DISSERTATION

MANIPULATION AND CHARACTERIZATION OF PROOPIOMELANOCORTIN (POMC) NEURONS IN THE HYPOTHALAMIC REGULATION OF ENERGY BALANCE

Submitted by

Christina Suzanne Dennison

Department of Biomedical Sciences

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Doctoral Committee:

Advisor: Shane T. Hentges
Co-advisor: Kathy M. Partin

Michael J. Pagliassotti
Michael M. Tamkun
Jozsef Vigh

ABSTRACT

MANIPULATION AND CHARACTERIZATION OF PROOPIOMELANOCORTIN (POMC) NEURONS IN THE HYPOTHALAMIC REGULATION OF ENERGY BALANCE

It is well documented that hypothalamic proopiomelanocortin (POMC) neurons are a critical component in the maintenance of energy balance. POMC neurons release peptide transmitters that modulate pathways involved in food intake, energy expenditure, and reward pathways. POMC peptide release can result in the inhibition of food intake and increased activation of these cells is thought to precipitate the development of anorexia in the activity-based anorexia (ABA) rodent model. Currently, the physiological underpinnings that drive the development of anorexia are not fully understood, but evidence suggests that POMC neurons are a likely contributor. The work presented in Chapter 2 of this dissertation provide further evidence that POMC neurons are activated during the early development of ABA and addresses whether this increase in POMC neuron activation is necessary for decreased food intake and body weight during ABA. POMC neurons were selectively inhibited during the onset of ABA. The results presented here indicate that POMC neuron activation facilitates suppression of food intake during the early stages of ABA. To determine if increased activation of POMC neurons is sufficient to induce lasting anorexia, in Chapter 3 POMC neurons were activated acutely and long-term to determine if activation of these cells alone is sufficient to initiate the development of anorexia. The data in Chapter 3 show that acute activation of POMC neurons decreases daily food intake. Prolonged activation of POMC neurons was able to give rise to long-term decreases in food intake under the proper conditions; but, the data from these experiments provide insight regarding

methodological considerations that are important for long-term behavioral work in rodents. Activation or inhibition of POMC neurons through the approach used here could lead not only to altered release of peptides from these neurons, but also altered amino acid (AA) transmitter release. Unlike, the heavily studied peptides, there is little information regarding AA transmitters in POMC neurons and their effects on energy balance are not known. To begin to build a more comprehensive understanding of POMC neuron physiology, the work described in Chapter 4 characterized the AA transmitter phenotype of POMC neurons during postnatal development and investigated the role of glutamate release from POMC neurons. Data shown here indicate that the AA transmitter phenotype of POMC neurons remains plastic during early postnatal development. Given that young mice are more vulnerable to developing ABA relative to adult mice and that POMC neurons are involved in developmentally regulated events such as reproduction and maturation of feeding circuits the research described here could provide insight into sensitive periods that are more amenable to manipulating POMC neurons. The work in Chapter 4 also shows that glutamate release from POMC neurons is involved in regulating body weight in a sex- and diet-specific manner, which is the first documented example describing the function of AA transmitter release from POMC neurons. Taken together the research described here expand current understanding of how POMC neurons participate in the dysregulation of energy balance. This work also highlights the importance of AA transmitters in POMC neurons and provides insight for future work.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Food consumption not commensurate with energy expenditure results in abnormal regulation of body weight. According to estimates, about 35% of adults in the United States are obese (Ogden et al., 2014) and just over 2% are underweight (Flegal et al., 2005). Obesity increases the risk of comorbidity with diseases such as diabetes, cardiovascular disease, and cancer (Guh et al., 2009). Furthermore, individuals who are obese or underweight have an increased risk of mortality relative to individuals with a normal body weight (Flegal et al., 2005). The economic burden of obesity continues to increase, with annual healthcare costs alone estimated to be about 42% higher than the cost for individuals with normal body weight (Finkelstein et al., 2009b). However, clinically significant weight loss is not associated with reductions in healthcare costs within 2 years following weight loss (Bilger et al., 2013; Finkelstein et al., 2009a); thus, underscoring the need for prevention and early intervention in energy balance dysregulation.

Proopiomelanocortin neurons are part of the neural control of energy balance

A large body of research aimed at understanding food intake and energy expenditure has focused on neural control of energy balance. Many of these studies have investigated neurons located in the arcuate nucleus (ARC) of the hypothalamus. The ARC is in the mediobasal hypothalamus next to the third ventricle and median eminence (ME, See Figure 1.1). Fenestrated endothelial cells in the ME and tanycytes lining the third ventricle increase permeability to circulating metabolic signals that then act on neurons within the ARC (Mullier et al., 2010; Langlet et al., 2013), therefore implicating this region as a key regulator of energy balance.

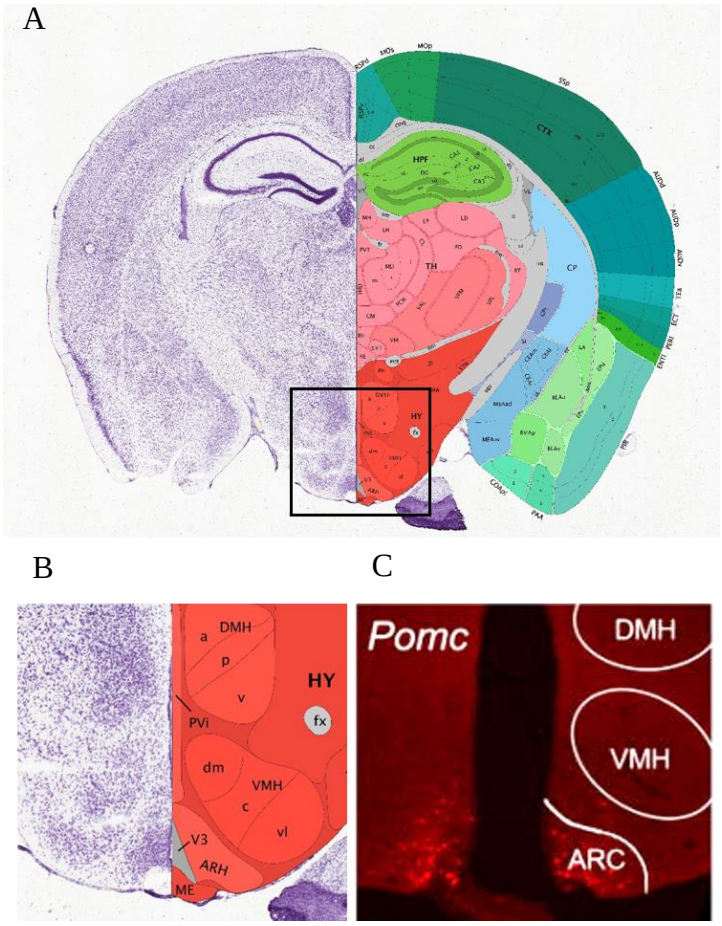

Figure 1.1. POMC neurons are located in the arcuate nucleus of the hypothalamus. A) Coronal section containing ARC from adult mouse brain **(**Allen Institute for Brain Science. Allen Mouse Brain Atlas [Internet]. Available from: http://mouse.brain-map.org/). **B)** Image refers to boxed area in A; enlarged to show hypothalamic nuclei. **C)** Pomc mRNA is localized to the arcuate nucleus. Image published in Jarvie and Hentges (2012). Reproduced with permission: see Appendix I.

A portion of early-onset obesity cases in humans can be attributed to genetic and epigenetic variations in the gene for proopiomelanocortin (POMC) (Krude et al., 1998; Clément et al., 2008; Mediratta MS et al., 2011; Kuehnen et al., 2012; Mencarelli et al., 2012) and the melanocortin system (Farooqi et al., 2003). POMC is the precursor propeptide for adrenocorticotrophin (ACTH), the melanocyte stimulating hormones (α-,β-, and γ- MSH), and beta-endorphin. POMC is expressed in the periphery, pituitary, and ARC (see Figure 1.1C) of the hypothalamus, and release of POMC peptides is regulated in a tissue specific manner such that beta-endorphin and alpha-MSH-but not ACTH-are released from POMC neurons in the ARC. In addition to peptide transmitters, POMC neurons also release amino acid (AA) transmitters (Dicken et al., 2012). Although POMC is expressed in non-neuronal cells, neuron-derived POMC peptides are sufficient to maintain body weight, meal

termination, and energy balance (Smart et al., 2006; Richard et al., 2011). Thus, ARC POMC neurons will be the focus of this dissertation.

Early evidence that hypothalamic POMC peptides are critical in the regulation of energy balance came from rodent studies showing alpha-MSH produces an anorexigenic effect through activation of centrally-expressed melanocortin receptor 4 (MCR-4 [Fan et al., 1997; Huszar et al., 1997]). Additional work has shown that the endogenous POMC peptide beta-endorphin has an inhibitory effect on body weight gain and mediates the hedonic aspects of food intake (Hayward et al., 2002; Appleyard et al., 2003). Moreover, POMC deficiency in mice produced a similar phenotype to that seen in humans, which was attributed to melanocortins (Yaswen et al., 1999; Challis et al., 2004). These data highlight the necessity of POMC peptides and POMC peptide signaling in inhibition of food intake, stimulation of energy expenditure, and the maintenance of appropriate energy balance in both humans and rodents.

Synthesis of POMC peptides is sensitive to energy state

The *Pomc* locus is on chromosome two (Owerbach et al., 1981) and is largely conserved across species (Deen et al., 1991; Dores and Baron, 2011). The mammalian *Pomc* gene contains three exons. Regulatory elements and proximal promoter are on exon one (Deen et al., 1991; Therrien and Drouin, 1991), exon two is required for translation, and exon three contains the coding region for the peptides (Krude et al., 1998; Yaswen et al., 1999). Transcription of neuron-derived *Pomc* is dependent on two neuronal POMC enhancers, nPE1 and nPE2, located upstream of the transcription start site (de Souza et al., 2005; Lam et al., 2015). Following transcription, precursor POMC moves to the Golgi where it is targeted to the regulated secretory pathway for

posttranslational processing and eventual release from dense core secretory vesicles (Stevens and White, 2009). As the prohormone POMC in the central nervous system is trafficked through the secretory pathway, prohormone convertases PC1/3 and PC2, carboxypeptidase E (CPE), peptidyl α-amidating monooxygenase (PAM) cleave and process the propeptide into beta-endorphin (Benjannet et al., 1991) and alpha-MSH (See Figure 1.2; Wardlaw, 2011).

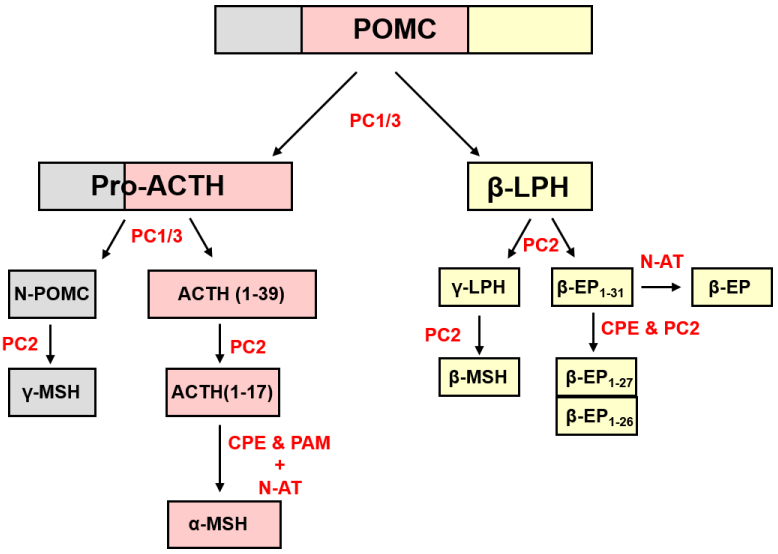

Figure 1.2. POMC posttranslational processing. Diagram showing the cleavage enzymes and major peptide products that are derived from posttranslational processing of POMC. Prohormone convertase (PC); carboxypeptidase E (CPE); peptidyl α-amidating monooxygenase (PAM); N-acetyltransferase (N-AT) is responsible for acetylation of α-MSH and β-endorphin (β-EP). Diagram recreated from Wardlaw (2011).

Synthesis of POMC peptides can be modulated by changes in energy state. For example, rodent studies have shown that acute high fat diet (HFD) increases *Pomc* mRNA (Fan et al., 2011; Marco et al., 2013; Mercer et al., 2014), but others have found that chronic HFD decreases or does not alter the levels of *Pomc* mRNA relative to *ad libitum* fed animals (Gout et al., 2008; Mercer et al., 2014), indicating differential short-term and long-term regulation of *Pomc* expression. During positive energy balance the adiposity signal leptin and the hormone insulin increase and both are associated with an increase in *Pomc* transcription (Schwartz et al., 1997; Mizuno et al., 1998; Benoit et al., 2002; Huo et al., 2006). Conversely, during a negative energy state, such as an overnight fast, *Pomc* mRNA is decreased

(Schwartz et al., 1997; Mizuno et al., 1998; Huo et al., 2006). However, transcription of *Pomc* increases in the refeeding period after fasting, most likely to terminate feeding in rodents (Fekete et al., 2012). A similar phenomenon has been observed in humans whereby underweight individuals have increased plasma levels of alpha-MSH following an afternoon meal, which is also thought to terminate meal-time feeding (Galusca et al., 2015). Although acute bouts of caloric deficits decrease *Pomc* transcription, Hillebrand and colleagues (2006) have shown that *Pomc* mRNA increases during the early stages of anorexia in the rodent model of activity-based anorexia (ABA). Furthermore, beta-endorphin increases during ABA (Aravich et al., 1993) and during physical exercise in rodents (Koehl et al., 2008), which is thought to mediate the rewarding aspects of wheel running in rodents (Garland et al., 2011). Early-onset exercise in rodents results in increased *Pomc* mRNA and resistance to obesity in adulthood (Patterson et al., 2008), suggesting that some energy states can have long-term effects on body weight regulation and *Pomc* gene expression. Together these data suggest that modulation of POMC neurons under certain conditions can promote short-term and long-term metabolic health and maintain a low body weight, but these data also indicate that POMC peptides can become dysregulated and lead to negative energy balance or obesity.

In addition to regulation by metabolic signals, POMC neuron activity is also affected by factors involved in reproduction (de Souza et al., 2011; Xu et al., 2011). Further, POMC neurons provide a link between energy state and reproductive competency and puberty might represent a period of increased plasticity for POMC neurons. The sex hormone estrogen aids in maintaining appropriate body weight and energy expenditure through estrogen receptor-α (ERα) signaling (Heine et al., 2000). About 20-30% of hypothalamic POMC neurons express ERα, which

mediates some of estrogen's effects on food intake and energy expenditure (Gao et al., 2007; de Souza et al., 2011; Xu et al., 2011). Additionally, de Souza and colleagues (2011) found a binding motif on nPE2 for ERα, suggesting a role for estrogen signaling in regulating *Pomc* transcription specifically in neurons. Given that POMC neurons are implicated in the ABA rodent model of anorexia and anorexia is associated with amenorrhea (Klenotich and Dulawa, 2012), it is possible that POMC neurons relay information about reproductive health during negative energy states.

As evidenced by the research described above, one of the ways POMC neurons mediate energy balance is through changes in gene expression and the release of peptides that occur in response to signals associated with energy state. While these data indicate that POMC can be regulated at the level of transcription, recent evidence suggests POMC processing can be altered without affecting *Pomc* transcription (Cakir et al., 2013) and individual POMC-derived peptides might be differentially trafficked and processed according to nutritional state (Mercer et al., 2014). Although it is enticing to think that changing the level of POMC peptides in accordance with energy state will simply counteract the caloric surplus during positive energy states and caloric deficit during negative energy states, the data implicating POMC neurons in anorexia suggest otherwise. Based on the aforementioned literature, it is clear that short-term and long-term metabolic states can differentially regulate *Pomc* gene expression (e.g. acute vs chronic HFD); however, to what extent AA transmitter expression in POMC neurons is influenced by energy states is not yet clear.

While expression of functional *Pomc* gene products is a necessary component in maintaining energy balance, it is the action of these transmitters at their target sites that confer physiological and behavioral effects. Mapping the connections between hypothalamic POMC neurons and synaptically coupled partners has not been a trivial undertaking. Unlike laminated structures such as the hippocampus or cerebellum, the ARC is composed of intermingled, molecularly distinct groups of neurons that make intra-ARC connections in addition to sending and receiving projections in a distributed manner (Wang et al., 2015). This complexity within the ARC has created technical difficulties that continue to be mitigated by new technologies and innovative use of existing tools. Nevertheless, understanding how transmitters released from POMC neurons regulate energy balance depends on how these cells integrate into the neural circuitry. Hence, to fully appreciate the involvement of POMC neurons in mediating food intake and energy expenditure an overview of the research elucidating projections from POMC neurons and the consequence of transmitter release follows.

POMC peptide release modulates energy state

Elucidating the potential effects of POMC peptide release onto neurons at various projection sites has been facilitated through the localization of neurons with receptors capable of responding to specific peptides. For example, within the hypothalamus POMC neurons project to the lateral hypothalamus (LH), supraoptic nucleus (SON), and paraventricular hypothalamus (PVN, [Bouret et al., 2004; King and Hentges, 2011; Wang et al., 2015]), and each of these areas contain neurons that are activated by exogenous alpha-MSH by way of MCR-4s (Sabatier et al., 2003; Ghamari-Langroudi et al., 2011; Cui et al., 2012; Siljee et al., 2013). Interestingly, Balthasar and colleagues (2005) found that MCR-4s in the PVN selectively modulate feeding

aspects of melanocortin signaling, therefore indicating that other areas are responsible for the effects of alpha-MSH on energy expenditure. Indeed, MCR-4s in sympathetic, but not parasympathetic, preganglionic neurons mediate energy expenditure and diet- and cold-induced thermoregulation (Berglund et al., 2014). Genetic deletion of MCR-3s, which are also expressed in the brain and activated by alpha-MSH, suggests that these receptors participate in regulating metabolism and energy expenditure (Butler et al., 2000). This study also found that, unlike mice lacking MCR-4s, mice without MCR-3s do not display hyperphagia but do appear to have an inability to regulate energy stores commensurate with HFD since these mice have increased fat mass. King and Hentges (2011) found that POMC neurons also project sparsely to regions outside of the hypothalamus including the ventral tegmental area (VTA), amygdala, and dorsal vagal complex (DVC). These brain regions are sensitive to melanocortins as evidenced by altered food intake following infusion of melanocortin receptor agonists and antagonists (Williams et al., 2000; Boghossian et al., 2010; Roseberry, 2013).

Work has also been carried out to understand the function of beta-endorphin-mediated opioid signaling in homeostatic and hedonic aspects of food intake and energy expenditure. Acute intracerebroventricular delivery of exogenous beta-endorphin increases food intake (Silva et al., 2001), but genetic removal of beta-endorphin also leads to increased food intake and body weight in mice (Appleyard et al., 2003). It was suggested in this later study that the discrepancy might be explained by acute and chronic delivery of beta-endorphin having differential effects on food intake. Work by Pennock and Hentges (2011) found that μ-opioid receptors on the pre- and post-synaptic compartments of POMC neurons are differentially regulated by agonists, whereby low concentrations activate presynaptic receptors but higher concentrations are required to

activate postsynaptic receptors. It was suggested that this type of mechanism might explain the disparate effects of beta-endorphin on food intake such that high concentrations inhibit POMC neurons but low concentrations disinhibit. Work by Hayward and colleagues (2002) indicates that chronic beta-endorphin increases the reinforcement value of food under *ad libitum* feeding conditions in mice. However, μ-opioid receptors, and presumably beta-endorphin, might also mediate energy balance during a deprived state given that increased physical activity in response to food anticipation is partly dependent on these receptors in rodents (Kas et al., 2004). Although POMC peptides are regarded as having an anorexigenic effect, it is clear from these data the effects of beta-endorphin on food intake are dependent on duration of opioid treatment and energy state.

The behavioral effects of activating POMC neurons are often attributed to peptide transmitters with little consideration for glutamate and GABA (gamma-Aminobutyric acid [see Aponte et al., 2011; Zhan et al., 2013]). For example, Zhan and colleagues (2013) found that long-term, but not acute, activation of POMC neurons reduces food intake and body weight, which was assumed to arise through activation of melanocortin pathways. Recent work has shown that distinct subsets of POMC neurons express GABAergic and glutamatergic markers, and a smaller population expresses markers for both transmitters (Jarvie and Hentges, 2012; Wittmann et al., 2013). POMC neurons also release GABA, as well as glutamate, in culture (Hentges et al., 2004; Hentges et al., 2009) and in an intact neural circuit (Dicken et al., 2012). While the function of AA transmitter release from POMC neurons is not yet known, it is possible that the discrepancies observed for acute versus chronic feeding states and short- versus long-term exposure to POMC peptides is attributable to differential regulation of energy balance by AA versus peptide

transmitters. Similar inconsistencies have been observed for AgRP/NPY/GABA releasing neurons in the ARC and recent work has increased current understanding of these transmitters in stimulating feeding acutely and over prolonged periods of time (Krashes et al., 2013). This study also increases awareness for the differential role of peptides and AAs in regulating food intake and body weight. Nevertheless, thoroughly dissecting the physiological consequences of endogenous peptide versus AA transmitter release from POMC neurons has not yet been accomplished.

Summary

The literature reviewed here provides a brief overview of how POMC neurons are modulated under different energy states and how POMC peptides mediate energy balance. While hypothalamic POMC neurons are necessary for managing appropriate food intake and body weight, it is clear that the manner in which they contribute to maintaining energy balance is incredibly complex. Moreover, current understanding of how transmitters released from these cells participate in specific feeding-related behaviors *in vivo* and over time is limited. Much attention has been given to understanding POMC neurons in terms of obesity, while considerably less focus has been on the role of POMC neurons in contributing to dysregulation of negative energy balance. Based on the research highlighted above, it is likely that POMC neurons participate in the physiology underlying anorexigenic states, but whether these cells initiate and/or maintain a negative energy balance through the release of AA or peptide transmitters is not known. The work presented in this dissertation is aimed at understanding the role of POMC neurons during negative energy balance and the plasticity of AA transmitters in POMC neurons during development.

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CHAPTER 2: INHIBITION OF POMC NEURONS TO DIMINISH ACTIVITY BASED ANOREXIA IN A RODENT MODEL

All work presented in Chapter 2 of this dissertation was carried out by Christina Dennison in the Hentges Lab and was overseen by Shane Hentges. This Chapter consists of unpublished data to be included in a future publication that will incorporate the work and contributions from other members in the lab.

- Previous work shows that neural activity and hypothalamic *Pomc* mRNA are transiently increased during the development of anorexia in the activity-based anorexia (ABA) rodent model
- Work in Chapter 2 will test the hypothesis that an increase in POMC neuron activity is necessary for the development of ABA
- Food restriction paired with voluntary wheel running (ABA) results in progressive loss of body weight, an increase in wheel running activity, and a transient increase in *Pomc* mRNA
- Inhibition of POMC neurons with the inhibitory DREADD during the development of ABA increases food intake.
- Findings presented here implicate a role for POMC neurons in feeding behavior during the development of ABA.

Box 2.1. Overview of Chapter 2

Introduction

A loss of appetite, or anorexia, is associated with many illnesses (i.e. cancer, HIV/AIDS and anorexia nervosa [AN]) and can occur under various conditions (i.e. amphetamine abuse and intense/prolonged exercise)-all of which, if left untreated, pose risks to an individual's health and are associated with increased mortality (Kaye et al., 2000; Morley et al., 2006). Currently

effective treatments are not available for individuals with AN and only about 40% of these individuals fully recover (Kaye et al., 2000); thus, highlighting the need to elucidate the early physiological changes that give rise to decreased food intake and increased hyperactivity.

Hypothalamic POMC neurons suppress food intake and aid in the maintenance of appropriate body weight (Krude et al., 1998; Yaswen et al., 1999). POMC peptides have also been implicated in AN and the rodent model of ABA. The ABA rodent model is a well-characterized paradigm for investigating anorexia in rodents (Dwyer and Boakes, 1997; Verhagen et al., 2011; Klenotich and Dulawa, 2012). In this model limited food access is paired with voluntary wheel running, which results in progressive body weight loss and increased wheel running over the course of three days. During ABA hypothalamic neurons have increased expression of c-Fos (Verhagen et al., 2011); furthermore, *Pomc* mRNA is transiently increased at the start of the dark cycle in rats during the first few days of ABA (Hillebrand et al., 2006). This increase in *Pomc* transcription is in contrast to the diurnal rhythm of *Pomc* mRNA in a normally fed mouse, which approaches nadir at the start of the dark cycle presumably to facilitate increased food intake that occurs at this time (See Figure 2.1; Stötz et al., 2007). Counterintuitive to the homeostatic role of POMC peptides, the increase in *Pomc* mRNA (and presumably peptide release) likely precipitates the decreased food intake despite increased energy output. Indeed, POMC peptides

***Pomc* mRNA:**	**Dark**	**Light**
Ad Libitum	**+**	**+++**
ABA	**+++**	**?**

Figure 2.1. *Pomc* mRNA at the start of the dark cycle in *ad libitum* and ABA mice. *Pomc* mRNA approaches nadir at the start of the dark cycle in *ad libitum* fed mice, but is increased in ABA mice. Under *ad libitum* feeding, *Pomc* mRNA slowly increases during the dark cycle and is highest at the start of the light cycle. Schematic created based on data from Stötz et al. (2007).

are capable of facilitating the progression of ABA since intracerebroventricular delivery of alpha-MSH exacerbates the weight loss and decrease in food intake during ABA (Hillebrand et al., 2005). Further evidence shows beta-endorphin increases during ABA (Aravich et al., 1993) and blocking endogenous opioids suppresses increased wheel running during food restriction in rats (Boer et al., 1990). Moreover genetic deletion of MCR-3s increases 24-hour wheel running in mice, thus suggesting a role of melanocortins in physical activity (Butler et al., 2000). In the human literature beta-endorphin has been shown to increase during exercise in an intensity-dependent manner (Goldfarb et al., 1990). Although both increased (Kaye et al, 1982; Brambilla et al., 1995) and decreased (Kaye et al., 1987) levels of beta-endorphin have been reported for individuals with AN, it appears there is a dysregulation of POMC during AN and ABA.

While it has been established that hypothalamic neurons increase cell activity (Verhagen et al., 2011) and that *Pomc* mRNA transiently increases during ABA in rats (Hillebrand et al., 2006), it is not known if these increases are necessary for the development of ABA. Inhibition of POMC neurons during the development of ABA would help elucidate the necessity of these cells in the development of ABA. While it has not been shown directly that changes in membrane potential and c-Fos expression alter *Pomc* transcription, c-Fos can enhance POMC promoter activity by binding to a response element on exon 1 (Boutillier et al., 1995) and it has been suggested that changes in synaptic activity can differentially regulate gene expression (Jain and Bhalla, 2014). Indeed, increased c-Fos expression in POMC neurons is associated with an increase in *Pomc* mRNA following leptin-induced activation of POMC neurons *in vivo* (Huo et al., 2006).

The inhibitory designer receptor exclusively activated by designer drug (DREADDs) is capable of suppressing POMC neuron activity and increasing 24-hour food intake in rodents (Atasoy et al., 2012). DREADDs are G-protein coupled receptors (GPCRs) engineered by mutating muscarinic acetylcholine receptors so they no longer have an affinity for their endogenous ligand acetylcholine, but rather respond to the synthetic ligand clozapine-N-oxide (CNO; [Armbruster et al., 2007; Nawaratne et al., 2008]), which is capable of crossing the blood-brain barrier (Rogan and Roth, 2011). Receptors coupled to $G\alpha_i$ (inhibitory DREADD or hM4Di), $G\alpha_s$ (Gs DREADD), and $G\alpha_q$ (stimulatory DREADD or hM3Dq) have been developed so that cell activity can be manipulated by signaling through each G-protein's respective pathway (Urban and Roth, 2015). Regardless of coupling each receptor is activated by CNO, which has been shown to initiate the same signaling cascade as the muscarinic acetylcholine receptors when activated by acetylcholine (Alvarez-Curto et al., 2011; Armbruster et al., 2007). The Cre-*lox*P system allows for site-specific recombination, which occurs when Cre-recombinase catalyzes the recombination of a gene flanked by *lox*P sites (also referred to as floxed [Sauer and Henderson, 1988]). Using Cre-*lox*P technology DREADDs can be targeted to Cre recombinase-expressing cells using replication deficient adeno-associated viral (AAV) vectors to deliver a Cre-dependent sequence of the gene containing DREADD and a fluorescent reporter.

The research presented here will use the inhibitory DREADD to test the hypothesis that an increase in POMC neuron activity is necessary for the development of ABA. Specifically, the work described here will address whether or not inhibition of POMC neurons increases food intake and decreases the body weight loss during ABA. The results of this study indicate that inhibitory DREADD-mediated inhibition of POMC neurons at the start of the dark cycle during

ABA increases food intake. These data provide further evidence for the role of POMC neurons in the development of ABA.

Methods and materials

Animals

Male and female transgenic mice expressing Cre recombinase driven by the POMC promoter (POMC-Cre) were used to selectively express the inhibitory DREADD in POMC neurons in the ARC. Transgenic animals were produced by standard techniques and validated elsewhere (Xu et al., 2005) and backcrossed >12 generations onto the C57Bl/6 strain (Jackson Laboratories, Bar Harbor, ME). Animals were 10-12 weeks old during the experiments. Mice were maintained on a 12 hr light/dark cycle and under controlled temperature (22–24°C). Mice had *ad libitum* access to standard rodent chow and water unless otherwise stated. All experiments met United States Public Health Service guidelines with the approval of the Colorado State University Institutional Animal Care and Use Committee.

Development of Activity-based anorexia (ABA)

Female wild-type (WT) and POMC-Cre mice were individually housed in cages equipped with running wheels (Columbus Instruments, Columbus, OH). Animals were acclimated for three days prior to collection of baseline data for body weight, 24-hour food intake, and wheel running activity. Body weight and food intake were measured each day one hour before lights out. Wheel revolutions were acquired in 15 minute bins using Multi Device Interface software from Columbus Instruments (Columbus, OH). Animals were assigned to one of three groups – *ad libitum* fed/running, food restricted/sedentary (FR/SED), and food restricted/running (also

referred to as ABA group or mice). Mice in the FR/SED group were individually housed in cages with running wheels, but the wheels were locked throughout the experiment. Animals in the two 'running' groups had voluntary access to running wheels throughout the experiment. Groups were matched for body weight and age. Baseline body weight and food intake were measured for five days before starting ABA/food restriction days (see Figure 2.2). ABA and *ad libitum* fed groups were also matched for baseline wheel running activity (WRA). During the ABA/food restriction days, food was presented during the first two hours of the dark cycle. Animals that developed ABA had the following features: 1) progressive weight loss during food restriction, 2) progressive increase in 24 hour WRA, and 3) progressive increase in wheel running four hours prior to food access (referred to as food anticipatory activity or FAA). FAA is a key feature of

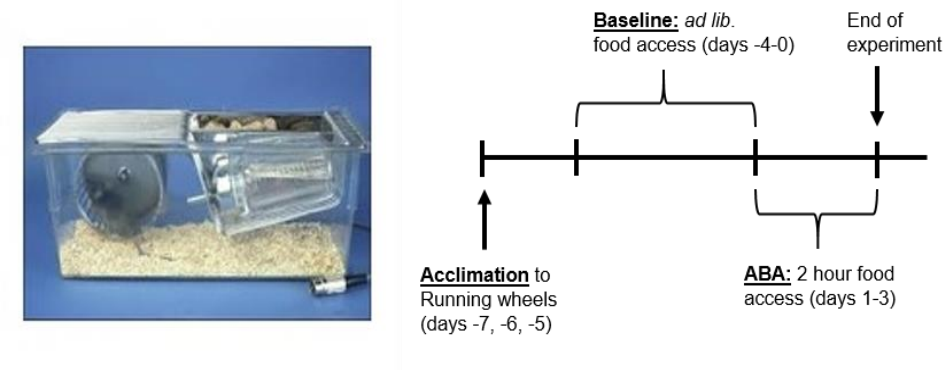

Figure 2.2. Activity-based anorexia (ABA) mouse model. Animals were individually housed in wheel cages 3 days before baseline data was collected. Animals had ad lib access to wheels and water throughout the experiment. Body weight and food intake was measured within 1 hour of the start of the dark cycle. During the ABA/food restriction days, food access was given during the first 2 hours of the dark cycle.

ABA and development of ABA is altered in the absence of FAA (Dwyer and Boakes, 1997; Pjetri et al., 2012). Animals not meeting this behavioral profile were excluded from analysis. Animals with weight loss exceeding 20% of baseline body weight were removed from the experiment. Mice did not experience more than three days of food restriction. Physical

appearance and ambulation of the mice were monitored throughout the experiment and mice were removed from the experiment if they appeared cold or exhibited weakness that prevented normal movement and eating behavior.

Detection of Pomc mRNA during ABA

Female WT mice aged 10-12 weeks were assigned to the control group (day 0) or one of three ABA groups (day 1, day 2, or day 3). All animals were sacrificed one hour before the start of the dark cycle and tissue was processed using fluorescent *in situ* hybridization (FISH) to detect *Pomc* mRNA as described below.

Stereotaxic microinjection

POMC-Cre transgenic mice were eight to nine weeks old at the time of stereotaxic injection. Mice were anesthetized with isoflourane (2-5%) under aseptic conditions. Animal health (e.g. breathing rate, vocalizations, and movement) was monitored at all times throughout the procedure and efforts were made to minimize distress (e.g. increased anesthesia or euthanasia). The coordinates for bilateral injections were calculated and adjusted as needed using the second edition of The Mouse Brain in Stereotaxic Coordinates by Paxinos and Franklin. Coordinates from bregma were as follows: X, ±0.27 mm; Y, -1.35 mm; Z, -6.05. Each animal received bilateral injections of 150-500 nl of a viral vector containing a double-floxed inverted ORF sequence of hM4D with an mCherry fluorescent protein tag on the receptor or a viral vector containing a double-floxed inverted ORF sequence of mCherry alone (rAAV2/hSyn-DIO-hM4D(Gi)-mCherry or rAAV2/hSyn-DIO-mCherry, respectively; University of North Carolina Vector Core, Chapel Hill, NC). To optimize viral transduction, the syringe was raised dorsal 0.02

mm three times over the course of 10 minutes and 50-165 nl of virus was injected at each site. The syringe remained at the most dorsal injection site for three minutes before slowly withdrawing the needle. Animals were sutured and allowed to recover for two weeks before the start of experiments unless noted otherwise.

Inhibition of POMC neurons during ABA

Animals were individually housed in cages with running wheels and exposed to the ABA paradigm as described above. During baseline, animals received intraperitoneal (i.p.) injections of saline (0.1 cc) two times per day. During ABA, CNO (1mg/kg) was delivered twice per day five hours apart-the first injection was given five and a half hours before lights out.

Tissue preparation

Mice were anesthetized and perfused transcardially with sucrose (10%) in water prior to paraformaldehyde (4%) in PBS. Brains were removed and postfixed overnight in paraformaldehyde (4%, 4°C). Coronal sections (50 μm) containing the ARC were prepared on a vibratome, collected in cold diethlpyrocarbonate (DEPC)-treated PBS for *in situ* hybridization or KPBS for immunohistochemistry, and processed for *in situ* hybridization or immunohistochemistry as previously described (Hentges et al., 2009; Jarvie and Hentges, 2012) and outlined below.

Fluorescent in situ hybridization

For FISH, sections were placed in 6% H_2O_2 for 15 minutes to quench endogenous peroxidase activity. Tissue was incubated for 15 minutes in proteinase K (10μg/ml) diluted in PBS

containing 0.1% Tween 20 (PBT). Proteinase K was deactivated by exposing tissue to glycine (2mg/ml) in PBT for 10 minutes. Following two five-minute washes in PBT, tissue was postfixed for 20 minutes in solution containing 4% paraformaldehyde and 0.2% gluteraldehyde. Tissue was washed in PBT, then dehydrated in ascending concentrations of ethanol diluted in DEPC-treated water (50, 70, 95, and 100%), and briefly rehydrated in PBT. Sections were transferred to vials and prehybridized in hybridization solution (66% deionized formamide 13% dextran sulfate, 260 mM NaCl, 1.3x Denhardt's solution, 13 mM Tris-HCL [pH 8.0], 1.3 mM EDTA [pH 8.0]) for one hour at 60°C. Probe was denatured at 85°C for five minutes and then added, along with 0.5mg/ml tRNA and 10 mM DTT, to the hybridization buffer. Fluorescein isothiocyanate (FITC)-labeled RNA probe for *Pomc* was made and used as described by Jarvie and Hentges (2012). Tissue hybridized with the *Pomc* probe in hybridization buffer at 70°C for 18-20 hours.

Three of the six 30-minute stringency washes at 60°C were in solution containing 50% formamide and 5X SSC followed by three washes in 50% formamide and 2x SSC. Tissue was then digested for 30 minutes at 37°C with RNAse A (20 µg/ml in 0.5 M NaCL, 10 mM Tris-HCL [pH 8.0], 1 mM EDTA) and subsequently placed in three 15-minute TNT (0.1 M Tris-HCL [pH 7.5], 0.15 M NaCl, 0.05% Tween-20). Sections were incubated in TNB for one hour (TNT plus 0.5% Blocking Reagent provided in the TSA kit; Perkin Elmer, Oak Brook IL) and then incubated overnight at 4°C in sheep anti-FITC (1:1,000; Roche Applied Sciences) antibody conjugated to horseradish peroxidase. The FITC-labeled probe was subsequently detected using a TSA PLUS DNP (HRP) system (Perkin Elmer). Tissue was exposed to three 15-minute TNT washes and then incubated for 30 minutes in a 1:50 dilution DNP Amplification Reagent. Tissue

was then washed in TNT and the FITC-labeled probe was visualized with 1:400 rabbit anti-DNP-KLH conjugated to Alexa Fluor 488 (1 hour; Invitrogen, Eugene, OR), both in TNT. Tissue was mounted and cover slipped with Aqua Poly/Mount (Polysciences, Inc., Warrington).

Immunohistochemistry

Tissue was prepared from mice 14-21 days after injection with AAV-hM4Di-mCherry or AAV-mCherry as described above. Coronal sections (50 μm) containing the ARC were prepared on a vibratome and incubated in normal goat serum (2% in 0.3% triton-x 100 in potassium phosphate-buffered saline [KPBS]) for one hour prior to the addition of rabbit anti-ACTH primary antibody to detect immunoreactivity (IR) of the POMC peptide ACTH (National Hormone and Peptide Program; 1:10,000, overnight at 4°C). Immunoreactivity against the primary antibody was detected and visualized with goat anti-rabbit conjugated to Alexa Fluor 635 (Invitrogen, Carlsbad, CA; 1:400).

Confocal laser microscopy

A Zeiss 510 Meta confocal microscope was used for imaging. Fluorescence from mCherry was imaged using 543 nm excitation filter and 560 nm longpass emission filter and far-red fluorescence (Alexa-635) was imaged using 633 nm excitation filter and 650 nm longpass emission filter. Green fluorescence (Alexa fluor 488) was imaged using 488 nm excitation filter and emissions was detected using a 505/530-nm bandpass filter. Images were taken sequentially at each wavelength to avoid crossover between channels. One-micron optical sections were taken in the z-plane every 3 μm for a total of 18-21 μm from all of the tissue along the rostral-caudal axis containing POMC neurons (~Bregman -1.2 mm to ~Bregma -2.3 mm).

Cell counting and analysis

Z-stacks with a tissue depth of 12-15 µm were constructed from sequential 1 µm-thick optical sections taken every 3 µm and were analyzed for cell number and colocalization and intensity above background using ImageJ software similar to that described in Jarvie and Hentges (2012). Two to three Z-stacks were constructed and analyzed for regions in the rostral, mid, and caudal ARC. Cells with a clear nucleus or cells completely contained in a 300 x 300 x 12-15 µm bounding box on the x-y-z plane were counted. Expression of mCherry in POMC cells is presented as a percent of ACTH labeled cells that were counted. Intensity of *Pomc*-FITC labeled cells was analyzed by comparing intensity of FITC labeling for each cell with the intensity of a background ROI devoid of FITC. Background ROIs were drawn in close proximity to *Pomc*-FITC labeled cells. Images used for figures were adjusted for brightness and contrast using Photoshop (Adobe Systems, San Jose, CA).

Live brain slice preparation

Sagittal brain slices (240 µm) containing the ARC were prepared (as described previously in Hentges et al., 2009) from POMC-Cre mice injected with AAV-hM4Di-mCherry or AAV-mCherry. Briefly, tissue was sliced in ice-cold artificial cerebral spinal fluid (aCSF) containing the following (mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 glucose (saturated with 95% O_2 and 5% CO_2). Slices rested prior to recording for 45-60 minutes in a vial containing MK-801 (15 µM) and aCSF at 37°C and saturated with 95% O_2 and 5% CO_2.

Electrophysiology

Whole-cell recordings were made using pipettes with 1.3-2.3 MΩ resistance when filled with internal solution containing the following (mM): 130 K-gluconate, 20 KCl, 1 $MgCl_2$, 10 Hepes, 1 EGTA, 2 ATP, 0.5 GTP, 10 Phosphocreatine, pH 7.3. Recording pipettes had a tip resistance of 1.5-2.6 MΩ when filled with internal solution. Cells expressing the hM4Di receptor were identified by mCherry fluorescence expressed as a fusion protein on the receptor. Whole-cell recordings were established in voltage-clamp configuration with a holding potential of -60 mV. Recordings were made through an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and data were collected using AxographX software (Axograph, Sydney, Australia). Data were collected at 10kHz and digitally filtered at 1kHz. Access resistance was monitored for several minutes before switching to current-clamp configuration. Integrity of the recording was determined by measuring access resistance at the end of current-clamp recordings and cells were accepted if access resistance did not exceed 15MΩ. Holding current was adjusted for each cell to set the membrane potential at -60 mV and data were collected from cells that had a stable holding current 5-10 minutes before drug perfusion. Cells without a stable holding potential during the baseline period were excluded from analysis. Action potential firing was determined by injecting 100 ms depolarizing current steps at 1.5 pA increments from the holding current every 200 ms (0-13.5 pA) before and after bath perfusion of CNO (10 μM). Membrane potential was measured before and after CNO just prior to current steps.

Drugs

Stock solutions of (+)-MK-801 (Sigma, St. Louis, MO), 6,7-Dinitroquinoxaline-2,3(1H4H) (DNQX, Sigma) were prepared with DMSO at no lower than 10,000 times the final

concentration. Stock solution of Clozapine N-Oxide (Enzo Life Sciences, Farmingdale, NY) was solubilized in water at no more than 5mg/ml.

Quantification and data analysis

Data are presented as mean ± SEM. Analysis and statistical significance of electrophysiology data were determined using paired *t*-tests. Development of ABA and the effect of inhibiting POMC neurons during ABA were analyzed using repeated measures analysis of variance (RMANOVA). Separate two-way RMANOVAs were used to determine if wheel running had an effect on relative body weight and food intake (% of baseline) over three days of food restriction (running x time). Two-way RMANOVA was used to determine if DREADD expression had an effect on relative food intake and body weight (% of saline) when CNO was administered during ABA. Post-hoc analysis using the Bonferroni's method was used to make comparisons between groups across time. The effect of wheel running on *ad libitum* food intake and body weight in *ad lib* fed mice was evaluated using one-way RMANOVA. Wheel running during the development of ABA was also analyzed using one-way RMANOVA and post-hoc comparisons using Bonferroni's method were made to determine if wheel-running behavior during each ABA day is different from baseline. The null hypothesis was rejected when $p < 0.05$.

Results

Development of ABA

Different strains of mice vary in their susceptibility to develop ABA (Gelegen et al., 2007; Pjetri et al., 2012), therefore the ABA rodent model was optimized for mice on the C57Bl/6 background used in this study. To minimize the stress of individual housing, preliminary studies

determined that three days of acclimation was sufficient for stable baseline data to be collected over the five days following acclimation. Over the course of three days the effect of restricting food access on relative body was significantly different between sedentary animals and running animals (running x day p=0.0002, 2-way RMANOVA, Figure 2.3A).

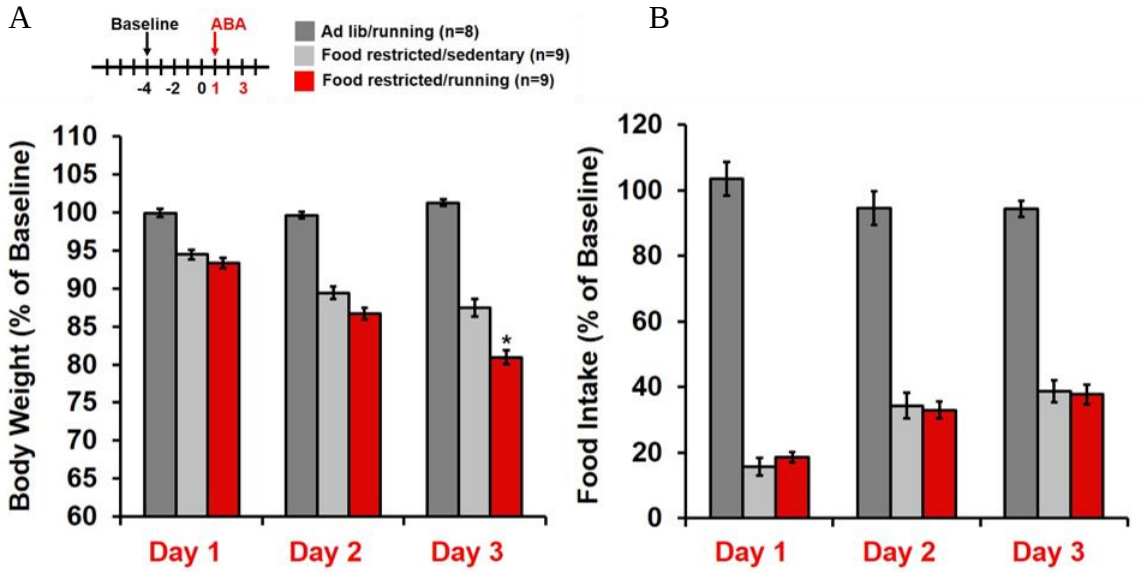

Figure 2.3. Development of ABA. A) Relative body weight during food restriction (FR) for sedentary and running mice is shown in the bar graph. Relative body weight during the 3 days following baseline for ad lib fed mice is also shown. Relative body weight loss between food restricted (FR)/running (ABA) and FR/sedentary (FR/SED) mice is significantly different across 3 days of FR. By day 3 of FR ABA mice had significantly lower relative body weight compared to FR/SED. Wheel running had no significant impact on body weight in ad lib fed mice. **B)** Relative food intake during 2hr food access is shown for sedentary and running mice is shown in the bar graph and food intake during the 3 days following baseline is shown for ad lib fed mice. Food intake is not significantly different between ABA and FR/SED mice during 3 days of FR. Wheel running does not significantly alter food intake in ad lib fed mice.*p<0.05.

During the development of ABA voluntary wheel running also had a significant effect on relative body weight independent of time (running, p=0.003). Post-hoc analysis using Bonferroni's method revealed that ABA mice lost significantly more weight by day three of food restriction relative to sedentary mice (ABA 80.96±0.94% vs FR/SED 87.46±1.14%; p < 0.0001; ABA

group on day 3 in Figure 2.3A). Over this same time course wheel running did not have a significant effect on body weight in *ad lib* fed mice (day 1: 100.02±0.52%, day 2: 99.73±0.45%, day3: 101.35±0.44; p=0.15; Figure 2.3A). As shown in Figure 2.3B, a similar pattern was observed for food intake in running, *ad lib* fed mice (day1: 103.45±5.14%, day 2: 94.66±5.16%, day3: 94.31±2.48; p=0.24). Three days of two hour food access had a significant effect on relative food intake independent of WRA, accounting for about 54.23% of the variance in food intake between days (day, p<0.0001; Figure 2.3B), but relative food intake was not significantly different between sedentary animals and running animals over three days of food restriction (running x time, p=0.58; day 3: ABA 37.77±3.03%, FR/SED 38.71±3.32%; Figure 2.3B).

WRA progressively increased during three days of two-hour food access (Figure 2.4A). Food restriction had a significant effect on 24-hour WRA (p<0.0001; Figure 2.4B) and wheel running in the four hours before lights out (p = 0.01, Figure 2.4C). Twenty-four hour wheel running was significantly increased all three days of food restriction (baseline: 21,093±3,122 vs day 1: 34,651±3,534 [p=0.003], day 2: 44,431±5,767 [p=0.005], and day 3: 51,821±5,419 [p=0.0004]) and WRA four hours before lights out was significantly increased on day 3 (wheel revolutions at baseline: 99.87±34.04 and day 3: 8,039±1,797; p=0.006; Figure 2.4B & C) . Over this same time course 24-hour WRA (baseline: 22,253±4,131 and day3: 28,725±6,061; p=0.09) and wheel running four hours before lights out did not significantly change (baseline: 66.28±13.39 and day3: 22.20±7.87; p=0.24).

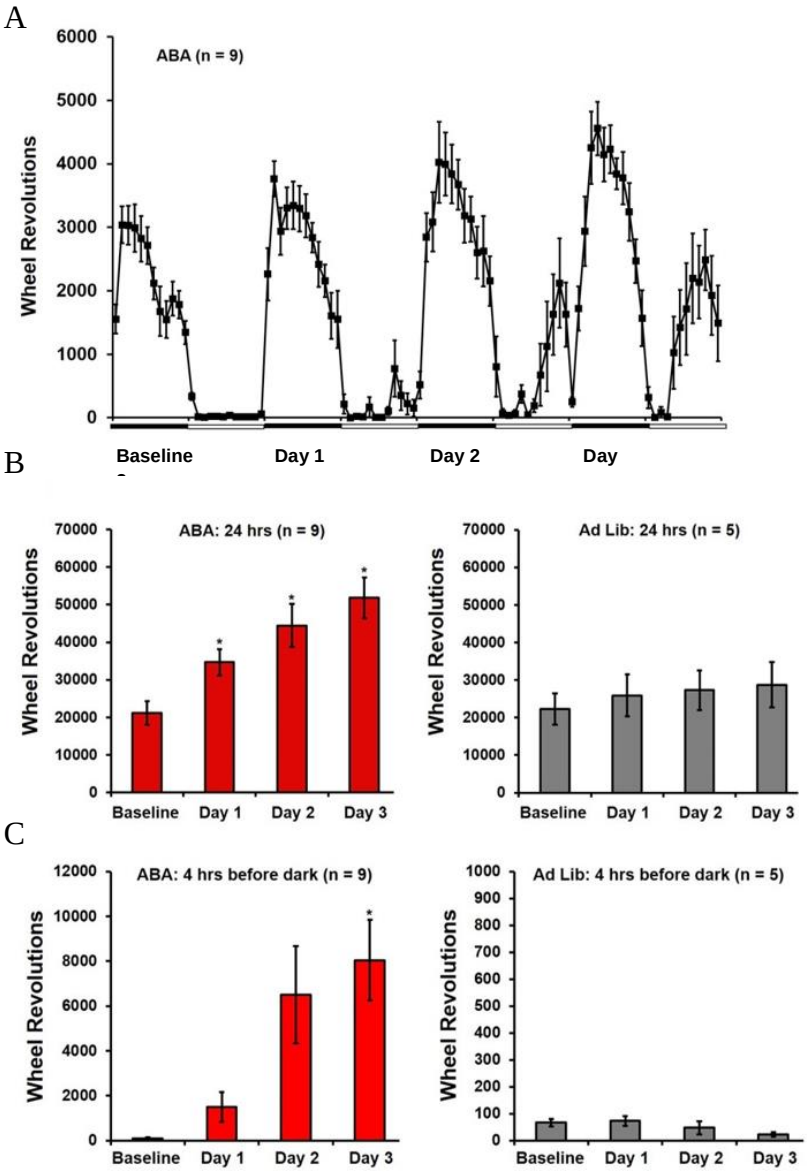

Figure 2.4. Wheel running activity increases during activity based anorexia. A) Hourly wheel revolutions during baseline and during 3 days of FR progressively increase during both the light and dark cycle. **B)** 24-hour wheel running was significantly different across 3 days of 22-hour FR and significantly increased at day 1, day 2, and day 3 of FR. Over the same time course 24-hour wheel running activity does not significantly change in ad lib fed mice. **C)** Scheduled feeding increases wheel running in the 4 hours prior to lights out during ABA and wheel running is significantly increased on day 3. Wheel running activity is not significantly altered in the 4 hours prior to lights out in ad lib fed mice. *$p<0.05$.

To determine if *Pomc* mRNA is transiently increased during the development of ABA, FISH was used to detect *Pomc* mRNA at day 0 (control), day 1, day 2, and day3 of ABA (Figure 2.5A). Consistent with previous reports (Hillebrand et al., 2006), data from the groups (n=2/group) analyzed here also show that *Pomc* mRNA was increased in mice during the first two days of ABA, but then declines by the third day (Figure 2.5B).

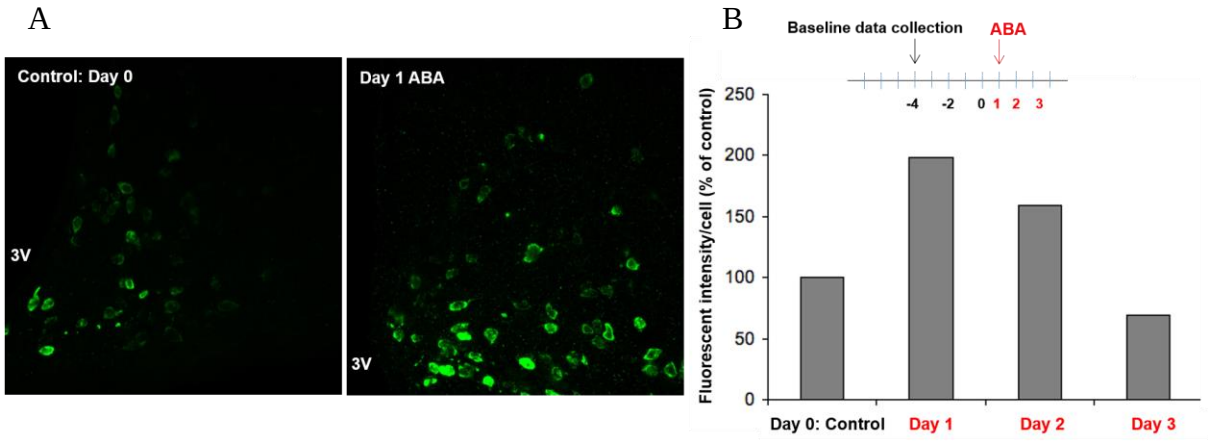

Figure 2.5. Hypothalamic *Pomc* mRNA is transiently increased during ABA. A) Representative image of *Pomc* mRNA measured 1 hour before lights out from an animal in the control group and an animal at day 1 of ABA. Fluorescent intensity of of *Pomc* mRNA appears more intense in the image from day 1 ABA. **B)** Data for the average fluorescent intensity per cell relative to background (expressed as a percent of day 0) is shown as an average for each group (n=2 animals/per group) in the bar graph. Average fluorescent intensity per cell relative to background was higher than control on days 1 & 2 of ABA, but declined by day 3 of ABA.

Expression of hM4Di:mCherry in POMC neurons

Selective expression of hM4Di:mCherry in POMC neurons was evaluated by colocalization of mCherry fluorescence and ACTH-IR 10-14 days after bilateral stereotaxic microinjections (150-500 nl/side) of the inhibitory DREADD into the ARC (see Figure 2.6A). Approximately 89% of ACTH-IR cells (531.83±33.10 POMC cells counted/animal) expressed mCherry and 84.54±2.44% of cells expressing mCherry (560.16±33.99 mCherry+ cells counted/animal) had ACTH-IR in animals with injections that hit the ARC (Figure 2.6B).

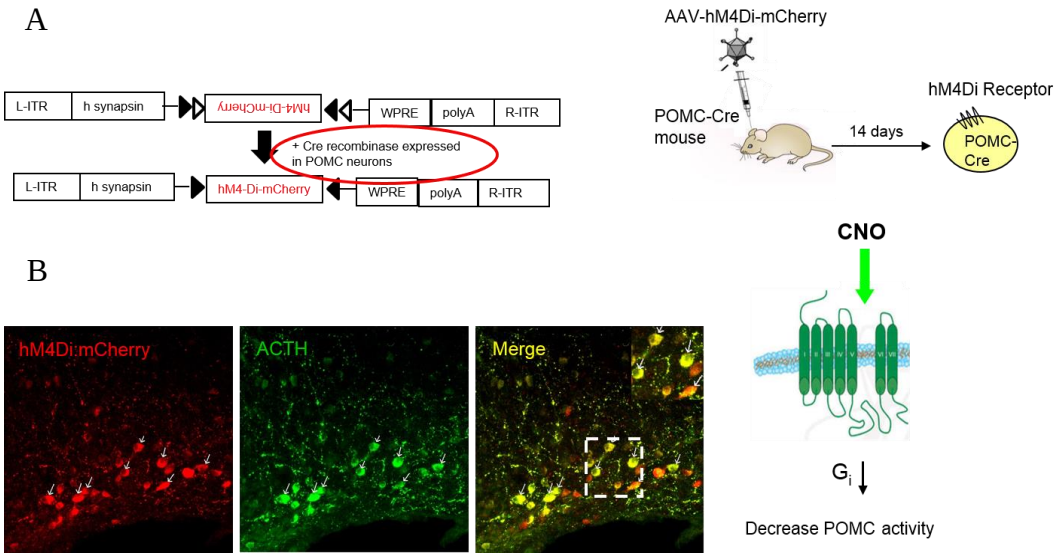

Figure 2.6. hM4Di:mCherry is expressed in hypothalamic POMC-Cre neurons. A) An AAV vector containing the hM4Di:mCherry construct is delivered to the bilateral arcuate nucleus via stereotaxic injections. Expression of hM4Di:mCherry is Cre-dependent. Expression of hM4Di:mCherry is observed 10-14 days after surgery as seen in panel B. Clozapine-N-Oxide (CNO) activates hM4Di and inhibits POMC neuron activity through an inhibitory G-protein signaling pathway. **B)** Representative image showing hM4Di:mCherry targets to POMC neurons as seen by colocalization of mCherry and ACTH immunoreactivity. Boxed area in merged is enlarged to show detail. 89.01 ± 2.21% of ACTH positive cells are colocalized with hM4Di:mCherry (n=6).

hM4Di:mCherry mediates CNO-induced inhibition of POMC neuron activity in vitro

Whole-cell current-clamp recordings were used to assess the ability of the inhibitory DREADD to suppress POMC neuron activity *in vitro*. As shown in Figure 2.7A bath perfusion of CNO resulted in a significant hyperpolarization of the cell membrane potential, relative to baseline, in POMC neurons expressing hM4Di: mCherry (baseline -58.59±1.45 mV, CNO -68.69±2.61 mV; p=0.001, paired t-tests), but had no significant effect on the membrane potential of cells lacking hM4Di:mCherry expression (baseline -58.13±1.18, CNO -59.72±1.17; p=0.11). To determine if the inhibitory DREADD desensitizes during prolonged exposure to agonist, CNO was bath perfused continuously for 20 minutes while recording in current-clamp configuration. In the presence of CNO the membrane potential hyperpolarized by -18.06 mV from baseline, but after

20 minutes of exposure to CNO the membrane potential was -2.45 mV from baseline (data not shown; n=1). Induced action potential firing was analyzed while injecting current steps in 1.5 pA increments (0-13.5 pA) before and after bath perfusion of CNO (10 μM). CNO significantly decreased action potential firing in cells expressing hM4Di:mCherry (number of action potentials fired at 6 pA step: baseline 5.12±1.12, CNO 1.87±0.58; p=0.005, paired t-tests; see Figure 2.7B), but had no significant effect on action potential firing in cells not expressing hM4Di:mCherry (baseline 4.29±0.92, CNO 4.43±0.92; p=0.36, Figure 2.7B). Cell firing and membrane potential recovers back to baseline following washout of CNO. Recovery of induced action potential firing occurred within 20 minutes of

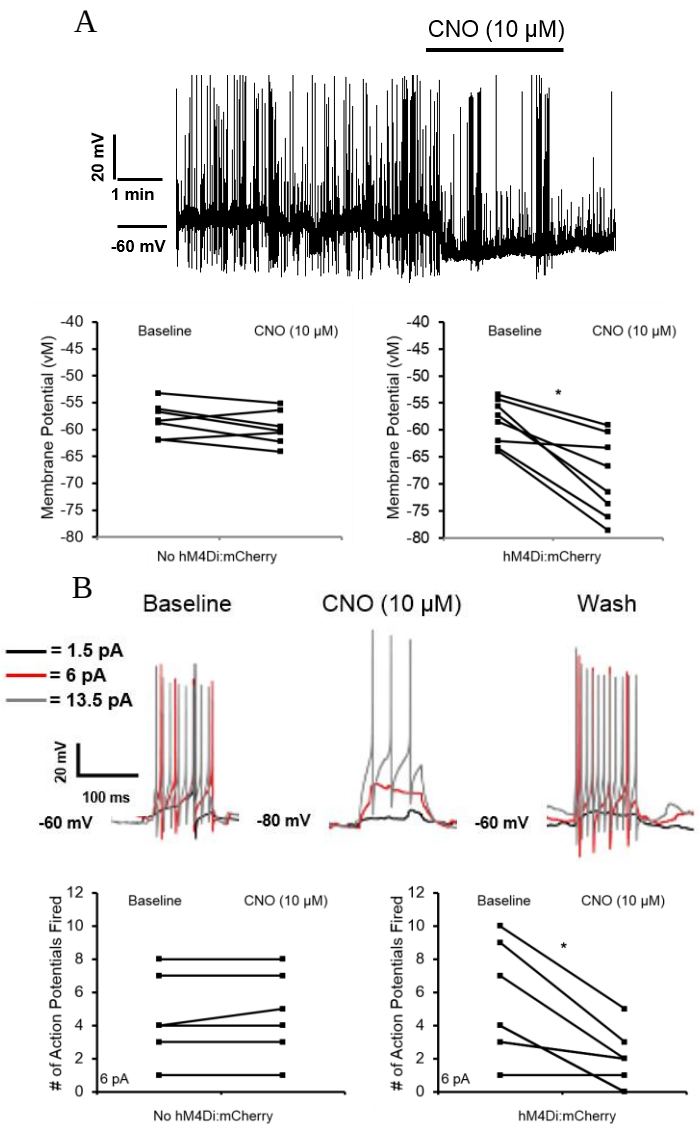

Figure 2.7. Activation of hM4Di decreases cell activity. A) Whole-cell current clamp recordings show hyperpolarization following bath application of 10 μM CNO in cells expressing hM4Di:mCherry (n=8) but not in cells lacking hM4Di:mCherry (n=7). **B)** 10 μM CNO decreases action potential firing during a current-step protocol (1.5 pA steps,10 steps; current injection was in addition to amount of current required to keep cell at -60 mV) in cells expressing hM4Di (n=8) but not in cells without hM4Di:mCherry (n=7). The decrease in firing induced by CNO is reversed upon washing with aCSF. *p<0.05

discontinuing CNO (see Wash in Figure 2.7B; n=1). The membrane potential also recovered during the wash as indicated by membrane potential observed at -12.74 mV from baseline in presence of CNO, but repolarized to 3.74 mV above baseline after 20 minutes of washout (data not shown; n=1).

Effect of CNO on food intake

Others have shown that in the presence of CNO *ad libitum* fed mice with bilateral expression of hM4Di:mCherry throughout the ARC consume more food (Atasoy et al., 2012). Therefore only animals with bilateral expression of hM4Di:mCherry throughout the ARC were used for data analysis. To determine if suppression of POMC neuron activity during ABA would increase food intake and decrease weight loss, male POMC-Cre mice expressing hM4Di:mCherry and control mice that were either uninjected or were expressing the control mCherry^{+} virus received CNO (2xdaily: 5.5 hrs before lights out and again 30 minutes before lights out). Expression of hM4Di:mCherry had a significant effect on food intake (DREADD, p=0.02; Figure 2.8) and accounted for 26.74% of the variance in food intake during ABA. Three days of limited food access also had a significant effect on food intake (day accounted for 27.65% of variance, $p<$ 0.0001), but the interaction between these factors was not significant (DREADD x day, p=0.55). Post-hoc analysis indicated that mice expressing the inhibitory DREADD ate significantly more food on day three of ABA relative to mice not expressing hM4Di (hM4Di+: 40.94±4.48%; no DREADD: 32.29±2.44%; p=0.02; Figure 2.8). Although three days of limited food access had a significant effect on body weight independent of DREADD expression (day, p<0.0001; data not shown), expression of hM4Di did not significantly alter body weight when CNO was given

during ABA (DREADD, p=0.76; body weight on day 3: hM4Di+ 88.45±1.93%, no DREADD 84.78±2.01%; data not shown).

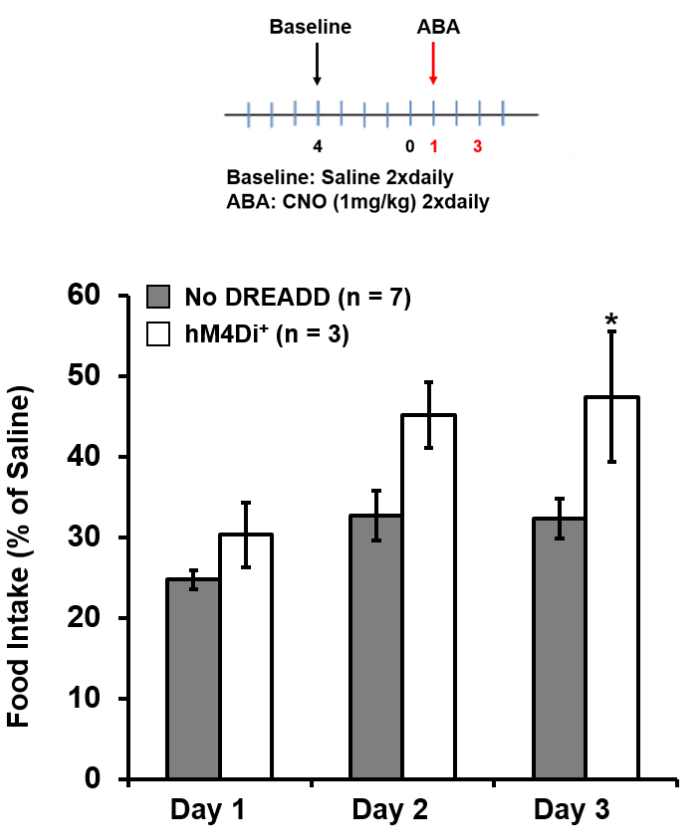

Figure 2.8. Inhibition of POMC neurons increases food intake during ABA. During baseline data collection all mice received saline (0.1 cc i.p. 2xdaily). All mice received CNO (1mg/kg i.p. 2xdaily) during ABA days. Relative food intake during ABA is shown as a percentage of food intake during saline in the bar graph. hM4Di expression had a significant effect on food intake and male POMC-cre:hM4Di+ mice consume significantly more food on day 3 of ABA as compared to food intake of mice not expressing hM4Di:mCherry. Food intake in the presence of CNO during ABA was not significantly different between POMC-Cre mice that were injected with a viral vector containing mCherry only (n=4) and uninjected POMC-Cre mice (n=3, p = 0.50; data not shown), therefore these animals were collapsed into one group – no DREADD expression. *p<0.05.

Discussion

The purpose of this study was to determine if the inhibitory DREADD could suppress POMC neuron activity and delay the development of ABA. The inhibitory DREADD has been shown to increase 24 hour food intake in mice (Atasoy et al., 2012), therefore this receptor was selected to inhibit POMC neurons and evaluate if increased POMC neuron activity was necessary for the decline in body weight and food intake during the early stages of ABA.

Inhibitory DREADD targets to POMC neurons and mediates CNO's effects on neuron activity

Data from the present study show that hM4Di:mCherry targets to POMC neurons in POMC-Cre mice. The ability of the viral vector mediated delivery of hM4Di (rAAV2/hSyn-DIO-hM4D(Gi)-mCherry) to transduce the majority of bilateral POMC-Cre neurons was dependent on precise injection of virus to the mid ARC on both sides of the third ventricle. The current study also found that CNO selectively inhibits the activity of neurons expressing hM4Di:mCherry *in vitro*, but shows no effect on cells without DREADD expression. Others have shown that hM4Di mediates CNO-induced hyperpolarization and decreased spontaneous and evoked firing in HEK293 cells through activation of G-protein coupled inwardly rectifying potassium channels (GIRK; [Armbruster et al., 2007]). While the intracellular pathways responsible for hM4Di-mediated attenuation of POMC neuron activity was not evaluated in the current study, previous work suggests this inhibition in POMC might also occur through GIRK activation. For example, POMC neurons express μ-opioid receptors, which are coupled to $G\alpha_i$, and in the presence of agonists induce hyperpolarization and decreased cell firing via GIRK activation (Pennock and Hentges, 2011). Regardless of the intracellular pathway mediating hM4Di inhibition of POMC neuron activity, these data show the inhibitory DREADD targets to POMC neurons where it acts to suppress neuronal activity in the presence of CNO.

Inhibitory DREADD mediates effects of CNO on food intake

Development of ABA is sensitive to the timing of food restriction, as well as the increase in WRA that occurs several hours before food access (Dwyer and Boakes, 1997; Verhagen et al., 2011; Klenotich and Dulawa, 2012). If the transient increase in POMC neuron activity during the first couple days of ABA drives the increase in WRA and decreased food intake-despite

increased energy output-which results in decreased body weight, then inhibition of POMC neurons should attenuate these behaviors and delay the onset of ABA. During the development of ABA the transient increase in *Pomc* mRNA and transient increase in hypothalamic neuron activity is detectable one hour before lights out (Hillebrand et al., 2006; Verhagen et al., 2011; Figure 2.5), but in a normally fed mouse *Pomc* mRNA approaches nadir at the start of the dark cycle (Stötz et al., 2007); therefore, inhibition of POMC neurons at the start of the dark cycle would be most sensitive for animals developing ABA. In the current study CNO was administered twice a day during the development of ABA-once five hours before lights out and then again 30 minutes before lights out. Given that CNO has been shown to alter behavior up to 8 hours after treatment (Alexander et al., 2009), this delivery protocol for CNO should be sufficient to suppress POMC neuron activity prior to the timing of food access used in this study. The results of this study show CNO-mediated inhibition of hM4Di-expressing POMC neurons significantly increases food intake by day three of ABA; thus, suggesting that increased POMC neuron activity precipitates, in part, the development of ABA. However, this increase in food intake failed to significantly thwart body weight loss and might indicate that inhibition of POMC neuron activity is insufficient to decrease *Pomc* mRNA or that other neuronal population are involved in the development of ABA.

Technical considerations

A limitation of the present study is the lack of data showing the inhibitory DREADD prevents the increase in POMC neuron activity *in vivo*. In this study decreased food intake was used as a proxy measurement for decreased POMC neuron activity *in vivo*, but future studies should determine if the inhibitory DREADD is capable of preventing the increase in *Pomc* mRNA or

decreases c-Fos expression. Although inhibiting POMC neurons increased food intake during ABA, no significant increase in body weight was observed. It is possible that body weight was not significantly altered in the present study due to insufficient suppression of POMC neuron activity *in vivo*. The ability of hM4Di to suppress the transient increase in *Pomc* message remains to be verified.

Wheel-running activity was not measured during the studies aimed at inhibiting POMC neurons during ABA, thus it is not known if physical activity was significantly affected by CNO treatment in hM4Di-expressing animals. It is possible the modest increase in food intake was not sufficient to counteract the increased wheel running in order to slow the rate of developing ABA, which could also explain why body weight loss was not attenuated. Although the effect of POMC neuron activity on wheel running behavior was not recorded in the present study, POMC neurons are involved in physical activity. For example, exercise has been shown to increase plasma levels of beta-endorphin in adolescent females (Gerra et al., 1992) and Patterson and colleagues (2008) reported that early-onset exercise in rodents is associated with increased *Pomc* mRNA in adulthood and is protective against diet-induced obesity. Future experiments should evaluate the effects of inhibiting POMC neurons on wheel running during ABA.

Moving forward, experiments should address whether or not the protocol for administering CNO is optimal in suppressing POMC neurons during the development of ABA. It is possible that administering CNO at a different time or a different dose would be more effective at inhibiting POMC neurons and delaying the progression of ABA. The inhibitory DREADD is reportedly less effective at silencing cell activity relative to cell activation induced by the stimulatory

DREADD, thus different dosing might be warranted for each of the DREADDs (Farrell and Roth, 2013). Indeed, Atasoy and colleagues (2012) used higher doses of CNO (5mg/kg) to inhibit POMC neurons and increase food intake relative to the dose (0.3mg/kg) used by Krashes and colleagues (2011) to activate AgRP/NPY neurons and increase food intake. Caution should be taken in considering a dosing protocol for the inhibitory DREADD given the evidence shown here for desensitization. Based on the likelihood of receptor desensitization a pulsatile dosing should be used over delivery via continuous infusion. Although *Pomc* mRNA is transiently increased during the early stages of ABA and decreasing POMC neuron activity is shown here to facilitate increased feeding during ABA, it is possible that ABA develops independent of POMC neurons. Currently it is not known if POMC neurons work independently to precipitate the development of anorexia or if they work in concert with other feeding neurons to give rise to decreased food intake.

Summary

The purpose of this study was to determine if an increase in POMC neuron activity during the early stages of ABA was necessary for the development of anorexia in this model. The research presented here shows the inhibitory DREADD targets to POMC neurons and can mediate CNO-induced suppression of cell activity *in vitro* and food intake *in vivo*. The ability to selectively modulate neuronal activity at definitive time points in the development of anorexia would be a valuable tool in probing the early physiological events that likely precipitate disease progression.

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CHAPTER 3: ACTIVATION OF POMC NEURONS TO INDUCE ANOREXIA IN MICE

All work presented in Chapter 3 of this dissertation was carried out by Christina Dennison in the Hentges Lab and was overseen by Shane Hentges. This Chapter consists of unpublished data to be included in a future publication that will incorporate the work and contributions from other members in the lab.

- POMC peptides decrease food intake and are involved in the development of anorexia, but it is not known if increased POMC neuron activity is sufficient to sustain anorexia.
- Work in Chapter 3 will test the hypothesis that prolonged activation of POMC neurons will result in a lasting anorexia.
- Activation of POMC neurons with the stimulatory DREADD increases cell activity and increases food intake
- Findings presented here indicate that acute activation of POMC neurons decreases food intake and preliminary data supports continued research into the sufficiency of POMC neurons to induce anorexia.

Box 3.1. Overview of Chapter 3

Introduction

As discussed in Chapter 2, POMC neurons are involved in the early development of ABA (Hillebrand et al., 2006; Figure2.5 in Chapter 2). Inhibition of POMC neurons during the early stages of ABA was used to determine if POMC neurons were necessary for the development of ABA. This approach revealed a potential role for POMC neurons in mediating feeding behavior during ABA. While these data suggest POMC neurons are a key component in the development of anorexia, it is not clear if activation of these neurons is sufficient to develop lasting anorexia.

Central infusion of MCR agonists decrease acute food intake and body weight in mice (Pierroz et al., 2002), whereas MCR antagonists increase short-term food intake and body weight (Fan et al., 1997; Kask et al., 1998). Zhan and colleagues (2013) found that continuously activating POMC neurons over three days using the stimulatory DREADD (also referred to as hM3Dq) results in ~40% decrease in food intake and ~5% decrease in body weight (Zhan et al., 2013). While these data indicate that declines in food intake and body weight can occur with activation of POMC neurons or exogenous treatment with MCR agonists, it is not known if food intake and body weight will decrease further with continued stimulation. Additionally, it is not clear if POMC peptides are mediating the effects on food intake since POMC neurons are capable of releasing both amino acid transmitters as well as peptides (Hentges et al., 2009; Dicken et al., 2012).

Similar to the inhibitory DREADD described in Chapter 2, the stimulatory DREADD has been shown to be a valuable tool for long-term manipulation of cell activity in freely moving animals (Alexander et al., 2009; Guettier et al., 2009; Krashes et al., 2011; Jain et al., 2013; Zhan et al., 2013). Evidence suggests that hM3Dq increases cell activity though PLC-dependent depolarization and increased firing (Alexander et al., 2009) as well as upregulation of c-Fos transcription *in vitro* (Kaufmann et al., 2013). The ability of this receptor to rapidly modulate neuronal activity and the potential for this receptor to maintain modulation over prolonged periods of time make hM3Dq an appropriate tool for investigating the effects of prolonged POMC neuron activation on food intake and body weight.

Parameters characteristic of developing anorexia (food intake and body weight) were monitored to test the hypothesis that activation of POMC neurons is sufficient to drive a persistent negative

energy state. The findings of this work show that the stimulatory DREADD is selectively expressed in POMC neurons, mediates CNO-induced activation of POMC cells, and decreases acute food intake. The data shown here also highlight some technical considerations when using DREADDs to modulate behavior.

Methods and materials

Materials and methods used for the experiments in this chapter are similar to those used for the experiments in Chapter 2. Therefore references to the subsections in the *Methods and Materials* section of Chapter 2 will be used for procedures described previously.

Animals

Male and female transgenic mice expressing Cre recombinase specifically in POMC cells (POMC-Cre) were used to selectively express the stimulatory DREADD in POMC neurons located in the ARC. These animals are identical to those described under *Animals* in Chapter 2. Male and female mice expressing discosoma red (dsRed [POMC-tdi]; Hentges et al., 2009) driven by the POMC promoter were maintained on the C57BL/6J background and were used in some control experiments described below. All animals were housed and cared for as described under *Animals* in Chapter 2. All experiments met United States Public Health Service guidelines with the approval of the Colorado State University Institutional Animal Care and Use Committee.

Stereotaxic microinjection

Procedure for stereotaxic microinjection of the stimulatory DREADD was identical to that described under *Stereotaxic microinjection* in Chapter 2 except for the following changes. Each animal received bilateral injections of 500 nl viral vector containing either a Cre-dependent sequence of hM3Dq with an mCherry fluorescent protein tag or a viral vector containing a Cre-dependent sequence of mCherry alone (rAAV2/hSyn-DIO-hM3D(Gq)-mCherry or rAAV2/hSyn-DIO-mCherry, respectively; University of North Carolina Vector Core, Chapel Hill, NC). To optimize viral transduction the syringe was raised dorsal 0.02 mm three times over the course of 10 minutes and ~166 nl of virus was injected at each site.

Measurement of POMC neuron activation in vivo

Animals expressing hM3Dq:mCherry or POMC-tdi received i.p. injections of CNO (1mg/kg in 0.1 cc saline) or saline (0.1cc) seven to nine hours into the light cycle and were perfused (as detailed in Chapter 2) 90 minutes after injection. Tissue was processed for IR against c-Fos. CNO-induced increases in c-Fos IR should be detectable within 90 minutes given that the dose of CNO used has been shown to maximally activate cells *in vivo* about 45 minutes after i.p. injection (Alexander et al., 2009) and 90 minutes has been shown to be sufficient for detecting changes in c-Fos expression in POMC neurons (Huo et al., 2006).

Measurement of food intake and body weight during POMC neuron activation

Female mice expressing hM3Dq and not expressing hM3Dq were individually housed in cages with access to food and water *ad libitum*. Food was weighed every 30 minutes using automated food hoppers and Fasting Plan software from IntelliBio (Seichamps, France). Animals were

allowed to acclimate to new caging for three days before data collection. To determine the effect of a single dose of CNO on food intake, animals received saline (i.p., 0.1cc) for three days prior to CNO (i.p. 2mg/kg). Cumulative food intake was analyzed in the 24 hours following injections. To evaluate prolonged activation of POMC neurons on food intake and body weight i.p. injections of saline (0.1 cc) were given five times per day at 8am, 1pm, 5pm, 10pm, and 3am for two days prior to receiving CNO (1mg/kg i.p.) five times per day. This same delivery paradigm and dose of CNO has been shown to reduce food intake and body weight with little evidence of receptor desensitization (Zhan et al., 2013). Body weight was measured daily at the time of the 5pm injection, which corresponded to about one hour before lights out. Experimental end point was defined as mice having lost 15-20% of starting body weight or body weight remained stable for three to five days.

Immunohistochemistry

Procedures for tissue preparation and immunodetection of ACTH are described under *Immunohistochemistry* in Chapter 2. Tissue processed for c-Fos IR was incubated in 5% horse serum and 0.3% triton-x 100 in potassium phosphate-buffered saline (KPBS) and then exposed to rabbit anti-cfos primary antibody overnight (Calbiochem, San Diego, CA; 1:2500, at 4°C). Rabbit anti-cfos signal was amplified using biotinylated horse anti-rabbit (Vector Laboratory, Burlingame, CA; 1:400, 1 hour at room temp) and visualized with streptavidin conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA; 1:600, 1 hour at room temp).

Confocal laser microscopy

Imaging was performed on a Zeiss 510 Meta confocal microscope as described under *Confocal laser microscopy* in Chapter 2.

Cell counting and analysis

Z-stacks with a tissue depth of 12-15 µm were constructed from sequential 1 µm-thick optical sections taken every 3 µm and were analyzed for cell number and colocalization using ImageJ software. Z-stacks were constructed and analyzed for regions in the rostral, mid, and caudal ARC. Cells with a clear nucleus or cells completely contained in a 300 x 300 x 12-15 µm counting box on the x-y-z plane were counted. Expression of mCherry in POMC cells is shown as a percent of ACTH labeled cells that were counted. Colocalization of c-Fos and mCherry or POMC-tdi is presented as a percent of total mCherry+ or POMC-tdi+ cells counted. Images used for figures were adjusted for brightness and contrast using Photoshop (Adobe Systems, San Jose, CA).

Live brain slice preparation

Sagittal brain slices (240 µm) containing the ARC from POMC-cre mice injected with AAV-hM3Dq-mCherry or AAV-mCherry were prepared and maintained as described under *Slice preparation* in Chapter 2.

Electrophysiology

Whole-cell recordings were made as described under *Electrophysiology* in Chapter 2. To prevent activation of postsynaptic AMPA receptors from confounding the interpretation of CNO-

mediated effects on POMC neuron activity, DNQX (10 μM) was bath perfused for 8-10 minutes prior to acquiring baseline action potential firing. Induced action potential firing was determined by injecting 100 ms depolarizing current steps at 1.5 pA increments from the holding current every 200 ms (0-13.5 pA) before and after bath perfusion of CNO (10 μM).

Drugs

Stock solutions of (+)-MK-801 (Sigma, St. Louis, MO), 6,7-Dinitroquinoxaline-2,3(1H4H) (DNQX, Sigma) were prepared with DMSO at no lower than 10,000 times the final concentration. Stock solution of Clozapine N-oxide (Enzo Life Sciences, Farmingdale, NY) was solubilized in water at no more than 5mg/ml.

Quantification and data analysis

Data are presented as mean ± SEM. Independent samples *t*-tests were used to determine statistical significance of CNO-induced activation of POMC neurons *in vivo.* Statistical significance was determined for all other experiments using paired *t*-tests. The null hypothesis was rejected when $p < 0.05$.

Results

Expression of hM3D:-mCherry in POMC neurons

Expression of hM3Dq:mCherry in POMC neurons is detectable 10-14 days following bilateral stereotaxic microinjections (500 nl/side) of AAV-hM3Dq-mCherry into the ARC. About 79% of ACTH-IR cells colocalized with hM3Dq:mCherry when bilateral injections hit the region of the ARC targeted with stereotaxic coordinates (390.14±39.28 ACTH-IR cells counted/animal; see

Figure 3.1B). This level of hM3Dq:mCherry expression in POMC cells is consistent with previous reports and is sufficient to induce increases in food intake (Zhan et al., 2013). About 89.13% of mCherry+ cells coexpressed ACTH-IR (314.75±12.69).

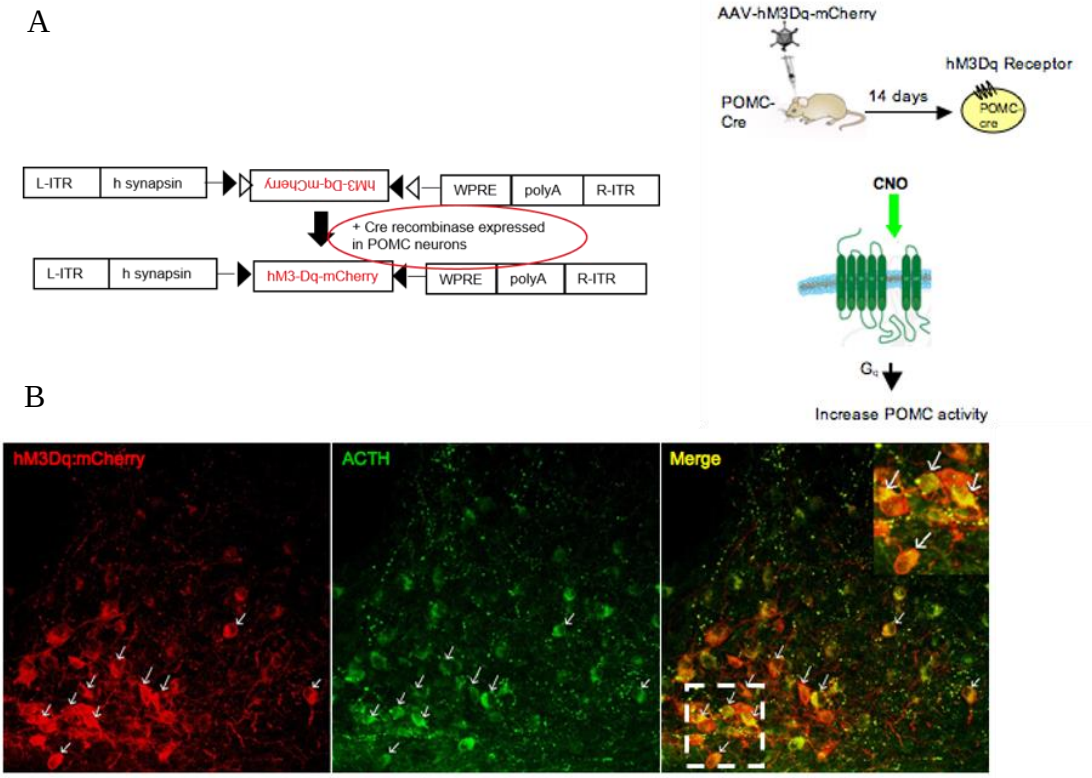


Figure 3.1. hM3Dq:mCherry is expressed in hypothalamic POMC-Cre neurons. A) An AAV vector containing the hM3Dq:mCherry construct is delivered to the bilateral ARC via stereotaxic injections. Expression of hM3Dq:mCherry is Cre-dependent. hM3Dq:mCherry is observed 10-14 days after surgery as shown in panel B. Clozapine-N-Oxide (CNO) activates hM3Dq through a Gq-protein signaling pathway. **B)** hM3Dq:mCherry targets to POMC neurons as seen by colocalization of mCherry and ACT-IR. 79.34±3.03% of ACTH positive cells are colocalized with hM3Dq:mCherry (n=7).

hM3Dq:mCherry mediates CNO-induced activation of POMC neurons in vitro

Whole-cell current-clamp recordings were used to assess the ability of the stimulatory DREADD to increase POMC neuron activity *in vitro*. Bath perfusion of CNO significantly depolarized the membrane potential, relative to baseline, in POMC neurons expressing hM3Dq:mCherry

(baseline -63.89±1.68 mV, CNO -53.27±2.24 mV; $p<0.0001$; Figure 3.2A). CNO-induced depolarization and increased basal firing persisted for 20 minutes after discontinuing perfusion of CNO, recovery to baseline membrane potential and basal firing was not observed in this recording (CNO: +14.05 mV from baseline, Wash: +14.88 mV from baseline, data not shown; n=1). CNO had no significant effect on cells lacking hM3Dq:mCherry expression (baseline -56.14±1.04, CNO -58.96±1.38; $p=0.10$). Action potential firing was analyzed while injecting current at 1.5 pA increments (0-13.5 pA) before and after bath perfusion of CNO (10 μM). As shown in Figure 3.2B CNO significantly increased the number of action potentials fired in cells expressing hM3Dq:mCherry (6 pA step: baseline 5.25±1.24, CNO 6.75±1.28; $p=0.01$), but had

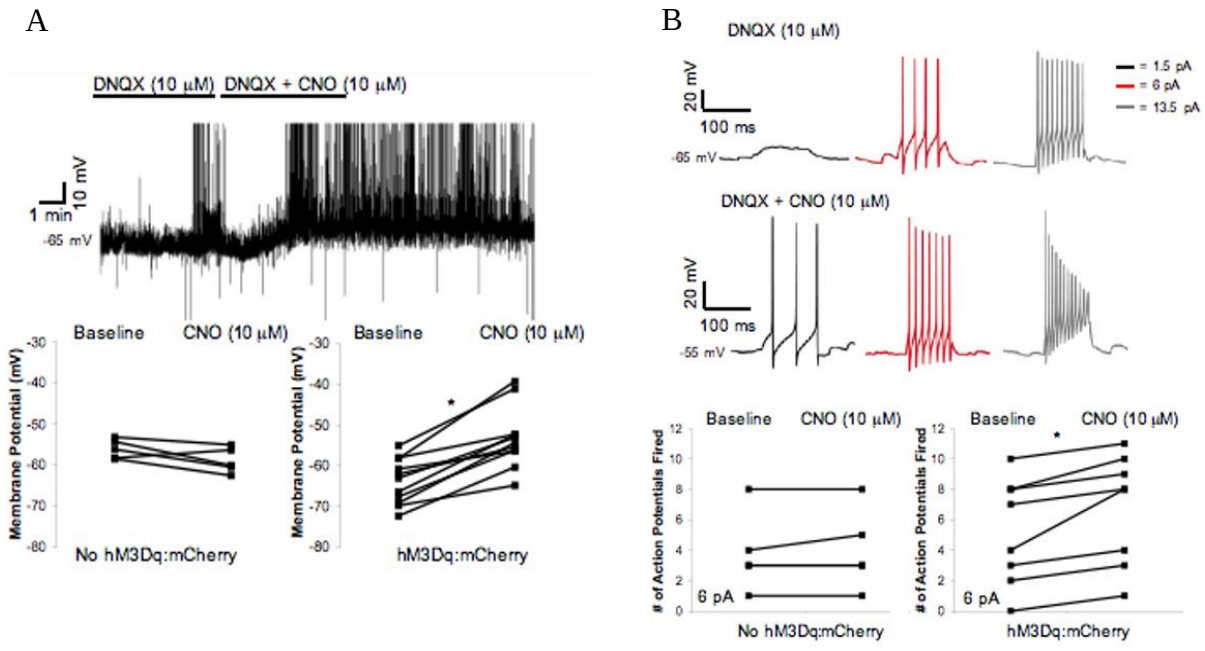


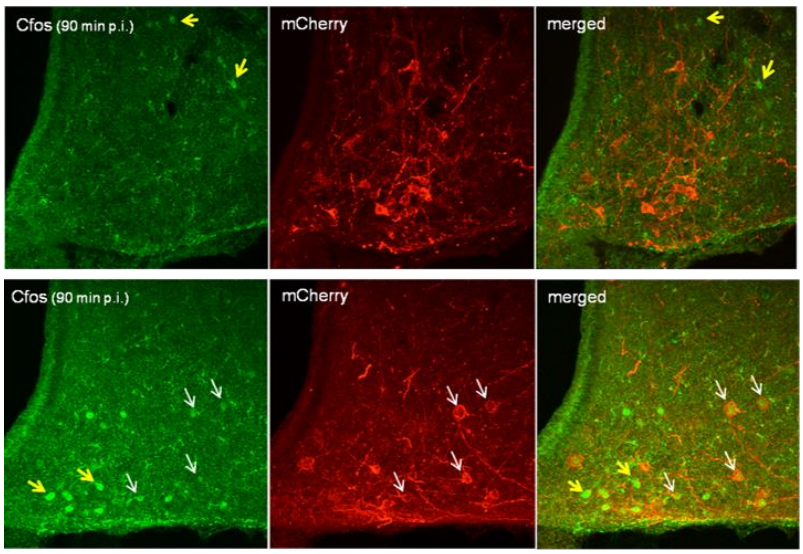
Figure 3.2. Activation of hM3Dq increases cell activity. **A)** Representative trace from a whole-cell current clamp recording showing depolarization following bath application of 10 μM CNO in cells expressing hM3Dq:mCherry. Compiled data in graphs show that hM3Dq mediates CNO induced depolarization (n=11). CNO has no significant effect on membrane potential in cells lacking hM3Dq:mCherry (n=5). **B)** Representative trace of action potential firing in hM3Dq+ cell before and after 10 μM CNO during a current-step protocol at 1.5pA, 6pA, and 13.5pA (1.5 pA steps,10 steps; current injection was in addition to amount of current required to keep cell at -60 mV). Compiled data for number of action potentials fired at 6pA step before and after CNO is shown in graph for cells expressing hM3Dq:mCherry (n=8) and cells without hM3Dq:mCherry (n=5). *$p<0.05$, paired t-tests.

no significant effect on action potential firing in cells not expressing hM3Dq:mCherry (6 pA step: baseline 3.80±1.15, CNO 4.0±1.18; p=0.37).

hM3Dq:mCherry mediates CNO-induced activation of POMC neurons in vivo

C-Fos is an early indicator of cell activation and has been used to identify activation of POMC neurons *in vivo* (Huo et al., 2006; Zhan et al., 2013). Increases in expression of c-Fos IR was used to determine if CNO could activate hM3Dq:mCherry+ neurons *in vivo*. Within 90 minutes of animals receiving an i.p. injection of CNO (1mg/kg), c-Fos IR was observed in 53.59±6.65% of mCherry+ cells (149.50±37.50 mCherry cells counted/animal; Figure 3.3A & B) , while 3.10±1.19% of mCherry+ cells expressed c-Fos IR 90 minutes after saline injections (238.0±57.38 mCherry cells/animal; Figure 3.3A & B). As seen in Figure 3.3B expression of c-Fos IR in mCherry+ cells was significantly higher following CNO relative to saline (p=0.003). To determine if CNO alone increases cell activity, c-Fos IR was evaluated in POMC-tdi mice following CNO. Ninety minutes after CNO injection 15.82±2.19% of POMC-tdi+ cells expressed c-Fos IR (341.25±35.20 total POMC-tdi+ cells counted/animal; Figure 3.3B). Although the relative number of hM3Dq:mCherry+ cells expressing c-Fos IR was significantly higher than the relative number of POMC-tdi+ cells expressing c-Fos IR (p=0.006), CNO also resulted in significantly higher c-Fos IR in POMC-tdi+ cells relative to c-Fos IR in hM3Dq:mCherry+ cells following saline (p=0.01; Figure 3.3B). Following CNO, c-Fos IR increased in non-POMC cells of the ARC in animals expressing hM3Dq:mCherry (c-Fos IR cells counted/animal, CNO: 180.75±44.50 vs Saline: 67.50±22.41) and in POMC-tdi animals (c-Fos IR cells counted/animal 157.50±17.79).

A



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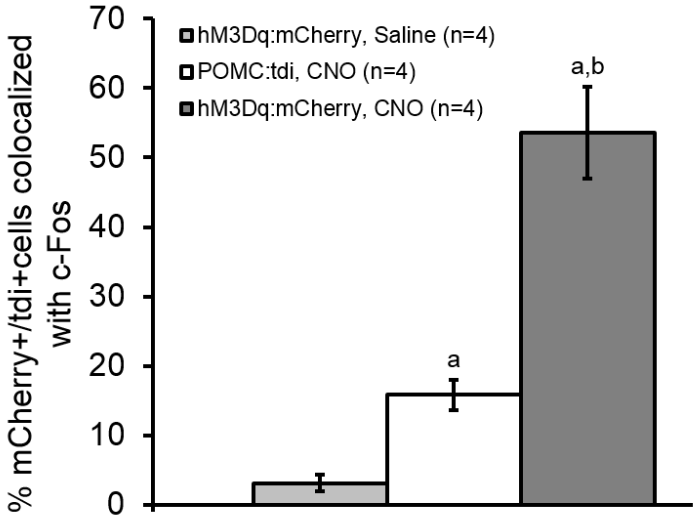

Figure 3.3. CNO activates hM3Dq:mCherry+ cells *in vivo*. A) Representative image showing c-Fos IR is increased in cells expressing hM3Dq:mCherry 90 min after CNO injection (1mg/kg i.p.) but this increase is not seen 90 min after saline injection (0.1 cc i.p.). Yellow arrows denote c-Fos+ cells without mCherry; white arrow denote c-Fos colocalized with mCherry. **B)** Relative number of mCherry+ or POMC-tdi+ cells expressing c-Fos IR 90 minutes after CNO (1mg/kg i.p.) or saline is shown in bar graph. a, significantly different than Saline; b, significantly different than POMC-tdi+ CNO; $p<0.05$.

Effect of CNO on food intake

To evaluate the ability of a single dose of CNO to inhibit food intake animals received saline (i.p.) at the start of the dark cycle for three days prior to CNO. As seen in Figure 3.4A Mice with bilateral expression of hM3Dq:mCherry throughout the ARC ate significantly less food in the 24 hours following CNO (2mg/kg, i.p.) relative to food intake in the 24 hours after saline (Saline: 3.76±0.06g vs CNO: 3.15±0.17g; $p=0.03$). Twenty-four hour food intake following CNO was not significantly different from saline in animals with

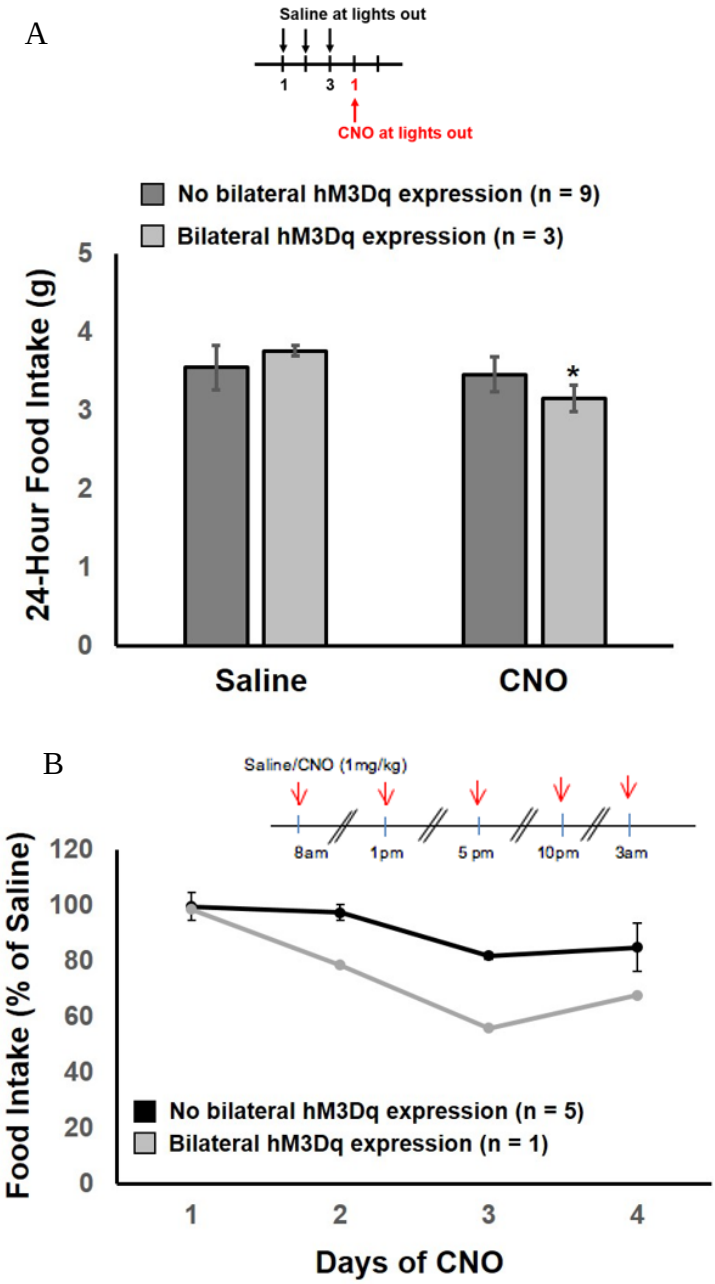

Figure 3.4. Effect of CNO on food intake. A) Mice without bilateral expression of hM3Dq and mice with bilateral expression of hM3Dq (>62% of ACTH-IR cells express mCherry) received saline (0.1cc i.p.) for 3 days at start of dark cycle before CNO (2mg/kg i.p.). 24-hour food intake was significantly less following CNO in mice with bilateral hM3Dq expression as compared to controls. **B)** Daily food intake (shown as a percentage of daily food intake during saline) across 4 days of CNO (1mg/kg 5xdaily, i.p. for animals without bilateral hM3Dq expression and bilateral expression of hM3Dq. *p<0.05, paired t-tests.

unilateral expression of hM3Dq:mCherry or no expression (Saline: 3.55±0.27g, vs CNO: 3.46±0.22g; p=0.69; data not shown). Body weight did not significantly decrease during CNO for hM3Dq expressing mice (saline: 19.74±0.59g, vs CNO: 20.13±0.60g, p=0.05; data not shown) or for animals without bilateral hM3Dq expression (saline: 20.35±0.38g, vs CNO: 20.36±0.40g, p=0.92).

To determine if prolonged activation of POMC neurons can sustain decreased food intake and induce body weight loss characteristic of anorexia, POMC-cre mice expressing hM3Dq:mCherry and mice without bilateral expression

were chronically treated with CNO (1mg/kg, i.p. 5xdaily) for four days. Analysis of hM3Dq:mCherry and ACTH-IR revealed that one animal had extensive DREADD expression in POMC neurons throughout the bilateral ARC. Animals with unilateral expression of hM3Dq:mCherry and animals with a lack of hM3Dq:mCherry expression ate similarly in the presence of CNO, therefore data from these animals were pooled into one group (no bilateral hM3Dq expression). Data shown in Figure 3.4B suggests that hM3Dq is mediating CNO's effect on food intake as indicated by the reduction of food intake on days 3 and 4 (55.90% and 67.73% of saline, respectively). Body weight over the four days of CNO treatment was relatively similar to body weight during saline (saline: 18.30g, CNO: 18.70g; data not shown). Food intake also declined for animals without bilateral hM3Dq expression. By day four of CNO food intake was 84.98±8.67% of food intake during saline (No bilateral expression group in Figure 3.4B) and body weight was relatively similar between saline and CNO (saline: 18.0±0.23g, CNO: 18.50±0.42g; data not shown). These results suggest that the repeated handling and injections alone also reduce food intake.

Discussion

The goal of this study was to use the stimulatory DREADD to activate POMC neurons over a prolonged period of time to determine if activation of these cells is sufficient to induce lasting anorexia. Previous research has shown that chronic exposure to CNO for three days significantly reduces food intake and body weight in animals expressing hM3Dq in POMC neurons (Zhan et al., 2013). Therefore this study would extend these findings by activating POMC neurons slightly longer than three days.

Stimulatory DREADD targets to POMC neurons and mediates CNO's effects on cell activity

The research described here found that hM3Dq:mCherry selectively targets to POMC neurons in a Cre-dependent manner. Transduction of hM3Dq:mCherry to POMC neurons bilaterally throughout the ARC required precise and accurate stereotaxic microinjections in the mid ARC region. The ability of AAV-hM3Dq-mCherry to spread away from the injection site appears to be similar to that of AAV-hM4Di-mCherry described in Chapter 2 given that no striking differences were observed in expression along rostral-caudal and medial-lateral axis between these receptors. The findings of the current work also show that CNO selectively increases cell firing and induces depolarization *in vitro* in cells expressing the stimulatory DREADD. Although desensitization of hM3Dq was not evaluated, CNO induces prolonged activation of POMC neurons even after perfusion of CNO is discontinued. While this observation does not preclude desensitization, it does indicate that hM3Dq-mediated activation of POMC neurons persists in the absence of CNO.

In the presence of CNO the stimulatory DREADD increases c-Fos expression, indicating the dosage of CNO used in this study is capable of activating POMC neurons *in vivo*. Expression of c-Fos was also elevated in animals without hM3Dq:mCherry expression relative to mice with hM3Dq:mCherry that were given saline (see POMC-tdi mice in Figure 3. 3). If CNO were highly selective for hM3Dq it would be expected that c-Fos expression in POMC-tdi+ mice following CNO would be similar to hM3Dq+ mice following saline. However, POMC-tdi+ animals that received CNO had significantly more c-Fos expression than hM3Dq+ mice that received saline. Expression of c-Fos was also increased in non-POMC neurons in the ARC following CNO injections in hM3Dq+ and POMC-tdi+ mice. While it is known that hypothalamic POMC

neurons form intra-arcuate projections (Wang et al., 2015) and the increased c-Fos observed in non-hM3Dq neurons might reflect activation of cells downstream of POMC neurons (Zhan et al., 2013), these data might also suggest that CNO is capable of altering neuronal activity in the absence of hM3Dq. Others have voiced concerns about CNO having off-target effects *in vivo*, particularly in regards to back-conversion of CNO to clozapine (Löffler et al., 2012). Clozapine acts at 5-HT receptors (Roth et al., 1994; Schmid et al., 2014) and given that POMC neuron activity is modulated directly by 5-HT2C and indirectly by 5-HT1B receptors (Heisler et al., 2006; Sohn et al., 2011; Doslikova et al., 2013), it is possible that retroconversion of CNO into clozapine is responsible for the increased c-Fos expression in the absence of hM3Dq. However, Guettier and colleagues (2009) found no significant retroconversion of CNO into clozapine in mice and formation of clozapine from CNO is inhibited by ascorbate (Pirmohamed et al., 1995), which is endogenously synthesized in rodents (Chatterjee et al., 1975). Based on these data it is not clear if CNO or clozapine is responsible for activation POMC neurons in the absence of hM3Dq, therefore future studies should carefully control for the possibility of CNO having off-target effects *in vivo*.

Stimulatory DREADD mediates effects of CNO on food intake

The data presented in this Chapter indicate that hM3Dq mediates the decrease in 24-hour food intake following a single dose of CNO at the start of the dark cycle. While liquid chromatography tandem mass spectrometry has shown that CNO is cleared from blood plasma within two hours of i.p. injection, the behavioral effects of CNO persist up to eight to nine hours (Guettier et al., 2009; Krashes et al., 2011). It is likely that changes in cell activity downstream of POMC neurons are involved in the prolonged behavioral effects of CNO. The prolonged

depolarization and increased basal firing observed *in vivo* and the observed increase in c-Fos expression in ARC cells not expressing hM3Dq are consistent with this rationale and might explain the prolonged behavioral effects of CNO seen here.

The decreased food intake following acute activation of POMC neurons observed in the present study is in contrast to the research by Zhan and colleagues (2013) showing a single dose of CNO has no significant effect on food intake. This discrepancy is likely due to the current study using a higher dose of CNO (2mg/kg vs 1 mg/kg). Although dose-dependent effects of CNO on food intake were not evaluated here, others have shown activation of hM3Dq+ pancreatic β cells with increasing doses of CNO elicits increasing hypoglycemia (Guettier et al., 2009). The present study shows the stimulatory DREADD can be used to modulate feeding behavior driven by hypothalamic POMC neurons. These data also provide preliminary evidence that acute activation of POMC neurons is sufficient to decrease short-term food intake.

Research shows that the stimulatory DREADD can be used for cell activation and to induce persistent physiological and behavioral changes with little evidence of desensitization over time (Jain et al., 2013; Zhan et al., 2013; Urban and Roth, 2015). Hence, this receptor appears to be an appropriate tool for investigating the effects of prolonged POMC neuron activation. The current study addressed the possibility that the stimulatory DREADD could activate POMC neurons for a prolonged period and sustain decreased food intake and body weight. The data shown here lends proof-of-concept information for using hM3Dq to sustain POMC neuron activation as evidenced by the reduction of food intake over four days in an animal expressing

hM3Dq:mCherry. These studies identified technical limitations and other considerations, which are discussed below.

Technical considerations

One concern raised by the results shown for prolonged POMC activation is that decreased food intake was observed in the control group, which lacked expression of the stimulatory DREADD. While back-conversion of CNO to clozapine could explain CNO having an effect in the absence of the DREADD receptor (Roth et al., 1994; Pirmohamed et al., 1995; Löffler et al., 2012; Schmid et al., 2014), it is also possible the reduction of food intake in the control group was induced by stress of the injection protocol. Animals received five daily i.p. injections for seven days and this procedure is likely to induce stress. Since Zhan and colleagues (2013) did not use a no-DREADD control group it is not possible to ascertain if they also saw putative stress-induced decreases in food intake. While hM3Dq could be a valuable tool for prolonged activation of POMC neurons, future studies need to explore less stressful methods for delivering CNO. For example, delivery of CNO through drinking water has been used for activating hM3Dq+ pancreatic β cells (Jain et al., 2013), however through a pilot experiment we did not find this approach effective for our studies. It is likely the concentration of CNO needed to elicit decreases in feeding behavior was not high enough (Farrell and Roth, 2013) since the concentration of CNO and absorption rates can be altered when drugs are administered orally (Burton et al., 2002). While osmotic pumps are commonly used to continuously deliver drugs to mice, control of dose and timing is not possible with this type of pump. Although limited evidence exists for desensitization of the stimulatory DREADD (Jain et al., 2013; Zhan et al., 2013; Urban and Roth, 2015), multiple endogenous GPCRs on POMC neurons are known to

desensitize (Pennock et al., 2012) and the continuous infusion of CNO through osmotic pumps raises concerns about the potential for desensitization. However, programmable infusion pumps have only recently become available and these pumps would allow control over the delivery of CNO such that a bolus of drug is given and allowed to clear before the next dose. Future studies investigating prolonged activation of POMC neurons – or DREADD-mediated modulation of any cell – should consider using programmable infusion pumps.

Behavioral consequences of endogenous release of peptides versus AA transmitters from POMC neurons is currently unknown. Furthermore, the frequency and intensity of stimulus required to elicit peptide versus AA transmitter release from POMC neurons or to elicit specific behaviors is also not fully understood. One of the limitations of the present study is the inability to distinquish which POMC-neuron-derived transmitter(s) is responsible for the increase in 24-hour food intake following POMC neuron activation. Although the data presented here show that hM3Dq mediates CNO-induced depolarization *in vitro* and increases in c-Fos expression *in vivo,* it is not clear if increased c-Fos expression can upregulate *Pomc* mRNA, peptide release, or AA transmitter release. Interpretation of the data shown here is based on the assumption that increased c-Fos expression likely leads to an increase in *Pomc* transcription (Huo et al., 2006). It has been shown that c-Fos enhances POMC promoter activity by binding to a response element on exon 1 of the *Pomc* gene (Boutillier et al., 1995) and it has been suggested that changes in synaptic activity can differentially regulate gene expression (Jain and Bhalla, 2014). Not knowing how POMC neuron activation alters transmitter output prevents thorough understanding of how these cells participate in energy balance. Though these data indicate that hM3Dq increases POMC neuron activity *in vivo,* future experiments should determine if c-Fos

expression is a good proxy measurement of increased peptide synthesis or changes in AA transmitter release.

It is possible that the G_q-coupled hM3Dq receptor used here is not capable of adequately increasing POMC neuron activity over a prolonged period of time or to the extent needed to induce weight loss. The G_s coupled DREADD receptor (GsD), which signals through G_s pathways and mediates cell activation through increases in cAMP (Guettier et al., 2009; Sternson and Roth, 2014; Urban and Roth, 2015), might be more effective in increasing POMC neuron activity relative to the G_q-coupled DREADD. Although this receptor has some constitutive activity that results in a basal phenotype when expressed in some cell populations in mice (Guettier et al., 2009), GsD has provided insight into various neural mediated-behaviors (reviewed in Sternson and Roth, 2014) and might be useful in understanding the consequences of POMC neuron activation.

As discussed in Chapter 2, WRA is important for the development of ABA and POMC peptides are involved in physical activity (Gerra et al., 1992; Dwyer and Boakes, 1997; Patterson et al., 2008). Currently it is not known if POMC neuron activation can initiate the physical activity associated with anorexia or if increased physical activity is a consequence of increased POMC neuron activity. WRA was not measured during POMC neuron activation in the present study, but will be an important variable to consider in future experiments. The relationship between physical activity and POMC neurons appears to be dependent on development since early-onset exercise increased *Pomc* mRNA in adulthood (Patterson et al., 2008). Younger animals might be more sensitive to the effects of prolonged POMC neurons activation relative to older mice, and

experiments using younger mice might uncover valuable insights into the underlying physiology of anorexia. Furthermore, evaluating changes in POMC peptides throughout the development of anorexia would provide clues about the role of peptides throughout the disorder. If POMC peptides are increased early but not late during prolonged activation, then it is possible that increased levels of POMC peptides are involved in initiating decreased food intake and body weight while circuit level changes might maintain anorexia. If POMC peptides are increased late but not early during the initial stages of anorexia, then it is possible that AA transmitters mediate the earlier effects of POMC activation on food intake and body weight loss while POMC peptides might mediate the sustained anorexia.

Summary

The purpose of this study was to determine if prolonged activation of POMC neurons induces lasting anorexia. The data presented here show hM3Dq:mCherry targets to POMC neurons where it mediates CNO-induced increase in cell activity *in vitro* and *in vivo* as well as decreased food intake. Although it was not definitively shown that prolonged activation of POMC neurons maintains decreased food intake and body weight, the findings presented here support continuing this line of inquiry. The underlying physiological contributors to maintaining anorexia and increased physical activity are not fully understood. Tools enabling the selective manipulation of cell populations thought to be involved in anorexia will be valuable in exploring the underlying physiology.

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CHAPTER 4: AMINO ACID PHENOTYPE SWITCHING AND THE ROLE OF GLUTAMATE RELEASE FROM HYPOTHALAMIC PROOPIOMELANOCORTIN NEURONS

Experiments using fluorescent *in situ* hybridization shown in Chapter 4 of this dissertation were carried out by Christina Dennison. Food and body weight experiments were completed by Connie King and electrophysiology was performed by Matthew Dicken. All work was conducted in the Hentges Lab and was overseen by Shane Hentges. This Chapter consists of data submitted for publication. Figures in the submitted article were altered to meet formatting requirements of the dissertation.

- The distribution of GABAergic and glutamatergic POMC neurons has not been evaluated during postnatal development. The role of AA transmitter release from POMC neurons is currently unknown.
- *In situ* hybridization was used to characterize the AA transmitter phenotype of POMC neurons in early development. Genetic deletion of *vGlut2* was used to investigate the role of glutamate release from POMC neurons.
- *vGlut2* mRNA is relatively low and *Gad67* mRNA is relatively high in POMC cells early in life compared to adulthood.
- Genetic loss of glutamate release from POMC neurons results in increased body weight in male mice fed a high-fat diet.
- These data show that POMC neurons exhibit plasticity in their AA transmitter phenotype early in life and that glutamate release has sex- and diet-specific effects on energy balance.

Box 4.1. Overview of Chapter 4

Overview

The work described in Chapters 2 and 3 was focused on understanding the necessity and sufficiency of POMC neurons in the development of anorexia. Young rodents are more susceptible to developing anorexia during ABA as compared to adult mice (Klenotich and Dulawa, 2012). POMC neurons have been implicated in reproduction and feeding circuit maturation, which are sensitive to development (Bouret et al., 2004a; b; Gao et al., 2007; de Souza et al., 2011; Xu et al., 2011). It is possible that POMC neurons exhibit increased plasticity around the time of puberty, which might increase susceptibility to a dysregulation of energy balance. Therefore Chapter 4 of this dissertation was focused on elucidating the AA transmitter phenotype of POMC neurons across postnatal development to determine if POMC neurons exhibit increased plasticity around the time of puberty.

Introduction

Hypothalamic proopiomelanocortin (POMC) neurons are necessary for the regulation of energy balance and exert many actions on food intake and energy expenditure through the release of their peptide transmitters (Mercer et al., 2013). However, recent work has shown that POMC neurons also release the amino acid transmitters (AA) GABA and glutamate (Dicken et al., 2012; Hentges et al., 2004; Hentges et al., 2009). While the role of GABA and glutamate release from POMC cells is not yet clear, AA transmitter release from other hypothalamic neurons is known to play a key role in regulating energy balance (Kong et al., 2012; Tong et al., 2008; Wu et al., 2009). The majority of the GABAergic and glutamatergic POMC neurons represent two distinct cell populations (Jarvie and Hentges, 2012), although a small subset of POMC neurons express a dual GABAergic and glutamatergic phenotype (Jarvie and Hentges, 2012). A dual AA

neurotransmitter phenotype has also been reported for cells in the hippocampus (Zander et al., 2010), basal ganglia (Shabel et al., 2014), ventral tegmental area (Root et al., 2014), preoptic area (Ottem et al., 2004), and presynaptic terminals from the supramammillary nucleus (Boulland et al., 2009). Mounting evidence suggests both GABAergic and glutamatergic markers in an individual cell may indicate a switch between phenotypes. Indeed, some neurons are capable of shifting the expression and distribution of phenotypes in response to ageing or environmental changes and it has been suggested that expression of multiple neurotransmitters and plasticity of neurotransmitter phenotype is a cellular adaptation that can be advantageous in a changing environment (Demarque and Spitzer, 2010).

Early postnatal life has been shown to be a malleable period in development for neurotransmitters. During this time period multiple brain regions, including the hypothalamus, show considerable expression of glutamatergic markers that then declines as rodents reach adulthood (Borgius et al., 2010; Boulland et al., 2004; Nakamura et al., 2005). Studies focusing on specific populations of neurons have shown that neurons in the ventral tegmental area (Berube-Carriere et al., 2009; Mendez et al., 2008), hippocampus (Boulland et al., 2004), and visual cortex (Berry et al., 2012) transiently express glutamatergic markers in early development. Expression of glutamatergic markers in these neurons declines as rodents reach adulthood. In line with these aforementioned studies, Gillespie and colleagues (Gillespie et al., 2005) show glutamate release from neurons in the medial nucleus of the trapezoid body during the first 10 days of postnatal development; subsequently, these neurons become increasingly inhibitory as animals reach adulthood. Although neurons expressing a dual AA transmitter phenotype early in development appear to differentiate into either inhibitory or excitatory neurons, evidence

suggests that activity-dependent re-expression of a dual phenotype is possible in mature neurons (Gomez-Lira et al., 2005).

While the specific function of upregulated glutamatergic signaling in postnatal development is likely to be dependent on brain region, evidence suggests glutamate plays a role in synapse formation (Berry et al., 2012; Berube-Carriere et al., 2009; He et al., 2012) and organization of neuronal circuits (Noh et al., 2010). Axonal projections from hypothalamic POMC neurons are formed during the second week of postnatal life (Bouret et al., 2004a), a timeframe coinciding with the increased plasticity of AA transmitters during development. Previous work has shown the distribution of GABAergic and glutamatergic POMC neurons in adult mice (Jarvie and Hentges, 2012), yet it is not known if the AA phenotype of these neurons exhibits plasticity during early postnatal development.

The purpose of this study was to determine if the distribution of glutamatergic and GABAergic POMC neurons changes during postnatal development relative to adulthood and to determine if altering AA phenotype of POMC neurons would affect energy balance regulation. Fluorescent *in situ* hybridization was used to detect mRNA for the vesicular glutamate transporter-2 (vGlut2) as an indicator of glutamatergic neurons. Putative GABAergic neurons were identified based on the presence of mRNA for the GABA synthetic enzymes glutamate decarboxylase 1 or 2 (referred to as Gad67 and Gad65, respectively). The results indicate that plasticity in the AA transmitter phenotype occurs in hypothalamic POMC neurons during postnatal development from day 1 to 8 weeks of age. Additionally, embryonic deletion of *vGlut2* from POMC neurons resulted in a sex-specific increase in body weight when mice were maintained on a high fat diet. Thus, it

appears that glutamate release from POMC neurons contributes to the ability of POMC neurons to regulate energy balance.

Methods and materials

Animals

Male and female mice (aged 1 day to 8 weeks, as indicated) expressing enhanced green fluorescent protein (eGFP; (Cowley et al., 2001)) driven by the POMC promoter and wild-type mice maintained on the C57BL/6J background were used for all *in situ* hybridization experiments. Deletion of the vesicular glutamate transporter type 2 (*vGlut2)* in POMC cells was accomplished by crossing vGlut2$^{flox+/-}$ mice (The Jackson Laboratory stock number 012898) with vGlut2$^{flox+/-}$;POMC-Cre (Xu et al., 2005) double transgenic mice. Standard PCR genotyping was used to detect the floxed alleles and Cre transgene. Animals were housed under controlled temperatures (22-24°C) and a 12-hour light/dark cycle. Mice were given standard rodent chow (except where noted in high-fat diet experiments) and tap water *ad libitum*. All of the animal protocols were approved by the Institutional Animal Care and Use Committee at Colorado State University and were in accordance with the United States Public Health Service guidelines for animal use.

Food intake and bodyweight studies

Upon weaning (3 weeks of age), mice lacking *vGlut2* in POMC-Cre cells and control litter mates were individually housed and given *ad libitum* access to water and standard rodent chow (Teklad 2018; 18% of kcal from fat) or high-fat rodent chow (Teklad 06414; 60% of kcal from fat).

Bodyweight and food intake measurements were collected weekly. Food intake and body weight studies in *vGlut2* knockout mice were performed by Connie King.

Tissue preparation

Mice were anesthetized and perfused transcardially with 10% sucrose in water prior to 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight at 4°C in 4% paraformaldehyde. Coronal sections (50-100 μm) containing the arcuate nucleus were prepared on a vibratome, collected in cold diethlpyrocarbonate (DEPC)-treated PBS, and processed for *in situ* hybridization as previously described (Jarvie and Hentges, 2012) and outlined below.

Antibody characterization

All antibodies used in this study are listed in table 1. Antibodies used to detect digoxigenin (DIG) labeled probes (RRID:AB_5145000) and fluorescein (FITC) labeled probes (RRID:AB_840257) for *in situ* hybridization produced a pattern that was unique to each gene target and matched work previously described (Jarvie and Hentges, 2012). Detection of GFP using the chicken anti-GFP antibody (RRID:AB_300798) is consistent with labeling pattern that has been previously described (Jarvie and Hentges, 2012). Western blot analysis has shown that the chicken anti-GFP antibody recognizes a single band around 27-30 kDa, but no band is detected in a WT control (technical information provided by the manufacturer). Negative controls included omitting primary and secondary antibodies as was done previously for these antibodies (Jarvie and Hentges, 2012).

TABLE 1

Antibodies Used in This Study

Antibody	Immunogen	Company, Cat#, RRID	Conc.
Primary			
Green Fluorescent Protein (GFP) Chicken, polyclonal	Recombinant full-length GFP	Abcam, Cat#ab13970 RRID:AB_300798	1:2,000
Digoxigenin conjugated to POD Sheep, polyclonal	digoxigenin (DIG)	Roche, Cat#11207733910 RRID:AB_5145000	1:1,000
Fluorescein conjugated to POD Sheep, polyclonal	fluorescein	Roche, Cat#11426346910 RRID:AB_840257	1:1,000
Secondary			
Anti-ckicken IgY(IgG) (H+L)Alexa647 Donkey, polyclonal	chicken IgY(IgG) (H+L)	Jackson ImmunoReseach, Cat#703605155	1:1,000
Streptavidin Alexa555 Polyclonal	Biotin	Life Technologies Cat#S32355, RRID:AB_2307336	1:1,000
Anti-DNP-KLH Alexa488 conjugate Rabbit, polyclonal	dinitrophenyl (DNP)	Molecular probes, Cat#A11097 RRID:AB_2314332	1:400

Fluorescent in situ hybridization

For fluorescent *in situ* hybridization, sections were placed in 6% H_2O_2 for 15 minutes to quench endogenous peroxidase activity. Tissue was incubated for 15 minutes in proteinase K (10µg/ml) diluted in PBS containing 0.1% Tween 20 (PBT). Proteinase K was deactivated by exposing tissue to 2mg/ml glycine in PBT for 10 minutes. Following two 5 minute washes in PBT, tissue was postfixed for 20 minutes in solution containing 4% paraformaldehyde and 0.2% gluteraldehyde. Tissue was washed in PBT, then dehydrated in ascending concentrations of ethanol diluted in DEPC-treated water (50, 70, 95, and 100%), and briefly rehydrated in PBT. Sections were transferred to vials and prehybridized in hybridization solution (66% deionized formamide 13% dextran sulfate, 260 mM NaCl, 1.3x Denhardt's solution, 13 mM Tris-HCL [pH

8.0], 1.3 mM EDTA [pH 8.0]) for 1 hour at 60°C. Probes were denatured at 85°C for 5 minutes and then added, along with 0.5mg/ml tRNA and 10 mM DTT, to the hybridization buffer.

Digoxigenin (DIG)-labeled and fluorescein isothiocyanate (FITC)-labeled RNA probes were made and used as described by Jarvie and Hentges (2012). Probes for *Pomc*, *vGlut2*, and glutamate decarboxylase 1 (*Gad67)* hybridize at 70°C, therefore combinations of these probes were hybridized simultaneously for 18-20 hours. The glutamate decarboxylase 2 (*Gad65)* probe hybridizes at 52°C and required sequential hybridization for dual *in situ*. For detection of *Gad67* and *Gad65*, the *Gad67* probe was hybridized first at 70°C and then the tissue was incubated for 20 hours at 52°C in new hybridization buffer with *Gad65*.

Three of the six 30-minute stringency washes at 60°C were in solution containing 50% formamide and 5X SSC followed by three washes in 50% formamide and 2x SSC. Tissue was then digested for 30 minutes at 37°C with RNAse A (20 µg/ml in 0.5 M NaCL, 10 mM Tris-HCL [pH 8.0], 1 mM EDTA) and subsequently placed in three 15-minute TNT (0.1 M Tris-HCL [pH 7.5], 0.15 M NaCl, 0.05% Tween-20) washes. Sections were blocked for 1 hour in TNB (TNT plus 0.5% Blocking Reagent provided in the TSA kit; Perkin Elmer, Oak Brook IL) and then incubated overnight at 4°C in either sheep anti-DIG (1:1,000; Roche Applied Sciences) or sheep anti-FITC (1:1,000; Roche Applied Sciences) antibodies, both conjugated to horseradish peroxidase. The DIG-labeled probes were detected using a TSA PLUS Biotin Kit (Perkin Elmer) and then incubated in 1% H_2O_2 to quench any remaining peroxidase activity. FITC-labeled probes were subsequently detected using a TSA PLUS DNP (HRP) system (Perkin Elmer). Tissue was exposed to three 15-minute TNT washes and then incubated for 30 minutes in a 1:50

dilution of either the Biotin Amplification Reagent or the DNP Amplification Reagent. Tissue was then washed in TNT and DIG-labeled probes were visualized with Streptavidin conjugated to Alexa Fluor 555 (30 minutes in 1:1,000; Invitrogen, Eugene, OR). FITC-labeled probes were visualized with 1:400 rabbit anti-DNP-KLH conjugated to Alexa Fluor 488 (1 hour; Invitrogen, Eugene, OR), both in TNT. Tissue was mounted and cover slipped with Aqua Poly/Mount (Polysciences, Inc., Warrington).

Immunodetection of GFP

Immunodetection of GFP in transgenic POMC-eGFP mice was used for detection of *vGlut2* and *Gad67* in POMC neurons. As shown previously (Jarvie and Hentges, 2012), GFP fluorescence is quenched during in situ but the antigenicity of GFP is maintained. Following detection of FITC-labeled probes, tissue was incubated for 2 hours in chicken anti-GFP antibody (1:2,000, Abcam, Boston MA), washed in TNT, and then placed in donkey anti-chicken secondary antibody conjugated to Alexa Fluor 647 for 1 hour (1:1,000, Jackson ImmunoResearch, West Grove, PA).

Imaging

Images were collected on a Zeiss 510 Meta confocal microscope. Green fluorescence (Alexa fluor 488) was imaged using 488 nm excitation filter and emissions was detected using a 505/530-nm bandpass filter. Red fluorescence (Alexa Fluor 555) was imaged using 543 nm excitation filter and 560/615 nm bandpass emission filter and far-red fluorescence (Alexa Fluor 647) was imaged using 633 nm excitation filter and a 650 nm longpass emission filter. Images were taken sequentially at each wavelength to avoid crossover between channels. Images were taken at an optical depth of 1 μm in the z-plane every 3 μm for a total of 18-21 μm from all of

the tissue along the rostral-caudal axis containing POMC neurons (~Bregma -1.2 mm to ~Bregma -2.3 mm). Images used for figures were adjusted for brightness and contrast using Photoshop (Adobe Systems, San Jose, CA).

Cell counts and analysis

Cell counts and analysis were similar to that described in Jarvie and Hentges (2012). Cells with a clear nucleus or cells completely contained in a 300 x 300 x 12-µm counting box on the x-y-z plane were counted. Z-stacks with a tissue depth of 12 µm were constructed from sequential 3 µm-thick sections of tissue with an optical depth of 1 µm and were analyzed for cell number, colocalization, and intensity above background using ImageJ software. *Pomc*-FITC labeled cells and POMC-eGFP cells were counted and analyzed for colocalization with *vGlut2, Gad67,* or *Gad65*. Presence of *vGlut2, Gad67*, or *Gad65* signal had to be in the same z-plane as POMC cells and be contained within POMC cells to be considered colocalized. Colocalization is reported as a percentage of POMC cells. To determine whether changes in *vGlut2* or *Gad67* label intensity were uniform or unique to POMC neurons, two circular ROIs were drawn in a region of the arcuate containing POMC cells and a region in the arcuate devoid of POMC cells and intensity of signal within the ROIs was determined. Since the distribution of *vGlut2* and *Gad67* is different across the arcuate, ROIs for *Gad67* were drawn in the dorsal-medial arcuate and *vGlut2* ROIs were drawn more towards the dorsal-lateral arcuate.

Evoked transmitter release

To determine whether the Cre-mediated excision of *vGlut2* effectively prevented glutamate release from POMC-Cre neurons, an optogenetic/electrophysiologic approach was used.

Channelrhodopsin2 was expressed in POMC-Cre neurons by injecting AAV2/9.EF1.dflox.hChR2(H134R)–mCherry.WPRE.hGH (obtained from the Penn Vector Core at the University of Pennsylvania School of Medicine, Philadelphia, PA) into the arcuate nucleus of POMC-Cre trangenic mice as previously described (Dicken et al., 2012). Brief (2 ms) pulses of blue light were used to evoke transmitter release from ChR2-expressing POMC-Cre neurons. Voltage-clamp (-60 mV holding potential) recordings were made in unlabeled neurons near the ChR2-mCherry neurons as previously described (Dicken et al., 2012). Pharmacologic blockade of AMPA receptors (6,7-dinitroquinoxaline-2,3(1*H*,4*H*), 10 μm; Sigma-Aldrich) or $GABA_A$ receptors (bicuculline methiodide, 10 μm; Tocris) was used to determine whether the postsynaptic current was mediated by glutamate or GABA, respectively. Electrophysiological recordings were performed by Matthew Dicken.

Statistics

For *in situ* hybridization and food intake data, statistical significance was determined using Student's t-tests or ANOVA (Bonferonni's post hoc analysis). No significant differences were found between sexes in any of the *in situ* hybridization experiments and thus, data from both sexes have been combined in the final analyses. Bodyweight data were analyzed using repeated measures ANOVA (RMANOVA) and Sidak's post-hoc tests. Results were considered significant if $p < 0.05$. Data are presented as mean ± SEM.

Results

Expression of vGlut2 in POMC neurons during postnatal development

Dual fluorescent *in situ* hybridization was used to evaluate the expression of *vGlut2* in *Pomc*-containing neurons in mice at the following ages: p1, 1 week, 3 weeks, 5 weeks, and 8 weeks. The relative number of *Pomc* cells expressing *vGlut2* was significantly different across all age groups (p1: 40.29 ± 3.18%, 1 wk: 35.29 ± 3.44%, 3 wk: 25.62 ± 3.36%, 5 wk: 17.98 ± 2.47%, 8 wk: 8.19 ± 1.98%; $p<0.0001$, Figure 4.1). Compared to 8-week-old mice, the percentage of *Pomc* neurons expressing *vGlut2* was significantly higher at p1, 1 week, 3 weeks, and 5 weeks of age (p1 vs. 8 wk, $p<0.0001$; 1 wk vs. 8 wk, $p<0.0001$; 3 wk vs. 8 wk, $p=0.0004$; 5 wk vs. 8 wk,

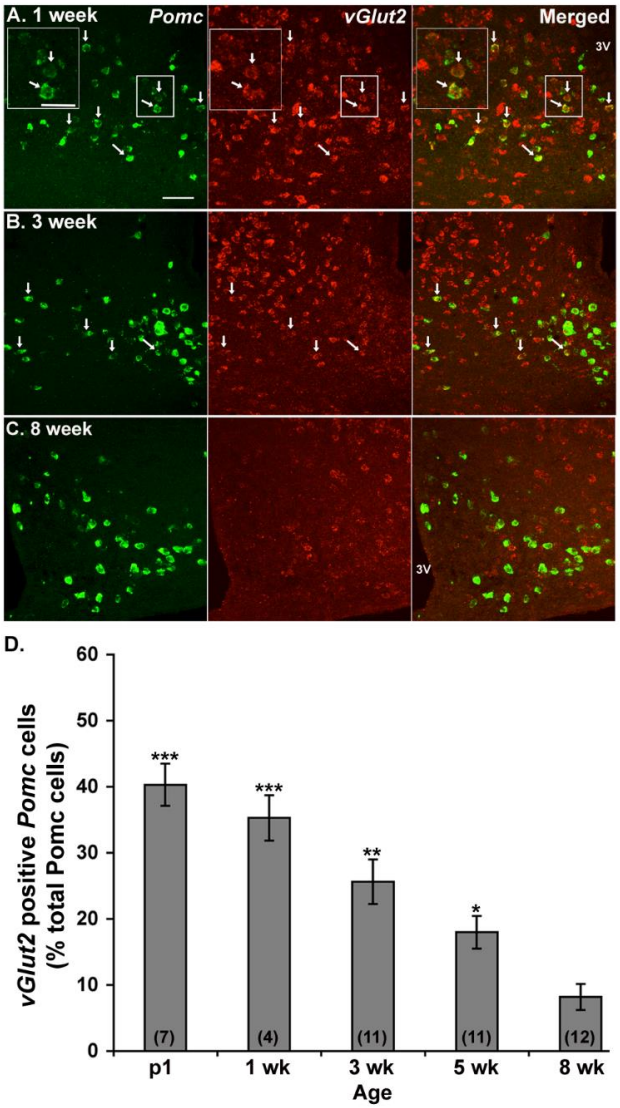

Figure 4.1. *vGlut2* expression in POMC neurons declines in early life. A-C) Representative images of *Pomc* (green) and *vGlut2* (red) mRNA in mice at ages 1 week, 3 weeks, and 8 weeks. White arrows denote cells coexpressing *Pomc* and *vGlut2* mRNA. 3V, third ventricle. Scale bar = 50 µm. Boxed areas in 1 week image are enlarged to show colocalization of *Pomc* and *vGlut2* mRNA. Scale bar = 10 µm for enlarged image**. D)** The percentage of POMC cells expressing *vGlut2* in the arcuate nucleus across postnatal development. Data are expressed as mean ± SEM. The numbers in parentheses represent the number of animals in each group. Significance was determined relative to 8-week-old mice. ***$p<0.0001$; **$p=0.0004$; *$p=0.03$; one-way ANOVA with Bonferroni's post-hoc test

p=0.03). Overall expression of *vGlut2* in the arcuate nucleus appeared to be higher in younger animals relative to older mice (Figure 4.1A-C center images). To determine if *vGlut2* expression is increased throughout the ARC or is unique to POMC neurons during early postnatal development, the intensity of *vGlut2* in a region lacking *Pomc* mRNA and in a region containing *Pomc* mRNA was evaluated at each age group (Figure 4.2). Age had a significant effect on the intensity of *vGlut2* in areas of the ARC without *Pomc* neurons (Figure 4.2A-C, dashed circle and

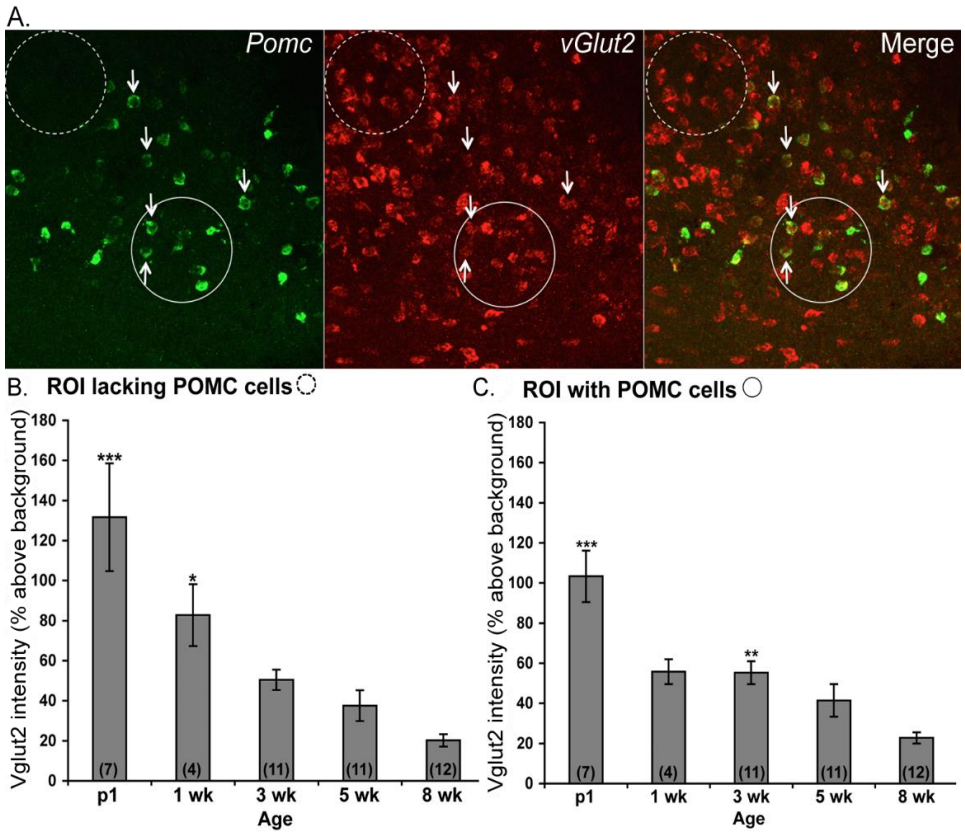

Figure 4.2. *vGlut2* expression decreases throughout the arcuate nucleus during postnatal development. A) Representative image from an animal at postnatal day 1 with analysis regions (ROIs) containing Pomc cells (green cells, solid circle) and lacking Pomc cells but containing *vGlut2* positive cells (dashed circle). White arrows indicate some cells coexpressing *Pomc* and *vGlut2* mRNA. 3V, third ventricle. Scale bar = 50 µm**. B)** Intensity of *vGlut2* fluorescence above background in ROIs lacking *Pomc*. ***$p < 0.0001$; *$p = 0.018$. **C)** Intensity of *vGlut2* fluorescence above background in ROIs with Pomc neurons. ***$p < 0.0001$; **$p = 0.006$. Data are expressed as mean ± SEM. The numbers in parentheses represent the number of animals in each group. Significance was determined relative to 8-week-old mice. Data analyzed using one-way ANOVA with Bonferroni's post-hoc test.

D, $p<0.0001$; intensity above background at 8 wk: 20.20±3.14%, at 1 wk: 82.76±15.43%) and with *Pomc* neurons (Figure 4.2A-C, solid circle & D, $p<0.0001$; intensity above background at 8 wk: 22.74±2.83%, at 1 wk: 55.79±6.22%).

Expression of Gad67 in POMC neurons during postnatal development

Dual fluorescent *in situ* hybridization was used to determine the number of *Pomc* neurons expressing *Gad67* at the following ages: p1, 1 week, 3 weeks, 5 weeks, and 8 weeks. Overall expression of *Gad67* in the ARC was similar across all age groups (Figure 4.3A, middle column) and age did not have a significant effect on the intensity of *Gad67* in POMC ($p=0.07$) and non-POMC cells ($p=0.93$). The relative number of *Pomc* cells expressing *Gad67* increased with age (Figure 4.3B, $p < 0.0001$). Compared to animals at 8 weeks of age (46.16±1.49%), the percentage of *Pomc* neurons expressing *Gad67* was significantly lower at p1 (8.04±1.57%), 1 week (29.15±2.23%), and 3 weeks (35.42±1.52%) of age (p1 vs. 8 wk, $p<0.0001$; 1 wk vs. 8 wk, $p=0.0004$; 3 wk vs. 8 wk, $p=0.0093$). By the time animals reached 5 weeks of age, the percent of *Pomc* cells expressing *Gad67* was not significantly different from 8-week-old mice (5 wk: 48.26±1.99%, 5 wk vs. 8 wk, $p=0.76$).

To determine whether the decreased *Gad67* expression in POMC neurons during early postnatal development might reflect a shift between *Gad67* and *Gad65* expression, dual fluorescent *in situ* was used to determine the number of *Pomc* neurons colocalized with *Gad65* in 1 week old mice. The percentage of *Pomc* neurons expressing *Gad65* was not significantly different than *Pomc* neurons expressing *Gad67* (Figure 4.3C; 32.37±4.78% colocalize *Gad65*, 29.15±2.23% colocalize *Gad67*, $p=0.57$) suggesting that *Gad65* and *67* have overlapping expression patterns.

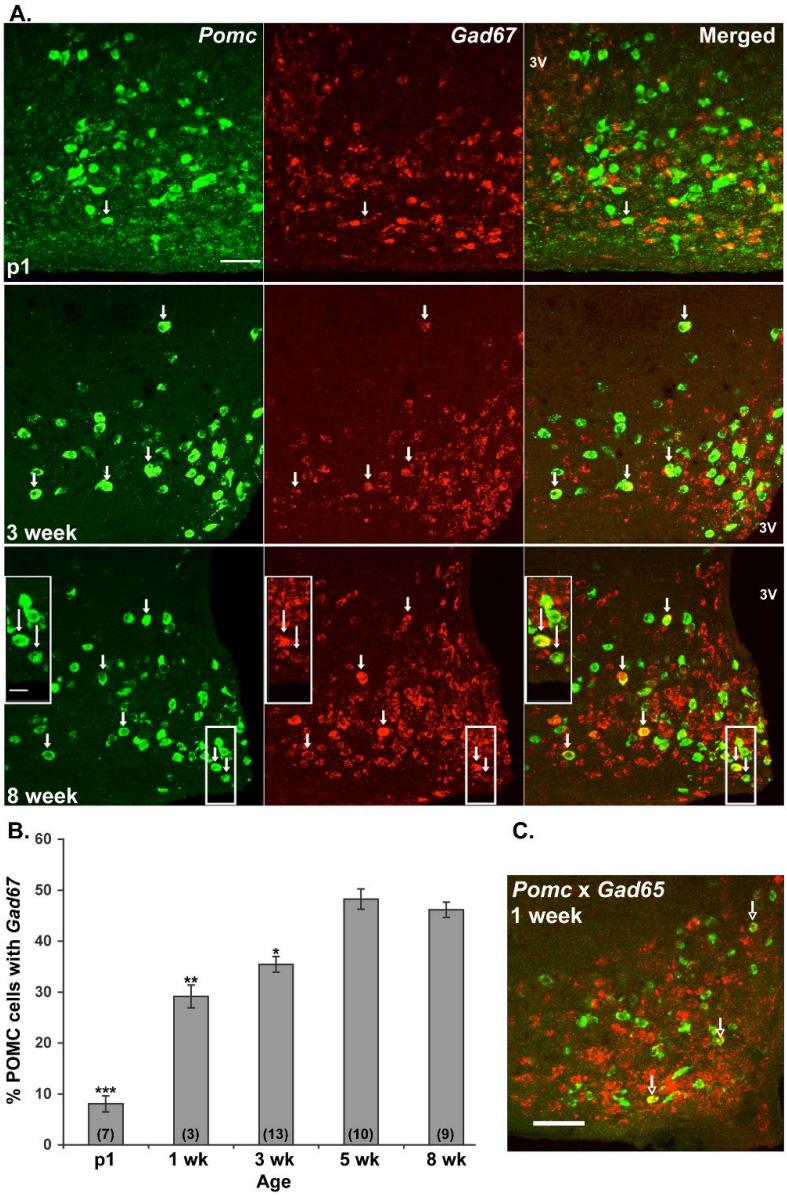

Figure 4.3. *Gad67* expression in POMC neurons increases with age. A) Representative images of *Pomc* (green) and *Gad67* (red) cells in the arcuate nucleus from mice 1day (top), 3 weeks (middle), and 8 weeks (bottom) of age. White arrows indicate cells coexpressing *Pomc* and *Gad67* mRNA. 3V, third ventricle. Scale bar = 50 µm. Boxed areas in 8 week images are enlarged to show colocalization of *Pomc* and *Gad67* mRNA. Scale bar = 10 µm for enlarged image. **B)** The percentage of POMC cells expressing *Gad67* in the arcuate nucleus across postnatal development. Data are expressed as mean ± SEM. The numbers in parentheses represent the number of animals in each group. Significance was determined relative to 8-week-old mice. ***$p<0.0001$; **$p=0.0004$; *$p=0.009$**. C)** Representative image of *Pomc* (green) and *Gad65* (red) mRNA in mice at 1 week. White arrows denote cells coexpressing *Pomc* and *Gad65* mRNA. Scale bar = 50 µm.

Dual phenotype POMC neurons during postnatal development

To determine if the relatively high number of glutamatergic POMC neurons during early postnatal development represents a population of cells expressing a dual AA transmitter phenotype during early development that later mature into GABAergic POMC cells, dual fluorescent *in situ* hybridization was used to detect *Gad67* and *vGlut2* mRNA in POMC-eGFP neurons. While eGFP fluorescence is quenched during tissue processing for the *in situ* hybridization , antigenicity of eGFP survives and can be used for immunodetection of POMC-eGFP cells (Jarvie and Hentges, 2012). Most POMC cells do not express a dual AA transmitter phenotype in animals aged 1 day, 3 weeks, or 5 weeks (9.7%, 7.6%, and 6.6%, respectively; Figure 4.4).

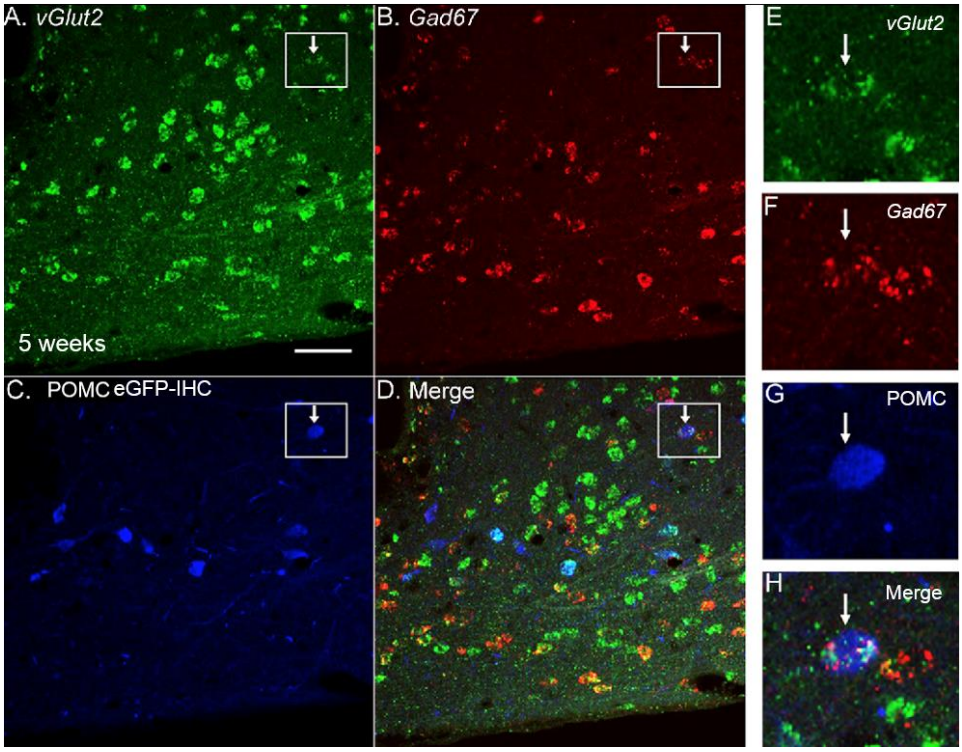

Figure 4.4. Detection of POMC neurons expressing both *vGlut2* and *Gad67* during postnatal development. Representative images of *vGlut2* (**A**, green) and *Gad67* (**B**, red) mRNA, and immunodetection of POMC-eGFP (**C**, blue) expression in the arcuate nucleus of an animal aged 5 weeks. D) Merged image of **A-C**. White arrows indicate a POMC cell containing *vGlut2* and *Gad67*. Scale bar = 50 µm. Boxed area in **A-D** is enlarged in **E-H.**

Effect of deletion of vGlut2 in POMC on body weight regulation

To determine whether glutamate release from POMC neurons contributes to energy balance regulation, Cre/lox technology was used to delete *vGlut2* from POMC-Cre expressing neurons. An optogenetic approach was used to demonstrate that deletion of *vGlut2* from POMC neurons prevented glutamate release from these cells. When ChR2 was expressed in POMC-Cre neurons, a brief pulse of blue light evoked transmitter release and a caused a postsynaptic current in downstream cells as previously reported (Dicken et al., 2012). In tissue from POMC-Cre;vGlut2$^{flox/flox}$ mice, the light evoked currents were mediated exclusively by GABA (9/9 recordings displayed GABA-mediated currents; 0/9 recordings that showed evoked currents displayed glutamate mediated currents, data collected and analyzed by Matthew Dicken). This is in contrast to previous reports showing that 30% of evoked currents from POMC neurons in control tissue are mediated by glutamate (Dicken et al., 2012). Thus, the Cre/lox approach effectively eliminated glutamate release from Cre-expressing neurons as shown previously for other hypothalamic neurons (Tong et al., 2007).

The deletion of *vGlut2* from POMC-Cre neurons did not alter the bodyweight of female mice maintained for 8 weeks after weaning on regular chow (final weight 20.2±0.9g for control, 19.3±0.6g for POMC-*vGlut2*-/-, p=0.27; RMANOVA for entire weight curve, p=0.29, n=10; Figure 4.5A) or high-fat chow (final weight 21.6±0.4g for control, 21.5±0.9g for POMC-*vGlut2*-/-, p=0.99; RMANOVA for entire weight curve, p=0.60, n=8-9; Figure 4.5B). The deletion of *vGlut2* from POMC-Cre neurons also did not alter the bodyweight of male mice maintained on regular chow (final weight 24.6±0.7g for control, 24.9±0.9g for POMC-*vGlut2*-/-, p=0.83; RMANOVA for entire weight curve, p=0.23, n=10-13, Figure 4.5C). When male mice were maintained on high-fat chow after weaning, the mice were significantly heavier at 12 weeks of

age (25.2±0.8g for control versus 30.2±1.3g for POMC-*vGlut2*-/-, p=0.007; n=8). There was a significant difference in the weight curves between control and POMC-*vGlut2*-/- males maintained on high-fat diet (RMANOVA, p=0.01 by genotype and p<0.0001 for interaction between age and genotype). Post-hoc analysis showed that bodyweights were significantly different between the genotypes beginning at 9 weeks of age (Figure 4.5D). The food intake curves were also significantly different between control and POMC-*vGlut2*-/- males maintained on high-fat chow (RMANOVA, p<0.0001, n=8) with the POMC-*vGlut2*-/- mice eating significantly more at weeks 9-12. The deletion of *vGlut2* from POMC neurons did not cause any apparent difference in the number of POMC neurons or the percentage of POMC neurons expressing *Gad67* in the arcuate nucleus of adult mice. Altogether, it appears that loss of glutamate release from POMC neurons selectively inhibited the ability of male mice to regulate their bodyweight on a high-fat diet as compared to control mice.

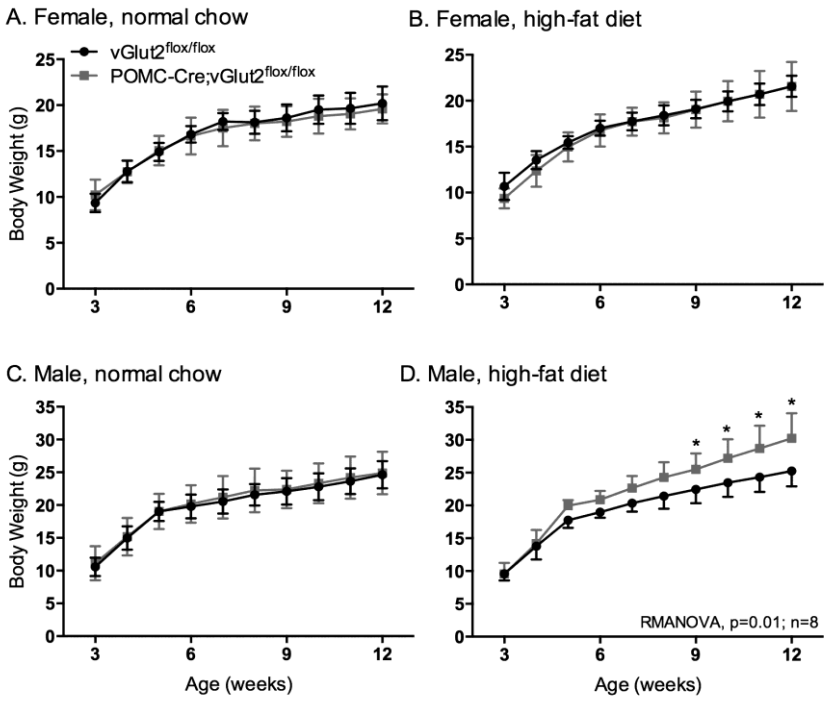

Figure 4.5. vGlut2 deletion in POMC neurons increases weight gain in males on a high-fat diet. **A-D)** Weight curves of control mice (black circles) and mice lacking *vGlut2* in POMC neurons maintained on normal chow (**A&C**) or high-fat diet (**B&D**). All data points are mean ± SEM. *=p<0.05 compared to same age control mice (*vGlut2*$^{flox/flox}$). Data collected and analyzed by Connie King

Discussion

While the AA transmitter phenotype of hypothalamic POMC neurons has been characterized in adult mice (Jarvie and Hentges, 2012), it was not known if the distribution of GABAergic and glutamatergic POMC neurons is stable from early postnatal development until adulthood. The present study used fluorescent *in situ* hybridization to show that POMC neurons exhibit plasticity in their AA transmitter phenotype during postnatal development and used genetic deletion of vGlut2 to examine the role of glutamate release from POMC neurons in energy balance regulation.

POMC neuron expression of vGlut2 during postnatal development

This investigation found that early in postnatal development mice have increased *vGlut2* expression in POMC neurons that progressively declines as animals approach 8 weeks of age. The pattern of *vGlut2* expression was not unique to POMC neurons since other cells in the arcuate nucleus devoid of POMC had a similar pattern of expression during postnatal development. These findings are in line with previous reports of relatively high *vGlut2* mRNA throughout the hypothalamus during postnatal development (Borgius et al., 2010; Boulland et al., 2004). Consistent with an increase in glutamatergic POMC neurons is the observation of increased expression of metabotropic glutamate receptor mGluR1 and mGluR5 in the hypothalamus during early postnatal development (van den Pol et al., 1994; van den Pol et al., 1995). Additionally, the activity of these receptors in the hypothalamus is highest at postnatal day 10 (Palmer et al., 1990; Sortino et al., 1990) indicating that hypothalamic neurons are capable of receiving increased glutamatergic signaling during early postnatal development. The

relative contribution of POMC-derived glutamate in enhanced hypothalamic glutamate signaling remains to be determined.

Although the significance of the transiently high *vGlut2* expression in the arcuate nucleus during postnatal development is unknown, in other brain regions developmentally regulated increases in glutamatergic signaling is involved in cell proliferation, neuron migration (Haydar et al., 2000; Komuro and Rakic, 1993; Luhmann et al., 2015) and guiding neurite outgrowth (Zheng et al., 1996). Interestingly, the transiently high *vGlut2* mRNA observed in the present study coincides with the development of axon projections from neurons in the arcuate nucleus to other hypothalamic regions, which occurs during the first three weeks of postnatal life in rodents (Bouret et al., 2004a; b). While proper development of these neural circuits depend on neonatal exposure to leptin (Bouret et al., 2004b; Bouyer and Simerly, 2013) and are sensitive to insulin and maternal nutrition (Vogt et al., 2014), some projections from POMC neurons appear to mature independent of leptin and insulin (Bouyer and Simerly, 2013; Vogt et al., 2014). Heterogeneity in the signals driving circuit development is known to occur for subpopulations of neurons within other hypothalamic nuclei (McClellan et al., 2008; Tong et al., 2007), thus it is plausible glutamatergic signaling during early postnatal developmental is important for a subset of projections from arcuate neurons.

POMC neuron expression of Gad67 during postnatal development

The current study found that *Gad67* expression in POMC neurons progressively increases during early postnatal development. Expression of *Gad67* did not significantly change in the arcuate nucleus overall during postnatal development. Importantly, the relatively low expression of

Gad67 mRNA in POMC neurons at young ages was not compensated for by *Gad65*. Consistent with previous data showing similarity in the number and pattern of POMC neurons expressing *Gad65* or *Gad67* in adult mice (Jarvie and Hentges, 2012), the current findings suggest the distribution of *Gad* expressing POMC neurons is also similar during early postnatal development.

The functional significance of GABAergic POMC neurons is currently unknown. Given that GABA signaling is important in maintaining energy balance (Kong et al., 2012; Tong et al., 2008; Wu et al., 2009), it is likely that this population of POMC neurons also have a role in mediating energy balance. Nevertheless, body weight does not appear to be affected by a loss of GABA until animals reach adulthood (Kong et al., 2012; Tong et al., 2008); thus, raising the possibility that GABA's effects on feeding circuits occurs later in development. This notion is supported by evidence showing that GABA can influence hypothalamic circuit development after the second week of postnatal life (Di Giorgio et al., 2014; Frahm et al., 2012). Interestingly, the current study found *Gad67* expression in POMC neurons progressively increases until adult levels are reached between the third and fifth week of postnatal life, which raises the possibility of GABAergic POMC neurons being involved in the later stages of postnatal neural development.

Switch in vGlut2 and Gad67 abundance in POMC neurons during postnatal development

Transient, widespread changes in expression of neurotransmitters, particularly AA transmitters, during development may precede neurotransmitter specification (Demarque and Spitzer, 2010). Additionally, expression of multiple neurotransmitters by an individual cell during development

is thought to be involved in determining neurotransmitter phenotype (Gomez-Lira et al., 2005; Root et al., 2014) and involved in adapting to changing environments (Demarque and Spitzer, 2010; Gomez-Lira et al., 2005). Given the shift in the distribution of glutamatergic and GABAergic POMC neurons from day 1 to 8 weeks of age found in the present study, it was speculated that *vGlut2*$^{+}$ POMC neurons in young animals might be part of the population of *Gad67*$^{+}$ POMC neurons in adult mice. However, it does not seem likely that glutamatergic POMC neurons in young mice become GABAergic in adulthood since the majority of POMC neurons do not express a dual AA transmitter phenotype at any of the ages examined in the present study. While it is possible that phenotype switching occurs rapidly in POMC neurons and might have been missed in the age groups evaluated, this seems unlikely since changes in neurotransmitter phenotype appear to occur over multiple days and stages of development (Gillespie et al., 2005; Gomez-Lira et al., 2005; Root et al., 2014). Taken together, these data suggest the majority of glutamatergic and GABAergic POMC neurons are non-overlapping subpopulations. In line with this idea is recent evidence showing transcription factors Ptf1a and Atoh1 are capable of controlling specification of GABAergic and glutamatergic cells in the cerebellum, respectively, and producing neurons with distinct AA transmitter phenotypes via mutual suppression of expression (Yamada et al., 2014). Deletion of *vGlut2* did not alter the percentage of POMC neurons expressing GABAergic markers in the arcuate nucleus of adult mice, further suggesting that the glutamatergic and GABAergic populations may develop independent of one another.

Deletion of vGlut2 and energy balance regulation

In the present study, *vGlut2* was deleted from POMC neurons to determine if glutamate release from these neurons may contribute to energy balance regulation. The finding that male mice lacking *vGlut2* in POMC-Cre neurons gained significantly more weight on a high-fat diet than control mice indicates that glutamate release from POMC-Cre neurons must normally contribute to energy balance regulation in a sex- and diet-specific manner. This study appears to be one of the first to indicate a physiologic role for POMC-neuron-derived AA transmitters. It may be that additional studies examining other parameters of energy balance regulation or looking at later ages may reveal additional consequences of the loss of glutamate release from POMC neurons. It is important to note that the current approach of using a transgenic mouse breeding strategy to remove *vGlut2* has some limitations and caveats that must be considered. First, given that *vGlut2* expression is relatively high in POMC neurons postnatally compared to later in age, it may be that constitutive deletion of *vGlut* from POMC neurons leads to development differences that could potentially underlie the altered regulation of energy balance in the adult animals. Studies that disrupt *vGlut2* expression or activity in POMC neurons of adult mice will be needed to distinguish potential developmental effects from the role of acute glutamate release from POMC terminals.

A second caveat to the transgenic approach is that the POMC-Cre transgene may be transiently expressed in neurons not fated to become adult POMC neurons. Previous studies show that during development, the POMC gene promoter is expressed in some cells in the arcuate nucleus and the nucleus of the solitary tract that do not express POMC peptides or *Pomc* mRNA in the adult (Padilla et al., 2010; Padilla et al., 2012). Thus, the approach used here could result in the

deletion of *vGlut2* in cells other than authentic POMC neurons. However, the overall contribution from non-POMC neurons is likely to be minimal given the relatively small number of neurons that transiently express POMC that are not authentic POMC neurons and the small number of glutamatergic neurons in the adult arcuate nucleus. Deleting *vGlut2* from POMC neurons in adult mice using an inducible approach could be a means to avoid contributions from putative non-POMC neurons. Despite the limitations of the current experiments, the data clearly show that vGlut2 deletion from neurons that express POMC-Cre during development can alter energy balance regulation and suggest that the physiologic consequence of AA transmitter release from POMC neurons and mechanisms of glutamate actions should be further explored.

Conclusion

It is well documented that hypothalamic POMC neurons exhibit heterogeneity in their neurotransmitter phenotype, receptor expression, and in the regions they innervate. The current results show that POMC neurons also exhibit plasticity in their AA transmitter phenotype during early postnatal development. It is not known if this plasticity represents a sensitive period for AA transmitter specification in POMC neurons, but future studies could help elucidate whether or not environmental perturbations alters AA transmitter phenotype of POMC neurons and the metabolic consequences later in life. It is also possible that the role for glutamate release from POMC neurons early in development is to help establish the circuitry needed for proper energy balance regulation. Future studies that abrogate POMC-neuron derived glutamate release in adulthood will help distinguish between developmental and sustained roles for glutamate release from these important neurons.

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CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

It is well documented that hypothalamic POMC neurons are a critical component in maintaining energy balance. POMC peptides are known for having an anorexigenic effect on food intake and energy expenditure, and previous work suggests that an increase in the transcription of *Pomc* and POMC peptide release during the early stages of the ABA rodent model of anorexia might precipitate the progression of weight loss and hypophagia (Hillebrand et al., 2005, 2006). Hence one of the aims of the research presented in this dissertation was to investigate the role of POMC neurons in the development of anorexia. In Chapters 2 and 3 DREADD technology was used to selectively manipulate POMC neuron activity *in vivo* and determine if POMC neurons are a necessary and sufficient component in developing ABA. The results shown in Chapter 2 suggest that POMC neurons have a role in feeding behavior during the development of ABA, which might give rise to the suppressed energy input relative to energy output observed for ABA mice as compared to food restricted/sedentary mice. The findings presented in Chapter 3 indicate that acute activation of POMC neurons decreases 24-hour food intake. While it has yet to be determined if POMC neuron activation alone is sufficient to induce lasting anorexia, the data shown here provide valuable insight for moving forward with this line of inquiry.

Young rodents are more susceptible to developing anorexia during ABA as compared to adult mice (Klenotich and Dulawa, 2012). A similar phenomenon has been observed for anorexia nervosa in humans, whereby individuals appear to be more vulnerable to a genetic predisposition around the time of puberty relative to other developmental periods (Scherag et al., 2010). Interestingly, POMC neurons have been implicated in developmentally regulated events such as

reproduction (Gao et al., 2007; de Souza et al., 2011; Xu et al., 2011) and maturation of feeding circuits (Bouret et al., 2004a; b), which occur around puberty in rodents. Therefore Chapter 4 of this dissertation was focused on elucidating the AA transmitter phenotype of POMC neurons across postnatal development to determine if POMC neurons exhibit increased plasticity around the time of puberty. Experiments in Chapter 4 characterized the AA transmitter phenotype of POMC neurons during postnatal development and used genetic deletion of *vGlut2* from POMC neurons to better understand the AA transmitter phenotype of POMC neurons and the role of glutamate release from POMC cells. Data from these studies suggest that POMC neurons express high levels of *vGlut2* and low levels of *Gad67* early in postnatal development relative to the levels observed in adult mice. Genetic deletion of *vGlut2* from POMC neurons revealed that glutamate release is involved in regulating energy balance in male mice fed an HFD.

While data from Chapters 2 and 3 support the use of DREADD technology in manipulating POMC neurons *in vivo*, these findings also corroborate previous work indicating the majority of POMC neurons need to be modulated to alter feeding (Atasoy et al., 2012; Zhan et al., 2013). One possible explanation for why a large number of POMC cells need to be engaged to induce changes in feeding behavior is the heterogeneity of POMC neurons. Previous work has shown that distinct and dispersed subpopulations of these cells express receptors for leptin (Münzberg et al., 2003; Huo et al., 2006; Williams et al., 2010; Lam et al., 2015), insulin (Williams et al., 2010), serotonin (Heisler et al., 2002; Xu et al., 2008; Sohn et al., 2011), and estrogen receptor-α (Xu et al., 2011). Additionally, separate populations of POMC neurons release glutamate or GABA in addition to their peptide products (Dicken et al., 2012; Jarvie and Hentges, 2012). Recruitment of most POMC neurons might be required if a widely distributed, but specific,

subset of these neurons need to be activated, or it is possible the entire population works in a coordinated manner to alter feeding. Evidence provided here and the work of others appears to suggest that selectively targeting subpopulations can have profound effects on energy balance (Xu et al., 2008; Sohn et al., 2011; data presented in Chapter 4). It would be interesting to determine if feeding behavior and wheel running are mediated by specific subpopulation of POMC neurons or the entire population during the development of ABA. Future work should focus on elucidating the physiological functions of these subpopulations as this insight could prove valuable in understanding how best to approach treatment and prevention for energy balance dysregulation. Until researchers have provided evidence for how these subpopulations work together and/or independently to give rise to specific behaviors, caution should be used when attributing certain behavioral effects to peptide versus AA transmitters.

As described previously and shown in Chapters 2 and 3, short- and long-term activation of POMC neurons has distinct effects on food intake. Zhan and colleagues (2013) found that acute activation of POMC neurons is not sufficient to decrease food intake; however, data shown in Chapter 3 of this dissertation indicate that increasing the intensity of the acute activation (relative to Zhan and colleagues) can suppress food intake. Thus stimulus intensity is likely to also have distinct effects on feeding behavior. A possible explanation for these differences is the release of AA versus peptide transmitters and the ratio of alpha-MSH and beta-endorphin released at a particular target site. Currently it is not clear if these peptides are released *in vivo* in equal ratios at each target site or preferentially released at some sites but not others (Mercer et al., 2013). Moreover, understanding how POMC neurons manage the release of AA transmitters and peptides-presumably at the same target sites-under physiological conditions is limited. Recent

work using DREADD technology to modulate activity of AgRP/NPY/GABA releasing neurons that lack specific combinations of these transmitters revealed the temporal effects of these transmitters on short- and long-term food intake (Krashes et al., 2013). Similar experiments in POMC neurons would be valuable to understanding what transmitters are released with different types of stimulation and the behavioral consequences. Indeed, as shown in Chapter 4 glutamate had a sex- and diet-specific effect on body weight regulation in male mice fed an HFD. It would not be surprising if preventing GABA release from POMC neurons also revealed a specific effect on energy balance regulation.

The functional significance of shifting AA transmitters during postnatal development has yet to be evaluated. It would be of interest to determine if changes in energy state early in postnatal development results in long-term changes in AA transmitter distribution and long-term consequences for energy balance regulation. In light of evidence suggesting that early postnatal nutrition can alter hypothalamic feeding circuits and POMC peptides (Chen et al., 2009; Ramamoorthy et al., 2015), it is possible that modulation of energy state during a time of increased plasticity might alter the distribution of AA transmitters in POMC neurons or induce changes in the organization of POMC neural circuitry. Moreover, POMC neurons appear to have an increased sensitivity to exercise around puberty (Patterson et al., 2008) and sensitivity to postnatal estradiol treatment in undernourished female rats (Carrillo et al., 2015). Future experiments could evaluate the possibility that increased plasticity of AA transmitters before adulthood is facilitative of the development of anorexia and increases the susceptibility to certain feeding disorders.

Summary

Hypothalamic POMC neurons are known for their anorexigenic effects on food intake and energy expenditure. In recent years it has become increasingly clear that these neurons might exert their effects on energy balance through AA transmitters as well as peptides. While the work presented in this dissertation highlight the importance of POMC neurons in the development of ABA and suppression of food intake, future work will need to determine if peptides alone mediate this process. Research presented here also characterizes the AA transmitter phenotype of POMC neurons during postnatal development and provides evidence for the functional role of glutamate release from these neurons. Increased plasticity of POMC neurons at a time in development associated with sensitivity to energy states and predisposition to energy balance dysregulation might augment future approaches to investigating the physiological underpinnings of feeding disorders. Overall, these data expand current understanding of POMC neurons and provide insight into future directions that would enhance this field of study.

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LIST OF ABBREVIATIONS, ACCRONYMS, AND TERMS

AA	Amino acid
AAV	Adeno-associated virus
ABA	Activity-based anorexia: refers to the rodent model used to induce anorexia and to mice that developed this specific type of anorexia
ACTH	Adrenocorticotrophin
Ad lib	*Ad libitum*
AgRP/NPY	Agouti-related peptide and neuropeptide Y
AN	Anorexia nervosa
ARC	Arcuate nucleus
β-endorphin	Beta-endorphin
c-Fos	Immediate early gene used as a marker of cellular activation *in vivo*
CNO	Clozapine-N-oxide
CPE	Carboxypeptidase E
DIO	Double-floxed inverted open reading frame
DREADD	Designer receptors exclusively activated by designer drug
eGFP	Enhanced green fluorescent protein
FAA	Food anticipatory activity
FISH	Fluorescent *in situ* hybridization
FR	Food restriction
FR/SED	Food restricted/sedentary: refers to mice that are food restricted but have locked running wheels
GABA	Gamma-Aminobutyric acid
Gad65/Gad67	Glutamate decarboxylase (synthetic enzyme for GABA; product of the genes *gad2* and *gad1* respectively)
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HFD	High-fat diet
hM3Dq	Stimulatory DREADD receptor

hM4Di	Inhibitory DREADD receptor
i.p.	Intraperitoneal
IR	Immunoreactivity
MCR	Melanocortin receptor
ME	Median eminence
MSH	Melanocyte stimulating hormones
PAM	Peptidyl α-amidating monooxygenase
PC1/3 or PC2	Prohormone convertases
POMC	Refers to propeptide proopiomelanocortin
Pomc	Refers to the murine gene coding for POMC
RMANOVA	Repeated-measures analysis of variance
vGlut2	Vesicular glutamate transporter-2
WRA	Wheel-running activity
WT	Wild-type