DISSERTATION

AMINO ACID TRANSMITTERS AND THE NEURAL CONTROL OF FEEDING AND ENERGY HOMEOSTASIS

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2016

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ABSTRACT

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Consuming the correct number of calories to maintain a healthy bodyweight is a delicate balancing act between intake and energy expenditure, and humans in modern society seem to have a keen knack for throwing the balance off-center. In the U.S. alone, more than 1/3 of adults are obese based on the body mass index scale, and $147 billion is the estimated annual medical cost for obesity in the United States. On the other end of the feeding spectrum, anorexia in the U.S. has been steadily rising since the 1960s, and has the highest mortality rate of any mental illness. While great strides have been made in understanding the neuronal regulation of energy balance, there is a need to more fully understand the homeostatic systems within the hypothalamus that are so powerful that they are able to drive individuals to poor health or death, often even in the face of consciously fighting their urges.

Two groups of functionally opposed neurons contained within the arcuate nucleus of the hypothalamus, Neuropeptide Y / Agouti-related peptide (NPY/AgRP) and proopiomelanocortin (POMC) cells (the so-called first order feeding neurons), have been extensively studied for their roles in energy homeostasis—mostly through research into the peptides they are named after. There is clear evidence that peptides play an essential role for the function of NPY/AgRP and POMC cells, but what about simple amino acid transmitters? While it is known that GABA is packaged and released by NPY/AgRP cells and that this release is relevant to feeding behavior,

there is still a dearth of information about this aspect of the circuitry, very much an area waiting to be mined.

This study focuses on better understanding the functional release and relevance of amino acid transmitters packaged in both NPY/AgRP and POMC cell populations. Evidence is presented here for the conclusive release of both GABA and glutamate from POMC cells within intact circuitry. For NPY/AgRP neurons, evidence is presented for a shift in functional release of GABA from these neurons onto POMC cells depending on feeding state, corroborated by concurrent *in situ* hybridization experiments. Using a combination of electrophysiology and *in situ* hybridization approaches, evidence is also provided that mRNA levels of glutamate decarboxylase can act as a general proxy for functional GABA release.

Altogether, these results indicate that amino acid transmitters play a significant role in first order feeding neuron physiology. Not only does this warrant further study on the significance of each transmitter alone and their purpose in comparison with the peptides released, but also the interplay between POMC cell and NPY/AgRP cell amino acid transmitters and their many shared downstream targets. Imbalances in proper glutamatergic and GABAergic signaling may significantly contribute to obesity, and advancing this area of study could lead to correcting those imbalances to restore healthy energy homeostasis.

ACKNOWLEDGEMENTS

Graduate school has been an amazing experience for me—mostly positive, but not always. Fortunately, I have always had a great accompaniment of individuals to help me achieve my successes in knowledge and publication, as well as give me courage in the face of my struggles. First and foremost, I could not have asked for a better mentor in Shane, whose wealth of knowledge, pragmatism, encouragement, humor, and honesty molded me into the scientist I am today. She maintains an amazing lab environment built for success and personal growth; after all—"every day in the Hentges lab is a holiday." I would also like to thank the rest of my committee, Mike, Greg, and Ron, for the support and constructive input they provided; there were more than a few times when I walked into their labs for some enlightenment. My good friends and labmates also deserve thanks, especially Connie, Reagan, Phil, Jacob, and Steven who helped me throughout my graduate career in a multitude of ways both research-related and not.

I would also like to express my gratitude for my girlfriend Caitlin, as well as my mom, Jane, the two most supportive and loving family members a person could have. They continuously kept me smiling and on track throughout the years of graduate work.

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1. INTRODUCTION

One of the largest current questions within the science of feeding and energy balance is this: with the multitude of targets discovered and much of the neural circuitry known, why hasn't a highly effective drug treatment for obesity been developed? More specifically, it has been decades since Jeffrey Friedman's group discovered the amazing adipogenic satiety peptide hormone they named leptin, so why do we currently have a dearth of pharmaceutical treatments for obesity, and those that do exist are only marginally better than their side-effects (e.g. glucagon-like peptide-1 receptor agonist Saxenda and 5-HT_{2C} receptor agonist Belviq)? The simple, as well as lazy, answer is that there is still much unknown in the field, and the search for the magic bullet continues. The more descriptive answer is that our current understanding of feeding, until recently, was based mostly on a simplistic model of yin and yang (signals of hunger and satiety), which lacked the depth of receptor complexity, asymmetric circuitry, pathway redundancy, and neurotransmitter variety present in a complete model. The field has not yet reached full maturity, but recent advances in technology, especially genetic and imaging tools, have allowed for great strides to be made in the past few years. Pertinent to the primary research in this study, one area largely ignored but now starting to become a topic of interest is amino acid transmitter release from first order feeding neurons. To understand the complex picture of where the knowledge gaps currently lie, where the primary research in this study fits, and where the field should be going next requires background detailing obesity and hypothalamic control of energy homeostasis, including the current prevailing paradigms and the biological players within them. The purpose of this introductory chapter is to provide this information, as well as to provide context for the primary research contained in chapters 2 and 3.

1.1 Obesity

Weighing too much is a problem individuals are battling worldwide. Obesity is now considered a chronic disease or metabolic syndrome and is defined by excess body fat and is accompanied by various comorbidities. The quantifiable measure for estimating the weight health of a person is by utilizing the body mass index (BMI = weight in kilograms divided by the square of height in meters [kg/m^2]). For adults a BMI of 18.5 to 24.9 is considered normal. BMI greater than 25 is considered as overweight, and a BMI larger than 30 and 40 indicate obesity and morbid obesity respectively (WHO, 2000). While BMI may occasionally be a poor measure of individual health (e.g. body builders or professional athletes), it is a good indicator of overweight on a population level. Although obesity may be plateauing in the United States, the obesity epidemic remains a public health crisis, with over 1/3 of adults and 16.9% of children being classified as obese in a 2011-2012 survey (Ogden et al., 2014). Worldwide, studies find that the prevalence and severity of obesity is increasing rapidly in children, adolescents, and adults (Wang and Lobstein, 2006; Chan and Woo, 2010).

It is known that obesity is associated with a number of chronic diseases spread across both adults and children including type 2 diabetes, hypertension, dyslipidemia, disturbed glucose tolerance, and some forms of cancer (Kelly et al., 2008; I'Allemand et al., 2008; Mauras et al., 2010). Perhaps unsurprisingly, this leads to a connection between obesity and a shortened lifespan (Biro and Wien, 2010). While inexpensive and seemingly simple life changes early on could prevent negative health outcomes, living with chronic obesity drives significant health care costs in order to treat the disease, its comorbidities, and prevent mortality (Ebbeling et al., 2002; Yach et al., 2006; John, 2010; Muller-Riemenschneider et al., 2008; Finkelstein et al., 2009). Knowing exactly what causes obesity and finding as many appropriate treatments as possible

would obviously be a boon to humanity worldwide, reducing suffering and excess spending. A frantic race in the scientific community is now underway to unravel the mechanisms involved and build potential therapeutic strategies, spurred on by the urge to understand the fundamental determinants of obesity and obesity-related disease.

So what is known about feeding circuitry, the disruption thereof, and how the body controls the homeostatic set point? Whereas some genetic loci have been clearly identified, and extensively studied as monogenic causes for obesity, it is widely accepted that the metabolic syndrome is in essence a multifaceted disease that encircles a complex network of molecular, cellular and physiologic alterations (Kopelman and Hitman, 1998; Kahn and Flier, 2000). Ultimately, the brain must coordinate the activity of peripheral tissue to insure the fate of some nutrients once ingested. The brainstem works at a basic and essential level to provide satiety behaviors (Grill and Norgren, 1978; DiRocco and Grill, 1979), but without a complete neural network, challenges to homeostasis cannot be countered by adjustments in physiology (Seeley et al., 1994; see Grill and Kaplan, 2001 for review). It is understood that the hypothalamus is where the core of much of this complex modulatory activity occurs, but it is conceptually easier to start with food actually entering the body and taking it from there. The following sections will start at the periphery and head into the central nervous system.

1.2 Endocrine and peripheral input into the central nervous system

Much of the regulation of food consumption in the short term is driven by gastrointestinal peptides that are released directly after food is consumed (see Havel, 2001). These hormones will travel through the blood and directly or indirectly (through the vagus nerve up the general visceral afferent pathway) interact with the nucleus tractus solitarius (NTS), an area at the base

of the brainstem known for forming circuits that contribute to autonomic regulation (see Holzer and Farzi, 2014; Svendsen and Holst, 2015). The NTS can be considered one of the major satiety centers in the CNS, collating and making sense of the peripheral signals coming from the stomach, liver, and both small and large intestines to regulate satiety. Through the interaction with the NTS, as well as direct interaction with neurons in the arcuate nucleus of the hypothalamus (ARC), these hormones, with the exception of ghrelin, induce satiety and cessation of food intake. Examples include cholecystokinin (CCK) from the duodenum and jejunum, glucagon-like peptide-1 (GLP-1) from the ileum and colon, gastrin-releasing peptide (GRP) from the stomach, and peptide YY (PYY) from the ileum and colon. The mechanisms by which CCK promotes satiety are through delaying gastric emptying and activating the vagal afferent nerves that innervate the NTS (Schwartz et al., 1999). GLP-1 functions in many ways, including acting as an insulin secretagogue, slowing gastric emptying and forcing food to stay in the stomach for longer periods of time, and binding to receptors on afferent nerves in the liver and GI tract, thereby relaying satiety signals to the NTS (Moran, 2009). GRP promotes satiety mostly through delaying gastric emptying (Merali ey al., 1999). PYY is released in response to a meal and reduces appetite by slowing gastric emptying (Moran, 2009). In contrast to the satiety-inducing (anorexigenic) actions of many gut hormones associated with the regulation of food intake, ghrelin is an octanoylated orexigenic peptide that is produced by the stomach, and to a lesser extent, the intestine and arguably the hypothalamus (Kojima et al., 1999; Havel, 2001). Plasma concentrations of ghrelin peak immediately before a meal and drop off quickly after a meal has been consumed (Cummings et al., 2001), acting on the G-protein coupled ghrelin receptors within the CNS to exert its metabolic effects (Guan et al., 1997; Mitchell et al., 2001; Zigman et al., 2006). In addition to the gastrointestinal peptides, mechanoreceptors in the

stomach and small intestines play a role in the short-term regulation of food intake. These receptors respond to the presence of food in the stomach and small intestines by transmitting signals via the vagal afferents to the hindbrain to initiate meal termination (Page et al., 2002; Carmagnola et al., 2005). Most of these gut signals are thought to act in the short term, while two other feeding-related hormones, insulin and leptin, are seen as longer-term players.

Whereas the short-term satiety signals often act upon the NTS in the brainstem, other circulating factors primarily interact with the various nuclei of the hypothalamus, including the ARC, paraventricular (PVN), ventromedial (VMH), and lateral (LH) nuclei (see Myers et al., 2008). One such factor is the adipogenic hormone, leptin. The idea of a signal from fat interacting with feeding centers in the hypothalamus goes back over half a century with Kennedy and what became to be known as the lipostatic hypothesis (Kennedy, 1953). It took until 1994, when the leptin gene was first cloned, to find Kennedy's adipogenic signal (Zhang et al., 1994). Leptin is produced by white adipose tissue, and plasma levels of leptin increase when the body has a surplus of energy available, especially when adipose tissue increases (Halaas et al., 1995; Friedman, 2009). One of the key signaling mechanisms driving the neuroendocrine, metabolic, and behavioral adaptations that promote a decrease in energy expenditure and increase food intake is a fasting-induced decrease in leptin (Ahima et al., 1996; see Myers et al., 2008). The importance of leptin in maintaining energy balance over the long term is highlighted by studies showing that leptin deficiencies or defects in the leptin receptors in the brain can cause hyperphagia and severe obesity (Montague et al., 1997; Clement et al., 1998; Strobel et al., 1998). Leptin-responsive neurons express the long-form of leptin receptor (LepRb), which is a single-trans-membrane-domain protein of the cytokine receptor family that operates through the JAK-STAT signaling cascade. This specific receptor variant is required for leptin's central

effects and is highly expressed within several CNS sites, including hypothalamic nuclei (Elmquist et al., 1998, 2005; Scott et al., 2009). One interesting aspect of leptin is its opposing role to ghrelin. Many of the same areas of the brain contain receptors for both hormones, leading naturally to a push/pull hypothesis because of the overlap (see Nogueiras et al., 2008). However, both receptors have quite distinct profiles within the CNS, and deletion of the ghrelin receptor does not affect leptin sensitivity, casting doubt on a simple relationship (Perello et al., 2012).

Insulin is another major player in energy homeostasis, but its role in the CNS is not as understood as in the periphery. Insulin is secreted from the beta cells of the pancreas in response to the ingestion of food, which promotes the absorption of glucose from blood to skeletal muscles and adipose tissue, causes fat to be stored rather than used for energy, and inhibits gluconeogenesis (Sonksen and Sonksen, 2000). Although insulin clearly has satiating effects in the CNS (Woods et al., 1979; Hallschmid et al., 2004; Brown et al., 2006), the physiological significance of these actions remains uncertain. For example, injecting an antisense oligonucleotide into the third ventricle in order to curtail insulin receptor expression in hypothalamic nuclei caused immediate and significant hyperphagia (Obici et al., 2002), but neural insulin receptor knockout strategies have found little to no phenotype in mice (Brüning et al., 2000; Choudhury et al., 2005). With a base of information on the link between peripheral signals and the CNS covered, this is an appropriate place to transition completely into the CNS, specifically regarding the hypothalamus and surrounding feeding circuitry.

1.3 Hypothalamic regulation of energy balance

Implication of the hypothalamus in feeding and energy balance goes as far back as 1840, when hypothalamic obesity was described by Mohr (1840). He noticed that a woman had

become obese within a year before her death, and upon autopsy of this woman, Mohr found a hypophysial tumor compressing and distorting the base of the brain. There was no attempt to connect the increase in adiposity to a hypophysial or hypothalamic injury, but this report and clinicians making other reports like it set the stage for new understanding of this brain region. Over decades, a controversy developed between those that thought pituitary dysfunction (Fröhlich, 1901) and those that thought purely neural dysfunction (Erdheim, 1904) was to blame for hyperphagia and increased adiposity in these case studies, and it was not until 1930 that enough empirical evidence was amassed to end the controversy for good (see Aschner, 1912; Smith, 1930 for best examples). Soon after the Horsley-Clarke stereotaxic instrument was adapted for rats in 1939, relatively more refined electrolytic lesion studies were performed to provide the first undeniable evidence that the hypothalamus is the central hub for feeding (Hetherington and Ranson, 1940, 1942). Lesions to the base of the hypothalamus produced such hyperphagia that rats recovering from the surgery were observed to begin eating voraciously even before the anesthesia had worn off, to the extent of dying from asphyxiation because their swallowing reflex had not yet returned (Brobeck et al., 1943). Later, somewhat more sophisticated, lesion studies would define specific areas of the hypothalamus, especially the ARC, as being responsible for feeding and energy balance (Nemeroff et al., 1978; Penicaud et al., 1983; Sims and Lorden, 1986).

Referring to the peptide hormones discussed in the latter portion of 1.2, ghrelin, leptin, and insulin, what makes the hypothalamus, and specifically the ARC, such an exquisite target for their actions? What makes this area specialized to its job is suggested by the unique anatomy within the region. The ARC is located in the ventral portion of the brain, at the bottom of the third ventricle, directly next to the median eminence (ME), which is one of the circumventricular

organs (CVO). The "leaky" blood-brain barrier at CVOs allows for selective exchange of substrates between in the blood and cerebrospinal fluid, eventually allowing contact to the neural tissue in the ARC. Regulation of food intake and energy homeostasis depends on the ability for hormones from the periphery to effect change directly at neurons charged with that task. These neurons, termed "first order" feeding neurons integrate signals circulating through the blood and encode energy signals into synaptic transmission that will affect downstream secondary neurons. Unsurprisingly, these downstream neurons have earned the moniker of "second-order" feeding neurons. Tanycytes, from the Greek word tanus, which means elongated, are the specialized hypothalamic glial cells that act as gatekeepers of the blood-brain barrier and allow for exchange across it (Gao et al., 2014). Tanycytes extend from the ependymal surface (thus making them a specialized type of ependymal cell) of the third ventricle to a bed of permeable fenestrated capillaries (Langlet et al., 2013). Energy state-signaling hormones, such as ghrelin and leptin, are able to quickly pass through and relay their relevant actions (Schaeffer et al., 2013; Balland et al., 2014). These features ultimately define why first order feeding neurons are so poised to rapidly adapt, making electrophysiological changes is response to hunger or satiety signals, and continue carrying out their functions.

First order feeding neurons: POMC cells and NPY/AgRP cells

The ARC contains (at least) two major populations of neurons that are crucial for monitoring energy signals and subsequently pushing for the suitable behavioral and metabolic responses to alterations of energy state (see Figure 1.1 for relative location in the brain). The first group of neurons is proopiomelanocortin (POMC) neurons, understood to promote satiety and named after the propeptide these cells produce and utilize (Xu et al., 2011; Myers and Olson,

2012). POMC is cleaved by processing enzymes in a tissue-specific manner, resulting in a handful of bioactive peptides. Outside of the ARC, in the corticotrophs located in the anterior pituitary, POMC is translated and cleaved to make adrenocorticotropic hormone (ACTH), which is released into the blood where it regulates stress hormone production and release from adrenal glands (Wardlaw, 2011). Relevant to feeding, in the mouse ARC, POMC is expressed in a fairly evenly spread group of a few thousand neurons (Cowley, 2001; Jarvie, 2012), where it is processed into α- and γ-melanocyte stimulating hormone (α-MSH and γ-MSH) and the opioid β-endorphin (Wardlaw, 2011). α-MSH, γ-MSH, and ACTH are all referred to as melanocortins because they agonize melanocortin receptors. All five melanocortin receptors are G-protein coupled receptors (GPCRs). The MC3R and MC4R are the receptors located in the CNS (Cone, 2005; Kim et al., 2014), while the other three carry out quite unrelated activities, such as skin pigmentation and exocrine gland function. The discovery that injection of melanocortins into the CNS inhibited both spontaneous and drug-induced feeding in rats suggested that POMC neurons might be able to sense and respond to indicators of feeding state (Poggioli et al., 1986). Although most of the peptides POMC cells release have an impact on feeding, effects on the suppression of food intake have been primarily attributed to α-MSH signaling (Brown et al., 1998; Thiele et al., 1998). Injections of α-MSH, as well as the α-MSH synthetic analogue melanotan-II (MTII), into the ventricles caused a highly significant decrease in food consumption (Thiele et al., 1998). MTII injection into the PVN also produces reduction in eating, which is prevented by coadministering an AgRP analogue (Fan et al., 1997).

The second ARC population of first order neurons which are essential for regulation of feeding are the neurons that make neuropeptide Y (NPY) and Agouti-related protein (AgRP) (from here on referred to as NPY/AgRP neurons). NPY was initially discovered in 1982

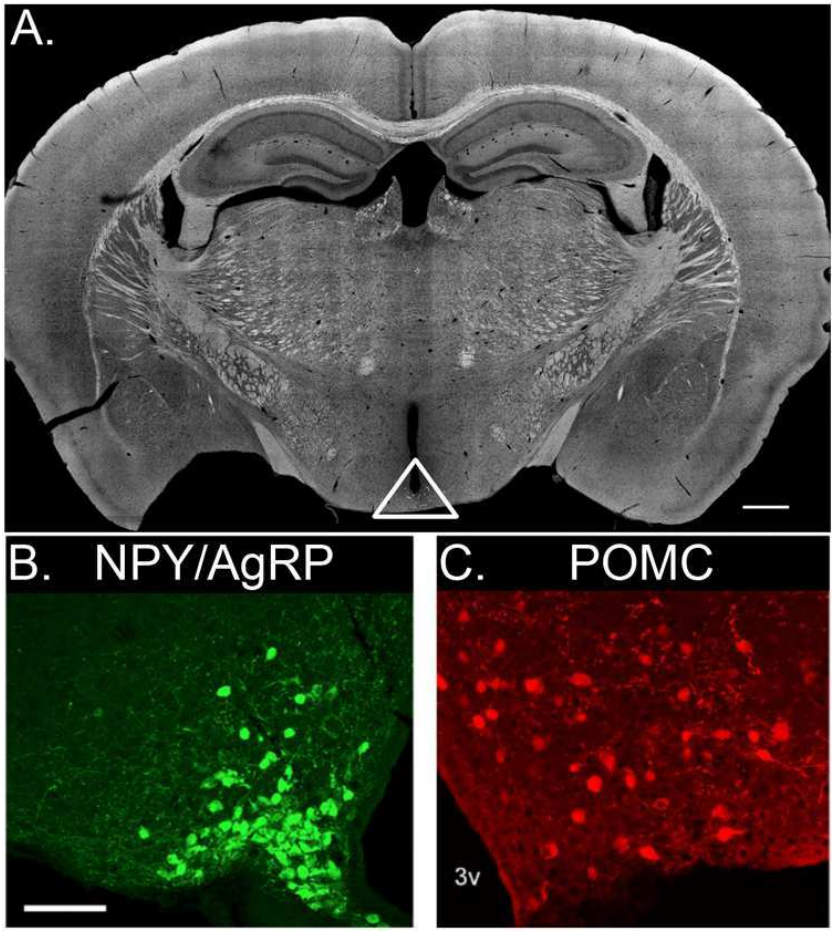

Figure 1.1. Opposing first order feeding neuron populations are located in the arcuate nucleus of the hypothalamus. (A) Coronal section of an adult mouse brain containing the ARC. White triangle encompasses arcuate nuclei and ME. NPY/AgRP cells are densely packed near the ME, visualized using an NPY-hrGFP animal and presented as a confocal Z-stack (B). POMC cells are relatively more spread through the ARC and slightly larger than NPY/AgRP cells, visualized using a POMC-DsRed animal (C). 3v, third ventricle. Scale bar for (A) is 500 μm, while scale bar for (B) is 100 μm.

(Tatemoto et al., 1982), but it wasn't until a couple years later that one of its functions was characterized as an orexigenic peptide (Clark et al., 1984). NPY agonizes a set of G-protein coupled NPY receptors (Linder et al., 2008). Infusion of NPY into the cerebral ventricles also causes an increase in eating (Clark et al., 1984; Levine and Morley, 1984; Stanley et al., 1985). Targeted infusion of NPY into subregions of the hypothalamus revealed the PVN, VMH, and LH as key areas involved in NPY-regulated eating (Stanley et al., 1985). AgRP was discovered as an

inverse agonist for the melanocortin receptors (Ollmann et al., 1997; Shutter et al., 1997), which led to the colocalization of the two peptides (Hahn et al., 1998).

Previous work with the agouti gene (different than the AgRP gene) actually led to the hypothesis of opposing feeding neuron populations harnessing the melanocortin system. The agouti gene was cloned in 1992 (Bultman et al., 1992) and was the first obesity gene to be characterized at the molecular level. Hair follicles express agouti, where it leads to the production of yellow and red pigments and inhibits the production of black pigments in melanocytes. The mechanism through which agouti acts on melanocytes is by antagonizing MC1Rs. The *lethal yellow* (A^y) mutation is one of five known agouti mutations that leads to ectopic agouti expression (Michaud et al., 1994). Heterozygotes (homozygous expression of this spontaneous mutation is lethal), in addition to their yellow hair color, develop obesity within the first few months of life. This is because ectopic expression of agouti in the hypothalamus functionally outcompetes α-MSH at MC3 and MC4Rs.

Like the effect of constitutively expressed agouti, infusion of AgRP into ventricles causes increases in food intake, which is prevented with simultaneous injection of melanocortin agonists (Hagan et al., 2000). NPY/AgRP cells not only release peptide neurotransmitters, they also release the amino acid transmitter γ-aminobutyric acid (GABA), providing NPY/AgRP neurons with another inhibitory mode of action (Horvath et al., 1997). Recent counts put the number of these neurons at about 10,000 in the mouse brain (Betley et al., 2013).

Satiety and POMC vs. hunger and NPY/AgRP: paradigm of opposition

The classical paradigm for opposing first order feeding neurons continues as the standard model with the decades' worth of literature behind it. Collectively, POMC and NPY/AgRP

neurons, their projecting fibers, target neurons expressing MC3R or MC4R, and a second small group of POMC-expressing neurons in the brain stem, define the neural melanocortin system (Mountjoy et al., 1994; Zhan et al., 2013). Through a combination of pharmacology experiments, electrophysiology, and knockout mouse models, a model of energy homeostasis was formed (Figure 1.2). Early hypotheses stated that two battling peptides, α-MSH and AgRP, act on the MC3 and MC4 melanocortin receptors throughout the brain to directly regulate food intake and energy balance. α-MSH-induced activation of melanocortin receptors decreases food intake and increases energy expenditure, and AgRP antagonizes these effects (Fan et al., 1997; Hagan et al., 2000). This idea is consistent with the finding that POMC and NPY/AgRP cells share common second order targets in the CNS that are located in the PVN, LH, VMH, and dorsomedial (DMH) nuclei of the hypothalamus, as well as extrahypothalamic regions like the NTS and parabrachial nucleus (PBN) (Bagnol et al., 1999; Wang et al., 2015). POMC-KO and MC4R-KO mice are hyperphagic and obese, and MC3R-KO mice have increased fat mass (Huszar et al., 1997; Yaswen et al., 1999; Butler et al., 2000). AgRP-KO mice have no metabolic phenotype (Qian et al., 2002), however, acute ablation of NPY/AgRP neurons in adulthood using targeted diphtheria toxin receptor causes starvation (Wu et al., 2008). This evidence of the importance of peptide transmitters was used to build and sustain the basic opposing force model.

Circulating hormones directly and indirectly modulate the two pools of first order feeding neurons. A lot of study has been devoted to leptin, which activates POMC neurons and increases transcription of POMC mRNA levels, at the same time inhibiting NPY/AgRP neurons and decreasing AgRP mRNA levels through direct activation of LepRb, which is expressed on both cell types (Schwartz et al., 1997; Mizuno and Mobbs, 1999; Elias et al., 1999; Lam et al., 2015). Electrophysiologically, there is some evidence, although not yet completely convincing, that

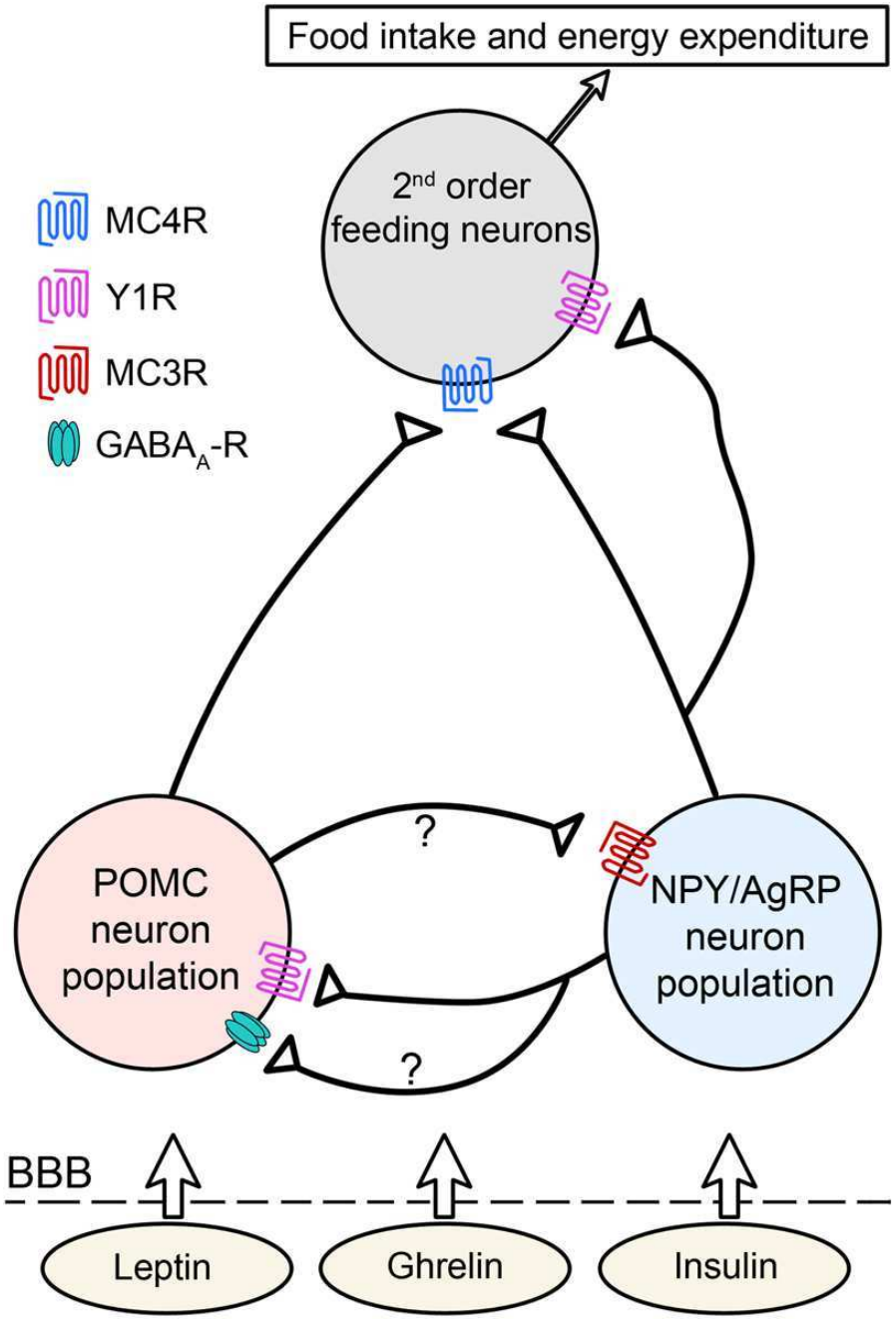

Figure 1.2. Classical paradigm of opposing first order feeding neurons. Circulating energy signals reach the median eminence where they are able to cross the weak blood-brain barrier (BBB). POMC and NPY/AgRP cells are excited or inhibited by these signals, which alters the balance between the populations of neurons and allows for appropriate autonomic and behavioral food intake and energy expenditure actions to occur. POMC neurons activate downstream 2nd order feeding neurons by releasing α-MSH onto melanocortin 4 receptors (MC4Rs). NPY/AgRP neurons fight this activation by releasing the MC4R antagonist AgRP, in addition to releasing NPY onto the inhibitory NPY type 1 receptors (Y1Rs). NPY/AgRP cells directly inhibit POMC cells through activation of Y1Rs and putative GABA release onto $GABA_A$ receptors, while POMC cells directly inhibit NPY/AgRP cells through putative activation of melanocortin 3 receptors (MC3Rs).

leptin signaling depolarizes POMC neurons via activation of TRPC channels (Qiu et al., 2010), whereas NPY/AgRP neurons are hyperpolarized by activation of the Kv2.1 potassium channel (Baver et al., 2014). Because POMC cells are innervated by NPY/AgRP neurons (Atasoy et al., 2012), leptin is also able to depolarize POMC neurons through reduction of inhibitory tone from AgRP NPY, as well as GABA, release (Cowley et al., 2001). Even though leptin inhibits NPY/AgRP neuron activity, it is actually required during early life development in order to properly wire. Mice genetically deficient of leptin (*ob/ob*) or its receptor (*db/db*) display abnormal projections to the PVN (Bouret et al., 2004; 2012) from ARC feeding neurons, pointing to a role as a trophic factor in neonates. Ghrelin also acts directly on first order neurons. It was shown to directly increase the firing rate of NPY/AgRP neurons, as well as indirectly inhibit POMC neurons by increasing inhibitory tone from NPY/AgRP cells onto POMC cells (Cowley et al., 2003). The ghrelin receptor is a $G_{\alpha 11}/G_{q11}$-coupled GPCR that activates phospholipase C (PLC). PLC increases the intracellular Ca^{2+} levels through inositol-3-phosphate (IP_3) and protein kinase C pathways (van der Lely et al., 2004; Ferrini et al., 2009). In addition to these peptides, POMC and AgRP neurons are now known to also respond to estrogen, glucose and fatty acids in an opposing manner (Ibrahim et al., 2003; Chang et al., 2005; Titolo et al., 2006; Jo et al., 2009; de Souza et al., 2011; Koch and Horvath, 2014).

Using the Cre-lox system with POMC- and NPY/AgRP-Cre animals continues to be a popular method to find out how these two populations of neurons function and interact. However, careful attention must be paid not to overstate results when using these strategies, especially in light of recent findings that POMC is transiently expressed during development in cells fated to become NPY/AgRP or Kisspeptin neurons (Padilla et al., 2010; Sanz et al., 2015). Any meaningful amount of Cre being expressed in unintended neurons will cause permanent

recombination of floxed sites. For examples of work that may warrant alternative interpretation, see Balthasar et al., 2004 and Xu et al., 2008. Some of the most convincing evidence for the basic idea of first order feeding neuron opposition comes from the development of optogenetic tools. Through expression of the light-gated nonselective cation channel, channelrhodopsin-2 (ChR2), it is possible to selectively activate molecularly marked neurons using a specific wavelength of light (Boyden et al., 2005; Arenkiel et al., 2007). Optogenetic stimulation of even a minority of NPY/AgRP cell bodies *in vivo* rapidly elicits an insatiable appetite and food consumption in mice, even if they have recently eaten a meal (Aponte et al., 2011). This effect scales with frequency of light stimulation. Conversely, light stimulation of POMC neurons decreases food intake and body weight, although a longer period of stimulation is needed to observe significant effects (Aponte et al., 2011). This difference between neuron types might be attributable to the timescales in which different neurotransmitters act. Interestingly, when A^y mice that are constitutively producing *agouti* protein, which binds melanocortin receptors and prevents changes in the natural melanocortin system to have any effect, are used, NPY/AgRP stimulation still causes feeding behavior and weight gain, while POMC cell stimulation has no effect on feeding (Aponte et al., 2011). This implies that, at least in that paradigm, melanocortin signaling is much more important for POMC cells than for NPY/AgRP cells, which could be relying on the fast actions of their amino acid transmitter, GABA. This is just one caveat to the simple idea of opposing actions of first order feeding neurons specifically at melanocortin receptors. More new findings and ideas are covered next.

Wrinkles in the classical paradigm

While the dueling melanocortin idea of feeding is still established and accepted, the field is rapidly growing beyond a simple model (for excellent contemporary reviews, see Mountjoy, 2015; Sternson and Atasoy, 2014). Advances in imaging and recording approaches have allowed for the development of *in vivo* calcium imaging. In line with *ex vivo* brain slice recordings, NPY/AgRP neurons are active during the fasted state and also respond with elevated calcium events to injections of ghrelin. The surprising and more novel finding, however, is that POMC and AgRP neurons respond rapidly to sensory food cues even before ingestion, and the response varies with nutritional state and depends on the palatability of the food presented (Betley et al., 2015; Chen et al., 2015); the fast response is caused by detection, not consumption. Thus, in addition to long-term integration of nutritional signals and maintenance of energy balance, the melanocortin system is poised to be dynamically regulated by sensory stimuli and may be directly involved in food-seeking behavior.

One conceptually simple finding to keep in mind is that POMC and AgRP neurons often, but not always, project to the same nuclei. This suggests that some areas of the brain utilize independent branches of the first order feeding neuron populations, implicating a possible role for each peptide independent of the other (Wang et al., 2015). It could be possible that there is constitutive activity of the melanocortin receptors in some brain regions, and there is some evidence to suggest that AgRP could act alone on a receptor as an inverse agonist rather than just a competitively binding antagonist (Tao, 2014). One hint at an alternate hypothesis lies with recent investigation into the signaling cascade downstream from the MC4R. Although α-MSH and opposing AgRP action on MC4Rs is transduced though a G_s-coupled pathway (Nickolls et al., 2005; Buch et al., 2009), recent findings are suggesting the somewhat controversial idea that

AgRP acts in a second, G_s-independent manner on the MC4R. Specifically, that there is coupling of the MC4R to the inward-rectifying potassium channel Kir 7.1 (Ghamari-Langroudi et al., 2015).

As previously discussed, AgRP-KO mice have no metabolic phenotype, while ablation of NPY/AgRP neurons in an adult results in starvation (Wu et al., 2008). This suggests developmental compensation, but in what manner? Further study revealed that the starvation was melanocortin-independent, and that proper hunger signaling and was mediated at least in part by the amino acid transmitter GABA (Wu et al., 2008; 2009; 2012). In addition, two methods of targeted neural activation (cell-type-specific light- or chemical-mediated activation) show that excitation of AGRP neurons leads to immediate and voracious feeding, also independent of melanocortin signaling (Krashes et al., 2011; 2013). Through additional experiments, it was determined that NPY and GABA released by NPY/AgRP neurons both play a significant role in the short-term feeding behavior, whereas AgRP might be more responsible for feeding on a longer time scale (Krashes et al., 2013).

POMC neurotransmitter release is not restricted to peptides; before beginning the primary research in this study, our lab had previously discovered that POMC cells release both GABA and glutamate in autaptic cultures (Hentges et al., 2009), although it was unknown if that phenotype held in intact circuitry or what physiological relevance amino acid transmitter release might hold. Further discussion will be found in later chapters, but to summarize, very little is known about the relevance of POMC amino acid transmitters. There is a small amount of evidence that there are two classes of POMC cell neurotransmitter release sites—one that releases a combination of neurotransmitters and peptides at conventional synapses, and the other being extrasynaptic sites that only release peptides (Atasoy et al., 2014). Clearly, the recent

findings described point toward a more complex feeding and energy homeostasis model that must account for separate mechanisms on separate timescales, redundancy, developmental adaptation, and importance of amino acid transmitters.

Beyond first order neurons

Where do POMC and NPY/AgRP neurons exert their largest effects? One way to answer this is to know in what brain regions melanocortin receptors are most necessary for proper feeding and energy balance. A group using the *Mc4r*$^{loxTB/loxTB}$ mouse line, a global MC4R knockout that will re-express MC4R if Cre is introduced, along with a *Sim1-Cre* line, MC4Rs were only expressed in the amygdala, nucleus of the lateral olfactory tract, DMH, and LH (Balthasar et al., 2005). This selective expression drastically diminished the increased body weight phenotype observed in the *Mc4r*$^{loxTB/loxTB}$ mouse model, but it was not a complete rescue, suggesting at least some relevant melanocortin activity outside of these brain regions. Further targeting of the PVN with a selective knockout *Mc4r*$^{lox/lox}$ mouse line, in conjunction with the use of the previous selective rescue line and some elegant use of Cre-expressing viral injections, showed that melanocortin activity specifically in the PVN is both necessary and sufficient for the majority of a proper weight phenotype (Shah et al., 2014). This study did a great job in essentially using a brute-force strategy to determine the relevance of other types of neurons to the melanocortin system. Loss or rescue of MC4Rs in glutamatergic neurons using the vGLUT2 glutamate transporter accounted for a complete loss or rescue of a weight phenotype, whereas re-expression in oxytocin, corticotropin-releasing hormone, arginine vasopressin, or prodynorphin neurons (all expressed within the PVN) had no measured effect. Interestingly, although the PBN is part of a known feeding pathway (Carter et al., 2013), regaining any expression of MC4Rs in

the PBN results in no change in phenotype, suggesting that a melanocortin-dependent role of function is lacking in the region, that GABA release is the relevant first order transmitter released in the pathway, and/or that the pathway is not relevant to feeding outside extreme physiological states e.g. being poisoned or very sick.

The PBN is actually a heavily researched area at this time, as a second group of neurons more lateral to those studied in Carter et al., 2013 is generating interest for being a major efferent target for the effects of MC4R-expressing PVN neurons. ChR2-mediated stimulation of terminals of MC4R-expressing PVN neurons located in the lateral PBN caused the same reduction in feeding behavior as activation at the soma level (Garfield et al., 2015). Although the pathway flowing through the glutamatergic PVN neurons is currently being heavily mined for information, there are other groups of relevant second order neurons that are innervated by both POMC and NPY/AgRP cells. However, results between groups have been mixed. Terminal field photostimulation of ChR2-expressing NPY-AgRP neurons in the anterior bed nucleus of the stria terminalis (aBNST) and LH promotes acute food consumption to comparable levels as that observed following NPY/AgRP soma activation (Atasoy et al., 2012; Betley et al., 2013; Sternson lab), but patch clamp recordings of MC4R-expressing cells in those regions while doing the same photostimulation of NPY/AgRP cells found no evidence for monosynaptic inhibitory connections (Garfield et al., 2015; Lowell lab). These pathways need further clarification. It may be that there is a complex system of interneurons between NPY/AgRP cells and their LH targets, or that the orexigenic nucleus in the LH is mostly under the control of POMC cells, but further work is needed to address these and other possibilities.

1.4 General hypotheses and aims of the study

With obesity being such a significant problem in Western society, there is an obvious need to more fully understand the homeostatic systems within the hypothalamus that drive feeding and energy balance. In reviewing some of the most important feeding literature currently published, context has been given for the primary research in this study. To summarize the ideas and concepts most relevant to the following chapters, NPY/AgRP and POMC neurons located in the ARC have been studied extensively for their opposing regulation of energy homeostasis. NPY/AgRP neurons express increased markers of activation, such as FOS during a fasted or hungry state (Wu et al., 2014). Conversely, POMC neurons show greater markers of FOS during a sated rather than a fasted state (Wu et al., 2014). The responsiveness of these cell types to changes in feeding state clearly points to their role as important players in feeding and energy balance circuits. Because of their exquisite placement next to a CVO and their ability to detect energy state signals in the bloodstream, it is no surprise that these two groups of cells are thought to represent the primary initial node in the feeding model of the mammalian brain.

So far, the majority of study has examined the peptide transmitters that these first order feeding neurons release and are named for, which are clearly important for normal control of feeding and weight. However, only a small portion of obesity can be explained by perturbations or mutations in the first order feeding neuron peptide systems (van Vliet-Ostaptchouk et al., 2009). While these peptides are clearly important for proper maintenance of body weight, classical amino acid transmitters may also play a significant role in POMC cell physiology (van den Pol, 2003; Meister, 2007), and definitely play an important role in NPY/AgRP cells' ability to modulate feeding behavior (Aponte et al., 2011; Wu et al., 2012; Krashes et al., 2013; Carter et al., 2013). GABA has been long known to affect feeding (Kelly et al., 1977; Tsujii and Bray,

1991), and the GABA receptor continues to be a potential target for eating disorder and obesity treatment (Guardia et al., 2011).

The first aim of this study is to test the hypothesis that both glutamate and GABA are released from POMC cell terminals, and that this release is relevant to the feeding circuit. Chapter 2 addresses release, and while there is still little evidence for relevance, new findings from our lab are discussed in Chapter 4.

The experiments in Chapter 3 test the hypothesis that GABA release from NPY/AgRP cells is regulated based on feeding state, focusing specifically on the non-reciprocated direct synaptic connection of NPY/AgRP cells to POMC cells. Additionally, through *in situ* hybridization experiments, glutamate decarboxylase mRNA (*Gad*) level as a proxy for functional GABA release is discussed as a broadly applicable tool. The importance of inhibitory tone from NPY/AgRP neurons onto POMC neurons is discussed in Chapter 4. Both aims rely heavily on a combination of electrophysiology and optogenetic techniques to obtain pseudo-paired recordings.

2. REGULATION OF GABA AND GLUTAMATE RELEASE FROM PROOPIOMELANOCORTIN NEURON TERMINALS IN INTACT HYPOTHALAMIC NETWORKS[1]

2.1 Summary

Hypothalamic proopiomelanocortin (POMC) neurons and their peptide products mediate important aspects of energy balance, analgesia, and reward. In addition to peptide products, there is evidence that POMC neurons can also express the amino acid transmitters GABA and glutamate, suggesting these neurons may acutely inhibit or activate downstream neurons. However, the release of amino acid transmitters from POMC neurons has not been thoroughly investigated in an intact system. In the present study, the light-activated cation channel channelrhodopsin-2 (ChR2) was used to selectively evoke transmitter release from POMC neurons. Whole-cell electrophysiologic recordings were made in brain slices taken from POMC-Cre transgenic mice that had been injected with a viral vector containing a floxed ChR2 sequence. Brief pulses of blue light depolarized POMC-ChR2 neurons and induced the release of GABA and glutamate onto unidentified neurons within the arcuate nucleus, as well as onto other POMC neurons. To determine if the release of GABA and glutamate from POMC terminals can be readily modulated, opioid and $GABA_B$ receptor agonists were applied. Agonists for mu and kappa, but not delta, opioid receptors inhibited transmitter release from POMC neurons, as did the $GABA_B$ receptor agonist baclofen. This regulation indicates that opioids and GABA released from POMC neurons may act at presynaptic receptors on POMC terminals in an autoregulatory

[1] Full article from Dicken MS, Tooker RE, and Hentges ST. (2012) The Journal of Neuroscience, 32:4042-4048. Shane Hentges contributed to experimental design and writing of the manuscript. Ryan Tooker performed initial ChR2 experiments and edited the manuscript. Matthew Dicken performed the majority of the experiments, analyzed collected data, assisted in experimental design, and contributed in writing the manuscript.

manner to limit continued transmission. The results show that in addition to the relatively slow and long-lasting actions of peptides, POMC neurons can rapidly affect the activity of downstream neurons via GABA and glutamate release.

2.2 Introduction

Hypothalamic proopiomelanocortin (POMC) neurons are thought to exert their effects on energy balance, motivation, pain and reward mainly through release of the mature POMC cleavage products α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (Coll, 2007; Bertolini et al. 2009; Hegadoren et al., 2009). While these peptides are clearly important for proper function, classical amino acid (AA) transmitters may also play a significant role in POMC cell physiology (van den Pol, 2003; Meister, 2007). Previous reports have shown both GABA and glutamate release from POMC cells that have formed recurrent synapses in primary culture (Hentges et al., 2004, 2009). In addition, electron micrograph data has shown that POMC terminals make both symmetric and asymmetric synapses, suggesting both GABAergic and glutamatergic POMC terminals, respectively (Wang et al., 2001; Reyes et al., 2006). However, glutamate release from POMC neurons in an intact circuit has not been demonstrated and some studies have failed to detect GABAergic markers in POMC neurons using immunohistochemical or transgenic approaches (Ovesjö et al., 2001; Vong et al., 2011). A complete understanding of how POMC neurons affect the larger circuitry and behavior requires knowing the amino acid phenotype of these neurons. Thus, the present study was designed to determine whether POMC neurons release GABA and/or glutamate to affect neuronal activity in intact hypothalamic networks.

To examine transmitter release from POMC neuron terminals in an intact system, a combined optogenetics-electrophysiology approach was used. This approach is similar to that proposed for circuit mapping and functional studies (Petreanu et al., 2007; Atasoy et al., 2008; Kohl et al., 2011). Cell type-specific expression of the light-gated ion channel channelrhodopsin-2 (ChR2) in POMC cells was used to excite POMC neurons as a population with light while recording from downstream neurons in slice preparations. With this method, amino acid (AA) transmitter release could be observed through native connections. The results show that both GABA and glutamate are released from POMC neurons onto downstream cells within the arcuate nucleus (ARC), indicating not only the phenotype of AA transmitter release, but also that POMC cells can terminate within the ARC. In addition, evoked transmitter currents could be dynamically modulated through G-coupled protein receptor (GPCR) activation, suggesting physiological regulation of GABA and glutamate release from POMC neurons may be another regulatory component of the POMC neuron system.

2.3 Materials and Methods

Animals

Male and female transgenic mice expressing Cre recombinase specifically in POMC cells (POMC-Cre) were used for all experiments. Transgenic animals were produced by standard techniques (see Xu et al., 2005) and backcrossed >12 generations onto the C57Bl/6 strain. Animals were 10-12-weeks-old when electrophysiological experiments were conducted. Mice were housed under controlled temperature (22-24°C) and a constant 12 hr light/dark cycle. Mice were fed standard rodent chow and tap water *ad libitum*. All experiments met United States

Public Health Service guidelines with the approval of the Colorado State University Institutional Animal Care and Use Committee.

Viral gene transfer in vivo

POMC-Cre transgenic mice (eight-weeks-old) were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments). A viral vector containing a floxed sequence for ChR2 with an mCherry tag (AAV2/9.EF1.dflox.hChR2(H134R)–mCherry.WPRE.hGH; obtained from the Penn Vector Core at the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania) was unilaterally injected into the ARC of POMC-Cre mice. For each animal, a 300 nl injection (3.52E+12 GC/ml) was made using a Hamilton syringe and the following coordinates from bregma: X, -0.27 mm; Y, -1.35 mm; Z, -6.14 mm. After microinjection, the wound was sutured and animals were left to recover for a minimum of 14, but no longer than 28 days before experiments to ensure a high level of ChR2 expression.

Immunofluorescence

18 days post-AAV injection, mice were anesthetized, perfused transcardially with 4% paraformaldehyde, and brains were removed and post-fixed as described previously (Hentges et al., 2009). Sagittal brain slices (50 μm) containing the ARC were prepared on a vibratome and were subsequently incubated in phosphate-buffered saline (PBS) with 3% normal goat serum and 0.3% Triton-X-100. The POMC peptide adrenocorticotropin (ACTH) was detected using a rabbit anti-ratACTH primary antibody (National Hormone and Peptide Program; 1:10,000, overnight at 4°C). Tissue was then washed in PBS and incubated with an Alexa Fluor 647-conjugated donkey anti-rabbit secondary antibody (Invitrogen; 1:400). ACTH-immunoreactivity was visualized

using a Zeiss-510-Meta laser-scanning confocal microscope based on the far-red fluor (633 nm excitation and 650 nm emission wavelengths) and ChR2-mCherry was visualized using 580 nm excitation and 600 nm emission filters. Colocalization of ACTH immunoreactivity and ChR2-mCherry expression was determined by counting the labeled cells in sections from 2 brains. Two investigators made independent counts of labeled cells in each brain to verify the results.

Electrophysiology

Sagittal brain slices containing the ARC were prepared as previously described (Pennock and Hentges, 2011). Whole-cell voltage clamp recordings were made at a temperature of 37°C with an internal recording solution containing the following (in mM): 57.5 KCl, 57.5 K-methyl sulfate, 20 NaCl, 1.5 MgCl2, 5 Hepes, 0.1 EGTA, 2 ATP, 0.5 GTP, 10 phosphocreatine, pH 7.3. Recording pipettes had a tip resistance of 1.5-2.2 MΩ when filled with internal solution. Slices were maintained in artificial cerebrospinal fluid (aCSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, and 11.1 glucose (saturated with 95% O_2 and 5% CO_2). Whole-cell recordings were conducted through the use of an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and data was collected using AxographX software (Axograph, Sydney, Australia) running on a Mac OS X operating system. POMC neurons expressing ChR2 were identified by the mCherry fluorophore expressed as a fusion protein on the channel. Cells were held at -60 mV for recording light-evoked postsynaptic currents (PSCs). When recording from unidentified cells within the ARC, the patched cells were within ~100 μm of POMC-ChR2 expressing cells. Cells were excluded if the access resistance increased significantly from the initial access (6.04 ± 1.17 MΩ for unidentified cells, 4.6 ± 0.75 MΩ for ChR2-expressing POMC neurons, and 4.9 ± 0.71 MΩ for POMC neurons without

ChR2). Postsynaptic currents were evoked using either a 25 or 100 ms blue light pulse delivered every 20 s to the slice from a mercury light source with a rapid shutter system (Lumen Pro 200, Prior). The light was adjusted to the minimum intensity required to evoke a consistent PSC, which was generally between 5-11 mW/mm^2. PSCs were evoked for at least 5 min under baseline conditions to ensure a steady recording. PSCs were collected at 10kHz and digitally filtered at 1kHz. Data were analyzed by comparing an average of 3 min of baseline to an average of 2 min of steady-state response to drug. Excitatory and inhibitory PSCs were identified pharmacologically by blocking $GABA_A$-mediated currents with bicuculline methiodide (BMI, 10 μM; Tocris) or AMPA- and NMDA-mediated currents with 6,7-dinitroquinoxaline-2,3(1H,4H) (DNQX, 10μM; Sigma) and (+)-MK-801 (15 μM;Sigma). Representative recordings were plotted by minute, averaging the peak amplitude of 3 sweeps spaced 20 seconds apart.

Drugs

Stock solutions of DNQX, (+)-MK-801, and (+)-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69593; Biomol International) were prepared with DMSO at least 10,000x more concentrated than the final solution. Stock solutions of [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO; Sigma), D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP; Tocris Bioscience), nor-binaltorphimine (nor-BNI; Sigma), [D-$Pen^{2,5}$]enkephalin (DPDPE; Bachem), bicuculline (Tocris Bioscience), [Met_5]enkephalin (Sigma), baclofen (Sigma), dynorphin A (Tocris Bioscience), naloxone (Sigma), (2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP 55845; Tocris Bioscience), and BMI were prepared with distilled water. All drugs were diluted to

working concentrations in aCSF and applied via bath perfusion except (+)-MK-801, which was applied to the collected slices prior to being moved to the recording chamber.

Data Analysis

All data are presented as mean ± SEM. Comparisons between groups were compared using *t*-tests. $P < 0.05$ was considered a significant difference.

2.4 Results

Functional expression of ChR2 in POMC cells

The selective expression of ChR2 in POMC neurons was verified in slices prepared from POMC-Cre mice that had been injected with the viral vector containing the floxed ChR2-mCherry ≥2 weeks earlier. Approximately 88% of cells expressing ChR2-mCherry also displayed ACTH-immunoreactivity (ACTH-IR). Cells expressing mCherry without detectable ACTH-IR most likely represent POMC neurons with low ACTH expression. Injection of the viral vector into the ARC of wild-type C57Bl/6 mice yielded no detectable mCherry fluorescence (data not shown), indicating that expression is limited to cells that express Cre recombinase. Approximately 79% of ACTH-IR cells in the injected side of the ARC expressed mCherry (Figure 2.1A). ChR2-mCherry was notable throughout the membranes of cell body and neuronal projections, indicating strong expression and widespread trafficking of the channel. These findings indicate specific targeting of strong ChR2 expression to POMC neurons.

Individual cells expressing high amounts of ChR2-mCherry were easily detected in live brain slices prepared from injected mice (Figure 2.1B). POMC cells with ChR2 had similar basal whole-cell properties as POMC neurons that do not express ChR2. The input resistance was

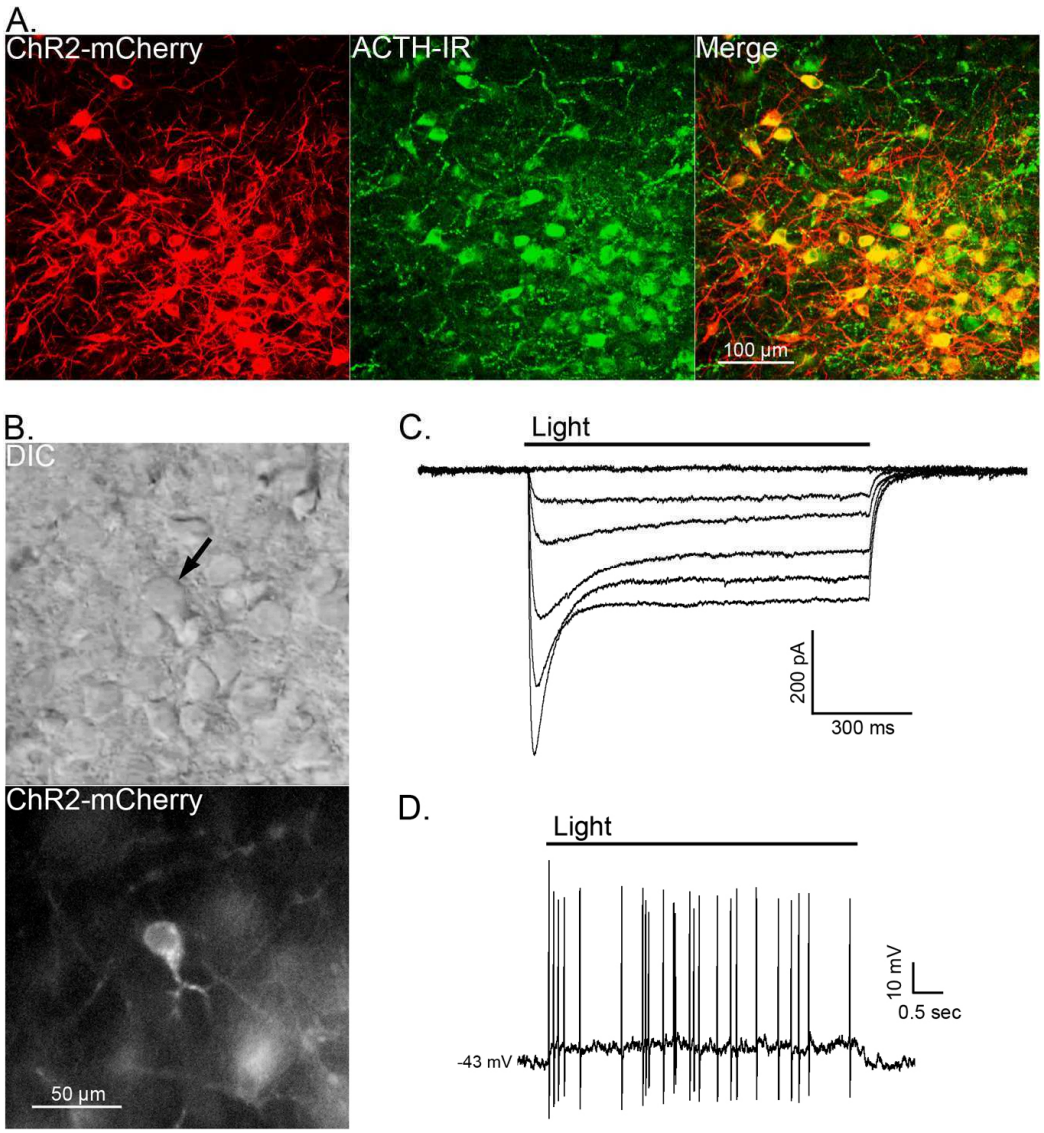

Figure 2.1. POMC cell-specific expression of functional ChR2. (A) Confocal z-stack image of a brain slice containing cells expressing ChR2-mCherry 18 days after AAV injection (left panel) and cells immunoreactive for ACTH (center panel). A high degree of colocalization can been seen in the merged image (right panel). (B) Images of the hypothalamus in a live brain slice from an AAV injected mouse shown in DIC (top panel) and fluorescence (bottom panel). Arrow indicates a neuron with a high level of ChR2 expression within the focal plane. (C) Whole cell currents from a ChR2-expressing neuron elicited by pulses of blue light at increasing intensities (0 at top trace; 28 mW/mm^2 elicited the largest current, bottom-most trace). (D) Light-induced depolarization of a ChR2-expressing neuron caused action potential firing in current clamp recordings.

1.3 ± 0.1 GΩ in ChR2 positive cells versus 1.6 ± 0.2 GΩ in ChR2 negative POMC neurons (n = 9, p = 0.31). Whole-cell capacitance was also not different between ChR2 expressing and non-expressing POMC neurons (29 ± 2.6 pF and 25.5 ± 1.4 pF, respectively; n = 9, p = 0.24). Whole-cell voltage clamp recordings made in POMC-ChR2 neurons indicate that ChR2 mediates an inward current in response to blue light (~ 470 nm, Figure 2.1C). Peak and steady-state current responses were stereotypical of currents mediated by ChR2(H134R) (Lin et al., 2009). In current clamp, blue light depolarized POMC-ChR2 neurons and caused action potential firing (Figure 2.1D).

GABA and glutamate release from POMC neurons

Activation of POMC neurons via ChR2 was used to determine if POMC cells release the AA transmitters glutamate and/or GABA in an intact neuronal network. Whole-cell voltage clamp recordings were made in unidentified cells (not expressing detectable mCherry) in the ARC near POMC neurons. The unidentified cells had basal properties similar to POMC neurons (input resistance, 1.1 ± 0.15 GΩ, capacitance, 23.3 ± 1.54 pF; n = 15, $p > 0.05$ for both properties compared to POMC neurons with or without ChR2). The likelihood that a pulse of light evoked neurotransmitter release from POMC-ChR2 cells varied from 50% to 8.33% (e.g. 1 out of 12 cells patched) depending on the accuracy of the injection and thus, the number of ChR2-expressing neurons. When light pulses evoked transmitter release from POMC terminals, the currents in the postsynaptic cell were mediated by both $GABA_A$ and AMPA receptors, indicating GABA and glutamate release from POMC neurons. Light-evoked inhibitory postsynaptic currents (IPSCs) were recorded in the presence of the AMPA receptor blocker DNQX (10 μM, Figure 2.2Ai) and were completely inhibited by the $GABA_A$ receptor antagonist BMI (10 μM,

Figure 2.2Aii). Upon washout of BMI, the current returned (Figure 2.2Aiii). Reversing the order of antagonist application showed the glutamatergic nature of some light-evoked currents. Currents evoked in the presence of BMI (Figure 2.2Bi) were blocked by the addition of DNQX to the bath (Figure 2.2Bii) and returned upon washout of DNQX (Figure 2.2Biii). In the presence of BMI, the onset of evoked currents was often substantially delayed from the initial light stimulus (as seen in Figure 2.2B), which may indicate a polysynaptic response. However, direct innervation was indicated in some instances, including where a brief light pulse evoked PSCs that were abolished only when both $GABA_A$ and AMPA receptors were blocked (Figure 2.2Ci-iv). Altogether, when a light-evoked PSC was detected in a non-POMC cell, it was most often GABAergic in nature (27/39 cells showed GABA-mediated PSCs, Figure 2.2D). Glutamate-mediated PSCs accounted for 23% of the PSCs observed (9/39 PSCs), and 3 cells appeared to receive input from both GABAergic and glutamatergic POMC cells (Figure 2.2D), although the possibility that individual terminals may release both GABA and glutamate cannot be ruled out based on the present experiments.

The majority of light-evoked PSC recordings were made in non-POMC neurons, since the direct inward current carried through ChR2 can obscure the current induced by GABA or glutamate release. However, some POMC cells expressing ChR2 displayed both a direct ChR2-mediated inward current and a slightly delayed light-evoked GABA-mediated IPSC ($n = 4$, Figure 2.2E). This result indicates that POMC neurons can innervate one another.

All light-evoked currents in cells downstream of POMC neurons could be completely blocked by BMI and/or DNQX indicating that GABA and glutamate were the only transmitters being released in response to the acute depolarization of POMC neurons. The light-evoked release of transmitter from POMC neurons appears to be dependent on action potential

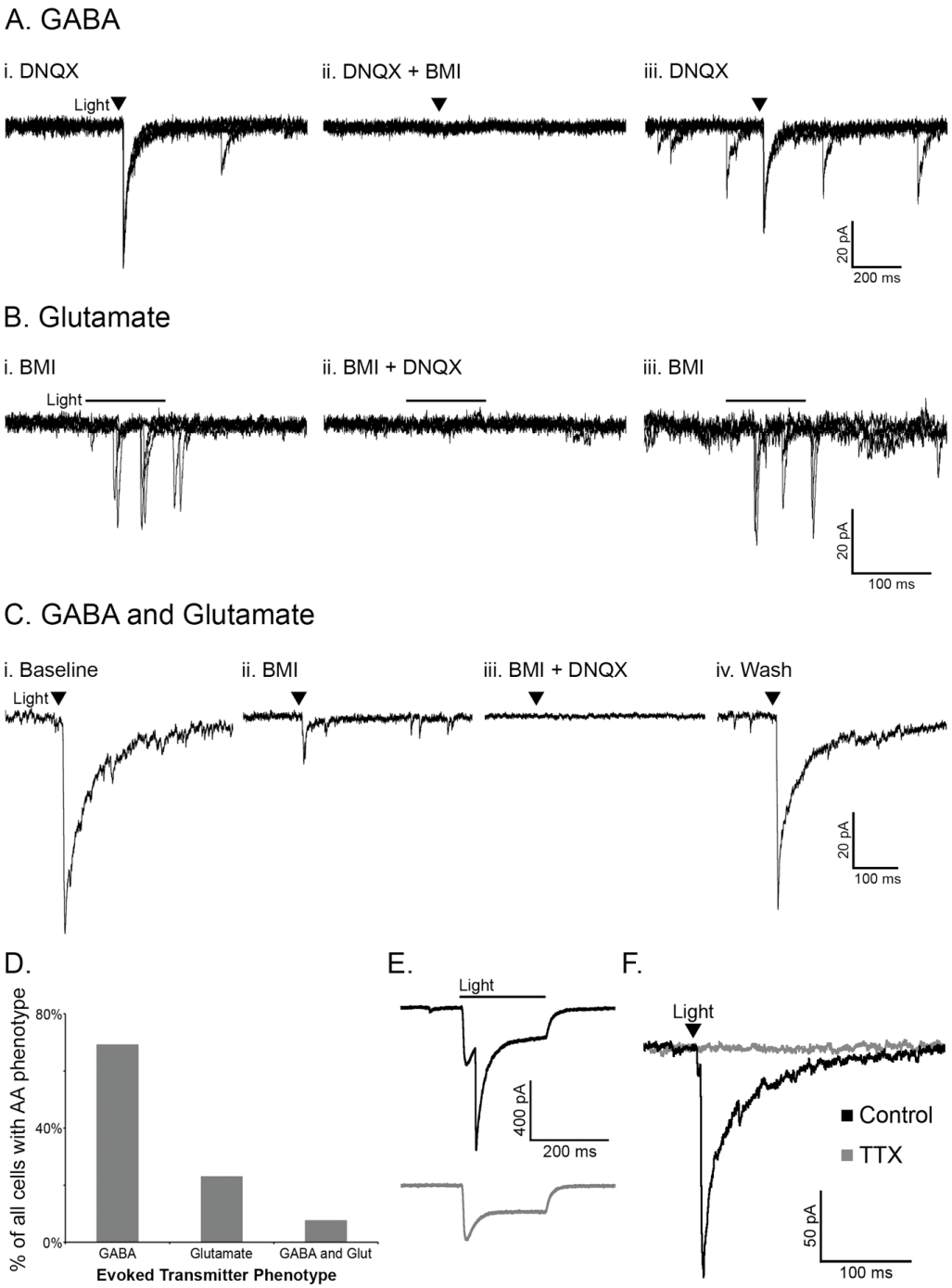

Figure 2.2. POMC neurons release GABA and glutamate onto cells within the arcuate nucleus. Light-evoked IPSCs observed in the presence of DNQX (10 μM, Ai) were abolished with the addition of BMI (10 μM, Aii). Light evoked EPSCs observed in the presence of BMI (Bi) were abolished with the addition of DNQX (Bii). (C) An example of a cell with light-evoked IPSCs and EPSCs. Upon washout of DNQX and/or BMI evoked currents returned (Aiii, Biii, Civ). Panels in A and B consist of three overlayed sweeps, whereas panels in C are each an average of three sweeps. The transmitter(s) mediating evoked currents was determined in all cases where a PSC was observed and is presented in (D). (E) A light-evoked IPSC in a POMC cell which also displays a direct ChR2 current (top trace). The evoked IPSC is ablated by BMI (bottom trace). (F) Currents evoked onto an unidentified cell are completely inhibited by treatment with TTX (1 μM).

propagation and not direct stimulation of terminal release based on the observation that the addition of tetrodotoxin (TTX, 1 μM) completely abolished light-evoked PSCs (98.68 ± 0.94% reduction, $n = 4$, $p < 0.001$; Figure 2.2F).

Opioids inhibit neurotransmitter release from POMC terminals via mu and kappa receptors

The ability of opioid receptor agonists to modulate the release of GABA and glutamate from POMC terminals was evaluated, since opioids reliably inhibit AA transmitter release from unidentified terminals within the ARC (Emmerson and Miller, 1999; Pennock and Hentges, 2011). The mu (MOR) and delta opioid receptor (DOR) agonist ME (10 μM) caused a dramatic decrease in light-evoked IPSCs (72.46 ± 4.00% reduction, $n = 9$, $p < 0.001$; Figure 2.3A-B) and EPSCs (72.45 ± 4.64% reduction, $n = 4$, $p < 0.001$; Figure 2.3C-D). This reduction in current was readily reversed by washing out of drug (103.73 ± 10.03% of baseline, $n = 5$, $p = 0.73$; Figure 2.3C) or by co-applying the MOR-selective antagonist, CTAP (500 nM, 97.22 ± 9.42% of baseline, $n = 8$, $p = 0.74$; Figure 2.3A).

The DOR selective agonist DPDPE (100 nM) had no effect on PSC amplitude in any cell tested (100.12 ± 3.41% of baseline, $n = 4$, $p = 0.49$), whereas the MOR selective agonist DAMGO (10 μM) reliably inhibited IPSC amplitude (72.70 ± 7.80% reduction, $n = 4$, $p = 0.001$; Figure 2.4A,B). The inhibition was reversed by CTAP (500 nM, 88.65 ± 5.95% of baseline, $n = 4$, $p = 0.15$; Figure 2.4A). Thus, mu but not delta receptor agonists can inhibit AA transmitter release from POMC neurons. Kappa opioid receptor (KOR) agonists inhibit IPSCs regulating POMC neurons (Pennock and Hentges, 2011). Although POMC neurons are not directly hyperpolarized by KOR activation (Pennock and Hentges, 2011), the possibility that POMC neurons express KORs presynaptically to inhibit transmitter release was examined. The KOR

selective agonist U69593 (500 nM) inhibited light-evoked IPSCs in unidentified downstream neurons (63.39 ± 8.33% reduction, $n = 6$, $p < 0.001$) and the KOR antagonist Nor-BNI (100 nM) reversed the inhibition (95.68 ± 8.57% of baseline, $n = 6$, $p = 0.64$; Figure 2.4C-D). Thus, KORs expressed in POMC neurons inhibit AA release from POMC neurons even though they do not mediate a detectable outward postsynaptic current in POMC neurons.

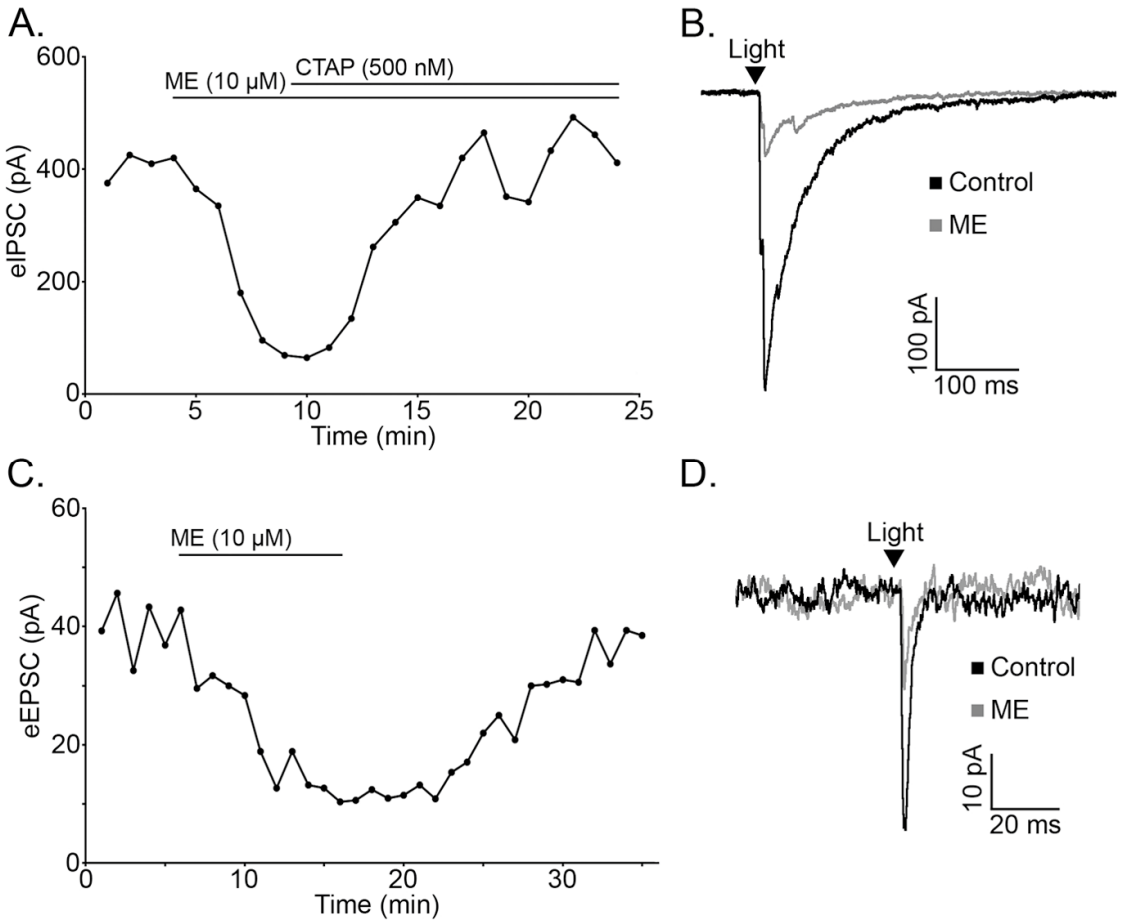

Figure 2.3. Light-evoked IPSCs and EPSCs are inhibited by opioid receptor activation. (A) Plot of light-evoked IPSC amplitudes over time shows an inhibition of light-evoked IPSCs by the nonspecific opioid agonist ME (10 μM). The IPSC inhibition is reversed by application of the MOR-selective antagonist, CTAP (500 nM). (B) Representative traces of IPSCs in control conditions and in the presence of ME. (C) ME inhibits light-evoked EPSC amplitude and EPSC amplitude returns to baseline upon washout of ME. (D) Representative traces of EPSCs in control conditions and in the presence of ME. The arrowheads in B and D indicate the timing of the light pulse.

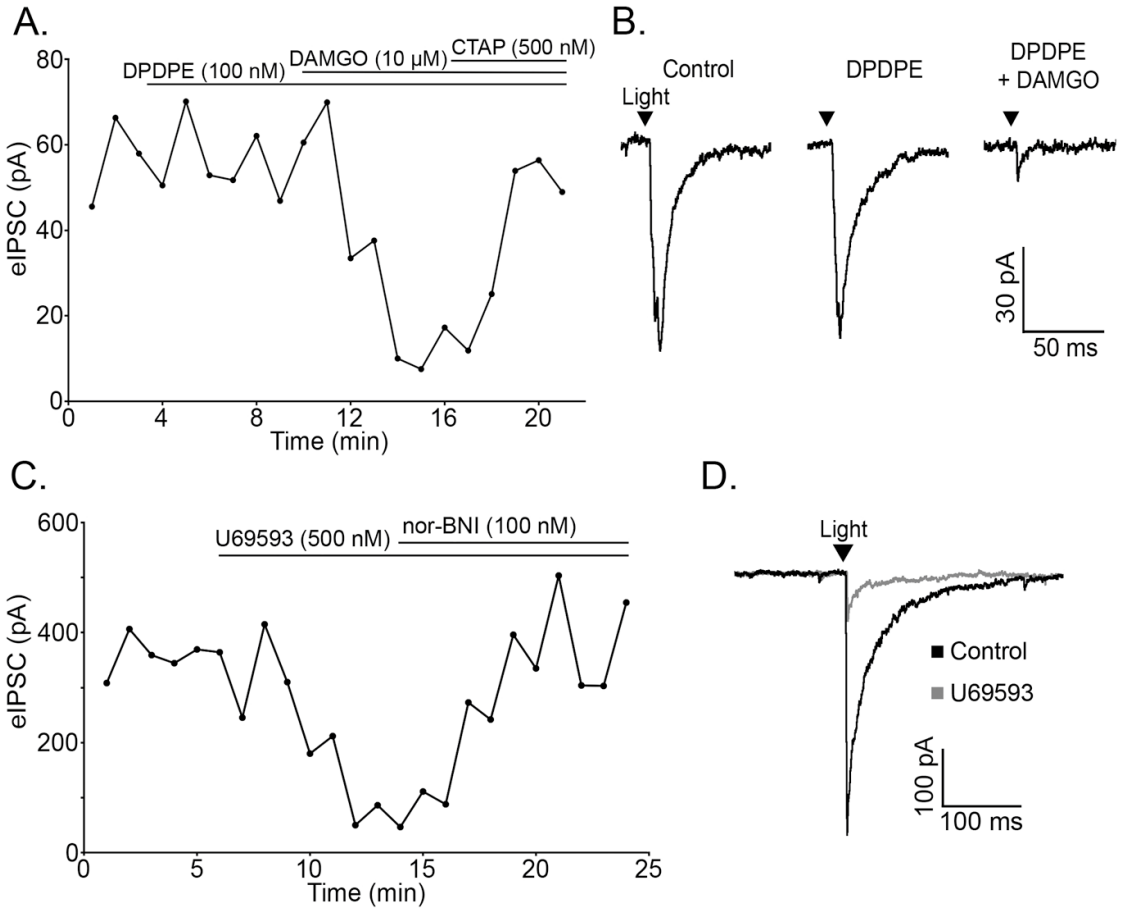

Figure 2.4. Mu and kappa opioid receptors mediate inhibition of evoked release from POMC terminals. (A) Plot of light-evoked IPSC amplitudes over time shows no effect of the DOR-selective agonist, DPDPE (100 nM) and inhibition of IPSC amplitude by the MOR-selective agonist, DAMGO (10 μM). The DAMGO-induced inhibition is reversed by application of the MOR-specific antagonist, CTAP (500 nM). (B) Representative traces taken during control and agonist treatment. (C) The specific KOR agonist U69593 (500 nM) inhibited evoked IPSC amplitude and was reversed by the addition of nor-BNI (100 nM). (D) Representative traces taken during control conditions and during U69593 treatment.

$GABA_B$ receptor activation inhibits transmitter release from POMC cells

In addition to opioid receptors, $GABA_B$ receptors can inhibit transmitter release from unidentified terminals in the ARC. To determine if $GABA_B$ receptors could modulate transmitter release from POMC neuron terminals in particular, light-evoked release was monitored in the presence of the $GABA_B$ receptor agonist baclofen. Baclofen (30 μM) caused a significant inhibition of light-evoked IPSCs in neurons downstream of POMC-ChR2 neurons (89.04 ± 3.20% reduction, $n = 6$, $p < 0.001$; Figure 2.5A-B). In 4 cells, baclofen (30 μM) completely

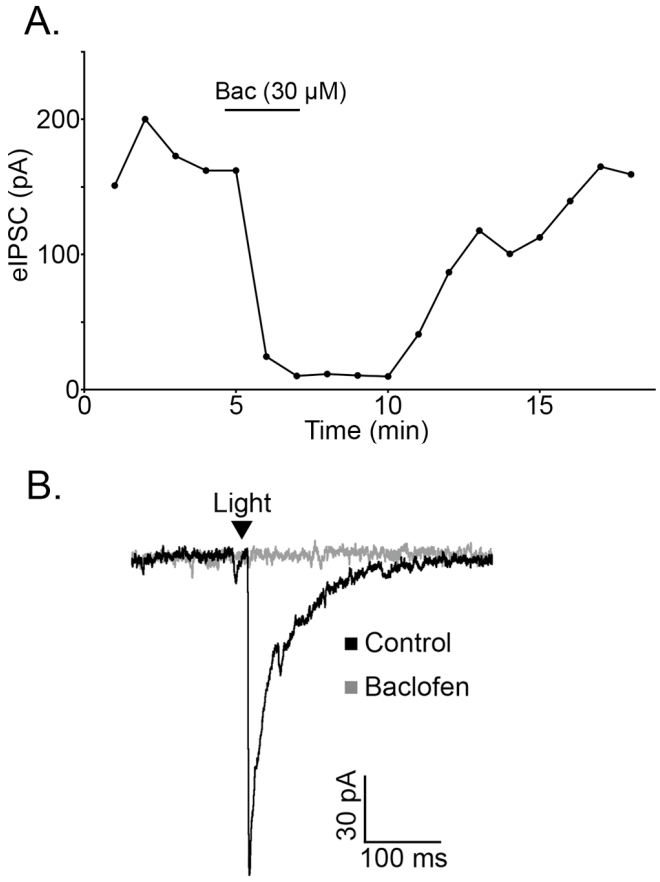

Figure 2.5. GABA-B receptor activation inhibits GABA release from POMC terminals. (A) Light-evoked IPSC amplitudes are inhibited by baclofen (30 μM). The inhibition is reversed by washout of drug. (B) Representative traces taken during control conditions and during baclofen treatment.

ablated the light-evoked IPSC. Baclofen-induced inhibition of the IPSC was fully reversed by washout or by co-applying the $GABA_B$ receptor antagonist CGP 55845 (1 μM, 92.44 ± 4.19% of baseline, $n = 6$, $p = 0.13$). This result indicates that POMC neurons express $GABA_B$ receptors, and these receptors can inhibit presynaptic GABA release from POMC terminals.

2.5 Discussion

The results of the present study show that POMC neurons are not only peptidergic, but release the classical amino acid transmitters GABA and glutamate. Considerable release of these transmitters occurs within the ARC and can be modulated dynamically by agents such as opioids and GABA. Although GABAergic and glutamatergic phenotypes had previously been suggested for POMC neurons based on immunolabeling and studies of transmitter release from cultured POMC neurons (Hentges et al., 2004, 2009; Meister, 2007), the finding that GABA and

glutamate release can be evoked from POMC cells in live brain slices indicates that theses transmitters are likely to play an important role in the intact circuitry.

Advantages of the optogenetic approach

The difficulty of determining transmitter phenotype in hypothalamic neurons using traditional means stems from 1) the fact that this structure lacks lamination precluding the use of paired recordings to detect released transmitters, and 2) the inability to directly detect GABA and glutamate reliably using immunohistochemical methods in this tightly-packed heterogeneous brain region. The optogenetic approach used in the present study allows for the activation of an entire population of identified neurons, which greatly increases the likelihood of detecting transmitter release onto downstream target neurons. Using this approach, Atasoy et al. (2008) recently demonstrated that POMC neurons could release GABA onto neurons in the paraventricular nucleus. The present data show that POMC neurons can also release glutamate, and that these neurons release both inhibitory and excitatory AA transmitters onto neurons within the arcuate nucleus. Thus, optogenetic activation of POMC neurons is a useful means of mapping distal projections and studying local innervation.

The use of combined optogenetic activation and electrophysiologic recording to detect transmitter release has many advantages over immunodetection and marker-based methods, including increased sensitivity and real-time assessment of release. Based on various labeling approaches, the AA phenotype of POMC neurons had been disputable. The inability to detect GABA immunoreactivity in POMC neurons (Ovesjö et al., 2001), and the presence of glutamatergic markers in a small population of POMC neurons (Collin et al., 2003; Kiss et al., 2005) suggested that POMC neurons are not GABAergic. However, when the GAD67 promoter

was used to drive expression of the green fluorescent protein in transgenic mice, labeling could be observed in a subpopulation of POMC neurons (Hentges et al., 2004; Yee et al., 2009), suggesting that some POMC neurons may be GABAergic. In addition, approximately one-third of POMC neurons express GAD mRNA (Hentges et al., 2004). The GABAergic nature of some POMC neurons was further indicated in studies showing evoked GABA release from POMC neurons in primary cultures (Hentges et al., 2004, 2009). Despite the evidence suggesting that POMC neurons can be either GABAergic or glutamatergic based on phenotypic markers and GABA and glutatmate release from cultured POMC neurons (Hentges et al., 2009), the ability of these neurons to release both transmitters in brain slices had not been demonstrated until the present study.

Interestingly, although the present data clearly show evoked GABA release from POMC terminals, a recent study failed to detect vesicular GABA transporter (vGAT) in POMC neurons (Vong et al., 2011). This transporter is the only protein identified to date that packages inhibitory transmitters into vesicles (McIntire et al., 1997; Sagne et al., 1997; Gasnier, 2004). Whether the apparent lack of vGAT expression in POMC neurons is due to the transgenic expression system used, or reflects that very low levels of vGAT are sufficient to package GABA in POMC neurons remains to be determined. Nonetheless, the expression of GAD67-gfp and GAD mRNA in POMC neurons, as well as the release of GABA from POMC neurons in culture and in brain slices indicate that at least a subpopulation of POMC neurons is indeed GABAergic. Further studies are needed to determine whether POMC neurons release GABA in a vGAT-independent manner or if vGAT expression is too low to be readily detected. Either way, vGAT may not a reliable indicator of the GABAergic nature of POMC neurons.

GABA and glutamate release from POMC neurons is consistent with synaptic morphology

The present data localize a portion of POMC cell AA transmitter release to the ARC. The release of GABA is consistent with ultrastructural studies demonstrating POMC terminals make symmetrical (GABAergic) synapses onto neurons within the ARC (Chen and Pelletier 1983; Kiss and Williams, 1983). Asymmetrical (glutamatergic) POMC synapses within the ARC have not been described, although glutamate release was detected locally in the present study. The low percentage of PSCs that were mediated by glutamate and the relatively low number of POMC neurons expressing vGLUT2 (Vong et al., 2011) may explain why, morphologically, glutamatergic POMC terminals have not been observed in the ARC. Interestingly, POMC neurons have been shown to form both symmetrical and asymmetrical synapses onto neurons in distal target sites including the dorsal raphe nucleus (Wang et al., 2001) and the locus coeruleus (Reyes, et al., 2006). Thus, GABA and glutamate release from POMC neurons may be important for regulation both locally and at distal target sites.

Amino acid transmitters and energy homeostasis

The studies of AA transmitters in hypothalamic circuits to date indicate a significant contribution of these transmitters in the regulation of food intake and metabolism. For example, glutamate release from neurons in the ventromedial hypothalamus is an important step in preventing hypoglycemia (Tong et al., 2007), the release of GABA from NPY/AGRP neurons in the ARC is also required for the normal regulation of energy balance (Tong et al., 2008), and GABA release from NPY/AGRP neurons into the parabrachial nucleus prevents starvation (Wu et al., 2009). It is likely that AA transmitters from POMC cells have important analogous

functions, although perhaps with more diversity, given the release of both GABA and glutamate from POMC neurons.

Presynaptic regulation of transmitter release

In the present study, the ability of $GABA_B$ and opioid receptor agonists to regulate AA transmitter release from POMC neurons was demonstrated. These studies were undertaken primarily to determine if AA release from POMC neurons could be dynamically modulated as would be expected if these transmitters play an important regulatory role. $GABA_B$ and opioid receptors were chosen, as both have been shown to potently modulate transmitter release from terminals within the hypothalamus (Mouginot et al., 1998; Emmerson and Miller, 1999; Pennock and Hentges, 2011). Since transmitter release was evoked only from POMC neurons and inhibited by $GABA_B$, MOR, and KORs, it is now clear that these receptors can inhibit release specifically from POMC terminals. The results raise the possibility that GABA and/or opioids released from POMC terminals could activate presynaptic receptors to inhibit further transmitter release.

Conclusion

The data collected here show that POMC neurons are not only peptidergic, but release the classical amino acid transmitters GABA and glutamate. Release of these transmitters can be dynamically modulated by agents such as opioids and GABA. Thus, in addition to the roles attributed to peptides, POMC neurons can cause rapid, inhibition or excitation of downstream neurons.

3. *Gad1* mRNA AS A RELIABLE INDICATOR OF ALTERED GABA RELEASE FROM OREXIGENIC NEURONS IN THE HYPOTHALAMUS[2]

3.1 Summary

The strength of GABA-mediated inhibitory synaptic input is a principle determinant of neuronal activity. However, because of differences in the number of GABA afferent inputs and the sites of synapses it is difficult to directly assay for altered GABA transmission between specific cells. The present study tested the hypothesis that the level of mRNA for the GABA synthetic enzyme glutamate decarboxylase (GAD) can provide a reliable proxy for GABA release. This was tested in a mouse hypothalamic circuit important in the regulation of energy balance. Fluorescent *in situ* hybridization results show that the expression of *Gad1* mRNA (encoding the GAD67 enzyme) was increased in hypothalamic neuropeptide Y/agouti related peptide (NPY/AgRP) neurons after an overnight fast, consistent with the ability of GABA from these neurons to stimulate food intake. Optogenetic studies confirmed that the observed increase in *Gad1* mRNA correlated with an increase in the probability of GABA release from NPY/AgRP neurons onto downstream proopiomelanocortin neurons. Likewise, there was an increase in the readily releasable pool of GABA in NPY/AgRP neurons. Selective inhibition of GAD activity in NPY/AgRP neurons decreased GABA release, indicating that GAD67 activity, which is largely dictated by expression level, is a key determinant of GABA release. Altogether, it appears that *Gad* expression may be a reliable proxy of altered GABAergic transmission. Examining changes in *Gad* mRNA as a proxy for GABA release may be particularly helpful when the downstream

[2] Full article from Dicken MS, Hughes AR, and Hentges ST. (2015) European Journal of Neuroscience, 42(9):2644-53. Shane Hentges contributed to experimental design, performed *in situ* hybridization, and contributed to writing of the manuscript. Alex Hughes performed *in situ* hybridization and edited the manuscript. Matthew Dicken contributed to experimental design, performed electrophysiology experiments, analyzed collected data, and contributed to writing of the manuscript.

targets are not known or when limited tools exist for detecting GABA release at a particular synapse.

3.2 Introduction

The amino acid transmitter GABA has long been recognized as a critical mediator of neuronal inhibition, yet the role of GABA in several circuits is just beginning to be explored in detail. For example, despite the fact that GABA was first detected in neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons of the hypothalamus nearly 2 decades ago (Horvath et al., 1997), it has only recently been discovered that synaptic GABA released from these neurons acutely stimulates food intake independent of peptide release (Tong et al., 2008; Wu et al., 2008; Krashes et al., 2013). The dynamic regulation of GABA release from this population of cells, particularly in response to altered energy state has not yet been explored. Several factors, such as sparse and spatially restricted afferent inputs and a potential lack of tools to detect transmitter release, hinder the ability to detect altered GABAergic transmission in various neuronal circuits. Therefore, the present study aimed to determine if the expression of GABA neuron specific mRNA could serve as a proxy for altered GABAergic transmission.

Several proteins are only found in neurons that can package and release GABA, including the vesicular GABA transporter (vGAT), the plasma membrane GABA transporters (GATs), and GABA synthetic enzymes glutamate decarboxylase (GAD) 65 and 67. While vGAT is generally necessary for vesicular packaging and release of GABA, several lines of evidence suggest that modest changes in vGAT expression will not affect GABA release (Edwards, 2007; Apostolides & Trussell, 2013). Indeed, heterozygous mice with a significant reduction in vGAT expression show no apparent changes in synaptic GABA release (Yamada et al., 2012). By contrast, the

levels of cytosolic GABA, which are derived primarily from glutamate uptake (Mathews & Diamond, 2003), greatly affect vesicular GABA content and synaptic strength (Apostolides & Trussell, 2013; Ishibashi et al., 2013; Wang et al., 2013). Cytosolic GABA content is largely controlled at the level of glutamate decarboxylation by the GAD enzymes, with GAD67 being responsible for >90% of brain GABA content and essential for synaptic GABA release and survival (Asada et al., 1997; Chattopadhyaya et al., 2007; Obata et al., 2008; Lazarus et al., 2013). The necessary nature of GAD67, and the fact that GAD67 protein and its mRNA (*Gad1*) levels are often more sensitive to a number of experimental conditions compared to GAD65 (Rimvall & Martin, 1992; 1994; McCarthy, 1995; Bowers et al., 1998; Mason et al., 2001), led to the current hypothesis that changes in *Gad1* expression could provide a means to assess overall changes in GABA transmission from a specific neuron type in response to a physiologic challenge. The results show that changes in energy state are sufficient to selectively increase or decrease *Gad1* mRNA in NPY/AgRP neurons and cause a concomitant change in synaptic GABA transmission from these neurons. Using *Gad1* mRNA as a proxy for altered GABA release has the advantages of examining the whole population of neurons at once and does not require recording inhibitory postsynaptic currents (IPSCs) from the postsynaptic neuron.

3.3 Materials and Methods

Animals

POMC-EGFP (C57BL/6J-Tg(Pomc-EGFP)1Low/J, stock 009593), NPY-hrGFP (B6.FVB-Tg(Npy-hrGFP)1Lowl, stock 006417), and AgRP-Cre mice (*AgRPtm1(cre)Lowl*, stock 012899) were obtained from The Jackson Laboratory. POMC-DsRed animals (Hentges et al., 2009) were originally obtained from Dr. Malcolm Low. In the arcuate nucleus, NPY expression

is restricted to cells that also express AgRP with overlap greater than 90% (Hahn et al., 1998), thus the transgenically-expressed NPY-hrGFP specifically labels cells also expressing AgRP in this region. All transgenic animals were maintained on the C57BL/6J background. Both male and female mice (8-12 weeks old) were used for experiments and were distributed evenly over treatment conditions. Mice were maintained on a 12-hour light/dark cycle and were given *ad libitum* access to water and standard rodent chow unless noted otherwise. All experimental protocols were reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee and were in accordance with the United States Public Health Service guidelines for animal use.

In situ hybridization

NPY-hrGFP mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with a 10% sucrose solution, followed by 4% paraformaldehyde in PBS. Brains were post-fixed overnight at 4°C in 4% paraformaldehyde solution in PBS. 50 μm sections containing the arcuate nucleus were cut on a vibratome, collected in cold PBS and processed for *in situ* hybridization to detect *Gad1* or *Gad2* mRNA as detailed previously (Jarvie & Hentges, 2012). The fluorescent signal of the hrGFP was quenched through the *in situ* hybridization procedure. Therefore, after completion of the *in situ* hybridization protocol, immunofluorescence was used to detect hrGFP by addition of a polyclonal antibody against hrGFP (1:1000, Agilent Technologies, Santa Clara, CA) overnight at 4°C and detection with goat anti-rabbit conjugated to Alexa 568 (1:400, 1 h room temperature). Confocal images were collected using a Zeiss 510-Meta confocal microscope. Z-stacks were initially constructed with 5-9 images 3 μm apart in depth for all tissue sections containing cells labeled for hrGFP. These stacks were pared down to

4 sequential images for analysis. Cell counts were made using a modification of the 3D counting method described by Williams and Rakic (1988) to limit oversampling (Williams & Rakic, 1988). Only gfp-positive cells with a clear nucleus and completely contained in a 300 x 300 x 12-μm counting box on the x-y-z plane were counted and the presence or absence of *Gad1* signal was determined for each gfp-positive cell. Average fluorescence intensity had to be greater than 10% above background for a cell to be considered positive for either signal and for *Gad1*, the signal had to be constrained within the somatic region of a gfp-positive cell for that gfp cell to be considered as expressing the *Gad1* signal. All images were analyzed using NIH ImageJ by an experimenter blinded to the treatment groups. NPY-hrGFP-positive cells were identified in a minimum of 7 slices per animal. For each hrGFP-positive cell, the assessor determined whether or not the cell also contained the *Gad* label while examining individual images from the image stack and label intensity was automatically determined for each cell.

In vivo gene delivery via adeno-associated virus

Adeno-associated virus (AAV) (3.56E+13 GC/ml) containing a double-floxed sequence for ChR2 with an mCherry tag (AAV2/9.EF1.dflox.hChR2(H134R)– mCherry.WPRE.hGH; obtained from the Penn Vector Core at the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania) was delivered bilaterally into the arcuate nucleus as previously described (38) and recordings were made 2-3 weeks post-injection.

Brain slice collection and electrophysiology

Sagittal brain slices (240 μm) containing the arcuate nucleus were prepared as previously described (Pennock & Hentges, 2011). Optogenetic stimulation and electrophysiologic

recordings were performed as previously described (Dicken et al., 2012). In brief, slices were maintained at 37°C in artificial cerebrospinal fluid (aCSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, and 11.1 glucose (saturated with 95% O_2 and 5% CO_2). Immediately after collection, slices were maintained in aCSF containing the NMDA receptor blocker MK-801 (15 μM) for at least 45 min before being transferred to the recording chamber. Whole-cell voltage clamp recordings were made with an internal recording solution containing the following (in mM): 57.5 KCl, 57.5 K-methyl sulfate, 20 NaCl, 1.5 $MgCl_2$, 5 Hepes, 0.1 EGTA, 2 ATP, 0.5 GTP, 10 phosphocreatine, pH 7.3. Recording pipettes had a tip resistance of 1.5–2.2 MΩ when filled with internal solution. For cell-attached recordings, the recording electrode contained aCSF. AgRP cells were identified by the fluorescence of the mCherry tag fused to ChR2. POMC cells were identified by EGFP fluorescence. Cells selected for patching were in the region containing mCherry positive fibers or cell bodies. Recordings were excluded from analysis if access resistance changed significantly during the recording or if access resistance increased above 18 MΩ. IPSCs were evoked either with paired 2 ms 470 nm light pulses 100 ms apart for paired-pulse ratio (PPR), or by 40 2 ms light pulses 100 ms apart for the depletion protocol through the use of an LED/LEDD1B driver (Thorlabs, Newton, NJ) connected to a TTL output on the ITC-18 data acquisition board (InstruTech, Longmont, CO). For PPR acquisition, sweeps were 20 s apart. A 40 s break between sweeps was used in experiments with depletion protocol. In all light-evoked-release studies, the light intensity was adjusted to the minimum level that would evoke consistent currents from ChR2-expressing cells, and currents were evoked for data collection no sooner than 4 minutes after break-in. Recordings were collected at 10kHz, digitally filtered at 1kHz, and at least 3 consecutive sweeps were averaged for presentation and analysis. To verify that evoked currents

were mediated by $GABA_A$ receptors, bicuculline methiodide (BMI; 10 μM; R&D Systems, Minneapolis, MN) was bath applied to the slice after experiments and always abolished the evoked currents. When recording from recurrent synapses in culture, GABA was evoked using a 2 ms depolarization to 0 mV (action potential artifacts are blanked in averaged traces).

Primary hypothalamic tissue culture

Tissue culture was performed as previously described (Hentges et al., 2004) from hypothalami of young (P2-P7) NPY-hrGFP mice with minor modifications. In brief, hypothalami were collected into ice-cold Hibernate-A medium (Life Technologies). Tissue was minced, and cells were dissociated after exposure to papain (20 U/ml; Worthington) by passing through glass pipettes with fire polished tips. Cells were plated onto glass coverslips pre-coated with poly-L-lysine in Neurobasal-A medium (Fisher Scientific) supplemented with B27 (Fisher Scientific), 0.4mM L-glutamine, and 1% fetal calf serum. The media were replenished every 3–5 d. Recordings from these cells occurred between 8-16 days of culture with either chelidonic acid alone (1 mM, Sigma) or with GABA (10 mM) included in the normal internal recording solution.

Recombinant mouse intraperitoneal leptin injections

Purified leptin (National Hormone & Peptide Program) was rehydrated by gentle mixing in 15 mM sterile HCl and the pH was adjusted to neutral. Leptin was brought to a final concentration of 1000 μg/ml with sterile saline. Mice received either a single intraperitoneal (IP) saline or leptin injection (6.0 μg/g body weight) 2 h prior to sacrifice.

Data analysis

All data are presented as mean ± SEM. Comparisons between two groups were evaluated using either *t*-tests, or in the case of the intensity distribution data, a Kolmogorov-Smirnov test. One-way ANOVA analyses followed by Tukey's HSD were used for comparisons between three groups. Repeated measures ANOVA with Dunnett post-hoc tests were used in the autapse experiments. For all experiments $p < 0.05$ was considered significant. Readily releasable pool estimates were performed as previously reported (Schneggenburger et al., 1999; Thanawala & Regehr, 2013).

3.4 Results

Fasting increases Gad1 mRNA in NPY/AgRP neurons

Since NPY/AgRP neurons are GABAergic, are activated by fasting (Takahashi & Cone, 2005; Yang et al., 2011) and may release more GABA in response to fasting (Vong et al., 2011) we sought to determine whether *Gad1* mRNA also increased in these cells in response to fasting as a putative indicator of enhanced GABA transmission. *In situ* hybridization for *Gad1* was performed in tissue from NPY-hrGFP mice under fed and fasted (17 h) conditions. Food restriction caused a significant increase in the number of NPY-hrGFP immuno-labeled cells that were also labeled with the probe for *Gad1* mRNA (fasted 1.41 ± 0.10 compared to normalized control values, $n = 6$ mice, $p = 0.01$ by unpaired *t*-test; Figure 3.1A-B). Additionally, the proportion of NPY/AgRP neurons with strong *Gad1* signal was increased in fasted mice (Kolmogorov-Smirnov test, $p = 7.3*10^{-21}$, $n = 608$ cells in fed, 882 cells in fasted from 6 mice per group; Figure 3.1C). The total number of hrGFP cells counted was the same for both groups

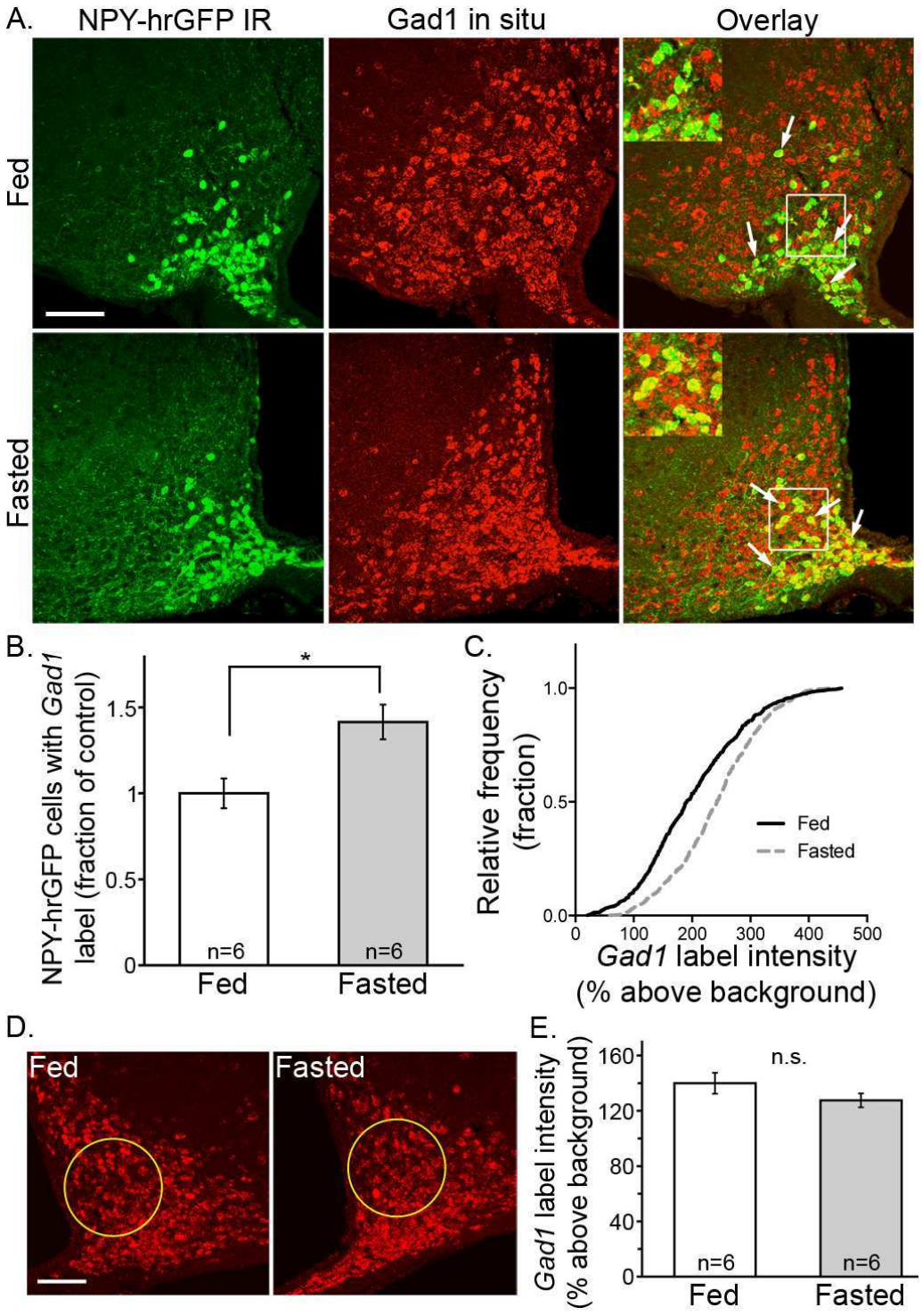

Figure 3.1. *Gad1* increases in NPY/AgRP cells after an overnight fast. NPY-hrGFP neurons were identified by immunoreactivity to hrGFP (A, left column, green) and *Gad1* was detected using a cRNA probe (A, middle column, red). Insets show an enlarged view of the area within the white box. White arrows point to some NPY-hrGFP cells containing *Gad1*. Fasting resulted in a significant increase in the percent of NPY cells expressing detectable levels of *Gad1* (B), and an increase in the proportion of cells with high levels of *Gad1* label intensity (C). The overall intensity of *Gad1* label in the area adjacent to the hrGFP-labeled cells (circled in D) was not changed by energy state (E). Scale bar=100 µm. Data are plotted as mean ± SEM. * = $p < 0.05$.

(fed = 300 ± 16 cells/animal, fasted = 314 ± 20 cells/animal, $p = 0.58$ by unpaired *t*-test). To determine if *Gad1* label intensity was broadly elevated in a majority of arcuate nucleus neurons, a large ROI was drawn that included a small number of hr-GFP-positive cells and a large number of *Gad1* labeled cells that were not hrGFP-positive (Figure 3.1D, region indicated by yellow circles). The average intensity above background was not significantly different between groups (139.9 ± 7.6% in fed, 127.5 ± 5.0% in fasted, $n = 6$, $p = 0.20$ by unpaired *t*-test; Figure 3.1E). Therefore, it appears that the fasting-induced increase in *Gad1* is relatively restricted to AgRP neurons in the arcuate nucleus, consistent with a previous report that also showed no overall difference in *Gad1* in the hypothalamic arcuate nucleus in response to fasting (Schwartz et al., 1993).

A separate *in situ* hybridization experiment to detect *Gad2* mRNA (encoding GAD65) was also performed. While the proportion of NPY/AgRP neurons with strong *Gad2* signal was increased in fasted mice (Kolmogorov-Smirnov test, $p = 4.4*10^{-18}$, $n = 1299$ cells in fed, 1430 cells in fasted from 4 mice per group), fasting did not significantly increase the number of *Gad2*-labeled hrGFP-positive cells (fasted 1.02 ± 0.03 compared to normalized controls, $n = 4$, $p = 0.41$ by unpaired *t*-test). Thus, it appears that *Gad1* expression is more sensitive to changes in energy balance compared to *Gad2*.

Fasting increases IPSCs in POMC neurons

Next, we wanted to determine whether the increase in *Gad1* expression after fasting might translate into increased synaptic GABA release from AgRP/NPY neurons. Thus, spontaneous IPSCs were recorded in hypothalamic POMC neurons, as POMC neurons are heavily innervated by AgRP/NPY cells (Cowley et al., 2001; Atasoy et al., 2012; Newton et al.,

2013). IPSCs were recorded using a pipette solution containing a high concentration of chloride causing GABA-mediated currents to be inward. POMC neurons from slices prepared from fasted animals (17 h) had a higher frequency of GABA-mediated IPSCs compared to the frequency in control mice with *ad libitum* access to food (7.3 ± 1.3 Hz in fed, 12.8 ± 1.9 Hz in fasted, $n = 9$ cells from 4 fed and 5 fasted animals, $p = 0.02$ by unpaired *t*-test; Figure 3.2A-C), consistent with a previous report (Vong et al., 2011). There was no significant difference in sIPSC amplitude between food-restricted and control mice (45.2 ± 3.6 pA in fed, 53.1 ± 4.2 pA in fasted, $n = 9$ cells from 4 fed and 5 fasted animals, $p = 0.17$ by unpaired *t*-test; Figure 3.2D), however the sample size may have precluded detecting an increase as other investigators have shown a significant increase in sIPCS amplitude in POMC neurons after fasting (Vong et al., 2011).

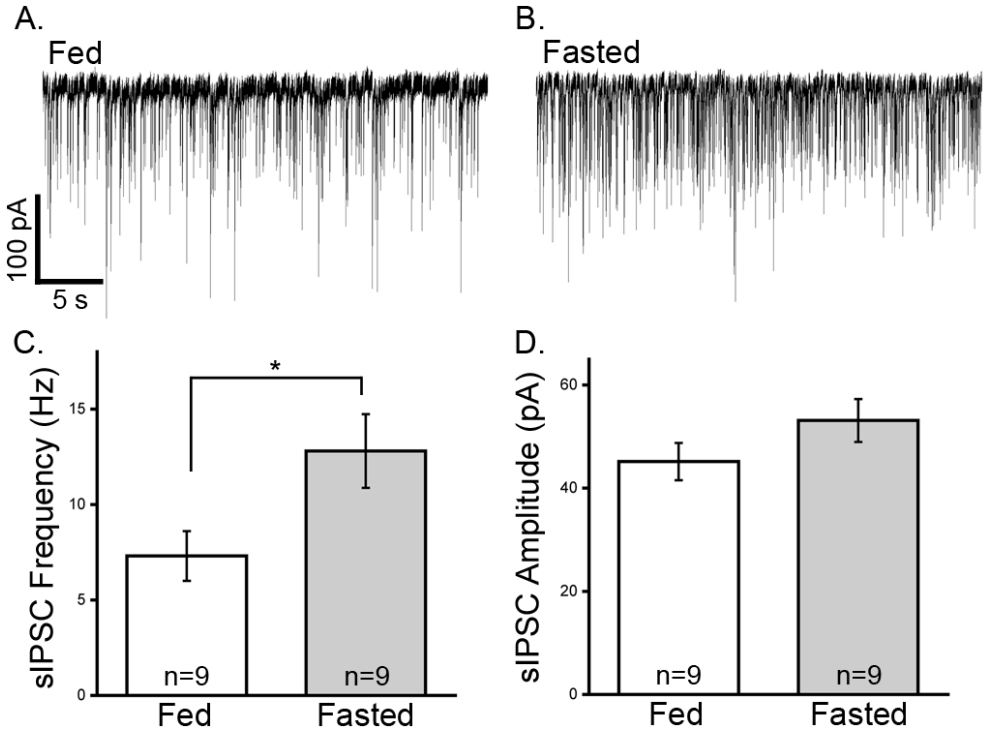

Figure 3.2. Spontaneous GABA release onto POMC cells is increased in fasted animals. Representative traces of spontaneous GABA-mediated IPSCs in POMC neurons in tissue from fed (A) and fasted (B) mice. Compiled results are shown in the graph (C). Current amplitudes were not significantly different between feeding states (D). Data are plotted as mean ± SEM. *=$p < 0.05$.

AgRP neuron activation is sufficient to acutely inhibit POMC neurons

Although POMC neurons are known to be postsynaptic to NPY/AgRP cells, it has been suggested that NPY/AgRP terminals do not account for a significant portion of the GABA inputs to POMC neurons (Tong et al., 2008). Therefore, the selectivity of functional coupling between AgRP and POMC neurons was examined. This was accomplished using specific activation of NPY/AgRP neurons with ChR2 expressed in NPY/AgRP cells. Strong expression of ChR2-mCherry in NPY/AgRP neurons was induced following injection of an AAV containing a Cre recombinase-dependent sequence for ChR2-mCherry into the arcuate nucleus of AgRP-Cre mice. To estimate the percentage of NPY/AgRP neurons expressing ChR2, NPY-hrGFP; AgRP-Cre double-transgenic mice were injected with the AAV and the tissue was processed for cell counting. Approximately 63% of the NPY-hrGFP cells expressed visible levels of ChR2-mCherry, whereas ChR2-mCherry was not expressed in NPY-hrGFP negative cells. Thus, the AgRP-Cre line provides reliable expression of ChR2-mCherry in AgRP neurons.

Next, AgRP-Cre mice were crossed to POMC-eGFP transgenic mice and double-transgenic offspring received injections of the virus containing floxed ChR2-mCherry. The mCherry tag on the ChR2 was used to detect NPY/AgRP cells while POMC neurons were identified by the presence of eGFP (Figure 3.3A). Light-stimulation of ChR2-expressing neurons caused an inward current (Figure 3.3B) and caused evoked IPSCs (latency to onset of 4.6 ± 0.2 ms) in POMC neurons (81 of 91 cells tested, Figure 3.3C, left). The $GABA_A$ antagonist BMI completely blocked the evoked IPSC in all cells tested (18/18 cells, Figure 3.3C, right). To determine whether the connection between AgRP and POMC neurons was specific or could also result from other groups of arcuate nucleus neurons, recordings were made in non-POMC neurons (cells lacking eGFP). Only 1 of 16 non-POMC neurons displayed an IPSC in response to

AgRP neuron stimulation. Thus, AgRP neurons preferentially innervate POMC neurons in the arcuate nucleus.

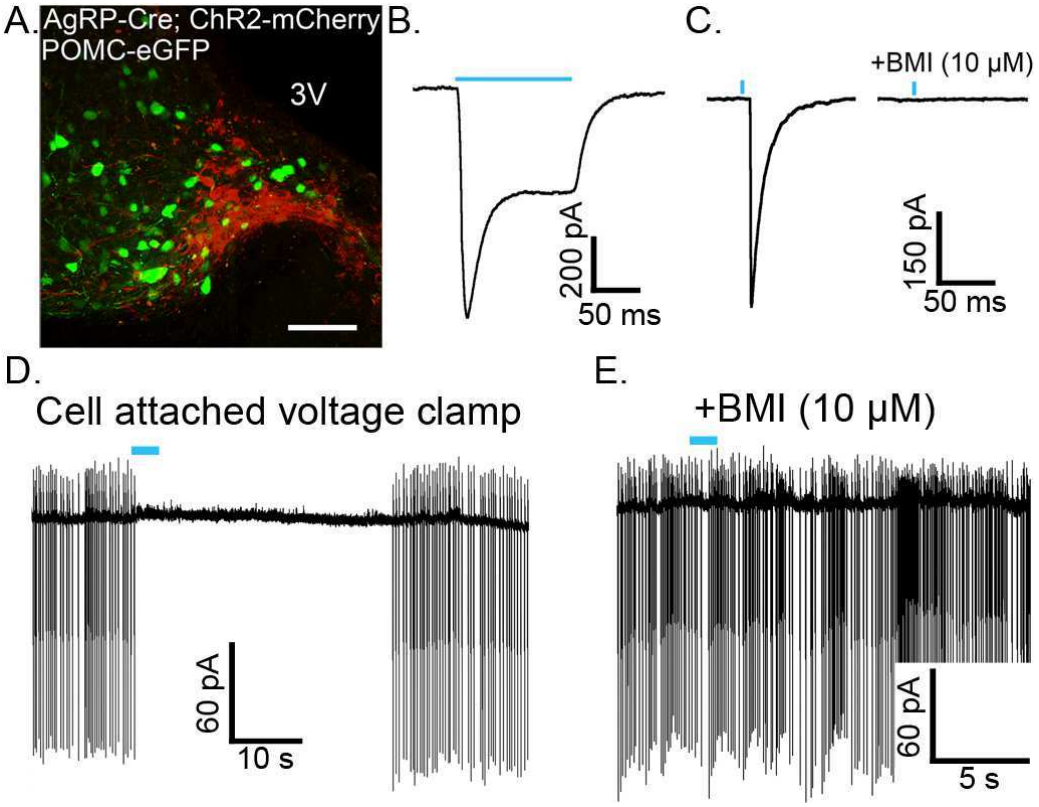

Figure 3.3. AgRP cells expressing ChR2 reliably release GABA onto POMC cells when stimulated. (A) In double transgenic AgRP-Cre;POMC-eGFP mice injected with the ChR2 construct, the POMC cells are visualized using the GFP tag (green) and AgRP neurons expressing ChR2 are visualized using the mCherry tag (red). A brief flash of blue light (indicated by the blue lines) causes a depolarization of ChR2-expressing cells and a stereotypical photocurrent in the AgRP cells (B), and evokes IPSCs in POMC cells (C, left) that are completely blocked by the $GABA_A$ antagonist bicuculline methiodide (BMI; C, right). In cell-attached voltage-clamp recording with external solution in the pipette, light-evoked GABA release inhibits POMC cell firing and action currents are lost (D). Addition of BMI to the bath prevents the light-evoked inhibition of cell firing (E). Scale bar in (A) is 100 μm.

To determine whether changing GABA release only from AgRP neurons is sufficient to alter the activity of POMC neurons, AgRP neurons were stimulated while measuring the firing of POMC neurons. Cell-attached recordings were made with external solution in the recording pipette such that the internal Cl^- concentration was maintained at physiological levels. Activation of AgRP neurons decreased spontaneous action potentials and action currents in POMC neurons

(0.006 ± 0.006 and 0.031 ± 0.031 of baseline firing, $n = 3$ cells from 2 animals, $p = 0.01$ and 0.04, respectively; Figure 3.3D). Application of BMI blocked the inhibition (Figure 3.3E). The results indicate that GABA release from AgRP neurons causes a profound inhibition of POMC neurons consistent with a previous report (Atasoy et al., 2012).

Fasting increases GABA release from NPY/AgRP to POMC neurons

To determine whether fasting increased the strength of the functional coupling specifically from NPY/AgRP to POMC neurons, optogenetic activation of NPY/AgRP neurons was examined in slices from fasted animals. The probability of GABA release determined by a change in the paired-pulse ratio was examined in slices from fed and fasted animals. Consistent with a higher probability of release, the PPR decreased in slices from fasted animals (0.94 ± 0.07 in fed, 0.75 ± 0.03 in fasted, 0.91 ± 0.06 in re-fed, $n = 11$ fed, 12 fasted, 11 re-fed cells from 6, 5, and 6 animals, respectively, one-way ANOVA, significant with Tukey's HSD; Figure 3.4A-B). Although slightly higher after fasting, the overall amplitude of the first evoked current was not significantly different between groups (410.8 ± 56.5 pA in fed, 533.0 ± 88.4 pA in fasted, 431.7 ± 79.2 pA in re-fed, $n = 11$ fed, 12 fasted, 11 re-fed cells from 6, 5, and 6 animals, respectively, one-way ANOVA, not significant with Tukey's HSD; Figure 3.4C). There was a weak negative correlation between PPR and initial IPSC amplitude ($r = -0.36$, $r^2 = 0.15$, $p = 0.02$).

The increase in probability of release could suggest that there is more GABA in the terminals overall. To test this possibility, the readily releasable pool (RRP) of GABA was examined in slices from fed and fasted animals using a depletion protocol (Schneggenburger et al., 1999). Repetitive pulses of light (40 at 10 Hz) were applied to evoke GABA release while recording from POMC neurons (Figure 3.5A-B, raw traces). The IPSC amplitudes were plotted

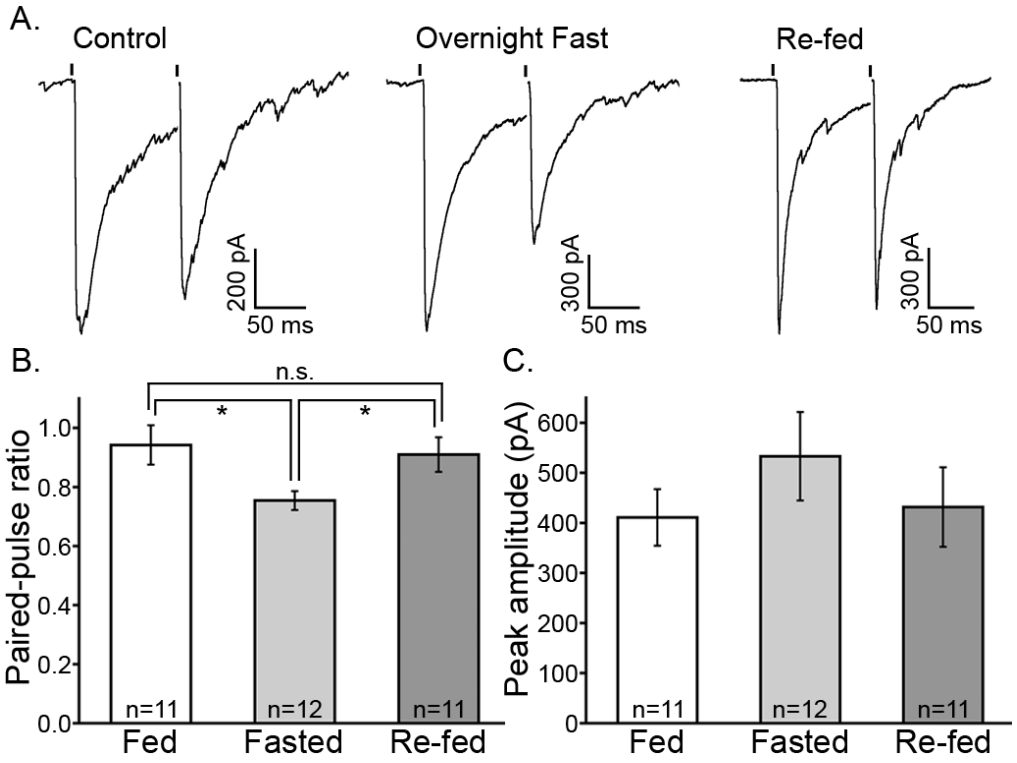

Figure 3.4. Fasting increases the probability of release from NPY/AgRP cell terminals presynaptic to POMC neurons. Representative traces (average of three consecutive sweeps) of GABA-mediated IPCSs light-evoked from NPY/AgRP neuron terminals in ad-lib fed (control, left), overnight fasted (middle), and re-fed (right) conditions are shown in (A). The paired-pulse ratio in mice allowed a 3 h re-feeding period after fasting is comparable to controls (B). There was not significant difference in peak GABA current amplitude between groups (C). Data are plotted as mean ± SEM. * = $p < 0.05$.

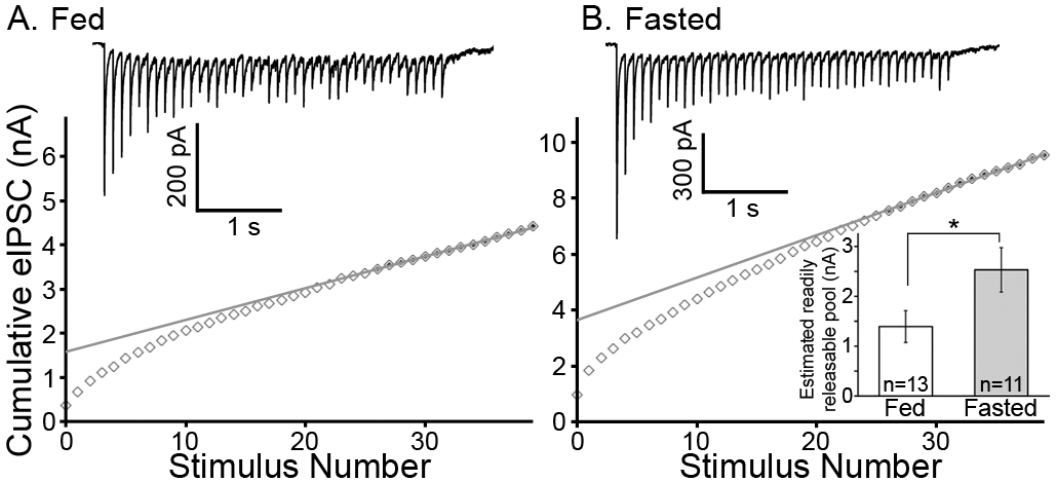

Figure 3.5. Fasting increases the readily releasable pool of GABA in NPY/AgRP cell terminals. Trains of light stimuli (40 stimulations at 10 Hz) were applied to brain slices prepared from fed (A) and fasted (B) mice. Cumulative current plots were made and the readily releasable pool was estimated by calculating the y-intercept from an extrapolated line drawn through the final 14 points of the plot. Estimated readily releasable pool is significantly increased in fasted animals (inset in B). Data are plotted as mean ± SEM. * = $p < 0.05$.

and a line was extrapolated from the linear portion of the curve and the Y-intercept was used to determine the RRP (Figure 3.5A-B, plotted data). A significantly higher RRP was observed in slices from fasted animals (1.4 ± 0.32 nA in fed, 2.5 ± 0.45 nA in fasted, n = 13 fed, 11 fasted cells from 6 fed and 5 fasted animals, p = 0.04 by unpaired t-test; Figure 3.5B inset). Additionally, when the initial current amplitudes were normalized to the last evoked current in the train, it became apparent that the peak (1st) current was relatively larger after fasting (fed = 7.7 ± 0.98; fasting = 12.4 ± 1.71, p = 0.02 by unpaired t-test). Importantly, the refilling rates (steady state currents at the end of the train) are not significantly different between the groups (fed = 97.9 ± 28.4 pA, fasted = 81.5 ± 13.1, p = 0.63 by unpaired t-test). Thus, by multiple estimates, amplitudes, PPR and RRP size, it appears that fasting increased GABA release from AgRP neurons onto POMC neurons, although the possibility of postsynaptic contributions cannot be fully excluded.

GAD activity is necessary for maintaining GABA release from NPY/AgRP neurons

We next asked whether the activity of GAD could be directly responsible for the increase in GABA release from AgRP/NPY neurons. This was examined in primary hypothalamic neuron cultures made from NPY-hrGFP transgenic mice and recordings were made in cells possessing recurrent synapse (autapses). When the conformationally restricted glutamate analogue chelidonic acid (1 mM), a competitive, potent, and fast-acting GAD inhibitor (Porter & Martin, 1985), was included in the pipette solution there was a significant inhibition of evoked autaptic GABA currents (17.5 ± 5.5% of baseline after 17 minutes, n = 5, $p < 0.0001$ by RMANOVA, minute 5-17 significantly lower than baseline using Dunnett's multiple comparisons test; Figure

3.6A,C). Thus, GAD activity is a pivotal determinant of GABA release in NPY/AgRP neurons consistent with previous reports at other GABAergic synapses (Apostolides & Trussell, 2013).

To verify that chelidonic acid was not reducing GABA release independent of its actions on GAD activity, GABA (10 mM) was included in the pipette along with the chelidonic acid in some recordings. The inclusion of GABA prevented the chelidonic acid-induced inhibition of the autaptic currents (117.8 ± 8.4% of baseline after 17 minutes, $n = 5$, $p = 0.38$ by RMANOVA, no minute significantly different than baseline using Dunnett's multiple comparisons test; Figure 3.6B-C), indicating that chelidonic acid inhibited GABA release by directly reducing GABA levels in the cytoplasm. The chelidonic acid-induced reduction in the evoked IPSC correlated with increased PPR over time consistent with decreased GABA release and this effect was absent when GABA was included in the pipette ($n = 5$, $p = 0.02$ by RMANOVA over time, minutes 14, 15, and 17 significantly different between groups using Sidak's multiple comparisons test; Figure 3.6D).

Decreased Gad1 expression correlates with reduced probability of GABA release

After observing the correlation between increased *Gad1* expression and enhanced functional GABA release, we tested the hypothesis that decreased *Gad* expression would reflect decreased GABA release from this synapse. IP leptin injections were used to approximate a satiated state. Compared to saline-injected controls, leptin caused a significant decrease in the number of NPY-hrGFP immuno-labeled cells that were also labeled with the probe for *Gad1* mRNA (0.75 ± 0.03 for leptin normalized to control, $n = 3$, 4 mice, $p = 0.035$ by unpaired *t*-test; Figure 3.7A). Correspondingly, the proportion of NPY/AgRP neurons with strong *Gad1* signal

was decreased in leptin-injected mice (Kolmogorov-Smirnov test, p=4.0012*10^{-18}, n = 390 saline, 565 leptin cells; Figure 3.7B).

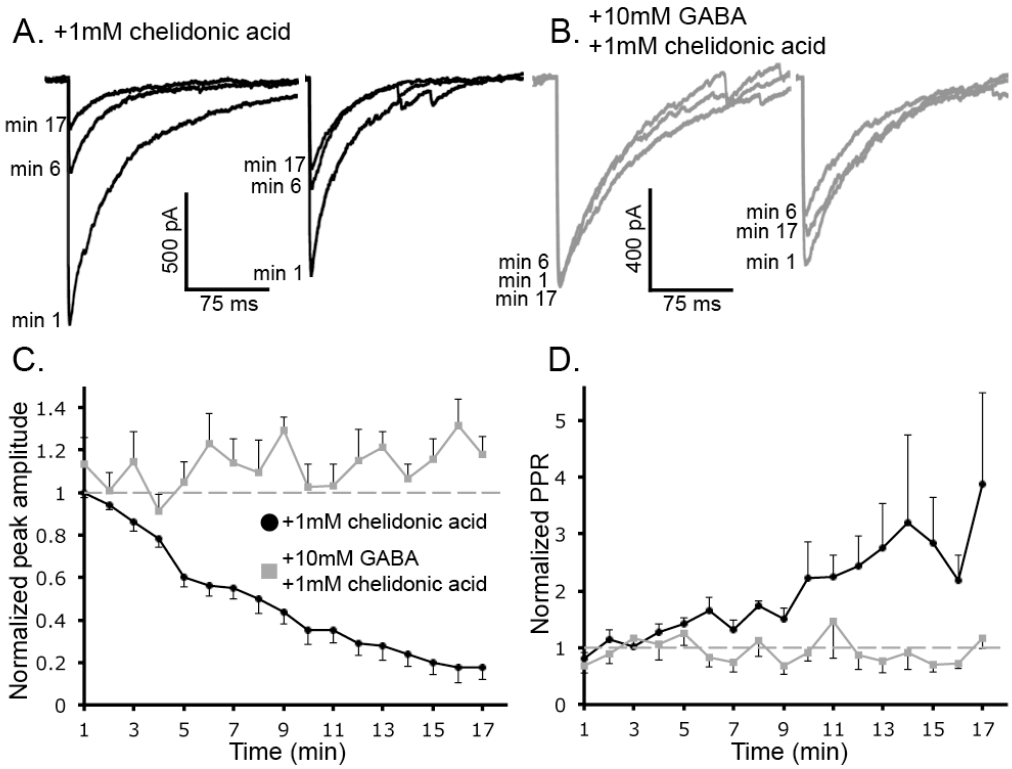

Figure 3.6. Inhibiting the GAD enzyme reduces autaptic GABA release from NPY/AgRP cells in culture. Whole-cell recordings were made in NPY/AgRP cells that had formed autapses in culture. Addition of chelidonic acid (1 mM) in the pipette solution caused a significant inhibition of evoked GABA release over time (A). Adding GABA into the pipette along with the chelidonic acid prevented the decrease in eIPSC amplitude (B). Average amplitudes for each condition are plotted in (C) and average paired-pulse ratios are plotted in (D). Data are plotted as mean ± SEM.

Optogenetic activation of NPY/AgRP neurons was again used to observe changes in probability of release. Leptin treatment caused a significant increase in PPR (0.90 ± 0.06 for saline, 1.30 ± 0.14 for leptin, n = 7 saline, 8 leptin from 3 saline-injected and 3 leptin-injected animals, p = 0.025 by unpaired t-test; Figure 3.7C) indicating a decrease in the probability of release. The peak amplitude of the first pulse in leptin-treated animals appeared to be less than in control tissue, but this was not statistically significant due to the high variance (324.63 ± 153.55

pA for saline, 86.71 ± 21.55 pA for leptin, n = 7 saline, 8 leptin from 3 saline-injected and 3 leptin-injected animals, p = 0.12 by unpaired t-test; Figure 3.7D). Together with the fasting results, the leptin-induced reduction in *Gad1* and corresponding decrease in the probability of GABA release indicates that *Gad1* is a dynamic indicator of both increases and decreases in GABA release.

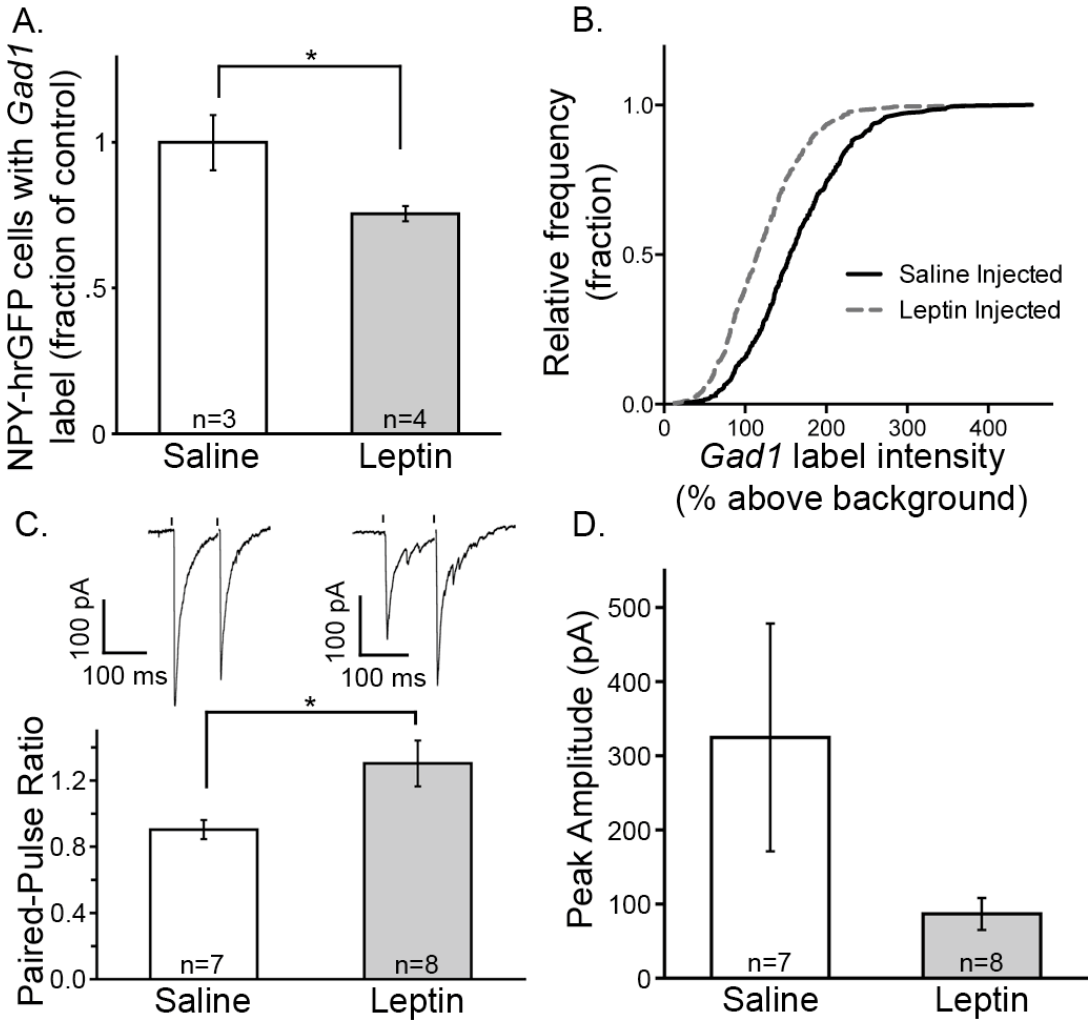

Figure 3.7. Leptin injection decreases *Gad1* in NPY/AgRP cells and decreases probability of GABA release. A single injection (IP) leptin 2 hours prior to tissue collection caused a significant decrease in the percent of NPY cells expressing detectable levels of *Gad1* (A), as well as a decrease in the proportion of cells with high levels of *Gad1* label intensity (B). Whole-cell recordings in POMC neurons in slice preparations show that leptin injection caused a significant increase in PPR when GABA release was light-evoked from NPY/AgRP terminals (C, averaged representative traces above respective bars). Peak amplitudes were not significantly different after leptin injection (D). Data are plotted as mean ± SEM. * = p <0.05.

3.5 Discussion

The objective of the present study was to determine if a physiologic perturbation could cause a change in GABA release that is reflected in the overall expression of a GABAergic marker *in situ*. Focusing on the NPY/AgRP population of neurons with a known GABAergic phenotype whose activity is sensitive to energy balance, fasting was found to increase the expression of *Gad1* mRNA in these neurons. Further, the enhanced mRNA expression correlated with an increase GABA transmission. It is likely that GABA release was increased at all terminals of NPY/AgRP neurons and that other conditions would also change *Gad* expression and GABA release from these neurons as indicated by the decrease in *Gad* expression and GABA release observed after leptin treatment. The expression of *Gad1* mRNA may prove to be a useful proxy for GABA release in other systems.

NPY/AgRP neurons and GABA release

The present study focused on GABA release from AgRP/NPY neurons. These neurons were chosen because of the availability of tools to examine and control them, the ability to identify and record from a postsynaptic target neuron and because of the important role these neurons play in the regulation of energy balance (Parker & Bloom, 2012). Although the current work focused on NPY/AgRP/GABA to POMC neuron connections, it seems most likely that the fasting-induced increase and leptin-induced decrease in GABA release observed would be consistent for other terminals from these neurons throughout the brain. Previous studies have indicated that NPY/AgRP terminals in the paraventricular hypothalamus (Atasoy et al., 2012) or the parabrachial nucleus (Carter et al., 2013) account for the ability of NPY/AgRP neuron-derived GABA to increase food intake, depending on the conditions studied. While NPY/AgRP

neuron-derived GABA can inhibit POMC neurons (Figure 3.3 and (Atasoy et al., 2012), this inhibition is reportedly not essential for the acute increase in feeding that results from experimental activation of NPY/AgRP neurons (Aponte et al., 2011; Atasoy et al., 2012). However, the possibility exists that under different conditions and/or timescales, changes in GABA input to POMC neurons may be an important factor in tilting energy balance in one direction or the other. What is clear at present is that GABA release from NPY/AgRP neurons is able to induce food intake and that both *Gad1* mRNA and GABA release in these cells is dynamically regulated in an energy-state dependent manner.

Correlating Gad67 mRNA and GABA tone

Numerous studies indicate that *Gad* mRNA, particularly *Gad1* mRNA, is sensitive to a variety of factors including steroids, stressors, glucose, insulin, seizure, lesions, cellular activity and GABA itself (Rimvall & Martin, 1992; 1994; McCarthy, 1995; Schwarzer & Sperk, 1995; Bowers et al., 1998; Mason et al., 2001; Pedersen et al., 2001; Patz et al., 2003). Separate studies have shown overall changes in regional GABA tone in response to many of these factors. However, we are aware of only one study showing a direct correlation between GABA release and *Gad1* expression and that study used a *Gad1* reporter system in cultured hippocampal slices as well as an olfactory bulb preparation (Lau & Murthy, 2012). Additional studies have shown that GAD activity or expression and corresponding changes in cytosolic GABA levels dictate the strength of GABAergic transmission in interneurons of the dorsal cochlear nucleus (Apostolides & Trussell, 2013) and in prefrontal cortex (Lazarus et al., 2013). Together with the present results in the hypothalamus using the physiological stimulus of fasting and leptin injection, it appears that changes in *Gad* mRNA and corresponding changes in protein levels for GAD may

closely reflect altered GABAergic transmission in many brain regions and cell types. Thus, at various synapses *Gad* may be a reasonable indicator of synaptic GABA levels.

It is important to note that, in addition to transcriptional regulation, the GAD enzymes are regulated by posttranslational modifications including coenzyme pyridoxal 5' –phosphate (PLP) binding and phosphorylation (Wei & Wu, 2008). While we cannot rule out that these additional levels of regulation may be important determinants of cytosolic GABA and GABA release, transcription appears to be a key point of regulation, particularly for *Gad1*. In many systems, *Gad1* mRNA levels are more sensitive to perturbations than *Gad2* (McCarthy, 1995; Bowers et al., 1998; Mason et al., 2001; Patz et al., 2003). This differential dependence on transcriptional regulation likely reflects the observation that the majority of the 65 kD form of GAD (transcribed from *Gad2*) is maintained in the inactive apoenzyme form and thus is dependent on posttranslational activation by PLP, whereas GAD67 is maintained primarily in the active holoenzyme state (Wei & Wu, 2008) and is therefore more dependent on the level of expression rather than posttranslational modification.

In addition to GAD activity, cytosolic GABA levels may also be affected via GABA uptake through the plasma membrane-bound GABA transporters. While the autaptic studies presented here suggest that GAD activity is a key determinant of GABA release from NPY/AgRP cells, a possible contribution from altered GABA uptake cannot be ruled out. The promotion of self-synapses necessitates that the neurons be grown under relatively sparse conditions, which may preclude the presence of substantial extracellular GABA concentrations. It is difficult to know if GABA uptake plays an important role in the intact system, although previous studies in intact circuits found that chemical inhibition of GAD was sufficient to reduce GABA release (Apostolides & Trussell, 2013) and the majority of GABA for release comes from

glutamate decarboxylation (Mathews & Diamond, 2003, Martin & Tobin, 2000) suggesting a limited role for GABA uptake in synaptic release. Consistent with the importance of GABA content as a primary driver of vesicular uptake and release are the present results showing an increase in PPR and decrease in evoked GABA currents as GAD activity is interrupted by chelidonic acid.

Broad potential for Gad mRNA as a proxy for GABAergic synaptic transmission

There are many conditions in which it may be desirable to detect changes in *Gad* mRNA as a proxy for plasticity in GABAergic transmission. For example, the use of single or multi-label *in situ* hybridization as used here allows for entire populations of cells to be examined in relatively intact tissue, which may be particularly useful in heterogeneous tissues or cell types. Further, semi-quantitative mRNA analysis of *Gad* could allow for an approximation of GABA tone when direct detection of GABA release is not technically feasible, such as when transgenic tools do not exist or when the postsynaptic target cannot be identified for paired recordings. While further studies will be needed to determine how generalizable the use of *Gad* mRNA will be as an indicator of GABA release from other cell types, the present studies together with past work indicate the potential for broad utility.

4. CONCLUSIONS, EXTENSIONS FROM FINDINGS, AND FUTURE DIRECTIONS

It is still a wonder to me how the essential act of eating, so basic in concept, is so mechanistically complex in mammals. Beyond the social and cultural factors that go into how people behave regarding food, understanding of the finely tuned biological processes required to signal healthy feeding and maintenance of a balanced energy set point is still an incomplete puzzle. The scientific community has come a long way since lesion experiments of the 1940s, when it was first determined that the hypothalamus played a large role in feeding behavior and energy balance, but there is still a lack of understanding when it comes to the true nature of the many relevant circuits present throughout the nervous system. Hopefully, this study has added a few good pieces to the puzzle to help understand the topic as a whole. After completion of the main project, the feeding and energy balance field now has a more robust understanding for the existence and regulation of amino acid transmitters released by first order feeding neurons, and we continue to investigate physiological roles in circuitry and subsequent phenotypes for which this release is most relevant.

4.1 Summary, weaknesses, and future directions for presented research

Results from Chapter 2 provide evidence for both glutamate and GABA release in the ARC from POMC cells. Using a combined optogenetics-electrophysiology approach, transmitter release could be assayed in pseudo-paired recordings with native synaptic connections still intact. ChR2 was used in POMC cells to find connections to nearby downstream cells. Just like the spontaneous input onto POMC cells, POMC glutamate and GABA release can be affected through activation of presynaptic GPCRs. One weakness of the recording paradigm used is that it

takes an enormous amount of effort to collect a data set of acceptable size. So far, it is not known if POMC neurons heavily innervate any one cell type in a given region. The ARC was chosen as a downstream target region mainly because it was convenient to do so, in addition to not finding a higher connection rate when randomly patching in the PVN. Through the inevitable development of future single synapse anterograde tracers, it will be interesting to see exactly on which neurons POMC cells synapse, and will provide an avenue for quickly finding many postsynaptic targets to patch onto or study in other ways. Alternatively, clever use of an anterograde tracer that is targetable and travels a known number of synapses per amount of time could be used similarly (e.g. the vesicular stomatitis virus vectors discussed in Beier et al., 2011). These strategies will allow for experiments like completed with NPY/AgRP cells in Chapter 3 to be completed in a timely manner, even if POMC cells innervate a sparse subset of any one type of cell. It would be interesting to know if glutamate or GABA release changes in a fed or fasted state, because this would be another avenue for feeding signals to be relayed, especially when considering juxtaposition with the fast amino acid GABA release from NPY/AgRP neurons. Opposing POMC amino acid transmitters of excitation and inhibition could be differentially regulated to appropriately modulate downstream feeding areas. Perhaps the two subpopulations of cells are completely distinct, and only rarely does glutamatergic POMC output overlap with GABAergic targets. After all, simultaneous glutamatergic and GABAergic release onto a downstream cell was a very rare occurrence in the data collected for Chapter 2.

Showing that POMC cells release both glutamate and GABA forces the scientific field to acknowledge both first order feeding neuron populations' amino acid transmitters when designing experiments. Current and previous studies that have failed to take into account these findings must accept potential weaknesses in their proposed models and be open to alternate

interpretations of their results. With this new information, GABA originating in the ARC is not confined to a unilaterally orexigenic force (Figure 4.1). As a nascent topic of research, there is still a dearth of information on POMC cell amino acid transmitter release, which leaves the subject poised to be mined for valuable contributions to energy homeostasis. It is exciting to hypothesize that adjusting amino acid transmitter release in POMC cells could help to regain healthy physiology from a far too negative or positive energy balance.

Results from Chapter 3 continued to add to the growing body of knowledge pertaining to amino acid transmitter release from first order feeding neurons. It was discovered that GABAergic input specifically from NPY/AgRP cells onto POMC cells is affected by feeding state. It had been shown before that in a fasted state, GABA tone increases onto POMC cells after a fast (Vong et al., 2011), but since it is not known what fraction of the total GABA release onto POMC cells is accounted for by NPY/AgRP cells, it was uncertain if the increase in inhibition actually comes specifically from these orexigenic neurons. As has been hypothesized before, this would be one mechanism by which satiety signals from POMC cells could be blunted, while at the same time exciting hunger circuits. I.e., not only can GABA be used by NPY/AgRP cells in a hungry state to acutely turn down the activity of POMC cells, it can also affect other downstream targets like anorexigenic second order neurons in the PVN.

While the discovery that there is a massive one-way connection of NPY/AgRP neurons to POMC neurons preceded the research in Chapter 3 (Atasoy et al., 2012), it was compelling to find this out first hand during my own research because it was still common to believe that there was direct reciprocal innervation between the neuronal populations (compare Figure 1.2 to Figure 4.1). In a healthy slice preparation with high viral penetrance and brightly labeled POMC cells, almost any eGFP-labeled cell would be downstream of GABA-releasing NPY/AgRP cell

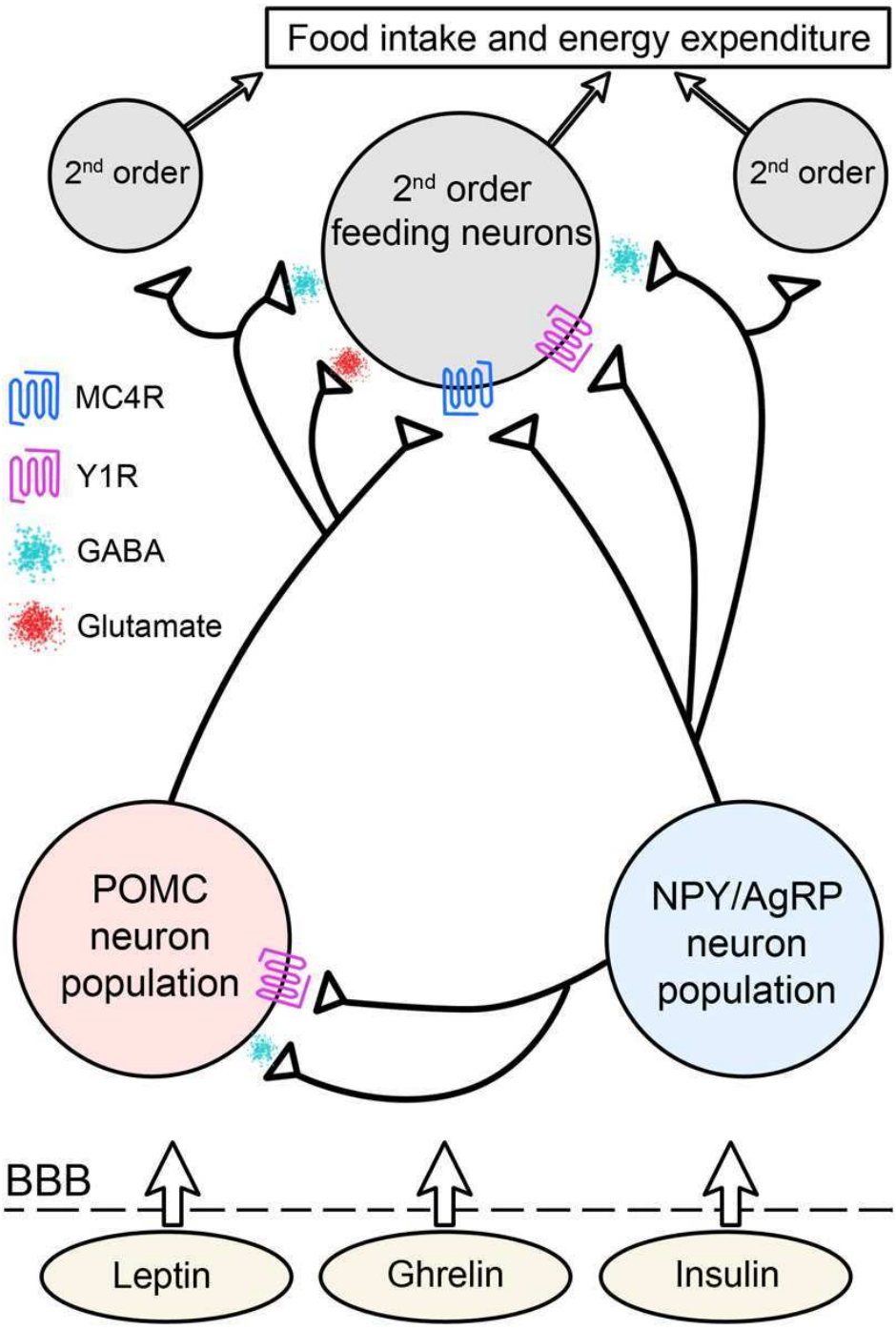

Figure 4.1. Adjusted paradigm of first order feeding neurons. In the new paradigm of first order neurons, reciprocal innervation between POMC cells has been removed. GABA release from NPY/AgRP cells is adjusted based on feeding state and release onto downstream targets is important for appropriate function of the circuit. The POMC population releasing glutamate and/or GABA onto downstream cells has been added to the model. In addition, either type of first order neuron may synapse onto a downstream cell without symmetrical connection by the opposing population. Abbreviations: blood-brain barrier (BBB), NPY type 1 receptors (Y1R), melanocortin 4 receptor (MCR4).

inputs. This is in contrast to expressing ChR2 in POMC cells and never finding a single glutamatergic or GABAergic current evoked onto NPY/AgRP cells. It seems that the older reciprocal feedback hypothesis is dying out. This is significant because in the old model, POMC and NPY/AgRP neurons are supposed to be battling for dominance both with each other and at downstream inputs. This finding continues to push the idea that these are often two distinctly separate pathways that often act asymmetrically from one another. POMC cells could still feed back onto their orexigenic neighbors, but it would have to be through indirect input or maybe paracrine effects of peptide release.

One limitation of the experiments in Chapter 3 is that all recordings were made at POMC→NPY/AgRP synapses. One set of future experiments would be to test other synaptic contacts located in other nuclei in order to determine if the increase in excitation observed after a fast translates to increased GABA release at all downstream inputs. Through these experiments, it could be determined if GABA release is regulated similarly at synaptic connections in both melanocortin -dependent and -independent pathways. It would also be important to extrapolate the synaptic physiology observed beyond the single synapse studied in order to continue validation of the *Gad* mRNA proxy hypothesis. The finding that the increase or decrease in *Gad1* message after a fast or leptin injection was a good correlate for increased functional GABA release might be a valuable tool for many projects. As a newer hypothesis, although as discussed in Chapter 3, not without rationale or additional support, this idea needs to be measured at the synaptic level and replicated across many areas in the brain. We also still do not know for certain if more *Gad1* mRNA actually means more GAD67, and that any additional GAD67 is responsible for the observed increase in GABA release. While we observed an increase in mRNA, and subsequently showed that depletion of functional GAD from a cell reduces GABA

release, it is still a correlative finding. While establishing both broad applicability and a solid mechanistic link are important, we focused more on showing that the mRNA-as-proxy could be used for both increases and decreases in GABA release (by using fasted state and leptin injections), and that *Gad1* tracks better with GABA release than *Gad2* (responsible for the GAD65 protein). As a future topic beyond the scope of the primary findings, knowing exactly what cells each group of opposing feeding neuron types synapse onto in downstream regions will provide valuable drug target information, as well as elucidate how both cell populations appropriately coordinate their amino acid transmitter release. For example, with the finding that both first order cell types use GABA as their primary amino acid transmitter, it would be odd if differential regulation of GABA occurred depending on feeding state in both POMC and AgRP cells, only to have projections from both cell types going to the same downstream cell. No matter what energy state the animal is in, the GABA would be released onto this hypothetical downstream cell. Future studies, perhaps using new tracer technology, could be used to fully explore this aspect of the circuitry.

4.2 Related experiments

GABA released onto POMC neurons

One of the questions left unanswered in Chapter 3 is how much of the total GABA input onto POMC neurons comes from NPY/AgRP neurons. It should be noted that some hypothesize that this source of POMC cell inhibition is relatively unimportant because selective activation of both NPY/AgRP and POMC neurons at the same time induces feeding to the same extent as activating NPY/AgRP cells alone (implying that inhibition of POMC neurons is not a significant contributor to increased food intake; Atasoy et al., 2012). While this was not necessarily a bad

experiment, the results do not warrant dismissing any relevance for acute inhibition of POMC neurons in food intake. It is not too surprising that acute maximal activation of both populations of first order feeding neurons, causes the biological imperative of energy consumption to win out; the effects of extraphysiological activation of NPY/AgRP neurons and subsequent transmitter release could trump POMC amino acid release, and POMC peptide release after chronic activation is not adequately considered. The interpretation of the specific experiment says more about the importance physiology puts on maintaining adequate energy stores than it does about the inhibition of POMC neurons by NPY/AgRP neurons. It is hard to believe that the significant effect that activation of NPY/AgRP cells expressing ChR2 has on POMC cell physiology (Figure 3.3) would not have physiological consequences if activation were not at a ceiling. A more nuanced set of experiments, where parameters are being manipulated within physiological dynamic ranges, is likely needed to test the true relevance of NPY/AgRP GABA tone on POMC cells. In any case, for the tuning of GABA release to have a relevant impact on POMC cell physiology, it is important to know that a large fraction of inhibitory tone onto POMC neurons is due to the release of GABA from NPY/AgRP cells. One early idea for answering this question involved expressing the hyperpolarizing light-sensitive Cl^--specific ion pump, halorhodopsin, in AgRP-Cre animals. Instead of exciting the cells with blue light so they would release GABA onto POMC cells, we could look at spontaneous GABA input onto POMC cells both before and after inhibition specifically of the NPY/AgRP cell population. We were apprehensive of this idea, though, as most of the GABA release onto POMC cells is action potential-independent i.e. most sIPSCs would remain as mIPSCs after application of TTX (Pinto et al., 2004; Pennock and Hentges, 2011), meaning hyperpolarization of NPY/AgRP somas with

halorhodopsin might just return inconclusive results. In lieu of this approach, we decided to use a vesicular GABA transporter (VGAT) knockout targeted to NPY/AgRP neurons.

Using a cross between an AgRP-Cre animal and a *Vgat*$^{flox/flox}$ animal, POMC cells could be recorded from in the same configuration as seen throughout Chapter 3 (requiring ChR2 expression and another cross so POMC cells could be visually targeted) and observed for spontaneous GABA input. After it was determined that GABA could not be light-evoked from NPY/AgRP cells (0/14 cells patched showed a light-evoked current), spontaneous IPSCs were compared between AgRP-Cre; *Vgat*$^{flox/flox}$; POMC-eGFP and AgRP-Cre; POMC-eGFP animals (Figure 4.2A-B). Interestingly, there was no difference between groups in either frequency or amplitude of spontaneous IPSCs (7.6 ± 1.5 Hz in control, 6.5 ± 2.0 Hz in experimental, $n = 9$ cells from 3 control and 3 experimental animals, $p = 0.66$ by unpaired *t*-test; Figure 4.2C. 50.7 ± 6.5 pA in control, 54.0 ± 7.7 pA in experimental, $n = 9$ cells from 3 control and 3 experimental animals, $p = 0.74$ by unpaired *t*-test; Figure 4.2D), suggesting that release from NPY/AgRP cells make up almost none inhibitory GABA tone seen by POMC cells. However, because this was not an inducible strategy and thus GABA release from a normal source was not available during development, there may have been some input compensation from other sources that prevented an accurate assessment of NPY/AgRP GABA release. This is a reasonable possibility given data to support to the existence of compensatory, redundant mechanisms in place to protect an animal from failing to consume a necessary caloric load. It is already accepted that global genetic deletions of NPY and/or AgRP, as well as directed knockout of *Vgat* from NPY/AgRP neurons has minimal effects on energy balance (Erickson et al., 1996; Qian et al., 2002; Tong et al., 2008).

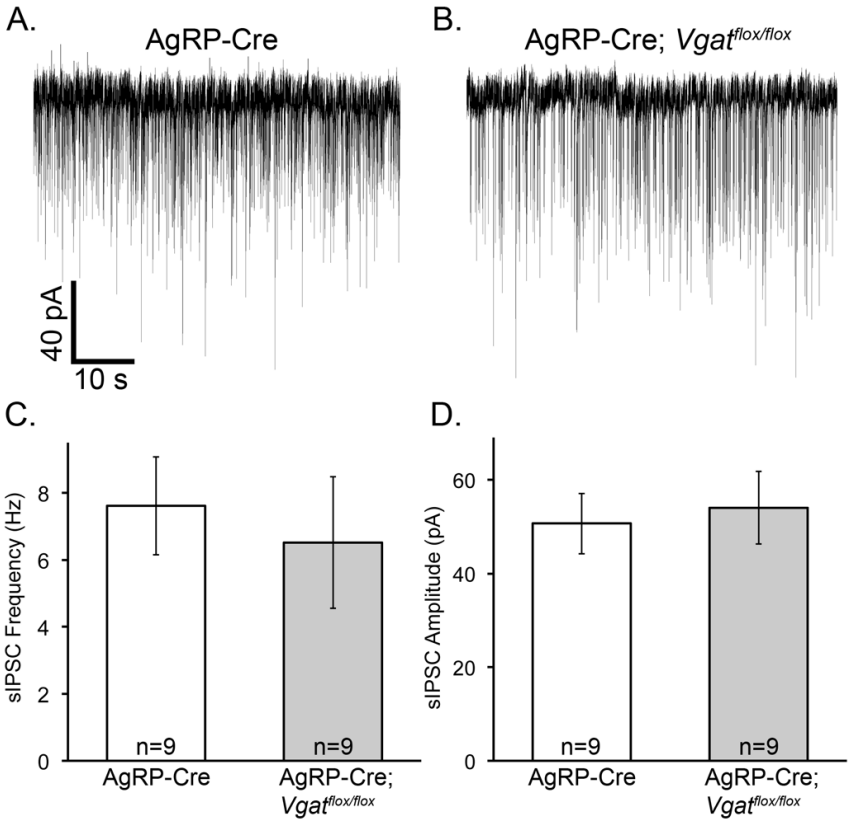

Figure 4.2. Spontaneous GABA release onto POMC cells is unaffected by removing the ability of NPY/AgRP cells to release GABA. Representative traces of spontaneous GABA-mediated IPSCs in POMC neurons in tissue from AgRP-Cre; POMC-eGFP (A) and AgRP-Cre; POMC-eGFP; *Vgat*$^{flox/flox}$ (B) mice. sIPSC frequency was not significantly different between groups (C), nor were current amplitudes (D). Data are plotted as mean ± SEM.

To get around developmental compensation, another transgenic approach was used. Instead of *Vgat*$^{flox/flox}$, a floxed μ-opioid receptor (MOR) animal was used. Previous work indicates that a saturating concentration of MOR agonist at this synapse in a brain slice preparation causes a ~75% reduction in spontaneous IPSCs (Pennock and Hentges, 2011). With MORs deleted specifically in NPY/AgRP cells, inhibition of presynaptic release can be compared between experimental and control animals, with less inhibition in the experimental condition meaning a larger contribution of GABA inputs from NPY/AgRP neurons. At the time

of this writing, these experiments are ongoing, but preliminary results indicate that there is a significant portion of GABA input onto POMC neurons by NPY/AgRP neurons.

Relevance of POMC cell amino acid neurotransmitters

While mice with POMC cells that are GABA release-deficient have not been developed yet, I was able to play a role in a study that looked at the physiological relevance of glutamate release (Dennison et al., 2015). This study was divided mainly into two parts. First, it was discovered that mRNA for the vesicular glutamate transporter vGlut2 is highly colocalized with *POMC* expression early in development, and then tapers off as mice age into adulthood. The second part of the study involved deletion of *vGlut2* specifically from POMC neurons. When fed a high-fat diet, male mice without vGlut2 expressed in their POMC neurons were not able to maintain their body weight, while female mice were unaffected (Figure 4.3). While this result suggests a physiologic role for glutamate release from POMC neurons in the maintenance of body weight on a high-fat diet, at least in males, care must be taken to consider the caveats of an interpretation limited to adult glutamate release. As discussed in Chapter 1, these genetic crosses that cause Cre expression whenever the POMC gene is turned on may make for convoluted results. The phenotype observed could be caused by glutamate acting as an important trophic factor early in development. Alternatively, neurons fated to be kisspeptin neurons, a subpopulation of ARC cells which express POMC early on, could have had their vGlut2 knocked out by Cre resulting from the transient expression of the *POMC* promoter in neurons that are not authentic POMC neurons (Padilla et al., 2012). While this study hasn't provided a fully satisfying answer for the importance of POMC neuron glutamate release in adult animals, it is an

interesting first step. Experiments are ongoing using an inducible POMC-Cre line of mice to delete vGlut2 from POMC neurons in adulthood.

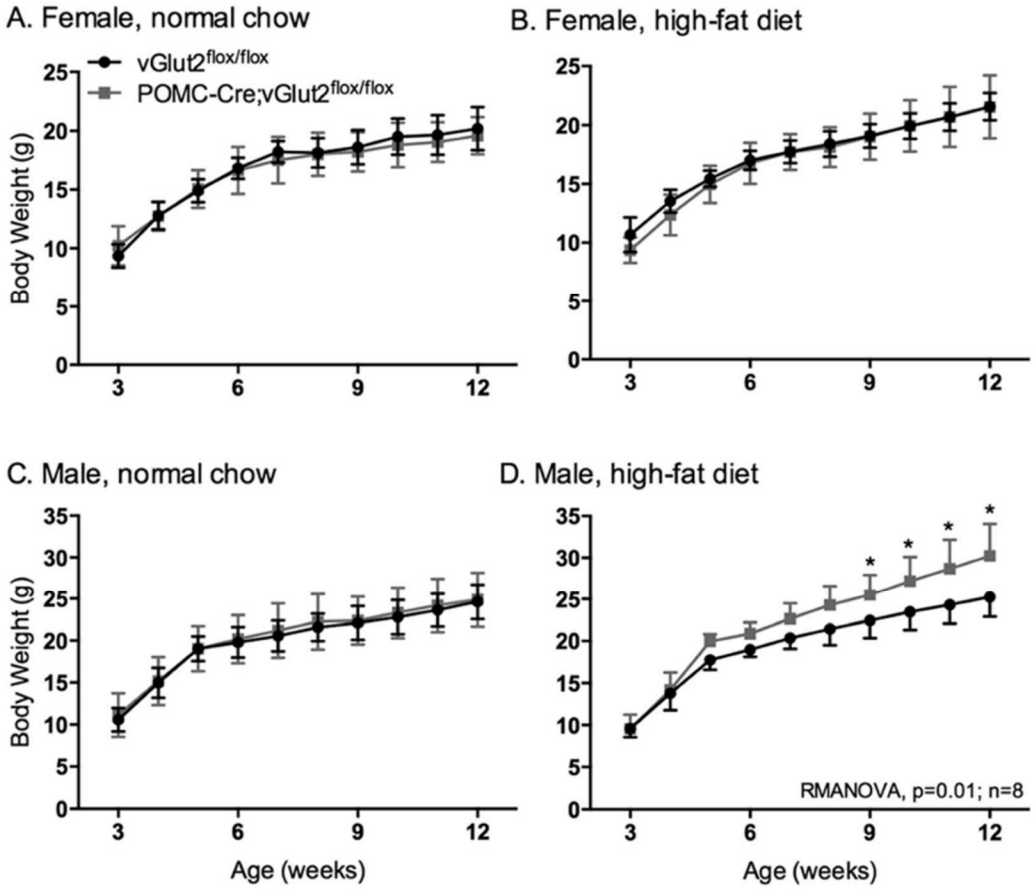

Figure 4.3. vGlut2 deletion in POMC neurons increases weight gain in males on a high-fat diet. A–D: Weight curves of control mice (blackcircles) and mice lacking vGlut2 in POMC neurons maintained on normal chow (A,C) or high-fat diet (B,D). All data points are mean ± SEM. * = $p < 0.05$ compared to same age control mice ($vGlut2^{flox/flox}$). Reproduced with permission from Dennison et al., 2015)

Amino acid transmitter release from POMC cells in the NTS

As mentioned in Chapter 1, in addition to the POMC cells located in the ARC, there is an additional neuronal population of POMC cells located in the medulla's NTS (Bronstein et al., 1992; Padilla et al., 2012). It is unknown, however, if this group of cells expresses POMC at any meaningful level in adulthood, since *POMC* mRNA cannot be detected past early postnatal

development. Nonetheless POMC-eGFP and POMC-Cre transgenes are expressed in the NTS, making this group of neurons targetable and able to be manipulated. This population of POMC cells' role in food intake and energy balance still is not completely clear, but recent research suggests that they play a role in cessation of feeding on a short-term timescale (Zhan et al., 2013). In light of this information and the knowledge that POMC neurons in the ARC release GABA and glutamate, we tested the hypothesis that amino acid transmitter release from POMC cells within the NTS, rather than peptide release, may mediate the physiologic response observed by Zhang et al. We used an approach similar to the blind patching in Chapter 2. An AAV containing the double-floxed inverted ChR2 construct was injected into the NTS of POMC-Cre animals, and after an incubation period, live sagittal slices were cut and were recorded from. Although exceedingly rare, both glutamate and GABA release were observed in this preparation (Figure 4.4). This might be one way in which these feeding neurons exert short-term feeding effects, but there exists one major caveat to these findings. Both in the case of Zhan et al., 2013 and our experiment, a POMC-Cre animal is used to express the proteins crucial to testing hypotheses about NTS POMC cells, whereas an *in situ* hybridization approach was used to verify that POMC neurons expressed markers for GABAergic and glutamergic phenotypes. The *in situ* hybridization approach relied on the detection of eGFP driven by the POMC promoter since it is not possible to readily detect endogenous POMC mRNA or protein. In attempting to verify that POMC-Cre and POMC-eGFP are expressed in the same cells, it became apparent that completely distinct populations of cells actually express POMC-Cre and POMC-eGFP —there is no overlap at all (unpublished findings). Although concerning, this is not completely surprising since the Padilla et al., 2012 study found similar results. In this corroborating instance, it was found that the population of cells labeled by performing immunohistochemistry for eGFP in a

POMC-eGFP animal was completely distinct from the population of cells labeled by the expression of a Cre-dependent fluorophore in POMC-neurons. Taken together, it appears that there are distinct populations of cells marked by the expression of POMC-Cre and POMC-eGFP. Based on functional studies, POMC-Cre neurons likely play a role in proper food intake, but the results are murky since biochemical studies have relied on POMC-eGFP to assess cellular properties.

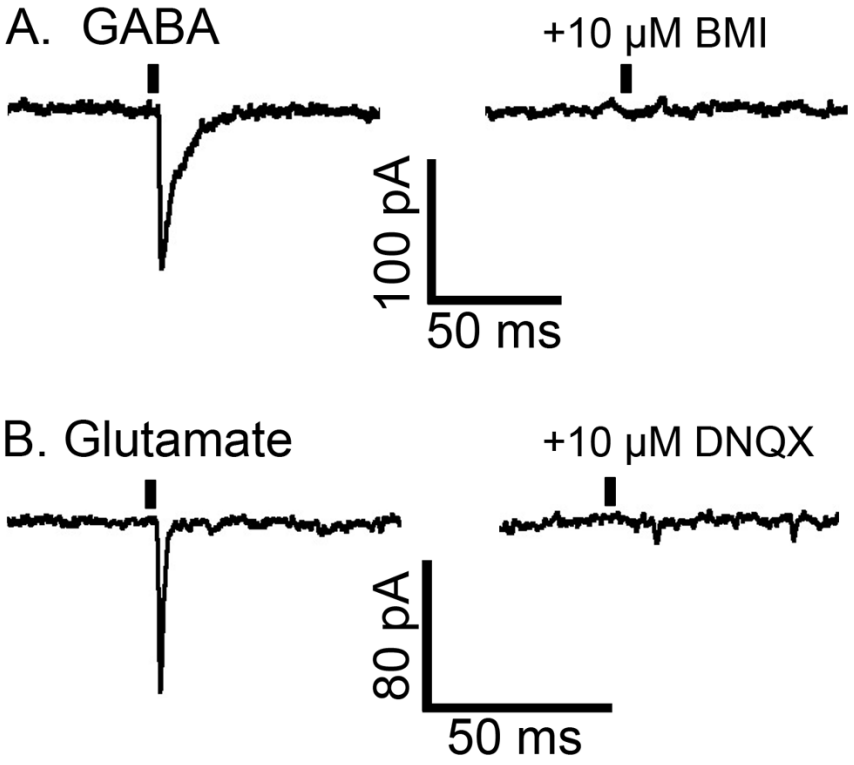

Figure 4.4. GABA and glutamate are released by NTS POMC-Cre neurons. Cells nearby ChR2-expressing NTS POMC-Cre cell bodies were patched while light evoking transmitter release. Both GABA and glutamate currents were observed (A-B, left). BMI and DNQX were able to ablate each current, respectively (A-B, right). Black marks indicate a short flash of blue light.

4.3 Final remarks

Altogether, this study adds substantial evidence to support the growing idea that amino acid transmitters are essential to proper function of the so-called first order feeding neurons. We have provided compelling evidence that glutamate and GABA are released from POMC cells within their native circuits, and are just beginning to understand the physiological relevance of this release. We also discovered that GABA release from NPY/AgRP cells is modulated based of feeding state, and that *in situ* hybridization for *Gad* mRNA matches these findings suggesting that mRNA may be a useful proxy for functional GABA release in this, and perhaps other brain regions. I speculate that the feeding and energy balance field will continue to discover the importance of amino acid transmitter release from first order feeding neurons. Sensing energy state through circulating factors and afferent inputs, and subsequently signaling for the proper behavioral and autonomic adjustments requires a finely tuned and complex system. The fast neurotransmission provided by amino acid transmitters is an important part of this system, and is exquisitely poised to work in concert with the relatively slower peptide release to maintain healthy physiology. With better understanding of every component that makes up the neural circuitry of energy homeostasis, we will be able to provide better tools to fight for the correction of a disrupted energy balance.

REFERENCES

Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, and Flier JS. (1996) Role of leptin in the neuroendocrine response to fasting. Nature. 18; 382(6588):250-2.

Aponte Y, Atasoy D, Sternson SM. (2011) AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat Neurosci, 14:351–355.

Apostolides PF and Trussell LO. (2013) Rapid, activity-independent turnover of vesicular transmitter content at a mixed glycine/GABA synapse. The Journal of neuroscience, 33, 4768-4781.

Arenkiel BR, Peca J, Davison IG, Feliciano C, Deisseroth K, Augustine GJ, Ehlers MD, and Feng G. (2007) In vivo light-induced activation of neural circuitry in transgenic mice expressing channelrhodopsin-2. Neuron, 54:205–218.

Asada, H., Kawamura, Y., Maruyama, K., Kume, H., Ding, R.G., Kanbara, N., Kuzume, H., Sanbo, M., Yagi, T. & Obata, K. (1997) Cleft palate and decreased brain gamma-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. Proceedings of the National Academy of Sciences of the United States of America, 94, 6496-6499.

Aschner B. (1912) Uber die Funktion der Hypophyse. Pflugers Arch 146: 1-146.

Atasoy D, Aponte Y, Su HH, Sternson SM (2008) A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. J Neurosci, 28:7025–7030.

Atasoy D, Betley JN, Li WP, Su HH, Sertel SM, Scheffer LK, Simpson JH, Fetter RD, and Sternson SM. (2014) A genetically specified connectomics approach applied to long-range feeding regulatory circuits. Nature Neuroscience, 17(12):1830-1839.

Atasoy D, Betley JN, Su HH, Sternson SM. (2012) Deconstruction of a neural circuit for hunger. Nature, 488:172–177.

Bagnol D, Lu XY, Kaelin CB, Day HEW, Ollmann M, Gantz I, Akil H, Barsh GS, and Watson SJ. (1999) Anatomy of an endogenous antagonist: Relationship between Agouti-related protein and proopiomelanocortin in brain. Journal of Neuroscience. 19(18):RC26.

Balland E, Dam J, Langlet F, Caron E, Steculorum S, Messina A, Rasika S, Falluel-Morel A, Anouar Y, Dehouck B, Trinquet E, Jockers R, Bouret SG, and Prévot V. (2014) Hypothalamic tanycytes are an ERK-gated conduit for leptin into the brain. Cell metab, 19:293–301.

Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V, Kenny CD, McGovern RA, Chua SC, Elmquist JK, and Lowell BB. (2004) Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron, 42(6):983-991.

Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD. (2005) Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell, 123:493–505.

Baver SB, Hope K, Guyot S, Bjørbaek C, Kaczorowski C, O'Connell KM. (2014) Leptin modulates the intrinsic excitability of AgRP/NPY neurons in the arcuate nucleus of the hypothalamus. J Neurosci, 34(16):5486-96.

Beier, KT, Saunders, A, Oldenburg, IA, Miyamichi, K, Akhtar, N, Luo, L, Whelan, SPJ, Sabatini, B, and Cepko, CL. (2011) Anterograde or retrograde transsynaptic labeling of CNS neurons with vesicular stomatitis virus vectors. PNAS, 108(37):15414-9.

Bertolini A, Tacchi R, Vergoni AV (2009) Brain effects of melanocortins. Pharmaco Res, 59:13–47.

Betley JN, Cao ZFH, Ritola KD, and Sternson SM. (2013) Parallel, redundant circuit organization for homeostatic control of feeding behavior. Cell, 155:1337–1350.

Betley JN, Xu S, Cao ZFH, Gong R, Magnus CJ, Yu Y, and Sternson SM. (2015) Neurons for hunger and thirst transmit a negative-valence teaching signal, Nature 521:180.

Biro, F.M. & Wien, M. (2010) Childhood obesity and adult morbidities. Am J Clin Nutr 91 [5], pp. 1499S-1505S.

Bouret SG, Draper SJ, and Simerly RB. (2004) Trophic action of leptin on hypothalamic neurons that regulate feeding. Science, 304:108– 110.

Bouret SG, Bates SH, Chen S, Myers MG Jr, Simerly RB. (2012) Distinct roles for specific leptin receptor signals in the development of hypothalamic feeding circuits. J Neurosci, 32:1244–1252.

Bowers, G., Cullinan, W.E. & Herman, J.P. (1998) Region-specific regulation of glutamic acid decarboxylase (GAD) mRNA expression in central stress circuits. The Journal of neuroscience : the official journal of the Society for Neuroscience, 18, 5938-5947.

Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. (2005) Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci, 8:1263–1268.

Brobeck JR, Tepperman J, Long CNH. (1943) Experimental hypothalamic hyperphagia in the albino rat. Yale J. Biol Med, 15: 831-853.

Bronstein DM, Schafer MK, Watson SJ, and Akil H. (1992) Evidence that beta-endorphin is synthesized in cells in the nucleus tractus solitarius: detection of POMC mRNA. Brain Res, 587:269–275.

Brown KS, Gentry RM, and Rowland NE. (1998) Central injection in rats of a-melanocyte-stimulating hormone analog: Effects on food intake and brain FOS. Regul Pept, 78:89–94.

Brown LM, Clegg DJ, Benoit SC, and Woods SC. (2006) Intraventricular insulin and leptin reduce food intake and body weight in C57BL/6j mice. Physiol Behav, 89: 687–691.

Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D, and Kahn CR. (2000) Role of brain insulin receptor in control of body weight and reproduction. Science, 289(5487):2122-5.

Buch TR, Heling D, Damm E, Gudermann T, and Breit A. (2009) Pertussis toxin-sensitive signaling of melanocortin-4 receptors in hypothalamic GT1-7 cells defines agouti-related protein as a biased agonist. J Biol Chem, 284:26411–26420.

Bultman SJ, Michaud EJ, Woychik RP. (1992) Molecular characterization of the mouse agouti locus. Cell, 71(7):1195-204.

Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M, and Cone RD. (2000) A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. Endocrinology, 141(9):3518-3521.

Carmagnola, S., Cantu, P., Penagini, R. (2005) Mechanoreceptors of the Proximal Stomach and Perception of Gastric Distension. The American Journal of Gastroenterology. 100, 1704–1710.

Carter, ME, Soden, ME, Zweifel, LS, and Palmiter, RD. (2013) Genetic identification of a neural circuit that suppresses appetite. Nature, 503(7474): 10.1038.

Chan, R.S. & Woo, J. (2010) Prevention of overweight and obesity: how effective is the current public health approach. Int J Environ Res Public Health 7 [3], pp. 765-83.

Chang GQ, Karatayev O, Davydova Z, Wortley K, and Leibowitz SF. (2005) Glucose injection reduces neuropeptide Y and agouti-related protein expression in the arcuate nucleus: A possible physiological role in eating behavior. Molecular Brain Research, 135(1- 2):69-80.

Chattopadhyaya, B., Di Cristo, G., Wu, C.Z., Knott, G., Kuhlman, S., Fu, Y., Palmiter, R.D. & Huang, Z.J. (2007) GAD67-mediated GABA synthesis and signaling regulate inhibitory synaptic innervation in the visual cortex. Neuron, 54, 889-903.

Chen Y, Lin Y, Kuo T, and Knight ZA. (2015) Sensory detection of food rapidly modulates arcuate feeding circuits. Cell, 160:829.

Chen YY, Pelletier G (1983) Demonstration of contacts between proopiomelanocortin neurons in the rat hypothalamus. Neurosci Lett 43:271–276.

Choudhury AI, Heffron H, Smith MA, Al-Qassab H, Xu AW, Selman C, Simmgen M, Clements M, Claret M, Maccoll G, Bedford DC, Hisadome K, Diakonov I, Moosajee V, Bell JD,

Clark JT, Kalra PS, Crowley WR, and Kalra, SP. (1984) Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology, 115:427–9.

Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P, and Guy-Grand B. (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature, 392: 398–401.

Coll AP (2007) Effects of pro-opiomelanocortin (POMC) on food intake and body weight: mechanisms and therapeutic potential? Clinical science (London, England□: 1979) 113:171–182.

Collin M, Backberg M, Ovesjo ML, Fisone G, Edwards RH, Fujiyama F, Meister B (2003) Plasma membrane and vesicular glutamate transporter mRNAs/proteins in hypothalamic neurons that regulate body weight. Eur J Neurosci 18:1265–1278.

Cone RD. (2005) Anatomy and regulation of the central melanocortin system. Nature Neuroscience, 8(5):571-578.

Cowley MA, Smart JL, Rubinstein M, Cordan MG, Diano S, Horvath TL, Cone RD, and Low MJ. (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature, 411(6836):480-484.

Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, and Horvath TL. (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron. 37(4):649-61.

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E., and Weigle, D.S. (2001) A Preprandial Rise in Plasma Ghrelin Levels Suggests a Role in Meal Initiation in Humans. Diabetes. 50 no. 8, 1714-1719.

Dennison CS, King CM, Dicken MS, and Hentges ST. (2015) Age-dependent changes in amino acid phenotype and the role of glutamate release from hypothalamic proopiomelanocortin neurons. J Comp Neurol, DOI: 10.1002/cne.23900.

de Souza FSJ, Nasif S, Lopez-Leal R, Levi DH, Low MJ, and Rubinsten M. (2011) The estrogen receptor alpha colocalizes with proopiomelanocortin in hypothalamic neurons and binds to a conserved motif present in the neuron-specific enhancer nPE2. European Journal of Pharmacology, 660(1):181-187.

Dicken, M.S., Tooker, R.E. & Hentges, S.T. (2012) Regulation of GABA and glutamate release from proopiomelanocortin neuron terminals in intact hypothalamic networks. The Journal of neuroscience : the official journal of the Society for Neuroscience, 32, 4042-4048.

DiRocco RJ and Grill HJ. (1979) The forebrain is not essential for sympathoadrenal hyperglycemic response to glucoprivation. Science, 204(4397):1112-4.

Ebbeling, C.B., Pawlak, D.B., and Ludwig, D. S. (2002) Childhood obesity: public-health crisis, common sense cure. Lancet 360 [9331], pp. 473-82.

Edwards, R.H. (2007) The neurotransmitter cycle and quantal size. Neuron, 55, 835-858.

Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, Bjorbaek C, Flier JS, Saper CB, Elmquist JK. (1999) Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron, 23(4):775-786.

Elmquist JK, Bjørbaek C, Ahima RS, Flier JS, Saper CB. (1998) Distributions of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol, 395(4):535-47.

Emmerson PJ, Miller RJ (1999) Pre- and postsynaptic actions of opioid and orphan opioid agonists in the rat arcuate nucleus and ventromedial hypothalamus in vitro. J Physiol 517 (Pt 2):431–445.

Erdheim J. (1904) Uber Hypophysenganggeschwülste und Hirncholesteatome. Sitz. Ber. Der Wiener Akad., III. Abt., Bd. 113: 537-726.

Erickson JC, Hollopeter G, Palmiter RD. (1996) Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. Science, 274:1704–1707.

Fan W, Boston BA, Kesterson RA, Hruby VJ, and Cone RD. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature, 385(6612):165-8.

Ferrini, F, Salio, C, Lossi, L, and Merighi, A. (2009) Ghrelin in Central Neurons. Curr Neuropharmacol, 7(1): 37-49.

Finkelstein, E.A., Trogdon, J.G., Cohen, J.W., and Dietz, W. Annual Medical Spending Attributable To Obesity: Payer-And Service-Specific Estimates. Health Affairs, 28, no.5 (2009) :w822-w831.

Friedman JM. (2009) Leptin at 14 y of age: an ongoing story. Am J Clin Nutr. 89(3):973S-979S.

Fröhlich A. (1901) Ein Fall von Tumor der Hypophysis cerebri ohne Akromegalie. Wiener klinische Rundschau, 15: 833–836; 906–908.

Gao Y, Tschop MH, Luquet S. (2014) Hypothalamic tanycytes: gatekeepers to metabolic control. Cell Metab, 19:173–5.

Garfield AS, Li C, Madara JC, Shah BP, Webber E, Steger JS, Campbell JN, Gavrilova O, Lee CE, Olson D, Elmquist JK, Tannous BA, Krashes MJ, and Lowell BB. (2015) A neural basis for melanocortin-4 receptor regulated appetite. Nat Neurosci, 18:863.

Gasnier B (2004) The SLC32 transporter, a key protein for the synaptic release of inhibitory amino acids. Pflügers Arch 447:756–759.

Ghamari-Langroudi, M, Digby, GJ, Sebag, JA, Millhauser, GL, Palomino, R, Matthews, R, Gillyard, T, Panaro, BL, Tough, IR, Cox, HM, Denton, JS, and Cone, RD. (2015) G-protein independent coupling of the MC4R to Kir 7.1 in hypothalamic neurons. Nature, 520(7545): 94-98.

Grill HJ, Kaplan JM. (2001) Interoceptive and integrative contributions of forebrain and brainstem to energy balance control. Int J Obes Relat Metab Disord, 25 Suppl 5():S73-7.

Grill HJ and Norgren R. (1978) Chronically decerebrate rats demonstrate satiation but not bait shyness. Science, 201(4352):267-9.

Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD. (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res. 48:23–29.

Hagan MM, Rushing PA, Pritchard LM, Schwartz MW, Strack AM, Van Der Ploeg LH, Woods SC, Seeley RJ. (2000) Long-term orexi- genic effects of AgRP-(83–132) involve mechanisms other than melanocortin receptor blockade. Am J Physiol Regul Integr Comp Physiol, 279:R47–R52.

Hahn TM, Breininger JF, Baskin DG, and Schwartz MW. (1998) Coexpression of agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci, 1:271–272.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. Science. 269(5223):543-6.

Hallschmid M, Benedict C, Schultes B, Fehm HL, Born J, Kern W. (2004) Intranasal insulin reduces body fat in men but not in women. Diabetes 53: 3024–3029.

Havel, P.J. (2001) Peripheral Signals Conveying Metabolic Information to the Brain: Short-Term and Long-Term Regulation of Food Intake and Energy Homeostasis. Experimental Biology and Medicine 226 963-77.

Hegadoren KM, O'Donnell T, Lanius R, Coupland NJ, Lacaze-Masmonteil N (2009) The role of beta-endorphin in the pathophysiology of major depression. Neuropeptides 43:341–353.

Hentges ST, Nishiyama M, Overstreet LS, Stenzel-Poore M, Williams JT, Low MJ (2004) GABA release from proopiomelanocortin neurons. J Neurosci 24:1578–1583.

Hentges ST, Otero-Corchon V, Pennock RL, King CM, Low MJ (2009) Proopiomelanocortin expression in both GABA and glutamate neurons. J Neurosci 29:13684–13690.

Hetherington A, and Ranson S. (1940) Hypothalamic lesions and adiposity in the rat. Anat Rec, 78:149–172.

Hetherington A, and Ranson S. (1942) The relation of various hypothalamic lesions to adiposity in the rat. J Comp Neurol, 76:475–499.

Holzer, P. and Farzi, A. (2014) Neuropeptides and the microbiota-gut-brain axis. Adv Exp Med Biol. 817:195-219.

Horvath TL, Bechmann I, Naftolin F, Kalra SP, and Leranth C. (1997) Heterogeneity in the neuropeptide Y-containing neurons of the rat arcuate nucleus: GABAergic and non-GABAergic subpopulations. Brain Res, 756:283–6.

Huszar D, Lynch CA, FairchildHuntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, and Lee F. (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell, 88(1):131-141.

I'Allemand, D., Wiegand, S., Reinehr, T., Muller, J., Wabitsch, M., Widhalm, K. and Holl, R. (2008): Cardiovascular risk in 26,008 European overweight children as established by a multicenter database. Obesity (Silver Spring) 16 [7], pp. 1672-9.

Ibrahim N, Bosch MA, Smart JL, Qiu J, Rubinstein M, Ronnekleiv OK, Low MJ, and Kelly MJ. (2003) Hypothalamic proopiomelanocortin neurons are glucose responsive and express K-ATP channels. Endocrinology, 144(4):1331-1340.

Ishibashi, H., Yamaguchi, J., Nakahata, Y. & Nabekura, J. (2013) Dynamic regulation of glycine-GABA co-transmission at spinal inhibitory synapses by neuronal glutamate transporter. The Journal of physiology, 591, 3821-3832.

Jarvie, B.C. & Hentges, S.T. (2012) Expression of GABAergic and glutamatergic phenotypic markers in hypothalamic proopiomelanocortin neurons. The Journal of comparative neurology, 520, 3863-3876.

John, J. (2010) Economic perspectives on pediatric obesity: impact on health care expenditures and cost-effectiveness of preventive interventions. Nestle Nutr Workshop Ser Pediatr Program 66. pp. 111-24.

Kahn BB, Flier JS. (2000) Obesity and insulin resistance. J Clin Invest. 106:473–81.

Kelly J, Alheid GF, Newberg A, Grossman SP. (1977) GABA stimulation and blockade in the hypothalamus and midbrain: effects on feeding and locomotor activity. Pharmacol Biochem Behav, 7, 523-541.

Kelly, T., Yang, W., Chen, C-S., Reynolds, K., and He, J. (2008) Global Burden of Obesity in 2005 and Projections to 2030. International Journal of Obesity 32.9, 1431-437.

Kennedy GC (1953) The role of depot fat in the hypothalamic control of food intake in the rat. Proc R Soc Lond B Biol Sci 140:578-92.

Kim, JD, Leyva, S, Diano, S. (2014) Hormonal regulation of the hypothalamic melanocortin system. Front Physiol, 9;5:480.

Kiss J, Csaba Z, Csáki A, Halász B (2005) Glutamatergic innervation of neuropeptide Y and pro-opiomelanocortin-containing neurons in the hypothalamic arcuate nucleus of the rat. Eur J Neurosci 21:2111–2119.

Kiss JZ, Williams TH (1983) ACTH-immunoreactive boutons form synaptic contacts in the hypothalamic arcuate nucleus of rat: evidence for local opiocortin connections. Brain Res 263:142–146.

Koch M and Horvath TL. (2014) Molecular and cellular regulation of hypothalamic melanocortin neurons controlling food intake and energy metabolism. Molecular Psychiatry, 19(7):752-761.

Kohl MM, Shipton OA, Deacon RM, Rawlins JNP, Deisseroth K, Paulsen O (2011) Hemisphere-specific optogenetic stimulation reveals left-right asymmetry of hippocampal plasticity. Nat Neurosci 14:1413–1415.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 402:656–660.

Kopelman PG, Hitman GA. (1998) Diabetes. Exploding type II. Lancet.352(Suppl 4):SIV5.

Krashes MJ, Koda S, Ye CP, Rogan SC, Adarns AC, Cusher DS, Maratos-Flier E, Roth BL, and Lowell BB. (2011) Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. Journal of Clinical Investigation, 121(4):1424-1428.

Krashes MJ, Shah BP, Koda S, and Lowell BB. (2013) Rapid versus delayed stimulation of feeding by the endogenously released AgRP neuron mediators GABA, NPY, and AgRP. Cell Metabolism, 18(4):588-595.

Lam DD, Attard CA, Mercer AJ, Myers MGJ, Rubinstein M, Low MJ. (2015) Conditional expression of Pomc in the Lepr-positive subpopulation of POMC neurons is sufficient for normal energy homeostasis and metabolism. Endocrinology, 156(4):1292-1302.

Langlet F, Levin BE, Luquet S, Mazzone M, Messina A, Dunn-Meynell AA, Balland E, Lacombe A, Mazur D, Carmeliet P, Bouret SG, Prevot V, and Dehouck B. (2013) Tanycytic VEGF-A boosts blood–hypothalamus barrier plasticity and access of metabolic signals to the arcuate nucleus in response to fasting. Cell Metab,17:607–17.

Lau, C.G. & Murthy, V.N. (2012) Activity-dependent regulation of inhibition via GAD67. The Journal of neuroscience : the official journal of the Society for Neuroscience, 32, 8521-8531.

Lazarus, M.S., Krishnan, K. & Huang, Z.J. (2013) GAD67 Deficiency in Parvalbumin Interneurons Produces Deficits in Inhibitory Transmission and Network Disinhibition in Mouse Prefrontal Cortex. Cerebral cortex.

Levine AS and Morley J. (1984) Neuropeptide Y: A potent inducer of consummatory behavior in rats. Peptides, 5:1025–1029.

Lin JY, Lin MZ, Steinbach P, Tsien RY (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. Biophys J 96:1803–1814.

Lindner D, Stichel J, and Beck-Sickinger AG (2008) Molecular recognition of the NPY hormone family by their receptors. Nutrition, 24 (9): 907–17.

Martin DL, Tobin AJ (2000) Mechanisms controlling GABA synthesis and degradation in the brain in GABA in the nervous system, eds Martin DL, Olsen RW (Lippincott Williams & Wilkins, Philadelphia), 25–41.

Mason, G.F., Martin, D.L., Martin, S.B., Manor, D., Sibson, N.R., Patel, A., Rothman, D.L. & Behar, K.L. (2001) Decrease in GABA synthesis rate in rat cortex following GABA-transaminase inhibition correlates with the decrease in GAD(67) protein. Brain research, 914, 81-91.

Mathews, G.C. & Diamond, J.S. (2003) Neuronal glutamate uptake Contributes to GABA synthesis and inhibitory synaptic strength. The Journal of neuroscience : the official journal of the Society for Neuroscience, 23, 2040-2048.

Mauras, N., Delgiorno, C., Kollman, C., Bird, K., Morgan, M., Sweeten, S., Balagopal, P. and Damaso, L. (2010) Obesity without established comorbidities of the metabolic syndrome is associated with a proinflammatory and prothrombotic state, even before the onset of puberty in children. J Clin Endocrinol Metab 95 [3], pp. 1060-8.

McCarthy, M.M. (1995) Frank A. Beach Award. Functional significance of steroid modulation of GABAergic neurotransmission: analysis at the behavioral, cellular, and molecular levels. Hormones and behavior, 29, 131-140.

McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. Nature 389:870–876.

Meister B. (2007) Neurotransmitters in key neurons of the hypothalamus that regulate feeding behavior and body weight. Physiol Behav, 92, 263–271.

Merali, Z., McIntosh, J., Anisman, H. (1999) Role of bombesin-related peptides in the control of food intake. Neuropeptides 33(5):376-86.

Michaud EJ, Bultman SJ, Klebig ML, van Vugt MJ, Stubbs LJ, Russell LB, Woychik RP. (1994) A molecular model for the genetic and phenotypic characteristics of the mouse lethal yellow (Ay) mutation. Proc Natl Acad Sci, 91(7):2562-6.

Mitchell V, Bouret S, Beauvillain JC, Schilling A, Perret M, Kordon C, Epelbaum J. (2001) Comparative distribution of mRNA encoding the growth hormone secretagogue-receptor (GHS-R) in Microcebus murinus (Primate, lemurian) and rat forebrain and pituitary. J Comp Neurol. 429:469–489.

Mizuno TM and Mobbs CV. (1999) Hypothalamic Agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. Endocrinology, 140(2):814-817.

Mohr B. (1840) Hypertrophie der Hypophysis cerebri und dadurch bedingter Druck auf die Hirngrundflache, insbesondere auf die Sehnerven, das Chiasma derselben und den linkseitigen Hirnschenkel. Wschr. ges. Heilk., 6: 565-71.

Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Ware- ham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, and O'Rahilly S. (1997) Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature, 387 903-8.

Moran, T.H. (2009) Gut peptides in the control of food intake. Int J Obes (Lond). 33 Suppl 1:S7-10.

Mouginot D, Kombian SB, Pittman QJ (1998) Activation of presynaptic GABAB receptors inhibits evoked IPSCs in rat magnocellular neurons in vitro. J Neurophysiol 79:1508–1517.

Mountjoy KG. (2015) Pro-Opiomelanocortin (POMC) neurones, POMC-derived peptides, melanocortin receptors and obesity: How understanding of this system has changed over the last decade. Journal of Neuroendocrinology, 27(6):406-418.

Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, and Cone RD. (1994) Localization of the melanocortin-4 receptor (MC4-r) in neuroendocrine and autonomic control circuits in the brain. Molecular Endocrinology, 8(10):1298-1308.

Muller-Riemenschneider, F., Reinhold, T., Berghofer, A. and Willich, S.N. (2008) Health-economic burden of obesity in Europe. Eur J Epidemiol 23 [8], pp. 499-509.

Myers, M.G., Cowley, M.A., and Munzberg, H. (2008) Mechanisms of Leptin Action and Leptin Resistance. Annual Review of Physiology 70.1, 537-56.

Myers, MG and Olson, DP. (2012) Central nervous system control of metabolism. Nature, 491(7424):357-363.

Nemeroff CB, Lipton MA, Kizer JS. (1978) Models of neuroendocrine regulation: use of monosodium glutamate as an investigational tool. Dev Neurosci, 1(2):102-9.

Newton, A.J., Hess, S., Paeger, L., Vogt, M.C., Fleming Lascano, J., Nillni, E.A., Bruning, J.C., Kloppenburg, P. & Xu, A.W. (2013) AgRP innervation onto POMC neurons increases with age and is accelerated with chronic high-fat feeding in male mice. Endocrinology, 154, 172-183.

Nickolls SA, Fleck B, Hoare SR, and Maki RA. (2005) Functional selectivity of melanocortin 4 receptor peptide and nonpeptide agonists: Evidence for ligand-specific conformational states. J Pharmacol Exp Ther, 313:1281–1288.

Nogueiras R, Tschöp MH, and Zigman, JM. (2008) Central nervous system regulation of energy metabolism: ghrelin versus leptin. Ann N Y Acad Sci. 1126:14–19.

Obata, K., Hirono, M., Kume, N., Kawaguchi, Y., Itohara, S. & Yanagawa, Y. (2008) GABA and synaptic inhibition of mouse cerebellum lacking glutamate decarboxylase 67. Biochemical and biophysical research communications, 370, 429-433.

Obici S, Feng Z, Karkanias G, Baskin DG, and Rossetti L. (2002) Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. Nat Neurosci, 5(6):566-72.

Ogden CL, Carroll MD, Kit BK, Flegal KM. (2014) Prevalence of Childhood and Adult Obesity in the United States, 2011-2012. JAMA. 311(8):806-814.

Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, and Barsh GS. (1997) Antagonism of central melanocortin receptors in vitro and in vivo by agouti- related protein. Science, 278:135–8.

Overstreet LS, Hentges ST, Bumaschny VF, de Souza FS, Smart JL, Santangelo AM, Low MJ, Westbrook GL, Rubinstein M. (2004) A transgenic marker for newly born granule cells in dentate gyrus. J Neurosci 24(13):3251-9.

Ovesjö ML, Gamstedt M, Collin M, Meister B (2001) GABAergic nature of hypothalamic leptin target neurones in the ventromedial arcuate nucleus. J Neuroendocrinol 13:505–516.

Padilla SL, Carmody JS, and Zeltser LM. (2010) Pomc-expressing progenitors give rise to antagonistic neuronal populations in hypothalamic feeding circuits. Nature Medicine, 16(4):403-405.

Padilla SL, Reef D, and Zeltser LM. (2012) Defining POMC neurons using transgenic reagents: impact of transient POMC expression in diverse immature neuronal populations. Endocrinology, 153:1219–1231.

Page AJ, Martin CM, Blackshaw LA. (2002) Vagal mechanoreceptors and chemoreceptors in mouse stomach and esophagus. J Neurophysiol. 87(4):2095-103.

Parker, J.A. & Bloom, S.R. (2012) Hypothalamic neuropeptides and the regulation of appetite. Neuropharmacology, 63, 18-30.

Patz, S., Wirth, M.J., Gorba, T., Klostermann, O. & Wahle, P. (2003) Neuronal activity and neurotrophic factors regulate GAD-65/67 mRNA and protein expression in organotypic cultures of rat visual cortex. The European journal of neuroscience, 18, 1-12.

Pedersen, A.A., Videbaek, N., Skak, K., Petersen, H.V. & Michelsen, B.K. (2001) Characterization of the rat GAD67 gene promoter reveals elements important for basal transcription and glucose responsiveness. DNA sequence : the journal of DNA sequencing and mapping, 11, 485-499.

Penicaud L, Larue-Achagiotis C, Le Magnen J. (1983) Endocrine basis for weight gain after fasting or VMH lesion in rats. Am J Physiol, 245(3):E246-52.

Pennock RL and Hentges ST (2011) Differential expression and sensitivity of presynaptic and postsynaptic opioid receptors regulating hypothalamic proopiomelanocortin neurons. J Neurosci 31:281–288.

Perello, M., Scott, M.M., Sakata, I., Lee, C.E., Chuang, J.-C., Osborne-Lawrence, S., Rovinsky, S.A., Elmquist, J.K., and Zigman, J.M. (2012). Functional Implications of Limited Leptin Receptor and Ghrelin Receptor Coexpression in the Brain. The Journal of Comparative Neurology, 520(2), 281–294.

Petreanu L, Huber D, Sobczyk A, Svoboda K (2007) Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. Nat Neurosci 10:663–668.

Pinto S, Roseberry AG, Liu H, Diano S, Shanabrough M, Cai X, Friedman JM, Horvath TL. (2004) Rapid rewiring of arcuate nucleus feeding circuits by leptin. Science 304:110–115.

Poggioli R, Vergoni AV, and Bertolini A. (1986) ACTH-(1-24) and alpha-MSH antagonize feeding behavior stimulated by kappa opiate agonists. Peptides, 7(5):843-848.

Porter, T.G. & Martin, D.L. (1985) Chelidonic acid and other conformationally restricted substrate analogues as inhibitors of rat brain glutamate decarboxylase. Biochemical pharmacology, 34, 4145-4150.

Qian S, Chen H, Weingarth D, Trumbauer ME, Novi DE, Guan X, Yu H, Shen Z, Feng Y, Frazier E, Chen A, Camacho RE, Shearman LP, Gopal-Truter S, MacNeil DJ, Van der Ploeg LH, and Marsh DJ. (2002) Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. Mol Cell Biol, 22:5027–5035.

Qiu J, Fang Y, Rnnekleiv OK, and Kelly MJ. (2010) Leptin Excites Proopiomelanocortin Neurons via Activation of TRPC Channels. Journal of Neuroscience, 30(4):1560-1565.

Reyes BA, Glaser JD, Magtoto R, Van Bockstaele EJ (2006) Pro-opiomelanocortin colocalizes with corticotropin- releasing factor in axon terminals of the noradrenergic nucleus locus coeruleus. Eur J Neurosci 23:2067–2077.

Rimvall, K. & Martin, D.L. (1992) Increased intracellular gamma-aminobutyric acid selectively lowers the level of the larger of two glutamate decarboxylase proteins in cultured GABAergic neurons from rat cerebral cortex. Journal of neurochemistry, 58, 158-166.

Rimvall, K. & Martin, D.L. (1994) The level of GAD67 protein is highly sensitive to small increases in intraneuronal gamma-aminobutyric acid levels. Journal of neurochemistry, 62, 1375-1381.

Sanz E, Quintana A, Deem JD, Steiner RA, Palmiter RD, and McKnight GS. (2015) Fertility-regulating Kiss1 neurons arise from hypothalamic Pomc-expressing progenitors. Journal of Neuroscience, 35(14):5549-5556.

Schaeffer M, Langlet F, Lafont C, Molino F, Hodson DJ, Roux T, Lamarque L, Verdié P, Bourrier E, Dehouck B, Banères JL, Martinez J, Méry PF, Marie J, Trinquet E, Fehrentz JA, Prévot V, and Mollard P. (2013) Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons. Proc Natl Acad Sci USA, 110:1512–7.

Schneggenburger, R., Meyer, A.C. & Neher, E. (1999) Released fraction and total size of a pool of immediately available transmitter quanta at a calyx synapse. Neuron, 23, 399-409.

Schwartz, M.W., Baskin, D.G., Kaiyala, K.J., and Woods, S.C. (1999) Model for the Regulation of Energy Balance and Adiposity by the Central Nervous System. The American Journal of Clinical Nutrition. 69, 584-96.

Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG. (1997) Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes, 46(12):2119-2123.

Schwartz, M.W., Sipols, A.J., Grubin, C.E. & Baskin, D.G. (1993) Differential effect of fasting on hypothalamic expression of genes encoding neuropeptide Y, galanin, and glutamic acid decarboxylase. Brain research bulletin, 31, 361-367.

Schwarzer, C. & Sperk, G. (1995) Hippocampal granule cells express glutamic acid decarboxylase-67 after limbic seizures in the rat. Neuroscience, 69, 705-709.

Scott MM, Lachey JL, Sternson SM, Lee CE, Elias CF, Friedman JM, and Elmquist JK. (2009) Leptin targets in the mouse brain. Comp Neurol, 514(5):518-32.

Seeley RJ, Grill HJ, Kaplan JM. (1994) Neurological dissociation of gastrointestinal and metabolic contributions to meal size control. Behav Neurosci, 108(2):347-52.

Shah BP, Vong L, Olson DP, Koda S, Krashes MJ, Ye C, Yang Z, Fuller PM, Elmquist JK, and Lowell BB. (2014) MC4R-expressing glutamatergic neurons in the paraventricular hypothalamus regulate feeding and are synaptically connected to the parabrachial nucleus. Proc Natl Acad Sci USA, 111:13193–13198.

Shutter JR, Graham M, Kinsey AC, Scully S, Lüthy R, and Stark KL. (1997) Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. Genes Dev, 11(5):593-602.

Sims JS and Lorden JF. (1986) Effect of paraventricular nucleus lesions on body weight, food intake and insulin levels. Behav Brain Res, 22(3):265-81.

Smith PE. (1930) Hypophysectomy and a replacement therapy in the rat. American Journal of Anatomy, 45(2): 205-273.

Sonksen P and Sonksen J (2000) Insulin: understanding its action in health and disease. Br J Anaesth 85 (1): 69–79.

Speakman JR, Batterham RL, Barsh GS, Ashford ML, and Withers DJ. (2005) The role of insulin receptor substrate 2 in hypothalamic and beta cell function. J Clin Invest, 115(4):940-50.

Stanley BG, Chin A, Leibowitz SF. (1985) Feeding and drinking elicited by central injection of neuropeptide Y: Evidence for a hypo- thalamic site(s) of action. Brain Res Bull, 14:521–524.

Sternson SM, Atasoy D. (2014) Agouti-Related Protein Neuron Circuits That Regulate Appetite. Neuroendocrinology, 100(2-3):95-102.

Strobel A, Issad T, Camoin L, Ozata M, and Strosberg AD. (1998) A leptin missense mutation associated with hypogonadism and morbid obesity. Nat Genet, 18: 213–5.

Svendsen, B. and Holst, J.J. (2015) Regulation of gut hormone secretion. Studies using isolated perfused intestines. Peptides, 11 pii: S0196-9781.

Takahashi, K.A. & Cone, R.D. (2005) Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. Endocrinology, 146, 1043-1047.

Tao Y-X. (2014) Constitutive activity in melanocortin-4 receptor: biased signaling of inverse agonists. Advances in pharmacology, 70:135-154.

Tatemoto K, Carlquist M, and Mutt V. (1982) Neuropeptide Y – a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature, 296:659–60.

Thanawala, M.S. & Regehr, W.G. (2013) Presynaptic calcium influx controls neurotransmitter release in part by regulating the effective size of the readily releasable pool. The Journal of neuroscience : the official journal of the Society for Neuroscience, 33, 4625-4633.

Thiele TE, van Dijk G, Yagaloff KA, Fisher SL, Schwartz M, Burn P, and Seeley RJ. (1998) Central infusion of melanocortin agonist MTII in rats: Assessment of c-fos expression and taste aversion. Am J Physiol 274:R248–R254.

Titolo D, Cai F, and Belsham DD. (2006) Coordinate regulation of neuropeptide Y and agouti-related peptide gene expression by estrogen depends on the ratio of estrogen receptor 120 (ER) alpha to ER beta in clonal hypothalamic neurons. Molecular Endocrinology, 20(9):2080-2092.

Tong, Q., Ye, C., Jones, J.E., Elmquist, J.K. & Lowell, B.B. (2008) Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. Nature neuroscience, 11, 998-1000.

Tong Q, Ye C, McCrimmon RJ, Dhillon H, Choi B, Kramer MD, Yu J, Yang Z, Christiansen LM, Lee CE, Choi CS, Zigman JM, Shulman GI, Sherwin RS, Elmquist JK, Lowell BB (2007) Synaptic glutamate release by ventromedial hypothalamic neurons is part of the neurocircuitry that prevents hypoglycemia. Cell Metab 5:383–393.

Tsujii S and Bray GA. (1991) GABA-related feeding control in genetically obese rats. Brain Research, 540, 48-54.

van den Pol AN. (2003) Weighing the role of hypothalamic feeding neurotransmitters. Neuron, 40, 1059–1061.

van der Lely AJ, Tschöp M, Heiman ML, and Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. (2004) Endocr Rev, 25(3):426-57.

van Vliet-Ostaptchouk JV, Hofker MH, van der Schouw YT, Wijmenga C, and Onland-Moret NC. (2009) Genetic variation in the hypothalamic pathways and its role on obesity. Obes Rev, 10, 593-609.

Vong L, Ye C, Yang Z, Choi B, Chua S, Lowell BB (2011) Leptin Action on GABAergic Neurons Prevents Obesity and Reduces Inhibitory Tone to POMC Neurons. Neuron 71:142–154.

Wang DQ, He XB, Zhao Z, Feng QR, Lin R, Sun Y, Ding T, Xu FQ, Luo MM, and Zhan C. (2015) Whole-brain mapping of the direct inputs and axonal projections of POMC and AgRP neurons. Frontiers in Neuroanatomy. 9:40.

Wang, L., Tu, P., Bonet, L., Aubrey, K.R. & Supplisson, S. (2013) Cytosolic transmitter concentration regulates vesicle cycling at hippocampal GABAergic terminals. Neuron, 80, 143-158.

Wang QP, Guan JL, Shioda S (2001) Immunoelectron microscopic study of beta-endorphinergic synaptic innervation of GABAergic neurons in the dorsal raphe nucleus. Synapse 42:234–241.

Wang, Y. & Lobstein, T. (2006) Worldwide trends in childhood overweight and obesity. Int J Pediatr Obes 1 [1], pp. 11-25.

Wardlaw SL. (2011) Hypothalamic proopiomelanocortin processing and the regulation of energy balance. European Journal of Pharmacology. 660(1):213-219.

Wei, J. & Wu, J.Y. (2008) Post-translational regulation of L-glutamic acid decarboxylase in the brain. Neurochemical research, 33, 1459-1465.

WHO (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser 894, pp. i-xii, 1-253.

Williams, R.W. & Rakic, P. (1988) Three-dimensional counting: an accurate and direct method to estimate numbers of cells in sectioned material. The Journal of comparative neurology, 278, 344-352.

Woods SC, Lotter EC, McKay LD, and Porte Jr D. (1979) Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature 282, 503–505.

Wu, Q, Boyle, MP, and Palmiter, RD. (2009) Loss of GABAergic Signaling by AgRP Neurons to the Parabrachial Nucleus Leads to Starvation. Cell, 137(7):1225-1234.

Wu, Q, Clark, MS, and Palmiter, RD. (2012) Deciphering a neuronal circuit that mediates appetite. Nature, 483(7391):594-U112.

Wu, Q., Howell, M.P., Cowley, M.A. & Palmiter, R.D. (2008) Starvation after AgRP neuron ablation is independent of melanocortin signaling. Proceedings of the National Academy of Sciences of the United States of America, 105, 2687-2692.

Wu, Q, Howell, MP, and Palmiter, RD. (2008) Ablation of neurons expressing agouti-related protein activates fos and gliosis in postsynaptic target regions. J Neurosci, 28(37):9218-26.

Wu, Q, Lemus, MB, Stark, R, Bayliss, JA, Reichenbach, A, Lockie, SH, and Andrews, ZB. (2014) The temporal pattern of cfos activation in hypothalamic, cortical, and brainstem nuclei in response to fast- ing and refeeding in male mice. Endocrinology, 155:840–853.

Xu Y, Elmquist JK, and Fukuda M. (2011) Central nervous control of energy and glucose balance: focus on the central melanocortin system. Ann N Y Acad Sci, 1243:1-14.

Xu Y, Jones JE, Kohno D, Williams KW, Lee CE, Choi MJ, Anderson JG, Heisler LK, Zigman JM, Lowell BB, and Elmquist JK. (2008) 5-HT(2C)Rs Expressed by Pro-Opiomelanocortin Neurons Regulate Energy Homeostasis. Neuron, 60(4):582-589.

Yach, D., Stuckler, D. and Brownell, K.D. (2006) Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. Nat Med 12 [1], pp. 62-6.

Yamada, M.H., Nishikawa, K., Kubo, K., Yanagawa, Y. & Saito, S. (2012) Impaired glycinergic synaptic transmission and enhanced inflammatory pain in mice with reduced expression of vesicular GABA transporter (VGAT). Molecular pharmacology, 81, 610-619.

Yang, Y., Atasoy, D., Su, H.H. & Sternson, S.M. (2011) Hunger states switch a flip-flop memory circuit via a synaptic AMPK-dependent positive feedback loop. Cell, 146, 992-1003.

Yaswen L, Diehl N, Brennan MB, and Hochgeschwender U. (1999) Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nature Medicine, 5(9):1066-1070.

Yee CL, Wang Y, Anderson S, Ekker M, Rubenstein JLR (2009) Arcuate nucleus expression of NKX2.1 and DLX and lineages expressing these transcription factors in neuropeptide Y(+), proopiomelanocortin(+), and tyrosine hydroxylase(+) neurons in neonatal and adult mice. J Comp Neurol 517:37–50.

Zhan C, Zhou J, Feng Q, Zhang JE, Lin S, Bao J, Wu P, and Luo M. (2013) Acute and long-term suppression of feeding behavior by POMC neurons in the brainstem and hypothalamus, respectively. J Neurosci, 33(8):3624-32.

Zhang Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994) Positional cloning of the mouse obese gene and its human homologue Nature 372:425-32.

Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK. (2006) Expression of ghrelin receptor mRNA in the rat and the mouse brain. J Comp Neurol. 494:528–548.

APPENDIX I

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October 2, 2014

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Pages	10
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Expected completion date	Dec 2015
Expected size (number of pages)	100
Requestor Location	Matthew Dicken 1917 S Shields St Apt. M8 FORT COLLINS, CO 80521 United States Attn: Matthew S Dicken
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Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 5
Will you be translating?	No
Expected completion date	Dec 2015
Expected size (number of pages)	100
Requestor Location	Matthew Dicken 1917 S Shields St Apt. M8 FORT COLLINS, CO 80521 United States Attn: Matthew S Dicken
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LIST OF ABBREVIATIONS

AA	Amino acid
AAV	Adeno-associated virus
aBNST	Anterior bed nucleus of the stria terminalis
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
AgRP	Agouti-related protein
ARC	Arcuate nucleus of the hypothalamus
BMI	Body mass index in introduction, Bicuculline methiodide, a $GABA_A$ receptor antagonist, elsewhere
CCK	Cholecystokinin, an anorexigenic gut peptide
CGP	(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid, a $GABA_B$ receptor antagonist
ChR2	Channelrhodopsin-2, a light-gated cation channel
CNO	Clozapine-N-oxide, main DREADD agonist
CTAP	D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2, a μ-opioid receptor-specific antagonist
CVO	Circumventricular organ
db/db	Leptin receptor-deficient mouse strain
DAMGO	[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin, μ-opioid receptor-specific agonist
DMH	Dorsomedial nucleus of the hypothalamus
DNQX	6,7-dinitroquinoxaline-2,3(1H,4H), an AMPA and kainite channel antagonist
DOR	δ-opioid receptor
DPDPE	[D-$Pen^{2,5}$]enkephalin, a δ-opioid receptor-specific agonist
DREADD	Designer receptor exclusively activated by designer drug
GABA	γ-aminobutyric acid

GAD	Glutamate decarboxylase, an enzyme that converts glutamate to GABA
GAT	Plasma membrane GABA transporter
Gad1 and *2*	Messenger RNA for GAD67 and 65, respectively
GLP-1	Glucagon-like peptide-1, an anorexigenic gut peptide
GPCR	G-protein coupled receptor
GRP	Gastrin-releasing peptide
hrGFP	Humanized Renilla reniformis green fluorescent protein
IP	Intraperitoneal
IP_3	Inositol trisphosphate
IR	Immunoreactivity
KOR	κ-opioid receptor
LepRb	Leptin receptor, active long-form version
LH	Lateral nucleus of the hypothalamus
ME	Median eminence
MOR	μ-opioid receptor
MSH	Melanocyte stimulating hormone
MTII	Melanotan-II, a melanocortin receptor agonist
Nor-BNI	Nor-binaltorphimine, a κ-opioid receptor-specific antagonist
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius or Nucleus of the solitary tract
ob/ob	Leptin-deficient mouse strain
PBS	Phosphate-buffered saline
PBN	Parabrachial nucleus
PLC	Phospholipase C
PLP	Pyridoxal 5' –phosphate
POMC	Proopiomelanocortin
PPR	Paired-pulse ratio
PSC	Postsynaptic Current—may be inhibitory (IPSC) or excitatory (EPSC)
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY or Peptide tyrosine tyrosine, an anorexigenic gut peptide
RRP	Readily releasable pool

TTX	Tetrodotoxin
U69593	(+)-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide, a κ-opioid receptor-specific agonist
vGAT	Vesicular GABA transporter
VMH	Ventromedial nucleus of the hypothalamus