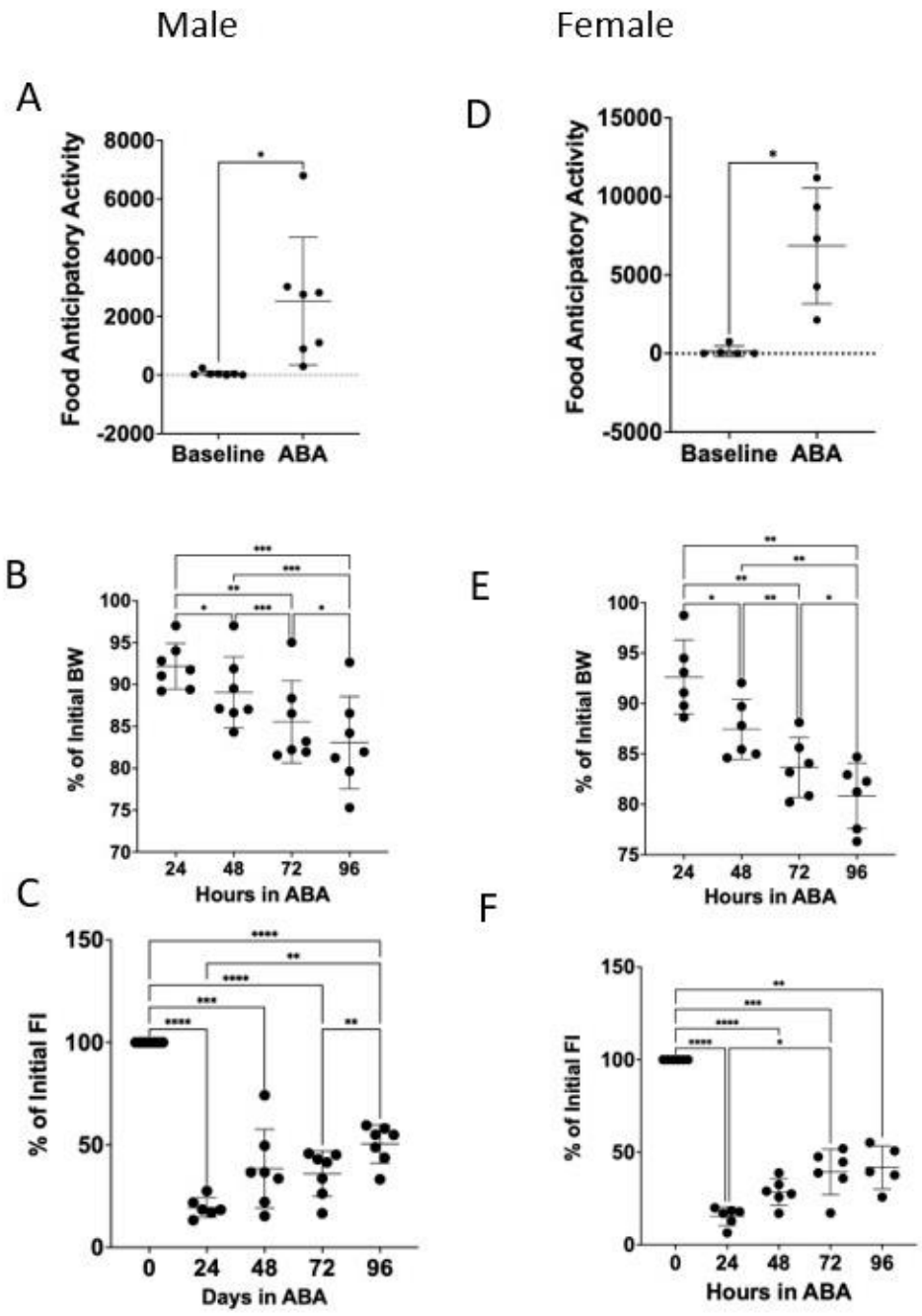

Figure 3.1. DREADD expression in POMC neurons does not interfere with the development of ABA. Development of the three hallmarks of ABA in both male and female mice expressing the inhibitory hM4Di DREADD are shown in panels A-F: 1) increased food anticipatory activity, or wheel running in the four hours preceding food presentation (A male, D female), 2) decreased bodyweight (B male, E female), and 3) decreased food intake over the first four days of ABA are shown (C male, F female). Data are presented as mean±SEM. Data were analyzed using repeated measures one-way ANOVA. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

as significant decreases in bodyweight and food intake (bodyweight males: Figure 3.1B, $F_{(1.690, 10.14)} = 54.4$, **** = $p < 0.0001$; bodyweight females: Figure 3.1E, $F_{(1.438, 7.192)} = 52.1$, **** = $p < 0.0001$, repeated measures one-way ANOVAs followed by Tukey's multiple comparisons tests; food intake males: Figure 3.1C, $F_{(1.604, 9.225)} = 71.2$ = **** = $p < 0.0001$; food intake females: Figure 3.1F, $F_{(1.671, 7.940)} = 117.8$, **** = $p < 0.0001$, mixed effects analysis followed by Tukey's multiple comparisons tests.

Inhibition of POMC neurons selectively inhibits FAA without impacting bodyweight or food intake in male mice

Given that there were no overt behavioral effects in the ABA paradigm associated with 4-hydroxytamoxifen administration, we proceeded to ask whether inhibition of POMC neurons via the inhibitory hM4Di DREADD would prevent the development or lessen the severity of ABA. Using both DREADD delivery methods we were able to significantly reduce the level of FAA in male mice expressing the DREADD (Figure 3.2A (transgenic; * = $p = 0.0500$, unpaired-t-test) and 3.2D (adenoviral vector; ** = $p = 0.00500$). No significant changes in bodyweight or food intake were observed with either DREADD delivery method in male mice (Figure 3.2B: Transgenic Bodyweight: two way ANOVA, $F_{(1, 12)} = 0.253$, $p = 0.624$; Figure 3.2C: Transgenic Food intake: mixed effects analysis, $F_{(1, 12)} = 0.322$, $p = 0.581$; Figure 3.2E Viral Bodyweight: mixed effects analysis, $F_{(1, 11)} = 0.980$; Figure 3.2F: Viral Food Intake: mixed effects analysis, $F_{(1, 11)} = 0.0135$, $p = 0.910$).

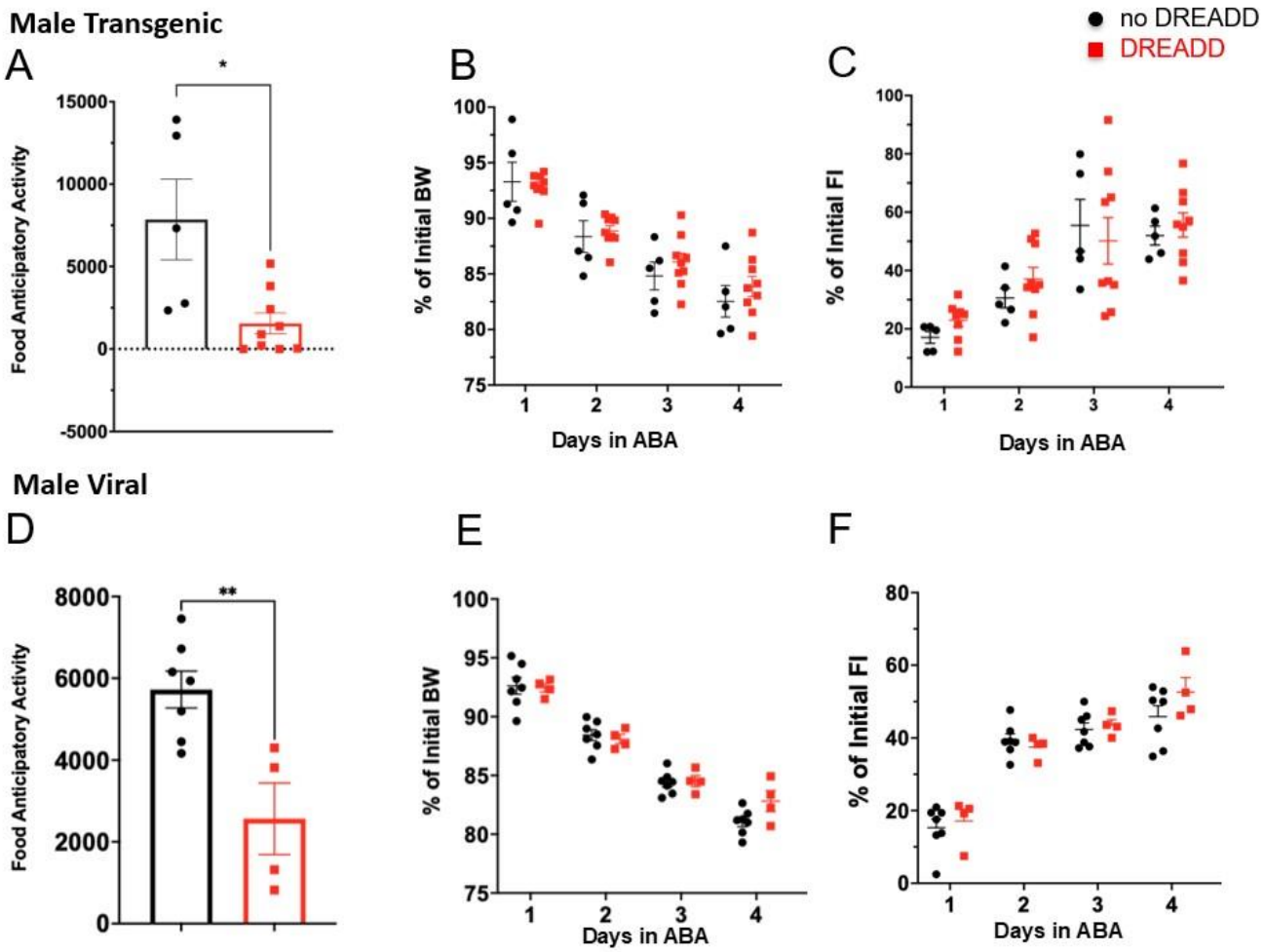

Figure 3.2. Inhibition of POMC neurons selectively inhibits FAA in male mice. The highest daily total of FAA for each animal is shown in panels A (transgenic approach) and D (viral approach). Bodyweight (B transgenic, E viral) and food intake data (C transgenic, F viral) are shown over the course of the first four days in ABA. Data are presented as mean±SEM. Data were analyzed using unpaired Student's t test (panels A, D) or two-way ANOVA or mixed effects analysis where appropriate (panels B-C and E-F). * = $p \leq 0.05$, ** = $p \leq 0.01$.

Using the transgenic approach in female mice, no significant differences were found with respect to FAA, bodyweight, or food intake between mice either with or without the inhibitory DREADD (Figure 3.3A-C (transgenic): FAA: paired t test, $p = 0.166$; Bodyweight: mixed effects analysis, $F_{(2, 17)} = 0.788$, $p = 0.471$; Food intake: mixed effects analysis, $F_{(1, 12)} = 0.0763$, $p = 0.787$). Using the viral delivery approach,

female animals with the DREADD exhibited less FAA compared to female animals without the DREADD (Figure 3.3D, unpaired t test, * = p = 0.0322). No significant changes were found in bodyweight (Figure 3.3E, mixed effects analysis, $F_{(1, 15)} = 1.190$, p = 0.293) or food intake (Figure 3.3F, mixed effects analysis, $F_{(1, 15)} = 1.659$, p = 0.217).

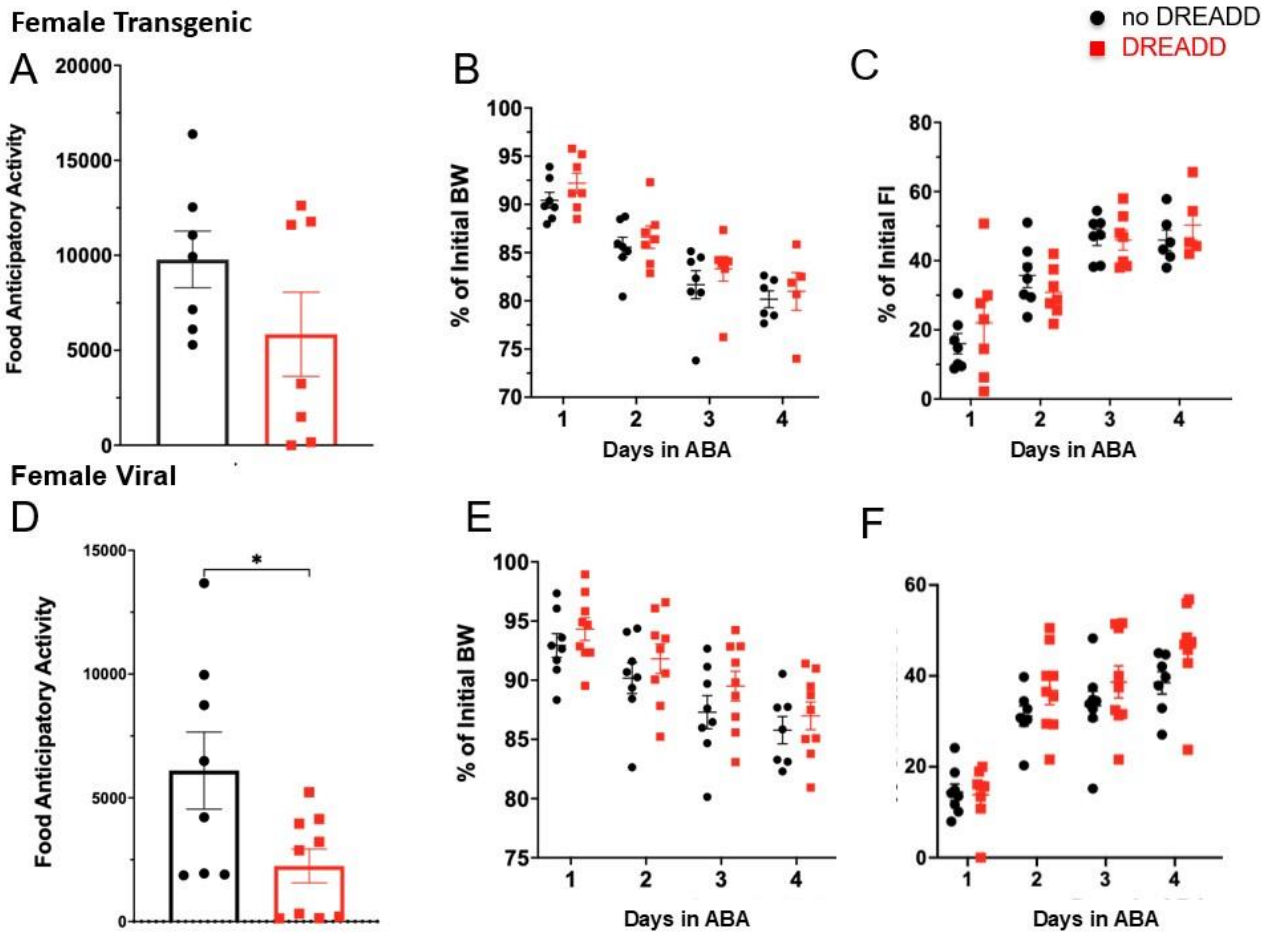

Figure 3.3. Inhibition of POMC neurons in female mice undergoing ABA selectively inhibits FAA via the viral DREADD approach. The highest daily total of FAA for each animal is shown in panels A (transgenic approach) and D (viral approach). Bodyweight (B transgenic, E viral) and food intake data (C transgenic, F viral) are shown over the course of the first four days in ABA. Data are presented as mean±SEM. Data were analyzed using either an unpaired Student's t test (panels A, D) or two-way ANOVA or mixed effects analysis where appropriate (panels B-C and E-F). * = p ≤ 0.05.

Inhibition of a mixed population of neurons in the ARC also results in selective inhibition of FAA in male mice

Based on the results shown in Figure 3.2, it appeared as though inhibition of POMC neurons most strongly impacts the strength of food anticipation response without causing noticeable alterations in bodyweight and food intake in male mice. If it is true that POMC neuron involvement in ABA is limited to the presentation of FAA, then concurrent inhibition of orexigenic AGRP neurons should have no impact on the observed results. To test this hypothesis, we utilized what is usually considered a disadvantage of cross-breeding animals constitutively expressing Cre recombinase driven by the *Pomc* promoter (*Pomc*$^{cre/+}$) to mice with the floxed hM4Di sequence tagged with human influenza hemagglutinin (B6.129-G*t(ROSA)26Sor*$^{tm1(CAG\ CHRM4*,mCitrine)Ute/J}$) used in the transgenic mice experiments. Based on the work of Padilla et al. (2010), roughly 25% of the neurons expressing the DREADD when using a constitutively active *Pomc* cre driver line will mature into antagonistic AGRP/NPY neurons and an additional 25% will mature into non-AGRP/NPY, non-POMC ARC neurons (Padilla, Reef, and Zeltser 2012). As shown in Figure 3.4, despite the mixed population of neurons being inhibited, we still observed only a selective decrease in FAA (Figure 3.4A, * = $p = 0.0491$, unpaired t-test). As expected, no significant differences were found with respect to bodyweight (Figure 3.4B, mixed effects analysis, $F_{(1,9)} = 0.00148$, $p = 0.970$) or food intake (Figure 3.4C, two-way repeated measures ANOVA, $F_{(1,9)} = 0.0539$, $p = 0.822$). Given that female mice only showed significant reductions in FAA using the viral approach (Figure 3.3A and 3.3D), we elected to only do this experiment in male mice.

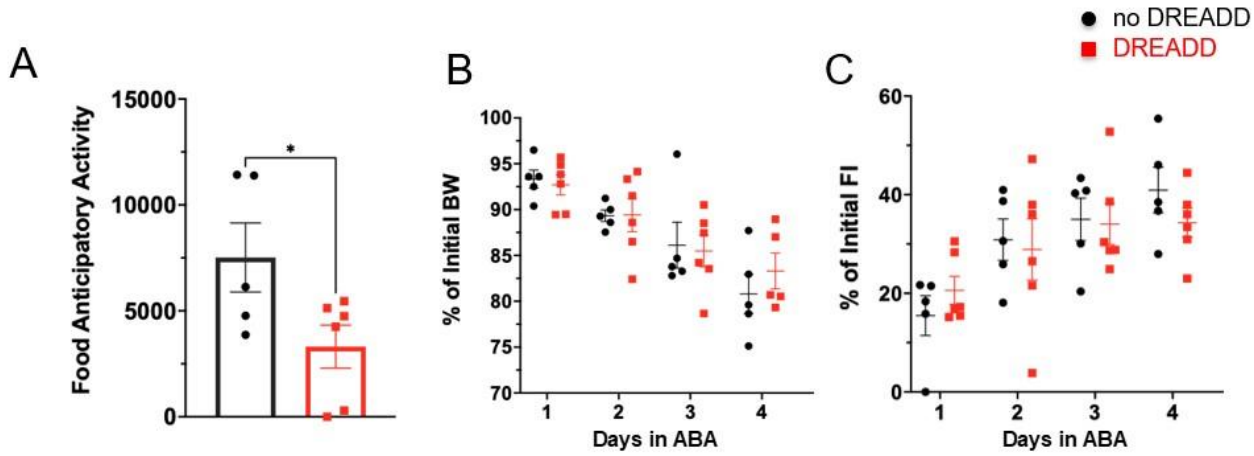

Figure 3.4. Inhibition of a mixed population of ARC neurons still results in selective inhibition of FAA in male mice. The highest daily total of FAA for each animal is shown in panels A. Bodyweight (B) and food intake data (C) are shown over the course of the first four days in ABA. Data are presented as mean±SEM. Data were analyzed using either an unpaired Student's t test (panels A) or two-way ANOVA (panels B-C). * = $p \leq 0.05$.

3.6 Discussion

In the current study, we show inhibition of POMC neurons causes a selective blunting of FAA in both male and female mice. Further, we show that this selective blunting of FAA occurs independently of any change in bodyweight or food intake. This is in line with our previous findings in which selective disruption of mu opioid receptor signaling and possibly β-endorphin function also resulted in blunted FAA without altering bodyweight or food intake in male mice (Daimon and Hentges 2021). Thus, it appears that manipulation of POMC neurons also contributes selectively to FAA in the ABA behavioral paradigm.

Transgenic versus viral DREADD delivery strategies

We employed two strategies to deliver DREADDs to POMC neurons and observed similar results between methods. While stereotaxic microinjection of DREADDs packaged in a viral vector has become the standard means of DREADD delivery in the field (Roth 2016), missed hits not discovered until post-hoc analysis is one disadvantage of this delivery method that can be avoided by utilizing a transgenic breeding strategy to deliver DREADDs. Additionally, while care is taken to ensure that mice receive nearly identical microinjections, some mice may inadvertently receive injections that are oriented on a slightly shifted rostral-caudal plane or dorsal-ventral plane, a scenario that is avoided when using the transgenic breeding strategy. Concerns have been raised over the necessity of administering the estrogen receptor antagonist 4-hydroxytamoxifen to activate Cre-recombinase in the transgenic mice, as locomotor and anxiety behaviors have been shown to be affected after 4-hydroxytamoxifen administration (Li et al. 2020). Based on this, we chose to control for these effects by administering 4-hydroxytamoxifen to all animals regardless of whether the mouse expressed the Cre transgene, as well as running the initial experiment discussed in Figure 3.1 to ensure that ABA can be reliably generated in 4-hydroxytamoxifen treated mice. The results from the current study suggest that in short-term behavioral experiments like ABA, it is acceptable to use the transgenic breeding strategy to deliver DREADDs.

Using the transgenic strategy, reduced FAA was observed in male mice regardless of whether a mixed ARC population was inhibited or POMC neurons alone were inhibited during ABA (Figures 3.2 and 3.4). Based on the work of Padilla et al. (2010), approximately 50% of neurons that express *Pomc* during development will

mature into a non-POMC cell type: their data show that if mice constitutively expressing Cre recombinase driven by the *Pomc* promoter are crossed to hM4Di-floxxed mice, roughly half of the neurons expressing the inhibitory DREADD are non-POMC cells. Of this 50%, roughly half were shown to be NPY/AGRP neurons; the other half was not identified (Padilla, Reef, and Zeltser 2012). One potential cell-type that might also have been inhibited is kisspeptin neurons, known to stimulate the pulsatile release of gonadotropin-releasing hormone as well as regulate metabolism (Harter, Kavanagh, and Smith 2018). Regardless of the identity of the non-POMC cells expressing the inhibitory hM4Di DREADD, the impact on FAA in the current study remained unchanged, bolstering support for the role of POMC neurons in selectively driving FAA.

Food Intake and Bodyweight

If POMC neurons contribute selectively to FAA and not bodyweight or food intake changes during ABA, it is likely that another neuronal population mediates feeding behavior and subsequent changes in bodyweight during ABA. It has previously been shown that administration of AGRP to rodents undergoing ABA show greater food intake compared to controls (Kas et al. 2003; Hillebrand et al. 2006). Moreover, a recent study found that ablating AGRP neurons resulted in significantly more weight lost and less food eaten during ABA compared to sedentary, AGRP-ablated controls (Miletta et al. 2020). Interestingly, Miletta et al. (2020) determined that it was specifically loss of the ability to mobilize free fatty acids and not simply lack of caloric intake that led to increased mortality in the mice with ablated AGRP neurons. Mice with ablated AGRP neurons also ran significantly less over each 24-h period in ABA compared to control animals, though it would have been interesting to examine FAA specifically, as our data

would suggest that increased wheel running in the hours immediately preceding food presentation might have been observed, especially since the AGRP neurons that directly modulate POMC neurons have been ablated (Tong et al. 2008).

Food Anticipatory Activity and the Food Entrainable Oscillator

Successfully finding and consuming food is critically important for survival in the wild. Animals use circadian clocks to predict food availability as well as other changes in environment; being able to generate these predictions is advantageous to an organism's survival, a concept known as the circadian resonance hypothesis (Spoelstra et al. 2016; Wyse et al. 2010). The mechanism that animals have evolved to anticipate food availability is known as the food entrainable oscillator (FEO) (Mistlberger 1994). Initially hypothesized to be under the control of the master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, experiments have subsequently shown that the FEO exists independent of SCN-mediated clock mechanisms (Mistlberger 2009). The observation that POMC inhibition only impacts FAA and not food intake or bodyweight in mice undergoing ABA could suggest that we uncovered a previously unrecognized component of the FEO. Future studies should further investigate this potential role of POMC neurons in the establishment of the FEO.

Conclusions

In the current study, we show that chemogenetic, DREADD-mediated inhibition of POMC neurons in mice undergoing ABA results in a selective blunting of FAA in male and female mice. No effect on either food intake or bodyweight was found, suggesting that POMC neurons appear to contribute specifically to increased activity in ABA. While

our findings contribute to our knowledge in understanding increased activity levels in individuals with AN, it appears that food intake and bodyweight are mediated by mechanisms other than POMC neurons.

Chapter 4: Chronic stimulation lasting one-month duration of proopiomelanocortin neurons causes paradoxical weight gain in mice

4.1 Overview

In the previous two chapters, the effect of manipulating proopiomelanocortin (POMC) neurons or their peptide products was examined in an animal model of anorexia nervosa (AN), a disorder of negative energy balance. Disorders reflecting a state of positive energy balance, I.e., a state of overweight or obesity, are far more common in the general population, however. An obese individual is at risk of increased mortality due to increased risk of developing type 2 diabetes, heart disease, and certain types of cancer (Barbieri et al. 2017). Especially pertinent to current world affairs, individuals who are obese have higher rates of hospitalization, require more intensive medical care, and have higher mortality rates compared to non-obese individuals infected with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Recalde et al. 2021).

Despite the commonly touted benefits of lifestyle changes for weight loss and the existence of FDA-approved anti-obesity drugs, obesity remains a public health crisis. Perhaps a more complete understanding of the neurobiology driving homeostatic regulation of energy balance will set the stage for new discoveries that could reduce the prevalence of obesity in the future. The current chapter investigates whether long-term stimulation of POMC neurons in mice fed an obesogenic diet is beneficial for reducing food intake in addition to inducing and sustaining weight loss. Whereas an inhibitory Designer Receptor Exclusively Activated by Designer Drug (DREADD) was used in Chapter 3 to dampen neuronal signaling in POMC neurons in mice undergoing

activity-based anorexia (ABA), in the current chapter POMC neurons will be stimulated via activation of the stimulatory hM3Dq DREADD. The hM3Dq DREADD couples through the Gq pathway and enhances neuronal signaling by increasing intracellular calcium levels.

I performed the studies described herein unless otherwise noted and analyzed the data under the supervision of Shane Hentges. These data will be combined with work from other lab members in a future publication. Portions of Figure 4.1 were included in a recent manuscript submission titled “Individual arcuate nucleus proopiomelanocortin neurons project to select target sites.” Appendix I Figure S1 contains the entire figure from this manuscript which has been accepted for publication as of this writing in the American Journal of Physiology- Regulatory, Integrative and Comparative Physiology.

4.2 Summary

Obesity is a global health crisis that is causing reductions in lifespan and vast increases in health care expenditures. Diet and lifestyle changes only yield modest results that are often temporary, as weight regain commonly occurs following initial weight loss. In light of these challenges, additional research into the neurobiology of feeding control may lead to new discoveries that have the potential to inform new therapeutics for obesity. The hypothalamic proopiomelanocortin (POMC) system plays a critical role in maintaining energy homeostasis, as POMC neurons are known to potently inhibit feeding via the actions of its peptide product α-MSH. Previous work has shown that both optogenetic and chemogenetic stimulation of POMC neurons can produce acute decreases in bodyweight and food intake in mice, yet it remains unknown

whether these findings could be extended over a longer period of time (weeks as opposed to days) as would be ideal of an anti-obesity drug target. Surprisingly, we found that chemogenetic stimulation of POMC neurons using the hM3Dq DREADD in both female and male mice led to weight gain rather than weight loss in mice on either a high-fat diet or normal chow diet. Data from a pilot study show this paradoxical weight gain is potentially avoided if POMC neurons are stimulated via the hM3Dq DREADD 3 times a day, 8 hours apart, though follow up study is needed. Overall, the results presented in this chapter suggest that chronic manipulation of POMC neurons to cause weight loss and decreased food intake may not be an effective strategy as readily hypothesized as there is the potential to cause weight gain rather than weight loss.

4.3 Introduction

The prevalence of obesity is rising across the world; in 1975, less than 1% of the world's population was obese; in 2016, that number rose to approximately 10% (Jaacks et al. 2019). In the United States, the situation is much worse: in all 50 states and territories, at least 20% of the population is considered obese (CDC 2021). Lifestyle interventions and/or use of anti-obesity drugs can result in modest weight loss, yet weight is often regained once these interventions are halted, highlighting an ongoing need for additional research into the neurobiology of feeding regulation.

POMC neurons in the arcuate nucleus (ARC) of the hypothalamus play a critical role in maintaining energy homeostasis as POMC neurons can strongly inhibit feeding via release of the peptide product α-MSH. Genetic missense mutations resulting in a lack of functional α-MSH peptide causes morbid obesity in humans, Labrador retrievers, and mice (Krude et al. 1998; Raffan et al. 2016; Yaswen et al. 1999). α-MSH exerts its

effects by binding to melanocortin receptors (MCRs) in the paraventricular nucleus (PVN) to cause cessation of feeding; if the ability of the MCR to bind to α-MSH is compromised, humans and mice become morbidly obese (Huszar et al. 1997).

Given their canonical role in anorexigenic signaling, several groups have investigated whether manipulation of POMC neurons can produce decreased food intake and bodyweight loss (Aponte, Atasoy, and Sternson 2011; Zhan et al. 2013). Both optogenetic and chemogenetic manipulation of POMC neurons required a considerable duration of application in order to produce physiological effects: Aponte and colleagues (2011) found that optogenetic stimulation of POMC neurons over 24 h can significantly reduce food intake and bodyweight, though the same stimulation protocol administered over 2 h was ineffective at reducing food intake (Aponte, Atasoy, and Sternson 2011). Using a chemogenetic approach, Zhan et al. (2013) found that POMC neurons required stimulation every 5 hours over the course of 3 days before observing significant reductions in food intake and bodyweight. In contrast, acute stimulation with 1 or 2 CNO injections in 5-hour intervals had no effect on food intake or bodyweight (Zhan et al., 2013).

While the results are no doubt encouraging, no studies to date have examined whether the reductions in food intake and bodyweight found by Aponte and colleagues (2011) and Zhan and colleagues (2013) can be sustained over longer durations of time. The goal of the current study was to take a chemogenetic approach similar to that used by Zhan and colleagues to determine if the weight loss and decreased food intake observed can be sustained over weeks in mice fed an obesogenic diet. Contrary to our initial hypothesis, we found that chemogenetic stimulation of POMC neurons via the

hM3Dq DREADD in both female and male mice led to weight gain rather than weight loss in mice. This was true whether mice were fed an obesogenic high-fat diet or normal chow. Data from a pilot study show this paradoxical weight gain is potentially avoided if POMC neurons are stimulated via the hM3Dq DREADD 3 times a day, 8 hours apart, though follow up study is needed.

4.4 Materials and Methods

Animals: All animal procedures were performed in accordance with protocols approved by Colorado State University's Institutional Animal Care and Use Committee. Animals were maintained on a 12/12-hour light/dark cycle (lights on at 0600 h in the breeding room; lights on at 0200 h in the experimental room). All animals were allowed to adjust to the modified light/dark cycle prior to experiment start. Temperature and humidity were kept within acceptable range in both the breeding facility and experimental procedure room. Ad libitum access to food and water was provided. The B6N;129-Gt(ROSA)Sor$^{tm2(CAG-CHRM3*,-mCitrine)1Ute/J}$ (hM3Dq$^{flox/flox}$) mouse strain was originally acquired from the Jackson Laboratory (stock # 026220). Pomc-cre:ER$^{T2/+}$ mice were originally a gift from Dr. Joel Elmquist at the University of Texas Southwestern (Berglund et al. 2013) and Pomc^{dsRED} mice were originally a gift from Dr. Malcolm Low (University of Michigan). Mice from these founding lines were cross-bred to create a hM3Dq$^{flox/flox}$ x Pomc-cre:ER$^{T2/+}$ x Pomc$^{dsRED/+}$ mice. Cre-recombinase was activated by administering 4-hydroxytamoxifen at approximately 8 weeks of age (see Chapter 3 Materials and Methods for additional details). Male and female mice aged 2-6 months were used in the experiments described. Animals were switched to a high-fat diet (HFD; 60% fat, Teklad) one week after 4-hydroxytamoxifen injections were performed. Animals

received HFD for at least one month prior to transcutaneous access button implantation.

Transcutaneous access button implantation: Multiple daily injections to the intraperitoneal (i.p) cavity can result in injection site pain and irritation as well as increase the animal's stress level. In place of repeated i.p. injections, the i.p. space was surgically catheterized with 25 gauge silicone tubing securely attached to a transcutaneous vascular access button made of silicone mesh that was implanted in the interscapular space (Instech Laboratories, Plymouth Meeting, PA). For surgical implantation of these devices, mice were induced at 3% Isoflurane and maintained at 2% throughout the surgery. Following induction, two incision sites (one on the ventral abdomen and one on the interscapular region) were clipped and scrubbed with betadine and 70% ethanol. As the first incision was made into the abdomen, a sterile piece of gauze was placed over the freshly scrubbed dorsal incision site so that the animal could be positioned on its back for the initial part of the surgery. A small midline incision was made and the 25 gauge silicone tubing inserted into the i.p. space. 5-0 suture was used to secure the tubing to the abdominal wall. Blunt dissection was used to create a tunnel for the silicone tubing from the ventral to dorsal aspect of the animal. Turning the animal to access the dorsal side, a midline incision was made and 3-0 suture was used to secure the skin around the transcutaneous access button. The catheter was flushed and placement verified prior to ending the surgery. Animals recovered on a heating pad and received pain medication for 3 days post-surgery. Catheters were kept patent with every other day flushing with 0.25 mL sterile saline solution. Animal weights were

monitored post-surgery and when 3 days of stable bodyweight achieved, mice were entered into the study.

Osmotic pump implantation: Osmotic pumps (Alzet, Cupertino, CA) that allow for continuous drug delivery were implanted into the i.p. space. Animals were induced and maintained on Isoflurane as described in the previous section. A small midline incision was made on the ventral abdomen after the animal's fur was removed and the area scrubbed for surgery. The osmotic pump filled with clozapine-n-ozide (CNO) was placed into the i.p. space and the layers of the abdominal wall and skin closed with 3-0 suture. Animals were recovered on a heating pad; as drug begins to continuously infuse, bodyweight and food intake data was collected immediately following surgery once a day for 7 days.

Chronic activation of POMC neurons: Prior to chronic activation of POMC neurons, bodyweight and food intake were measured for 3 days and then averaged to get a baseline value. All animals received clozapine-n-oxide (CNO; 1 mg/kg). For twice daily dosing, one dose was given 0.5 hours prior to lights out (start of dark cycle) and a second dose given 5 hours later. Bodyweight and food intake were measured daily at the time of the first dose administration. For three times daily dosing, CNO doses were given approximately 8 hours apart. Bodyweight and food intake were measured daily at the same time.

Overnight fast/refeed: The evening prior to the test, animals were singly housed in fresh cages with no food (water was freely provided). The next morning, after fasting 18-20 h, a single injection of CNO was administered (1 mg/kg, i.p.) and a premeasured amount of food returned to the animal 30 minutes after injection. Food was measured

again 2 h later and amount of food eaten by the animal calculated. Food and water remained freely available following the test.

Fluorescent in situ hybridization: Pomc mRNA was detected using fluorescent in situ hybridization as previously described (Daimon and Hentges 2021; Jarvie et al. 2017; Dennison et al. 2016). Brains stored in 4% paraformaldehyde were sliced coronally in 50 μm sections spanning the rostral-caudal axis of the ARC. Slices were then incubated at room temperature sequentially in: 6% hydrogen peroxide, Proteinase K (10 mg/ml), glycine (2 mg/ml), post-fixation solution containing 4% paraformaldehyde and 0.2% glutaraldehyde and finally ascending concentrations of ethanol prior to incubation in hybridization solution for 1 h at 60°C (66% (v/v) deionized formamide, 13% (w/v) dextran sulfate, 60 mM NaCl, 1.3x Denhardt's solution, 13 mM Tris-Hcl, pH 8.0, 1.3 mM EDTA, pH 8.0). The *Pomc* probe (0.25 pg/ml, corresponding to bases 531-1000 of GenBank sequence NM_08895.3) was denatured for 5 minutes at 85°C, added to the hybridization solution, and hybridized at 70°C for 18-20 hours. Brain slices were washed in saline sodium citrate buffer post-hybridization before detection of the fluorescein isothiocyanate-labelled *Pomc* probe with a secondary antibody conjugated to Alexa Fluor 488 (1:400, Invitrogen/Thermo Fisher Scientific, Waltham, MA). Tissue sections were mounted on glass slides, cover-slipped, and stored at 4°C for later image collection and analysis.

Image collection and analysis: All images were collected on a Zeiss 800 confocal microscope at 40x. Imaging parameters were kept consistent between experiments and each experiment contained both control and experimental animals. *Pomc*-expressing cells labeled with AlexaFluor-488 were identified using masks generated in ImageJ. The

fluorescent intensity of each *Pomc*-expressing cell was expressed as a percentage of background fluorescence intensity for that given image. An overall average of fluorescent intensity above background was generated for each animal by averaging the values collected from individual z-stack images.

Statistical Analyses: All data were analyzed using Prism (GraphPad Software, Inc., San Diego, CA). Data are presented as mean ± SEM. Differences were considered significant when $p \leq 0.05$.

4.5 Results

hM3Dq DREADDs activate POMC neurons in vivo

Prior to starting long-term stimulation experiments, we first set out to confirm that we can observe behavioral effects of neuronal activation via the hM3Dq stimulatory DREADD in our lab (Figure 4.1). Given that animals are highly motivated to consume food after a period of fasting, we decided to test the strength of the hM3Dq DREADD's

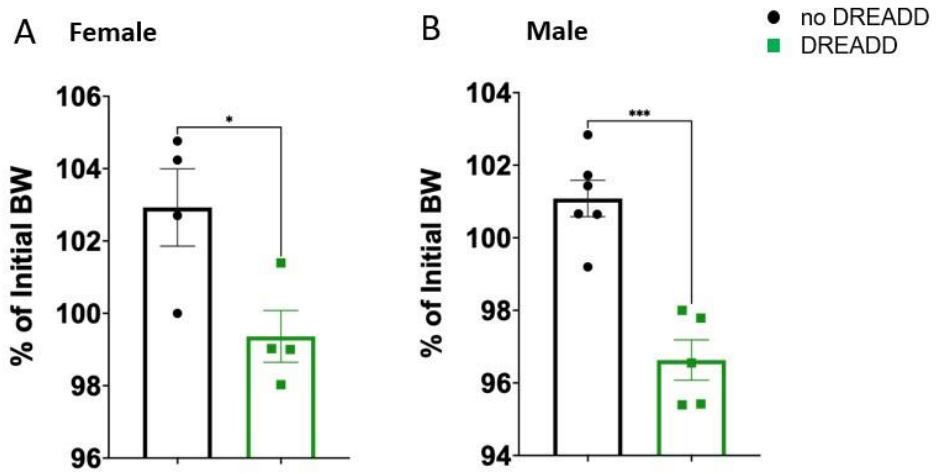

Figure 4.1. Activating hM3Dq DREADDs on POMC neurons lessens food intake. Food consumption data from female (A) and male (B) mice in an overnight fast-refeed test following CNO administration. Data are presented as mean ± SEM. * = $p \leq 0.05$.

ability to activate POMC neurons to dampen this refeeding response. As shown in Figure 4.1A and B, both female and male mice expressing the hM3Dq DREADD ate significantly less food during the refeed compared to mice not expressing the hM3Dq DREADD in POMC neurons (Females: Figure 4.1A, Males: Figure 4.1B; unpaired student's t-test, * = $p \leq 0.05$).

Continuous CNO administration via osmotic pump is not an effective means of drug delivery

Knowing that we could successfully activate POMC neurons expressing the hM3Dq DREADD in vivo, we next examined whether continuous CNO delivery via an osmotic pump inserted into the i.p. space is a feasible method of DREADD activation. Given that DREADDs are modified GPCRs, which are known to readily desensitize in response to sustained agonist binding (Rajagopal and Shenoy 2018), we were skeptical of the feasibility of continuous drug administration. However, osmotic pumps require a less invasive surgery compared to the transcutaneous access button approach and would be a convenient method by which to chronically administer CNO if a viable option. We ran a small pilot study to determine whether osmotic pump would be a feasible means of CNO delivery. As shown in Figure 4.2A, animals in both the control and treatment group lost about 10% of their bodyweight over the course of 7 days. There was no significant difference between the two groups (two-way repeated measures ANOVA; $F_{(1,5)} = 0.0381$, $p = 0.853$). As there is no way to delay drug administration using an osmotic pump, drug delivery begins immediately upon

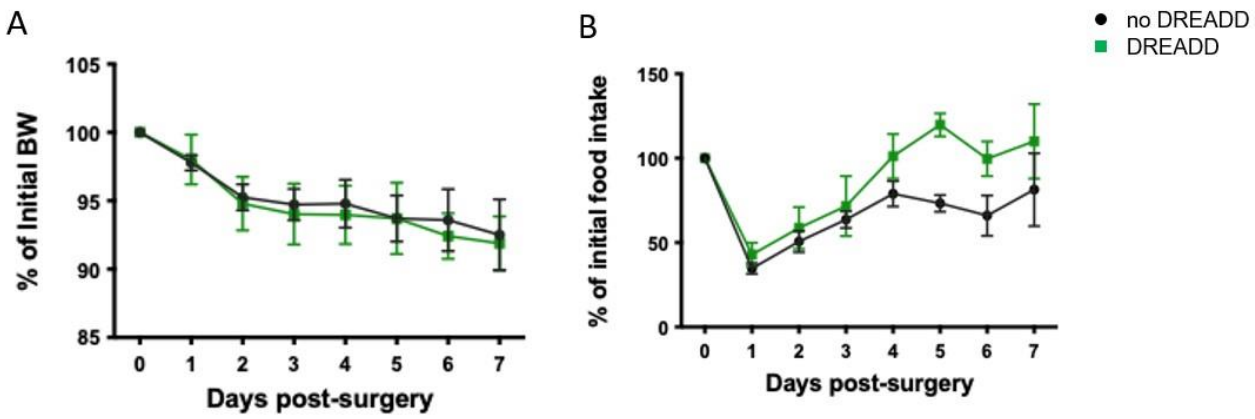

Figure 4.2. Continuous CNO administration via osmotic pump is not an effective means of drug delivery. Bodyweight (A) and food intake (B) data from male mice expressed as a percentage of presurgical bodyweight or food intake values is shown over the course of one week following implantation of an osmotic pump continuously releasing CNO into the i.p. space. Data are presented as mean±SEM.

implantation of the device; therefore, the bodyweight loss observed in both groups is likely due to incomplete recovery from surgery. Food intake dropped to approximately 50% of pre-surgical daily food intake in both groups and while the DREADD-containing treatment group did resume eating approximately 100% of their pre-surgical 24 h food intake, DREADD-negative control animals never did return to baseline food intake levels (Figure 4.2B). These data strengthen the argument for delivering CNO in discrete boluses and justifies the more invasive surgical approach required to catheterize the i.p. space.

Chronic CNO administration results in paradoxical weight gain in female and male mice fed an obesogenic diet without altering Pomc mRNA

Daily bodyweight and food intake data during one month of twice daily CNO administration are shown for females in Figure 4.3A and B and for males in Figure 4.3C and D. Surprisingly, no decrease in bodyweight was observed and in fact, after one week of CNO treatment, hM3Dq-positive DREADD mice treated with CNO were

consistently heavier than mice receiving CNO that lacked the hM3Dq DREADD (Figure 4.3A and 4.3C). This unexpected effect was significant in both females and males (females: Figure 4.3A, mixed effects analysis, $F_{(1,8)} = 53.6$, **** $p \leq 0.0001$; males: Figure 4.3C, mixed-effects analysis, $F_{(1,7)} = 42.9$, *** = $p = 0.0003$). Food intake was variable from day to day and no significant effect was found in either sex, though males were close to significance (hM3Dq-positive animals treated with CNO generally ate more than hM3Dq-negative animals treated with CNO) (females: Figure 4.3B, mixed

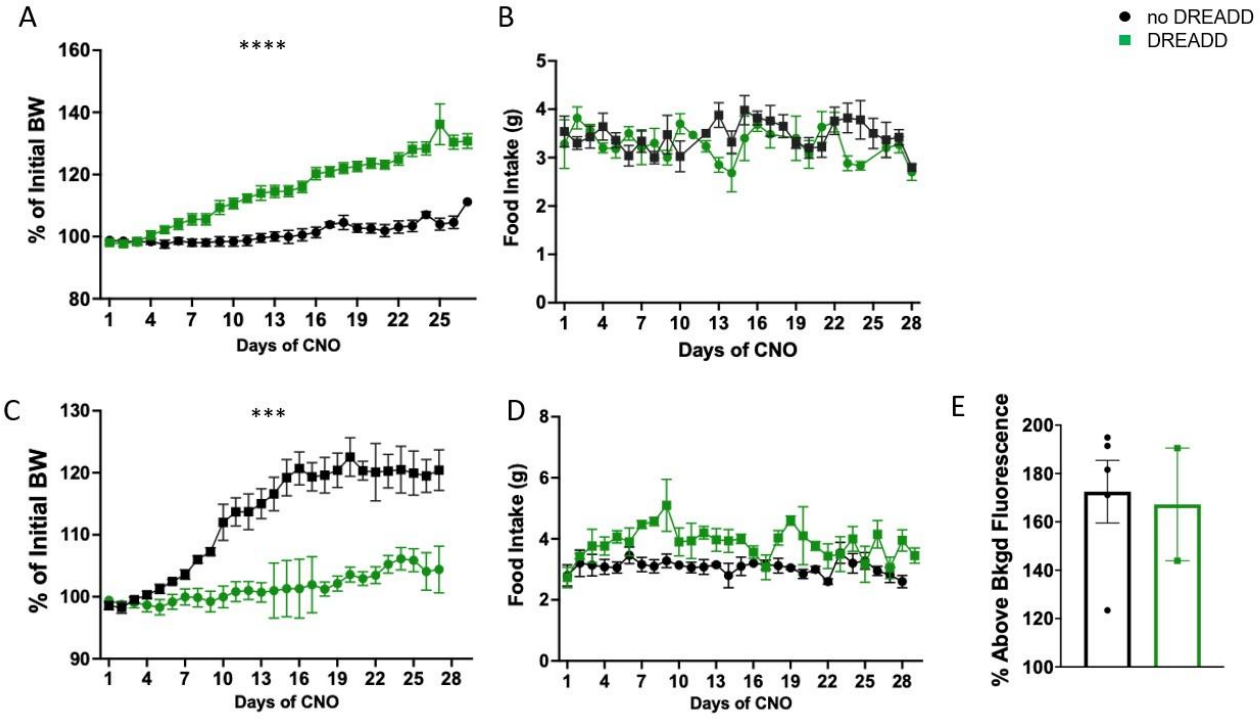

Figure 4.3. Chronic CNO administration results in paradoxical weight gain in female and male mice fed an obesogenic diet without altering Pomc mRNA. The effect on bodyweight and food intake during one month of twice daily CNO injections administered 5.5 h apart during the dark cycle are shown for both females (Bodyweight: Figure 4.3A; food intake: Figure 4.3B) and males (Bodyweight: Figure 4.3C; food intake: Figure 4.3D) on an obesogenic high-fat diet. After one month of chronic CNO administration, *Pomc* mRNA was detected using fluorescent in situ hybridization and the percent fluorescent intensity over background determined (Figure 4.3E). Data are presented as mean±SEM.

effects analysis, $F_{(1,8)} = 2.87$, $p = 0.129$; males: Figure 4.3D, mixed effects analysis, $F_{(1,6)} = 5.93$, $p = 0.051$). The unexpected results could not be explained by alterations in *Pomc* mRNA in response to chronic POMC neuron activation (Figure 4.3E, unpaired student's t-test, $p = 0.841$).

Chronic CNO administration also leads to paradoxical weight gain in male and female mice fed regular chow

As POMC neuron dysregulation in disorders of energy balance is incompletely understood, it was unclear whether the paradoxical weight gain we observed was a function of simply chronically stimulating POMC neurons or whether there was some undetected interaction between the obesogenic diet and chronic stimulation.

We therefore chose to repeat this experiment in female and male mice receiving regular chow. As shown in Figure 4.4, while both female (Figure 4.4A) and male (Figure 4.4B) animals with the hM3Dq DREADD initially showed slight decreases in bodyweight, after a month of stimulation the animals were consistently heavier than animals not expressing the hM3Dq DREADD; this was not, however, a statistically significant finding, perhaps due to considerable variability in the data (Female bodyweight: Figure 4.4A, mixed effects analysis, $F_{(1,9)} = 1.047$, $p = 0.3329$; Male bodyweight: Figure 4.4C, two-way repeated measures ANOVA, $F_{(1,6)} = 0.2658$, $p = 0.6246$). Similar to the food intake data in animals fed an obesogenic diet (Figure 4.3), food intake data from animals fed normal chow was quite variable and significance was not achieved (Female food intake: Figure 4.4B, mixed effects analysis, $F_{(1,10)} = 2.076$, $p = 0.1802$; Male food intake: Figure 4.4D, mixed effects analysis, $F_{(1,8)} = 3.244$, $p = 0.1094$).

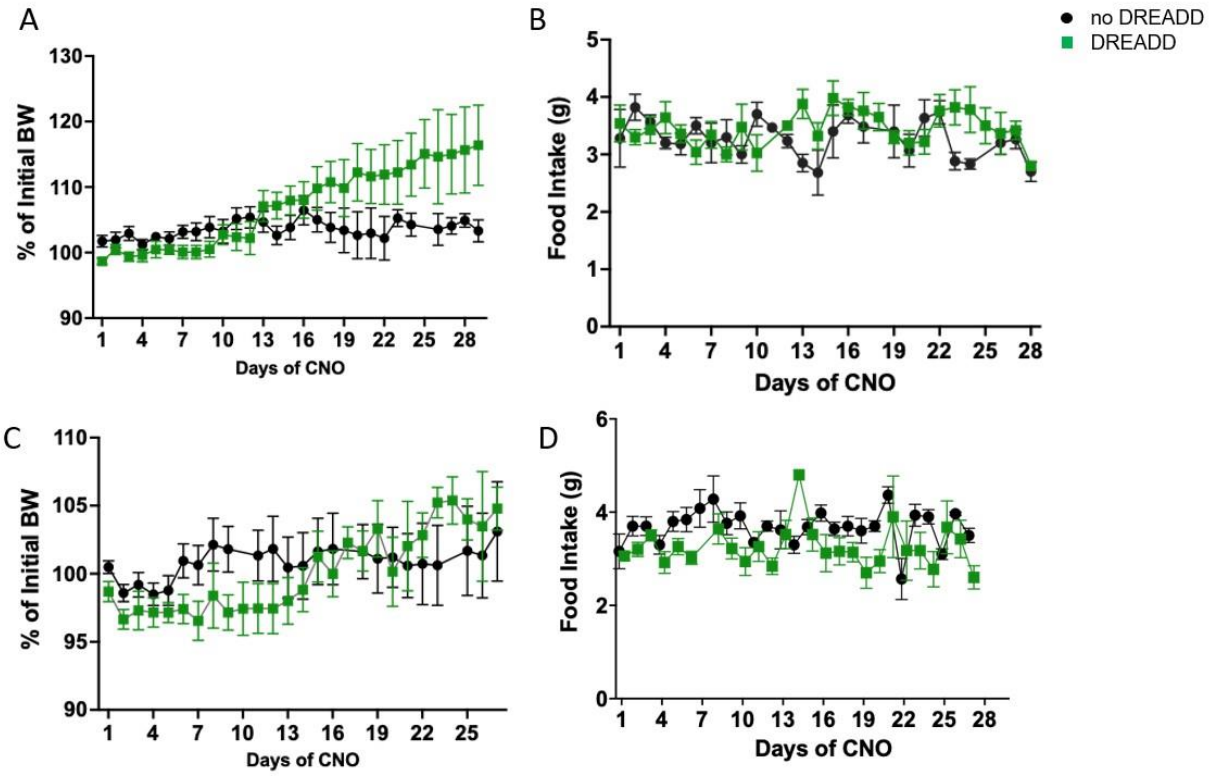

Figure 4.4. Effects of chronic CNO administration on bodyweight and food intake in female and male mice fed normal chow. The effect on bodyweight and food intake during one month of twice daily CNO injections administered 5.5 h apart during the dark cycle are shown for both females (Bodyweight: Figure 4.4A; food intake: Figure 4.4B) and males (Bodyweight: Figure 4.4C; food intake: Figure 4.4D) on a normal chow diet. Data are presented as mean ± SEM.

Altering the dosing scheme could potentially stave off paradoxical weight gain: a pilot study in mice fed normal chow

From the data presented in Figures 4.3 and 4.4, it appears that paradoxical weight gain is not unique to animals fed a high-fat diet as both normal chow-fed and high-fat diet fed animals showed trends of increasing bodyweight. It could be possible that the dosing scheme that we adopted from Zhan and colleagues (two doses given 5.5 h apart during the dark cycle) is not suited for long-term activation of DREADDs given concerns about receptor desensitization. Therefore, we decided to adopt an

alternative dosing scheme in a pilot study whereby CNO was given approximately every 8 h to mice on normal chow. Given that this is a pilot study, transcutaneous access buttons were not implanted; instead, CNO was administered via i.p. injection. Due to the high number of injections given, this study was limited to a two-week duration, as by two weeks it would be evident whether hM3Dq-expressing animals were beginning experience weight gain relative to non-hM3Dq-expressing controls. As shown in Figure 4.5, it does not appear as though the bodyweights of mice expressing the hM3Dq DREADD would increase relative to controls at the two-week time point as was seen in previous experiments. As this data is merely exploratory, statistical methods were not used to analyze this dataset.

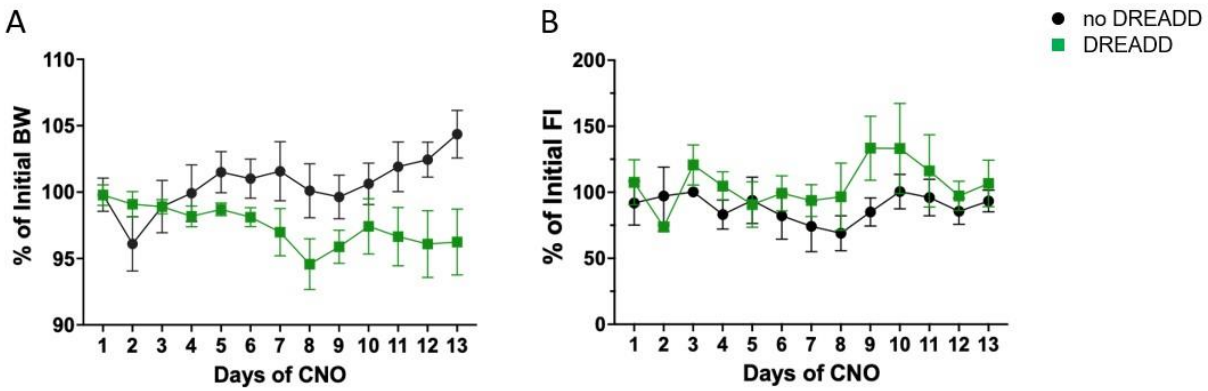

Figure 4.5. Pilot data from mice fed normal chow treated with CNO 3 times a day with an interdose interval of approximately 8 hours. Bodyweight (A) and food intake (B) data from male and female mice (combined) either with or without the hM3Dq DREADD on POMC neurons following 3x/day CNO administration. Data are presented as mean ± SEM.

4.6 Discussion

The experiments discussed in the current chapter set out to test the hypothesis that chronic stimulation of POMC neurons produces weight loss and decreased food

intake in mice expressing the stimulatory hM3Dq DREADD. We found the following results: a) decreased food intake can be produced following POMC neuron stimulated via the hM3Dq stimulatory DREADD (Figure 4.1); b) continuous CNO administration via an osmotic pump is not a feasible drug delivery method, at least in the current studies (Figure 4.2); c) after approximately two weeks of twice daily CNO treatment, mice of both sexes fed normal chow or a high-fat diet began to exhibit unexpected weight gain rather than weight loss (Figure 4.3 and 4.4); and d) data from a pilot study might indicate that this paradoxical weight gain can be avoided if CNO is administered throughout both the light and dark phases of the light/dark cycle and a longer interdose interval is used (Figure 4.5).

One of the assumptions critical to our hypothesis is that DREADD activation via CNO administration facilitates synaptic transmission in the cells expressing the DREADD (Roth 2016). In addition to the in vivo food intake data presented in the current chapter, our lab has also performed calcium imaging studies in brain slices to assess the ability of CNO to activate DREADD receptors on POMC neurons (Metz et al. conditionally accepted at the American Journal of Physiology Regulatory, Integrative and Comparative Physiology). Following bath application of CNO to brain slices containing POMC neurons, we observed an increase in fluorescence of the calcium indicator gCaMP6f, indicating that POMC neurons were can be activated in vitro (Metz et al. conditionally accepted). Thus, we feel confident in our interpretation that we have successfully activated POMC neurons via hM3Dq DREADDs in the current study.

We based our initial dosing scheme of twice-daily injections spaced 5.5 h apart in the dark cycle on the work of Zhan and colleagues, as this group had previous success

in generating decreased bodyweight and food intake using this dosing scheme (Zhan et al. 2013). However, it appears that after one week (on high-fat diet) or two weeks (on normal chow), animals begin to gain weight, an effect that continued for the duration of the study. *Pomc* mRNA and presumably peptide release is modulated in response to an organism's energy state (Schwartz et al. 1997; Mizuno et al. 1998; Benoit et al. 2002) and previous work has shown *Pomc* mRNA levels to be decreased following exposure to a high-fat diet (Souza et al. 2016; Huang et al. 2003). Thus, we hypothesized that chronic POMC neuron stimulation might alter *Pomc* mRNA levels relative to controls. However, we did not observe a difference in *Pomc* mRNA levels in mice fed a high-fat diet, suggesting that regulation of POMC neurons in response to chronic stimulation is likely not occurring at the mRNA level. Future studies should examine POMC peptide release following chronic POMC neuron stimulation, as it is possible that with repeated neuronal stimulation that POMC neurons no longer release maximal amounts of α-MSH. Indeed, both DREADDs and the melanocortin receptor that the α-MSH binds to are prone to desensitization as GPCRs (Sharma et al. 2019; Shinyama et al. 2003). If desensitization were to have occurred in the current study, this might explain the paradoxical weight gain observed in the latter half of the experiment.

Peptide transmitters are often released in a pulsatile manner from dense core vesicles within the cell (Vilim et al. 1996). A large increase in α-MSH can be observed following intake of a meal, yet if release is again stimulated by consumption of a second meal prior to α-MSH levels returning to baseline, a less pronounced peak in α-MSH is observed (Enriori et al. 2016). While the dosing scheme was chosen to mimic previous literature, it is possible that the 5.5 h interdose interval was not long enough to allow

peptide stores to fully replenish, especially under the chronic stimulation protocol. Thus, we performed a pilot study where we increased the interdose interval from 5.5 h to 8 h; though the data are preliminary, it is possible that this increase in interdose interval was sufficient to allow for peptide stores to fully replenish as bodyweights were not appearing to increase at the two-week timepoint. However, weight loss was still not observed; therefore, the data presented in this chapter suggest that chronic manipulation of POMC neurons to cause weight loss and decreased food intake is a more complex undertaking than initially hypothesized. Additional experiments on this topic are needed if we are to successfully manipulate the POMC system long-term.

Chapter 5: Conclusions

5.1 Summary

Proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) of the hypothalamus are well known for their role as critical mediators of energy balance. Thus, treatment strategies for disorders of energy balance have focused on POMC cells or their targets. To advance this line of research, the overarching goal of the work presented herein was to determine whether manipulation of POMC neurons could improve pathophysiological alterations in bodyweight and food intake in mouse models of energy balance disorders. Energy balance disorders can be divided into those of negative energy balance and positive energy balance. To study the role of POMC neurons in both of these conditions, chapters 2 and 3 address anorexia nervosa (AN) as an example of a disorder of negative energy balance, while chapter 4 addresses obesity as an example of a disorder of positive energy balance.

Chapters 2 and 3 provide support for the idea that POMC neurons are selectively involved in generating food anticipatory activity (FAA) in mice undergoing activity-based anorexia (ABA), with minimal contributions to the excessive weight loss and severely restricted food intake observed in animals undergoing the behavioral paradigm. Disruption of β-endorphin signaling as well as inhibition of the entire POMC neuron resulted in decreased FAA, suggesting that POMC neurons mediate FAA expression via its peptide product β-endorphin. Chapter 4 provides evidence to suggest that chronic stimulation of POMC neurons, at least at the dosing scheme used in the current experiments (twice daily for one month, doses administered 5.5 h apart during the dark

cycle), might cause more harm than good given that animals gained weight compared to controls after two weeks of stimulation.

There are at least two ways in which these results could be interpreted. One interpretation of the results is that manipulation of POMC neurons during diseased metabolic states is inconsequential at best (minimal effects on bodyweight and food intake in chapters 2 and 3) and deleterious at worst (increased bodyweight in chapter 4). While it is understandable how one would come to this conclusion, I would argue that this conclusion overlooks some of the nuances inherent to these neurons. Perhaps it was a little naïve to, in essence, apply Joliffe's "appestat" hypothesis to a single population of neurons (see chapter 1 for original discussion of the "appestat" concept) in thinking that one could simply "dial up" POMC neuron activity to improve outcomes in an animal model of obesity and "dial down" POMC neuron activity to improve outcomes in an animal model of AN. A more realistic interpretation would acknowledge that evolutionary pressures predispose an organism to optimize chances of survival by favoring 1) a healthy appetite and 2) the ability and motivation to seek out food. Each of these points will now be addressed.

5.2 Evolutionary Considerations: Hunger wins out over satiety

While a satiety signal certainly has its benefits, such as initiating meal termination to avoid overfilling and potentially rupturing parts of the gastrointestinal tract, in times of food scarcity it would be disadvantageous to be able to easily manipulate neurons that signal satiety. Moreover, it could be detrimental if the satiety signal network always responded maximally to activation cues; in the event of aberrant activation signals, it would be advantageous from an evolutionary standpoint to be able to ignore and not

respond to these cues in order to maintain a state of hunger. Viewed this way, perhaps it is not surprising that repeated chemogenetic stimulation of POMC neurons as discussed in chapter 4 not only led to a lack of bodyweight loss but also over time caused weight gain.

Consuming food is essential to life, so it makes intuitive sense that when hunger and satiety are pitted against one another that hunger will win out. Indeed, Wei and colleagues performed exactly this experiment by optogenetically stimulating POMC and appetite stimulating agouti-related peptide (AGRP) neurons simultaneously; the fact that the mice ate voraciously indicates that hunger signals will overrule satiety signals (Wei et al. 2018).

The deleterious impact of a regulatory system built to let hunger prevail is evident in the world around us. Humans are not the only species with alarmingly high rates of obesity; companion animals like dogs and cats (see chapter 1 for more information) and even zoo animals such as Asian elephants (Chusyd et al. 2021) and lemurs (Mellor et al. 2020) are experiencing unprecedented levels of obesity amongst their respective populations. Clearly, our ancient homeostatic regulatory systems are no match for the modern world where food is always available and often delivered right to our doors (or food bowls or enclosures, in the case of our pets and zoo animals). What can be done about this?

While we may not be able to alter the predisposition of our physiology towards weight gain and energy surplus, perhaps there are ways that we could counteract these tendencies. However, in order to do this we need to better understand how it is that POMC neurons appear to dampen their response to repeated stimulation. While *Pomc*

mRNA levels are sensitive to energy state, as shown in chapter 4 it does not appear that chemogenetic stimulation of POMC neurons alters the amount of mRNA generated. Bioactive POMC peptides are generated by post-translational processing via cleavage enzymes such as the prohormone convertases PC1/3 and PC2 (Cawley, Li, and Loh 2016), therefore it is possible that while mRNA levels remain stable, less cleaved peptide is produced. Following cleavage, POMC peptides must also be trafficked and eventually released from dense core secretory vesicles (Harno et al. 2018); therefore, another possibility is that less peptide is released in response to repeated POMC neuron stimulation. Future studies should determine whether either or both of these scenarios are true.

While filling these gaps in knowledge will help move the field toward a way to manipulate POMC neurons to produce and maintain sustained weight loss, the fact remains that obese humans and animals require help today. In my opinion, this underscores the importance of lifestyle interventions including consumption of a nutritionally balanced and appropriately portioned diet, as well as regular exercise. Many would appreciate the convenience and ease of a magic weight loss pill, yet it does not appear that evolution will allow such an endeavor to succeed.

5.3 Evolutionary considerations: POMC and the food entrainable oscillator

As mentioned previously in section 5.1, it appears that evolution has selected for the motivation and ability of an organism to seek out its food in addition to a robust appetite. If food sources tend to be more readily available during a certain time of day, it would be advantageous to be able to predict the impending arrival of one's food source so that the organism can prepare accordingly. The food entrainable oscillator (FEO)

allows for just that, first described by Richter nearly a hundred years ago (Richter 1922). Despite decades of subsequent study, the neuroanatomical location of the FEO has yet to be discovered, leading some to postulate that unlike the light entrainable oscillator located singularly in the suprachiasmatic nucleus (SCN) of the hypothalamus, the FEO might be comprised of multiple cell types and signals (Mistlberger 2020). The main behavioral output of the FEO is food anticipatory activity (FAA); thus, because inhibiting either β-endorphin (chapter 2) or POMC signaling (chapter 3) had minimal effects on bodyweight and food intake yet significantly reduced FAA, it could very well be that β-endorphin released from POMC neurons is a key component of the FEO.

To explore this possibility, future experiments could determine whether β-endorphin knockout mice are incapable of developing FAA; this experiment would be as simple as performing the same ABA paradigm used in chapters 2 and 3. If this hypothesis turned out to be true there are interesting implications for individuals suffering from AN, in particular AN patients that present with excessive exercise habits. It is likely that the increased activity observed in AN patients is how FAA is exhibited in modern times. Inhibition of β-endorphin could be beneficial in reducing the excessive exercise/activity observed in up to 80% of AN patients (Casper et al. 2020); indeed a precedent has been set for this as the β-endorphin receptor antagonist naloxone has been given to AN patients receiving inpatient therapy, though the primary outcome measured in this trial was bodyweight and not excessive activity (Moore, Mills, and Forster 1981). The fact that Moore and colleagues observed improvements in weight gain with naloxone is encouraging.

5.4 Closing Remarks

The data presented in this dissertation serve to broaden the field's understanding of POMC neuron functionality in disorders of energy balance. The POMC gene sequence evolved over 500 million years ago in times when food was scarce; the oldest animal to have a POMC system like those found today are lampreys, an ancient jawless fish (Navarro et al. 2016; Takahashi and Kawauchi 2006). Given its long history of existence and high degree of homology between species, a strong evolutionary advantage must be conferred by this system otherwise it would not have persisted as long as it has. Data from chapters 2 and 3 suggest that the role of POMC neurons, and in particular the peptide product β-endorphin, is likely to drive the development of FAA. This uncovers a potential new therapeutic avenue for excessive activity observed in AN patients. Data from chapter 4 indicate that long-term stimulation of POMC neurons can lead to paradoxical weight gain over time, highlighting the need for future research in this area if stimulation of POMC neurons is to be a viable option for weight loss.

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Appendix I

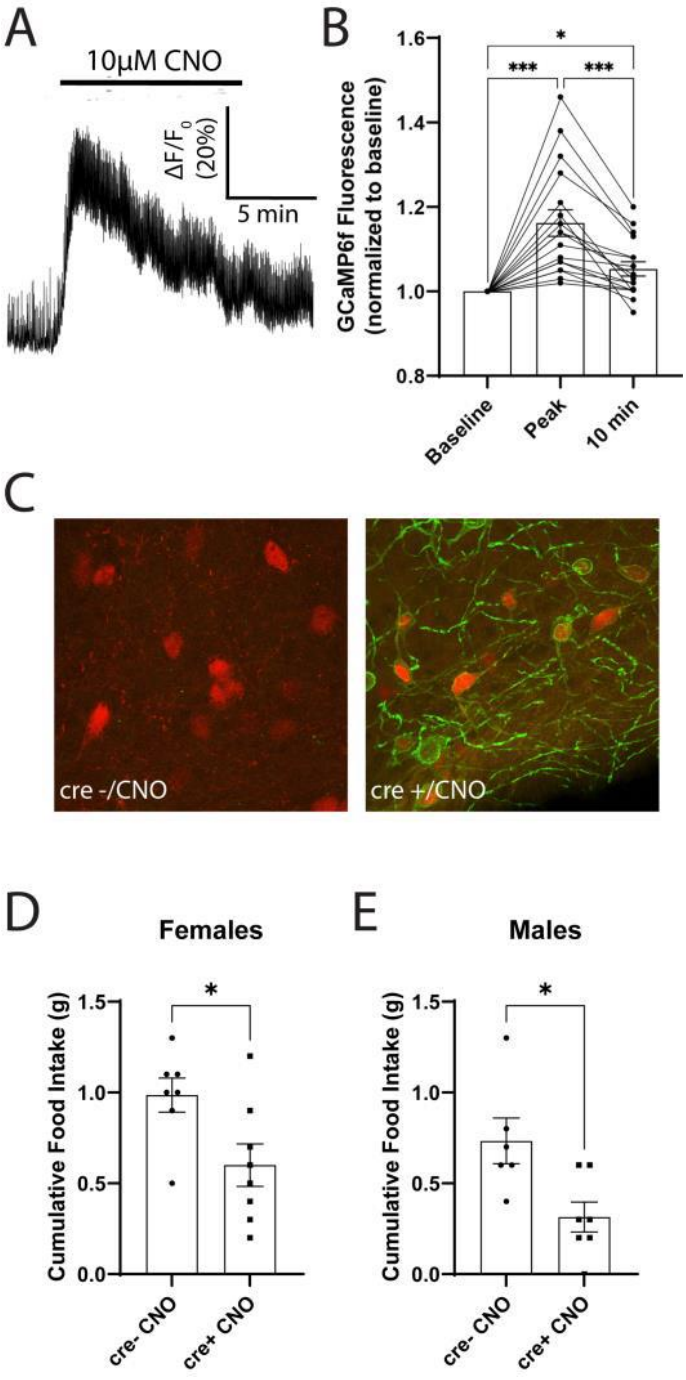

Figure S1. Gq DREADDs activate POMC neurons in vitro and in vivo. A) Representative trace of GCaMP6f fluorescent activity in an ARH POMC neuron virally expressing AAV-hM3Dq (Gq DREADDs) after 10 μM CNO application. B) GCaMP6f

fluorescence increased soon after CNO application and decreased within 10 min of continuous CNO application but remained significantly above baseline fluorescence. Each point represents a single POMC cell. C) Immunohistochemical detection of HA-tagged Gq DREADDs (green) in ARH POMC neurons (red). No detectable Gq DREADD expression was observed in tissue from POMC cre- mice (left), but Gq DREADDs were detected in tissue from POMC cre+ mice (right). D) Cumulative food intake during a 2 h refeed after an overnight fast was decreased in POMC cre+ mice compared to POMC cre- mice for females and E) males. *$p > 0.05$, **$p > 0.001$.

Figure 3 in: Metz, Marissa J., Daimon, Caitlin M., King, Connie M., Rau, Andrew R., and Shane T Hentges. (2021) “Individual arcuate nucleus Proopiomelanocortin neurons project to select target sites.” Accepted for publication in the American Journal Physiology – Regulatory, Integrative and Comparative Physiology.