Identification of hypothalamic neural circuits regulating glucose and energy homeostasis

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Abstract

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Diabetes and obesity are among the most common and costly health issues facing modern humans. The World Health Organization (WHO) estimates that obesity rates have tripled in the last 50 years: as of 2016, nearly 40% of adults are overweight, and 13% are obese. Given the strong association between obesity and type 2 diabetes (T2D), a compelling need exists for improved understanding of their pathophysiology. Accumulating evidence shows that the brain plays a critical role in the regulation of energy and glucose homeostasis. However, the underlying neurocircuitry by which the brain regulates energy balance and glycemia is narrowly understood, due to the exceedingly complex and highly interconnected nature of mammalian neurocircuitry. In this dissertation, I will discuss how, using a combination of engineered mouse models and genetically encoded tools, along with sophisticated metabolic phenotyping, we advanced our understanding of brain neurocircuits regulating energy homeostasis.

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Chapter 1 Overview

Obtaining sufficient energy is imperative to survival. Given this basic, fundamental need shared by all organisms, the intake, storage, and utilization of energy are highly regulated processes. Yet many questions remain as to how these processes are coordinately regulated to maintain energy balance. For example, how does a hungry animal determine whether to defend its territory against an intruder, or whether to flee? Or, when faced with harsh climate and cold conditions, what allows an animal to adapt and increase its heat production to maintain core body temperature? For an organism to survive and reproduce in the face of energetically costly demands of a constantly changing environment, there is a need for both assessment of available energy, and redirection of energy to needed tissues. The rapidity and flexibility with which adaptations in animal behavior must occur in the face of external challenges clearly implicates a significant role for the central nervous system (CNS).

The CNS is uniquely positioned to receive afferent information regarding energetic status via circulating nutrients and hormones, as well as via sensory neural input from peripheral organs. Thus, the CNS has the unique capacity to i) sense the available energy in the circulation and stored in peripheral tissues, ii) rapidly integrate external cues of metabolic demand (e.g., predator danger, cold exposure), and iii) transduce this information into coordinated neuroendocrine and autonomic outflow to partition energy amongst the organs needed to counter the challenge. Such a powerful system ensures survival and fitness by mitigating energy wasted on nonessential processes. Moreover, the CNS is highly plastic, enabling an organism to learn and continuously adapt to new challenges, aided by the memory of prior experience. When framed in such a way, it should come as no surprise that dysregulations in CNS systems may contribute to the pathophysiology of metabolic disease, including diabetes and obesity.

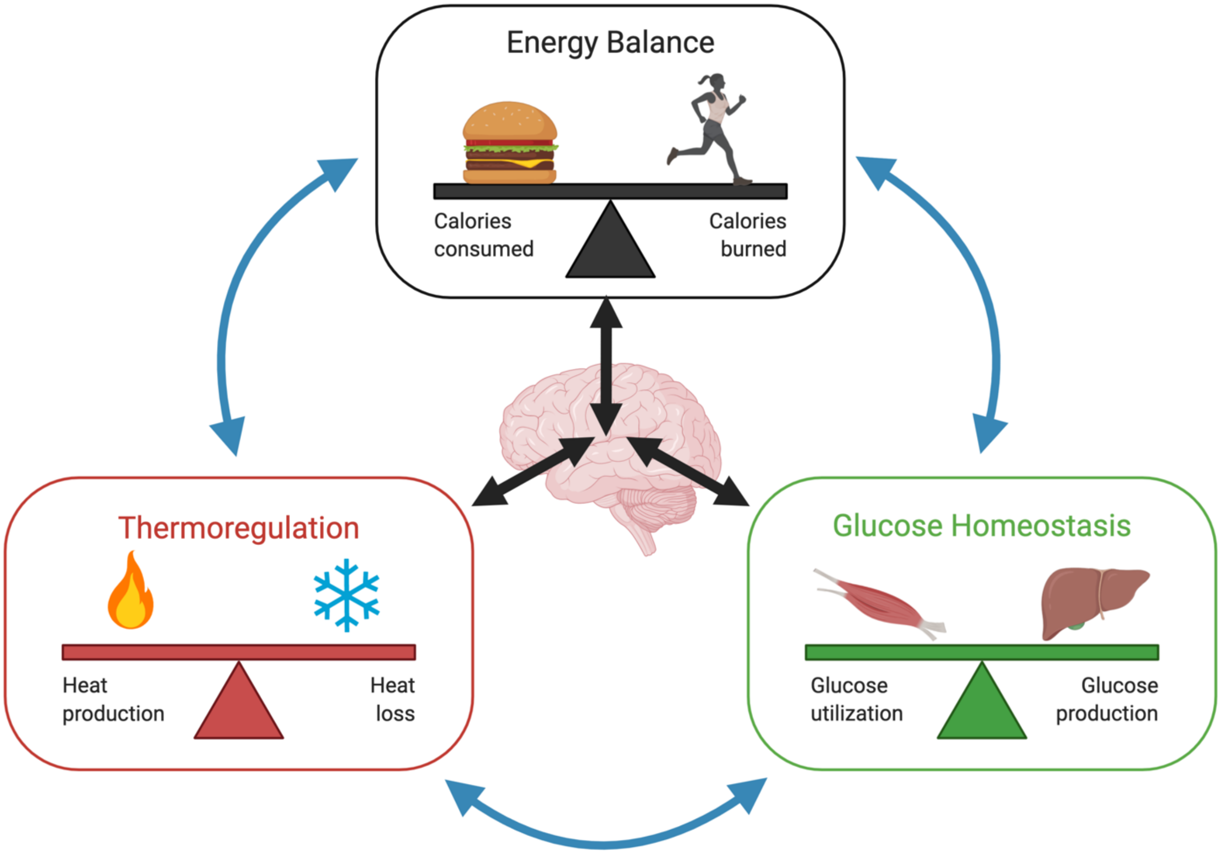

Figure 1.1 Integrative control of energy balance, glucose homeostasis, and thermoregulation by the CNS.
Maintenance of body adiposity, blood glucose levels, and core temperature within narrow physiological limits requires balance within each system that is adaptable, and informed by the status of the other systems. A cold challenge, for instance, requires increased glucose to thermogenic tissues (increasing glucose utilization) to maintain core temperature. To avoid hypoglycemia, glucose production must correspondingly increase, thus preserving glucose homeostasis. Energy homeostasis is preserved in a similar manner, as food intake increases to offset the increased energetic demands of the body. Adapted from Brown J, Scarlett J, and Schwartz MW (2019) Rethinking the role of the brain in glucose homeostasis and diabetes pathogenesis. *Journal of Clinical Investigation*. Created with BioRender.com.

Indeed, seminal work from physiologists over a century ago pointed to the brain as a critical regulator of both adiposity and blood sugars, with lesions of the brain producing defects in either or both. However, the discovery of insulin and the characterization of the pancreatic islet at the turn of the 20th century spawned a new islet-centric view of diabetes, and interest in a role for the brain dwindled. In contrast, a role for the CNS in the control of feeding and adiposity remained prominent, owing in part due to early findings that the brain is a major target for the adipocyte-derived hormone leptin, deficiency of which produces robust hyperphagia and obesity. Interestingly, the subsequent observation that intracerebroventricular (icv) leptin corrects not just hyperphagia and adiposity in both genetic and acquired models of leptin deficiency, but also diabetic hyperglycemia independently of changes in food intake, has helped renew interest in the idea of targeting the brain to treat diabetes. In fact, this observation further suggests that the brain circuits regulating adiposity, energy expenditure, and glucose homeostasis, may at least partially overlap at the level of individual neuronal populations. While evidence supporting this hypothesis continues to accumulate, the precise mechanisms by which the brain not only detects and integrates input on the status of energy balance, but also coordinates efferent autonomic and neuroendocrine responses, are only beginning to be functionally mapped and characterized.

Greatly aiding in this endeavor is the rapid development of sophisticated neuroscience tools that enable reversible, cell-type specific interrogation of circuit function. The advent of genetically-encoded molecular sensors, in combination with optical photometry approaches (e.g., fiber photometry, two-photon imaging), has enabled in vivo measurement of neuronal, and astrocytic, activity patterns. In addition, manipulating cellular activity can be achieved via optogenetic and pharmacogenetic approaches. Further, the highly specific and targeted delivery of these neuroscientific tools is made possible by the continuously expanding genetic toolkit, including genetically engineered mouse models, DNA recombinases (e.g., Cre and Flp recombinases), and pseudotyped viruses to deliver a genetic payload to nearly any cell of interest. The specificity of these approaches is further informed and refined by information gleaned from single-cell transcriptional, translational, epigenetic, and proteomic profiling studies. The collaborative, open-access platforms in which these data are accessible have profoundly altered the landscape of science, making interdisciplinary approaches more possible and more powerful than ever. Thus, while the complex heterogeneity and interconnectedness of neural circuits within the brain present a major challenge to understanding animal behavior, ever-evolving and advancing techniques have provided the means to meet this challenge.

Equipped with these powerful tools, I embarked on my thesis work nearly 5 years ago with the primary goal of identifying hypothalamic neurocircuits regulating glycemia. Specifically, I sought to characterize the neurocircuitry by which neurons expressing nitric oxide synthase-1 in the hypothalamic ventromedial nucleus (VMN^{NOS1} neurons) contribute to the defense against hypoglycemia. Upon completion of this body of work, and guided by serendipitous findings, I shifted my focus to characterizing hypothalamic neurocircuits that regulate feeding behaviors, with a particular focus on leptin-receptor neurons within the dorsomedial hypothalamus (DMH^{LepR} neurons). In the following chapters, I relate my findings and expound on their impact, clinical implications, and context within the greater field of the central regulation of energy balance. I begin in Chapter 2 by providing a literature review summarizing the state of knowledge regarding the central regulation of energy homeostasis, particularly in feeding and appetite. In

Chapter 3, I provide a brief overview of the role of the CNS in the regulation of glycemia by modulating islet secretion, and consider whether disruption in the coordination between brain and islet contributes to the pathogenesis of diabetes. Chapter 4 details my work demonstrating a physiological role for VMN^{NOS1} neurons in the glucagon response to hypoglycemia, and characterizing the neurocircuitry by which these neurons drive glycemic and defensive effects. Chapter 5 details work expanding on these findings, in which I assisted with a project exploring whether activity of a parabrachial nucleus (PBN) to VMN neurocircuit contributes to diabetic hyperglucagonemia. In Chapter 6, I relay ongoing, unpublished findings demonstrating a novel role for DMH^{LepR} neurons in circadian feeding behaviors. Finally, in Chapter 7 I summarize and discuss the impact and limitations of these findings, and important future directions for the field. Together, this work has uncovered novel insights into the hypothalamic regulation of both energy and glucose homeostasis that may contribute to the development of new therapies for the prevention and treatment of diabetes and obesity.

Chapter 2 Central Regulation of Feeding

Text and figures modified from Faber and Morton, Encyclopedia of Biological Chemistry, 2020.

Introduction

While it seems intuitive to ascribe decisions about when, what, and how much to eat to the perception of hunger with which we are all familiar, such decisions are in fact informed by numerous biological, environmental, and social cues, most of which we lack conscious awareness of. Variables ranging from emotional state and cultural values to circadian rhythm and learned flavor preferences, in coordination with humoral and neural signals that reflect the internal nutritional state, can all influence the number of calories we consume in any given meal or any given day. Consequently, the number of calories consumed on a day-to-day basis tends to be quite variable. Yet body weight and fat content tend to remain relatively stable over time, owing to the biological process known as energy homeostasis, which precisely coordinates energy intake and energy expenditure such that body fat stores remain stable.

The central nervous system (CNS) plays a key role in energy homeostasis through its unique ability to sense and integrate myriad hormonal, autonomic, and nutritive signals regarding both long-term energy stores and short-term energy availability, and transduce them into coordinated adjustments in energy expenditure and energy intake. An example of this process is the adaptive response to energy excess, or positive energy balance. During such periods (*e.g.,* overfeeding leading to short-term weight gain), the brain engages responses that both suppress food intake and stimulate energy expenditure, so as to limit excess weight gain and return body fat mass to its original level. Conversely, during times of negative energy balance (*e.g.,* prolonged fast or caloric restriction), the brain triggers adaptive responses that increase food intake while also reducing energy expenditure, limiting weight loss and promoting the recovery of fat stores to their original value. This response is not only relevant to the physiology of body weight control – it underlies the frustration dieters experience trying to maintain weight loss.

In normal-weight individuals, the energy homeostasis system robustly defends a healthy level of adiposity, yet the high prevalence of obesity worldwide illustrates that this system is susceptible to pathophysiological disruption, such that increased levels of body adiposity are defended in obesity (Fothergill *et al.*, 2016). While the molecular mechanisms underlying regulatory dysfunction remain unclear, an evolutionary basis has been suggested. Namely, in early humans, weight loss during times of food scarcity posed a threat to survival and reproductive success. Consequently, natural selection tended to favor genetic traits that enabled our ancestors to consume and store calories as efficiently as possible to safeguard against future periods of food scarcity (Neel, 1962). However, as humans and our way of life rapidly evolved, these once protective homeostatic survival systems became a liability, being poorly-adapted to our modern sedentary lifestyle with its abundant and readily-available nutrient dense foods. As the health and economic burdens of obesity continue to rise (Blüher, 2019), they highlight the need for an improved understanding of mechanisms underlying the physiological control of feeding.

In this chapter, we describe the mechanisms whereby the CNS senses and responds to hormonal and nutrient-related information and engages pathways that regulate food intake and energy expenditure to maintain energy homeostasis.

Mechanisms Regulating Energy Homeostasis

Adiposity Negative Feedback

More than 60 years ago, Kennedy first proposed the concept that energy homeostasis is achieved by a system whereby signals of energetic status circulate in proportion to body fat mass and inform the brain of current energy stores via a negative feedback loop (Kennedy, 1953). To date, two hormones have been postulated to provide this adiposity-related negative feedback to the brain: the pancreatic hormone, insulin, and the adipocyte hormone, leptin (see Figure 2.1). In support of this concept, both insulin (Bagdade, Bierman and Porte, 1967) and leptin (Considine *et al.*, 1996) circulate in proportion to body fat stores and therefore, in conditions of negative energy balance such as prolonged fasting and/or weight loss, plasma insulin and leptin levels are reduced. Furthermore, leptin and insulin enter the CNS in proportion to their plasma level (Baura *et al.*, 1993; Schwartz *et al.*, 1996), where they act on their receptors in key brain areas that regulate

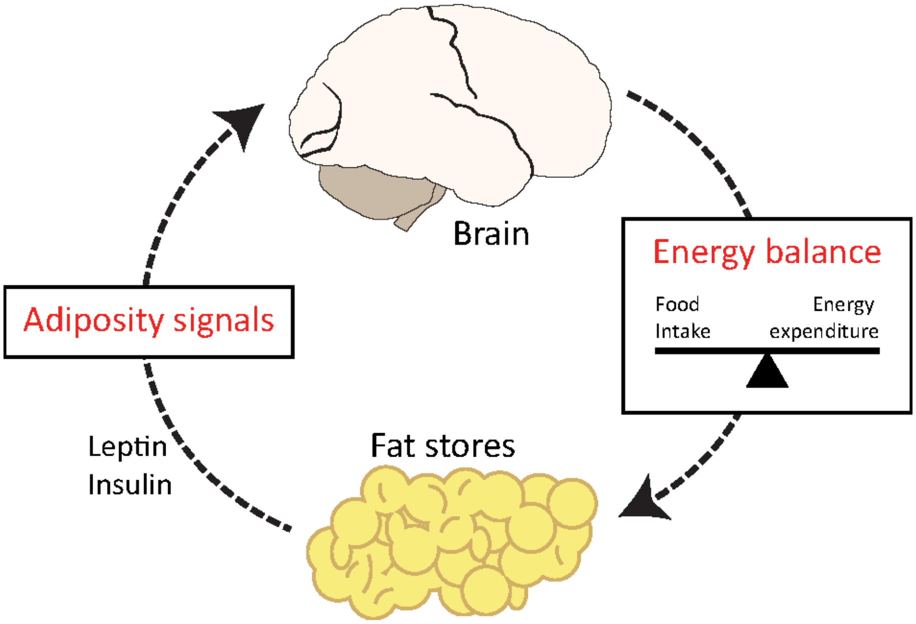

Figure 2.1 Negative feedback regulation of body fat mass.

energy intake and energy expenditure (Baskin, Breininger and Schwartz, 1999; Baskin, Figlewicz Lattemann, *et al.*, 1999). Finally, administration of either leptin (Campfield *et al.*, 1995) or insulin (Woods *et al.*, 1979) directly into the brain reduces food intake and induces weight loss while conversely, deficiency of either hormone or impairment in their respective neuronal signaling pathways promotes hyperphagia and obesity (Zhang Y *et al.*, 1994; Sipols, Baskin and Schwartz, 1995). As an adiposity signal, however, leptin appears to play the dominant role.

The discovery of leptin and the findings that leptin deficiency (i.e. *ob/ob*) caused obesity in both mice (Zhang Y *et al.*, 1994) and humans (Montague *et al.*, 1997), an effect ameliorated by leptin treatment (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Weigle *et al.*, 1995; Farooqi *et al.*, 2007), established its role in the control of food intake and body weight, and sparked hopes for leptin as a new therapeutic for obesity treatment. However, clinical trials of leptin as a weight loss agent were largely unsuccessful (Heymsfield *et al.*, 1999). Since leptin is secreted in proportion to body fat, and since most forms of obesity are associated with hyperleptinemia, obesity is associated with leptin resistance, rather than a lack of leptin. Although several possible explanations have been put forth for the development of obesity-associated leptin resistance, including an impaired ability of circulating leptin to cross the blood-brain barrier (BBB) (Myers *et al.*, 2010) or altered signal transduction as a consequence of hypothalamic inflammation and gliosis (Thaler and Schwartz, 2010; Thaler *et al.*, 2012), both the mechanisms underlying leptin resistance and its role in obesity pathogenesis remain active areas of investigation.

Satiety Perception

While adiposity negative feedback signals provide input to the brain regarding stored energy availability, the CNS also detects and responds to short-term, meal-related signals. Among these nutrient- and gut-derived satiety signals are peptides such as peptide YY (PYY) (Batterham *et al.*, 2002), glucagon-like peptide 1 (GLP1) (Turton *et al.*, 1996), and cholecystokinin (CCK) (Gibbs, Young and Smith, 1973) which are secreted from specialized gastrointestinal epithelial cells in response to ingested nutrients. Whereas CCK is secreted from the proximal gut (Wren and Bloom, 2007), both GLP1 and PYY are secreted predominantly from enteroendocrine cells in the distal intestine (Psichas, Reimann and Gribble, 2015) as food passes through the gastrointestinal tract. Consequently, the primary effect of each of these satiety peptides is to reduce food intake by, as their name suggests, promoting satiety after a meal (Waise, Dranse and Lam, 2018).

In contrast to the satiating effects of these peptides, the orexigenic hormone ghrelin is secreted from enteroendocrine cells of the stomach. Administration of ghrelin either centrally or peripherally increases food intake, and increases fat mass when administered chronically (Tschop, Smiley and Heiman, 2000). Moreover, plasma ghrelin levels increase with fasting, rising sharply before a meal, and are suppressed by feeding (Cummings *et al.*, 2001), suggesting a critical role for this hormone to promote the drive to eat.

Ultimately, these various humoral signals along with other stimuli (*e.g.,* mechanical stretch and distention of the gut) activate afferent fibers of the vagus nerve that communicate information about the type and volume of ingested nutrients to the brain via projections to the nucleus of the solitary tract (NTS) in the hindbrain (Waise, Dranse and

Lam, 2018). However, while satiety peptides reliably promote meal termination, the effect is relatively short-lived. Thus, repeated administration of CCK has no effect on body weight despite its potent effect on satiety, as the reduction of individual meal size is offset by a compensatory increase in the number of meals, such that the total amount of calories consumed is not affected (West, Fey and Woods, 1984). These findings highlight the inherently limited potential of meal-related satiety peptides *per se* to promote therapeutic weight loss, although GLP-1 analogues can be effective if their duration of action is increased from a matter of minutes to hours or days (Knudsen and Lau, 2019).

How then is energy intake adjusted to meet long-term energy needs on a meal-to-meal basis? Growing evidence suggests that brain sensitivity to satiety signals can be adjusted in response to changes of nutritional state (Figlewicz *et al.*, 1986; Emond *et al.*, 1999), such that meal size is sensitive to long-term changes in body adiposity. Consistent with this idea is evidence that leptin-mediated food intake suppression is characterized by a reduction of meal size (Kahler *et al.*, 1998) via its ability to augment the satiety response to CCK (Barrachina *et al.*, 1997). Conversely, in conditions of reduced (*e.g.,* during prolonged fasting or weight loss) or deficient leptin signaling (*e.g.,* in *ob/ob* mice or Koletsky rat models of obesity), the sensitivity to satiety signals such as CCK is reduced, leading to increases of both meal size and overall energy intake (McMinn *et al.*, 2000; Morton *et al.*, 2005). In parallel, during weight loss elevated plasma ghrelin levels, in combination with reduced insulin and leptin signaling, contribute to the regain of lost weight (Cummings *et al.*, 2002). This coupling of leptin to satiety perception involves the activation of leptin receptors not only in the hindbrain (Grill *et al.*, 2002), but also in hypothalamic regions which in turn project to the same hindbrain areas, modulating their response to satiety-related input (Morton *et al.*, 2005). Together, these observations highlight the process whereby adiposity signals regulate food intake by modulating brain sensitivity to short-acting satiety signals, thereby linking changes of fat stores to adaptive changes of intake on a meal-to-meal basis.

Reward

The palatability of food is also a key determinant of the decision to eat, irrespective of energy status. This type of feeding has been described as “non-homeostatic,” or hedonic, (Berthoud, 2006). However, as obtaining sufficient food is vital for the survival of all animals, it should come as no surprise that physiological mechanisms exist to ensure that food intake is a highly motivated and rewarding behavior. In this sense, food reward is considered an integral component of energy homeostasis, with increasing evidence suggesting that coordination of homeostatic and hedonic systems is fundamental to feeding control (Rossi and Stuber, 2018).

Concordant with this hypothesis, the absence (or anticipation) of food heightens food-related reward perception whereas conversely, reduced perception of food reward is a fundamental component of satiety. In line with this, brain imaging studies in both rodents (Kenny, 2011) and humans (Schur *et al.*, 2009) have shown that visual presentation of highly hedonic foods activates brain areas involved in energy homeostasis, reward, and cognitive processing. Conversely, after a meal, the effect of visual food cues to activate regional reward centers within the brain is greatly reduced (Mehta *et al.*, 2012), suggesting that satiety signals suppress feeding in part by blunting the rewarding properties of palatable foods.

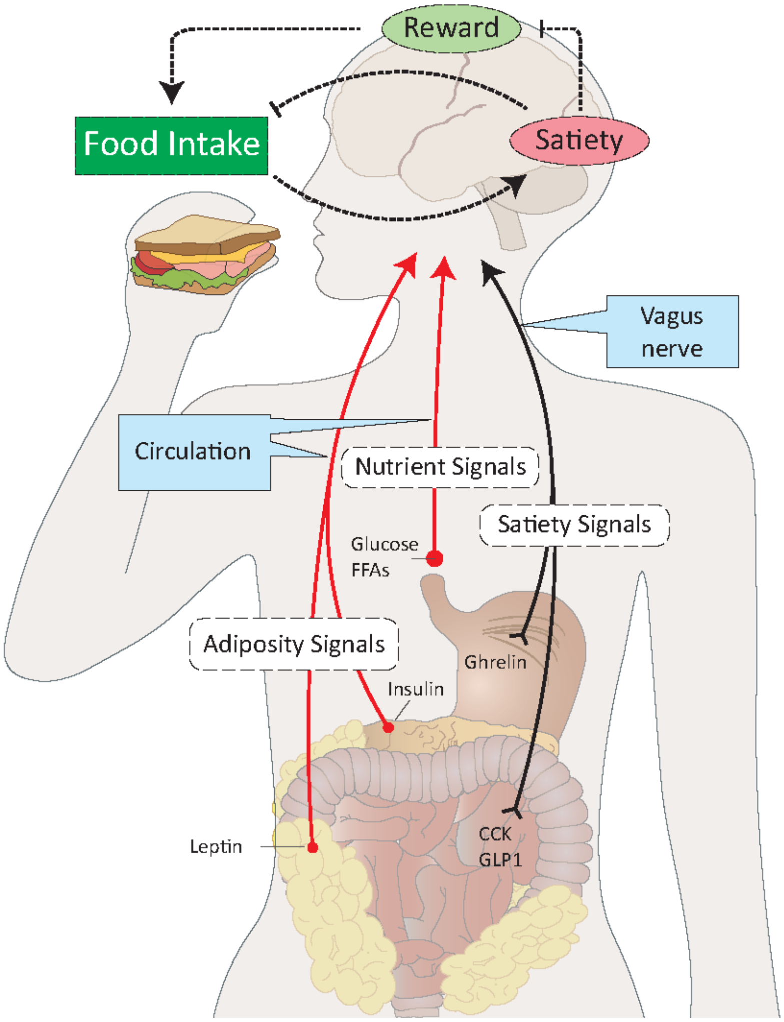

Figure 2.2 CNS regulation of energy homeostasis.

Adiposity signals also modulate the rewarding properties of food. For instance, in both rodent and human studies, reduced or deficient leptin signaling increases both the rewarding properties of food and the motivation to work for food (Figlewicz, Naleid and Sipols, 2007). Notably, these responses are blunted by intracerebroventricular (icv) injection of leptin (Fulton, Woodside and Shizgal, 2000) or insulin (Figlewicz *et al.*, 2004). Furthermore, clinical findings show that in humans with congenital leptin deficiency, leptin treatment decreases food intake in part by diminishing the perception of food reward, an effect associated with reduced neuronal activity in brain areas that regulate food reward (i.e. ventral striatum) in response to food-related stimuli (Farooqi *et al.*, 2007). Rooted within these collective observations is a highly integrative and powerful control system by which the brain modulates feeding behavior: in response to increased input from adiposity negative feedback signals, the CNS dampens reward perception while enhancing sensitivity to satiety signals, while the opposite is true of conditions in which adiposity negative feedback to the brain is reduced. In this way, changes of body fat stores are compensated for over long time intervals by coordinated, reciprocal adjustments of both food reward and satiety perception.

Allostasis

While adiposity negative feedback signals play a critical role in energy homeostasis and the biological defense of body fat mass, energy balance and feeding behaviors can also be regulated by anticipatory, feed-forward mechanisms, commonly referred to as allostasis. Allostasis is defined as the process whereby an organism engages adaptive changes that defend the level of a regulated parameter in response to new or changing environments – before the regulated parameter itself has changed. It is a more efficient type of control system than homeostasis, in that it *predicts* metabolic needs in response to changing external input and adjusts behavior to meet these anticipated needs; by comparison, homeostatic responses are typically engaged in response to a change of the defended parameter, rather than in anticipation of this occurring (Ramsay and Woods, 2014; Schulkin and Sterling, 2019). For example, in response to cold exposure, heat production (and thus energy expenditure) must increase rapidly to prevent a destabilizing fall in body temperature; to avoid weight loss, energy intake must rise in parallel to fuel this increased metabolic demand. Both responses appear to involve allostasis, since the increases of both heat production and food intake during cold exposure occur so quickly, and with such coordination, that changes of body temperature and fat mass are not detected (Kaiyala *et al.*, 2015). Although further study is needed, these observations support the idea that changing thermal input from the skin elicits anticipatory changes in energy balance that provide the first line of defense when exposure to a changing thermal environment challenges the stability of body temperature and fat stores.

As our understanding of these brain systems continues to grow, it is becoming increasingly apparent that both allostatic and homeostatic systems function in coordination not only to predict metabolic needs, but to also correct any errors in response to negative feedback. The integration of these two systems is proposed to greatly increase not just the survival of an animal, but overall fitness by minimizing wasted energy.

Hypothalamic Neurocircuits Regulating Energy Balance

The hypothalamus has long been recognized as a key region governing energy homeostasis, as lesions of various hypothalamic regions were shown to cause profound disturbances of food intake and body weight (either hyperphagia and obesity or reduced food intake and body weight, depending on the specific hypothalamic area targeted), while electrical stimulation of the same brain areas elicited the opposite effects. While these seminal studies gave rise to testable hypotheses regarding the role of distinct hypothalamic areas in the control of energy balance, early neuroscience techniques lacked the specificity, precision, and temporal control to parse underlying mechanisms.

Recent years have seen the rapid development of new neuroscience technologies, enabling a deeper understanding of the neurocircuits that regulate feeding. Among these advances are the combination of mouse genetics with optogenetic (Boyden *et al.*, 2005) and chemogenetic (*e.g.,* Designer Receptors Exclusively Activated by Designer Drugs (DREADD)) technologies that utilize light and drugs, respectively, to activate or inhibit defined neuronal subsets in free-living, conscious, adult mice (Zhang *et al.*, 2007; Roth, 2016). Moreover, greater insights into how these neuronal populations are regulated *in vivo* has been revealed with the use of fiber photometry, a system that uses genetically encoded calcium indicators to record the neuronal activity of genetically defined subpopulations in freely moving animals.

Arcuate Nucleus

The hypothalamic arcuate nucleus (ARC) emerged early as a brain area important in the regulation of energy homeostasis. Situated adjacent to the floor of the third ventricle and the median eminence (a circumventricular organ that lacks a functional blood-brain barrier), the ARC is well-positioned to sense cues of energy status, including the humoral signals leptin and insulin, both directly via the circulation and indirectly via synaptic input from other brain areas. Within the ARC are two distinct neuronal populations that exert potent, opposing effects on food intake and energy expenditure in response to hormonal- and nutrient-related signals.

POMC Neurons

Among leptin-sensitive ARC neuronal populations involved in energy homeostasis are those that express proopiomelanocortin (POMC). POMC is a precursor polypeptide that is cleaved to generate, among other products, the anorexigenic neuropeptide α-melanocyte stimulating hormone (α-MSH). On release from axon terminals, α-MSH binds to and activates downstream melanocortin receptors (MC3R/MC4R) (Fan *et al.*, 1997) in the ventromedial (VMN) and paraventricular (PVN) nuclei, as well as hindbrain areas such as the nucleus of the solitary tract (NTS), to inhibit food intake and increase energy expenditure, thereby promoting weight loss. A role for melanocortin neurons in energy homeostasis is further substantiated by the observations that 1) POMC neurons are potently stimulated by leptin (Cheung, Clifton and Steiner, 1997; Cowley *et al.*, 2001), 2) hypothalamic expression of *Pomc* is reduced in conditions of reduced or deficient leptin signaling, an effect reversed by leptin treatment (Schwartz *et al.*, 1997), 3) leptin-induced anorexia requires melanocortin signaling (Seeley *et al.*, 1997), and 4) genetic or pharmacological inhibition of either POMC neurons or downstream signaling via melanocortin receptors (e.g. MC4R deficiency) causes obesity in humans (Krude *et al.*, 1998; Yeo *et al.*, 1998) as well as rodents (Fan *et al.*, 1997; Anderson *et al.*, 2016). In fact, genetic variants within the leptin-melanocortin signaling pathway are the most frequent cause of monogenic obesity in humans (Fairbrother *et al.*, 2018). Collectively, these observations raise the possibility that the melanocortin pathway has promise as a novel therapeutic target for obesity prevention and treatment, and MC4R agonists have been used to treat human obesity secondary to reduced melanocortin signaling. Ongoing research is identifying other leptin-responsive neurocircuits that also contribute to normal energy homeostasis.

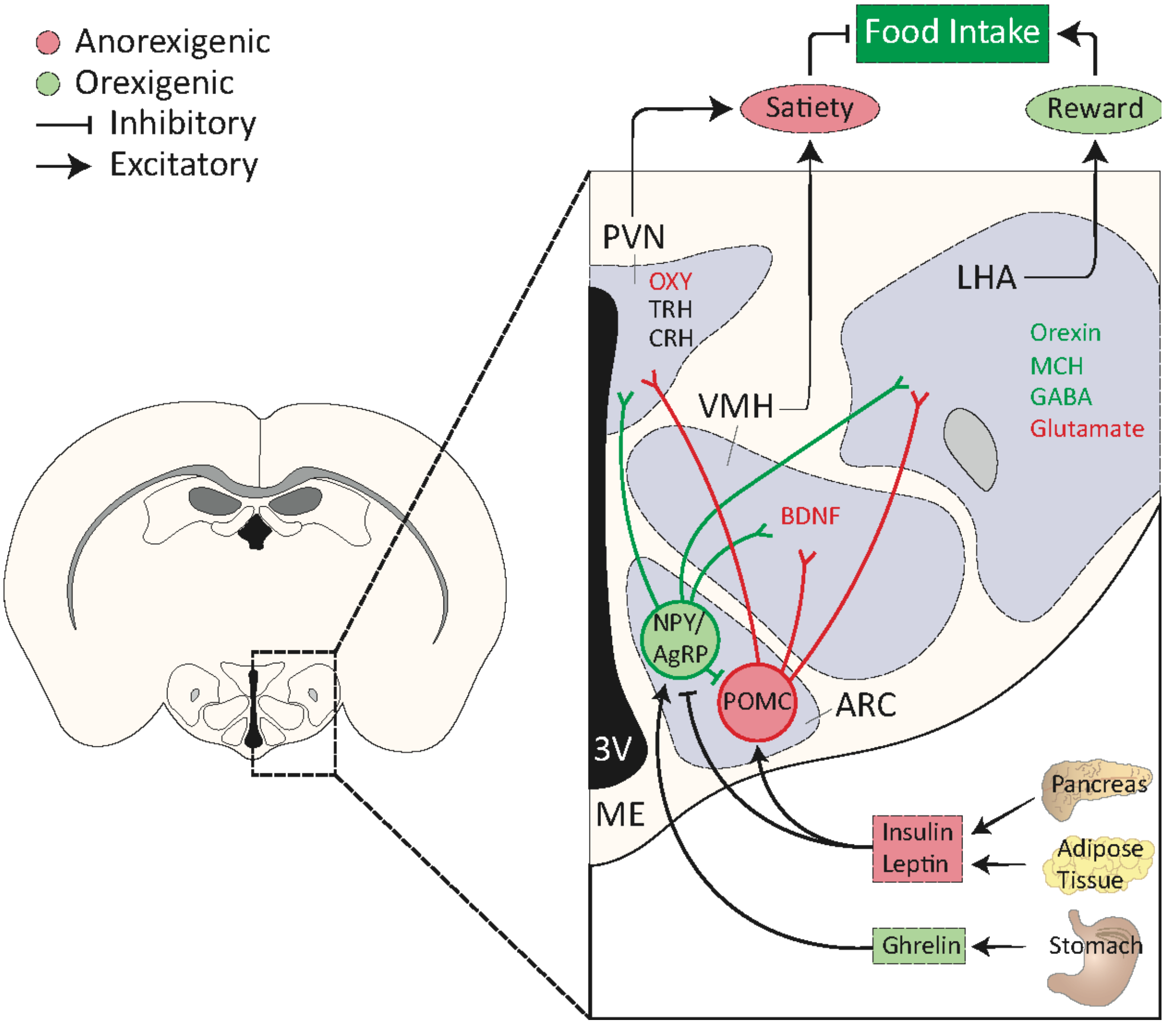

Figure 2.3 Hypothalamic neurocircuitry regulating energy homeostasis

AgRP Neurons

Situated adjacent to POMC neurons in the ARC is a distinct neuronal subset that co-expresses neuropeptide Y (NPY), agouti-related protein (AgRP, an endogenous antagonist of melanocortin signaling), as well as the inhibitory neurotransmitter γ-aminobutyric acid (GABA), (hereafter referred to as NPY/AgRP neurons) (Hahn *et al.*, 1998). NPY is among the most potent orexigenic agents and acts via NPY Y1 and Y5 receptors to rapidly (*i.e.,* within minutes) and robustly increase food intake (Clark *et al.*, 1984), and repeated or continuous icv administration of NPY induces sustained hyperphagia and increased body fat accumulation (Stanley *et al.*, 1986). The co-secreted peptide AgRP is an inverse agonist of MC3R/MC4R that reduces melanocortin signaling by directly competing with α-MSH binding. Thus, in contrast to α-MSH, icv administration of either AgRP or pharmacological melanocortin receptor antagonists induces a sustained increase in food intake, albeit of slower-onset than NPY, along with a reduction of energy expenditure (Hagan *et al.*, 2000) that also promotes positive energy balance and weight gain.

Unlike POMC neurons, NPY/AgRP neurons are inhibited by adiposity negative feedback signals leptin and insulin (Schwartz *et al.*, 2000), and are activated by ghrelin (Cowley *et al.*, 2003). Therefore, in conditions of negative energy balance, NPY/AgRP neurons are activated, whereas POMC neurons are inhibited. In addition, NPY/AgRP neurons also inhibit POMC neurons directly through the release of the inhibitory neurotransmitter, GABA (Cowley *et al.*, 2001). Together, the effect of weight loss to activate this combination of responses serves to reduce neuronal melanocortin signaling, thus promoting a state of positive energy balance until lost weight has been recovered.

With recent advances in neuroscience technologies, our understanding of the physiological role of these ARC neuronal populations in feeding and behavior has continued to evolve. As might be predicted, optogenetic (Aponte, Atasoy and Sternson, 2011) and chemogenetic (Krashes, Koda, ChianPing Ye, *et al.*, 2011) activation of NPY/AgRP neurons is sufficient to rapidly and potently stimulate feeding, irrespective of nutritional state. Moreover, activation of NPY/AgRP neurons increases food seeking behavior as well as the motivation to work for food (Krashes, Koda, ChianPing Ye, *et al.*, 2011), while conversely, silencing NPY/AgRP neurons reduces food intake in fasted mice (Krashes, Koda, ChianPing Ye, *et al.*, 2011). Concordant with the timescale of feeding induced by icv delivery of these peptides, recent opto- and chemogenetic studies suggest that both NPY and GABA mediate the rapid food intake associated with NPY/AgRP neuron activation (Chen *et al.*, 2019), while AgRP signaling at downstream melanocortin receptors is implicated in a slower, sustained increase in food intake (Krashes, Koda, ChianPing Ye, *et al.*, 2011; Krashes *et al.*, 2013). Taken together, these findings demonstrate that NPY/AgRP neuron activation is sufficient to drive an anabolic, energy-sparing behavioral output through multiple mechanisms. In contrast, activation of POMC neurons using opto- or chemogenetic techniques, as expected, reduces food intake, an effect that requires intact melanocortin receptor signaling (Aponte, Atasoy and Sternson, 2011), whereas inhibition of these neurons has the opposite effect. Notably, these feeding effects occur over hours, not minutes, further supporting a role for AgRP and α-MSH signaling at downstream melanocortin receptors in the slower output, long-term control of feeding (Krashes, Lowell and Garfield, 2016).

While both NPY/AgRP and POMC neurons are regulated by feedback signals related to energy stores, recent evidence suggests that these neurons are also regulated by feed-forward mechanisms that promote homeostasis by anticipating future need. Among feed-forward signals are cues from the environment (e.g. the sight or smell of food), as well as the presence of nutrients within the gut (Chen *et al.*, 2015; Beutler *et al.*, 2017). Illustrating this point, in fasted mice, NPY/AgRP neurons are inhibited upon food presentation, prior to meal initiation, whereas POMC neurons are activated (Chen *et al.*, 2015). While such a finding may initially seem at odds with the orexigenic and anorexigenic drive of these neurons, respectively, it suggests that, in anticipation of caloric sufficiency after a meal, NPY/AgRP and POMC neuronal activities are inversely modulated by sensory detection of food cues, consistent with an allostatic regulation of their function to restrict meal-size in accordance with overall energy status. In support of this idea, in sated mice, the effect of food presentation to inhibit NPY/AgRP neurons and to activate POMC cells is abolished (Chen *et al.*, 2015). Interestingly, sated mice still respond to the presentation of highly palatable food (e.g chocolate)(Chen *et al.*, 2015), suggesting that hedonic or rewarding aspects of feeding behaviors can, in some cases, override satiety and adiposity feedback signals to drive food intake. Taken together, these findings demonstrate that homeostatic, negative feedback, and allostatic feed-forward mechanisms coordinately influence NPY/AgRP and POMC neuronal function such that moment-to-moment activity of these cell types is modulated both in response to changes in nutritional state and body fat mass and in anticipation of such changes.

Paraventricular Nucleus

Among brain regions contacted by ARC projections, the paraventricular nucleus (PVN) is of particular interest given that bilateral PVN lesions cause hyperphagia and obesity (Leibowitz, Hammer and Chang, 1981), while electrical stimulation of this brain area inhibits food intake. Moreover, activation of NPY/AgRP terminals in the PVN mimics the feeding observed with cell body activation in the ARC (Atasoy *et al.*, 2012). This effect is attributed to NPY/AgRP GABAergic inhibition of PVN neurons, as chemogenetic activation of PVN neurons conversely reduces food intake and blunts the hyperphagic effect of upstream NPY/AgRP neuron activation (Atasoy *et al.*, 2012), suggesting a physiological role for PVN neurons to constrain food intake and body weight gain by acting downstream of ARC neurons. In support of this concept, the PVN not only receives dense innervation from both NPY/AgRP and POMC neurons, but also contains a dense population of MC3R/MC4R neurons (Mountjoy *et al.*, 1994). Furthermore, administration of MC4R agonists directly into the PVN reduces food intake (Giraudo, Billington and Levine, 1998), and selective re-expression of MC4R in the PVN of congenital MC4R knockout mice normalizes their hyperphagia (Balthasar *et al.*, 2005). By comparison, MC4R-expressing neurons within the hindbrain appear to mediate melanocortin-dependent effects on energy expenditure rather than food intake (Rossi *et al.*, 2011).

Multiple distinct neuropeptides expressed by PVN neurons have been hypothesized to participate in the regulation of energy balance. Among these are thyrotropin-releasing hormone (TRH), corticotropin releasing factor (CRF), and oxytocin (Hill, 2012). Of these, neurons expressing oxytocin have emerged as components of an anorectic neurocircuit engaged by leptin downstream of ARC neurocircuits. In support of this notion, icv administration of oxytocin reduces food intake and increases energy expenditure (Arletti, Benelli and Bertolini, 1990) even in leptin-resistant animal models, whereas leptin-induced anorexia is blocked by administration of an oxytocin-receptor antagonist (Blevins, Schwartz and Baskin, 2004). These effects appear to involve projections to the NTS, where oxytocin release is postulated to modulate sensitivity to satiety signals such as CCK (Blevins, Schwartz and Baskin, 2004). Moreover, in individuals with Prader-Willi syndrome, a human genetic disorder characterized by severe hyperphagia and obesity, the numbers and size of leptin-responsive oxytocin neurons in the PVN is markedly decreased (Swaab, 1997). However, while mice deficient in either oxytocin (Camerino, 2009) or oxytocin receptors (Takayanagi *et al.*, 2008) exhibit a late-onset obesity phenotype, these mice are not hyperphagic. Thus, the precise role for oxytocin signaling in the regulation of food intake remains to be established.

Ventromedial Hypothalamus

Early endeavors to identify key brain regions implicated in the regulation of food intake discovered that lesions targeting the hypothalamic ventromedial nucleus (VMH), situated dorsolateral to the ARC, produced hyperphagia and obesity (Hetherington and Ranson, 1942). Conversely, electrical stimulation of the VMH reduced food intake (Anand and Dua, 1955), leading researchers to describe the VMH as the “satiety center” of the brain. As the VMH expresses leptin receptors (Elmquist, Bjørbæk, *et al.*, 1998), and as leptin activates neurons in this brain area (Elmquist, Ahima, *et al.*, 1998), the VMH emerged as a key brain region mediating leptin’s anorexic effects. In support of this concept, leptin microinjection directly into the VMH suppresses food intake in rodents (Jacob *et al.*, 1997; Meek *et al.*, 2013), whereas selective deletion of leptin receptors from VMH neurons elicits hyperphagia and obesity (Dhillon *et al.*, 2006).

Within the VMH, neurons expressing brain-derived neurotrophic factor (BDNF) are among those implicated in the regulation of energy balance. Thus, central administration of BDNF reduces food intake and increases energy expenditure in both diet-induced obese (Nakagawa *et al.*, 2000) and leptin-receptor deficient (*db/db*) mice (Tonra *et al.*, 1999). Conversely, mice with genetic deficiency of BDNF or its receptor, TrkB tyrosine kinase, exhibit hyperphagia and obesity on a high fat diet (Xu *et al.*, 2003). Moreover, BDNF neurons in the VMH are activated by leptin and regulated by both nutritional state and input from melanocortin signaling (Xu *et al.*, 2003), as VMH expression of BDNF is markedly reduced in MC4R-null mice and during fasting (Xu *et al.*, 2003), which is associated with reduced melanocortin signaling (owing to the combined effects of NPY/AgRP activation and POMC neuron inhibition). Moreover, loss-of-function mutations that disrupt BDNF signaling cause severe obesity in humans (Yeo *et al.*, 2004) as well as in mice (Ernest Lyons *et al.*, 1999). Taken together, these observations suggest that neurons in the VMH transduce input from leptin and melanocortin signaling into regulation of feeding, and that such effects are required for normal control of food intake and body weight.

Lateral Hypothalamus

In contrast to the VMH "satiety center", the lateral hypothalamus (LHA) has traditionally been known as the "hunger center," as LHA lesioning causes profound hypophagia and weight loss (Anand, Dua and Shoenberg, 1955), whereas electrical stimulation increases food intake and food reward (Delgado and Anand, 1953). Within the LHA are at least two distinct neuronal populations that promote feeding, one that expresses the neuropeptide melanin concentrating hormone (MCH) and the other expressing orexin (also known as hypocretin). Both neuronal populations are orexigenic, as central administration of either MCH (Qu *et al.*, 1996) or orexin (Sakurai *et al.*, 1998) drives feeding. Moreover, expression of both MCH and orexin is regulated by nutritional status, as their expression increases with fasting and in genetic models of leptin deficiency (Qu *et al.*, 1996; Sakurai *et al.*, 1998), whereas in vivo electrophysiology studies demonstrate increased activity during food consumption (Mileykovskiy, Kiyashchenko and Siegel, 2005). However, while mice deficient in MCH are characterized by hypophagia and weight loss (Shimada *et al.*, 1998), a definitive role for orexin and MCH in energy homeostasis and feeding behavior remains elusive, as these neurons are implicated in a wide range of behaviors additional to feeding. For instance, both LHA MCH and orexin populations are activated during various points in the sleep-wake-cycle (Izawa *et al.*, 2019), and targeted deletion of orexin results in narcolepsy (Hara *et al.*, 2001), a sleep disorder characterized by disrupted circadian rhythms and sudden daytime sleeping, suggesting that orexin signaling plays a role in both sleep and feeding.

In addition, the LHA is enriched in neurons that express both the inhibitory and the excitatory neurotransmitters GABA and glutamate, respectively. A further role has been implicated for these populations in the regulation of feeding and reward given that optogenetic activation of GABAergic LHA neurons potently stimulates feeding and rewarding phenotypes (Jennings *et al.*, 2015), whereas activation of glutamatergic LHA neurons has the opposite effect (Jennings *et al.*, 2013). Consistent with this notion, selective ablation of GABAergic LHA neurons reduces food intake, weight gain, and food reward (Jennings *et al.*, 2015), while ablation of glutamatergic LHA neurons has the opposite effect (Stamatakis *et al.*, 2016). Thus, these experiments revealed a broad division of LHA neuronal populations with counteracting effects on feeding and reward (Sternson and Eiselt, 2017).

Recent in vivo imaging studies have demonstrated LHA neuronal populations exhibit heterogenous responses during feeding behaviors. For instance, while one subset of LHA GABAergic neurons is excited during food consumption, separate groups are excited during a nose-poke food-seeking paradigm (Jennings *et al.*, 2015), and after exposure to food-predictive cues (Nieh *et al.*, 2015). These findings suggest that within LHA GABAergic cells, distinct ensembles are differentially active during various aspects of feeding behavior (i.e., food seeking vs. food consumption). Similarly, LHA glutamatergic neurons exhibit responses during sucrose consumption that appear to be dynamically modulated by both satiety state (i.e., fed vs. fasted) and long-term adiposity signals (Rossi *et al.*, 2019). Collectively, while these observations implicate the LHA as an important regulatory area in appetitive feeding and reward, the vast heterogeneity of cell types and inputs from diverse brain areas suggest broader functions in various aspects of energy balance and behavior.

Extra-Hypothalamic Regulation of Feeding Behavior

A physiological role for the hindbrain in food intake control was established initially by seminal work showing that in 'decerebrate' rats, in which forebrain connectivity to spinal cord and caudal brain regions is severed surgically, the functionally isolated hindbrain is sufficient for satiation to occur in response to ingested nutrients (Grill and Norgren, 1978), as well as for mounting neuroendocrine and autonomic responses to declining blood glucose levels (DiRocco and Grill, 1979). Within the hindbrain, the NTS detects and responds to multiple cues of energetic status. These include 1) hormonal cues (e.g. leptin) (Grill *et al.*, 2002), 2) circulating nutrients (e.g. glucose) (Ritter, Slusser and Stone, 1981), and 3) gut-derived satiety signals (e.g. CCK, GLP-1) via vagal afferents (Travers, Travers and Norgren, 1987; Hayes, Bradley and Grill, 2009). This information is in turn relayed to higher brain areas to influence autonomic, behavioral, and endocrine responses (Grill and Hayes, 2012). In addition, the NTS receives input via descending projections from forebrain areas (e.g., hypothalamus) that convey information regarding energy stores.

Another relay station for visceral and gustatory signals in the hindbrain is in the parabrachial nucleus (PBN), which plays important roles both to protect against the consumption of novel or potentially toxic substances, and to act downstream of neurons in the NTS to mediate anorexic responses to satiety signals from the gut. Implicated in these responses is a subset of neurons in the PBN that express calcitonin-gene related peptide (CGRP). These neurons are well-positioned to integrate information about long- and short-term energy availability, as they receive both descending inhibitory GABAergic projections from NPY/AgRP neurons in the ARC, as well as ascending projections from excitatory glutamatergic neurons in the NTS (Wu, Clark and Palmiter, 2012). Activation of CGRP neurons via either opto- or chemogenetic strategies robustly suppresses food intake and reduces body weight (Carter *et al.*, 2013), and activation of these neurons underlies the dramatic and potentially lethal anorexia induced by ablation of NPY/AgRP neurons, an effect reversed by blocking either excitatory, glutamatergic output from the PBN, or excitatory input from the NTS (Wu, Clark and Palmiter, 2012). Conversely, inhibition of CGRP neurons increases short-term food intake, in part, by blunting responses to meal-related satiety signals, such as CCK (Campos *et al.*, 2016). Inhibition of CGRP neurons also prevents lethargy, anxiety, and malaise associated with cancer (Campos *et al.*, 2017). Together, these findings identify CGRP neurons as a hub in which hunger and satiety circuits interact with aversive stimuli to control feeding behavior.

Summary

To summarize, the brain integrates information from the periphery regarding both long- and short-term energy availability and, in accordance with the body's metabolic needs, transduces this information into coordinated autonomic, neuroendocrine, and behavioral outputs that promote energy homeostasis while also anticipating future energy needs. Energy stored within fat tissue is conveyed to the brain by the adiposity signals leptin and insulin, where they act to fine-tune brain sensitivity to both the rewarding properties of nutrients and cues of short-term energy availability, including circulating nutrients, gut-derived satiety signals, and sensory information from the environment.

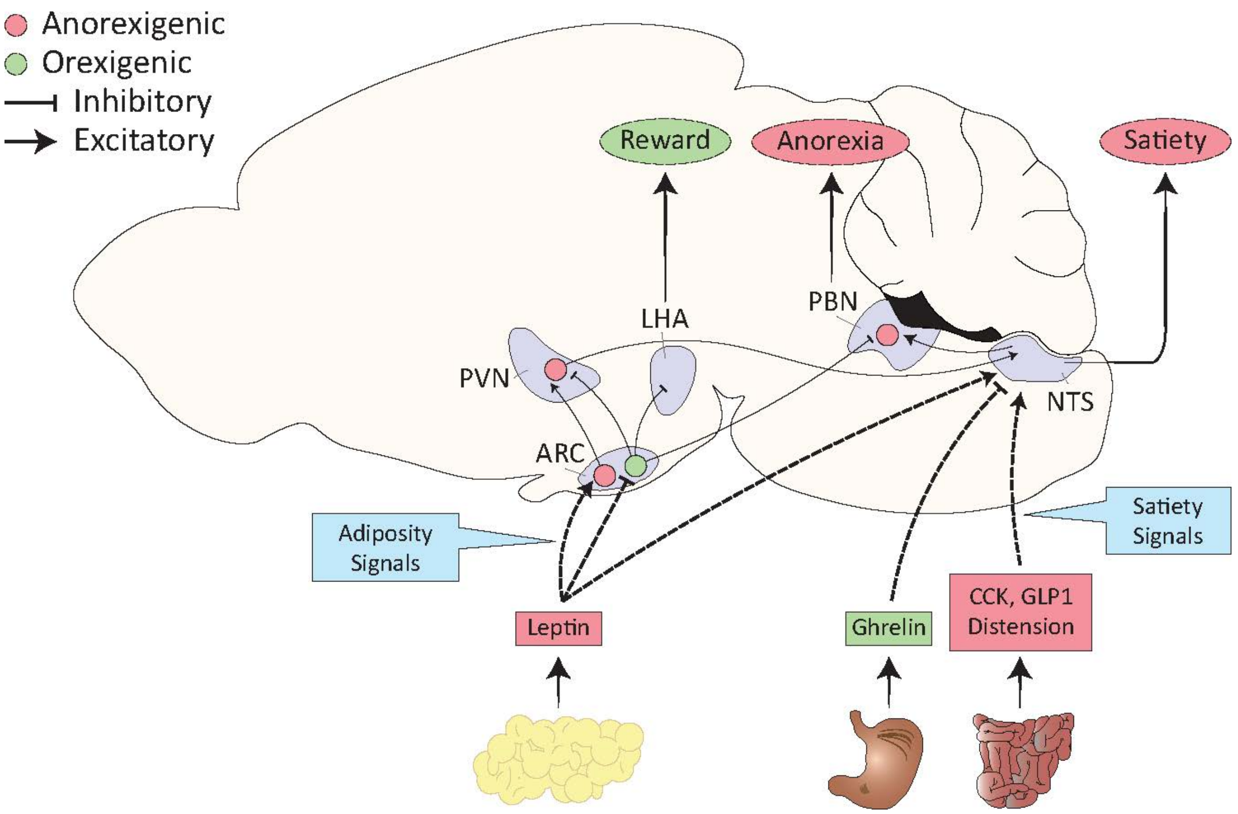

Figure 2.4 CNS integration of long-term adiposity signals and short-term satiety signals

Chapter 3 Central Regulation of the Endocrine Pancreas

Text and figures modified from invited review, in resubmission: Faber et al, Diabetologia 2020.

Abstract

Increasing evidence suggests that although pancreatic islets can function autonomously to detect and respond to changes in the circulating glucose level, the brain cooperates with the islet to maintain glycemic control. Here, we review the role of the central (CNS) and the autonomic nervous system (ANS) in the control of the endocrine pancreas, mechanisms whereby the brain senses circulating blood glucose levels, and examine whether dysfunction in this system might contribute to complications of type 1 (T1D) and the pathogenesis of type 2 diabetes (T2D).

Introduction

Blood glucose levels are maintained within narrow physiological limits. Whenever glucose levels deviate from their defended level, adaptive metabolic responses are engaged to ensure glucose levels return to the normal range. Critical to these responses are the capacities of the pancreatic islet α- and β-cells to coordinately adjust glucagon and insulin secretion, respectively, in response to changes in blood glucose concentrations. However, accumulating evidence suggests that the CNS works in tandem with the islet to maintain glucose homeostasis. Here, we review key evidence suggesting that: *i)* the brain can regulate islet function via direct innervation by both parasympathetic (PNS) and sympathetic (SNS) branches of the autonomic nervous system (ANS), and indirectly via neuroendocrine mechanisms, *ii)* the brain senses circulating glucose levels both directly and indirectly, transducing glycemic information into adaptive glucoregulatory responses, and *iii)* interventions targeting the brain can regulate glycemic control, in part, by modulating islet function. Finally, we discuss the implications of these concepts for the pathogenesis and treatment of T2D.

Autonomic Innervation of the Endocrine Pancreas

The rich autonomic innervation of the islet, first described in 1869 by Langerhans, has since been characterized in multiple species by several independent research groups (Rodriguez-Diaz and Caicedo, 2014). While rodent islets are extensively innervated by both efferent cholinergic PNS and adrenergic SNS fibers (Figure 3.1), initial reports in human islets suggested sparse PNS, and less SNS, innervation than seen in mice (Rodriguez-Diaz *et al.*, 2011). However, using state-of-the-art tissue clearing and 3D reconstructive imaging to reduce background and improve clarity and resolution of islet morphology, more recent work (Tang *et al.*, 2018; Chien *et al.*, 2019) revealed dense PNS innervation of the human islet, and more SNS fibers than previously reported (Rodriguez-Diaz *et al.*, 2011).

Despite differences in the distribution and innervation of islet endocrine cells, autonomic activation affects islet hormone secretion similarly in rodents and humans. Whereas SNS activation inhibits both basal and glucose-stimulated insulin secretion (GSIS) through release of norepinephrine acting via the α2-adrenergic receptor on pancreatic β-cells, PNS activation augments GSIS via the release and subsequent binding of acetylcholine to β-cell muscarinic receptors (Ahrén, 2000). In contrast, both PNS and SNS activation stimulates glucagon secretion via activation of muscarinic and

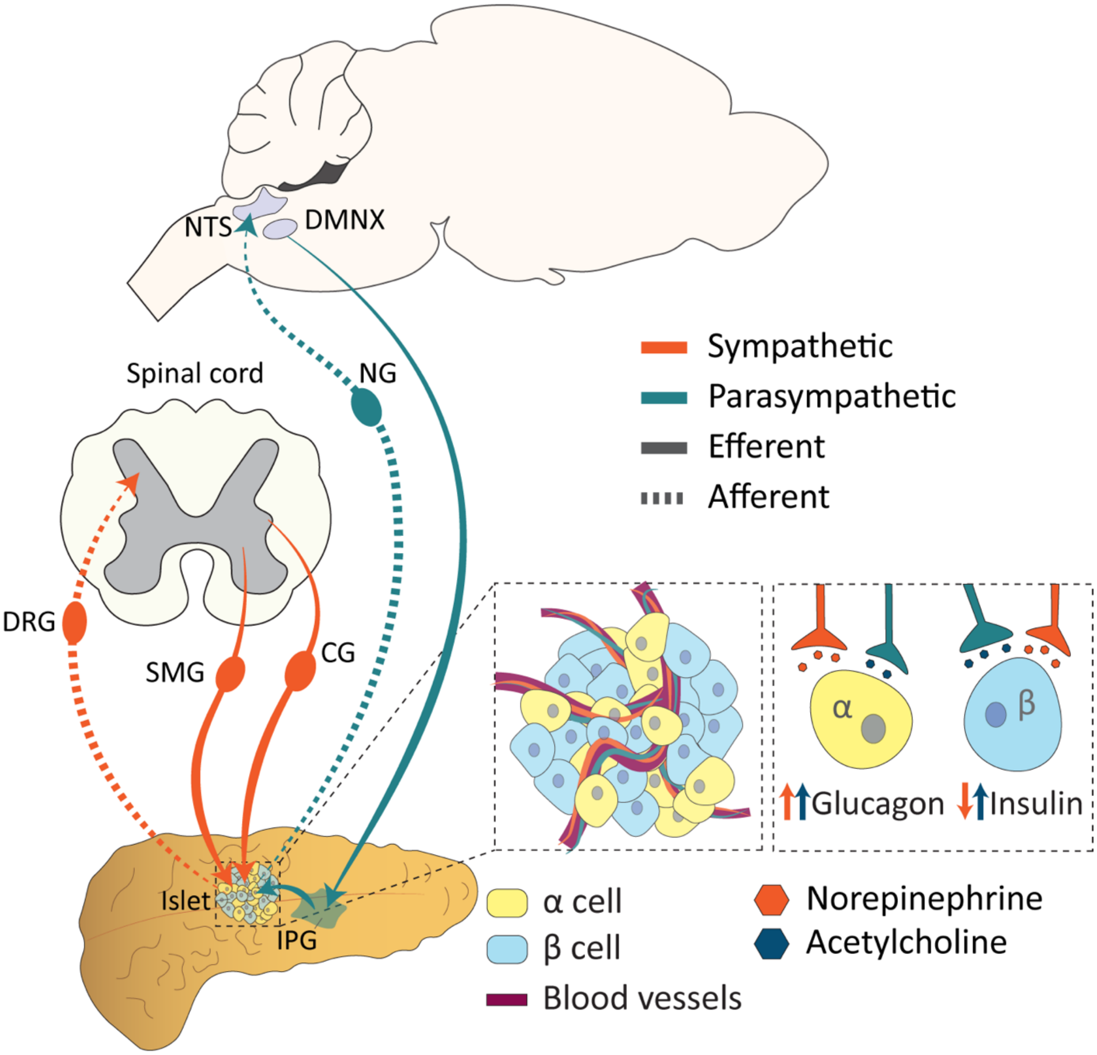

Figure 3.1 Sensory and autonomic innervation of the endocrine pancreas. The islet receives efferent innervation (solid lines) from both sympathetic (orange) and parsympathetic (teal) branches of the ANS, as well as sensory afferent fibers (dashed lines). Projecting from the lateral horn of the spinal cord, the cell bodies of sympathetic efferent fibers are positioned within the celiac ganglia (CG) and superior mesenteric ganglia (SMG), enter the islet along blood vessels (inset), and release norepinephrine from their terminals that stimulates glucagon secretion through binding to b-adrenergic receptors on a-cells and inhibits insulin secretion through activation of b-cell a2-adrenergic receptors. Afferent sympathetic fibers have their cell bodies in the dorsal root ganglia (DRG) and project to the laminae I and IV of the spinal cord. Efferent parasympathetic fibers originate in the dorsal motor nucleus of the vagus (DMNX) and innervate intrapancreatic ganglia (IPG), which in turn sends cholinergic input to the islet to stimulate increased glucagon secretion from a-cells and to potentiate insulin secretion through local release of acetylcholine via muscarinic receptors on b-cells (inset). Pseudounipolar afferent parasympathetic neurons have their cell bodies within the nodose ganglion (NG), and terminals in the islet and nucleus of the solitary tract (NTS). In response to hypoglycemia, increased sympathetic activity inhibits insulin secretion, while both increased sympathetic and parasympathetic activity stimulates glucagon secretion.

β2-adrenergic receptors, respectively, expressed on islet α-cells, which are further regulated by insulin secreted from adjacent b-cells (Ahrén, 2000).

The neural pathways regulating autonomic outflow to the pancreas have been mapped by multiple retroviral tracing studies. These brain-to-pancreas neurocircuits include efferent PNS pathways consisting of preganglionic neurons in the dorsal motor nucleus of the vagus (DMNX), whereas SNS motor neurons are located in the interomediolateral (IML) column of the spinal cord (Buijs *et al.*, 2001; Rosario *et al.*, 2016) (Figure 3.1**)**. However, in addition to a direct brain-to-pancreas neurocircuit, the SNS can also affect islet secretion indirectly by both i) increasing outflow to the adrenal medulla, thereby stimulating epinephrine secretion into the circulation, resulting in inhibition of insulin and stimulation of glucagon secretion via α2 and β2-adrenergic signaling, respectively (Thorens, 2014), and ii) regulating glucose utilization by peripheral tissues such as muscle, liver and fat (Thorens, 2014). Importantly, the activity of both PNS and SNS outflow to the pancreas is regulated by neural input from multiple overlapping hindbrain, midbrain, and forebrain structures (Buijs *et al.*, 2001; Rosario *et al.*, 2016) (Figure 3.2). To illustrate the importance of this CNS control of islet function, we next consider how the body responds when glucose homeostasis is challenged.

Central Control of the Endocrine Pancreas

Perhaps the most robust example of central control of the endocrine pancreas is the counter-regulatory response (CRR) to hypoglycemia. In response to a fall in blood glucose levels (i.e., insulin-induced hypoglycemia (IIH)), a coordinated set of adaptive responses is engaged to restore normoglycemia, including increased secretion of glucagon, glucocorticoids, and epinephrine, along with inhibition of GSIS (Cryer, 1981). These effects are driven largely by hypoglycemia-induced activation of both PNS and SNS outflow to the islet, and increased SNS outflow to the adrenal medulla to drive epinephrine secretion. Surgical or pharmacological ablation of autonomic outflow markedly reduces the glucagon and epinephrine responses to IIH, which in turn leads to impaired glucose recovery (Ahrén, 2000). Moreover, recurrent iatrogenic hypoglycemia (common during intensive insulin therapy) produces a brain adaptation termed hypoglycemia-associated autonomic failure (HAAF), which results in reduced autonomic outflow during subsequent IIH, thereby increasing susceptibility to, and severity of, future episodes of hypoglycemia (Cryer, 2006). HAAF is of particular concern for humans with T1D, which is also associated with an impaired glucagon response to IIH. While the precise explanation for impaired glucagon secretion is unclear, putative mechanisms include the loss of not only β-cells, but also SNS fibers selectively within the islet (Mundinger *et al.*, 2016), although evidence suggests important role for the brain as well.

Within the brain, subsets of neurons located within the hypothalamic ventromedial nucleus (VMN) are implicated in mediating CRRs, as *i)* VMN-specific glucopenia triggers CRRs during systemic normoglycemia, while *ii)* infusion of glucose within the VMN blunts CRRs during IIH (Sherwin, 2008). Moreover, recent work using advanced neuroscience approaches have identified several subsets of neurons in the VMN whose activity is not only sufficient to robustly raise blood glucose levels of normoglycemic mice to the diabetic range by activating hormonal CRRs, but is also required for intact CRR hormonal responses and recovery from IIH (Meek *et al.*, 2016; Stanley *et al.*, 2016; Faber *et al.*, 2018). Whether these neurons play a physiological role in primary sensing of circulating glucose levels or are secondarily engaged as part of the brain's efferent limb to drive CRRs, are questions awaiting further study.

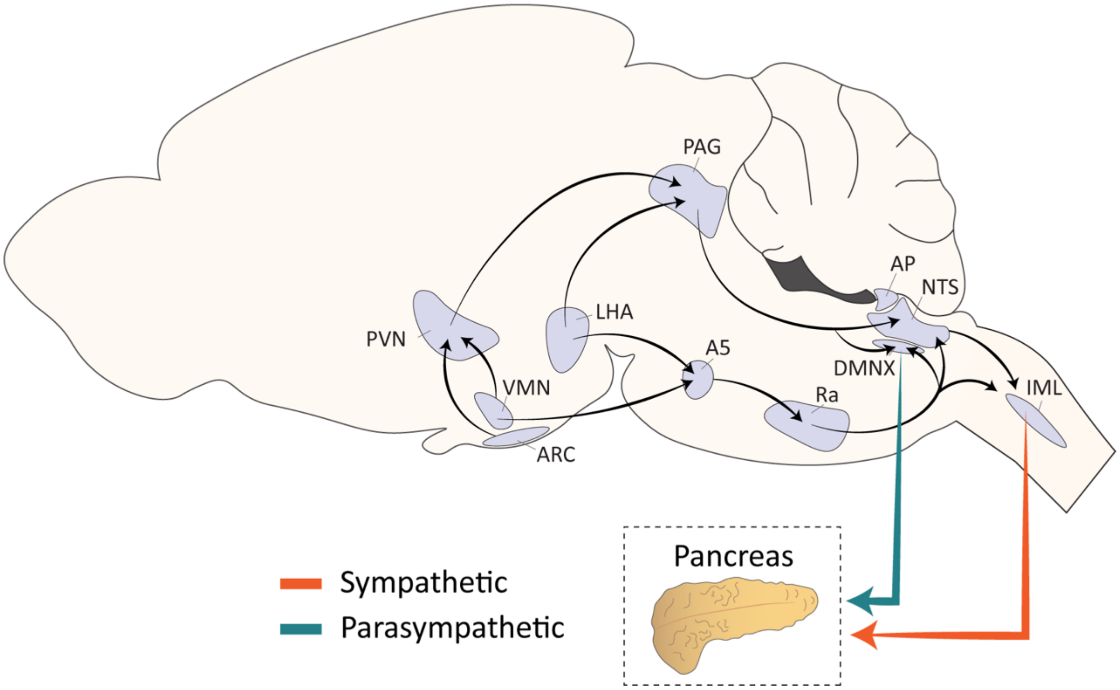

Figure 3.2 Central neurocircuits implicated in efferent outflow to the islet. Motor neurons of the SNS and PNS receive input from extensively overlapping brain nuclei, including both hypothalamic and hindbrain regions. Motor neurons of the SNS lie within the IML of the spinal cord and the receive synaptic input directly from premotor neurons within the NTS. These sympathetic premotor neurons receive input from hypothalamic regions, including the PVN and LHA, which in turn, receive input from the VMN and ARC, amongst other brain areas. In contrast, efferent PNS pathways consist of preganglionic neurons in the DMNX, intrapancreatic ganglia, and postganglionic neurons in the pancreas. The DMNX, in turn, receives input from hypothalamic areas, including the PVN, LHA, and VMN, via the PAG and/or Ra and A5. Abbreviations: A5, noradrenergic cell group 5; AP, area postrema; ARC, arcuate nucleus; DMNX, dorsal motor nucleus of the vagus; IML, interomediolateral column; LHA, lateral hypothalamic area; NTS, nucleus of the solitary tract; PAG, periaqueductal gray; PVN, paraventricular nucleus; Ra, raphe pallidus; VMN, ventromedial nucleus.

The defense of core body temperature during cold exposure represents another major physiological challenge where the substantial energy needs of thermogenic tissues pose a challenge to glucose homeostasis (Shaun F. Morrison, 2016). This challenge is met through coincident, adaptive reductions in insulin secretion that allow glucose to be directed to thermogenic tissues, while avoiding a fall in blood glucose levels (Morton *et al.*, 2017). The brain is implicated in these effects since *i)* cold exposure increases sympathetic tone to both the pancreas and thermogenic tissues (Young and Landsberg, 1979; Shaun F. Morrison, 2016), and *ii)* pharmacological blockade of α-adrenergic receptors rapidly reverses the cold-induced inhibition of insulin secretion (Morton *et al.*, 2017), implying that SNS tone to the islet helps maintain normoglycemia despite increased demand for glucose.

Evidence also suggests a neural link to the cephalic and first phases of insulin secretion, wherein insulin is secreted prior to nutrient absorption during sensory stimulation of the oral cavity as food is ingested (Teff, 2011). A role for the PNS is implicated in this cephalic phase insulin release since it is suppressed by vagotomy, ganglionic blockade, and by antagonism of muscarinic receptors in rodents (Berthoud *et al.*, 1981). However, the extent to which these findings apply in humans remains uncertain, owing in part to differences in paradigms used (Teff, 2011; Veedfald *et al.*, 2016). Taken together, these observations support a model in which the brain integrates and transduces afferent information regarding fuel availability and cooperates with the islet to maintain glycemic control in the face of anticipated metabolic demand. Yet critical questions remain as to how the brain senses circulating blood glucose levels to mediate these adaptive responses.

Mechanisms of Central Glucose Sensing

The brain has the capacity to detect and respond to glycemic status via: *i)* primary sensory fibers innervating peripheral organs which transmit glycemic information to the brain, and *ii)* intrinsic sensing of glucose levels within the brain (Figure 3.3). Regarding the former, sensory fibers are found extensively throughout the periphery, including the pancreas, gastrointestinal tract, portal-mesenteric vein (PMV), and carotid body (Routh *et al.*, 2014). Evidence suggests that some sensory neurons innervating the gastrointestinal tract detect glucose and other nutrient-related inputs (Williams *et al.*, 2016), while those that innervate the PMV are critical for establishing CRRs to slow-onset hypoglycemia (Bohland *et al.*, 2014), with each transmitting this information to the nucleus of the solitary tract (NTS) in the hindbrain. Sensory fibers innervating the pancreas may also play a role in glucose homeostasis, as their ablation increases β cell mass and GSIS (Riera *et al.*, 2014), and protects against diabetes progression in multiple rodent models (Razavi *et al.*, 2006; Gram *et al.*, 2007; Rodriguez-Diaz and Caicedo, 2014). Whereas rodents exhibit dense sensory innervation within the islet, human sensory fibers predominantly localize to intrapancreatic ganglia and the peri-islet area, but do not penetrate the islet core (Chien *et al.*, 2019). Thus, the extent to which sensory innervation of the pancreas contributes to CNS control of glycemia, and whether afferent fibers sense glucose or other signals within the islet microenvironment, remain undetermined.

Within the brain, evidence suggests that not only neurons, but also astrocytes and other glial cell types play a role in glucose-sensing (see (Garcia-Caceres *et al.*, 2016)), although neurons have received the most attention. Glucose-sensing neurons (GSNs) are broadly subdivided into either glucose-excited (GE) or glucose-inhibited (GI) subsets, which increase their firing rates when extracellular glucose concentrations increase or decrease, respectively (Routh *et al.*, 2014). Although GSNs express the critical cellular machinery (e.g., GLUT2, glucokinase (GCK), and the Kir6.2 subunit of the K_{ATP} channel) needed to respond to changes in ambient glucose levels (Routh *et al.*, 2014), the majority are located behind the blood-brain barrier (BBB), where glucose concentrations in the brain interstitial fluid are nearly 7-fold lower and temporally decoupled from changes in circulating glucose levels (Abi-Saab *et al.*, 2002). Conversely, neurons in circumventricular organs (CVOs), such as the arcuate nucleus-median eminence (ARC-ME) of the hypothalamus or the area postrema (AP) of the hindbrain, are not protected by the BBB and therefore have greater inherent potential to directly sense circulating blood glucose levels and relay this information to glucoregulatory systems behind the BBB (Bentsen, Mirzadeh and Schwartz, 2019).

To better understand the physiology of brain glucose sensing, we consider how the brain detects and responds to a change of ambient temperature. For instance, during cold exposure, thermal information, detected by temperature-sensitive thermosensory receptors in the skin, is transmitted via a well-mapped peripheral-to-central relay that is integrated by neurons in the hypothalamic preoptic area (POA) and transduced into adaptive responses (e.g., vasoconstriction, thermogenesis) that maintain core temperature (Shaun F. Morrison, 2016). According to this model, neurons within the POA comprise the integrative/efferent limb of the brain's thermoregulatory system, and while they are not involved in directly sensing ambient temperature under physiological conditions, they retain intrinsic thermal sensing properties that enable them to respond to changes in local hypothalamic temperature. Therefore, while subsets of POA neurons have the capacity to directly sense temperature change, just as subsets of neurons behind the BBB have the capacity to sense glucose, it is unclear if meaningful changes in local brain temperature or interstitial glucose levels occur unless a challenge to homeostasis is prolonged or severe.

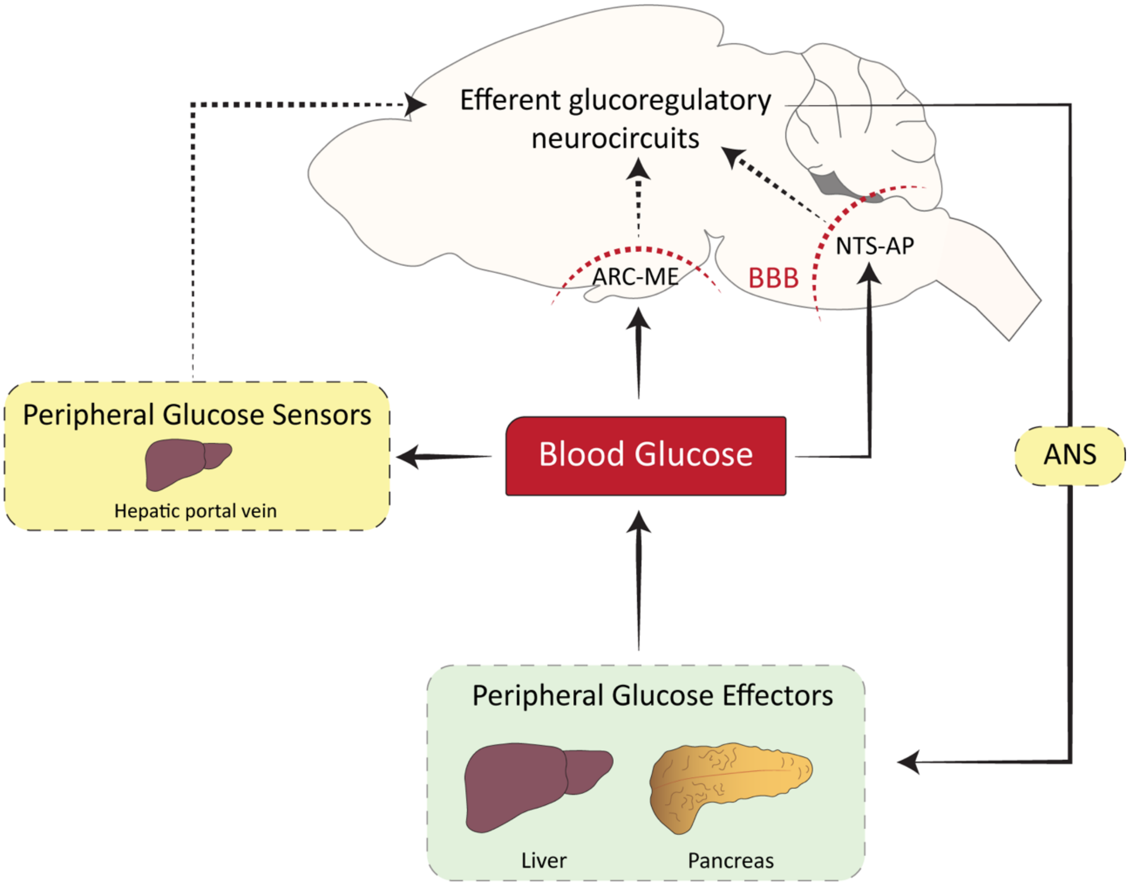

Figure 3.3 Model for central glucose sensing.
Circulating blood glucose levels are detected in both the periphery by sensory afferent fibers (e.g., that innervate the hepatic portal vein) and central circumventricular organs, including the arcuate nucleus-median eminence (ARC-ME) and the nucleus of the solitary tract-area postrema (NTS-AP). This afferent information is relayed to neural centers located behind the blood-brain-barrier (BBB) that comprise the efferent limb of the brain's glucoregulatory system. These neurons also have the capacity to detect concentrations of glucose in brain interstitial fluid (ISF) and when activated, regulate both neuroendocrine and autonomic mechanisms through peripheral tissues via both direct, and indirect mechanisms to regulate circulating blood glucose levels.

Instead, just as afferent input regarding external temperature is transmitted primarily by neurons innervating the skin and other peripheral tissues, we anticipate that afferent information regarding the circulating glucose level is provided by GSNs that lie outside the BBB (i.e., within CVOs and/or peripheral glucose-sensors) and is subsequently transmitted to glucoregulatory circuits sheltered behind the BBB. While these latter neurons putatively comprise the integrative/efferent limb, rather than the sensory/afferent limb of the brain's glucoregulatory system, many maintain intrinsic glucose sensing properties, such that changes of local glucose availability can activate these neurons with potent consequences for systemic glucose homeostasis (Bentsen, Mirzadeh and Schwartz, 2019). For example, pharmacological glucoprivation of NTS neurons activates both ascending (Flak *et al.*, 2015) and descending (Lamy *et al.*, 2014) circuits, with the net outcome being a potent CRR analogous to that induced by hypoglycemia. These findings are consistent with a model in which GSNs situated behind the BBB do not function as primary sensors of the circulating glucose level, but can detect and respond to changing glucose levels in brain ISF, should they deviate from the normal range. Future studies are needed to identify and characterize the physiological roles played by different populations of GSNs (Figure 3.3).

Implications for the Pathogenesis of Type 2 Diabetes

Maintenance of normal glucose tolerance hinges on the capacity of the β-cell to adjust insulin secretion in response to changes not only of glycemia, but of insulin sensitivity (Kahn, Hull and Utzschneider, 2006). During conditions of increased insulin sensitivity (e.g., cold exposure, exercise), insulin secretion must be reduced to avoid a fall of the blood glucose concentration while conversely, during insulin-resistant conditions (e.g., pregnancy, puberty, and obesity), insulin secretion must increase proportionately to maintain glucose tolerance. Failure of the β-cell to compensate for insulin resistance is a characteristic finding in patients with T2D, and is associated with a gradual rise in the defended level of glycemia (Alejandro *et al.*, 2015).

Given that functional β-cells normally regulate neighboring α-cells and suppress glucagon secretion, hyperglucagonemia resulting from unregulated α-cells is also implicated in the pathogenesis of both T1D and T2D as the β-cells are destroyed or fail, respectively (Unger and Cherrington, 2012). Based on additional evidence that glucagon receptor-null mice are protected from streptozotocin-induced diabetes, suppression of glucagon secretion has been suggested as a strategy for treatment for T2D (D'alessio, 2011; Unger and Cherrington, 2012).

Taken together, these observations suggest that the capacity of the β-cell to adapt to conditions of insulin resistance constitutes a primary defense against T2D (Alejandro *et al.*, 2015). Consistent with this idea, genome-wide association studies (GWAS) indicate that the majority of gene variants associated with T2D regulate β-cell function or mass (Alejandro *et al.*, 2015), although environmental factors also play a significant role in determining β-cell resilience and susceptibility to T2D (Alejandro *et al.*, 2015). As the mechanism that mediates the crosstalk between insulin-sensitive tissues and the islet has yet to be identified, we consider here the possibility that the brain plays a role.

The most compelling evidence of a direct role for brain glucose sensing in glucose homeostasis is derived from work examining the role of GLUT2, the glucose transporter utilized by glucose-sensing cells in pancreas, liver and brain. Mice with chronic inactivation of *Glut2* specifically in the brain (NG2KO) exhibit impaired cephalic and first-phase insulin secretion and impaired glucose tolerance (Tarussio *et al.*, 2014), as well as reduced β-cell mass mediated in part, via reduced PNS activity, (Tarussio *et al.*, 2014). In addition, NG2KO mice also exhibit elevated plasma glucagon levels (Tarussio *et al.*, 2014). GLUT2 neurons within the NTS are implicated in this effect, as acute activation of

NTS GLUT2 neurons increases vagal nerve activity and drives glucagon secretion in vivo (Lamy *et al.*, 2014).

While GLUT2 is important for cellular glucose import, GCK activity is required for intracellular glucose metabolism and neuronal glucose sensing (Kang *et al.*, 2006). Indeed, GSNs are absent in mice with *Gck* knockdown, while conversely, increased *Gck* expression heightens sensitivity of GSNs to fluctuations of ambient glucose (Kang *et al.*, 2006). Furthermore, brain-specific GCK inhibition in mice impairs glucose tolerance and blunts GSIS (Osundiji *et al.*, 2012), while conversely, increasing *Gck* expression in the ARC (and thus brain sensitivity to glucose) has the opposite effect (de Backer *et al.*, 2016). Combined with additional evidence that *Gck*-expressing neurons in the ARC are a source of polysynaptic innervation of the pancreas (Rosario *et al.*, 2016), these findings suggest that hypothalamic GSNs have the capacity to regulate islet secretion via neurocircuits modulating ANS flow, raising the question of whether dysfunctional brain glucose sensing impairs glucose homeostasis. The relevance of these findings to human T2D is bolstered by evidence that i) both *GCK* and *GLUT2* are expressed in human as well as rodent brain (Roncero *et al.*, 2004), and ii) *GLUT2/GCK* gene variants are associated with impaired fasting glucose and increased risk of T2D (Laukkanen *et al.*, 2005; Chakera *et al.*, 2018). Indeed, loss of function *GCK* mutations, including GCK, are a cause of in maturity-onset diabetes of youth (MODY) in humans (Chakera *et al.*, 2018). However, additional studies are required to determine the extent to which glucose homeostasis is perturbed by the impact of *GLUT2/GCK* variants on the function of pancreatic islets versus GSNs.

Could impaired glucose-sensing in the brain contribute to elevated blood glucose levels in T2D, and if so, can the brain be targeted to treat this disease? Several observations support this possibility. First, intracerebroventricular (icv) administration of leptin to leptin-deficient *ob/ob* mice ameliorates their hyperglycemia independently of changes in energy balance, suggesting that deficient leptin signaling in the brain contributes to their diabetes phenotype. Central leptin administration can similarly normalize glycemia in rodent models of severe insulin-deficient T1D (which is also associated with leptin deficiency), an effect accompanied by normalization of elevated plasma glucagon and corticosterone levels. In addition, icv leptin administration *i)* modulates GSIS via the SNS in rats (Park, Ahn and Kim, 2010), and ii) improves glucose homeostasis in high-fat fed mice (Pocai *et al.*, 2005). Moreover, systemic administration of leptin to a polygenic model of T2D normalizes fasting plasma glucose levels, in association with decreased glucagon concentrations, improved insulin sensitivity, and elevated pancreatic insulin content (Cummings *et al.*, 2011). However, growing evidence suggests that the degree of hyperleptinemia necessary to achieve adequate brain levels can exert effects that actually undermine leptin's central action (Zhao *et al.*, 2019). It is perhaps for this reason that in a pilot study in humans, subcutaneous metreleptin therapy was not efficacious in improving glycemic control in patients with T1D, although it did reduce daily insulin requirements (Vasandani *et al.*, 2017). Additional work is warranted to investigate whether central leptin administration can ameliorate hyperglycemia without the need for insulin in patients with T2D.

The capacity of the brain to normalize diabetic hyperglycemia is also clear from studies examining the glucose-lowering actions of fibroblast growth factor (FGF) peptides. In particular, recent work demonstrates that a single icv injection of fibroblast growth factor-1 (FGF1) is sufficient to induce sustained diabetes remission across several rodent models of T2D (Scarlett *et al.*, 2016), an effect which involves FGF1 action in the ARC-ME (Brown *et al.*, 2019) and is mediated in part by preservation of β-cell mass and function, thus delaying the progressive decline of basal insulin levels that parallels hyperglycemia in controls (Scarlett *et al.*, 2019). Importantly, rather than simply lowering blood glucose levels, FGF1 appears to act in the brain to lower the defended level of glycemia, such that icv FGF1 has no effect on glycemia in nondiabetic rodents (Scarlett *et al.*, 2016). This observation suggests that FGF1 resets the glycemic set-point to normal, without increasing the risk of iatrogenic hypoglycemia, a critical factor limiting tight glycemic control in people with diabetes. While the mechanisms underlying these effects remain to be elucidated, these observations raise the possibility that therapeutic interventions that target both the brain and islet may be more effective for the treatment of diabetes than current treatments targeting the islet alone, which, though effective acutely, fail to sustainably preserve β-cell function over the long-term in either adults or children with T2D (RISE Consortium, 2019).

Conclusions

In conclusion, cooperation between brain and islet is fundamental to glucose homeostasis, and involves both neuroendocrine and autonomic mechanisms. While the brain contains GSNs and certain brain regions have the capacity to sense circulating glucose levels, the brain also receives glycemic information from peripheral glucose sensors. Key next steps in the field are to *i)* distinguish the neuronal subsets that comprise the afferent vs. efferent limb of the brain's glucoregulatory system, and *ii)* examine whether defective activity in either the afferent or efferent limb occurs in human T2D. Recent advances in neuroscience technology (including cell-type specific viral tract tracing, chemo- and optogenetics to selectively activate or inhibit neuronal populations, and *in vivo* fiber photometry techniques for monitoring activity of discrete neuronal subsets in conscious, free-living mice) create numerous exciting avenues and opportunities to advance this field.

Chapter 4 Distinct Neuronal Projections from the Hypothalamic Ventromedial Nucleus Mediate Glycemic and Behavioral Effects

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Abstract

The hypothalamic ventromedial nucleus (VMN) is implicated both in autonomic control of blood glucose and in behaviors including fear and aggression, but whether these divergent effects involve the same or distinct neuronal subsets and their projections is unknown. To address this question, we used an optogenetic approach to selectively activate the subset of VMN neurons that express neuronal nitric oxide synthase 1 (VMN^{NOS1} neurons) implicated in glucose counterregulation. We found that photoactivation of these neurons elicits 1) robust hyperglycemia achieved by activation of counterregulatory responses (CRRs) usually reserved for the physiological response to hypoglycemia, and 2) defensive immobility behavior. Moreover, we show that the glucagon response to insulin-induced hypoglycemia is selectively blunted by photo-inhibition of the same neurons. To investigate the neurocircuitry by which VMN^{NOS1} neurons mediate these effects, and to determine whether these diverse effects are dissociable from one another, we activated downstream VMN^{NOS1} projections in either the anterior bed nucleus of the stria terminalis (aBNST) or the periaqueductal gray (PAG). While glycemic responses are fully recapitulated by activation of VMN^{NOS1} projections to the aBNST, freezing immobility occurred only upon activation of VMN^{NOS1} terminals in the PAG. These findings support previous evidence of a VMN→aBNST neurocircuit involved

in glucose counterregulation and demonstrate that activation of VMN^{NOS1} neuronal projections supplying the PAG robustly elicits defensive behaviors.

Introduction

Although glucose is sensed in peripheral tissues including pancreatic beta cells and the hepatic portal vein, the brain has the capacity to sense glucose both directly and indirectly (Ritter, Slusser and Stone, 1981; Saberi, Bohland and Donovan, 2008; Routh, Donovan and Ritter, 2012). The brain also plays a key role in driving the counterregulatory responses (CRRs) to hypoglycemia, which include inhibition of insulin secretion and enhanced secretion of glucagon, corticosterone, and epinephrine (Beall, Ashford and McCrimmon, 2012). Hypoglycemia is both the most frequent complication of diabetes treatment and the major obstacle to achieving tight glycemic control in people with diabetes (Cryer, 2013). Furthermore, a single severe bout of hypoglycemia increases the risk not only of cardiovascular disease and mortality, but also of subsequent hypoglycemic episodes (Heller and Cryer, 1991). Improving our understanding of the mechanisms by which hypoglycemia is sensed and responded to by the brain is therefore critical to the development of new, more effective strategies for diabetes treatment.

Although glucose counterregulation involves multiple brain areas (Ritter, Slusser and Stone, 1981), neurons in the hypothalamic ventromedial nucleus (VMN) appear to play an important role. This assertion is based on evidence that *i)* the VMN is activated during hypoglycemia (Canabal *et al.*, 2007), *ii)* electrical stimulation of the VMN activates CRRs and raises blood glucose levels (Frohman and Bernardis, 1971; Dubuc, Leshin and Willis, 1982), and *iii)* glucose delivery specifically within the VMN blunts CRRs during systemic hypoglycemia (Borg *et al.*, 1997). Moreover, using an optogenetics approach, we recently demonstrated that activation of the subset of VMN neurons that express steroidogenic factor 1 (VMN^{SF1} neurons) activates CRRs and thereby raises blood glucose levels, whereas their inhibition blocks recovery from hypoglycemia (Meek *et al.*, 2016). These data are in line with previous studies showing an impaired CRR to insulin-induced hypoglycemia when glutamate is deleted from VMN^{SF1} neurons (Tong *et al.*, 2007), and with evidence that projections from the lateral parabrachial nucleus to the VMN are essential to effective glucose counter-regulation (Garfield *et al.*, 2014).

In addition to its role in glucose homeostasis, the VMN is implicated in the “fight or flight” response and associated behaviors including fear/anxiety and aggression (Anderson, 2012; Kunwar *et al.*, 2015; Hashikawa *et al.*, 2017). In accordance with this concept, activation of VMN^{SF1} neurons elicits defensive behaviors, including freezing immobility and/or activity bursts, characteristic of a fearful emotional state (Kunwar *et al.*, 2015; Wang, Chen and Lin, 2015). Since psychological stress could potentially contribute to hyperglycemia induced by VMN^{SF1} neuron activation, the current work was undertaken to determine if neurocircuitry downstream of the VMN involved in glucose counterregulation can be distinguished from that involved in defensive behaviors. Specifically, we hypothesized that these divergent responses involve discrete neuronal subsets within the VMN that project to distinct downstream brain regions. Consistent with this hypothesis, neurons located in the ventrolateral (vl) portion of the VMN are known to be involved in reproductive and aggressive behaviors (Wang, Chen and Lin, 2015; Falkner *et al.*, 2016), while neurons in the central (c) and dorsomedial (dm) VMN are implicated in both metabolic regulation (Chan and Sherwin, 2013; Choi *et al.*, 2013) and defensive behaviors (Kunwar *et al.*, 2015). Moreover, work from us and others has shown that VMN neurons project heavily to multiple brain areas including the aBNST, an area implicated in autonomic control of homeostatic functions and stressor integration (Choi *et al.*, 2007; Crestani *et al.*, 2013), and the periaqueductal gray (PAG), a brain region classically known for its role in fear and pain (Benarroch, 2012).

Based on work from Routh and colleagues, we hypothesized a glucoregulatory role for neurons in the VMN expressing neuronal nitric oxide synthase-1 (VMN^{NOS1} neurons), a subset of VMN^{SF1} neurons. This hypothesis is supported by published evidence that *i)* VMN^{NOS1} neurons are responsive to a fall in glucose levels (Canabal *et al.*, 2007; Murphy *et al.*, 2009), *ii)* hypoglycemia induces phosphorylation and activation of NOS1 (Canabal *et al.*, 2007; Fioramonti *et al.*, 2010), and *iii)* pharmacological and genetic inhibition of NOS1 impairs epinephrine and glucagon responses to hypoglycemia and slows the recovery of blood glucose levels to normal (Fioramonti *et al.*, 2010). Based on these findings, we sought to determine if *i*) activation of VMN^{NOS1} neurons is sufficient and/or required for intact CRRs to hypoglycemia, and *ii*) if distinct projections from VMN^{NOS1} neurons mediate glycemic and behavioral responses or if instead the two responses are inextricably linked, as would be expected if the hyperglycemia is a manifestation of the behavioral response.

Research design and methods

Animals.

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee at the University of Washington. All studied animals were individually housed in a temperature-controlled room with a 12:12-h light:dark cycle under specific pathogen free conditions and provided with ad libitum access to water and chow unless otherwise noted. Nos1-Cre mice and C57Bl/6J mice were purchased from The Jackson Laboratory (stock no. 017526 and 000664, respectively).

Viral Microinjection and Fiber Placement Surgeries.

The viral vectors AAV5-EF1a-DIO-hChR2(H134R)-EYFP, AAVDJ8-EF1a-DIO-SwiChR$_{CA}$-TS-EYFP-WPRE, and AAV5-EF1a-DIO-EYFP used in this study have been previously described (Meek *et al.*, 2016). All viruses were purchased from the Gene Therapy Center at the University of North Carolina except the SwiChR virus, which was kindly provided by Karl Deisseroth (Stanford University, Stanford, CA) and packaged into a DJ8 vector by the University of Washington Diabetes Research Center Viral Vector and Transgenic Mouse Core. For viral microinjection, animals were placed in a stereotaxic frame (Kopf 1900; Cartesian Research Inc., CA) under isoflurane anesthesia. The skull was exposed with a small incision, and a small hole was drilled for unilateral 200-nL injection volume of AAV5-EF1a-DIO-hChR2(H134R)-EYFP, or 200-nL bilateral injection of the inhibitory AAVDJ8-EF1a-DIO-SwiChR$_{CA}$-TS-EYFP-WPRE, to the VMN of Nos1-Cre male mice based on slightly modified coordinates from the Mouse Brain Atlas:

anterior-posterior (AP) -1.0 mm; dorsal-ventral (DV) -5.7mm; lateral 0.45mm. AAV was delivered using a Hamilton syringe with a 33-gauge needle at a rate of 50 nL/min (Micro4 controller), followed by a 7- minute wait at the injection site, and a 1-minute wait 0.1 mm dorsal to the injection site before needle withdrawal. After viral injections, a fiberoptic ferrule (Doric Lenses, Quebec, CA) was implanted above either the VMN (AP: -1.0mm; DV: -5.3mm; lateral 0.45mm), the aBNST (AP: 0.86 mm; DV -4.4 mm, lateral 0.65 to the ipsilateral side), or the PAG (AP -4.2 mm; DV -2.2 mm; lateral 0.15 mm to the ipsilateral side) in separate cohorts of mice. For bilateral inhibition, fiberoptics were implanted at a 15 degree from center angle. Animals received a peri-operative subcutaneous injection of buprenorphine hydrochloride (0.05 mg/kg; Reckitt Benckiser, VA). Following surgery, mice were allowed two (for cell body stimulation) to six weeks (for terminal stimulation) to recover and to maximize viral expression. Mice were acclimated to handling and experimental conditions three times prior to the start of any in vivo studies. Viral expression and fiber placement were verified post hoc in all animals, and any data from animals in which the virus or fiber was located outside the targeted area were excluded from the analysis.

Optogenetic Photoactivation and Photoinhibition.

Optogenetic studies were supported by the Nutrition Obesity Research Center (NORC) Energy Balance Core at the University of Washington. Light was delivered to the target area from a diode laser (473nm, DPSS continuous wave laser system; Laserglow, Toronto, CA) controlled via pulse generator (Master-9; A.M.P.I., Jerusalem, IL) as previously described (Meek *et al.*, 2016). Briefly, the output beam from the laser system was delivered through a single fiber port connected to a fiberoptic rotary joint (Doric Lenses, Quebec, CA). A terminal fiber attached to the rotary joint was connected to the indwelling fiberoptic cannula via a ceramic mating sleeve (Thor Laboratories, NJ). Unless otherwise noted, all photostimulation experiments utilized 5-ms pulses at 40 Hz with an estimated 3 mW light power exiting the fiber tip. Photoinhibition experiments used a constant beam of light for 1-2h as indicated for each experiment. Irradiance at target regions is estimated at 7.82 mW/mm^2 based upon previously described light penetration through neural tissues (web.stanford.edu/group/dlab/cgi-bin/graph/chart.php).

Metabolic Studies and Tissue Processing.

To investigate the effect of selective activation or inhibition of VMN^{NOS1} neurons on glycemic control, we used a 3-h approach with alternating laser off/on/off sequences in Nos1-cre mice in a randomized, cross-over manner, as previously described (Meek *et al.*, 2016). Tail vein blood was collected for blood glucose levels at indicated times using a handheld glucometer (Abbott FreeStyle, CA). Tail blood for plasma hormonal measurement was collected at the end at the end of the “Laser on” photo-activation (Stim) or “Laser off” (Mock) period (t=60 min); additional blood was collected at the end of the baseline period (t=0) for photo-inhibition studies as indicated. Tail blood was collected in an EDTA-coated capillary tubes, centrifuged (10000 rpm, 7 min) and plasma subsequently removed and stored at -80 °C for subsequent assay. Plasma insulin (Crystal Chem, IL), corticosterone (Alpco, NH), and glucagon (Mercodia, NC) were determined by ELISA.

Intraperitoneal insulin tolerance tests (ITTs) (1.0U/kg body weight) were conducted in 4h fasted animals. Tail vein blood was collected for measurement of blood glucose levels at -15, 0, 15, 30, 45, 60, 90 and 120 min using a hand-held glucometer (Abbott Laboratories, IL).

Insulin-Induced Hypoglycemia.

For studies evaluating c-Fos expression during insulin-induced hypoglycemia, C57Bl/6J mice were acclimated to handling and intraperitoneal (i.p.) injections. On the study day, animals were fasted for 4 hours prior to receiving an i.p. injection of insulin (1.5 U/kg body weight; Humulin, Eli Lilly, IN) or saline control. After 60 minutes, animals were anesthetized and perfused as described below. For quantification of c-Fos expression, Fiji was used to define a standard region of interest (ROI) for three representative sections per animal spanning the aBNST (bregma +0.62 to 0.30mm) and the PAG (bregma -4.16 to -4.36), respectively. Briefly, images were converted to 16-bit and the threshold adjusted to minimize nonspecific background fluorescence. Cells were then identified and counted using the analyze particles feature, such that consistent fluorescence and size thresholds were used throughout, as previously described (Grishagin, 2015).

Immunohistochemistry.

For brain immunohistochemical (IHC) studies, after 1 hour of mock, photo-activation or -inhibition, animals were overdosed with ketamine:xylazine and perfused with PBS followed by 4% (v/v) paraformaldehyde (PFA) in 0.1M PBS. Brains were removed and post-fixed for 2-6 hours, sucrose (30%) embedded, and snap frozen in OCT blocks. Free-floating coronal sections were obtained via Crysotat at 30 microns and stored in PBS with azide at 4°C for IHC staining.

c-Fos immunostaining was carried out in free-floating sections. Briefly, sections were put into PBS with Tween 20 (PBST) overnight at 4°C. Sections were then washed at room temperature in PBST (3 x 8 minutes), followed by a blocking buffer (5% normal goat serum, 1% bovine serum albumin in 0.1M PBST with azide) for 60 minutes with rocking. Sections were then incubated overnight at 4°C with goat anti-cFos (Santa Cruz Biotechnology, TX; Cat# sc-52-G, RRID:AB_2629503) diluted 1:500 in blocking buffer. Next sections were washed (3 x 8 minutes) in PBST before incubating in secondary donkey anti-goat Alexa 594 (Jackson ImmunoResearch Laboratories, PA) diluted 1:300. Sections were then mounted to slides and imaged using a Nikon Eclipse E600 upright microscope equipped with a Diagnostic Instruments Spot RT Color digital camera.

Behavioral Quantification.

To avoid confounding stress responses, behavioral sessions were performed in the animals' home cages. All sessions were video recorded and subsequently analyzed with Ethovision (Noldus, VA) tracking software as previously described (Sanford *et al.*, 2017).

Statistical Analyses.

All results are presented as means ± SEM. *P* values for pairwise comparisons were calculated by two-tailed Student's *t* test. Time course comparisons between groups were analyzed using a two-way repeated measures ANOVA with main effects of treatment (mock vs. stimulation or inhibition) and time. All post hoc comparisons were determined using Sidak's correction for multiple comparisons. All statistical tests indicated were performed using Prism (version 7.4; GraphPad, CA) software.

Results

Activation of VMN^{NOS1} neurons induces hyperglycemia

To test the hypothesis that photoactivation of VMN^{NOS1} neurons is sufficient to engage CRRs and thereby raise blood glucose levels, Nos1-Cre+ mice received a unilateral microinjection to the VMN of an adeno-associated virus (AAV) encoding either a YFP control or a cre-dependent sodium-conducting channelrhodopsin fused with a fluorescent

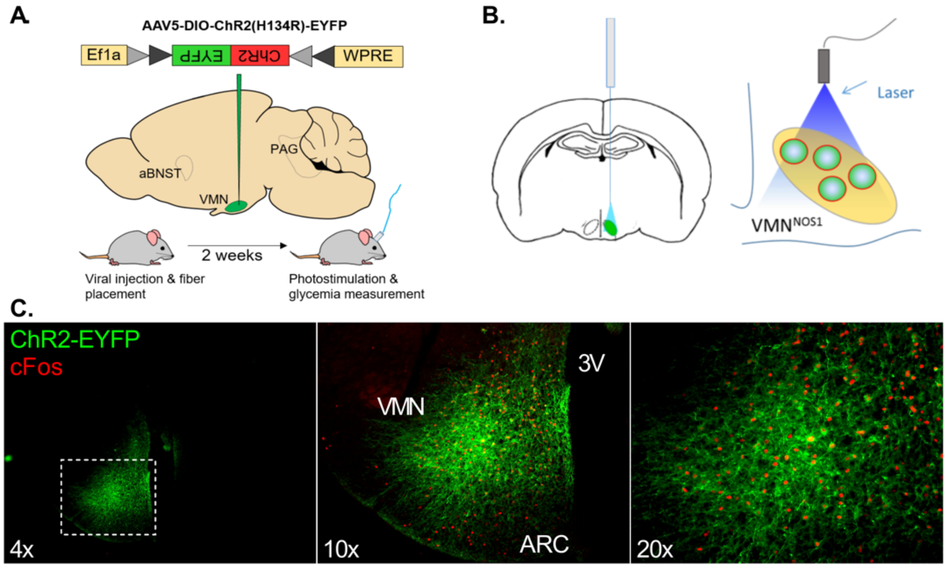


Figure 4.1 Strategy for photoactivation of VMN^{NOS1} neurons and verification of VMN targeting.
A) Schematic demonstrating unilateral microinjection of the cre-dependent ChR2-YFP or YFP control virus targeting the VMN of Nos1-Cre+ mice and B) fiberoptic placement dorsal to the injection site. C) Representative images indicating unilateral infection and expression of ChR2-EYFP and light-induced cFos restricted to the VMN of Nos1-Cre mice. 3V, third ventricle; arc, arcuate nucleus.

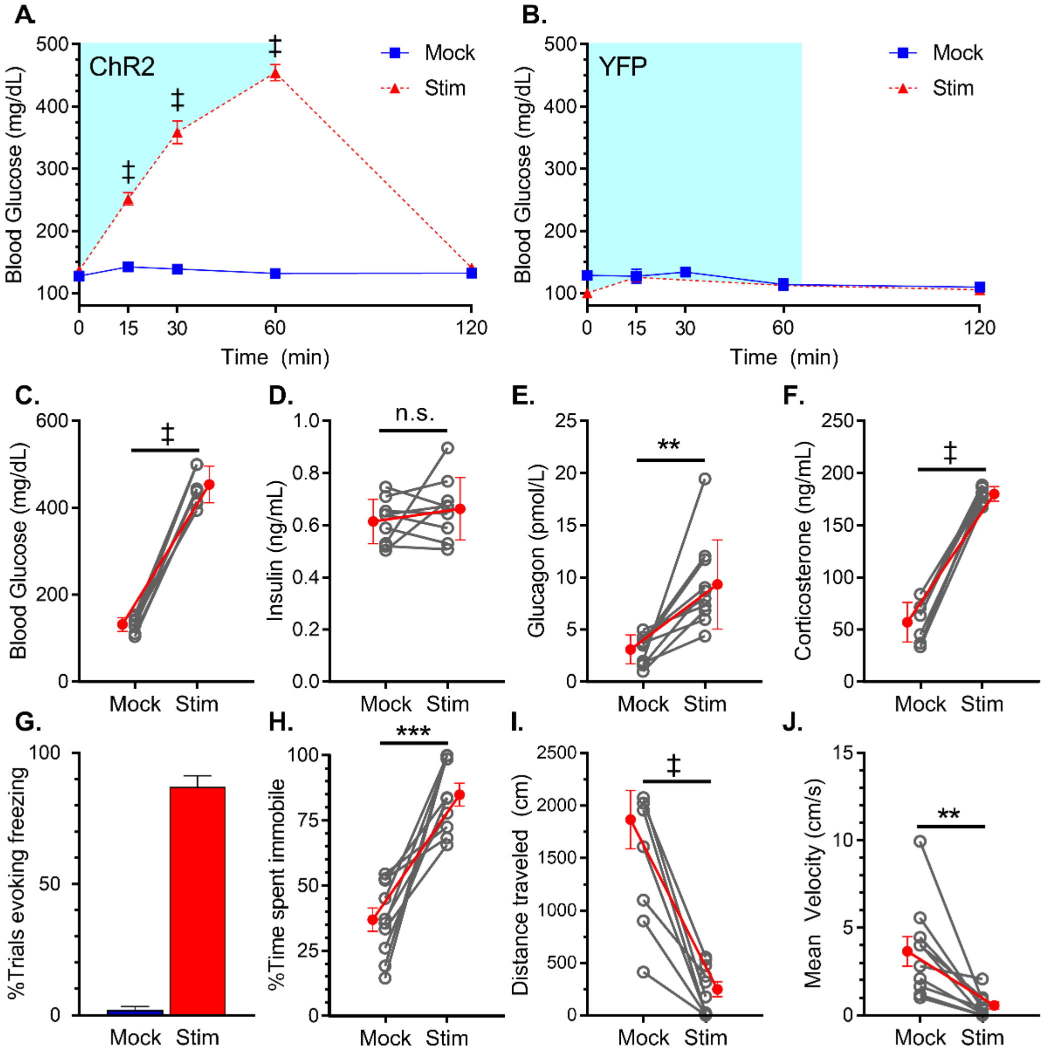


Figure 4.2 Photoactivation of VMN^{NOS1} neurons elicits both glucose counterregulatory responses and defensive freezing immobility.
Blood glucose levels during unilateral laser off (Mock) and laser-induced stimulation (Stim) of VMN^{NOS1} neurons in **A)** ChR2-YFP injected (n=10) or **B)** YFP control injected (n=3) animals; blue shading represents the duration of laser stimulation. **C)** Blood glucose values from ChR2-YFP animals at the 60 minute time point during which tail blood was collected for measurement of **D)** insulin, **E)** glucagon, and **F)** corticosterone. **G)** Percentage of photoactivation trials evoking freezing. **H)** Percentage of time spent immobile, **I)** total distance traveled, and **J)** average velocity during mock- and photostimulation. Values are mean ± SEM; p values by two-way ANOVA (A,B) or two-tailed, paired Student's *t*-test (C-J); $^{**}p<0.01$, $^{***}p<0.001$, ‡$p<0.0001$, n.s. not significant.

reporter (AAV5-EF1a-DIO-hChR2(H134R)-EYFP, hereafter called ChR2-YFP), followed by implantation of an optic fiber above the injection site (Figure 4.1A and B). As expected, ChR2-EYFP expression was largely restricted to the VMN and not adjacent nuclei (Figure 4.1C). Activation of VMN neurons following photostimulation was further confirmed by increased expression of the immediate-early gene, c-Fos (Figure 4.1C). As predicted, photoactivation of VMN^{NOS1} neurons rapidly elevated blood glucose levels, with levels returning to baseline within 1 h of termination of the light stimulus (Figure 4.2A), an effect that recapitulates the response to photoactivation of VMN^{SF1} neurons (of which VMN^{NOS1} neurons are a subset) (Meek *et al.*, 2016). Because this effect was not observed in Nos1-Cre+ animals that underwent the same light stimulation procedure, but had received a YFP control virus (Figure 4.2B), we conclude that the observed glycemic responses were not due to nonspecific effects of blue light delivery to the VMN, and instead reflect a specific response to activation of VMN^{NOS1} neurons. Moreover, our findings that there was little if any effect on blood glucose levels following photoactivation of Nos1 neurons in which viral and/or fiberoptic targeting was detected predominantly outside the VMN (data not shown) further strengthen our hypothesis that NOS1 neurons in the VMN, but not those in adjacent brain areas, have potent glucoregulatory effects.

Autonomic and neuroendocrine effects of VMN^{NOS1} photoactivation

Based on the response to photoactivation of VMN^{SF1} neurons (Meek *et al.*, 2016), we hypothesized a role for autonomic and neuroendocrine mechanisms engaged during glucose counterregulation in the hyperglycemic response to activation of VMN^{NOS1} neurons. Consistent with this hypothesis, we found that the effect of photoactivation of VMN^{NOS1} neurons to induce hyperglycemia (Figure 4.2A, 60 minute, shown in Figure 4.2C as line graph) was associated with a marked rise in plasma levels of the counterregulatory hormones corticosterone and glucagon (Figure 4.2E and F). The increase of glucagon levels is particularly notable in that while it plays a key role in glucose counterregulation, it is not typically a component of the nonspecific response to fear-inducing stimuli (Ulrich-Lai and Herman, 2009). Further, plasma insulin levels failed to increase, despite marked hyperglycemia, indicating potent autonomic suppression of glucose-stimulated insulin secretion (GSIS) (Figure 4.2D). These cardinal features of the CRR fully recapitulate the effects of VMN^{SF1} activation.

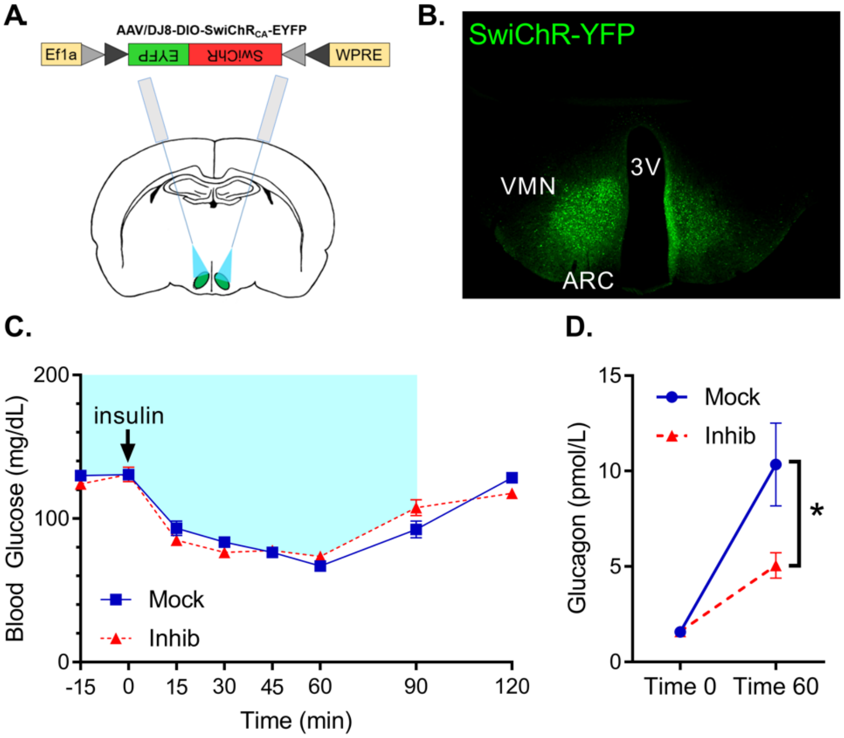


Figure 4.3 Photoinhibition of VMN^{NOS1} neurons selectively impairs glucagon responses during insulin-induced hypoglycemia.
A) Schematic demonstrating bilateral microinjection of the cre-dependent inhibitory SwiChR-YFP virus, and fiberoptic placement dorsal to the injection site, targeting the VMN of Nos1-Cre+ mice. **B**) Blood glucose levels in the basal state during bilateral laser off (Mock) or laser-induced inhibition (Inhib) of VMN^{NOS1} neurons; blue shading represents duration of laser-induced inhibition. **C**) Blood glucose levels in Nos1-Cre+ mice with mock- or photoinhibition of VMN^{NOS1} neurons during insulin-induced hypoglycemia. Changes in **D**) glucagon and **E**) corticosterone levels during insulin-induced hypoglycemia during mock and photo-inhibition of VMN^{NOS1} neurons (n=12 for all). Values are mean ± SEM; p values by two-way ANOVA; $*p<0.05$.

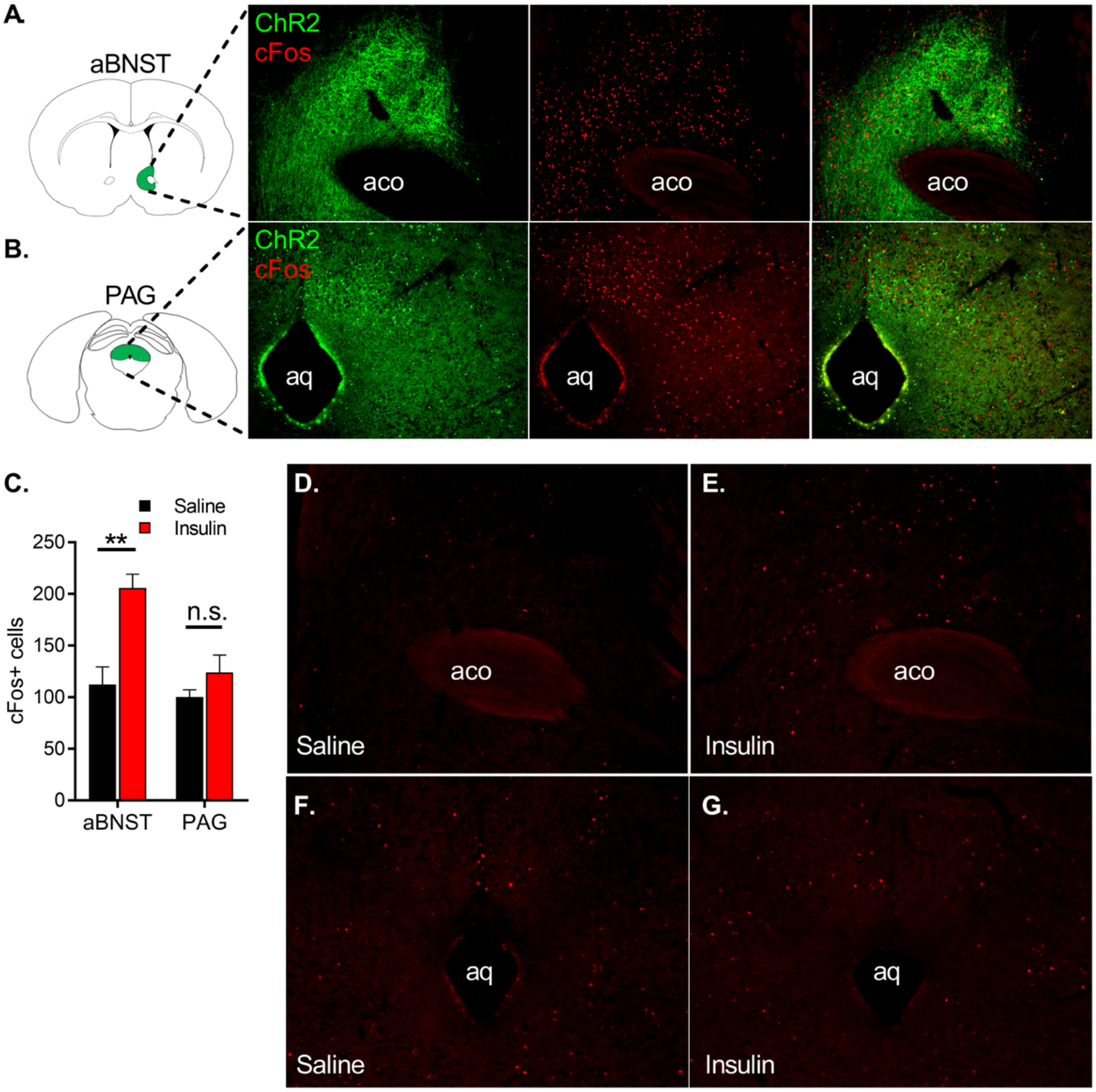


Figure 4.4 VMN^{NOS1} neurons project to and activate neurons in both the aBNST and the PAG, while insulin-induced hypoglycemia increases cFos expression within the aBNST.
Fluorescently labeled projections of ChR2-expressing VMN^{NOS1} neurons in the aBNST **(A, left panel)** and PAG **(B, left panel).** Photoactivation of upstream VMN^{NOS1} neurons for 60 minutes elicits robust cFos expression in these regions **(A and B, middle panel)**. **C)** Quantification of cFos+ cells in the aBNST and PAG of C57Bl/6J male mice following i.p. saline (n=4) or insulin (n=6; 1.5U/kg). Representative 10x magnification of cFos induction in the aBNST **(D, E)** and PAG **(F,G)** following i.p. saline (left) or insulin (right). aco, anterior commissure; aq, cerebral aqueduct. Values are mean ± SEM; two-tailed, unpaired Student's *t*-test for each brain region; **p<0.01, n.s. not significant.

Behavioral effects of VMN^{NOS1} photoactivation

Consistent with the known role of the VMN in mediating defensive behaviors, we found that in addition to glycemic effects (Figure 4.2A-F), freezing immobility behavior was observed throughout the entire period during which VMN^{NOS1} neurons were activated (Figure 4.2G). This effect was quantified as an increase in overall time spent immobile (Figure 4.2H) and a corresponding decrease in the total distance traveled and average velocity (Figure 4.2I and J). Importantly, these behaviors were not observed in Nos1-Cre+ animals that received a YFP control virus. Taken together, these data suggest that VMN^{NOS1} neuron activation elicits defensive freezing behaviors characteristic of generalized VMN stimulation and suggestive of the response to an underlying fearful emotional state.

VMN^{NOS1} neurons are required for an intact glucagon response to insulin-induced hypoglycemia

To investigate the physiological role of VMN^{NOS1} neurons in the CRR, we asked whether activation of these neurons is required for the ability to recover from insulin-induced hypoglycemia. To this end, we utilized a complementary optogenetic approach in which a light-activated chloride-conducting channel was selectively expressed in VMN^{NOS1} neurons, such that light delivery hyperpolarizes and thereby reduces the firing of these neurons (Berndt *et al.*, 2014; Meek *et al.*, 2016). Specifically, AAVDJ8-EF1-DIO-SwiChR$_{CA}$-TS-EYFP (hereafter called SwiChR-YFP) was microinjected bilaterally to the VMN of Nos1-Cre+ mice, followed by angled bilateral fiberoptic placement dorsal to each injection site (Figure 4.3A and B). We report that photoinhibition of VMN^{NOS1} neurons had no effect on blood glucose levels following saline injection (p=ns), suggesting that, like VMN^{SF1} neurons, activation of VMN^{NOS1} neurons is not required for maintenance of

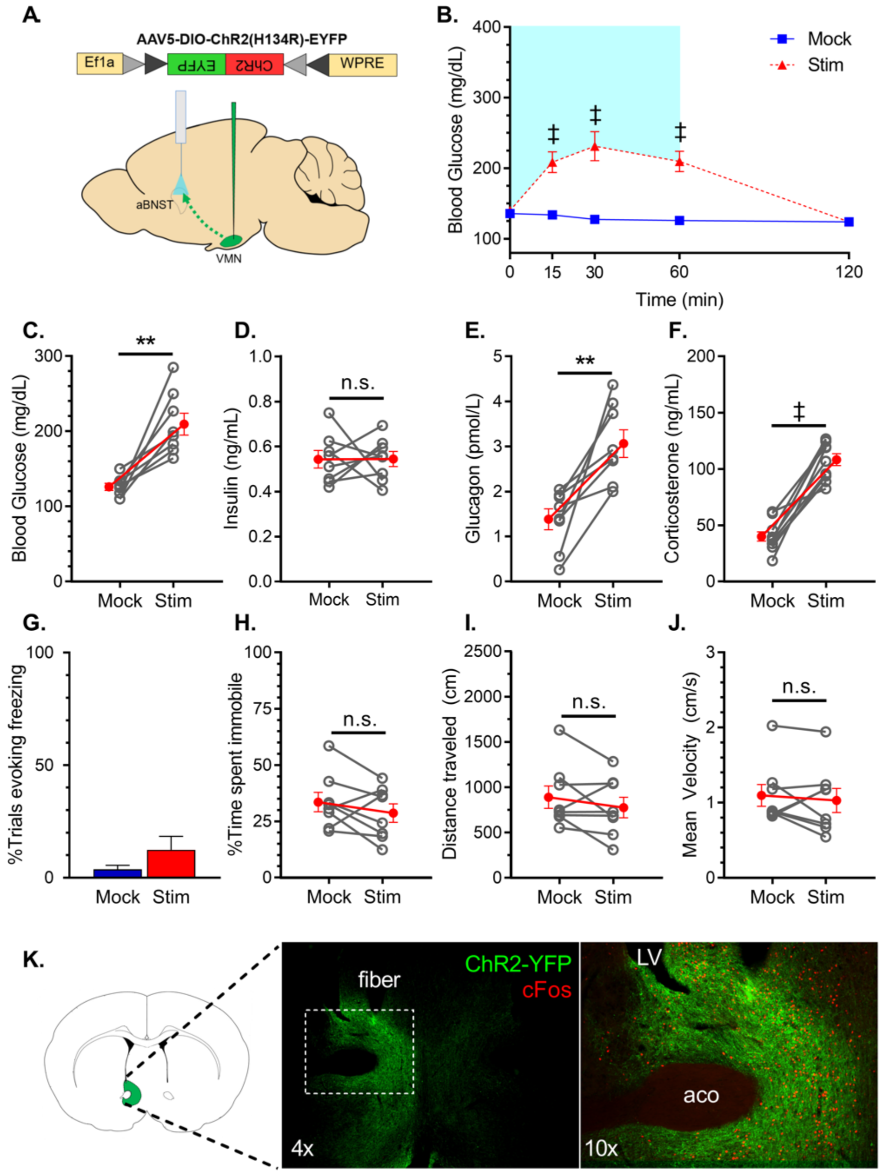


Figure 4.5 Photoactivation of VMN^{NOS1}→aBNST projections selectively promotes hyperglycemia by activating CRRs, without eliciting freezing immobility.

A) Schematic for laser off (Mock) or laser-induced stimulation (Stim) of VMN^{NOS1} terminals in the aBNST (VMN^{NOS1}→aBNST). **B)** Blood glucose levels during ipsilateral mock- and photoactivation of VMN^{NOS1}→aBNST terminals; blue shading represents the duration of laser-induced stimulation. **C)** Blood glucose values at the 60 minute time point during which tail blood was collected for measurement of **D)** insulin, **E)**, glucagon, and **F)** corticosterone. **G)** Percentage of photoactivation trials evoking freezing. **H)** Percentage of time spent immobile, **I)** total distance traveled, and **J)** average velocity during mock- and photostimulation (n=8 for all). **K)** Representative image indicating terminals of ChR2-expressing VMN^{NOS1} neurons within the ipsilateral aBNST and light-induced cFos within the aBNST. LV, lateral ventricle; aco, anterior commissure. Values are mean ± SEM; p values by two-way ANOVA (B) and two-tailed, paired Student's *t*-test (C-J); **p<0.01, ‡p<0.0001, n.s. not significant.

normal blood glucose levels in the basal state. Unlike photoinhibition of VMN^{SF1} neurons (Meek *et al.*, 2016), however, photoinhibition of VMN^{NOS1} neurons did not impair overall recovery from insulin-induced hypoglycemia (Figure 4.3C), relative to mock controls. Nevertheless, the increase of circulating glucagon levels that normally occurs during hypoglycemia was selectively blunted when VMN^{NOS1} neurons were silenced (Figure 4.3D), while corticosterone levels (Mock: 118.1 ± 35.15 ng/mL vs. Inhib: 122.2 ± 31.21 ng/mL) during hypoglycemia were unaffected. Taken together, these data demonstrate that activation of VMN^{NOS1} neurons is required for an intact glucagon response to insulin-induced hypoglycemia.

Identification of downstream projections of VMN^{NOS1} neurons mediating glycemic and behavioral responses

As a first step to determine whether divergent glycemic and behavioral responses associated with VMN^{NOS1} activation can be dissociated from one another, we sought to identify projection fields of VMN^{NOS1} neurons tagged with fluorescently labeled channelrhodopsin virus. Consistent with previous data (Canteras, Simerly and Swanson, 1994; Meek *et al.*, 2016), fluorescently labeled projections were detected in both the ipsilateral aBNST (Figure 4.4A, left) and both Figure 4.4A, left) and both ipsilateral and contralateral PAG (Figure 4.4B, left), areas implicated in autonomic control of metabolism and defensive behavior, respectively (Niimi *et al.*, 1995; Benarroch, 2012; Crestani *et al.*, 2013; Flak *et al.*, 2017). Moreover, neuron activation (as judged by c-Fos induction) was observed in both areas (Figure 4.4A and B) following photoactivation of VMN^{NOS1} soma.

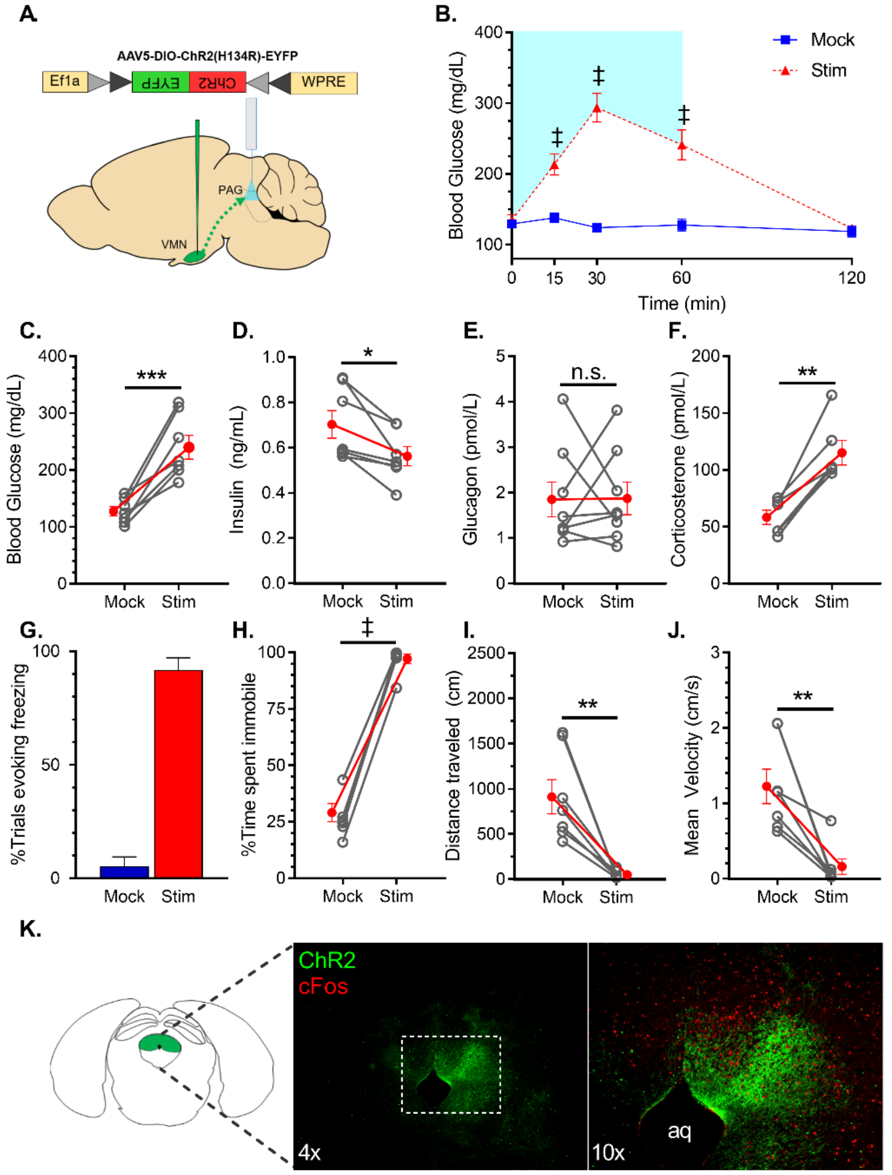


Figure 4.6 Photoactivation of VMN^{NOS1}→PAG projections elicits defensive freezing immobility.

A) Schematic for laser off (Mock) or light-induced stimulation (Stim) of VMN^{NOS1} terminals in the PAG (VMN^{NOS1}→PAG). **B**) Blood glucose levels during ipsilateral mock- and photoactivation of VMN^{NOS1}→PAG terminals; blue shading represents the duration of laser-induced stimulation. C) Blood glucose values at the 60 minute time point during which tail blood was collected for measurement of **D**) insulin, **E**), glucagon, and **F**), and corticosterone. **G**) Percentage of photoactivation trials evoking freezing. **H**) Percentage of time spent immobile, **I**) total distance traveled, and **J**) average velocity during mock- and photostimulation (n=8 for all). **K**) Representative image indicating terminals of ChR2-expressing VMN^{NOS1} neurons in the PAG and light-induced cFos within the PAG. aq, cerebral aqueduct. Values are mean ± SEM; p values by two-way ANOVA (B) and two-tailed, paired Student's *t*-test (C-J); *p<0.05, **p<0.01, ***p<0.001, ‡p<0.0001, n.s. not significant.

Innervation of the PAG was particularly robust within its dorsolateral portion superior to the cerebral aqueduct.

Electrophysiological studies of the VMN have shown the presence of both glucose-excited (GE) and glucose-inhibited (GI) neurons in this area, which decrease and increase their firing in response to a fall in ambient glucose levels, respectively (Routh, 2010). Importantly, work from Routh and colleagues suggests that NOS1 neurons are predominantly GI (Fioramonti *et al.*, 2010), and activation of these neurons, alongside inhibition of GE neurons, in response to hypoglycemia may be required for the full CRR (Fioramonti *et al.*, 2010). Based on these data, and on evidence that VMN neurons are predominantly glutamatergic (Tong *et al.*, 2007; Chachlaki *et al.*, 2017), we hypothesized that brain regions downstream of GI VMN^{NOS1} neurons would be activated during insulin-induced hypoglycemia.

To interrogate whether either the aBNST or PAG are activated during hypoglycemia, we exposed C57Bl/6J mice to insulin-induced hypoglycemia and evaluated c-Fos induction within these regions. Our data reproduce previous evidence that insulin-induced hypoglycemia activates neurons within the aBNST (Niimi *et al.*, 1995), but not within the PAG (Figure 4.4C-G). We therefore hypothesized that selective activation of the VMN^{NOS1}→aBNST neurocircuit activates CRRs, whereas activation of the VMN^{NOS1}→PAG neurocircuit elicits the defensive freezing behaviors associated with VMN^{NOS1} activation.

To test this hypothesis, we used an optogenetics approach to stimulate the projection fields of VMN^{NOS1} neurons supplying the aBNST and the PAG. Specifically, AAV encoding cre-dependent ChR2-YFP was microinjected unilaterally to the VMN of

Nos1-Cre+ mice, followed by ipsilateral implantation of an optic fiber above projection fields within either the aBNST (Figure 4.5*A*) or PAG (Figure 4.6*A*) in separate cohorts of mice. In extension of our previous data (Meek *et al.*, 2016), we found that photoactivation of VMN^{NOS1}→aBNST terminals recapitulated the effects of VMN^{NOS1} cell body activation to rapidly and reversibly raise blood glucose levels (Figure 4.5B), a response accompanied by elevated plasma levels of both glucagon and corticosterone (Figure 4.5E and F) and suppression of GSIS (Figure 4.5D). Moreover, this effect occurred in the absence of the freezing immobility elicited by VMN^{NOS1} cell body stimulation (Figure 4.5G-J). Collectively, these findings corroborate our previous data implicating the subset of aBNST-projecting VMN neurons as components of a neurocircuit involved in glucose counterregulation.

We also report that, as predicted, photoactivation of VMN^{NOS1}→PAG projections reliably and robustly reproduced the freezing immobility seen upon VMN^{NOS1} activation (Figure 4.6G-J). While we also found that photoactivation of VMN^{NOS1}→PAG projections elicited hyperglycemia (Figure 4.6B), activation of these projections failed to increase glucagon secretion (Figure 4.6E). Although activation of this circuit is clearly capable of inducing both hyperglycemia and behavioral fear responses, therefore, it does not fully engage the prototypic CRR, as seen following activation of the VMN^{NOS1}→aBNST projection.

Discussion

The brain requires a continuous supply of glucose to support its energy demands and, in response to an acute energy deficit (*e.g.* hypoglycemia), it engages rapid, potent, and highly integrated neuroendocrine and autonomic responses that return blood glucose into the normal range. Although many brain areas participate in this response (Ritter *et al.*, 2011), we recently reported that activation of the subset of VMN neurons that express SF1 is both necessary and sufficient for intact CRRs in response to insulin-induced hypoglycemia (Meek *et al.*, 2016). Here, we extend this work by showing that the subset of these VMN neurons that express NOS1 contributes to this effect.

Our findings show that while inhibition of VMN^{NOS1} neurons selectively blunts glucagon responses during insulin-induced hypoglycemia, the recovery of blood glucose levels remains intact, thereby providing further evidence that multiple, redundant central and peripheral pathways contribute to the defense against hypoglycemia (McCrimmon, 2008). Conversely, we found that selective photoactivation of VMN^{NOS1} neurons in otherwise normal mice causes robust hyperglycemia characterized by activation of CRRs normally reserved for the response to hypoglycemia (inhibition of GSIS and increased secretion of both glucagon and corticosterone – epinephrine also plays a key role in glucose counterregulation, however, we were unable to measure epinephrine in the current studies). Since photoactivation of VMN^{NOS1} neurons also induces defensive behaviors (i.e. freezing immobility), however, the question is raised as to whether the behavioral and metabolic responses are inextricably linked to one another or instead involve neuronal projections to distinct brain areas. Our findings support the latter hypothesis. Specifically, we found that activation of VMN^{NOS1} projections to the aBNST ($\text{VMN}^{\text{NOS1}}\rightarrow$aBNST) induces CRRs and hyperglycemia, while defensive behavioral responses were only observed following activation of VMN^{NOS1} terminals in the PAG ($\text{VMN}^{\text{NOS1}}\rightarrow$PAG). Together, these findings suggest that the subset of VMN^{NOS1} neurons that project to the aBNST is involved in glucose counterregulation, while the VMN^{NOS1}→PAG pathway is linked to fear-induced behavioral responses.

Although compelling evidence implicates the VMN in both autonomic and neuroendocrine CRRs that drive recovery from hypoglycemia (Frohman and Bernardis, 1971; Dubuc, Leshin and Willis, 1982; Borg *et al.*, 1994, 1995, 1997), activation of the VMN also elicits “fight or flight” (Spiteri *et al.*, 2010; Silva *et al.*, 2013), defensive (Kunwar *et al.*, 2015; Wang, Chen and Lin, 2015), and aggressive (Lin *et al.*, 2011; Falkner *et al.*, 2014; Lee *et al.*, 2014) behaviors, and the extent to which the glycemic response is secondary to or is an inherent component of the behavioral stress response is unknown. Here, we considered the possibility that these divergent biological functions reflect the activity of distinct subsets of VMN neurons that project to discrete downstream brain regions. Our focus on VMN neurons expressing NOS1 is based on evidence that decreased glucose activates NOS1 and increases NO production in mediobasal hypothalamus (MBH) (Canabal *et al.*, 2007) and that NOS1 is required for intact glucose sensing by GI neurons in the VMN (Fioramonti *et al.*, 2010). Moreover, MBH injection of a non-selective NOS inhibitor impaired the CRR and blunted the recovery from insulin-induced hypoglycemia (Fioramonti *et al.*, 2010), while the CRR to hypoglycemia is impaired in NOS1-deficient mice (Fioramonti *et al.*, 2010). Lastly, VMN^{NOS1} neurons are a subset of VMN^{SF1} neurons, activation of which robustly engages CRRs. We therefore interpret our findings that i) photoinhibition of VMN^{NOS1} neurons selectively impairs glucagon responses during insulin-induced hypoglycemia, and ii) photoactivation of VMN^{NOS1} neurons induces diabetes-range hyperglycemia in otherwise normal mice via a mechanism involving activation of CRRs to suggest an important role for VMN^{NOS1}

neurons to drive specific components of the CRR to hypoglycemia. While the mechanism(s) that mediate each of these responses remain to be elucidated, long-standing evidence suggests that the islet is under autonomic control (Begg and Woods, 2013), and increased sympathetic activity both increases glucagon (Taborsky and Mundinger, 2012) and inhibits insulin secretion (Thorens, 2014).

Several lines of evidence also link activation of VMN neurons to “fight or flight” and other behavioral responses to fearful or threatening stimuli. For example, neurons within the VMNvl are activated during escape and defensive responses to an aggressive conspecific, and predator exposure activates neurons in the VMNdm; conversely, painful stimuli (e.g. foot shock) does not activate VMN neurons (Silva *et al.*, 2013). The hypothesis that specific VMN neuronal populations are recruited during predator and social fear, and that similar fear behaviors recruit different brain circuits, is also supported by data from pharmacogenetics studies showing that inhibition of the VMNvl decreases defensive responses to an aggressive conspecific (Silva *et al.*, 2013), whereas activation of a specific subset of VMNvl neurons (marked by expression of estrogen receptor 1) induces mating behavior and fighting in rodents (Falkner *et al.*, 2014; Lee *et al.*, 2014). By comparison, pharmacogenetic inhibition of the VMNdm impairs defensive responses to predators (Silva *et al.*, 2013), while photoactivation of VMNdm neurons induces flight and freezing behavior (Kunwar *et al.*, 2015; Wang, Chen and Lin, 2015). These behaviors also appear to occur in a scalable manner, with less intense stimulation inducing freezing and more intense stimulation evoking activity bursts (Kunwar *et al.*, 2015). Taken together, these studies suggest that the VMNdm, a region also implicated in the control of energy homeostasis and metabolism (Dhillon *et al.*, 2006; Choi *et al.*, 2013), is involved in predator fear and other defensive behaviors, whereas the VMNvl is a region involved in sexual and aggressive behavior and social fear (Spiteri *et al.*, 2010; Lin *et al.*, 2011; Falkner *et al.*, 2014; Lee *et al.*, 2014).

As a first step to investigate whether the subset of $\mathrm{VMN}^{\mathrm{NOS1}}$ neurons involved in glucose counterregulation are causally linked to these behavioral responses, we characterized the downstream projection fields of these neurons using a fluorescently-labeled channelrhodopsin. Consistent with work from us and others, the aBNST and PAG receive the heaviest projections from $\mathrm{VMN}^{\mathrm{NOS1}}$ neurons (Canteras, Simerly and Swanson, 1994; Gross and Sabino Canteras, 2012; Meek *et al.*, 2016). Our finding that photo-stimulation of the subset of $\mathrm{VMN}^{\mathrm{NOS1}}$ neurons that project to the aBNST mimics the glycemic response elicited by $\mathrm{VMN}^{\mathrm{NOS1}}$ neuronal stimulation (including increases of plasma glucagon and corticosterone levels and inhibition of GSIS), but does not induce defensive or other detectable behaviors, provides compelling evidence that the $\mathrm{VMN}^{\mathrm{NOS1}}$→aBNST projection constitutes a neurocircuit involved in glycemic control, but not in behavioral regulation. Implicit in this conclusion is not only that a behavioral response is not required for VMN neuron activation to elicit robust hyperglycemia, but also that a distinct $\mathrm{VMN}^{\mathrm{NOS1}}$ neuronal projection must drive the behavioral response.

Among various VMN projection fields with the potential to mediate defensive behaviors, the PAG stands out because *i)* this brain area is activated in response to a wide variety of threats (Vianna and Brandão, 2003; Motta *et al.*, 2009); *ii)* impaired PAG function diminishes the expression of defensive behaviors including freezing, risk assessment and flight (Hunsperger, 1963; Silva *et al.*, 2013); *iii)* stimulation of the PAG is sufficient to induce defensive responses including freezing, escape and flight (Quintino-dos-Santos *et al.*, 2014) and *iv)* this brain area (particularly, its dorsal columns) receives input from the VMN (Canteras, Simerly and Swanson, 1994; Gross and Sabino Canteras, 2012; Meek *et al.*, 2016). Our finding that photo-stimulation of the subset of VMN^{NOS1} neurons that project to the PAG elicits defensive behaviors is consistent with previous evidence linking projections from the VMNdm to the dorsal region of the PAG in this behavior, whereas projections to the anterior hypothalamic area are implicated in risk assessment and flight (Wang, Chen and Lin, 2015).

Given this evidence linking the VMN→PAG projection in defensive behavior, it came as something of a surprise that hyperglycemia is also induced by photo-stimulation of VMN^{NOS1} neurons that project to the PAG, especially since activation of VMN^{SF1}→PAG projections does not have this effect (Meek *et al.*, 2016). The mechanism driving this response is not identical with that engaged by projections of VMN^{NOS1} neurons to the aBNST, however, since although it raised corticosterone levels (reflecting activation of the hypothalamic-pituitary-adrenal (HPA) axis), glucagon secretion was not increased. Furthermore, we report that insulin-induced hypoglycemia activates neurons in the aBNST but not the PAG. We interpret these collective findings to suggest that activation of the VMN^{NOS1}→PAG projection induces a nonspecific stress response marked by defensive behaviors, activation of the HPA axis and hyperglycemia, but does not participate in glucose counterregulation *per se*. This hypothesis warrants additional investigation, particularly given recent evidence suggesting a role for the PAG in mobilizing glucose during exposure to noxious or painful stimuli (Flak *et al.*, 2017).

In conclusion, we report that activation of the subset of VMN neurons that express NOS1 rapidly induces diabetes-range hyperglycemia owing to the combination of a marked increase in glucagon and corticosterone secretion and inhibition of GSIS, characteristic of the CRR to hypoglycemia. We further show that activation of the specific subset of VMN^{NOS1} neurons that project to the aBNST is sufficient to engage the same glucoregulatory response, while having no detectable behavioral phenotype, whereas VMN^{NOS1} neurons that project to the PAG are implicated in defensive behaviors. Further insight into these VMN neurocircuits will inform our understanding of both hypoglycemia and its complications in patients with diabetes (Frier, 2014) and mechanisms underlying the “fight or flight” response and other defensive behaviors.

Chapter 5 In Uncontrolled Diabetes, Hyperglucagonemia and Ketosis Result from Deficient Leptin Action in the Parabrachial Nucleus

Published previously in (Meek *et al.*, 2018).

Abstract

Growing evidence implicates neurons that project from the lateral parabrachial nucleus (LPBN) to the hypothalamic ventromedial nucleus (VMN) in a neurocircuit that drives counterregulatory responses to hypoglycemia, including increased glucagon secretion. Among LPBN neurons in this circuit is a subset that expresses cholecystokinin ($LPBN^{CCK}$ neurons) and is tonically inhibited by leptin. Since uncontrolled diabetes is associated with both leptin deficiency and hyperglucagonemia, and since intracerebroventricular (icv) leptin administration reverses both hyperglycemia and hyperglucagonemia in this setting, we hypothesized that deficient leptin inhibition of $LPBN^{CCK}$ neurons drives activation of this LPBN→VMN circuit and thereby results in hyperglucagonemia. Here, we report that although bilateral microinjection of leptin into the LPBN does not ameliorate hyperglycemia in rats with streptozotocin-induced diabetes mellitus (STZ-DM), it does attenuate the associated hyperglucagonemia and ketosis. To determine if LPBN leptin signaling is required for the anti-diabetic effect of icv leptin in STZ-DM, we studied mice in which the leptin receptor was selectively deleted from $LPBN^{CCK}$ neurons. Our findings show that although leptin signaling in these neurons is not required for the potent anti-diabetic effect of icv leptin, it is required for leptin-mediated suppression of diabetic hyperglucagonemia. Taken together, these findings suggest that leptin-mediated effects

in animals with uncontrolled diabetes occurs through actions involving multiple brain areas, including the LPBN, where leptin acts specifically to inhibit glucagon secretion and associated ketosis.

Introduction

The recent finding that systemic (Yu *et al.*, 2008; Wang *et al.*, 2010) or intracerebroventricular (icv) (Hikada *et al.*, 2002; Lin *et al.*, 2002; Da Silva *et al.*, 2006; Fujikawa *et al.*, 2010; German *et al.*, 2011) administration of the adipocyte hormone leptin can fully normalize blood glucose levels in rodent models of uncontrolled, insulin deficient diabetes mellitus (uDM) suggests that mechanisms driving hyperglycemia in this setting are sensitive to leptin action in the brain. Among these mechanisms is increased hepatic glucose production (HGP) and reduced glucose utilization, both of which are reversed by icv leptin (German *et al.*, 2011). Also normalized in this setting are elevated plasma levels of glucagon, corticosterone and ketone bodies (Fujikawa *et al.*, 2010; German *et al.*, 2011). Since this action of leptin occurs despite the absence of any detectable effect on pancreatic insulin synthesis or secretion, leptin action in the brain appears to have the capacity to regulate glucose metabolism independent of insulin action. A key priority for future work is to identify the neuronal substrates underlying this effect.

The biological actions of leptin are mediated through the long form of the leptin receptor (LepRb) (Robertson, Leinninger and Myers, 2008) that is expressed widely in the brain, including both hypothalamic and extra-hypothalamic areas (Elmquist, Bjørbæk, *et al.*, 1998; Baskin, Schwartz, *et al.*, 1999). Within the hypothalamus, leptin action in the ventromedial nucleus (VMN) appears to be sufficient, but not required, for leptin’s glucose-lowering effects in rodent models of type 1 diabetes (T1D) (Meek *et al.*, 2013). Similarly, leptin receptor signaling in pro-opiomelanocortin (Pomc) neurons, situated in the adjacent arcuate nucleus (ARC), is also not required for the anti-diabetic effect of leptin, since 1) re-activation of leptin receptors in these neurons does not recapitulate the anti-diabetic effects of leptin in rodent models of T1D, and 2) leptin's glucose-lowering effects are only mildly attenuated in mice lacking leptin receptors in Pomc neurons (Fujikawa *et al.*, 2013). By comparison, leptin signaling in γ-aminobutyric (GABAergic) neurons appears to mediate much of the glucose-lowering effects of leptin in insulin-deficient mice (Fujikawa *et al.*, 2013). Yet neither glutamate nor GABA release from LepRb (+) neurons is required for leptin's anti-diabetic effects, whereas STAT3, a key signal transduction molecule involved in leptin signaling, is required (Xu and Tong, 2017). Taken together, these observations suggest that leptin actin within a distributed neuronal network, rather than in a particular brain area or neuronal subset, may mediate reversal of hyperglycemia in uncontrolled diabetes.

Among other leptin-regulated neurons implicated in glucose homeostasis are those situated in the lateral parabrachial nucleus (LPBN) that co-express the leptin receptor and cholecystokinin (CCK) (Garfield *et al.*, 2014; Flak *et al.*, 2015). These LPBN $\text{LepRb}^{\text{CCK}}$ neurons project to the VMN, and activation of this LPBN→VMN neurocircuit is implicated in the counter-regulatory response (CRR) to hypoglycemia responsible for returning low blood glucose levels (e.g., during insulin-induced hypoglycemia) back to the normal range. Specifically, LPBN neurons that express both CCK and LepRb are activated by hypoglycemia and, using a DREADD-based pharmacogenetic approach, activation of LPBN $\text{LepRb}^{\text{CCK}}$ neurons is sufficient to activate the CRR and thereby raise blood glucose levels, while conversely, inhibition of the same neurons blunts the glycemic response to glucoprivation (Garfield *et al.*, 2014; Flak *et al.*, 2015). Since this circuit is engaged to increase blood glucose in response to noxious stimuli (Flak *et al.*, 2017), it's activation may serve to raise blood glucose levels across a wide range of physiological and pathophysiological conditions.

Many of the same behavioral, neuroendocrine and autonomic responses are engaged in response to either deficiency of immediately available fuel (i.e. low glucose) or stored energy (i.e. low leptin) (Garfield *et al.*, 2014; Morton, Meek and Schwartz, 2014; Flak *et al.*, 2015, 2017; Xu and Tong, 2017). Since activation of this $\text{LepRb}^{\text{PBN}}$→VMN circuit is both strongly implicated in the CRR to hypoglycemia and sufficient to raise blood glucose levels (Meek *et al.*, 2016), its activation could also potentially contribute to hyperglycemia and associated neuroendocrine abnormalities characteristic of uDM. To test this hypothesis, we determined whether leptin action limited to the LPBN is capable of blunting either hyperglycemia or its underlying neuroendocrine mediators in rodent models of T1D. Our findings implicate deficient leptin action in this brain area in the genesis of increased glucagon levels (and associated ketosis) in this setting.

Research design and methods

Animals

All procedures were performed in accordance with NIH Guidelines for Care and Use of Animals and were approved by the Animal Care Committee at the University of Washington. All studied animals were individually housed under specific-pathogen free (SPF) conditions in a temperature-controlled room with a 12:12h light:dark cycle and provided with ad libitum access to water and chow unless otherwise stated (PMI Nu trition, MO).

Adult male Wistar rats were obtained from Harlan Laboratories, IN. To determine if leptin's anti-diabetic effects in uDM is dependent on leptin receptor (LepRb) expression in the lateral PBN (LPBN), we generated mice with deletion of leptin receptors from this brain region. Since a variety of cell types in the LPBN express LepRb (Flak *et al.*, 2015), this goal was achieved by crossing Cck^{cre} mice (which express *cre* recombinase in CCK neurons) with $Lepr^{flox/flox}$ animals to generate mice lacking LepRb expression specifically in this neuronal subset (i.e., $LepRb^{CCK}$ knockout (KO) animals) as previously described (Flak *et al.*, 2015). Cck^{cre} mice and $Lepr^{flox/flox}$ animals were kindly provided to us by Drs. Martin Myers, Jr., (University of Michigan, Ann Arbor, MI) and Streamson Chua, Jr. (Albert Einstein College of Medicine, New York, NY). Mice were genotyped for the Cck^{cre} and $Lepr^{flox}$ alleles as described previously (Flak *et al.*, 2015).

Surgery

Rats underwent implantation of a bilateral cannula directed to the LPBN (Plastics One, VA) under isoflurane anesthesia at stereotaxic co-ordinates: 9.4 mm posterior to bregma; ±2.1 mm lateral, and 4.8 mm below the skull surface. The cannula was secured to the skull with stainless steel screws and dental cement. Buprenorphine hydrochloride (0.3 mg/kg; Rickett Colman Pharmaceuticals) was administered at the completion of the surgery and animals were allowed to recover for at least a week prior to experimentation.

For mouse studies, both LepRb^{CCK} KO animals and their littermate controls were implanted with a single cannula placed in the lateral ventricle (Alzet; DURECT Corp., Cupertino, CA) at stereotaxic co-ordinates: 0.7 mm posterior to bregma; 1.3 mm lateral, and 2.3 mm below the skull surface as previously described (Meek *et al.*, 2013, 2014). After allowing the animals at least one week to recover from surgery, STZ-DM animals were implanted sc with an osmotic minipump that was connected to the lateral ventricle cannula to enable direct infusion of either vehicle (veh; PBS, pH 7.9) or leptin (1 µg/d) directly to the brain.

Effect of intra-LPBN leptin on diabetic hyperglycemia in STZ-DM rats

Following a recovery period, adult male Wistar rats bearing a cannula directed to the LPBN received either 2 consecutive daily subcutaneous (s.c.) injections of STZ (40 mg/kg body weight) to induce uDM or vehicle (NaCit, pH 4.5) as previously described (German *et al.*, 2010, 2011). Five days later, animals received daily bilateral intraparenchymal injections into the LPBN of either vehicle (veh; PBS, pH 7.9) or leptin (0.1 µg/d; Dr. Parlow; National Hormone Peptide Program) using a microinjection needle that extended 1 mm beyond the tip of the cannula. In this way, the following 3 groups of rats were studied: 1) s.c. veh/LPBN veh; 2) s.c. STZ/LPBN veh; and 3) s.c. STZ/LPBN leptin. Intraparenchymal injections were performed over a 60 s period, with leptin or its vehicle dissolved in a final volume of 500 nl (German *et al.*, 2011; Meek *et al.*, 2013, 2014). Body weight, food intake and blood glucose were measured daily in the fed state at 10 am.

Role of leptin receptors in the lateral PBN on diabetic hyperglycemia in STZ-DM mice

Both male Cre (+) (LepRb^{CCK} KO) and Cre (-) LepR$^{flox/flox}$ (LepRb WT) mice were allowed to recover for at least 1 wk following cannulation of the lateral ventricle. Each animal subsequently received two s.c. injections spaced 3 d apart of either STZ (150 mg/kg body weight) to induce uDM (Meek *et al.*, 2013, 2014). Animals were defined as diabetic when after 2 consecutive days, the fed blood glucose level was >200 mg/dl. Animals meeting this criterion were then implanted s.c. with an osmotic minipump connected to the lateral ventricle cannula to enable continuous infusion of either vehicle (veh; PBS, pH 7.9) or leptin (1 μg/d) directly into the brain. Body weight, food intake and blood glucose were measured daily in the fed state at 10 am.

Blood collection and tissue processing

Daily blood glucose levels were measured using a hand-held glucometer (Accu-Chek, IN) on blood obtained from tail capillary samples. At study completion, blood samples for plasma hormonal measures was collected from trunk blood into appropriately treated tubes (5), and centrifuged with the plasma removed, aliquoted and stored at -80°C for subsequent assay. Plasma insulin levels were determined by ELISA (Crystal Chem, IL), ketone bodies using a colorimetric kit (Wako Chemicals, VA), thyroxine (T4) levels using an enzyme immunosorbent assay (MP Biomedicals), plasma corticosterone (Alpco, NH) levels using an ELISA and glucagon levels for rat and mouse using a RIA kit (Linco Research, MO) and an ELISA (Mercodia, NC), respectively.

For immunohistochemical (IHC) studies to verify cannula placement and injectate spread in rats, anesthetized animals were perfused with ice-cold phosphate buffered saline (PBS; Diamedix, FL) followed by 4% paraformaldehyde (PFA; Electron Microscopy Sciences, PA) in 0.1M PBS. Brains were removed, post-fixed in 4% PFA overnight, and transferred to a 25% sucrose solution in PBS for 48 h at 4°C before being frozen in isopentane cooled on dry ice. Coronal sections (14 µm) were taken throughout the hindbrain and hypothalamus, slide-mounted and stored at -80°C for IHC staining and cannula verification.

Verification of cannula and injection site

Cannula placement in rats was confirmed postmortem using standard histological techniques in which Chicago Sky Blue ink was microinjected in the LPBN of euthanized animals, the brains were removed, sectioned on a cryostat, mounted on glass slides, and analyzed for placement. To further verify the spread of injectate, we examined leptin-induced activation of pSTAT3, a marker of leptin receptor activation in a subset of animals that received an intraparenchymal injection of leptin directly into the PBN. Immunoreactivity for pSTAT3 was visualized in the hypothalamus and LPBN using a Nikon Eclipse E600 microscope fitted with a grid reticule with the investigator blinded to study conditions. A representative image of immunostaining of pSTAT3 in the LPBN and ARC (as a negative control) is depicted in Figure 5.1. Rats with injection sites located outside of the LPBN were excluded from further analysis.

Immunohistochemistry

Immunohistochemical identification of pSTAT3 was used to measure direct leptin signaling in the brain using a protocol described previously (Morrison *et al.*, 2005; Meek *et al.*, 2013). Briefly, sections were washed in Phosphate Buffered Saline (PBS) at room temperature and were subsequently incubated 0.1% Hydrogen Peroxide for one minute.

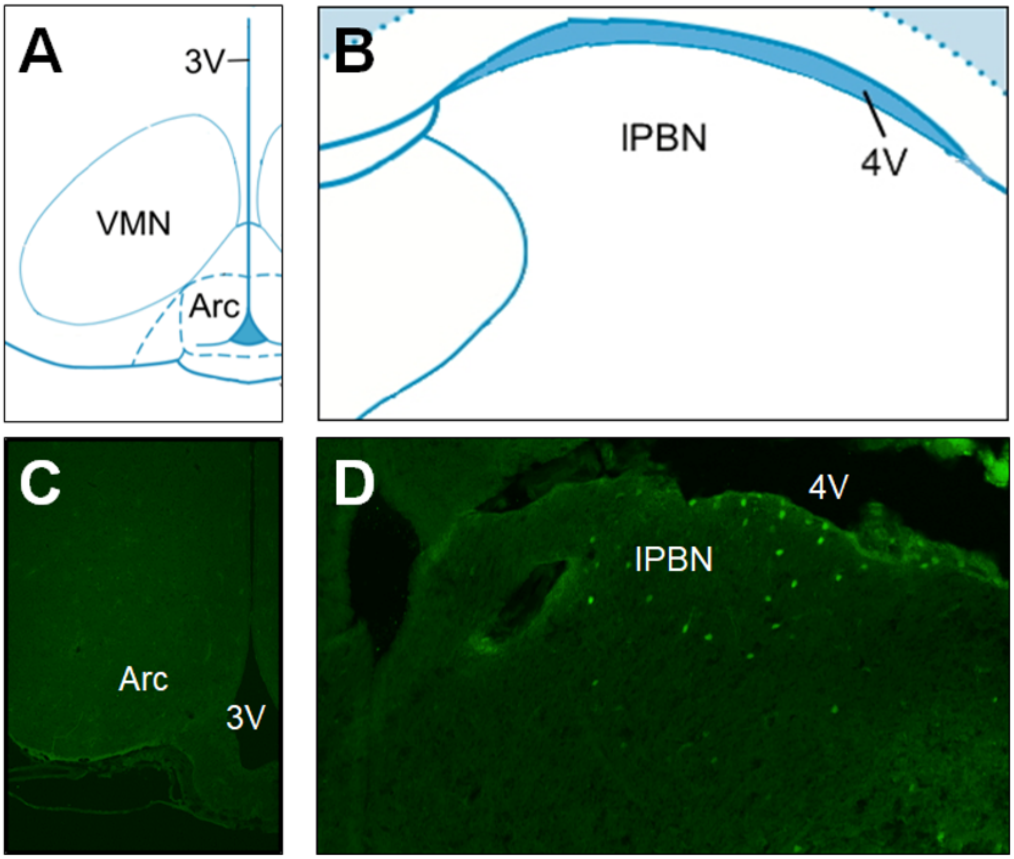


Figure 5.1 Validation of lateral parabrachial nucleus injection site. Schematic and representative image of immunostaining for pSTAT3, a downstream marked of leptin receptor activation following intraparenchymal injection of leptin into the lateral parabrachial nucleus in the (**A, C**) hypothalamic arcuate nucleus (negative control) and (**B,D**) lateral parabrachial nucleus. 3V = third ventricle; 4V = fourth ventricle; Arc = arcuate nucleus; lPBN = lateral parabrachial nucleus; VMN = ventromedial nucleus.

Sections were then washed and permeabilized for 10 minutes with 0.5% Triton in PBS, containing 0.1% BloxAll (Vector Laboratories, Burlingame, CA) and subsequently incubated in 1% Normal Serum blocking solution plus rabbit anti-pSTAT3 antibody (1: 1,000; Sigma-Aldrich, St. Louis, MO; Research Resource Identifier (RRID): AB_10620527) overnight at 4°C. Sections were then washed and blocked for 1 hour in blocking solution containing biotinylated anti-rabbit antibody (Vectastain Elite ABC kit; RRID: AB_2336820), and incubated for 30 minutes with Vector avidin/biotin complex (ABC) reagent (Vector Laboratories, Burlingame, CA). Slides were washed in PBS and incubated with SA-488 (Streptavidin Alexa 488; RRID: AB_2336881) for 30 minutes and then mounted with Fluoromount-G (ThermoFisher Scientific). Confocal images were taken on a Leica SP8X (Leica Microsystems Inc., Buffalo Grove, IL).

Statistical analysis

All results are expressed as mean ± SEM. For rat studies, a one-way ANOVA with Tukey post hoc tests were used to compare mean values between multiple groups. For mouse studies, two-way repeated measures ANOVA with Tukey post hoc tests were used. Hormonal data were analyzed by standard two-way ANOVA with Tukey post hoc tests. Statistical analyses were performed using Statistica (version 7.1; StatSoft, Inc., Tulsa, OK) or Prism (version 7.4; GraphPad Software, Inc., La Jolla, CA). In all instances, probability values of less than 0.05 were considered significant.

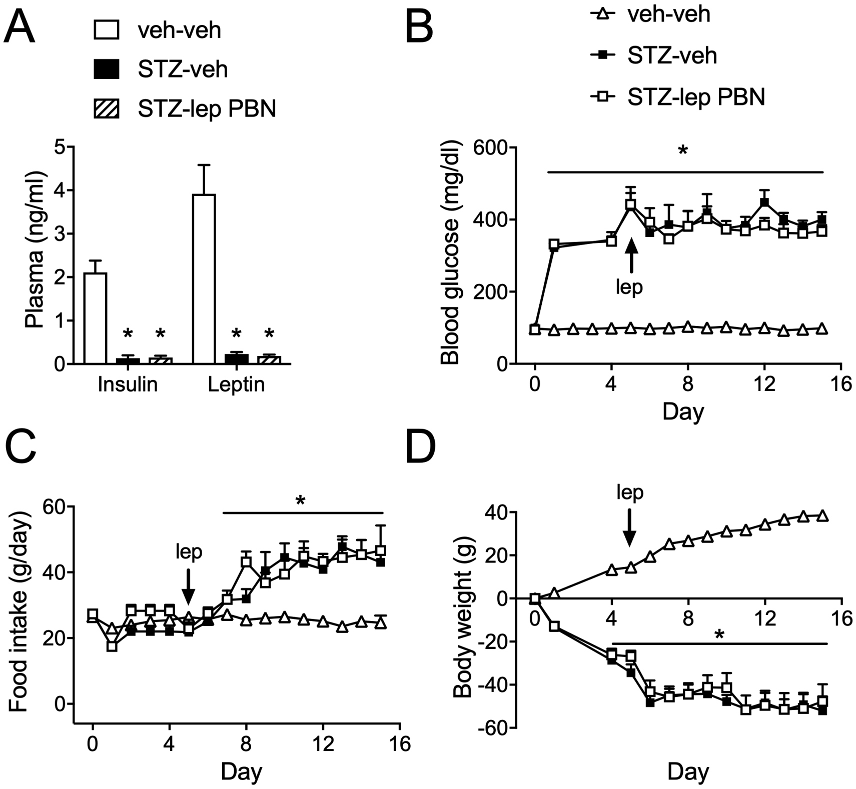


Figure 5.2 Intra-lateral PBN leptin is not sufficient to ameliorate diabetic hyperglycemia or hyperphagia in STZ-diabetic rats.
(**A**) Plasma insulin and leptin levels, mean daily (**B**) fed blood glucose levels, (**C**) food intake and (**D**) body weight change in either non-diabetic (veh-veh) or STZ-induced diabetic animals receiving daily intraparenchymal injections of either vehicle (STZ-veh) or leptin (STZ-lep PBN) administered directly into the PBN (n = 6-7 per group). The arrow represents the start of daily leptin injections. STZ was administered on Day 0 and 1 to induce uDM. Data represent mean ± SEM. * $p<0.05$ vs. veh-veh.

Intra-LPBN leptin administration is not sufficient to reverse diabetic hyperglycemia in STZ-DM rats

As expected, relative to non-diabetic controls, all animals that received STZ exhibited marked reductions of both plasma insulin and leptin levels (Figure 5.2A), consistent with previous evidence that STZ-DM is characterized by insulin and leptin deficiency (Havel *et al.*, 1998). Consequently, STZ-DM animals that received vehicle in the LPBN exhibited a profound hyperglycemia that was maintained throughout the duration of the study, and leptin administration directly into the LPBN failed to attenuate this effect (Figure 5.2B). Although a higher dose may have had a greater effect, the dose of leptin we used was previously demonstrated to normalize blood glucose levels when administered directly into the VMN of STZ-DM rats, while having no effect when administered into the lateral ventricle (Meek *et al.*, 2013). As expected, STZ-veh treated rats were also characterized by increased food intake relative to non-diabetic controls, and this diabetic hyperphagia was similarly not attenuated by intra-LPBN leptin (Figure 5.2C). Despite the increase of food intake, however, STZ-veh treated animals lost body weight, since the excess calories cannot be stored into adipose tissue as result of the insulin deficiency and are

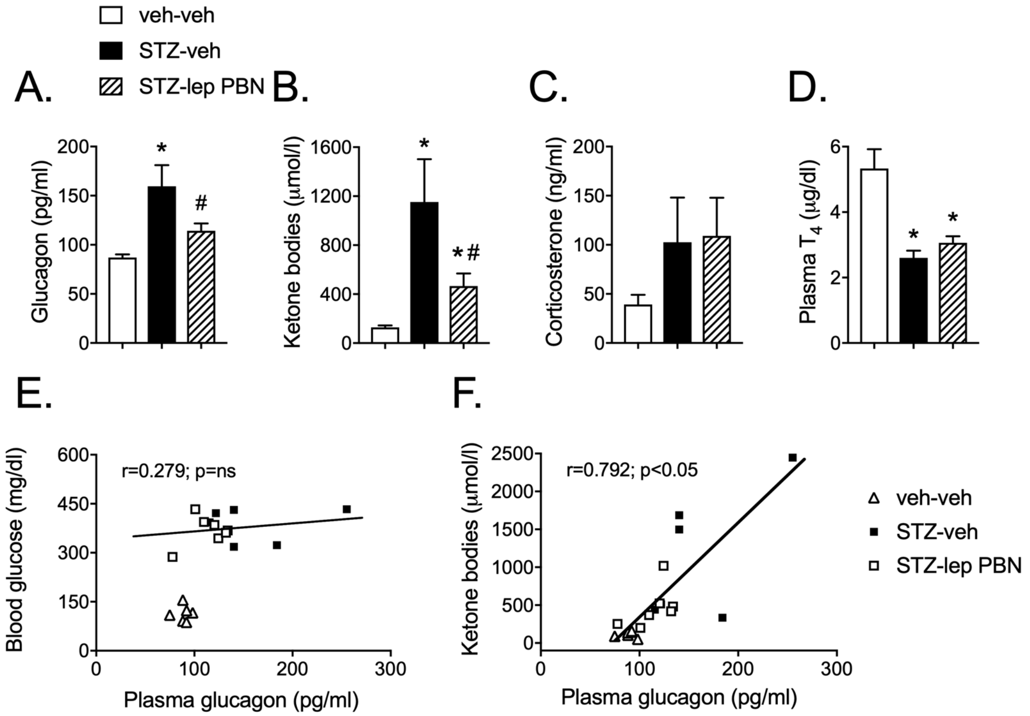


Figure 5.3 Intra-lateral PBN leptin is sufficient to ameliorate diabetic hyperglucagonemia in STZ-diabetic rats.
(**A**) Plasma glucagon, (**B**) plasma ketone bodies, (**C**) plasma corticosterone, and (**D**) plasma thyroxine levels (T4). Correlations between plasma glucagon and blood glucose levels (**E**) and plasma glucagon and ketone bodies (**F**) in either non-diabetic (veh-veh) or STZ-induced diabetic animals receiving daily intraparenchymal injections of either vehicle (STZ-veh) or leptin (STZ-lep PBN) administered directly into the PBN (n = 6-7 per group). Data represent mean ± SEM. * $p<0.05$ vs. veh-veh; # $p<0.05$ vs. STZ-veh.

lost in the urine. Since STZ-DM rats that received intra-LPBN leptin also exhibited similar weight loss (Figure 5.2D), these data suggest that leptin signaling in the LPBN is insufficient to reverse the hyperphagia, weight loss and hyperglycemia characteristic of STZ-DM rats.

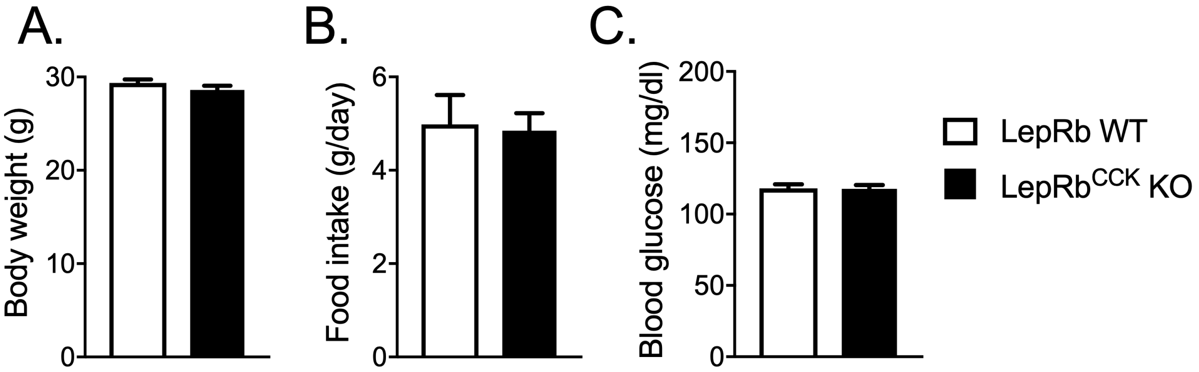


Figure 5.4 Baseline phenotype of mice deficient in leptin receptor in lateral PBN^{CCK} neurons.
(**A**) Body weight (**B**) mean daily food intake and (**C**) blood glucose levels, in mice with leptin receptors deleted from lateral PBN^{CCK} neurons (LepRb^{CCK} KO) or their littermate controls (LepRb WT). (n = 7-10 per group). Data represent mean ± SEM.

Effect of intra-LPBN on metabolic and neuroendocrine parameters in STZ-DM rats

Although leptin administration directly into the LPBN failed to attenuate the diabetic hyperglycemia in STZ-DM rats, it significantly blunted the increase of plasma glucagon levels characteristic of STZ-DM (Figure 5.3A), and this effect was accompanied by reduced plasma ketone body levels (Figure 5.3B). These findings are consistent with previous evidence that 1) leptin action in the brain of rats with uDM ameliorates hyperglucagonemia, and 2) ketosis, but not hyperglycemia, in this setting is dependent on elevated plasma glucagon levels (German *et al.*, 2011; Meek *et al.*, 2013, 2014, 2015). Further support for this concept is found in the observation that variation in plasma glucagon levels across study groups was strongly predictive of plasma ketone body levels, but not blood glucose levels amongst diabetic rats (Figure 5.3C, D) (Meek *et al.*, 2015). In contrast, leptin microinjection into the LPBN had no effect on either plasma corticosterone or T4 levels (Figure 5.3E, F). This finding, like the absence of an effect on hyperglycemia, stands in contrast to the effect of icv leptin in STZ-DM (German *et al.*, 2011; Matsen *et al.*, 2013).

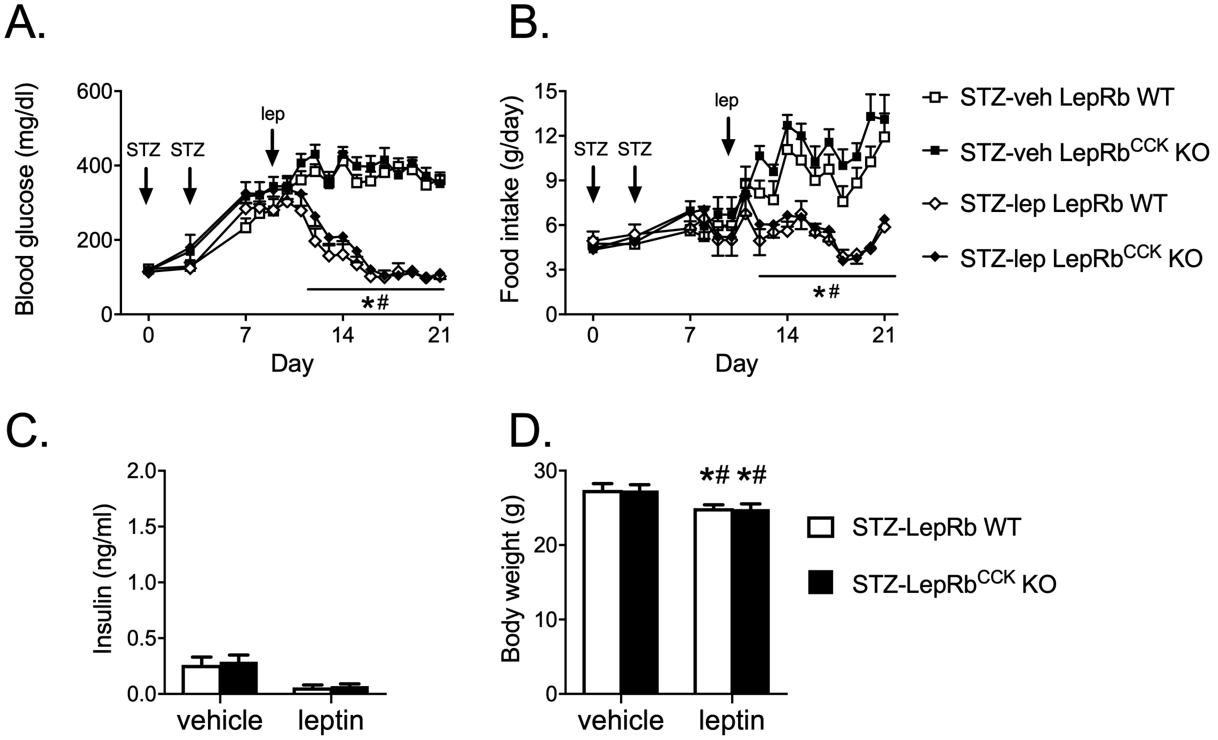


Figure 5.5 Anti-diabetic effects of leptin in STZ-DM do not require leptin signaling through lateral PBN^{CCK} neurons.
Mean daily fed blood glucose levels (**A**) and food intake (**B**). (**C**) Plasma insulin levels, and (**D**) body weight in STZ-induced diabetic mice with leptin receptors deleted from lateral PBN^{CCK} neurons (LepRb^{CCK} KO) or their littermate controls (LepRb WT) treated with either icv vehicle or leptin. (n = 7-10 per group). Data represent mean ± SEM. * p<0.05 vs. STZ-veh WT; # p<0.05 vs. STZ-veh KO.

Role of leptin receptor-expressing LPBN^{CCK} neurons in the control of diabetic hyperglycemia in mice

To determine whether leptin signaling in LPBN^{CCK} neurons is required for leptin's glucose-lowering effects in uDM, we studied mice with selective deletion of leptin receptors from this neuronal subset (Flak *et al.*, 2015). Consistent with available evidence

(Flak *et al.*, 2015), we found no differences in body weight (29.36 ± 0.38 vs. 28.60 ± 0.46 g), mean daily food intake (4.98 ± 0.63 vs. 4.85 ± 0.37 g/day), or blood glucose levels (118.1 ± 2.8 vs. 117.7 ± 2.7 mg/dl) in LepRb^{CCK} KO mice relative to their littermate controls at baseline (p=ns for each) (Figure 5.4). Similarly, all animals were characterized by markedly reduced plasma insulin levels, elevated blood glucose levels and hyperphagia following STZ administration (Figure 5.5A-C). Specifically, because the increases of both blood glucose and food intake induced by STZ did not differ between LepRb^{CCK} KO and littermate controls (LepRb WT mice), we infer that leptin signaling in LPBN^{CCK} neurons is not required for diabetic hyperglycemia and hyperphagia (Figure 5.5B, C). The more relevant question for this study is whether leptin signaling in this neuronal subset is required for the ability of icv leptin to ameliorate hyperglycemia, ketosis and other neuroendocrine manifestations of uDM. Here, we found that continuous icv infusion of leptin normalized both blood glucose levels (Figure 5.5B) and food intake (Figure 5.5C) to a similar extent and a similar rate (Days 12-21; $p<0.05$ for each) in both LepRb WT and LepRb^{CCK} KO mice. These data suggest that leptin action in LPBN^{CCK} neurons is not required for leptin's ability to normalize both glycemia and food intake in STZ-DM. Our findings also showed a significant main effect of icv leptin to lower plasma glucagon levels ($p<0.05$). Further analysis revealed that icv leptin infusion significantly reduced plasma glucagon in diabetic controls (LepRb WT; $p<0.05$), but not in STZ-DM LepRb^{CCK} KO mice (p=ns) (**Error! Reference source not found.**A), although there was no significant interaction between leptin and genotype. Taken together, these observations suggest that leptin action in LPBN^{CCK} neurons is required for the full effect of leptin to suppress hyperglucagonemia in uDM, although leptin action in other brain areas also likely contributes. Our findings also revealed that while there was a significant main effect of icv leptin to markedly reduce plasma ketone body levels in both STZ-DM LepRb^{CCK} KO and LepRb WT mice ($p<0.05$ for each) (Figure 5.6B), the variation in plasma glucagon levels was predictive of plasma ketone bodies ($r=0.471$; $p<0.05$). In a similar manner, we found that the effect of continuous icv leptin infusion on plasma levels of corticosterone and T4 did not differ by genotype (Figure 5.6C, D).

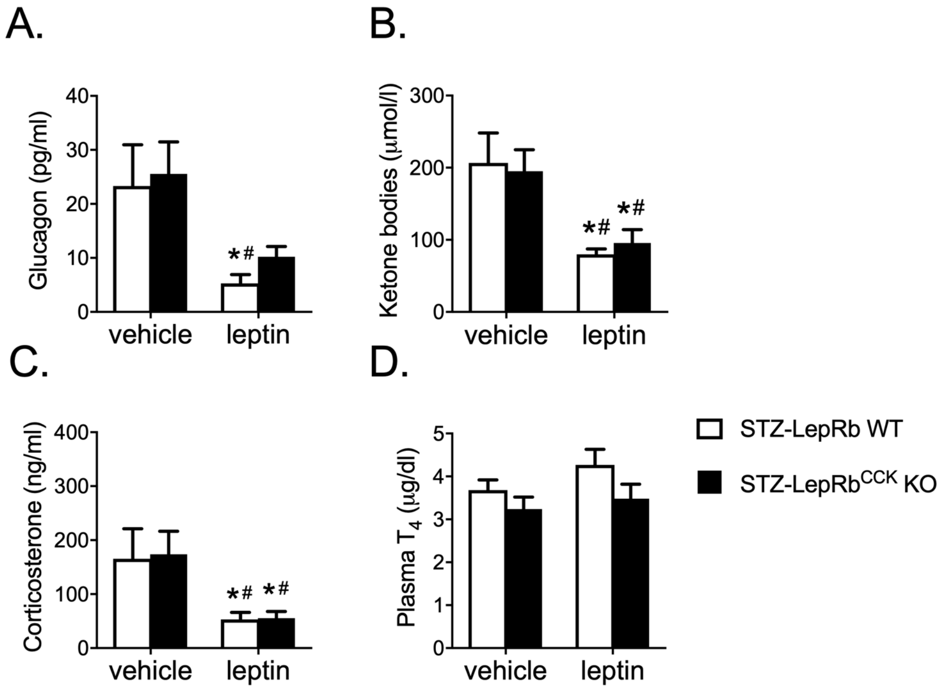


Figure 5.6 Leptin-mediated normalization of hyperglucagonemia in STZ-DM mice requires leptin signaling through lateral PBN^{CCK} neurons.
(**A**) Plasma glucagon levels, (**B**) ketone bodies, (**C**) plasma corticosterone and (**D**) plasma thyroxine (T4) levels in STZ-induced diabetic mice with leptin receptors deleted from lateral PBN^{CCK} neurons (LepRb^{CCK} KO) or their littermate controls (LepRb WT) treated with either icv vehicle or leptin. (n = 7-10 per group). Data represent mean ± SEM. * $p<0.05$ vs. STZ-veh WT; # $p<0.05$ vs. STZ-veh KO.

Discussion

Since its discovery nearly a century ago, insulin has remained the cornerstone of medical management of T1D, including the prevention of acute (e.g., ketoacidosis) and chronic diabetes complications (e.g., nephropathy, neuropathy, retinopathy and cardiovascular disease) (The Diabetes Control and Complications Trial Research Group, 1997). However, recent studies have demonstrated that leptin action in the CNS can restore euglycemia in rodent models of T1D via mechanisms that are independent of insulin action (Hikada *et al.*, 2002; Lin *et al.*, 2002; Da Silva *et al.*, 2006; Fujikawa *et al.*, 2010; German *et al.*, 2011). Based on the recent identification of a leptin-regulated circuit involved in glucose control (Garfield *et al.*, 2014; Flak *et al.*, 2015), which appears to involve projections from neurons in the LPBN to the VMN, and subsequently to the anterior bed nucleus of the stria terminalis (aBNST) (Meek *et al.*, 2016), we hypothesized that leptin action on discrete components of this circuit underlies leptin's anti-diabetic action in T1D. Our data in STZ-DM rats show that whereas leptin action limited to the LPBN is insufficient to reverse the effect of uDM on levels of blood glucose, corticosterone and T4 levels, it nevertheless attenuated the associated hyperglucagonemia and ketosis. We also show that while the anti-diabetic effects of leptin remain intact in mice in which the leptin receptor was deleted from LPBN CCK neurons (LepRb^{CCK} KO), leptin failed to normalize elevated plasma glucagon levels in these mice, but did so effectively in controls. Collectively, these findings suggest that in uDM, reduced leptin action in the LPBN drives hyperglucagonemia in a selective manner, without substantially altering hyperglycemia or associated neuroendocrine defects.

To place these findings in context, consider that in response to conditions of either acute or chronic energy deficit, the brain engages a series of autonomic, behavioral and neuroendocrine responses designed to increase the availability of fuel to the CNS while simultaneously conserving fuel stores. These responses include an increase in both the drive to eat and in HGP, with the latter being mediated through increased secretion of the counterregulatory hormones, glucagon and corticosterone. Since fasting and STZ-DM are each characterized by deficiency of both insulin and leptin, we and others have argued that the adaptive CNS response to the two conditions overlap in ways that resemble the CRR to hypoglycemia (Morton, Meek and Schwartz, 2014; Xu and Tong, 2017). Inherent in this concept is the possibility that neurocircuits that drive the response to hypoglycemia are tonically inhibited by input from leptin, and recent work supports this possibility (Morton, Meek and Schwartz, 2014; Xu and Tong, 2017).

Also consistent with this concept is the observation that hyperglucagonemia is a feature of both uDM (Müller, Faloona and Unger, 1971; Dobbs *et al.*, 1975) and the CRR to hypoglycemia (Cryer, 2004). In T1D, hyperglucagonemia contributes to both ketosis and diabetic hyperglycemia by driving hepatic production of both glucose and ketones (Unger and Cherrington, 2012). Conversely, suppression of glucagon hypersecretion (e.g., with somatostatin) lowers HGP, reduces blood glucose levels and reverses diabetic ketoacidosis in uDM (Dobbs *et al.*, 1975; Gerich *et al.*, 1975; Raskin and Unger, 1978; Unger and Cherrington, 2012), and similarly blunts the increase of HGP characteristic of the response to hypoglycemia (Rizza, Cryer and Gerich, 1979). Moreover, since the anti-diabetic effects of leptin in uDM are associated with normalization of plasma glucagon levels (Fujikawa *et al.*, 2010; German *et al.*, 2011), and leptin blunts the effect of neuroglucopenia to stimulate glucagon secretion (Müller, Faloona and Unger, 1971; Dobbs *et al.*, 1975), it suggests that leptin's glucose lowering effects in uDM might involve inhibition of a neurocircuit normally activated by hypoglycemia.

This hypothesis is consistent with our finding that leptin administration directly into the LPBN of STZ-DM rats attenuates the hyperglucagonemia of uDM, and thereby attenuates diabetic ketosis. That this intervention fails to attenuate diabetic hyperglycemia suggests further that leptin-responsive LPBN neurons are but one node in a complex and distributed circuit that, when activated, drives the complex but highly integrated behavioral, metabolic, autonomic and neuroendocrine manifestations of uDM.

In support of this concept, evidence suggests that leptin acts through a distributed network, whereby multiple leptin-responsive neurons carry out different aspects of the overall leptin effect, rather than leptin acting on one specific brain area or neuronal subset. Consistent with this model, we found that repeated daily microinjection of a low dose of leptin directly into the VMN is sufficient to ameliorate hyperglycemia in uDM, in effect mimicking the anti-diabetic effect of icv leptin (Meek *et al.*, 2013). However, we also observed that icv leptin fully reverses hyperglycemia in STZ-DM mice lacking leptin receptor specifically in VMN^{SF1} neurons (Fujikawa *et al.*, 2013; Meek *et al.*, 2013). These findings suggest that leptin signaling in the VMN is not required for leptin's anti-diabetic effects and implicate leptin-responsive neurocircuits outside the VMN in this effect.

Given that VMN neurons receive excitatory axonal projections from leptin-sensitive LPBN LepRb^{CCK} neurons (Garfield *et al.*, 2014; Flak *et al.*, 2015; Meek *et al.*, 2016) as part of a LPBN→VMN^{SF1}→aBNST neurocircuit, we hypothesized in the current work that leptin action in the LPBN, as well as in the VMN, underlies its anti-diabetic actions and our data support this hypothesis. Specifically, our data suggest that leptin action in the LPBN plays a specific role to inhibit glucagon secretion, and because uDM is a state of leptin deficiency, activation of leptin-inhibited LPBN neurons drives hyperglucagonemia in this setting. However, additional leptin-responsive neurocircuits are likely to be involved as glucagon secretion was not fully normalized following LPBN leptin administration.

Further, while leptin significantly reduced plasma glucagon levels in WT, but not LepRb^{CCK} KO mice, the difference between groups did not reach statistical significance. One possibility is that hypothalamic pro-opiomelanocortin (Pomc) neurons play a role, since most of these neurons express leptin receptors (Schwartz *et al.*, 1997) and we have previously shown that a melanocortin-dependent mechanism contributes to leptin-mediated inhibition of glucagon secretion in uDM (Meek *et al.*, 2014).

We note that unlike what has been previously reported following icv leptin administration in uDM, leptin microinjection into the LPBN was without effect on plasma levels of either thyroxine or corticosterone in rats with STZ-DM (German *et al.*, 2011; Matsen *et al.*, 2013). This outcome is not surprising given that leptin regulation of the thyroid axis is believed to involve a both direct effect on TRH neurons in the hypothalamic PVN and an indirect effect mediated via activation of Pomc neurons that project onto TRH neurons (Lechan and Fekete, 2006; Ghamari-Langroudi *et al.*, 2010; Nillni, 2010). Similarly, the effect of leptin to regulate the HPA axis involves a melanocortin-dependent pathway, and we are unaware of links between leptin-responsive neurons in the LBPN and the melanocortin system (Malendowicz *et al.*, 2007).

Chapter 6 Activation of dorsomedial hypothalamic LepR-expressing neurons is required for normal circadian feeding rhythms

Abstract

Neurons in the dorsomedial hypothalamic nucleus (DMH) are implicated in thermoregulation, circadian rhythms, and energy balance. Of relevance to food intake control is a distinct subset of leptin receptor (LepR)-expressing neurons in the ventral DMH (DMH^{LepR} neurons), while LepR neurons in the dorsal DMH are implicated in thermogenesis. To determine the physiological importance of these neurons, we chronically silenced them via stereotaxic microinjection into the DMH of an AAV encoding a cre-dependent tetanus-toxin light chain (TeTx), which blocks neurotransmission to downstream neurons, in both LepR-IRES-Cre mice and WT littermate controls. We found that TeTx-mediated silencing of DMH^{LepR} neurons resulted in transient hyperphagia, weight gain and increased adiposity that persisted despite the eventual return of food intake to control levels. Unexpectedly, we also observed a rapid and profound disruption of diurnal feeding rhythms, associated with suppression of locomotor activity, and elevated respiratory exchange ratio (RER) indicative of a shift in peripheral substrate utilization towards *de novo* lipogenesis. In contrast, thermoregulation was entirely normal in these mice. Restoration of feeding to the dark cycle via a time-restricted feeding (TRF) paradigm normalized RER, but failed to fully restore feeding and locomotor behaviors. Taken together, our findings identify a physiological role for a specific subset of DMH^{LepR} neurons in the regulation of circadian feeding.

Introduction

The homeostatic control of energy balance is vital to ensure that the metabolic demands of the body are met by sufficient energy intake. In healthy, nonobese individuals, the energy homeostasis system robustly defends a relatively stable body weight, such that acute deviations in either energy expenditure or energy intake are counterbalanced to equalize the energy balance equation. Conversely, obesity is characterized by pathological elevation in the defended level of adiposity (Schwartz *et al.*, 2017), suggesting that sustained positive energy balance contributes to the development and maintenance of overweight and obesity.

The regulation of feeding behaviors is subject to integrative control by a variety of both short-term and long-term signals of energetic status, including satiety signals from the gut (e.g., ghrelin, cholecystokinin), as well as input from adiposity negative feedback signals such as leptin (Morton, Meek and Schwartz, 2014). Indeed, accumulating evidence suggests a critical role for leptin-responsive hypothalamic neurocircuits in the regulation of energy balance (Pan and Myers, 2018). However, in all organisms, food intake is also highly dependent upon time-of-day, with most mammals exhibiting highly predictive rhythmicity in feeding behaviors. Synchronization of endogenous behavioral rhythms with signals from the external world enables animals to predict food availability, and optimize metabolic processes to prepare the body for daily periods of fasting and feeding, respectively (Challet, 2019).

In mammals, oscillations in metabolism and behavior are organized by the “master clock” molecular oscillator in the hypothalamic suprachiasmatic nucleus (SCN), whose activity is phase-controlled by the detection of light by the retina (Ramsey and Bass, 2011). The importance of synchronized circadian rhythms to energy homeostasis is increasingly appreciated, as ample evidence in both rodents and humans has shown that mistimed feeding (i.e., when eating occurs during the normal resting period) impairs metabolic health (Yasumoto *et al.*, 2016; McHill *et al.*, 2017). Moreover, disrupted circadian rhythms are evident in a variety of rodent models of obesity, including high-fat-diet induced obesity (DIO) (Kohsaka *et al.*, 2007), as well as in leptin-deficient models (Laposky *et al.*, 2006, 2008), while restoring feeding to the appropriate time of day protects against obesity (Hatori *et al.*, 2012), and presents a promising strategy to mitigate, or even reverse, weight gain in humans. Therefore, studying the circadian regulation of feeding behaviors can provide valuable insights into not just the mechanisms regulating energy balance, but also the pathophysiology of metabolic disease (Challet, 2019).

The dorsomedial hypothalamus (DMH) is broadly implicated in several distinct aspects of metabolic homeostasis, including energy balance and circadian rhythms. The DMH receives neural input from hypothalamic brain regions implicated in energy homeostasis, but is also a major target of the SCN (Thompson and Swanson, 1998). The DMH, in turn, projects to brain areas critical for regulation of feeding (Garfield *et al.*, 2016), glucocorticoid secretion (Elmquist, Ahima, *et al.*, 1998), and thermogenesis (Zhang *et al.*, 2011). Here, we show that the activity of a specific subset of leptin-receptor (LepR)-expressing neurons in the DMH are required for the maintenance of normal circadian feeding rhythms, such that inactivating these neurons robustly and repeatably alters the phase of feeding rhythms, resulting in increased light cycle feeding, increased adiposity, and reduced dark cycle locomotor activity. Furthermore, we demonstrate that animals with inactivation of DMH^{LepR} neurons fail to entrain to a restricted feeding window, indicating a potential role for these neurons in meal-entrainment.

Research Design and Methods

Mice

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee at the University of Washington. Following stereotaxic surgery, all studied animals were individually housed with ad libitum access to standard chow diet (LabDiet 5053) in a temperature and humidity-controlled facility with 14:10 light:dark cycles. Heterozygous adult male LepR-IRES-Cre/+ mice (Jackson Laboratory no. 008320) were used for all experiments, unless otherwise noted.

Stereotactic Surgeries

The viral vector AAV1-CMV-DIO-GFP-2A-TetTox (TeTx) was generated as previously described (Campos *et al.*, 2016), and generously provided by Dr. Richard Palmiter (University of Washington, Seattle, WA), while vector AAV5-EF1a-DIO-GFP (GFP) was purchased from the Gene Therapy Center at the University of North Carolina. For viral microinjection, animals were placed in a stereotaxic frame (Kopf 1900; Cartesian Research Inc., Tujunga, CA) under isoflurane anesthesia. The skull was exposed with a small incision, and two small holes were drilled for bilateral 200-nL injection volume of TeTx or GFP control targeting the DMH of LepR-Cre, or Cre-negative littermates (WT) male mice based on coordinates from the Mouse Brain Atlas (Franklin and Paxinos, 2008): anterior-posterior (AP) -1.6, dorsal- ventral (DV) -5.6 mm, and lateral 0.45 mm. Adeno- associated virus (AAV) was delivered using a Hamilton syringe with a 33-gauge needle at a rate of 50 nL/min (Micro4 controller), followed by a 5-min wait at the injection site and a 1-min wait 0.05 mm dorsal to the injection site before needle withdrawal. Animals received a perioperative subcutaneous injection of buprenorphine hydrochloride (0.05 mg/kg) (Reckitt Benckiser, Richmond, VA). After surgery, mice were allowed 2-3 weeks to recover and to maximize viral expression. Mice were acclimated to handling and experimental conditions three times prior to the start of any in vivo studies. Viral expression and fiber placement were verified post hoc in all animals, and any data from animals in which the virus or fiber was located outside the targeted area were excluded from the analysis.

Body Composition Analysis

Measurements of body lean and fat mass were determined in live, conscious mice by use of quantitative magnetic resonance spectroscopy (QMR; EchoMRI-700TM; Echo MRI, Houston, TX) by the University of Washington Nutrition Obesity Research Center (NORC) Energy Balance Core.

Body Temperature Measurements

Mice underwent implantation of a body temperature transponder in the peritoneal cavity (Starr Life Sciences Corp., Oakmont, PA) before study. Animals were allowed at least 1 wk to recover and were then acclimated to metabolic cages enclosed in temperature- and humidity-controlled cabinets (Caron Products and Services, Marietta, OH). Signals emitted by body temperature transponders were sensed by a receiver positioned underneath the cage and analyzed using VitalView software as previously described (Kaiyala *et al.*, 2016).

Leptin effects on food intake

To assess the ability of leptin to suppress the compensatory hyperphagia that normally follows a prolonged fast, mice were fasted for 24h from ZT2 – ZT2'. On the second day, leptin (3 mg/kg; Dr. Parlow; National Hormone Peptide Program) or vehicle-control (PBS, pH 7.9) was injected intraperitoneally in mice 15m before preweighed food was placed back in the cage, and intake was monitored as indicated for the following 24h.

Indirect Calorimetry, Food Intake, and Activity

Mice were acclimated to calorimetry cages prior to study and data collection. Energy expenditure measurements were obtained by a computer-controlled indirect calorimeter System (Promethion, Sable Systems, Las Vegas NV) with support from the EBCore of the NORC at the University of Washington, as previously described (Kaiyala *et al.*, 2015). Oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured for each mouse for 1-min at 10-min intervals, and food and water intakes were measured continuously while mice were housed in a temperature- and humidity-controlled cabinet (Caron Products and Services, Marietta, OH NV). Ambulatory activity was determined simultaneously and beam breaksin the x-, y- and z-axes were scored as an activity count, and a tally was recorded every 10 min. Data acquisition and instrument control were coordinated by MetaScreen v.1.6.2, and raw data were processed using ExpeData v.1.4.3 (Sable Systems, Las Vegas, NV) using an analysis script documenting all aspects of data transformation.

Meal Patterning

Food and water intakes were continuously measured during indirect calorimetry, as described above. Meal patterning was analyzed post hoc by extracting and processing raw data using ExpeData v.1.4.3 (Sable Systems, Las Vegas, NV). Briefly, a feeding bout was defined as a meal if the animal interacted with the food hopper for at least 30s, and was separated from another meal by at least 150s.

Time Restricted Feeding (TRF)

Food was removed each morning at the start of the light cycle (ZT0) and returned at the start of the dark cycle (ZT14); body weight was also measured at both ZT0 and ZT14 daily. To eliminate the effects in fed status of animals, 1d before TRF animals were fasted for 24h from ZT14 (on Day -1) to ZT14’ (on Day 0) before TRF began. Animals were then subjected to indirect calorimetry for 5 additional days during TRF before returning to ad lib feeding for the remaining 3 days of study (Figure 4A).

Immunohistochemistry

For brain immunohistochemical (IHC) analyses, animals were terminally anesthetized with ketamine:xylazine and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1mol/L PBS. Brains were removed and postfixed overnight, then transferred into 30% sucrose overnight or until brains sunk in solution. Brains were subsequently sectioned on a freezing-stage microtome (Leica) to obtain 30μm coronal sections in four series. A single series of sections per animal was used in histological studies, and the remainder stored in -20C in cryoprotectant. Brain sections were washed in PBS with Tween-20, pH 7.4 (PBST) overnight at 4C. Sections were then washed at room temperature in PBST (3x8 min), followed by a blocking buffer (5% normal donkey serum (NDS), 1% bovine serum albumin (BSA) in PBST with azide) for 60 minutes with rocking. Sections were then incubated overnight at 4C in blocking buffer containing primary antiserum (goat anti-GFP, Fitzgerald, 1:1000; rabbit anti-pSTAT3, Sigma-Aldrich, St Louis, Missouri, 1:1000). Next, sections were washed (3 x 8 min) in PBST before incubating in secondary donkey anti-goat IgG Alexa 488 (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:1000 in blocking buffer. Sections were washed (3 x 8 min) in PBST before incubating with DAPI for 8 minutes, followed by a final wash (3 x 10 min) in PBS. Sections were mounted to slides and imaged using a Nikon Eclipse E600 upright microscope equipped with a Diagnostic Instruments Spot RT Color digital camera.

Tissue Processing, Blood Collection

Tail blood for plasma hormonal measurement was collected at indicated times. Blood was collected via EDTA-coated capillary tubes and centrifuged at 4 °C (7,000 rpm, 4 min) and plasma was subsequently removed and stored at -80 °C for subsequent assay. Plasma corticosterone (Crystal Chem, Elk Grove Village, IL; #80556) was determined by ELISA.

Statistical Analyses

All results are presented as means ± SEM. *P* values for unpaired comparisons were calculated by two-tailed Student's *t* test. Time course comparisons between groups were analyzed using a two-way repeated measures ANOVA with main effects of treatment (control vs. TeTx) and time. All post hoc comparisons were determined using Sidak's correction for multiple comparisons. All statistical tests indicated were performed using Prism (version 7.4; GraphPad, CA) software.

Results

Chronic inhibition of DMH^{LepR} neurons elicits transient hyperphagia and increased adiposity

To test the hypothesis that DMH^{LepR} neurons play a physiological role in feeding, we utilized a viral loss-of-function strategy (Figure 6.1A). In LepR-Cre+ adult male mice, DMH^{LepR} neurons were permanently inactivated following bilateral microinjection of an adeno-associated virus (AAV) encoding a Cre-dependent tetanus toxin light chain (AAV1-DIO-TeTx:GFP) directed to this brain area (Kim *et al.*, 2009). As expected, viral expression was limited largely to the ventral and dorsal aspects of the DMH, but was not detected in the dorsal hypothalamic area (DHA) (Figure 6.1B and S1). The specificity of TeTx:GFP to LepR+ neurons in the DMH was further confirmed by the detection of leptin-induced pSTAT3 immunoreactivity in most virally-transduced cells. In addition, we observed extensive GFP+ fiber terminals in the ARC (Figure 6.2B), consistent with previous evidence of a DMH^{LepR} → AgRP neurocircuit (Krashes *et al.*, 2014; Garfield *et al.*, 2016). In contrast, TeTx:GFP was not detected in Cre- littermate controls (not shown).

Our findings show that, relative to controls, mice with DMH-specific inactivation of LepR neurons (denoted DMH^{TeTx} mice) exhibited a transient hyperphagia that persisted for 8 days before returning to back to the level of controls (Figure 6.1C, D). This increase in food intake was accompanied by increased body weight (Control: 105.9% ± 1.86% vs. TeTx: 118.5% ± 2.92%, p = 0.0081; Figure 6.1E) that was due to a selective increase in

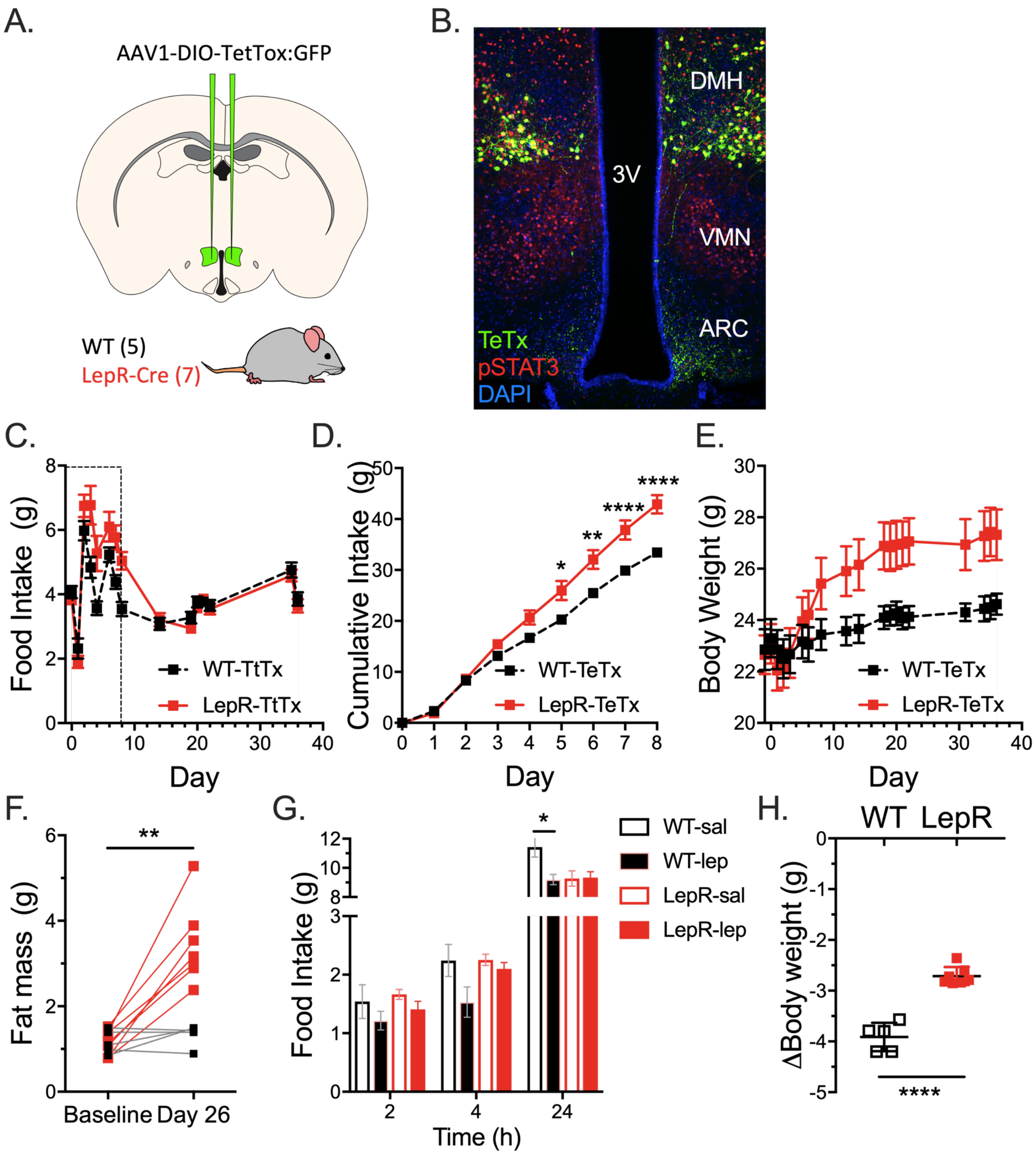


Figure 6.1 Inactivation of DMH^{LepR} neurons elicits transient hyperphagia and increased adiposity.

A) Strategy for chronic inhibition of DMH^{LepR} neurons by bilateral microinjection on Day 0 of a cre-dependent TeTx:GFP delivered to LepR-Cre+ males (LepR-TeTx; n=7) and Cre- littermate controls (WT-TeTx; n=5). **B**) Representative image indicated bilateral infection and co-expression of TeTx and leptin-induced pSTAT3, a marker of leptin receptor activation, selectively in the DMH. **C**) Daily food intake of LepR-TeTx and WT-TeTx mice. **D**) Cumulative food intake from week 1 in C (inset). Repeated measures ANOVA, main effect of treatment: $F_{(1,10)} = 8.44$; $p = 0.0157$; interaction: $F_{(8,80)} = 11.47$, $p < 0.0001$. **E**) Body weight. Main effect of treatment: $F_{(1,10)} = 2.175$, $p = 0.1711$; interaction: $F_{(18,180)} = 15.98$; $p < 0.0001$. **F**) Fat mass 26 days after microinjection surgery. **G**) Refeeding following a 24h fast and i.p. administration of saline (sal; PBS pH 7.4) or leptin (lep; 3mg/kg) in WT-TeTx and LepR-TeTx animals. **H**) Change in body weight following the 24h fast in G. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

body fat mass (Figure 6.1F), with no significant change of lean body mass (not shown). Together, these findings suggest that permanent inhibition of $\mathrm{DMH}^{\mathrm{LepR}}$ neurons is sufficient to induce transient hyperphagia and sustained obesity in otherwise normal mice.

Silencing $\mathrm{DMH}^{\mathrm{LepR}}$ neurons blunts leptin-induced suppression of refeeding

We next investigated whether leptin-mediated suppression of fasting-induced hyperphagia requires leptin signaling in $\mathrm{DMH}^{\mathrm{LepR}}$ neurons. Here, we found that fasting-induced refeeding responses remained intact in both control saline- and $\mathrm{DMH}^{\mathrm{TeTx}}$ saline-treated mice (Figure 6.1H). In contrast, however, the ability of leptin to suppress fasting-induced hyperphagia was blunted in mice in which $\mathrm{DMH}^{\mathrm{LepR}}$ neurons were permanently inhibited. We note that the refeeding response in controls was greater than in $\mathrm{DMH}^{\mathrm{TeTx}}$ animals, irrespective of treatment, consistent with the elevated daily intake at this timepoint in controls relative to $\mathrm{DMH}^{\mathrm{TeTX}}$ (Figure 6.2B), although the more severe weight loss in control animals following the 24h fast (Figure 6.1H) may also play a role. Taken together, these findings support a model in which leptin action in a subset of DMH neurons is required to suppress feeding; future studies are needed to determine the extent to which downstream AgRP neurons mediate this effect (Xu *et al.*, 2018a).

Inhibition of $\mathrm{DMH}^{\mathrm{LepR}}$ neurons blunts diurnal variation in feeding

To further characterize the energy homeostasis phenotype following chronic inhibition of $\mathrm{DMH}^{\mathrm{LepR}}$ neurons, and based on evidence that a subset of glutamatergic $\mathrm{DMH/DHA}^{\mathrm{LepR}}$

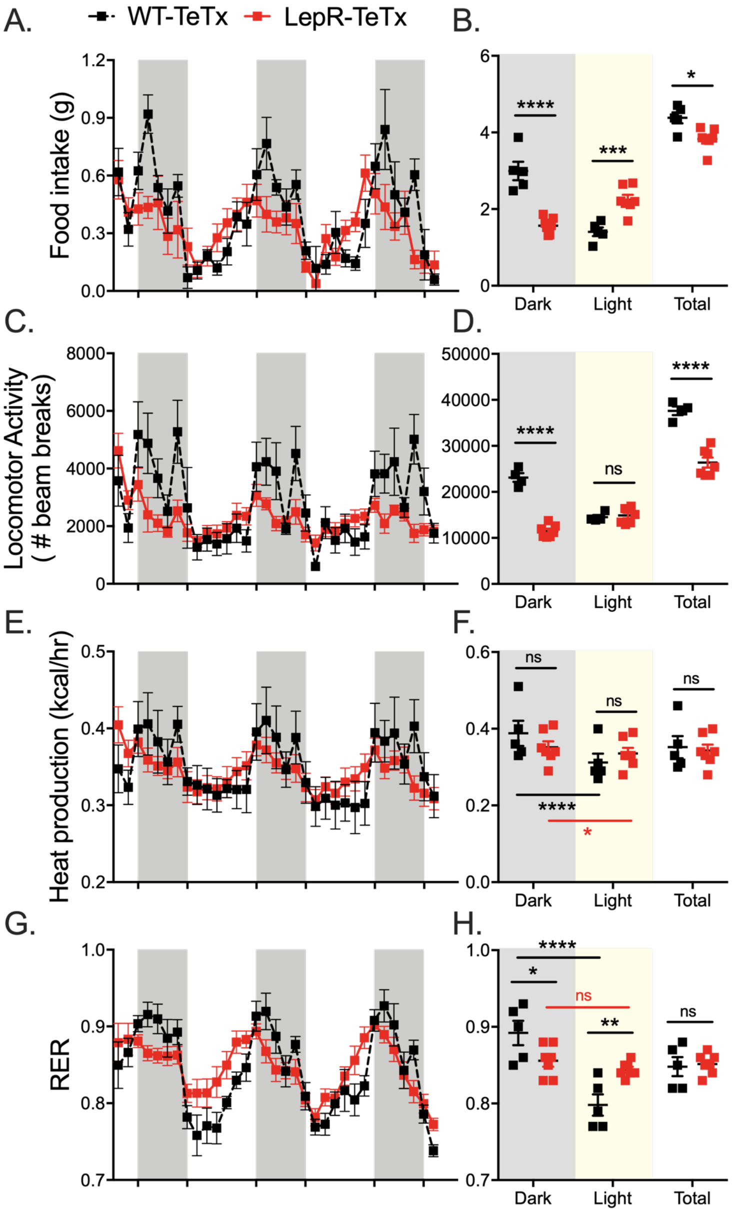


Figure 6.2 Inhibition of DMH$^{\text{LepR}}$ neurons phase-shifts feeding rhythms and blunts dark-cycle locomotor activity.
2h-binned continuous measures and mean values during the dark, light, and 24h-period throughout full calorimetry session in WT-TeTx and LepR-TeTx animals three weeks after microinjection surgery. **A,B**) Food intake; **C,D**) locomotor activity; **E,F**) heat production, and **G,H**) respiratory exchange ratio (RER). Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

neurons are implicated in thermogenesis (Rezai-Zadeh *et al.*, 2014; Zhao *et al.*, 2017), we subjected animals to indirect calorimetry three weeks after microinjection. Somewhat surprisingly, we found no difference in energy expenditure between control and DMH$^{\text{TeTx}}$

mice, although we only detected a limited number of cells targeted with the virus in the DMH/DHA region which is implicated in leptin-regulation of thermogenesis (Figure S1). In contrast, although the hyperphagia had subsided by the time of calorimetry, we observed a striking disruption of circadian feeding and behavioral rhythms (Figure 6.2). Specifically, relative to control mice which consumed the majority of their food intake in the dark cycle, $\mathrm{DMH}^{\mathrm{TeTx}}$ mice exhibited a significant reduction in their dark cycle food intake, while light cycle feeding was increased (Figure 6.2A,B), such that a comparable amount was consumed in each. A similarly shifted and blunted diurnal rhythm was observed with RER, likely reflective of the observed changes in food intake (Figure 6.2G,H). Moreover, while control mice displayed typical diurnal patterns in locomotor activity, $\mathrm{DMH}^{\mathrm{TeTx}}$ animals exhibited a robust reduction of locomotor activity, which failed to increase selectively during the dark cycle and remained low in the light cycle (Figure 6.2C,D). Taken together, these findings suggest that circadian patterns of feeding and locomotor behavior require $\mathrm{DMH}^{\mathrm{LepR}}$ neuronal activity.

Increased RER and blunted locomotor activity precede detectable changes in feeding rhythms

To determine if the transient weight gain induced by $\mathrm{DMH}^{\mathrm{LepR}}$ neuron inactivation involves changes of energy expenditure or body temperature, we studied a new cohort of animals in which Cre+ male mice bearing body temperature transponders received either AAV-DIO-TeTx ($\mathrm{DMH}^{\mathrm{TeTx}}$) or AAV-DIO-GFP virus (control) directed to the DMH as a control. Our findings indicate that neither core temperature nor energy expenditure were different between $\mathrm{DMH}^{\mathrm{TeTx}}$ and control mice throughout the initial period of excessive weight gain

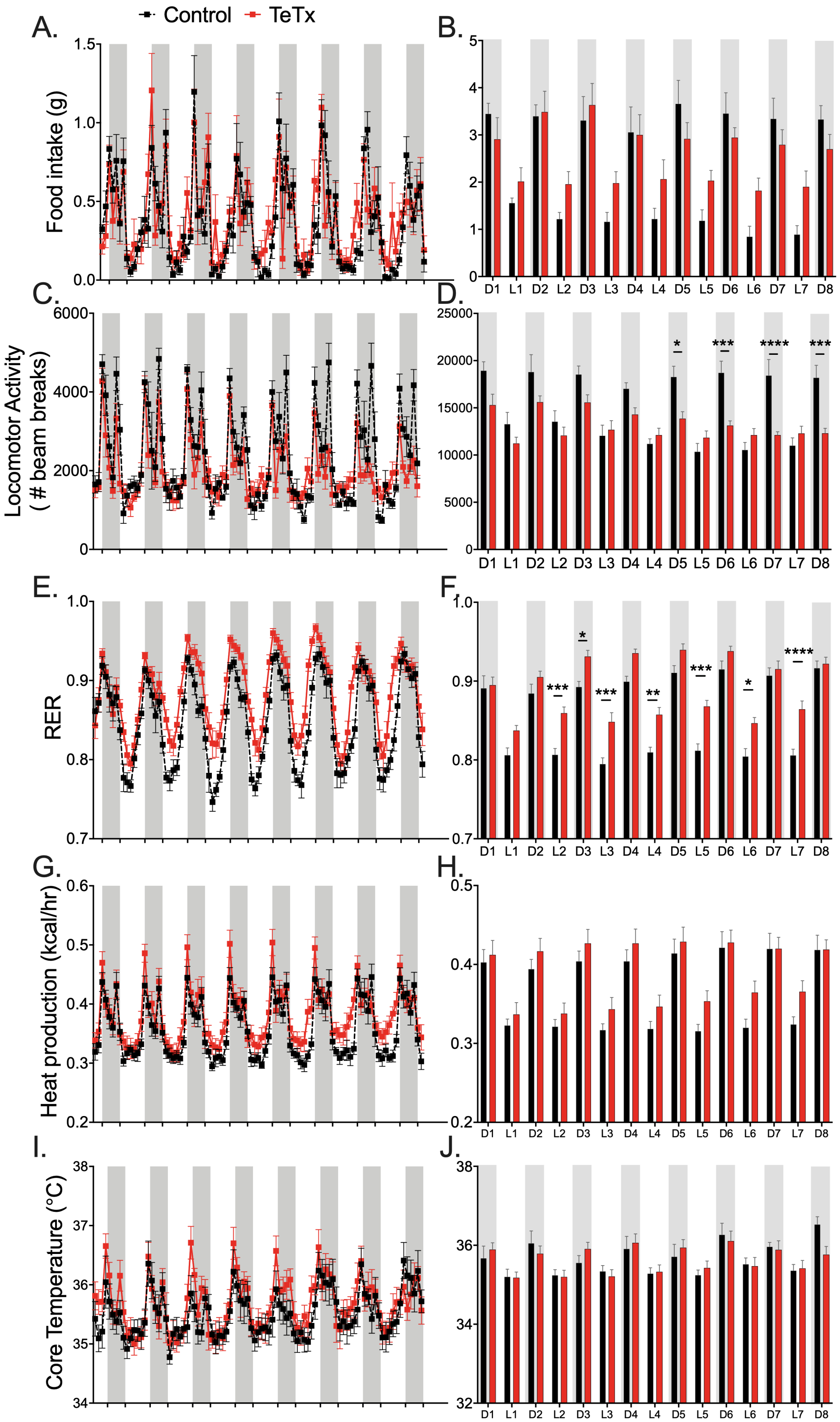


Figure 6.3 Rapid disruption of RER and locomotion precede changes in feeding.

2h-binned continuous measures and mean values during the dark and light period in GFP control and TeTx-treated animals beginning 48h after microinjection surgery. **A,B**) Food intake; **C,D**) locomotor activity; **E,F**) respiratory exchange ratio (RER); repeated measures ANOVA, main effect of treatment: $F_{(1,14)} = 23.23$; $p = 0.0003$; **G,H**) heat production; and **I,J**) core body temperature. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

in the former mice. In contrast to our earlier findings, the increase of daily food intake induced by chronic inactivation of DMH^{LepR} neurons did not achieve statistical significance (Figure 3A,B; cumulative week 1 food intake: 36.35g ± 2.144g for GFP controls vs. 39.08g ± 1.469g for TeTx; $p = 0.3244$), although values were consistently markedly elevated during the light cycle relative to controls (% light cycle feeding on Day 7: 21.2 ± 3.877 for GFP controls vs. 40.8 ± 4.684 for TeTx; $p = 0.0061$). We also observed a significant increase of RER during the light cycle in DMH^{TeTx} mice relative to controls that persisted throughout the study, suggesting that inactivation of DMH^{LepR} neurons alters substrate oxidation independently of changes in feeding (Figure 6.3E,F). In addition, we found that by Day 5 of calorimetry, locomotor activity was also significantly altered, with DMH^{TeTx} animals exhibiting a selective reduction of dark cycle activity.

Three weeks following microinjection, body weight was increased in LepR-Cre+ mice that received intra-DMH TeTx, relative to GFP controls, as expected (Day 21: 28.35g ± 0.78g for GFP control vs. 32.26g ± 0.80g for TeTx; $p<0.01$; Figure 6.5B). Moreover, we found once again that chronic inhibition of DMH^{LepR} neurons disrupted diurnal patterns of feeding and behavior. Specifically, animals exhibited both a decrease in their dark cycle and an increase in their light cycle food intake, reduced dark cycle locomotor activity, and increased RER during the light cycle (Supplemental Figure 2), replicating our previous findings (Figure 6.2) that chronic inhibition of DMH^{LepR} neurons causes a persistent disruption in circadian feeding and locomotor behaviors.

Meal size is unaffected by inhibition of DMH^{LepR} neurons

Evidence that DMH^{LepR} neurons are acutely activated by food cues (Garfield *et al.*, 2016) suggests a conceivable role for these neurons in responding to endogenous meal-related satiety signals to regulate meal termination. To explore this possibility, we studied meal patterning in animals with inactivated DMH^{LepR} neurons, after the initial hyperphagia had subsided and body weight had stabilized at a new, elevated level. We found no difference in average meal size between controls and DMH^{TeTx} animals, irrespective of the time of day (Figure 6.4A), although a small but significant reduction in meal duration was apparent during the dark cycle (Figure 6.4B). In contrast, the increased food intake during

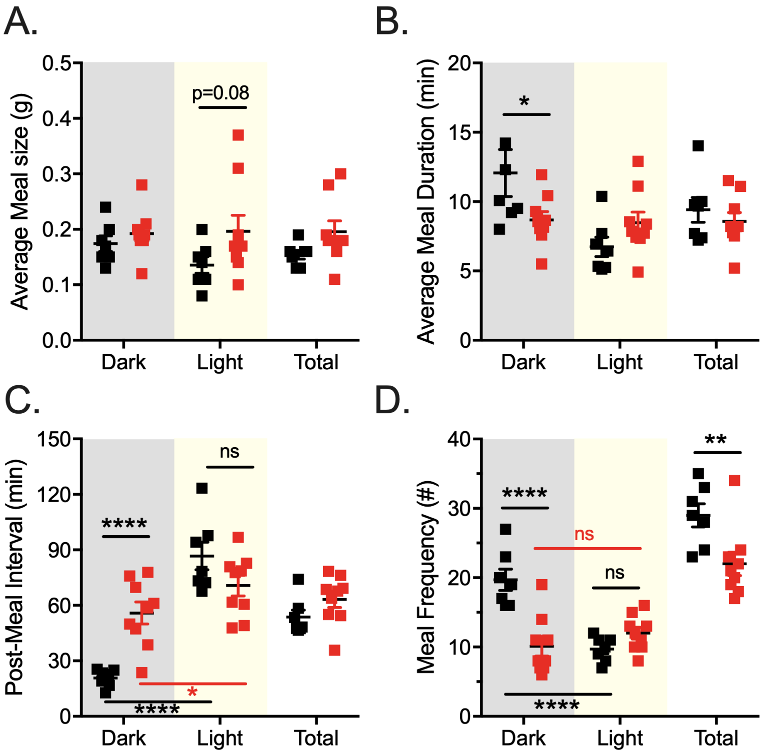


Figure 6.4 Effect of DMH^{LepR} inactivation on meal patterning.
A) Average meal size, **B**) average meal duration, **C**) inter-meal interval, and **D**) meal frequency measured continuously 21d after microinjection in control and TeTx animals. Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

the dark cycle in controls (Figure 6.2A,B) was associated with reduced time between each meal (Figure 6.4C), and thus greater number of total meals consumed (Figure 6.4D). These findings suggest that acute satiety responses or meal initiation do not require activation of DMH$^{\text{LepR}}$ neurons, and further imply that the underlying defect in meal timing is driven by a selective defect in the circadian control of food intake.

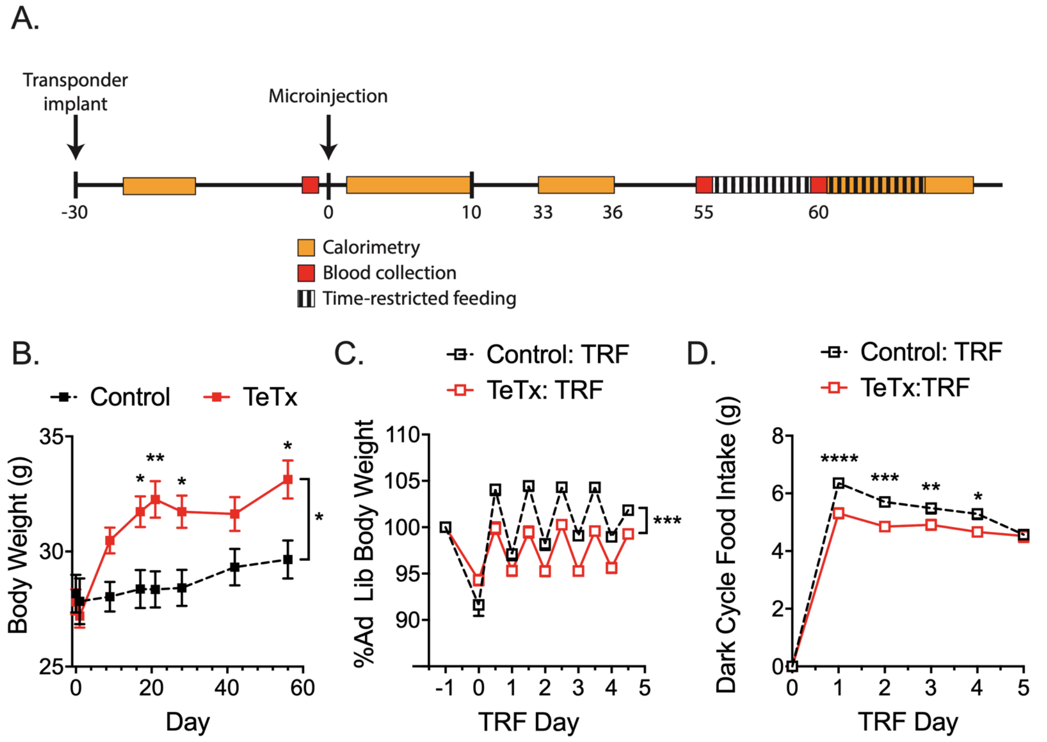


Figure 6.5 Time-restricted feeding experimental paradigm and lead-in data. **A**) Schematic of study design. **B**) Morning-fed body weight of controls and TeTx animals following microinjection on Day 0. Repeated measures ANOVA, main effect of treatment: F$_{(1,14)}$ = 5.091; p = 0.0406. **C**) Body weight response to (TRF) as a percentage of pre-TRF, ad lib fed weight at light-onset (ZT0; peaks) and dark-onset (ZT14; troughs) after TRF begins on TRF Day 0. Repeated measures ANOVA, main effect of treatment: F$_{(1,14)}$ = 22.07; p = 0.0003. **D**) Dark cycle (ZT14 – ZT24) food intake in control and TeTx animals during the first 5 days of TRF. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Silencing DMH$^{\text{LepR}}$ neurons prevents behavioral adaptation to a restricted feeding window

Based on growing evidence implicating the DMH in circadian feeding and behavioral rhythms (Cedernaes, Waldeck and Bass, 2019), we next sought to determine whether DMH^{LepR} neurons are required for adaptive responses to time-restricted feeding (TRF). This was achieved by determining whether disruptions in diurnal feeding and locomotor activity in mice with chronic inhibition of DMH^{LepR} neurons are corrected by restricting food intake to the 10h dark cycle only. To test this hypothesis, GFP control and DMH^{TeTx} animals were acclimated to TRF for 5 days, before being subjected to indirect calorimetry and meal pattern analysis for 5 additional days during TRF, followed by 3 days of ad lib feeding (Figure 6.5A).

During the TRF acclimation period, as expected, body weight oscillated on a daily basis in GFP-treated control mice, being increased at the end of the dark cycle (after ad lib access to food) and lower at the end of the light cycle (no food access), but was essentially maintained throughout the 5-day TRF period (Figure 6.5C). While a similar diurnal pattern was observed in TeTx-treated mice, these mice were unable to maintain their body weight (Figure 6.4D), suggestive of an impaired ability to appropriately increase food intake during the dark cycle (Figure 6.5D).

Consistent with this possibility, we found that during TRF, DMH^{TeTx} mice failed to increase either food intake or locomotor activity during the dark cycle, unlike what was observed in GFP-controls. Nevertheless, these mice did exhibit normalized RER levels without effects on heat production. Moreover, when mice were returned to ad lib feeding, DMH^{TeTx} animals rapidly and selectively elevated their light cycle food intake (Figure 6.6A,B), and subsequently regained lost body weight (data not shown). Taken together, these findings demonstrate that while mice with chronically silenced DMH^{LepR} neurons are able to entrain RER to a TRF schedule, they are unable to adapt their food intake and

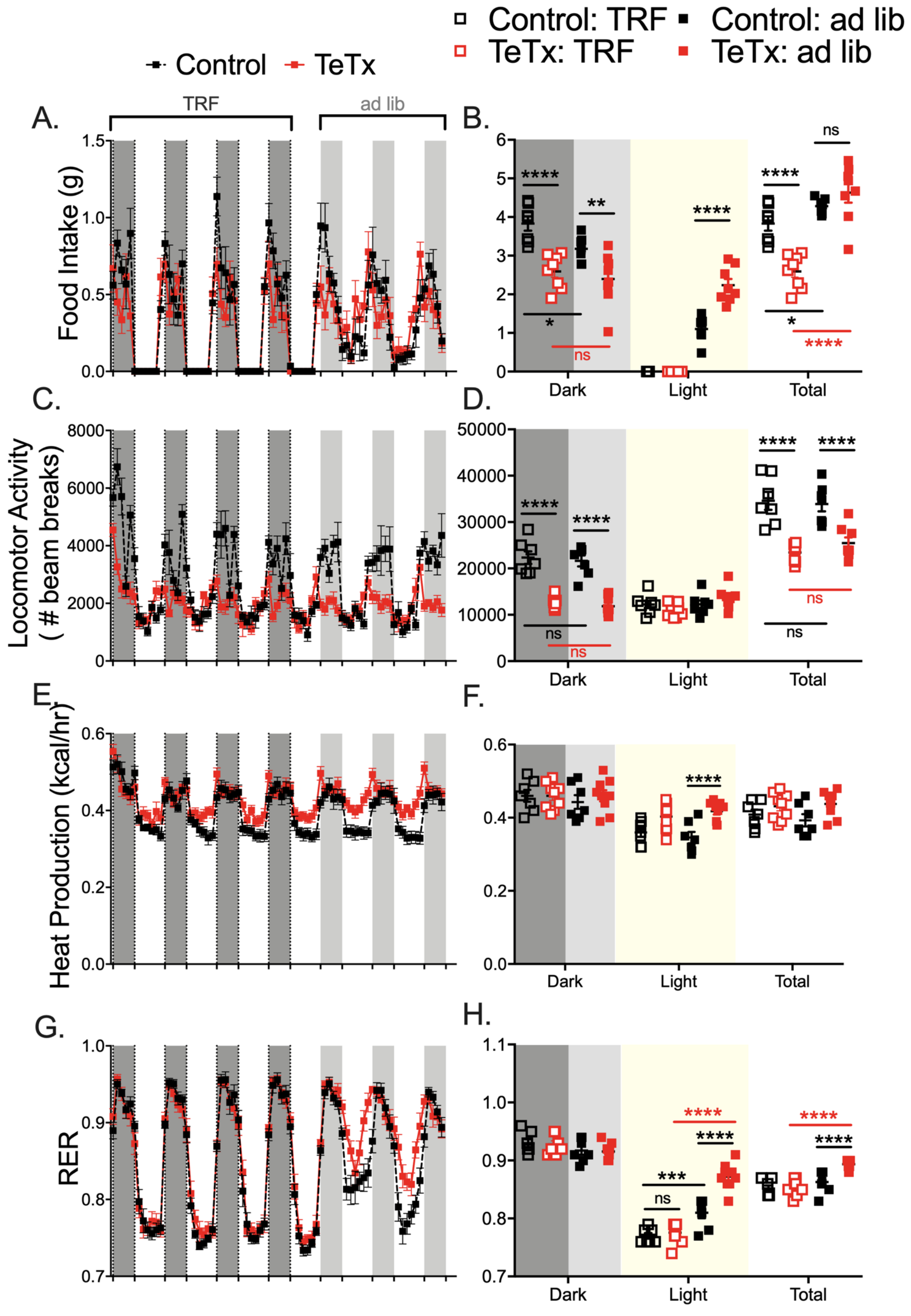


Figure 6.6 DMH^{TeTx} animals fail to adapt feeding behaviors under TRF. 2h-binned continuous measures and mean dark:light measures in GFP control and TeTx-treated animals after 5 days of TRF. Mice were maintained on TRF for 4 additional days (dark gray shaded dark cycles; open squares), then restored to ad lib feeding (light gray shaded dark cycles; filled squares). **A,B**) Food intake, **B, F.** locomotor activity, **C, G.** heat production, and **D, H.** respiratory exchange ratio (RER). Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

hence experience loss of body weight. Furthermore, the reduction of dark-cycle locomotor activity induced by inhibition of these neurons is unaffected by the altered feeding schedule.

Intact adaptive thermogenic responses to mild cold exposure with DMH^{LepR} inhibition

Based on previous evidence that leptin action in the DMH and DHA play a physiological role in thermoregulation (Enriori *et al.*, 2011; Rezai-Zadeh *et al.*, 2014), we sought to determine if the specific subset of DMH^{LepR} neurons targeted by our intervention also participate thermoregulation either at baseline or in the adaptive response to a thermal challenge. To this end, we studied control and DMH^{TeTx} mice housed both at room temperature (22°C) and during subsequent mild cold exposure (14°C) and recorded food intake, heat production and core temperature over time. As expected, control animals increase their heat production when subjected to mild cold exposure (Figure 6.7A-C), such that core temperature remains unchanged (Figure 6.7D-E). Somewhat to our surprise, DMH^{TeTx} animals mount a similarly robust adaptive increase in heat production (Figure 6.7A-C) and also maintain core temperature during the same cold challenge (Figure 6.7D-F). However, we observed large variation in core temperature in DMH^{TeTx} animals, with one potential outlier that may warrant exclusion upon post hoc analysis of viral expression, which will be completed prior to submitting this work for publication.

To offset the increased caloric needs to fuel thermogenic responses, animals must increase food intake to avoid depletion of stored energy (i.e., within fat depots). Accordingly, we found that while control animals increased their dark cycle and overall

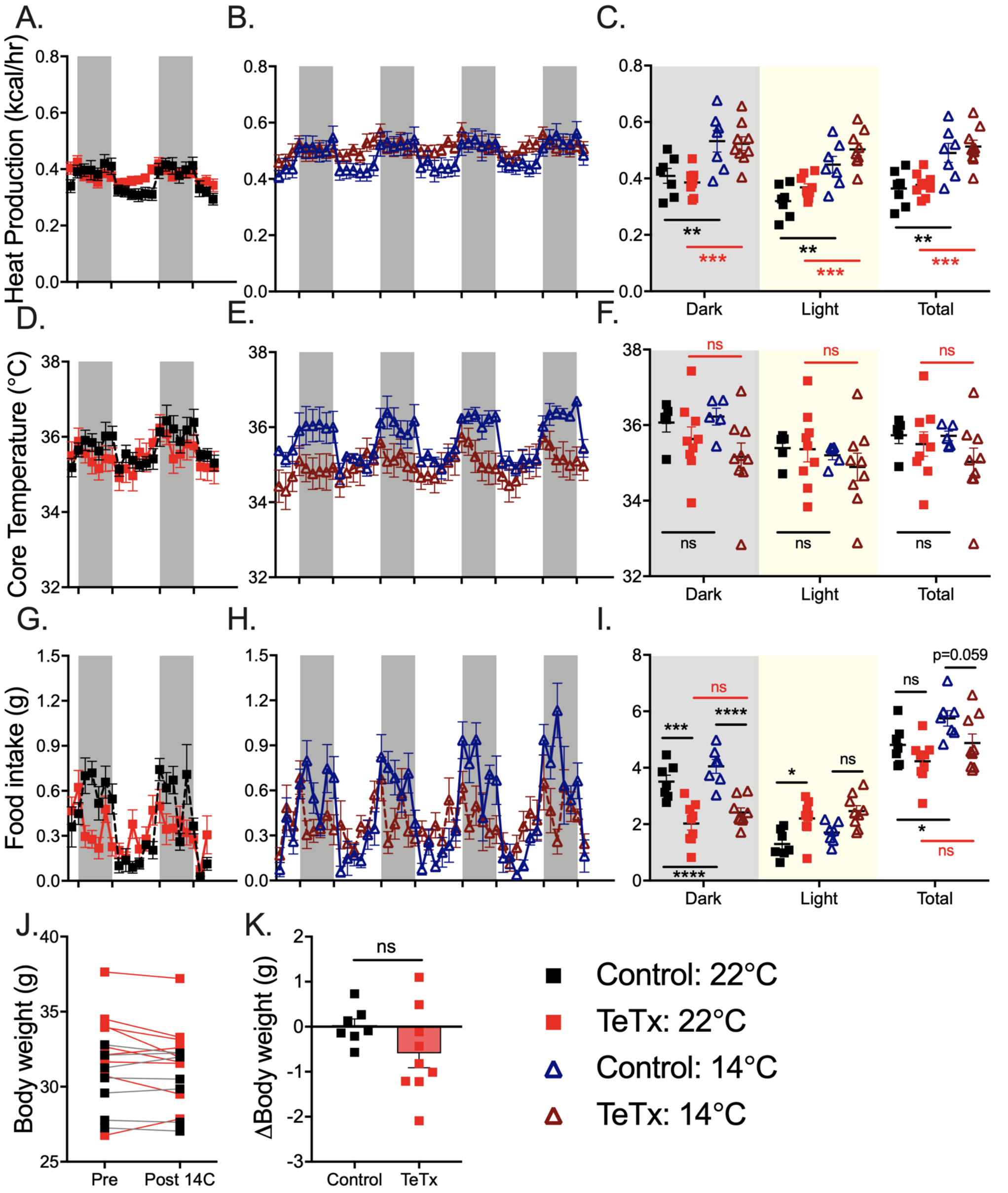


Figure 6.7 Activation of DMH^{LepR} neurons is not required for thermogenic responses during mild cold exposure.

2h-binned continuous measures and mean values during the dark, light, and 24h-period throughout full calorimetry session in control (GFP) and TeTx animals four weeks after microinjection surgery. Mice were housed at 22°C for 2 baseline days (left panels), before the temperature was gradually reduced to 14°C for 4 days (middle panels). A) Heat production during 22°C and B) 14°C. Repeated measures ANOVA, main effect of treatment: F(1,14) = 0.2812; p = 0.6042. C) Mean values from A and B. D) Core body temperature during 22°C and E) 14°C. Main effect of treatment: F(1,12) = 1.901; p = 0.1931. F) Mean values from D and E. G) Food intake during 22°C and H) 14°C. I) Mean values from G and H. J) Body weight before and after cold exposure. K) Change in body weight after cold exposure. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

total food intake in response to cold exposure (Figure 6.7G-I), this cold-induced hyperphagia was blunted in DMH^{TeTx} mice, such that their overall intake remained unchanged in response to mild cold exposure (Figure 6.7I). Nonetheless, body weight in both controls and DMH^{TeTx} animals was unaffected by mild cold exposure (Figure 6.7J,K). Taken together, these observations suggest that activation of DMH^{LepR} neurons is not required for mice to effectively thermoregulate in response to a mild cold challenge.

Discussion

Neurons in the DMH are implicated in metabolic processes relevant to energy homeostasis ranging from feeding behavior to thermoregulation and circadian rhythms. Leptin signaling appears to play a role in these effects, as leptin injection directly into this brain area stimulates brown adipose tissue (BAT) thermogenesis (Enriori *et al.*, 2011; Rezai-Zadeh *et al.*, 2014) and suppresses food intake (Enriori *et al.*, 2011). In the current work, we sought to identify the physiological role of DMH^{LepR} neurons in energy homeostasis. We report that chronic inactivation of DMH^{LepR} neurons induces a biphasic phenotype, with the first phase characterized by increased both body weight gain and body adiposity, effects associated with hyperphagia, increased RER, indicative of reduced fatty acid oxidation, and reduced dark cycle locomotor activity. Whereas the period of increasing food intake and body weight was transient, lasting only 10-20 days, we subsequently observed a progressive and sustained disruption of circadian feeding and behavioral rhythms, such that food intake, locomotor activity, and RER are markedly reduced in mice with inactivated DMH^{LepR} neurons during in the dark cycle, whereas food intake and RER are both increased in the light cycle compared to controls. Further, these effects were not corrected by time-restricted feeding, and were not associated with thermoregulatory defects. Collectively, these findings identify a physiological role for DMH^{LepR} neurons in the regulation of both energy homeostasis and circadian control of feeding behavior and locomotor activity.

The finding that inactivation of DMH^{LepR} neurons causes transient hyperphagia and obesity implies a physiological role for these neurons to defend against excessive food intake and weight gain. A role for reduced leptin signaling in this phenotype is suggested by our evidence that leptin-induced activation of DMH^{LepR} neurons is required to suppress re-feeding hyperphagia in fasted animals, consistent with previous studies (Xu *et al.*, 2018a). Moreover, DMH^{LepR} neurons in the ventral compartment of the DMH are GABAergic, and they are implicated as physiological inhibitors of orexigenic AgRP neurons. Specifically, 1) vDMH^{LepR} neurons provide direct GABAergic tone to AgRP neurons (Garfield *et al.*, 2016; Xu *et al.*, 2018a), 2) activation of this circuit inhibits AgRP neurons and suppresses food intake during the dark cycle (Garfield *et al.*, 2016), and 3) vDMH^{LepR} →ARC projections are activated by sensory detection of food (Garfield *et al.*, 2016). Ample evidence has also shown that acute activation of AgRP drives voracious food intake and food-seeking behaviors (Krashes, Koda, ChianPing Ye, *et al.*, 2011) and that sustained activation of AgRP neurons induces hyperphagia and increases weight gain (Krashes, Koda, ChianPing Ye, *et al.*, 2011). Together, these observations warrant future studies to determine the role played by activation of AgRP neurons in the hyperphagia induced by chronic inhibition of DMH^{LepR} neurons.

In addition to food intake control, AgRP neurons are also implicated in control of peripheral substrate metabolism. Specifically, Agrp neuron activation leads to an increase of carbohydrate and coincident decrease of fat utilization, even when neither total calories consumed nor diet composition are allowed to change (Cavalcanti-de-Albuquerque *et al.*, 2019). Thus, while AgRP-induced increases of food intake can clearly affect RER, this effect can also occur in a food intake-independent manner (Cavalcanti-de-Albuquerque *et al.*, 2019). Moreover, sustained AgRP activation promotes fat accumulation in a manner that is independent of changes in food intake (Padilla *et al.*, 2017; Cavalcanti-de-Albuquerque *et al.*, 2019). With this background, our finding of increased RER induced by chronic inactivation of $\mathrm{DMH}^{\mathrm{LepR}}$ neurons is suggestive of increased de novo lipogenesis arising from activation of downstream Agrp neurons. This effect, in combination with the modest hyperphagia we observed, likely contributes to the increased body weight gain and body adiposity exhibited by these mice.

The DMH is also an important component of the brain's thermoregulatory system (DiMicco and Zaretsky, 2007; Shaun F Morrison, 2016). The DMH receives input from the hypothalamic preoptic area (POA), which integrates afferent thermal information from temperature-sensitive thermoreceptors from the skin via a well-mapped peripheral-to-central relay (Shaun F Morrison, 2016). Specifically, the activity of neurons in the dorsal aspect of the DMH (dDMH) is regulated by changes in ambient temperature (Zhao *et al.*, 2017), and activation of either GABAergic or glutamatergic neuronal subsets in the dDMH is sufficient to raise core temperature, while conversely, inhibition of these same neurons induces hypothermia (Zhao *et al.*, 2017). Moreover, DMH leptin signaling is implicated as a key mediator of these thermogenic responses. In support of this concept, i) transsynaptic retrograde tracing from BAT labels $\mathrm{DMH}^{\mathrm{LepR}}$ neurons (Zhang *et al.*, 2011), ii) cold exposure induces cFos in LepR neurons in the DHA/dDMH (Zhang *et al.*, 2011),

iii) intra-DMH leptin microinjection increases core body temperature (Enriori *et al.*, 2011), iv) loss of LepR from DMH neurons is associated with complete loss of leptin-induced thermogenesis (Dodd *et al.*, 2014), and v) activation of LepR-expressing DMH/DHA neurons is sufficient to increase thermogenesis (Rezai-Zadeh *et al.*, 2014). Based on these observations, it was somewhat surprising that we observed no defect in either thermoregulation or heat production in mice with chronic $\mathrm{DMH}^{\mathrm{LepR}}$ inhibition, whether studied at room temperature or in response to a mild cold challenge, although individual responses were somewhat more variable in these mice than in controls. These findings suggest that the subset of $\mathrm{DMH}^{\mathrm{LepR}}$ neurons targeted in the current study are not directly involved in the maintenance of core body temperature, unlike lepr (+) neurons in the dorsal aspect of the DMH.

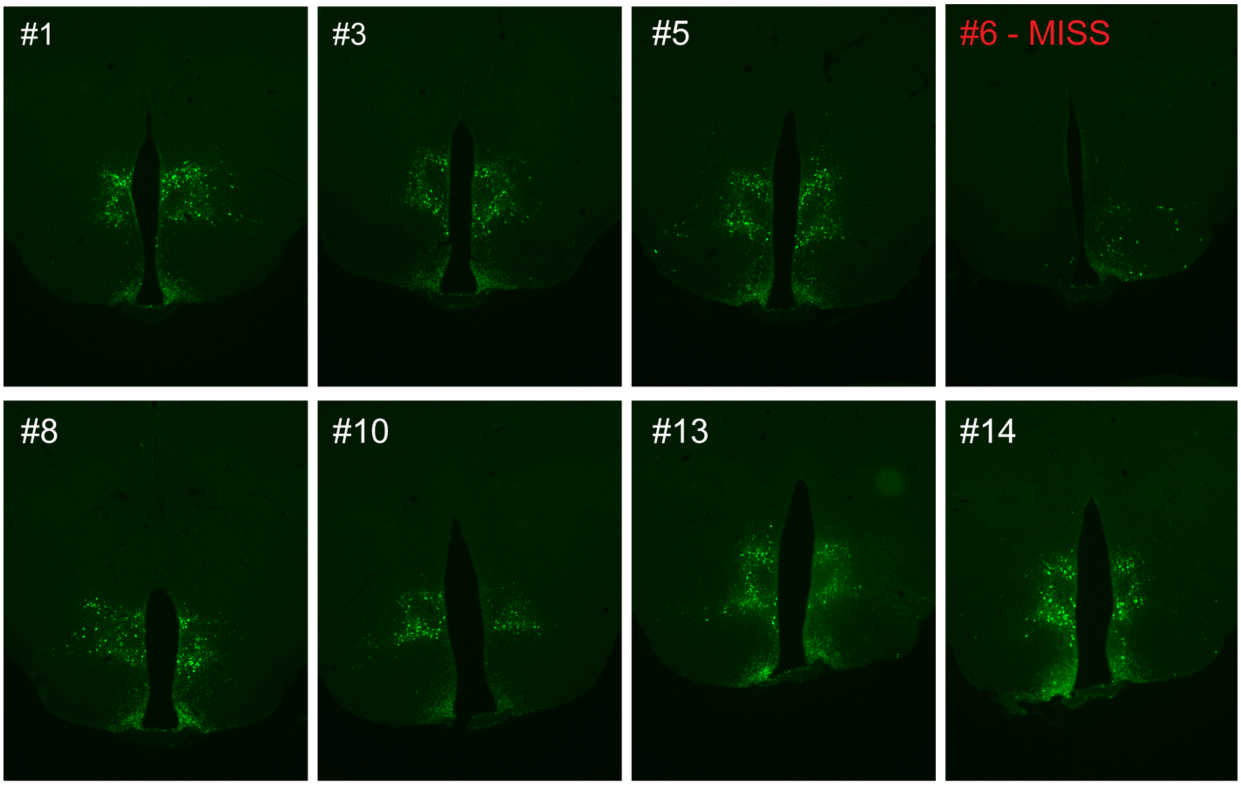
We also found that chronic inhibition of $\mathrm{DMH}^{\mathrm{LepR}}$ neurons induces a biphasic phenotype, characterized initially by a rapid increase in body weight and fat accumulation during the first 10-20 days of study. After this dynamic period of weight gain and hyperphagia subsides, however, body weight remains fairly stable for the remainder of the study, despite the persistently disrupted circadian feeding rhythms. From these observations we infer that while the requirement for $\mathrm{DMH}^{\mathrm{LepR}}$ neuronal activity in the regulation of circadian feeding behavior is not readily compensated by other components of the system governing circadian rhythmicity, the initial hyperphagia and weight gain may recruit adaptive responses that normalize food intake and prevent further expansion of the adipose depot (Schwartz *et al.*, 2017). A plausible explanation for the latter is that the obesity associated elevation of plasma leptin levels engages compensatory homeostatic circuits that limit further weight gain, but is unable to counter the change in circadian feeding behavior.

While many studies suggest a role for the DMH in the regulation of circadian rhythms, the role(s) played by specific DMH cell types is largely unknown (Challet, 2019). Nonspecific lesions targeting the DMH produce a marked reduction of locomotor activity and blunted rhythms in food consumption, plasma corticosterone, and body temperature (Chou *et al.*, 2003). Further work has implicated the DMH in the ingestive and behavioral adaptations to time-restricted feeding (TRF), although this subject has been somewhat controversial (Challet, 2019). Our findings add to this body of work by demonstrating that $\mathrm{DMH}^{\mathrm{LepR}}$ neuronal activity is required for normal circadian feeding rhythms and associated behaviors. Specifically, we observed that mice eat more during the light cycle and less during the dark cycle following inactivation of $\mathrm{DMH}^{\mathrm{LepR}}$ neurons, thereby eliminating the difference in intake between dark and light cycles characteristic of normal mice. This intervention also blunted dark cycle locomotor activity, but had no detectable effect on either meal size or duration. This observation suggests that satiety responses during individual meals are not affected by $\mathrm{DMH}^{\mathrm{LepR}}$ inactivation, and hence that the underlying defect is relatively specific for circadian control of food intake (rather than resulting from impaired control over meal onset or termination). Furthermore, when we restricted food availability to the dark cycle (i.e., a time when nocturnal rodents consume the majority of their calories), we observed an impairment in the ability of mice with $\mathrm{DMH}^{\mathrm{LepR}}$ inactivation to adjust their daily intake so that they consume sufficient food during the dark cycle to maintain their body weight. Taken together, these findings suggest that $\mathrm{DMH}^{\mathrm{LepR}}$ neurons are required both for normal circadian feeding rhythms and for the ability to adapt to a restricted feeding window. Additional studies are warranted to clarify whether the timing or duration of the feeding window is tied to the activity of DMH^{LepR} neurons or whether their activation is only required during certain times of the day.

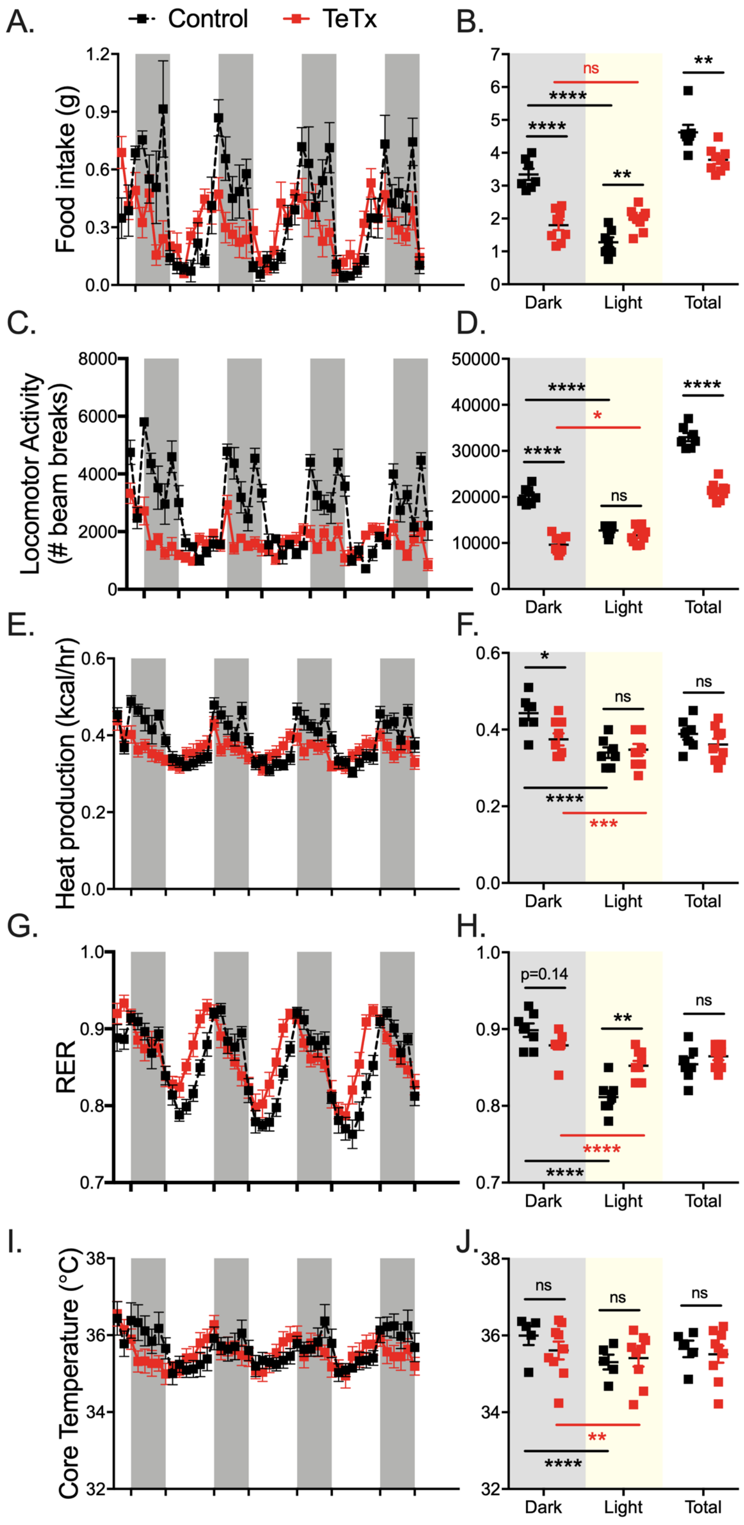
Indeed, previous evidence has demonstrated rhythmic cFos expression in the DMH over a 24 h period in animals fed *ad libitum*, with maximal expression occurring during the dark cycle (when feeding is maximal) and very little detected at midday during the rest period (Gooley, Schomer and Saper, 2006). Interestingly, during TRF, maximal cFos induction occurs just before the scheduled mealtime – even if this meal is “mistimed” during the light cycle (Gooley, Schomer and Saper, 2006). While these findings suggest that the DMH exhibits endogenous rhythmic activity that is subject to modulation by food availability, the identity of these cyclically-responsive neurons is unknown. Also unknown are the neural and/or humoral factors that lie upstream of DMH^{LepR} neurons to establish these rhythms, but projections from the suprachiasmatic nucleus (SCN) are an obvious possibility, given the key role of this brain area as the “master controller” of circadian rhythms, including in the DMH (Chou *et al.*, 2003; Huang *et al.*, 2011). A priority for future studies is to identify both pre- and postsynaptic cells in this circuit using, for example a Cre-dependent, modified (delta G) rabies tracing strategy to identify monosynaptic inputs to these DMH^{LepR} neurons. This strategy, in combination with optogenetics and fiber photometry technology to manipulate and record the endogenous activity of these cells in vivo, will shed valuable new light on mechanisms underlying circadian regulation of DMH neuronal activity.

In conclusion, we report that LepR-expressing DMH neurons play a physiological role in the control of both overall energy homeostasis and of circadian rhythms of feeding behavior and locomotor activity. These findings have important translational implications given evidence from both humans and rodents that mistimed feeding can predispose to obesity and T2D (Huang *et al.*, 2011; Challet, 2019). In humans, even a short-term circadian misalignment is sufficient to impair glucose tolerance and insulin sensitivity in healthy individuals (Gonnissen *et al.*, 2012) and may underlie the increased risk of T2D associated with long-term shift work (Knutsson and Kempe, 2014). Conversely, restricting food intake to narrow windows during the active cycle has been suggested to improve metabolic health and longevity (Hatori *et al.*, 2012; Longo and Panda, 2016; Chaix *et al.*, 2019). An improved understanding and identification and characterization of the neural circuits underlying endogenous rhythms in behavior, feeding, and metabolism may facilitate the development of new therapeutic and dietary strategies for the treatment of humans with metabolic dysfunction.

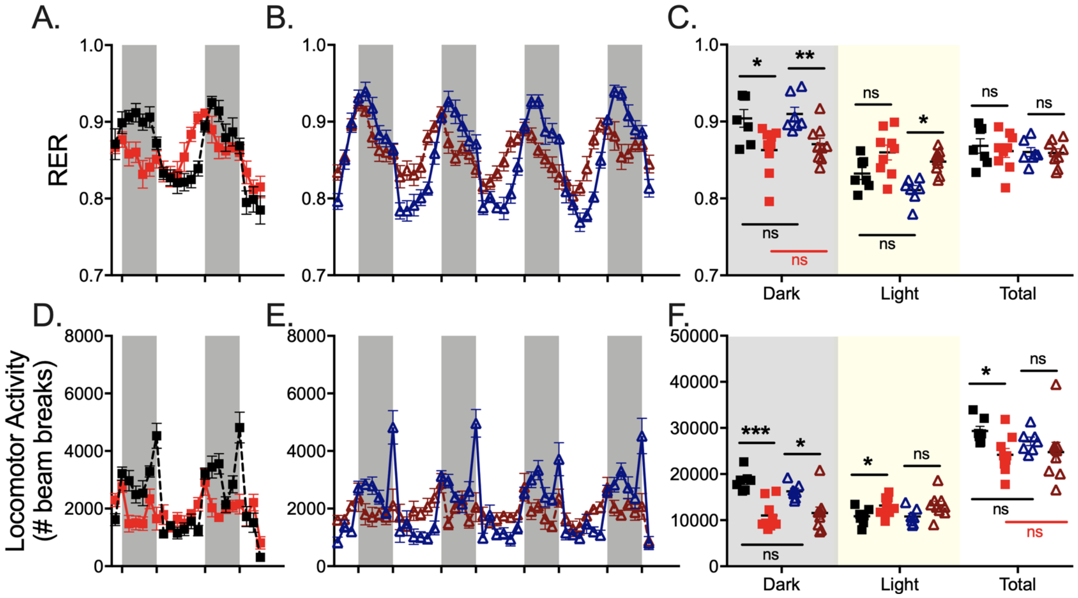
Supplemental Information



Supplemental Figure 1 Representative viral expression in included and excluded (missed) animals (associated with Figures 1 and 2).



Supplemental Figure 2 Steady-state calorimetry in animals 4 weeks after microinjection (associated with Figures 3-7).



Supplemental Figure 3 Additional calorimetry measures during mild cold exposure (associated with Figure 7).

2h-binned continuous measures and mean values during the dark, light, and 24h-period throughout full calorimetry session in control (GFP) and TeTx animals four weeks after microinjection surgery. Mice were housed at 22C for 2 baseline days (left panels), before the temperature was gradually reduced to 14C for 4 days (middle panels). **A,B,C**) respiratory exchange ratio (RER); **D,E,F**) locomotor activity. Data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Chapter 7 An Adaptable Angled Stereotactic Approach for Versatile Neuroscience Techniques

Publication (Faber *et al.*, 2020) in press at (https://www.jove.com/video/60965/adaptable-angled-stereotactic-approach-for-versatile-neuroscience). Copyright 2020 by the Journal of Visualized Experiments.

Abstract

Stereotactic surgery is an essential tool in the modern neuroscience lab. However, being able to precisely and accurately target difficult to reach brain regions still represents a challenge, particularly when targeting brain structures along the midline. These challenges include avoiding the superior sagittal sinus and third ventricle and being able to consistently target selective, discrete brain nuclei. In addition, more advanced neuroscience techniques (e.g., optogenetics, fiber photometry, and 2-photon imaging) rely on targeted implantation of significant hardware to the brain and spatial limitations represent a common hindrance.

Here we present a modifiable protocol for stereotactic targeting of rodent brain structures using an angled coronal approach that may be adapted for 1) either mouse or rat; 2) various neuroscience techniques and 3) for multiple brain regions. As a representative example, we include calculation of stereotactic coordinates for targeting the mouse hypothalamic ventromedial nucleus (VMN) for an optogenetic inhibition experiment. This procedure begins with the bilateral microinjection of an adeno-associated virus (AAV) encoding a light-sensitive chloride channel (SwiChR++) to a cre-dependent mouse model, followed by the angled bilateral implantation of fiberoptic cannulae. Using this approach, our findings show that activation of a subset of VMN neurons is required for intact glucose counterregulatory responses to insulin-induced

hypoglycemia.

Introduction

Neural control of behavior, feeding, and metabolism involves coordination of highly complex, integrative, and redundant neurocircuits, and a driving goal of the neuroscience field is to dissect the relationship between neurocircuit structure and function. Although classical neuroscience tools such as lesioning, local pharmacological injections, and electrical stimulation uncovered vital knowledge regarding the role of specific brain regions in control of behavior and metabolism, these tools were limited by their lack of specificity and reversibility(King, 2006).

Recent advances in the neuroscience field have greatly improved our ability to interrogate and manipulate circuit function in a cell-type specific manner with high spatiotemporal resolution. Optogenetic (Boyden *et al.*, 2005) and chemogenetic (Roth, 2016) approaches, for instance, allow the rapid, reversible manipulation of activity in genetically defined cell types of freely moving animals. Optogenetics involves the use of light-sensitive ion channels, termed channelrhodopsins, to control neuronal activity. Key to this technique is gene delivery of the channelrhodopsin, and a source of light to activate the opsin. A common strategy for gene delivery is through a combination of 1) genetically-engineered mice expressing Cre-recombinase in discrete neurons, and 2) Cre-dependent viral vectors encoding channelrhodopsin. While optogenetics provides an elegant, highly precise means to control neuronal activity, the method is contingent upon successful stereotactic microinjection of the viral vector and fiberoptic placement into a defined brain region. Although stereotactic procedures are commonplace within the modern neuroscience lab, and there are several excellent protocols describing this procedure (McSweeney and Mao, 2015; Fricano-Kugler *et al.*, 2016; Richevaux *et al.*, 2019), being able to consistently, reproducibly, and reliably target discrete brain regions along the midline, such as the mediobasal hypothalamus, a brain are critical in the regulation of homeostatic functions (Lowell, 2019), represents significant additional challenges. These challenges include avoiding the superior sagittal sinus, the third ventricle, and adjacent hypothalamic nuclei. In addition, there are significant spatial limitations for the bilateral implantation of hardware that is required for inhibition studies. With these challenges in mind, we herein present a modifiable procedure for targeting discrete brain regions via an angled stereotactic approach.

Protocol

All procedures should be approved in accordance with the National Institutes of Health Guide for the Care and Use of Animals and be approved by both the Institutional Animal Care and Use Committee and Environmental Health and Safety.

1. **Calculate angled coordinates**
   1.1. Using a coronal brain atlas, mark a right triangle so that the hypotenuse passes through the target region of interest. In the representative example (Figure 7.1), the hypothalamic ventromedial nucleus (VMN) is targeted at a 15° angle from the coronal midline).

NOTE: The placement of the axis of rotation depicted in Figure 1 (and thus, the length of side C) is arbitrary and can be modified to target any brain region. Although this may seem counterintuitive, later steps in the protocol will adjust the position of the head in the

Z axis such that this point aligns with the stereotactic center of rotation (see part **6**). However, it is recommended not to exceed a coronal rotation angle of 15° due to physical constraints of the head holder apparatus.

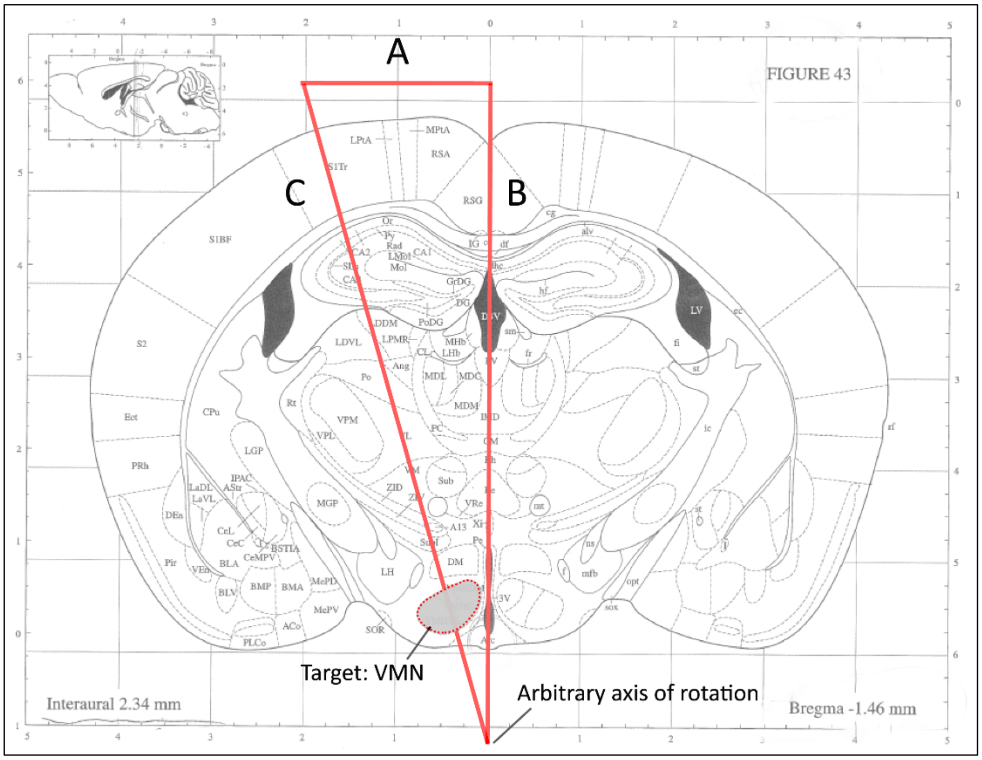


Figure 7.1 Representative example for calculating angled coordinates targeting the hypothalamic ventromedial nucleus.
A. This length should be calculated using basic trigonometry. Per this example, A = 2.03mm. B. Estimated length based upon assignment of arbitrary axis of rotation. Per this example, B = 7.576mm. C. Calculated hypotenuse. Note that the depth of fiberoptic/needle insertion used will depend upon desired proximity to target region and requires optimization.

1.2. Establish the desired angle, **a**, and the estimated length of side **B** and use trigonometry to calculate the length of sides **A** and **C**. This step will be important for properly positioning the head during rotation.

1.2.1. In the example in Figure 7.1, atlas gridlines were used to approximate the length of side B, giving 7.576mm; this information was used to calculate the length of side A thus:

$$tan(15°) = \frac{A}{B} = \frac{A}{7.576mm}$$

$$A = tan(15°) * 7.576mm = \mathbf{2.03mm}$$

NOTE: In this example, 2.03mm indicates the R/L distance from midline where the fiberoptic cannula will enter the brain when the head is rotated by 15°.

1.2.2. Optionally, calculate the length of side C in order to approximate the D/V coordinate:

$$A^2 + B^2 = C^2$$

$$\sqrt{2.03^2 + 7.576^2} = C = \mathbf{7.84mm}$$

NOTE: The length of the hypotenuse, C, does not represent the depth of injection, but will be helpful in determining the D/V coordinate, which may need to be adjusted to accommodate for the increased length vs. side B for straight-in injections. It is therefore recommended to perform test injections to optimize the D/V coordinate.

In this example targeting the VMN, we therefore have two sets of coordinates, one for the microinjection that is non-angled (A/P: -1.4, R/L: 0.4 at 0°, D/V: -5.7) and one for the angled fiberoptic implantation (A/P: -1.4, R/L: 0.0 at 15°, D/V: -5.4).

2. **Prepare the stereotax for angled procedure**

    2.1. Confirm that the stereotactic frame and micromanipulator have been calibrated (see Kopf Manual for full protocol).

    2.2. Place the Center Height Gauge into the socket of the head holder base plate.

2.3. Secure the Centering Scope in the tool holder, then sight down the scope. Adjust the position of the micromanipulator until the crosshairs are aligned and focused on the gauge crosshairs.

NOTE: During this step, the scope is being positioned into the focal plane of the head holder's center of rotation. Once established, the micromanipulator should not be moved during the remaining steps..

2.4. Place the ear bars into the holders and center them such that the indicator lines on both sides are at 0 (Figure 7.3A).

2.5. Use the Medial-Lateral and Anterior-Posterior knobs on the head holder (Figure 2) to center-align the ear bars in the X and Y planes above the crosshair of the Center Height Gauge (Figure 7.3A).

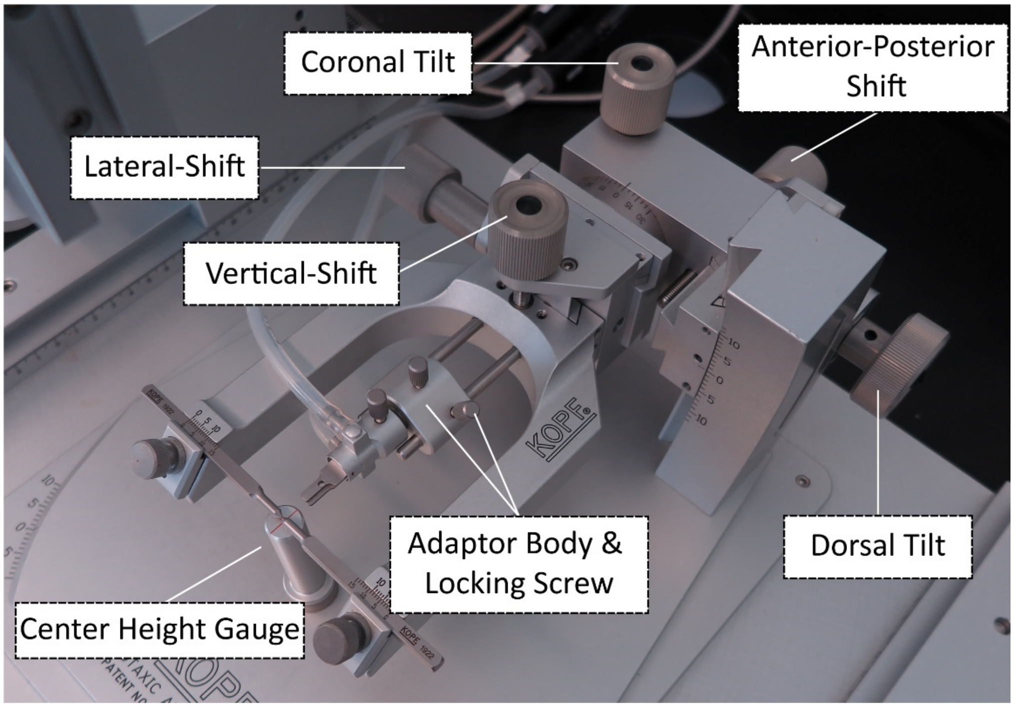


Figure 7.2 Adjustment knobs for the stereotactic head holder apparatus.

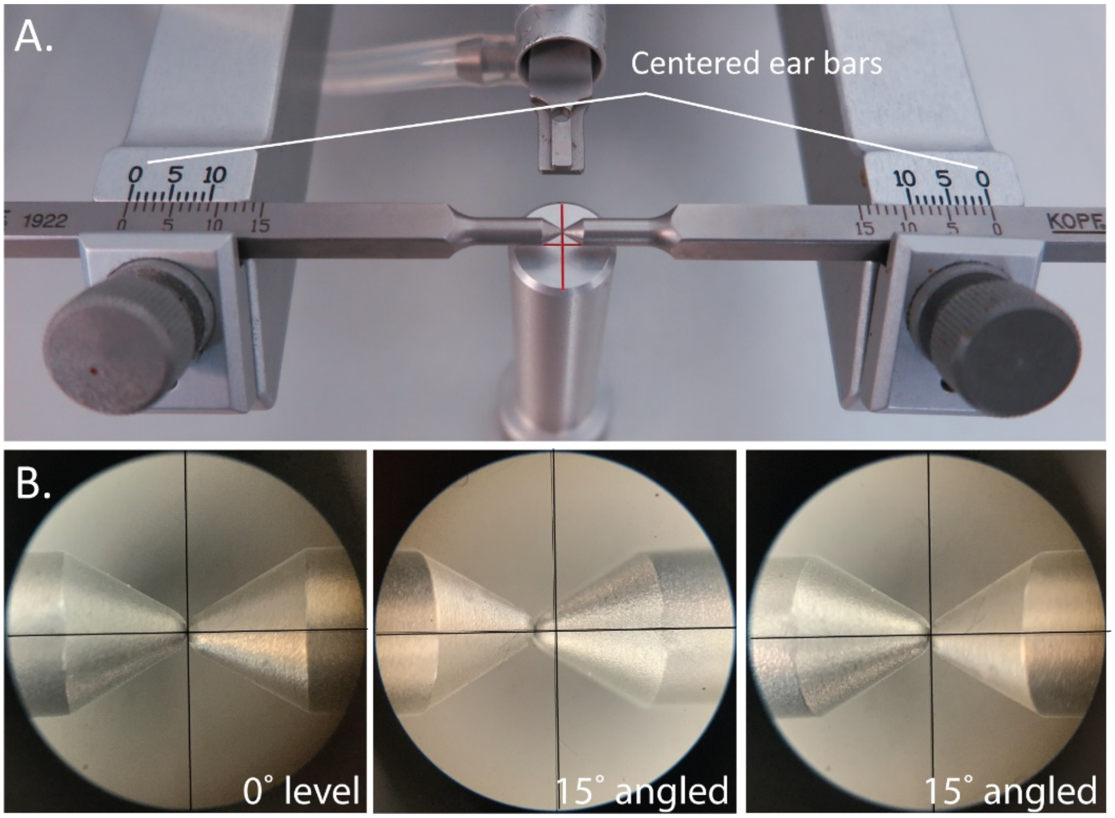


Figure 7.3 Aligning the head holder center of rotation.
A. Positioning the ear bars. B. Sighting down the scope during 0° level coronal rotation (left), during 15° rotation before adjusting the Vertical Shift and the center of rotation is misaligned (middle), and during 15° rotation after adjusting the Vertical Shift, and the center of rotation is properly aligned (right)

2.6. To align the ear bar position in the Z axis, remove the ear bars from the holder and remove the Center Height Gauge. Replace the ear bars and again center them at 0.

2.7. Sight down the scope. Use the Vertical Shift knob (Figure 7.2) and Coronal Tilt knob, respectively to lower and rotate the ear bars until the scope crosshairs remain centered between the ear bars throughout coronal rotation.

2.8. The stereotax is now calibrated and ready. **Do not make any further adjustments to the position of the head holder.**

3. **Prepare materials for injection/implantation.**

3.1. Ensure all instruments, surgical tools, and materials are sterilized and placed in a sterile surgical field next to the stereotax.

3.2. Handle and store viral constructs according to their biosafety level and recommended guidelines.

3.3. Draw up virus into the syringe, taking care to use proper handling practices and personal protective equipment.

4. Anesthesia.

4.1. Record body weight prior to surgery.

4.2. Deeply anesthetize mouse using isoflurane.

4.3. Ensure animal is deeply anesthetized by performing toe pinch test until the flinching response is absent. If the animal continues to show strong reflexes, increase the concentration and/or duration of anesthesia.

4.4. Shave the scalp from just behind the ears to just behind the eyes with a hair clipper.

4.5. Apply eye ointment to each eye to keep them moist during surgery.

4.6. Continuously monitor the animal throughout the surgical procedure and provide thermal support, if required.

5. Surgical Procedure.

5.1. Place the head into the head holder by placing the upper incisors into the gap in the bite bar, making sure that the tongue is below the bite bar.

5.2. Secure the head in the ear bars by gently inserting the ear bars into the external auditory meatus, taking care that the ear bars are symmetrically placed (typically between 3 and 4 for an adult mouse). This step is critical to ensure the head is stable and centered for rotation.

5.3. Sterilize the shaved incision area with 3 alternating scrubs of betadine and alcohol swabs.

5.4. Expose the skull by making an incision along the sagital midline of the scalp. Gently scrape the surface of the skull to remove any fascia and expose the sutures.

NOTE: If suture lines are difficult to visualize, hydrogen peroxide can be applied to the skull using a sterile cotton-tipped applicator to improve suture visualization.

5.5. Place the Centering Scope into the holder and center the crosshairs on bregma (Figure 7.4, left panel). Zero the micromanipulator.

5.6. Move the crosshairs caudally to lambda, noting the bregma-lambda (B-L) distance.

NOTE: If the suture lines do not follow a straight line along the midline, it is recommended that the midline should be established by the “line of best fit” through both bregma and lambda. However, if the above steps are followed, the initial placement of the scope reticle should be halfway between the ear bars and closely approximate the B-L midline suture.

5.7. If B-L distance is significantly less or greater than 4.21mm, incrementally adjust the assigned bregma to obtain a B-L distance of 4.21 +/- 0.2 mm.

5.8. Replace the centering scope with the Alignment Indicator. by Place the probes on lambda and bregma and adjust the Dorsal Tilt knob on the head holder to level in the sagital plane (nose-up or down); use the Centering Scope to reassign bregma.

5.9. Use the Alignment Indicator to level in the coronal plane using the Coronal Tilt knob. Measure at multiple points throughout the rostral/caudal axis to account for surface deformations in the skull.

5.10. Make sure to note the position on the dial of the coronal tilt knob – this is the 0° rotation position.

6. Aligning central axes of rotation for angled coordinate

6.1. Secure the Centering Scope in the tool holder and position the micromanipulator to the calculated coordinate from part 1. Note that the R/L coordinate for the angled implantation corresponds to the length of side A.

  6.1.1. In the example in Figure 1, the angled coordinates for fiberoptic placement targeting the VMN are (A/P: -1.4, R/L: [2.03] at 0° coronal rotation, [0.00] at 15° coronal rotation, D/V: -5.4.

6.2. Sighting down the scope and mark this coordinate (R/L 2.03mm from the midline per the VMN example; Figure 4, middle panel); this mark represents where the cannula will enter the brain once the head has been rotated.

6.3. Reposition the micromanipulator over the midline (R/L: 0.00). Use the Coronal Tilt knob to rotate the head to the angle calculated in part 1.

  6.3.1. If the scope crosshairs already line up with the mark, proceed to part 7.

  6.3.2. If the scope crosshairs do not line up with the reference mark, adjust the head position in the Z axis using the Vertical-Shift knob (Figure 7.2) until the crosshairs line up as close as possible to the mark.

6.4. Rotate the head back to the 0° coronal position; if the Vertical Shift was adjusted in step 6.3, reassign bregma using the Centering Scope.

6.5. Repeat steps 6.3 and 6.4 until the crosshairs consistently hit the reference mark when the head is rotated (Figure 7.4C).

6.6. At this point, the arbitrary point of rotation established in part 1 should now be aligned with the stereotactic center of rotation.

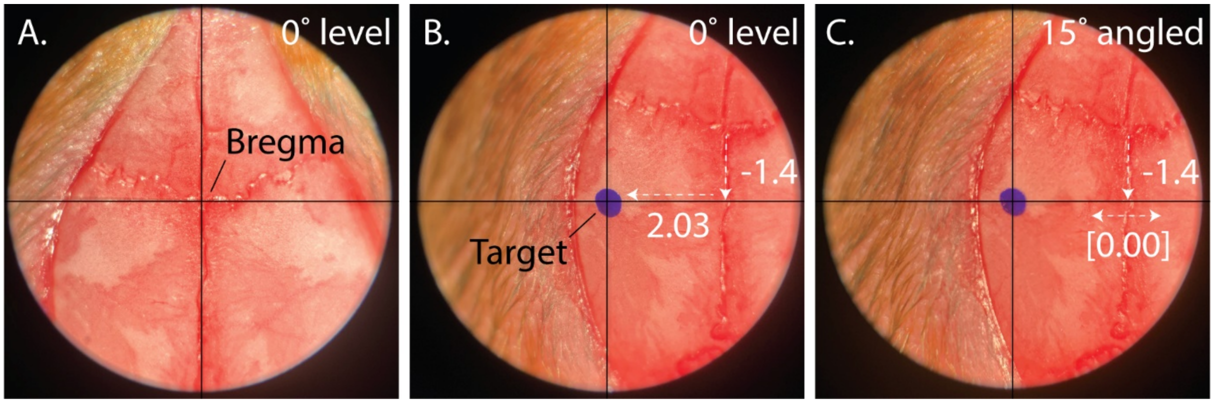


Figure 7.4 Assigning bregma and aligning the animal head with the central axes of rotation.
Representative image indicating typical bregma placement. B. Drawing a reference mark while head is level, before alignment. C. Properly aligned axis of rotation, after adjusting the Vertical Shift and readjusting bregma.

7. Microinjection

7.1. Place the Stereotactic Drill in the holder and maneuver the micromanipulator to the first injection coordinate.

7.1.1. Per the example for targeting the VMN, drill at A/P: -1.4, R/L: 0.4 while head is level.

7.2. Lower the drill until the bit is just above the skull. Turn on the drill, and gently lower until the bit has just drilled through the skull (not the dura).

7.3. Repeat for the contralateral injection site.

7.4. Gently poke through the dura mater using the tip of a sterile 0.5mL insulin syringe that has been bent to 90°.

NOTE: If bleeding occurs, apply pressure with a sterile cotton-tipped applicator and clean with sterile water until the bleeding has stopped.

7.5. When ready to inject, carefully place filled Hamilton syringe into stereotactic holder.

NOTE: The coordinates on the micromanipulator no longer apply after switching to a new tool. Use the center of the burr hole as the new target for injection.

7.6. Carefully position the needle above the burr hole.

7.7. Lower the needle until it just touches the dura within the center of the burr hole.

CRITICAL STEP: Zero the micromanipulator **only in the Z axis,** such that the coordinates on the micromanipulator for the stereotactic centering scope and drill are maintained.

7.8. Slowly lower the needle into the brain, watching closely to ensure the needle does not deflect on the edge of the burr hole. Continue to lower until 0.05mm ventral to the D/V injection coordinate and wait 1 minute; this extra step creates a small “pocket” to minimize viral backflow on needle removal.

7.9. Slowly raise the needle to the D/V coordinate and start the injection.

NOTE: Flow rate and volume will vary depending on target region and experimental design. For optogenetic silencing of the VMN neurons, sufficient coverage was desired, and so 200nL virus was injected at a rate of 1nL/s.

7.10. Following microinjection, wait 10 minutes at the injection site to minimize efflux of virus during withdrawal.

7.11. Slowly withdraw the micropipette from the brain at an approximate rate of 1mm/minute.

7.12. Once the needle is clear of the skull, eject a small volume of virus to ensure the needle has not clogged with blood or tissue. Use a sterile cotton-tipped applicator to remove the virus before continuing.

7.13. Repeat steps 7.6 – 7.12 for the contralateral side.

7.14. Seal the microinjection burr holes with bone wax to improve healing (Figure 7.5B).

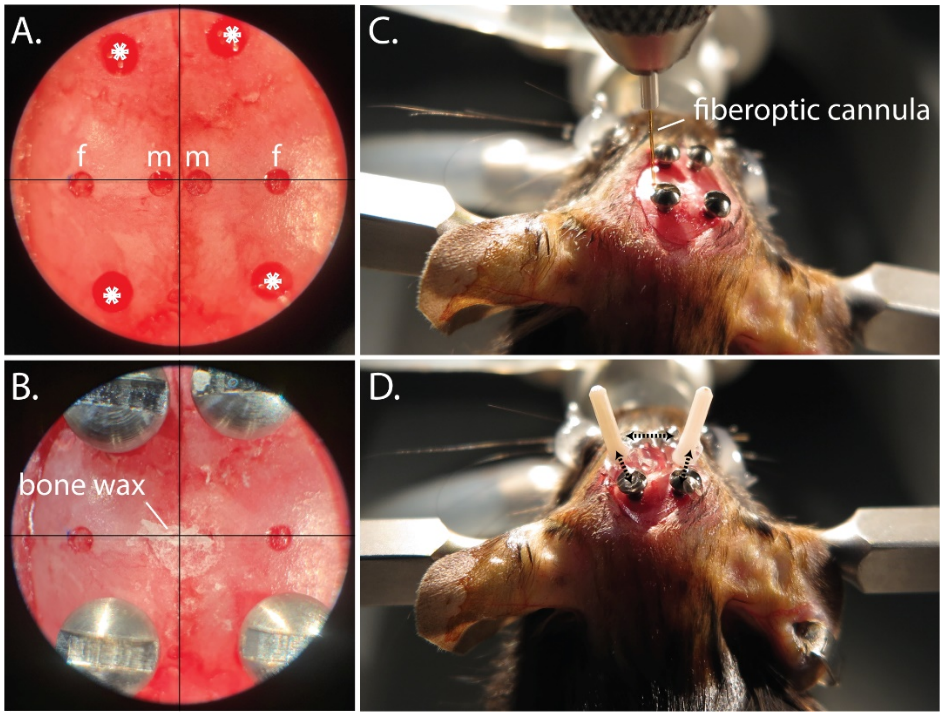


Figure 7.5 Fiberoptic implantation procedure.
A. Centering scope view of pilot holes for microinjection (m), fiberoptic (f), and anchor screws (*). B. Centering scope view of implanted anchor screws, and bone wax covered microinjection drill holes. C. Positioning the fiberoptic into place during angled implantation. D. Representative bilateral angled fiberoptic placement; dotted black arrows indicate areas where super glue is used to anchor the fiberoptic to the anchor screws and to the ipsilateral fiberoptic.

8. Fiberoptic implantation

After viral injection, bilateral fiberoptic cannulas are implanted at the calculated angle per **part 1**. Note that these coordinates should already be marked on the skull from **part 6**.

8.1. Follow steps 7.1 – 7.4 as described above for the angled coordinates.

8.2. Return the head to the level 0° position.

8.3. Next, use the hand drill to make 4 additional holes for the bone screws: 2 should be placed anteriorly, and 2 posteriorly (Figure 7.5A). These will serve as anchors to affix the fiberoptics to the skull (Figure 7.5D).

NOTE: Make sure to space these far enough away from the angled coordinate burr holes to accommodate the ferrule portion of the fiberoptic that sits above the skull.

8.4. As gently as possible, use the small flathead screwdriver to set the bone screws such that they sit firmly in the skull but do not penetrate into the brain.

8.5. Clamp a fiberoptic cannula into the cannula holder and place into the stereotactic holder.

8.6. Rotate the head to the calculated angle, noting again that the coordinates on the micromanipulator do not apply to the new tool; use the center of the angled burr holes as the implantation target.

8.7. Lower the fiberoptic until it just touches the dura within the center of the burr hole (Figure 7.5C). Zero the micromanipulator in the Z axis, then slowly slower the fiberoptic to the D/V angled coordinate (-5.4 per the VMN example).

8.8. Use cyanoacrylate gel to connect the fiberoptic ferrule to the ipsilateral anchor screws, then apply an accelerant with a micropipette tip (Figure 7.5D).

8.9. Once the cyanoacrylate gel has completely hardened, gently loosen the cannula holder and raise until clear of the fiberoptic ferrule.

8.10. Repeat steps 8.5 – 8.9 for the contralateral angled coordinate, then level the head. For extra security, make an additional connection between the two angled fiberoptic cannulas with the cyanoacrylate gel and accelerant (Figure 7.5D).

8.11. Prepare a small, relatively thin, amount of dental cement. Apply to the surface of the skull, making sure to thoroughly cover the anchor screws and the base of the fiberoptic cannulas but leaving enough of the ferrule clean for subsequent mating with the fiberoptic patch cables.

8.12. Once the cement is completed dry, remove mouse from the stereotactic apparatus.

8.13. Inject mouse subcutaneously with analgesic (buprenorphine; 0.5mg/kg)

8.14. Place mouse in recovery cage with thermal support. Allow animal to recover and transfer them to their home cage once they appear alert, mobile and are grooming.

9. Post-surgical Care

9.1. Monitor animals daily for three days post-operatively for both behavior, posture, activity and grooming and keep records of food intake and body weight.

9.2. If animals exhibit any general indicators of pain or poor health, consult with Veterinarian Services.

9.3. Allow mice at least 2 weeks for recovery and for viral expression before starting behavioral studies.

10. Optogenetics studies

10.1. For the performance of optogenetics studies, please refer to *Sidor et al., 2015*(Sidor *et al.*, 2015).

10.2. Validate viral expression and fiber placement at completion of studies.

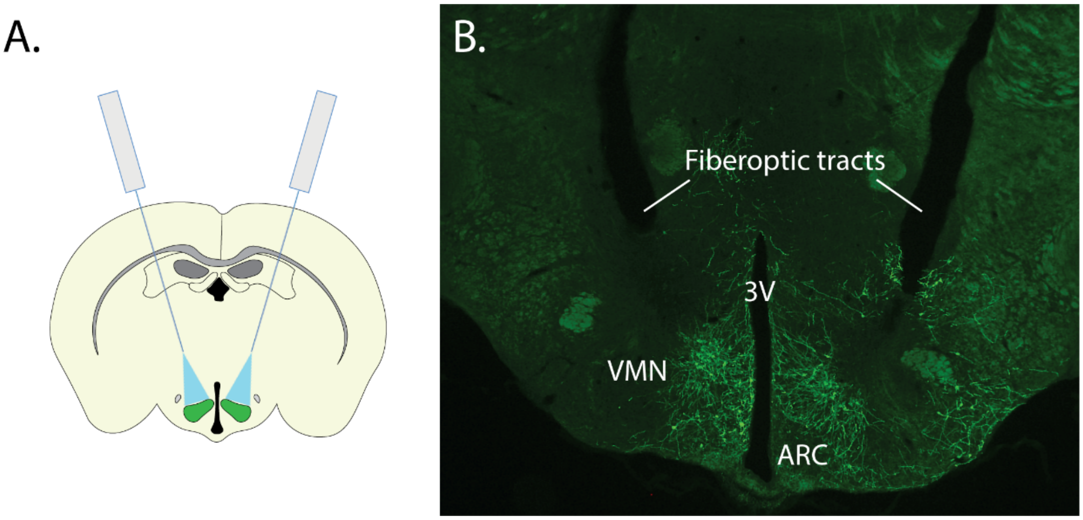


Figure 7.6 Representative results for bilateral targeting of the ventromedial hypothalamus.
A. Schematic representing bilateral microinjection and angled fiberoptic strategy for targeting the VMN. B. Representative image showing bilateral expression of SwiChR-GFP and tissue damage from angled fiberoptic tracts. 3V: third ventricle, ARC: arcuate nucleus, VMN: ventromedial nucleus.

Representative Results

In this example, we describe the surgical procedure for performing optogenetics studies to interrogate the role of hypothalamic VMN neurons in glycemic control (Faber *et al.*, 2018). In this study, we first utilized a standard (non-angled), stereotactic approach for the bilateral microinjection of an inhibitory channelrhodopsin virus to the VMN. While an angled approach would also be suitable, the standard (non-angled) approach was selected given that it was sufficient to target the brain region of interest and is an easy, reliable and consistent approach. However, given the VMN's proximity to the midline, space constraints did not permit the non-angled implantation of bilateral fiberoptics, necessitating the development of a surgical strategy for precisely implanting fiberoptics at an angle (see Figure 7.6). Using this surgical strategy, we microinjected a cre-dependent AAV expressing a modified channelrhodopsin anion-conducting channel fused with the fluorescent reporter, referred to as a ”SwiChR++” virus(Berndt *et al.*, 2016) bilaterally to the VMN of Nos1-cre mice, followed by implantation of an optic fiber dorsolateral to each injection site at a 15° angle from the midline. As expected, viral expression was restricted to the VMN and not detected in other brain areas.

Discussion

Recent advances in neuroscience have supported advanced insight and understanding into the activity and function of brain neurocircuits. This includes the application of optogenetic and chemogenetic technologies to activate or silence discrete neuronal populations and/or their projection sites *in vivo* and more recently, the development of genetically-encoded calcium indicators (e.g. GCaMP, RCaMP) and other fluorometric biosensors (e.g. dopamine, norepinephrine) for in vivo recording of neuronal activity in a defined cell type in freely moving animals. Effective employment of these technologies, however, relies upon successful stereotactic surgery to target the region of interest. While there are several established protocols describing these procedures and are suitable for targeting many different brain regions, targeting deep brain regions along the midline represents significant additional challenges. Here we demonstrate a detailed surgical technique for targeting discrete brain regions via an angled stereotactic approach. Importantly, this technique can be adapted and applied to a diverse range of neuroscience techniques, including optogenetics, chemogenetics, and fiber photometry approaches.

Using this approach, we showed that acute optogenetic silencing of VMN neurons expressing neuronal nitric oxide synthase (VMN^{NOS1} neurons) blunts glucagon responses to insulin-induced hypoglycemia in mice (Faber *et al.*, 2018). Using a slightly modified approach, we further demonstrated that unilateral activation of VMN^{NOS1} neurons is 1) elicits robust hyperglycemia that is driven by counterregulatory responses that are normally reserved for the response to hypoglycemia and 2) defensive immobility behavior. Furthermore, these behavioral and metabolic responses involve neuronal projections to distinct brain areas such that activation of VMN^{NOS1} neurons that project to the anterior bed nucleus of the stria terminalis are involved in glycemic responses whereas VMN^{NOS1} neurons that project to the periaqueductal gray are linked to fear-induced behavior responses (Faber *et al.*, 2018).

We note that the protocol described is highly specific to the Kopf Model 1900 stereotax and its accompanying accessories. While this system enables precise, reproducible implantation and/or microinjection to discrete brain regions, with common centerline position across multiple tools, the strategy and approach can be adapted to suit other stereotaxic frames. Specifically, instead of rotating the head to perform angled microinjections and implantations, an alternative approach is to utilize the same principles and rotate the dorsal-ventral manipulator instead (see *Correia et al.*(Correia, Matias and Mainen, 2017)).

As with any new method, it is critical for each individual to optimize his/her technique to improve their reliability, consistency, and accuracy. In addition, it is important to include the necessary appropriate controls for proper analysis and interpretation of data. These include the use of Cre-negative littermate controls, viral reporter controls (i.e. AAV-GFP), verification of light-dependent neuronal firing modulation using electrophysiology and, upon study completion validation of viral targeting and fiberoptic placement in the region of interest. Please refer to *Cardozo & Lammel* (Cardozo Pinto and Lammel, 2019) for a detailed review of technical considerations and suggested controls

In summary, the advent of more advanced and precise neuroscience techniques has supported a significant advancement and understanding of the role of the brain in behavior, cognition and physiology, and these advancements may one-day lead to potential therapies for CNS-related disorders.

Chapter 8 Summary of Findings, Limitations, and Future Directions

The rising prevalence of obesity and obesity-associated pathologies presents a major health concern. In particular, obesity is highly associated with the development of type 2 diabetes (T2D). T2D is further associated with a host of serious complications such as retinopathy, neuropathy, limb amputation, cardiovascular disease, and premature death (Smyth and Heron, 2006). Current treatment options for diabetes to improve glycemic control include lifestyle and dietary interventions, as well as pharmaceutical treatments (Smyth and Heron, 2006). Although tight glycemic control reduces the risk of diabetes complications, it also increases the risk of hypoglycemia, especially among individuals treated with insulin (Diabetes Control and Complications Trial Research Group, 1993; The Diabetes Control and Complications Trial Research Group, 1997). Among the most concerning clinical disorders associated with hypoglycemia in patients with diabetes is the impaired ability to mount counterregulatory responses (CRRs) that can result from recurrent bouts of hypoglycemia. Termed "hypoglycemia-associated autonomic failure" (HAAF), this condition increases risk for severe, prolonged bouts of hypoglycemia because of the inability to return low blood glucose levels into the normal range (Cryer, 2013). Thus, a compelling need exists for an improved understanding of diabetes pathogenesis and complications that can inform new strategies for its treatment.

A role for VMN^{NOS1} neurons in counterregulatory responses and defensive behaviors

I began my thesis work with the specific goals of expanding our knowledge of the role of the hypothalamic ventromedial nucleus (VMN) in glucose homeostasis by identifying i) the subsets of VMN neurons required for intact CRRs, and ii) the neurocircuitry by which CRRs are driven. While previous work identified the VMN as a key brain region in the establishment of CRRs, the VMN is a highly heterogeneous region implicated in not just glycemic control, but also other aspects of energy balance including feeding (Xu *et al.*, 2003; Sternson, Shepherd and Friedman, 2005), energy expenditure (Bingham *et al.*, 2008), and thermogenesis (Perkins *et al.*, 1981; Kim *et al.*, 2011). Moreover, the VMN is implicated in social (Lee *et al.*, 2014) and defensive behaviors (Falkner *et al.*, 2014; Kunwar *et al.*, 2015). We hypothesized that these divergent responses involved i) discrete neuronal subsets within the VMN and/or ii) projections to discrete downstream brain regions.

Previous work in our lab had begun to interrogate this hypothesis by characterizing the functional role of VMN neurons expressing steroidogenic factor 1 (VMN^{SF1} neurons). While this work established a key role for VMN^{SF1} neurons in driving counterregulatory responses (CRRs) during insulin-induced hypoglycemia (IIH) (Meek *et al.*, 2016), defensive behaviors (i.e., activity bursts and freezing) are consistently observed during activation of these neurons, as well (Kunwar *et al.*, 2015). We thus sought to extend our work with the goal of identifying the subset of VMN^{SF1} neurons that selectively mediated glycemic effects. Based on a variety of evidence generated by Dr. Vanessa Routh's research group (see Chapter 4: Introduction), we hypothesized that those VMN neurons expressing neuronal nitric oxide synthase 1 (NOS1) were a promising candidate population for selective activation of glycemic effects.

In partial support of this hypothesis, my findings indicate that activation of

VMN^{NOS1} neurons is i) sufficient to produce CRRs, including increased glucagon and corticosterone secretion and suppression of GSIS, and ii) required for an intact glucagon, but not corticosterone, response to IIH. Although sympathoadrenal output is an important component of CRRs to hypoglycemia as well, due to technical limitations we were unable to measure epinephrine in our studies. Thus, the extent to which selective activation of VMN^{NOS1} neurons is required for sympathoadrenal responses during IIH remains unknown.

While previous work has suggested VMN nitric oxide (NO) production by NOS1 is important for epinephrine responses to IIH (Fioramonti *et al.*, 2010), there are several technical considerations when comparing the methodologies used. Firstly, studies addressing the role of NOS1 in CRR generation relied on i) pharmacological NOS inhibitors and ii) genetic mouse models of complete neuronal NOS1 deletion. Regarding the former, pharmacological NOS inhibitors are inherently "dirty," as they are not specific to the neuronal NOS isoform, but also targeted endothelial NOS (Fioramonti *et al.*, 2010). In addition, the administration of NOS inhibitors targeted not just the VMN, but also the adjacent arcuate nucleus (ARC). Thus, different effects could be attributed to alternate NOS isoforms, or to an effect in the neighboring ARC. The tenetic mouse models utilized are also problematic, as they lack cell-type, or even brain region, specificity in their pan-neural NOS1 deletion (Fioramonti *et al.*, 2010). By comparison, our optogenetic strategy selectively targeted NOS1 neurons within the VMN, but not the ARC, and enabled rapid and reversible modulation of neuronal firing. Nonetheless, evidence still supports an important role for nitric oxide (NO) production in CRRs, and it is unclear the extent to which the photoinhibition paradigm used in our studies would affect NOS1 function itself, if at all.

Photoinhibition is expected to reliably blunt action potential firing (APF) and neurotransmitter release in VMN^{NOS1} neurons via the opening of light-sensitive chloride channels (Berndt *et al.*, 2016). By comparison, the activation of NOS1 depends upon phosphorylation by AMP-activated protein kinase (AMPK), and thus is indirectly regulated by intracellular AMP:ATP levels, which are further subject to modulation by glucose, leptin, and insulin signaling cascades (Canabal *et al.*, 2007; Fioramonti *et al.*, 2011a). In response to decreased glucose, AMPK activation in glucose-inhibited (GI) neurons promotes both i) increased APF via closure of membrane chloride channels (Murphy *et al.*, 2009), and ii) NO production (Canabal *et al.*, 2007), which is proposed to further amplify AMPK activation through the binding of soluble guanylyl cyclase (sGC) (Fioramonti *et al.*, 2011a), and, upon diffusion from the cell, may have signaling effects in adjacent glia and endothelial cells (Canabal *et al.*, 2007; Fioramonti *et al.*, 2011b). Thus, while pharmacological inhibition of NOS1 may be less precise, it results in both reduced NO production and APF. In contrast, photoinhibition may only reduce APF but leave AMPK:NO signaling intact, such that NO-dependent effects on CRRs could persist.

While this possibility has not been directly tested, such an idea would provide additional explanation of differences in outcome between photoinhibition of VMN^{NOS1} neurons as opposed to pharmacological NOS1 inhibition. Nonetheless, optogenetics remains a powerful tool to visualize and stimulate the projection sites of the opsin-expressing target cell, enabling functional, projection-specific interrogation of local circuitry.

Indeed, this strategy was particularly useful in clarifying how VMN^{NOS1} activation drove the disparate defensive behaviors coincident with CRRs. Firstly, ChR2-eYFP labeled cell bodies and axon terminals provide visualization of the brain regions contacted by VMN^{NOS1} neurons, and thus, potential efferent drivers of the counterregulatory and freezing behaviors elicited with upstream cell body stimulation. The projection patterns of VMN^{NOS1} neurons largely recapitulated those we reported for VMN^{SF1} neurons, with the most densely labeled fibers appearing in both the anterior bed nucleus of the stria terminalis (aBNST) and the periaqueductal gray (PAG). Independent illumination of PAG or aBNST projections resulted in a clear delegation of freezing behaviors to the PAG pathway, while CRRs without freezing were driven by projections to the aBNST. Notably, VMN^{NOS1}→PAG stimulation was also associated with components of CRR activation, including elevated corticosterone (reflecting HPA axis activation), hyperglycemia, and suppression of GSIS, but increased glucagon secretion, characteristically attributed to activation of the parasympathetic outflow to the islet (Taborsky and Mundinger, 2012), was absent. That freezing and glycemic effects both occurred upon VMN^{NOS1} → PAG stimulation indicates that either i) an unidentified subset of VMN^{NOS1} neurons projecting to the PAG initiates defensive behaviors, while another produces glycemic effects, or ii) hyperglycemia, presumably by HPA activation, is intrinsically linked to defensive behaviors.

The latter hypothesis makes sense from a teleological perspective, as situations in which fight or flight responses are activated (e.g., in response to a predator or intruder) would necessitate mobilizing glucose to fuel the tissues driving these behaviors, while diverting fuel away from nonessential behaviors (e.g., feeding or reproduction). Consistent with such a hypothesis, evidence from David Anderson's group indicated that stimulation of VMN^{SF1} neurons, in addition to driving defensive behaviors, interrupted ongoing consummatory behaviors (Kunwar *et al.*, 2015). While this report did not include assessment of glycemic effects associated with VMN^{SF1} activation, the findings, in combination with our own, establish the VMN as a critical node in driving defensive behaviors, while simultaneously mobilizing fuel to meet this demand and reducing the drive for nonessential consummatory behaviors.

However, we are still limited in our understanding as to which specific cell types are associated with these divergent effects. Nonetheless, some clarification is gleaned by comparing the outcome of NOS1 vs SF1 stimulation. Key differences include observations that i) whereas SF1 activation is associated with *both* freezing and activity burst behaviors (Kunwar *et al.*, 2015), NOS1 activation selectively induced freezing, with minimal hyperactivity observed (Faber *et al.*, 2018), and ii) activation of SF1 neurons was require for both corticosterone and glucagon secretion during IIH, while NOS1 activation was required only for intact glucagon responses. Collectively, these findings suggest a more specific role for VMN^{NOS1} neurons in aspects of counterregulation and freezing behaviors. Moving forward, it is important for additional studies to investigate the physiological context in which these neurons are activated, for instance, using in vivo calcium imaging or electrophysiology approaches.

Leptin signaling in the LPBN is required to normalize diabetic hyperglucagonemia

We were intrigued by the observation that the same neuroendocrine and autonomic patterns engaged during hypoglycemia are also elevated in rodent models of energy deficiency and in diabetic hyperglycemia. Based on this reasoning, we next shifted our attention to the role for brain regions upstream this VMN counterregulatory circuit. Recent work had identified a leptin-sensitive neuronal population within the lateral parabrachial nucleus (LPBN^{LepR} neurons) that i) projects to SF1 neurons, ii) is required for intact CRRs during hypoglycemia, and iii) is under tonic inhibition by leptin (Garfield *et al.*, 2014; Flak *et al.*, 2015). We reasoned, in the context of uncontrolled diabetes, hyperglycemia and hyperglucagonemia may be driven, in part, by a failure of leptin to suppress activation of this LPBN^{LepR}→VMN neurocircuit, thus aberrantly driving tonic glucagon and corticosterone secretion. To explore this hypothesis, we studied STZ-diabetic mice in which leptin receptor was deleted from LPBN neurons. In these mice, we found that icv leptin was unable to fully normalize hyperglucagonemia, but normoglycemia was restored. Conversely, leptin administration directly to the PBN was sufficient to reduce hyperglucagonemia and ketosis, but unable to normalize glycemia fully. Taken together, these findings provide partial support for our hypothesis that pathological overactivity of defined neurocircuits may contribute to the diabetic phenotype, and suggest that leptin likely mediates its effects in rodent models of T1D through a distributed network of leptin-responsive neurocircuits.

Is the brain a key player in glucose homeostasis? Hints from the arcuate nucleus.

Although the brain is clearly capable of affecting blood glucose levels, the extent to which it participates in day-to-day regulation of glucose levels remains controversial. However, accumulating evidence suggests that interventions targeting the brain can normalize glycemia in a variety of rodent models of diabetes. A powerful example of this is the diabetes remission elicited by icv leptin administration (Fujikawa *et al.*, 2010; German *et* *al.*, 2011). Central melanocortin signaling is required for the antidiabetic effects of icv leptin, as pretreatment with a MC3R/MC4R antagonist blocks glucose-lowering by leptin (Da Silva *et al.*, 2009; Meek *et al.*, 2014). However, pharmacological activation of central melanocortin signaling is not sufficient to ameliorate diabetic hyperglycemia in streptozotocin-induced diabetic (STZ-DM) rodents, presumably due to the combative antagonist effects of elevated AgRP levels on melanocortin signaling observed in this model, masking the otherwise beneficial effects of increased melanocortin signaling (Meek *et al.*, 2014). Indeed, a key effect of central leptin administration in STZ-DM is a reduction in *Agrp* expression, as well as increased *Pomc* expression (Fujikawa *et al.*, 2010), which likely contributes to the leptin-induced normalization of STZ-DM hyperphagia in these models, as well.

Furthermore, icv leptin fails to reduce blood sugars in mice with selective deletion of LepR from AgRP neurons (Xu *et al.*, 2018b), suggesting that AgRP neurons may contribute to diabetic hyperglycemia. Additional support for this hypothesis includes the observations that i) STZ-DM rodents exhibit elevated AgRP neuronal activity, chemogenetic inhibition of which robustly lowers (but does not completely normalize) blood glucose levels (Xu *et al.*, 2018b), and ii) acute activation of AgRP neurons induces peripheral insulin resistance (Steculorum *et al.*, 2016).

Moreover, recent evidence generated in our own lab also suggests an important role for AgRP neurons and melanocortin signaling in glucose homeostasis. Specifically, our lab reported that a single icv dose of the peptide fibroblast growth factor 1 (FGF1) induced sustained diabetes remission in multiple rodent models of diabetes, including both *ob/ob* and *db/db* mice, diabetic diet-induced obese (DIO) mice, and Zucker diabetic fatty (ZDF) rats (Scarlett *et al.*, 2016). This effect of FGF1 appears to require an action in the arcuate nucleus-median eminence (ARC-ME) (Brown *et al.*, 2019), and is mediated, in part, by preservation of β cell function (Scarlett *et al.*, 2019). Furthermore, recent unpublished evidence from our group also supports a key role for AgRP neuronal suppression and melanocortin signaling in the antidiabetic effects of FGF1, as i) AgRP neurons are rapidly inhibited by FGF1 in slice culture, ii) single-cell transcriptomic analysis shows a rapid and robust suppression of AgRP neurons that is sustained for 42 days, and iii) genetic or pharmacological disruption of melanocortin signaling precludes FGF1 from inducing diabetes remission (Bentsen *et al*., in review – see CV).

However, a role for the melanocortin system in glycemia cannot be examined in isolation, as these same circuits, and indeed, often the same neuronal subsets, have additional roles in the regulation of feeding (see Chapter 2: Central Regulation of Feeding). Indeed, in humans, MC4R variants are associated with both obesity (Speliotes *et al.*, 2010) and T2D, even after correcting for BMI (Xi *et al.*, 2012). Taken together, these observations provide a strong rationale for studies to identify the afferent input regulating AgRP neuronal activity, with the ultimate goal of determining whether disruptions in this circuit contributes to T2D and/or obesity.

A role for the dorsomedial hypothalamus in circadian feeding behaviors

Recent findings demonstrated, somewhat paradoxically, that despite the predominant output of AgRP activation to increase feeding (Aponte, Atasoy and Sternson, 2011; Krashes, Koda, Chianping Ye, *et al.*, 2011), AgRP neurons are rapidly *inhibited* upon the sensory detection of nutrient-related cues (Chen *et al.*, 2015). These findings suggest that, in addition to the slower input provided AgRP neurons by homeostatic feedback signals of energy status (i.e., leptin, ghrelin), AgRP neurons are also affected by fast, anticipatory feed-forward cues. Such a system is proposed to allow meal-to-meal regulation of feeding in accordance with how much energy is available, and how much is needed to maintain energy balance. While very little is understood regarding how this rapid inhibition of AgRP neurons is achieved, recent work has identified one candidate neuronal population in the dorsomedial hypothalamus that is marked by expression of LepR (DMH^{LepR} neurons) (Garfield *et al.*, 2016). DMH^{LepR} neurons in the ventral compartment are GABAergic, and selectively provide inhibitory tone to AgRP, but not POMC, neurons (Garfield *et al.*, 2016), such that photoactivation of DMH^{LepR} neurons acutely inhibits AgRP neurons and reduces food intake (Garfield *et al.*, 2016). Furthermore, food-related cues rapidly activate DMH^{LepR} neurons (Garfield *et al.*, 2016), suggesting that activation of this circuit may mediate the rapid inhibition of downstream AgRP neurons. However, whether or not DMH^{LepR} neurons play a physiological role in feeding behaviors is unknown.

In Chapter 6, I addressed this question by chronically silencing DMH^{LepR} neurons and assessing the impact on feeding behaviors and glycemia. We hypothesized that, based on the evidence for an inhibitory DMH^{LepR} → AgRP neurocircuit, inactivation of DMH^{LepR} neurons would disinhibit AgRP neurons and promote hyperphagia and weight gain. In support of this hypothesis, we observed an acute, transient hyperphagia and rapid fat accumulation and body weight gain in response to DMH^{LepR} inactivation. While we have not yet assessed the direct contribution of AgRP neurons to these effects, the additional effects of DMH^{LepR} inhibition to cause rapid elevation in RER, coincident with fat accumulation, further point to a role for increased activation of AgRP neurons (Cavalcanti-de-Albuquerque *et al.*, 2019). Future studies are warranted to address the effects of DMH^{LepR} inhibition on AgRP neuronal activity.

We also observed a more slowly-emerging disruption in circadian rhythm of feeding behaviors. Mice with inactivated DMH^{LepR} neurons exhibit a significant increase in their light cycle food intake, relative to controls, and marked reduction in dark cycle activity. Interestingly, mice with DMH^{LepR} inhibition, when deprived of food during the "mistimed" light cycle, failed to increase their dark cycle intake to compensate. In fact, they began to lose body weight. It would be interesting to determine the extent to which adaptation to other TRF windows is affected, and whether the DMH is required for entrainment to scheduled feeding, an idea that has been highly controversial in the field of circadian rhythms.

Finally, our preliminary analysis failed to detect any defect in heat production or core body temperature in response to a cold challenge. This is somewhat surprising, given the literature describing increased thermogenesis and core temperature with leptin administration to the DMH. Nonetheless, the absence of any defect in thermoregulation in our studies indicate that DMH^{LepR} activation is not required for adaptive thermogenesis during cold exposure, although the possibility remains that an inadequate number of cells were targeted to observe an impairment. This possibility will be explored in future studies targeting selectively the dorsal LepR+ compartment of the DMH, which is hypothesized to be the region of the DMH important for thermoregulation (Shaun F. Morrison, 2016).

Although this body of work is preliminary in its conclusions, I have learned a great deal about the value of chasing unexpected findings. Indeed, this project seems to prompt more questions than it answers. Therefore, the story doesn't end here. If, as previously published (Garfield *et al.*, 2016), DMH^{LepR} neurons provide presynaptic inhibition of AgRP neurons during meal initiation, are they the only source of this input? What is the contribution of presynaptic inhibition on satiety, and indeed, in overall energy homeostasis? Moreover, what neural and/or humoral factors regulate the endogenous activity of DMH^{LepR} neurons? Presumably, their activity displays an endogenous circadian rhythm, but what establishes this rhythm? An intriguing possibility is that defective leptin action in DMH^{LepR} neurons contributes to the altered circadian feeding rhythms observed in high-fat diet (HFD) fed rodents. If such a role exists, we might predict that, just as female mice are protected from weight gain and circadian disruption during HFD feeding (Palmisano, Stafford and Pendergast, 2017), perhaps they will be similarly protected from the metabolic impairments arising from DMH^{LepR} inactivation.

Closing Remarks

Overall, my graduate studies have advanced our knowledge of several neurocircuits by which the brain regulates aspects of energy and glucose homeostasis. My findings from the early stage of my training support a specific role for VMN^{NOS1} neurons in the glucagon response to hypoglycemia, but also suggests that these neurons may play a more broad role in glucose mobilization during defensive behaviors. During the latter part of my training, my focus shifted to characterizing circuits regulating feeding behaviors, and my findings, while preliminary in mechanism, demonstrate a significant and novel role for DMH^{LepR} neurons in circadian feeding rhythms. Ultimately, these studies provide valuable insight into the potential mechanisms by which simple changes in feeding patterns shift animal metabolism, and may inform the development of therapeutic or dietary interventions for the prevention and treatment of metabolic disease.

Chapter 9 Curriculum Vitae

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Areas of Expertise:

Sterile rodent surgery
- Advanced stereotaxic surgeries
- Osmotic minipumps

In vivo optogenetics and chemogenetics

Fiber photometry

Metabolic phenotyping
- Glucose/insulin tolerance tests
- Indirect calorimetry

Molecular biology
- Western blot
- ELISA
- qPCR

Immunohistochemistry

Microscopy

Scientific writing

Peer review

OBJECTIVE

To establish an independent scientific program exploring CNS regulation of metabolism and energy balance.

EDUCATION

University of Washington

Sep 2015 - present
PhD in Molecular Medicine and Mechanisms of Disease (M3D)
Thesis: ***Identification of neural circuits regulating glucose homeostasis***
Supervisors: Dr. Gregory J. Morton and Dr. Michael W. Schwartz

The goals of my thesis work are to delineate neurocircuits that function to control blood glucose and to investigate their mechanisms of action. My work demonstrates that neurons within the hypothalamic ventromedial nucleus that express neuronal nitric oxide synthase (VMN^{NOS1} neurons) play a physiological role in mediating the glucagon response to insulin-induced hypoglycemia.

University of Michigan

Sep 2010 - May 2014
BS *cum laude* in Cellular and Molecular Biology

EXPERIENCE

Research Technician Associate | University of Michigan

July 2013 – May 2015
Supervisor: Dr. Martin M. Myers Jr.

During my undergraduate studies and the year that followed, I worked in the lab of Dr. Martin Myers. The goals of my studies here were to 1) map neuronal circuits involved in metabolism, particularly with regard to the hormone leptin and its receptor and 2) discern the cellular mechanisms of leptin action, including its involvement in the development of diabetes and other metabolic disorders.

Research Assistant | University of Michigan

Sep 2011 – July 2013
Supervisor: Dr. Peter Dempsey

I investigated the role of ADAM10 (a disintegrin and metalloprotease) in Notch signaling in the intestinal stem cells of mice. I largely did in vitro work here, using stem cell cultures to assess the effects of the loss of ADAM10 on crypt homeostasis.

HONORS AND AWARDS

Funding

- National Research Service Award (NIH 1F31DK113673-01A1)
- Molecular Medicine Training Grant (NIH T32 GM095421)
- Graduate School Top Scholar Award

Awards

- Keystone Symposia Scholarship
- Samuel and Althea Stroum Endowed Graduate Fellowship
- Brunzell Fellow Award for Best Poster Presentation, Diabetes Research Center, University of Washington
- James B. Angell Scholar, University of Michigan
- University Honors, University of Michigan

Presentations

- 2019: American Diabetes Association Scientific Sessions, Poster Presentation.
- 2018: Keystone Symposium, Poster Presentation.
- 2017: American Diabetes Association Scientific Sessions, Oral Presentation.

FIRST-AUTHORED PUBLICATIONS

Faber CL, Deem JD, Ogimoto K, Larsen S, Nelson JT, Doan T, Phan BA, Damian V, Kaiyala KJ, Lighton J, Schwartz MW, Morton GJ. Activation of dorsomedial hypothalamic LepR neurons is required for normal circadian feeding behavior. [in preparation]

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Faber CL and Morton GJ. Metabolic roles of orexigenic and anorexigenic neuropeptides. *Encyclopedia of Biological Chemistry, 3rd Edition.* 2020. [in press]

TEACHING EXPERIENCE

Speaker & Organizer | NRSA Grant Writing Workshop

Jan 2017 – present

Coordinated and taught annual four-part grant writing workshop for graduate students and post docs.

Teaching Assistant | PATH 550

Sep 2016 – Dec 2016

LEADERSHIP

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Dec 2017 – Dec 2019

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