TrkB NEURONS OF THE PARAVENTRICULAR HYPOTHALAMUS
SUPPRESS FEEDING THROUGH PROJECTIONS TO THE
PARABRACHIAL NUCLEUS

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By

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Obesity is the result of failed energy homeostasis, and is characterized by the progressive accumulation of body weight, with the majority of weight gain due to increased fat mass. The regulation of body weight through energy homeostasis is carried out primarily within the central nervous system. The brain responds to an excess or deficit in caloric availability, and corrects these imbalances by adjusting physiological processes that utilize calories and by influencing cognitive processes such as motivations, decision making, and behavior. However, the biological substrates that perform these corrective actions are susceptible to disruptions from genetic and environmental factors that can reduce their effectiveness. Within the brain, the paraventricular nucleus of the hypothalamus (PVH) is a region highly involved in the integration of energy signals and the coordination of corrective outputs. Here I show that tropomyosin receptor kinase B (TrkB), the receptor for brain-derived neurotrophic factor, is expressed by neurons of the PVH (PVH$^{TrkB}$ neurons). I also show that TrkB receptors within this nucleus are critical for the suppression of food intake. Using the Cre-Lox system, I demonstrate that the specific post-developmental deletion of $TrkB$ from the PVH produces severe hyperphagic obesity, but not reduced energy expenditure. Moreover, I show that this effect is mediated by the specific population of PVH$^{TrkB}$ neurons that project to the parabrachial nucleus (PBN) by selectively deleting TrkB only from this population, which recapitulated the hyperphagic obesity produced by whole PVH $TrkB$ deletion.
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CHAPTER ONE: INTRODUCTION
Obesity and Health Impact

Human obesity is defined as the state where the value for body mass index (BMI) is greater than or equal to 30, and is generally accompanied by the accumulation of excess fat stores. BMI is calculated by dividing a person’s weight in kilograms by the square of their height in meters. This index was created as a means of normalizing statistical data; and was based on the observation that body weight increases proportional to the square of height after adolescence, though this was nearly a century before modern obesity was significantly manifest within the population. As it was not within the scope of purpose for this metric at the time of its inception, BMI does not account for body composition and can therefore classify an otherwise healthy lean individual that is highly muscular as overweight or obese. Despite this limitation, it has been consistently demonstrated that, for the majority of humans, BMI is highly correlated with the development of debilitating secondary conditions such as type 2 diabetes and cardiovascular diseases, most prominently; but also osteoarthritis, cholelithiasis (gall stones), infertility, and certain forms of cancer (Willett 1999). Due in part to these secondary conditions, obese individuals lose approximately 6 to 10 years of their lifespan (Peeters 2003, Whitlock 2009); and can also suffer diminished quality of life, characterized by an increased incidence & severity of pain, and deficits in mood, motivation, mobility, and sexual function (Kolotkin 2001).

According to the Centers for Disease Control and Prevention (CDC), the incidence of obesity in the United States has been rising rapidly over the last three decades, from approximately 11.6% in 1990 to 30.1% in 2016 (CDC 2016). Obesity is also increasing at the global level, having “nearly tripled since 1975,” according to the World Health Organization (WHO 2018). These time frames are insufficient in length to be accounted for purely by genetic changes on a population level. Therefore, these changes are likely due to a combination of behavioral factors
such as diet and levels of physical activity (Bortolin 2017, Bray 1977, Gibbs 2013, Kwon 2013, Li 2016, Williams LM 2014); environmental influences such as socioeconomic status, gastrointestinal flora composition, and in utero exposure to the effects of maternal obesity (Al-Emrani 2013, Barlow 2015, Oken 2003, Portela 2015); and the intersection of these factors with genetic susceptibilities (Aguilera 2013, Horn 2015, Merritt 2018, Nelson 1999). Childhood obesity is also on the rise, having more than tripled since 1971 (Fryar 2014). In addition to carrying increased risks for the same secondary conditions as adults, obese children often suffer from psychological insults due to an increased incidence of teasing & bullying (Haflon 2013), and have a higher frequency of social isolation and low self-esteem (van Geel 2014, Griffiths 2015). The increasing prevalence of obesity has led to a parallel increase in obesity-related medical spending, primarily for the treatment of obesity-related illness rather than prevention, leading to a total of $147 billion in 2008 (Finkelstein EA 2009). Considering that overweight and obesity has become the second leading cause of preventable death in the United States (Danaei 2009), and has greatly surpassed under-nutrition as a global cause of death since 1990 (Lim 2012), efforts to understand the cause of this growing epidemic and discover methods for prevention and treatment are more crucial than ever.

In order to more effectively understand obesity, in addition to human studies, animal models of obesity are also utilized for determining the biological substrates that regulate body weight. Since obesity is a term defined using a human-specific metric, the generation of criteria for defining animal obesity has been elusive. However, obesity in mice can be loosely defined as significantly increased body weight compared to appropriate sex-matched littermate controls, where body weight is at least approximately double that of controls, and increased fat mass accounts for the majority of the overall increase in body weight.
Energy Homeostasis

Body weight in mammals is regulated through the process of energy homeostasis (Figure 1), which is mediated by a complex interconnected network of signal relays that balance energy intake and energy expenditure. These relays involve sensors that detect relevant changes in energy availability, and effectors that induce a homeostatic response intended to prevent significant deviations in body weight. Intermediate components of these relays are responsible for integrating sensor inputs and coordinating the appropriate effector outputs.

Low energy availability during conditions such as fasting leads to a reduction in energy expenditure, and activates sensors of orexigenic relays that promote hunger and feeding. Conversely, food ingestion and products of digestion lead to increased energy expenditure, and activate sensors of anorexigenic relays that suppress hunger, activate satiety, and terminate feeding. While these anti-parallel effects on energy intake and expenditure are coincident, they are generally performed by separate relays; though sometimes paired by sharing a particular triggering mechanism. However, the regulation of energy expenditure operates in relative co-dependence with that of thermoregulation, which continuously expends calories through thermogenesis to maintain body temperature within a range that is typically higher than the environment. Thus, thermoregulation prevents energy homeostasis from making drastic changes to caloric flux through thermogenesis, as compared to energy intake where feeding creates a large spike in caloric flux. As such, endogenous energy homeostasis effects on thermogenesis usually appear as modest, proportional responses to changes in energy availability. Nevertheless, even a modest decrease in energy expenditure due to aberrant regulation, not balanced by reduced energy intake, can accumulate over time into significant weight gain.
The sensing of energy availability is achieved by directly detecting the presence of food and nutrients in the gastrointestinal tract or bloodstream (Marty 2007, Williams 2016); indirectly detecting these measures through mediators such as the hormone insulin, which is secreted by the pancreas in proportion to glucose levels (Kleinridders 2014, Unger 1971); or by sensing the content of stored energy encoded by hormones such as leptin, which is secreted in proportion to adiposity (Maffei 1995). The activity of effectors is modulated by this information, resulting in homeostatic changes in behavior and metabolic expenditure. These effectors regulate the motivational states of hunger & satiety within the brain, and physiological processes in the periphery that consume metabolic fuel such as brown adipose tissue thermogenesis (Krashes 2014b, Rosenbaum & Leibel 2012, von Essen 2017).

The complexity of the pathway through the intermediate components of these relays varies greatly, from simple reflex loops to networks of distributed integration. This complexity provides numerous insertion points for other systems to coordinate the simultaneous use of effectors. As such, there is considerable overlap between energy homeostasis and other homeostatic systems such as thermoregulation, which also controls thermogenesis (Morrison and Nakamura 2011, Pitoni 2011); glucose homeostasis, for which novel glucose must be periodically supplied through food intake (Jouvet & Estall 2017); and the neuroendocrine hormone axes, some of which are partially within the energy homeostasis network such as the thyroid axis, while others simply coordinate with the network to carry out their co-dependent functions (Lechan & Fekete 2006, Schriock 1984, Seres 2004, Uehara 1998). Additionally, certain non-homeostatic systems can insert overriding control over relevant parts of the network. Hunger is strongly suppressed by acute stress (fight-or-flight response) (Jiang 2016), and thermogenesis is significantly increased in pyrexia (fever) as a defense mechanism against pathogens (Malvar 2011).
Energy homeostasis is not only complex due to the overlap and interplay between pathways, but also to the diversity and interactions of the genes required to carry out their functions. However, since individual genes can have variants of different effectiveness expressed within a population, and are also susceptible to loss-of-function mutations; this creates the opportunity for genetic pathologies to cause disruptions in energy homeostasis. Human and animal studies have established a homeostatic role for many genes by studying the effects of specific mutations, polymorphisms, and chromosomal deletions in obesity. Genes for which genetic alterations have shown the highest association with human obesity include the fat mass- and obesity-associated gene (FTO) (Meyre 2010, Rovite 2014), leptin and the leptin receptor (Clément 1998, Wabitsch 2015); the melanocortin 4 receptor (MC4R) (Rovite 2014, Yeo 1998), and more recently the genes for brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB) (Gray 2006, Mou 2015, Rios 2001, Yeo 2004, Yükekkaya 2016).
Figure 1: Whole body circuit of energy homeostasis.

Diagram depicting communication pathways between the brain and peripheral organs. Shown are the brain (top left), and specifically a cluster of nuclei in the hypothalamus within the brain (top middle) including the arcuate nucleus (ARC), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), lateral hypothalamic area (LH), and paraventricular nucleus (PVH). Collectively, these nuclei have output pathways that either positively or negatively regulate hunger and food intake (top right), and additional pathways that connect to and communicate with peripheral organs through the autonomic nervous system (bottom). These peripheral organs regulate glucose and lipid metabolism (liver, pancreas, skeletal muscle, and white adipose tissue [WAT]) or energy expenditure (brown adipose tissue [BAT]; via calorie consumption for heat production [thermogenesis]). The gastrointestinal tract (G.I. tract; bottom right) is responsible for the digestion of ingested food, but also signals the presence of food and the nutrient products of digestion. Collectively, these peripheral organs send hormonal and nutritional signals to the brain, completing the circuit.

[Adapted from: López 2013]
Central Nervous System Regulation of Energy Homeostasis

Between the sensors and effectors of energy homeostasis, the action of processing energy availability signals and coordinating outputs is predominantly executed within the central nervous system (CNS). However, the regions of the hypothalamus and dorsal vagal complex have been the most heavily implicated and studied, with a recently increased focus on circuits that connect with the parabrachial nucleus and nuclei of and related to the amygdala. Together, these regions have been shown to play a role in the regulation of energy intake and expenditure, with more complex regulation in the suppression of feeding through the entire duration of food ingestion and much of the inter-meal period.

Meal-Related Regulatory Mechanisms

Ingestion begins as food enters the mouth, where taste information is relayed through the gustatory pathway. Taste sensations travel from the tongue along the seventh cranial nerve (facial) to the rostral segment of the nucleus of the solitary tract (r-NTS). They are then transmitted to the “waist area” of the parabrachial nucleus (PBN); from which contralateral projections to the gustatory cortex in the insula provide taste perception, allowing food acceptance or rejection, and taste satiation; and bilateral projections to the gustatory region of the thalamus relay taste information to the striatum for taste learning (Norgren & Leonard 1973).

Bipolar neurons in the nodose ganglia of the vagus nerve extend axons that terminate as mechanosensitive receptors within the mucosal lining of the stomach. Once ingested material reaches the stomach, these receptors are stimulated by the stretching of the stomach lining, and the signals are transmitted to the NTS by separate axons on the opposite ends of these neurons (Williams 2016). In addition to these signals being used to slow gastric emptying and aid
digestion, activation of the NTS through this mechanism leads to a modest but transient satiety (Grill & Hayes 2009, Trapp and Cork 2015), though the subsequent circuitry remains to be characterized.

As partially digested food moves into the proximal intestine, enteroendocrine I-cells within the duodenum and proximal jejunum are activated by carbohydrates, proteins & amino acids, and most strongly by long-chain free fatty acids. Activated I-cells then secrete cholecystokinin (CCK), which activates CCK receptors expressed on the terminals of bipolar nodose ganglia neurons that innervate the proximal intestines (Nilaweera 2010). These vagal neurons transmit the signals to the NTS and area postrema (Lo 2012), leading to satiety. Though dietary fat is the strongest activator of satiety through this mechanism, I-cell activation appears to be saturable. In this case, increasing dietary fat content would produce diminishing returns in satiety as I-cell activation approaches this saturation. CCK-induced satiety is partially mediated by activation of Glucagon-like peptide-1 (GLP-1) neurons in the NTS, which project to the hypothalamus and promote satiety through a mechanism that is not fully understood (Grill & Hayes 2009, Rinaman 2007, Trapp & Cork 2015, Williams DL 2014).

As food moves further along the intestines, enteroendocrine L-cells residing primarily in the ileum & colon become activated and secrete peptide YY (PYY). This hormone circulates to the brain and antagonizes type-2 orexigenic neuropeptide Y (NPY) receptors (Y2Rs), notably within the mediobasal hypothalamus (Ueno 2008). The segment of intestine that secretes PYY is considerably longer than that of CCK, and thus the passage of food through this segment leads to a prolonged PYY-induced satiety between meals.
As macronutrients are broken down along all segments of the gastrointestinal tract, the resulting micronutrients are absorbed into the hepatic portal vein and taken directly to the liver. A majority of the incoming micronutrients are then absorbed by liver hepatocytes; which modify these nutrients to improve their transport and absorption by other tissues, store excess glucose and amino acids as glycogen, or utilize them for the production of other biological molecules such as cholesterol and bile acids. However, a portion of the incoming nutrients pass through the liver and into the bloodstream, possibly as a result of saturated hepatocyte absorption, as a sizeable increase in the levels of circulating glucose, amino acids, and fat-transporting chylomicrons can be detected shortly after a meal (Ahmed 1976). Increased blood glucose stimulates a proportional increase in insulin secretion, and both have been shown to produce centrally mediated effects on food intake. However, these effects are somewhat transient, as insulin also stimulates the movement of excess glucose out of the bloodstream. However, glucose-sensing neurons have been identified within the hypothalamus, two populations of which has been found in the arcuate nucleus that express either pro-opiomelanocortin (POMC) or interleukin-1 (IL-1) in the arcuate nucleus (Mizuno 2013, Parton 2007); and within the dorsal vagal complex nuclei of area postrema and the NTS (Adachi 1991, Ritter 2000). The regulation of food intake mediated by glucose-sensing neurons is not well characterized. However, it likely involves orexigenic neurons that stimulate hunger and are activated under fasting conditions when glucose levels are low, but are relatively silent in the fed state; and/or anorexigenic neurons that suppress hunger by exerting an inhibitory tone in the fed state when glucose levels are maintained within the normal physiological range. Insulin induces satiety via the mediobasal hypothalamus and dorsal vagal complex, though NPY-expressing neurons mediate the effect of insulin in the hypothalamus.
(Adachi 1991, Loh 2017). Following food ingestion, insulin levels do not fall as rapidly as glucose; thereby providing a somewhat extended satiety signal.

As glycogen stores approach saturation, further excess calories are stored as triglyceride fats within adipose tissue depots; and the brain senses the level of whole body adiposity via circulating levels of the adipose-derived hormone leptin. In the fed state, this hormone is secreted by adipocytes in proportion to the total amount of stored fat (Considine 1996, Lönnqvist 1997). Evidence suggests that leptin may also be secreted by fibroblasts, which share a common stem cell lineage with adipocytes (Glasow 2001, Lin 2011). Leptin receptors (LepRs) are expressed by white adipocytes (Priego 2009), fibroblasts (Glasow 2001), liver hepatocytes (Cohen 2005), cells of the pancreas including insulin-producing β cells (Morioka 2007), peripheral blood mononuclear cells (Tsiotra 2000), and a portion of brain vascular endothelial cells that form blood vessels (cerebrovascular cells), with tight junction that create the blood brain barrier (Bjørbaek 1998, Yuan 2018). LepRs are also expressed by neurons in many regions of the brain including the NTS (Garfield 2012), area postrema (Liberini 2016), granular layer of the cerebellum (Savioz 1997), Edinger-Westphal nucleus (Xu 2011) ventral tegmental area (Leshan 2010), hippocampus (Guo 2013), and the hypothalamic ventral premammillary nucleus (Donato 2011), lateral hypothalamic area (Leinninger 2009), dorsomedial nucleus (Zhang 2011), ventromedial nucleus (Dhillon 2006), arcuate nucleus (Balthasar 2004), and medial preoptic area (Donato 2011). These central LepRs are activated by circulating leptin that is transported across the blood brain barrier (Kastin 2000), likely mediated by LepR-expressing cerebrovascular cells (Banks 2004, Di Spiezieo 2018); or directly diffuses into the brain through special fenestrated capillaries within select regions of the brain including circumventricular organs and choroid plexus. Specific populations of LepR-expressing neurons in the hypothalamus and NTS have
been shown to suppress food intake and increase energy expenditure (Balthasar 2004, Dhillon 2006, Garfield 2012, Kanoski 2012, Leinninger 2009).

In addition to effects on food intake suppression, food ingestion also stimulates an increase in energy expenditure proportional to the caloric load, but with stronger responses to alcohol and protein than to fats and carbohydrates (Westerterp 2004). This “thermic effect of food” is the result of increased basal metabolism and thermogenesis due to the rapid increase in metabolic substrate. Additionally, leptin has been shown to promote energy expenditure (Fischer 2016), and leptin levels fluctuate in relation to the fed or fasted state (Kolaczynski 1996). Thus, leptin levels increases following food ingestion, potentially accounting for a portion of meal-related energy expenditure. Glucose-sensing neurons may also contribute to increased thermogenesis, as they directly detect meal-related increases in glucose; and certain brain regions known to regulate thermogenesis have been shown sensitive to glucose levels (Claret 2007).

**Fasting-Related Regulatory Mechanisms**

Once adequate time has passed following a meal, and all meal-related satiety pathways become sufficiently quiescent, orexigenic relays become active to promote hunger and provoke the next meal. Two populations of neurons have been shown to strongly evoke feeding, both residing within the hypothalamus: one in the arcuate nucleus that express agouti-related peptide (AgRP neurons), and one in the lateral hypothalamus that express orexin (LH\(^{Orexin}\) neurons). Optogenetic and pharmacogenetic activation of AgRP neurons has been shown to produce rapid, voracious feeding in sated animals (Aponte 2011, Betley 2013, Krashes 2014a). However, this population of neurons has also been shown to reduce energy expenditure by suppressing the browning of white adipose tissue (Dodd 2017, Ruan 2014). AgRP neurons are directly activated by the
orexigenic hormone ghrelin, which generally exhibits a circadian secretion pattern from the stomach (Wang 2013); dopamine, which may originate from dopamine neurons also residing within the arcuate nucleus (Zhang & van den Pol 2016); and several recently-identified glutamatergic projections from other hypothalamic nuclei including the dorsomedial nucleus (DMH) and paraventricular nucleus (PVH) (Krashes 2014b). AgRP neurons are inhibited by leptin (Baver 2014), PYY (Batterham 2002), and by GABAergic projections from the DMH and from other arcuate neurons (Garfield 2016). LH$_{Orexin}$ neurons, however, have not been as extensively studied. Despite this, activation of these neurons has been shown to also strongly evoke feeding (Inutsuka 2014), with glucose and ghrelin acting as direct activators, and leptin and PYY acting as direct inhibitors (Sheng 2014).

With progressive extension of the inter-meal period and into outright fasting, orexigenic relays become increasingly more active to further promote hunger and food intake; and relays that promote energy expenditure become less active to conserve energy through reduced caloric utilization (Shibata & Bukowiecki 1985). Since thermogenesis and basal metabolism continue during this period, glycogen stores are gradually broken down into glucose (glycogenolysis) in order to maintain sufficient blood glucose levels. As glycogen depletion progresses, the overall rate of glucose production through glycogenolysis and gluconeogenesis (glucose production from other metabolites) is gradually reduced. Eventually, the rate of production becomes lower than the rate of whole-body glucose utilization, at which point blood glucose levels begin to decrease, and thus insulin levels also decrease. Additionally, the levels of circulating leptin also exhibit a similar reduction (Boden 1996, Grinspoon 1997, Kolaczynski 1996, Perry 2018), and evidence suggests that the adiposity-proportional secretion of leptin is also gated by glucose, insulin, cortisol, and catecholamines. Glucose, insulin, and cortisol increase adipocyte leptin
mRNA levels and leptin secretion (Malmstrom 1996, Wang 1998, Zakrzewska 1997), while catecholamines such as norepinephrine released by neurons of the sympathetic nervous system innervating adipose tissue reduce these parameters (Ricci & Fried 1999). Additional evidence suggests a connection with fasting-induced ketogenesis, where the dynamics of circulating leptin during fasting-refeeding inversely mirror the dynamics of circulating ketones (butyrate), though exogenous ketone administration does not directly affect leptin levels (Kolaczynski 1996).

Since glucose, insulin, and leptin have effects that suppress feeding; fasting-induced decreases in their circulating levels results in reduced satiety and hunger-suppression, thereby partially driving food intake. Fasting-induced reductions in energy expenditure are likely due, in part, to reduced energy availability, which results in reduced metabolism and thermogenesis in brown and beige adipose tissue. However, since leptin has been shown to promote energy expenditure, it is also likely that reduced expenditure is due to decreased circulating leptin. Recent evidence suggests that leptin raises the low-end thermoregulatory temperature threshold at which active heat dissipation mechanisms becomes completely inhibited and thermogenesis becomes significantly activated to prevent further reductions in body temperature. This effectively increases the lowest acceptable body temperature within the defended range of thermoneutrality (Kaiyala 2016, Fischer 2016). Thus, leptin only increases energy expenditure when the ambient temperature is below this range; though for humans, the ambient temperature of both indoor and outdoor environments is generally lower than regulated body temperature. The fasting-induced decrease in circulating leptin reduces this low-end threshold, allowing body temperature to drop further than when leptin is present in sub-thermoneutral environments, and thus also reduces the amount of energy expended to maintain body temperature.
Central BDNF-TrkB Signaling Regulates Energy Homeostasis

Brain-derived neurotrophic factor (BDNF) is a neurotrophin of the nerve growth factor family, and binds with high affinity to tropomyosin receptor kinase B (TrkB). Signaling through this interaction is important for many biological processes including brain and lung development, cell survival and differentiation, synaptic plasticity, learning and memory, and energy homeostasis (An 2008, Gao 2014, Hofer & Barde 1988, Intlekofer 2013, Knüsel 1991, Rios 2001). Disruptions in this signaling have been observed as a result of DNA mutations and polymorphisms (Hohenadel 2014, Mou 2015, Yeo 1998, Yeo 2004), complex genetic syndromes due to chromosomal deletions (Han 2008, Han 2010), in many neuropsychiatric and neurodegenerative disorders (Andero 2014, Polyakova 2015, Zuccato & Cattaneo 2009), and obesity (Mou 2015, Yeo 1998, Yeo 2004).

Central BDNF Opposes Weight Gain

The first evidence that BDNF-TrkB signaling within the brain regulates energy homeostasis comes from a study in rats where BDNF was injected into the third ventricle, which reduced weight gain compared to vehicle injected controls (Lapchak & Hefti 1992). A later study in mice deleted BDNF from much of the post-developmental brain, which produced a severe obesity phenotype driven by increased food intake (hyperphagia); though energy expenditure was not reported (Rios 2001). It was then discovered that Bdnf expression within the ventromedial nucleus of the hypothalamus (VMH) responds to nutritional state, whereby Bdnf mRNA levels were found to be reduced by 60% under fasting conditions (Xu 2003).

It was later discovered by our lab that the Bdnf gene produces mRNA transcripts of different lengths due to the existence of two separate (polyadenylation) transcription termination
sequences at different locations within the 3’ untranslated region (An 2008). The shorter transcript is generally found within the cell body, while the longer transcript is preferentially trafficked to the dendritic compartment where it is used for local BDNF translation and secretion. It was then determined that this dendritic BDNF secretion affects the process of synaptic refinement; suggesting that dendritic secretion may be stimulated by incoming presynaptic activity and acts to strengthen active synapses specifically (Orefice 2013). In mice rendered incapable of producing the long Bdnf mRNA transcript, the total levels of Bdnf mRNA within the VMH were substantially reduced; suggesting that the long mRNA transcript is the main form expressed in this nucleus. These animals displayed a severe hyperphagic obesity phenotype, and the ability of leptin to suppress food intake was significantly impaired; though energy expenditure was not reported (Liao 2012).

Another hypothalamic site where BDNF-TrkB signaling has been shown to regulate energy homeostasis is the paraventricular nucleus (PVH). Direct injections of BDNF into this nucleus decrease food intake and increase energy expenditure measured over 24 hours; which results in reduced body weight (Wang CF 2007), rather than mere suppression of weight gain as seen in third ventricle delivery. This strong energy homeostatic effect of BDNF in the PVH led us to focus on this nucleus.

The PVH exists as a heterogeneous collection of distinct, essentially non-overlapping neuronal populations including those that express oxytocin, vasopressin, corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), somatostatin (SS), growth hormone-releasing hormone (GHRH), and tyrosine hydroxylase (TH). Oxytocin and vasopressin neurons are composed of separate groups of magnocellular neurons, with large cell bodies that project to
the pituitary and release large quantities of their respective hormone directly into the bloodstream; and parvocellular neurons, with smaller cell bodies that project to locations within the brain (Simmons & Swanson 2009, Swanson & Sawchenko 1980). CRH, TRH, SS, and GHRH neurons, all parvocellular, send projections to the median eminence and release smaller quantities of their respective hormone into the hypophyseal portal system that envasculates both the median eminence and the pituitary. These hormones then stimulate cells of the pituitary to secrete second order hormones into the bloodstream (Dabrowska 2013, Lechan & Fekete 2006, Swanson & Sawchenko 1980).

Recently, our lab has identified an additional population of PVH neurons that express BDNF (PVHBDNF neurons). We determined through immunohistochemical staining in Bdnf reporter mice that PVHBDNF neurons are a distinct population that does not significantly colocalize with other known cell types. Specifically, we showed that BDNF-expressing cells are essentially non-overlapping with oxytocin, vasopressin, CRH, TRH, SS, GHRH, tyrosine hydroxylase (TH), or the melanocortin 4 receptor (MC4R). Selectively deleting Bdnf from the PVH causes a moderate hyperphagic obesity phenotype, a reduced capacity to increase thermogenesis in response to cold exposure (adaptive thermogenesis), and a nearly significant (p = 0.059) reduction in dark cycle energy expenditure (An 2015).

**Potential energy homeostatic role for TrkB-expressing PVH neurons**

TrkB receptors have also been implicated in the energy homeostasis regulation through the use of mice harboring a novel TrkB allele where global expression of the receptor in homozygous animals is reduced to 24% of wild-type. These animals exhibit moderate-to-severe hyperphagic obesity; though the hyperphagia is dependent on the caloric density of the diet. Homozygous
mutant mice consume similar amounts of low fat diet compared to controls, but show a greatly reduced capacity to adjust feeding in response to increases in this density when switched to a moderate fat diet. However, energy expenditure was not reported in this study (Xu 2003).

Here I show the existence of TrkB-expressing neurons in the PVH (PVH\textsuperscript{TrkB} neurons). Similar to PVH\textsuperscript{BDNF} neurons, these neurons are generally distinct from other neuronal populations within the nucleus, including PVH\textsuperscript{BDNF} neurons (Figure 2), with the exception that \textasciitilde 20\% of PVH\textsuperscript{TrkB} neurons also express oxytocin (parvocellular). Bdnf-expressing neurons in other regions of the brain such as the hippocampus coexpress TrkB and secrete BDNF from the cell body and dendritic compartment to activate these TrkB autoreceptors (Harward SC 2016). However, PVH\textsuperscript{BDNF} neurons do not appear to have this capacity. Thus, the proximity of PVH\textsuperscript{BDNF} and PVH\textsuperscript{TrkB} neurons suggests that these populations may communicate with each other, either by a direct projection or through somatodendritic release of BDNF.

Experiments performed by Juanji An in our lab suggest an energy homeostatic role for PVH\textsuperscript{TrkB} neurons. In these experiments, the expression of TrkB receptors was ablated using the Cre-Lox system in mice harboring loxP-flanked TrkB coding sequences, with or without an additional allele that expresses Cre recombinase (Cre) driven by the promoter for single-minded homolog 1 (SIM1). SIM1 is a transcription factor that is expressed by essentially all PVH neurons, and is critical for the development of the PVH. SIM1-mediated TrkB deletion causes obesity due to hyperphagia and reduced energy expenditure (Figure 3). However, SIM1 expression begins early in development; and expression is not restricted to the PVH. Therefore, SIM1-mediated TrkB deletion is not specific to the PVH, and may be confounded by developmental compensation.
Thus, it remains unknown whether PVH^{TrkB} neurons specifically are involved in the regulation of energy homeostasis after development.

Due to this lack of spatial and temporal specificity, the goal of my thesis was to determine the role of PVH^{TrkB} receptors in the regulation of energy homeostasis. To do this, I used the Cre-Lox system to selectively ablate TrkB expression in cells of the PVH. Using mice homozygous for a loxP-flanked TrkB coding sequence, I stereotaxically injected a virus that expresses Cre recombinase (Cre) bilaterally into the PVH. I then measured the body weight of these animals weekly, and also measured body composition, energy expenditure, and food intake at two time points: one early in phenotype progression and another late in progression. Furthermore, the goal was also to identify specific projection targets of these PVH^{TrkB} neurons, and to determine which, if any, were involved in energy homoeostasis regulation. To do this, I first injected a Cre-dependent tdTomato-expressing virus into one hemisphere of the PVH of mice that express Cre in all TrkB-expressing cells. This induced the expression of the red fluorescent tdTomato protein within cell bodies, axons, and axon terminals. I analyzed the locations of what appeared to be terminal-like tdTomato signal, and unilaterally injected these locations with retrotracer in separate animals to confirm the presence of PVH^{TrkB} neurons terminals within these regions. I then used a two-virus manipulation employing both the Cre-Lox and FLP-FRT systems to selectively delete TrkB only from PVH neurons projecting to individual specific targets, separately in different animals. In these animals, I measured weekly body weight, and additionally body composition and food intake at the late time point to determine if any of these projection-specific TrkB deletions affects energy homeostasis.
Figure 2: The majority of PVHTkrä neurons are distinct from other populations.

Immunohistochemical staining of PVH neurons in TrkB<sup>CreaER<sup>+</sup>;Ai9/</sup> mice. Shown alone in panels on the left, and again within merged images in panels on the right, are TrkB-expressing cells visualized by the red fluorescent TrkB reporter tdTomato. Staining for oxytocin (A), vasopressin (B), corticotropin-releasing hormone (C), tyrosine hydroxylase (D), the melanocortin 4 receptor (E), and transgenic Bdnf-driven β-galactosidase (F) are shown alone in panels in the middle, and again within the merged images on the right.

tdTomato expression is displayed in red, and the fluorescence of secondary antibodies from staining in green.

[All immunohistochemical staining and imaging for this figure were performed by Juanji An.]
Figure 3: Sim1-mediated deletion of TrkB causes obesity.

A,B: Histograms of weekly body weight for female (A) and male (B) SIM1-Cre;fB/fB mice and fB/fB littermate controls. Two-way ANOVA with repeated measures and Bonferroni post-tests.

C,D: Post-mortem fat pad weights harvested from male (C) and female (D) mice in A and B, respectively. Two-way ANOVA with Bonferroni post-tests.

E: Oxygen consumption separately averaged across the light and dark cycle for female mice in B. Two-way ANOVA with Bonferroni post-tests.

F: Average daily food intake for female mice in B. One-tailed t-test.

Error bars indicate standard error. (*, p<0.05; **, p<0.01; ***, p<0.001)

[All experiments, data collection, analysis, and creation of graphs for this figure were performed by Juanji An.]
CHAPTER TWO: MATERIALS AND METHODS
Materials

Animals

A transgenic mouse harboring a TrkB allele, where Cre recombinase recognition sequences (LoxP) have been inserted to flank the coding region (floxed TrkB; fB), has been previously generated by our lab on a C57BL/6 background (Baydyuk M et al. PNAS, 2011). Mice expressing a fusion protein of Cre recombinase and a modified estrogen receptor that responds only to tamoxifen (Cre-ERT2) from the Ntrk2 promoter (TrkB\textsuperscript{CreER} mice) were a generous gift from David Ginty, Ph.D. (Harvard University). Mice expressing CAG promoter-driven tdTomato behind a floxed STOP-cassette within the ROSA26 locus were obtained from The Jackson Laboratory (Stock No. 007909). All mice were housed within the Animal Resources Center (ARC) at The Scripps Research Institute – Florida and maintained on a 12h/12h light-dark cycle with regular rodent chow and water provided \textit{ad libitum}. All animal procedures used for this dissertation were approved by the Animal Care and Use Committee at The Scripps Research Institute – Florida.

Surgical Equipment

SurgiVet Classic T3\textsuperscript{TM} Isoflurane Vaporizer (Patterson Veterinary); Dual Manipulator Small Animal Stereotaxic Instrument with Digital Display Console & Mouse Gas Anesthesia Head Holder (Kopf Instruments, Model 942); UMP3-4 Microinjection Syringe Pump (World Precision Instruments); 10 μL NANOFIL glass syringe (World Precision Instruments); 33 gauge beveled NanoFil\textsuperscript{TM} needle (World Precision Instruments); 36 gauge blunt NanoFil\textsuperscript{TM} needle (World Precision Instruments).
**Reagents**

Chemicals: avertin [1g 2,2,2-tribromoethanol (Alfa Aesar) to 1mL t-amyl alcohol (Fisher)], tamoxifen (Sigma) in corn oil (Sigma) [2 mg / 300 µL], 4’,6-diamidino-2-phenylindole (DAPI) (Sigma).

Antibodies: Primary antibodies raised against tyrosine hydroxylase (Millipore) were diluted 1:1000 in blocking buffer (described in immunohistochemistry section). Fluorescent dye-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and used according to manufacturer’s instruction.

Viruses and Tracers: rAAV2/CMV-Cre-GFP & rAAV2/TRUFR(CMV)-eGFP (UNC Vector Core), rAAV2/CAG-FLEX-tdTomato (UNC Vector Core), rAAV2/fDIO-mcherry2ACre & rAAV2/fDIO-mcherry (UNC Vector Core), CAV2/CMV-flpE-GFP & CAV2/CMV-flpO (Institut de Génétique Moléculaire de Montpellier), and green Retrobeads™ (Lumafluor Inc.).
Methods

Genotyping

The genotype of all mice used for experiments was determined using polymerase chain reaction (PCR) with the following primers:

<table>
<thead>
<tr>
<th>Allele</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ai9</td>
<td>5’-ctgttccctgtacggtgcatg-3’</td>
<td>5’-ggcattaacgcgctcagtc-3’</td>
<td>196 bp</td>
</tr>
<tr>
<td>WT ROSA26</td>
<td>5’-aagggagtgctgctgggaag-3’</td>
<td>5’-ccgaaatctgtggaagtc-3’</td>
<td>297 bp</td>
</tr>
<tr>
<td>fB</td>
<td>5’-actgacatccgtaagcagc-3’</td>
<td>5’-atgtcgccctgctagtc-3’</td>
<td>~450 bp</td>
</tr>
<tr>
<td>WT TrkB</td>
<td>5’-actgacatccgtaagcagc-3’</td>
<td>5’-atgtcgccctgctagtc-3’</td>
<td>369 bp</td>
</tr>
<tr>
<td>TrkBCreER</td>
<td>5’-ggatgagttcgcaagaacc-3’</td>
<td>5’-ccatgagaacgacacctgg-3’</td>
<td>~390 bp</td>
</tr>
</tbody>
</table>

For Ai9 genotyping, the Ai9 amplicon was generated from within the Ai9 transgene, residing within the ROSA26 locus. The wild-type amplicon was generated across the region where the Ai9 transgene would reside. However, when Ai9 is present, the distance between the two primer binding sites is increased to the extent that no amplicon can be generated within the PCR cycle.

For fB genotyping, both amplicons are generated by the same pair of primers. However, with the additional base pairs inserted in the fB allele, the fB amplicon is larger than the wild-type TrkB amplicon.

For TrkBCreER genotyping, the amplicon is generated within the Cre recombinase transgene, and is a binary readout of presence or absence.
Stereotaxic Surgery

The following coordinates were used for all stereotaxic injections:

Relative to Bregma:

- **c-PVH**: AP bregma – 0.70 mm / ML ± 0.28 mm / DV skull – 5.52 mm
- **c,p-PVH**: AP bregma – 0.80 mm / ML ± 0.28 mm / DV skull – 5.52 mm
- **a-LPBN**: AP bregma – 4.39 mm / ML ± 1.03 mm / DV skull – 3.60 mm
- **p-LPBN**: AP bregma – 5.25 mm / ML ± 1.10 mm / DV skull – 3.55 mm
- **a-MPBN**: AP bregma – 4.39 mm / ML ± 1.03 mm / DV skull – 4.05 mm
- **p-MPBN**: AP bregma – 5.25 mm / ML ± 1.10 mm / DV skull – 4.03 mm
- **LC**: AP bregma – 5.40 mm / ML ± 0.91 mm / DV skull – 4.10 mm
- **vl-PAG**: AP bregma – 4.72 mm / ML ± 0.68 mm / DV skull – 2.70 mm

[central division of the paraventricular nucleus of the hypothalamus (c-PVH), transition segment between central and posterior divisions of the PVH (c,p-PVH), anterior and posterior segments of the lateral division of the parabrachial nucleus (a-LPBN and p-LPBN, respectively), anterior and posterior segments of the medial division of the PBN (a-MPBN and p-MPBN, respectively), locus coeruleus (LC), and ventrolateral part of the periaqueductal gray (vl-PAG)]

Relative to Calamus Scriptorius:

- **r-NTS**: AP CS + 0.85 mm / ML ± 0.58 mm / DV surface – 0.42 mm
- **m-NTS**: AP CS + 0.35 mm / ML ± 0.50 mm / DV surface – 0.35 mm
- **c-NTS**: AP CS + 0.00 mm / ML ± 0.00 mm / DV surface – 0.32 mm
- **c-NTS**: AP CS + 0.00 mm / ML ± 0.26 mm / DV surface – 0.40 mm

[rostral segment of the nucleus of the solitary tract (r-NTS), middle or central segment of the NTS (m-MTS), and caudal segment of the NTS (c-NTS)]
**Instrument Zero**

This procedure was performed, using a 45x or 90x stereo microscope, each time a microsyringe was mounted onto a manipulator arm, with no animal present in the stereotaxic frame. However, this was not done for syringes intended to inject the brainstem using calamus scriptorius, subsequently defined. Once installed into the nanopump syringe holder on the manipulator arm, the syringe body was independently rotated to orient the beveled face of the attached needle away from the U-frame, and toward the investigator, with the point of the needle tip precisely centered to align with the midline of the needle shaft.

The numerical value on the digital display readout for the x-axis (mediolateral) was manually set to zero after aligning the midline of the needle with the location known as “**Instrument Zero.**” This location is defined here as ‘**the point on the midline plane of a stereotaxic instrument where the tips of straight, symmetrically-installed ear bars touch.**’ To perform this alignment, the ear bars must first be precisely installed into the clamps on the frame using the markings on both the ear bars and the clamps. Ear bars were placed into the clamps, and tips were made to contact near the center. The positions of the ear bars were then adjusted such that the alignment between the ear bar markings and frame clamp markings for both sides was symmetrical. Clamps were fully tightened, and the right ear bar was removed. At the tip of the left ear bar, the right-most vertical surface corresponds to the midline plane of the stereotaxic instrument with the same degree of accuracy & precision as the ear bar installation. To align the midline of the needle with this plane, the anteroposterior (AP; forward/backward), mediolateral (ML; left/right), and dorsoventral (DV; up/down) adjustment knobs of the manipulator arm were maneuvered to bring the needle tip close to the ear bar tip. The straightness of the needle was then checked by moving the needle up & down (DV direction), and noting any change in the
distance to the ear bar (ML direction) or movement in the AP direction. When necessary, curved forceps were used to straighten the needle shaft by gently squeezing, with one pincer on either side of the needle shaft. With the needle tip brought to rest above the ear bar, the ML was then adjusted to align the needle point with the vertical surface at the end of the ear bar tip. Next, the DV was adjusted to move the needle downward until the left half of the needle tip made contact with the ear bar tip, and deflected to the right with further downward movement. Visualizing this contact and deflection either confirms correct alignment or demonstrates the need for adjustment. The precision of the alignment between needle shaft midline (represented by a properly centered needle point) and instrument midline (represented by the vertical tip surface at the end of either ear bar from a precisely, symmetrically installed pair) is generally a function of microscope power. Once sufficiently aligned, the display readout for the x-axis (ML axis) was reset to 0.000.

This procedure was performed in order to use the midline of the stereotaxic instrument as the midline of the skull, and thus the brain; as opposed to using the midline skull sutures. Given that the sutures can manifest up to 0.5 mm on either side of the instrument midline, using instrument zero for this purpose greatly increases bilateral accuracy and reduces inter-animal variability, particularly for injections of smaller nuclei.

Following this procedure, the syringe was charged with the liquid solution to be injected. To do this, the needle was moved away from the ear bars and U-frame to an arbitrary location that is still within the working distance of the stereo microscope, and also has sufficient manual working space. The syringe plunger was positioned at nearly full ejection, leaving a small air pocket to separate the liquid from directly contacting the plunger. A drop of the injection liquid (no more than 5 µL) was first transferred onto the wax surface of a small square of paper-backed
paraffin wax film (Parafilm M). This square was maneuvered using curved forceps to carry the liquid drop to the needle tip; and held in place while the liquid was fully drawn up into the syringe. This was performed while viewing through the stereo microscope. Afterwards, the plunger was further retracted to draw the liquid completely into the syringe body, leaving the needle shaft and a small space within the syringe body, near the needle, devoid of liquid.

Preoperative
Mice were initially anesthetized with an i.p injection of 2.5% avertin using a dosing paradigm of 15 µL per gram body weight in males and 12 µL per gram body weight in females. Once ambulation ceased, the fur on the head was shaved using electric clippers. Animals were mounted onto the ear bars of the stereotaxic frame, and the head was precisely centered using the markings on the ear bars and ear bar clamps. A platform was then inserted under the animal to level the body. The nose was secured onto the head holder, and the head tilt was adjusted to -2.25 mm below horizontal for C57BL/6-based mouse strains. An ointment (50/50 mix of petroleum jelly and mineral oil) was applied to the eyes to prevent them from drying out, and the scalp was prepared for incision by applying isopropanol followed by iodine-povidone.

Intraoperative
Anesthesia was maintained using a flow of isoflurane in oxygen gas from an isoflurane vaporizer delivered to the nose cone of the head holder, with excess gas and expirate flowing out to an isoflurane scrubber. The oxygen flow was held at 1.0 L/min “O₂ AT 14.7 PSIA (1.0 BAR) AND 70°F (21°C),” and the concentration of isoflurane was initially set to 1.5, and decreased to ~0.8 once the animal was fully anesthetized. A surgical level of anesthesia was confirmed by the complete absence of the toe pinch reflex, bilaterally. Given the variability in anesthetic
sensitivities between animals, maintenance of anesthesia within the therapeutic window was accomplished by observing the quality of breathing, or repeating the toe pinch reflex test; and adjusting the concentration of isoflurane accordingly. An increase in respiratory rate, usually accompanied by a decrease in depth of ventilation, or an increase in the labor of breathing was used as a sign of excess anesthesia. If insufficient anesthesia was suspected, the toe pinch reflex was retested, and any amount of reflex was a sign of insufficiency.

Injections Relative to Bregma

Once stable anesthesia was achieved, a midline incision was made to the scalp using a sterile No. 10 carbon steel scalpel blade, with a slight lateral offset to avoid damaging the underlying sagittal and interfrontal midline sutures. A sterile cotton swab was then used to open the incision, expose the skull, and clean the skull surface of blood and loose connective tissues. Sterile 15% hydrogen peroxide on a sterile cotton swab was then applied only to the sutures that are used to determine the landmark “bregma,” defined here as ‘the point on the dorsal skull surface at which a virtual parabola, formed by the trend of the coronal sutures as they approach the midline, intersects the trend of the midline suture’ (adapted from: Blasiak 2010). Hydrogen peroxide applied to the connective tissues at the sutures turns them white. Sharpey’s fibers reside within the narrow gap between skull plates, and dense pericranium covers the suture itself. The pericranium was removed by lightly scraping with a scalpel blade; exposing the thin, white Sharpey’s fiber line, making bregma selection more precise. Of the three axes that define bregma, only the AP value was used to determine the location of the target injection site. The ML and DV values at bregma were not used since the midline of the instrument more accurately represents the midline of the animal, and the DV of the injection site is determined more
successfully and consistently from the DV at the skull surface directly above it. Once bregma was selected, the display readout for the y-axis (AP axis) was reset to 0.000.

The needle was then moved to a position directly above the injection site using experimentally-verified coordinates for AP relative to bregma and ML relative to the midline of the brain. The needle was then carefully lowered until only the tip point made contact with the skull. The DV value was recorded from the readout, and a mark was made on the skull at the point of contact using an ultra-fine permanent marker. For unilateral injections, the DV value of the skull surface at the corresponding location on the contralateral side was also measured. For bilateral injections using one or multiple sets of coordinates, the process of measuring and marking was performed at each separate AP-ML location. The DV values between corresponding cross-hemisphere pairs were compared, and if they were different by an acceptable amount, the two values were averaged and used to determine the DV at the injection site in both hemispheres. For injection sites near the midline, the tolerance for paired values was 0.02 mm. If the difference in the values was larger than this tolerance, the location of the midline suture was taken into consideration, as its proximity can affect the measured value independently. In this case, a weighted average, biased away from the suture, was used for both hemispheres. For injection sites farther from the midline, the tolerance for paired values was on a gradient between 0.02 mm and 0.08 mm, depending on the lateral distance. For paired values not within tolerance, and not affected by the midline suture, improper mounting or additional causes such as aberrant skull development were investigated, and exclusion of the animal was considered. Once a DV value for the skull surface was determined, the DV at the injection site was calculated using an experimentally-verified relative DV value.
Craniotomies were opened using a high-speed electric drill, centered on the skull markings, exposing the dura mater but not rupturing it. At each craniotomy, the meninges were carefully punctured with the tip of a fresh, sterile 30 ga. needle, friction fit onto the end of a cotton swab; with bevel facing up, and using a shallow stroke to prevent damaging the underlying brain tissue or surface blood vessels. Once inserted, the needle was moved in lateral sweeping motions while maintaining an upward pressure to cut the meninges into a flap, which was then removed using ultra-fine forceps. The needle was lowered until the tip rested above the plane of the skull surface, and the syringe plunger was advanced until a small amount of injection liquid protruded from the needle tip, as both a test for good flow and to ensure that the needle was primed for delivery. The slight excess liquid was blotted using the twisted corner of a ‘delicate task wipe’ (Kimwipes), and the needle was lowered until it punctured the surface of the brain. If any needle deflection was observed, the needle was retracted, and the point of needle insertion was examined for residual pieces of bone or meninges. Once the needle was able to insert into the brain tissue without deflection, it was slowly lowered at an approximate rate of 0.05 mm per second. At every 1.00 mm above the injection site, descent was halted and the needle was retracted 0.20 mm before descent was resumed. This was done to reduce the pressure being delivered to the brain through friction along the needle shaft. Once the needle was lowered to the calculated depth, it was retracted by 0.01 mm.

Once the needle tip reached the correct depth, a period of three minutes was allowed to pass before initiating the infusion. A rate of 50 nL/min was used for all injection sites relative to bregma. Following each infusion, a period equal to the duration of the infusion itself was allowed to pass, and the needle was then fully retracted from the brain.
For angled injections, the angle adjustment on the manipulator arm carrying the injection syringe was aligned at 40° within the mediolateral plane. A second manipulator arm on the opposite side, with a disposable needle attached and oriented vertically, was used as a reference needle. The procedures for instrument zero, bregma selection, and marking the skull above the injection site were performed using this reference needle. However, the skull marking was done in a more precise manner by applying marker ink to the reference needle tip and touching the skull, creating an especially fine mark. Once this was accomplished, the reference needle manipulator arm was removed, and the angled manipulator arm was installed on the opposite side. The point of the angled needle was aligned with the skull mark such that the midline of the needle shaft was aimed directly at the center of the mark. The DV was then adjusted until the needle made contact with the skull surface. The DV was then further lowered, causing the needle to deflect slightly, until the center of the needle tip opening was aligned with the center of the mark. All three readout displays were then reset to 0.000, and pre-calculated movements were performed to move the needle to a location above the lateral skull surface on the contralateral hemisphere. At this point, the injection procedure became essentially synonymous with vertical injections, beginning at the measurement of skull surface DV. However, the depth of the injection site was calculated relative to the point above the lateral skull surface.

**Injections Relative to Calamus Scriptorius**

Once stable anesthesia was achieved, a midline incision was made to the skin covering the back of the neck and posterior skull using a sterile No. 10 carbon steel scalpel blade, beginning a few millimeters rostral to the interparietal-occipital ridge and ending approximately one inch caudally. Blunt dissection was used to separate the skin from the underlying muscle. The thin, transparent connective tissue between the back of the skull and the sheet of superficial trapezius
muscle was teased apart, and the trapezius was drawn caudally to create space for accessing the deeper cleidomastoid and sternomastoid muscles. Rather than resecting medial portions of these muscles, the connective tissue holding the left and right bundles together were teased apart along the natural midline division; and fine tissue spreaders were used to hold them apart, exposing the occipital bone and meninges covering the brainstem. At this point, the platform under the animal was removed, allowing the hind limbs to rest on the stage of the stereotaxic frame. During this action, the trapezius muscle sheet followed the torso down, which pulled it further away from the brainstem, increasing visibility within the working space.

A section from the lower half of the occipital bone, approximately 0.7 mm wide, was then removed using an electric drill to score a channel around the section. The bone fragment was then removed using forceps by pulling in a strictly caudal motion. This caused the dura mater to peel away with the bone, leaving behind only the thin, more transparent pia mater. A horizontal incision was then carefully made to the pia mater; just below the edge of the cerebellum, where there is a cerebrospinal fluid-filled space separating the pia from the brainstem surface. To expose the segment of brainstem that contained the nucleus of the solitary tract, the cerebellum was retracted anteriorly. This was done using a Paton Spatula (World Precision Instruments) that had been modified to form a C-shaped channel across the width of the spatula at the end of the flattened portion. The recess created by this channel was used to support the cerebellum and hold it in an anteriorly translocated position. Once the relevant portion of the brainstem was exposed, the parameters of the manipulator arm were adjusted such that the needle was angled at 40° within the sagittal plane, towards the caudal end of the animal. Thus, the needle was oriented in a dorsocaudal to rostroventral direction. The syringe used for these injections was fitted with a blunt 36 ga. needle, selected to reduce or eliminate leakage that occurs when using a beveled
needle to inject a particularly shallow target. The needle tip was brought to rest on “calamus scriptorius,” defined here as ‘the point along the midline of the brainstem at which the lateral edges of the fourth ventricle converge at the caudal end.” This location corresponds to the caudal tip of area postrema. Once in position, all three display readouts were reset to 0.000, and the needle tip was moved to a position directly above the injection site using experimentally-verified coordinates relative to calamus scriptorius. The syringe and needle were loaded, primed, and prepared for delivery in the same manner as vertical injections. The needle was lowered to rest on the surface of the brainstem directly above the injection site, and the DV value was recorded. The contact point was visually noted, and after raising the needle, the surface of the brainstem at this location was gently teased open using a fresh, sterile 30 ga. needle, friction fit onto the end of a cotton swab. This was done using strokes parallel to the visible grain of axon fibers at the brainstem surface. The needle was then lowered into the opening, and slow but repetitive in & out movements were made, gradually increasing in depth until arriving at the final depth. For these brainstem injections, an infusion rate of 25 nL/min was used.
Behavior

Energy Expenditure and Locomotor Activity

At 9 weeks post-injection in males, and 2 & 9 weeks post-injection in females, PVH TrkB deletion animals were individually housed within the Columbus Instruments “Comprehensive Lab Animal Monitoring System” (CLAMS) unit near the beginning of the dark cycle (6 pm). Lean body mass for each animal was entered into the computer for normalization, and measurements of oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were taken for a 1 minute period per cage, continuously cycling through all activated cages for the duration of the session. Independent of periodic gas analysis, the computer monitored locomotor activity by continuously counting breaks in infrared beams spaced evenly along the longer dimension of the cage, aimed across the shorter dimension, and situated near the floor. The room was fitted with an automatic light timer, which turned lights off at 6 pm and on at 6 am, and did not undergo a one hour shift during daylight saving time. The animals remained within the CLAMS unit for approximately 60 hours (3 dark cycle & 2 light cycles), beginning with the first dark cycle, and were removed the morning after the third dark cycle.

Food Intake

Immediately following CLAMS measurements, animals remained singly housed, but were transferred to individual home cages. Body weights were measured, and a pre-weighed amount of normal chow was placed into each home cage. Body weight and remaining chow was measured each day for seven days; and additional pre-weighed chow was added when necessary. Cages were not changed by the animal facility staff during this measurement period. Only the data from the last four daily measurements was used to calculate daily food intake.
**Body Composition**

For PVH *TrkB* deletion mice, at 9 weeks post-injection for males and at 3 & 9 weeks post-injection for females, body composition was measured using a Bruker “The minispec mq 7.5” NMR analyzer, optimized for live animals. Awake mice were placed inside the hydrocarbon-free containment tube, and immobilized by inserting the tube plunger and advancing until gently snug. The tube was placed inside the port within the analyzer, through the core of the magnet, and the proportion of fat mass, lean mass, and fluid were analyzed by nuclear magnetic resonance (NMR), and computed from whole body weight measured immediately before analysis.

**Histology**

Mice were anesthetized with an i.p injection of 2.5% avertin, and a dosing paradigm of 20 µL per gram body weight. Once ambulation ceased and animals showed no response to toe pinch or strong tail pinch, they were transcardially perfused with tris-buffered saline (TBS), followed by 4% paraformaldehyde in TBS (4% PFA). Brains, pituitaries, and whole spinal cords (cervical to cauda equina) were removed and post-fixed overnight in 4% PFA at 4ºC. Tissues were then transferred to a solution of 30% sucrose in TBS and stored at 4ºC for 2-3 days before being sectioned at 40 µm using a freezing stage microtome (SM2000R, Leica Biosystems). Sections were stored in TBS at 4ºC until processing. For long-term storage, sections were transferred to a TBS-based antifreeze solution (30% glycerol, 30% ethylene glycol, in TBS) and sealed with Parafilm M before being stored at -20ºC.
**Immunohistochemistry**

Free-floating sections were gently agitated in blocking buffer (10% donkey serum, 2% bovine serum albumin, 1% glycine, 0.4% Triton-X 100, in TBS) for 60 min at room temperature. Sections were then incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Three subsequent washes were performed by agitation in blocking buffer at room temperature for 10 min each. Sections were then incubated with fluorescently labeled secondary antibodies diluted in blocking buffer for 2 hrs at room temperature. Three subsequent washes were performed by agitation in TBS at room temperature for 20 min each. Finally, sections were incubated in a solution of DAPI in TBS for ≥30 min at room temperature to stain all cell nuclei.

**Imaging**

Sections were mounted onto gelatin-coated slides and allowed to gently dry. Slides were then cover-slipped using a TBS-based mounting medium, and imaging was not performed for at least 24 hrs. All imaging was performed using a Nikon Eclipse Ti inverted microscope outfitted with a C2 scanning confocal laser system. Tiled large-scan multichannel epifluorescence images of whole brain sections were captured using a 4x objective. In regions of interest (ROIs), confocal z-stack images were captured using either a 10x or 20x objective, and a maximally closed pinhole. When it was necessary to display high-magnification images containing signal from the full thickness of the tissue within ROIs, maximum intensity projection (MaxIP) images were generated by the software. These MaxIP images are the product of summating the signal intensity from all individual confocal planes within a z-stack, or simply a flattening of the 3-dimensional stack of images.
**Injection Site Verification**

**PVH subdivisions**

Injection site analysis within the PVH was performed separately for the anterior, central, and posterior divisions. Analysis was further separated according to subdivisions created within the cross-sectional area of each division. Anterior PVH is least in the mediolateral dimension; therefore anterior PVH was separated into dorsal and ventral subdivisions. Central PVH is similar to anterior PVH in the dorsoventral dimension, but larger in the mediolateral dimension, especially in the dorsal half. Therefore central PVH was separated into dorsomedial, dorsolateral, and ventral subdivisions. Posterior PVH is essentially devoid of an analogous ventral subdivision; therefore, posterior PVH was separated into medial and lateral subdivisions (Figure 4).

**PVH TrkB deletions**

Virally-induced GFP expression was used to analyze the coverage of the bilateral injections within the PVH, non-PVH nuclei within the injection site, and nuclei along the needle tract. Microscope images for all animals were coded using unique animal identifiers that do not contain information about treatment; therefore, I scored the infections blinded to treatment. At minimum, every third brain section that contained the PVH was imaged and used for analysis, and this frequency guarantees at least one section from each of the three PVH divisions. Two separate scoring systems were used for analysis; however, both systems utilize the same scale to represent the abundance of GFP-expressing cells (GFP-cells) within the analyzed area. Trivial numbers of GFP-cells (≤ 2 cells) was denoted by a minus sign (-) regardless of scoring system, and the abundance of GFP-cells was graded on a scale from one to five. A score value of five represents full saturation of the analyzed area; and the remaining score values represent evenly
distributed abundance levels between the two extremes. For the first scoring system (system 1), a score for the analyzed area was generated by averaging the individual scores for each hemisphere. This “total score” represents the total bilateral abundance, and was denoted with one to five interpunct symbols (•). For the second scoring system (system 2), which was used exclusively for analyzing the PVH, a score for each subdivision was generated by reporting the lesser of the two individual hemisphere scores. This “bilateral score” represents the amount of infection that can be considered as bilateral, and was denoted with one to five plus signs (+). All non-PVH regions and nuclei were analyzed using system 1; and female AAV-GFP injected control mice were additionally analyzed using system 1 due to the use of system 2 producing a score of zero for certain control animals with strong unilateral injections, masking the totality of the infection. Scores for unilateral infections in AAV-Cre injected mice were purposefully allowed to remain suppressed due to the potential confound of contralateral compensation.

**Anterograde Tracing**

Virally-induced tdTomato expression was used to analyze the coverage of the unilateral injections within the PVH, non-PVH nuclei within the injection site, and nuclei along the needle tract. Half of every brain was imaged for the purpose of TLS analysis; therefore, every other brain section that contained the PVH was included and used for analysis. Scoring in these animals followed the procedure that is common for both system 1 and system 2, where scores that represent the abundance of tdTomato-expressing cells (tdT-cells) within each PVH subdivision were generated for each hemisphere. However, since these injections were unilateral, only two animals contained a small amount of infection in the contralateral hemisphere due to diffusion, individual hemisphere scores were tabulated without any averaging.
PVH projection-specific TrkB deletions

The injection site of the projection targets were analyzed similarly for the parabrachial nucleus (PBN) and nucleus of the solitary tract (NTS), though methodological differences were present. For the PBN, a small area of autofluorescence was created by minor tissue damage at the infusion site. Additionally, a small number of cells within the injection site became infected and began expressing GFP. The neighboring region of the locus coeruleus (LC) was visualized by staining all PBN-containing brain sections for tyrosine hydroxylase. This staining, and the markers of infusion site autofluorescence and the local GFP-expressing cells, were used to determine the success of PBN injections. Any animals containing either of the two markers within the LC, or lacking both markers in both hemispheres of the PBN and showing negative results in PVH analysis, were removed from the study. For the NTS, green Retrobeads™ (GRBs) were mixed with the retrovirus to mark the location of the infusion. Tyrosine hydroxylase staining was also performed on all NTS-containing brain sections to aid in the determination of the NTS boundaries. Any animals lacking GRB signal in both hemispheres of the NTS, or containing signal in the dorsal motor nucleus of the vagus (DMV), were removed from the study.

Virally-induced mCherry expression was used to analyze the coverage of the bilateral injections within the PVH, non-PVH nuclei within the injection site, and nuclei along the needle tract. However, mCherry expression as a reporter for viral infection is also dependent on injections of retrograde virus into the respective projection target. Thus, the abundance of mCherry-expressing neurons (mCherry-neurons) was analyzed within the PVH, and used in conjunction with the success of retrograde virus injections to determine overall success of the manipulation for each animal. Blinding and frequency of analyzed sections was the same as in whole PVH TrkB deletion analysis. For all animals, only trivial or near-trivial (3 to 10) numbers
of mCherry-expressing cells (mCherry-cells) were found within nuclei along the needle tract and in non-PVH nuclei within the injection site.

From the results of the tracing studies, projections of PVH\textsuperscript{TrkB} neurons to the PBN or NTS preferentially reside within different PVH subdivisions. Therefore, the success of projection-specific \textit{TrkB} deletions was determined using the abundance of mCherry-cells, weighted differently for the separate projection targeting injections according to the proportion of colocalized cells found within the different subdivisions from the retrotracer injections. Any animals containing only trivial numbers of mCherry-cells within the relevant PVH subdivisions were removed from the study. All remaining animals, with successful projection target injections and non-trivial numbers of relevant mCherry-cells, were retained in the study.
**Figure 4: Analytical subdivisions of the PVH.**

Cartoon depictions of the anterior (A, red), central (B, green), and posterior (C, blue) divisions of the PVH, with separate analytical subdivisions filled using different luminosities of the respective segment color. [Modified from Paxinos and Franklin, 2001]

d, dorsal; v, ventral; m, medial; l, lateral; dm, dorsomedial; dl, dorsolateral
Terminal-Like Signal Discrimination and Analysis

Virally-induced tdTomato expression was used to analyze the entire brain for identifying regions that contain tdTomato signal that resembled axon terminals (terminal-like signal; TLS). Every other section throughout the brain, from the olfactory bulb to at least the level of the NTS, was imaged for analysis. To distinguish between tdTomato signal that represented axons and TLS, I generated the following criteria. tdTomato signal that appeared as smooth regular strings, regardless of length or curvature, was considered to be bouton-deficient axonal signal. The appearance of this type of signal was typically thicker and brighter than that defined as TLS. Signal that appeared as thinner strings scattered with dots that were brighter and larger than the apparent diameter of the string-like structures along which the dots were integrated, which was generally not as bright as axonal signal, was considered to be TLS. Furthermore, TLS often appeared as a web-like structure that spread across a nucleus or subnuclear compartment, but tended to stay within a discrete area.

A list of all nuclei or non-nuclear regions (such as cortical area) containing TLS was generated for each animal, with the intensity and coverage of the area scored on the same 6-point scale as other virus injections. Specifically, trivial or less signal was again represented by a minus sign; and signal strength, as a combination of coverage and density within each analytical area, was represented by one to five interpunct symbols. However, the maximum score value of five for this scale was set by the largest amount of signal found in a nucleus across all animals (essentially axon terminal saturation), with the remaining score values representing evenly distributed signal strengths between the two extremes.
Half of the injections were performed using a vertical needle path, and the other half using an angled needle path within the coronal plane approaching from the lateral brain surface. This was done to eliminate TLS-containing regions from the list that were due to projections from TrkB-expressing neurons along the needle tract. Therefore, the lists for successful vertically injected animals (4x) was compared to those of successful angle injected animals (5x); and any regions that were only present on one set of lists were not included in the final list (first pass exclusions). Additionally, one animal received an injection that was a shallow miss of the PVH; thus, all TLS signal found within the brain of this animal was from TrkB-expressing neurons along the needle tract. Therefore, the regions on the list for this animal were also not included in the final list (second pass exclusions). However, since it is possible that regions may receive simultaneous projections from both $PVH^{TrkB}$ neurons and TrkB-expression neurons along the needle tract, the exclusion of regions from the missed animal could potentially create false negatives. Therefore, only regions for which the signal strength in the missed animal was greater than or equal to that in all other animals were excluded through this second pass. Notably, none of the regions excluded by either the first or second pass are known to receive projections from non-TrkB or generic PVH neurons.
Data Analysis

Energy Expenditure and Locomotor Activity

The CLAMS software reports the data within a spreadsheet template, with a single value for all measured and calculated variables reported for each ~15 min time point, with time and date stamps included. The data for the second light cycle and third dark cycle were isolated and analyzed, with the first three cycles (dark, light, dark; 36 hours) accounting for acclimation. For oxygen consumption (VO₂) and calculated respiratory exchange ratio (RER; VCO₂/VO₂), the values for each animal were averaged across each cycle. For locomotor activity (beam breaks), the values for each animal were summated across each cycle.

Food Intake

The daily change in food weight for each animal was averaged across the last five days of the seven day measurement period, with the first two days accounting for acclimation. Normalized food intake (food intake / body weight) was calculated by dividing average food intake for the five post-acclimation days by the average body weight during that same period.
Statistics

Data for each gender was analyzed separately due to the differences between respective control groups. For whole PVH *TrkB* deletion animals, initial body weight at the time of stereotaxic injection was analyzed between control and treatment groups by two-tailed unpaired t-test in order to demonstrate that there was no significant difference between groups, initially. The amount of weight gain for these animals, calculated as the within-subject difference between body weight at 0 weeks (initial) and 10 weeks post-injection, was analyzed by one-tailed unpaired t-test due to the directional hypothesis of increased body weight. The data for weekly body weight (at all 11 time points) and female food intake (at both time points) was analyzed by two-way ANOVA with repeated measures, and additionally with Bonferroni post-tests when a significant main effect was detected. Body composition data at each of the two time points was also measured by two-way ANOVA, though without repeated measures, as fat and lean mass are separate dependent variables; and additionally with Bonferroni post-tests when a significant main effect was detected. Metabolic cage data was separately analyzed for males at the late time point, females at the early time point, and females at the late time point. Data for cycle-averaged oxygen consumption, cycle-averaged respiratory exchange ratio, and cycle-summated locomotor activity were each analyzed by two-way ANOVA with repeated measures; and Bonferroni post-tests when a main effect was detected. Data for all ungrouped, single time point measurements was analyzed by unpaired t-test: one-tailed for male food intake and female normalized feeding due to the directional hypothesis of increased food consumption, and two-tailed for male feeding efficiency due to the lack of a directional hypothesis. The correlation between female food intake and body weight at the early time point was analyzed by linear regression.
CHAPTER THREE: RESULTS AND DISCUSSIONS
PVH-specific TrkB deletion

SIM1-mediated deletion of TrkB receptors, which includes the deletion of TrkB from neurons of the PVH, causes in obesity driven by hyperphagia and reduced energy expenditure. However, since the deletion is not confined to the PVH, the contribution of TrkB receptors specifically within the PVH to this effect remains unclear. Additionally, SIM1 expression is initiated early in development, as this gene encodes a transcription factor that is critical for the development of the PVH. Therefore, SIM1-mediated deletion may be confounded by developmental compensation. To determine the post-developmental role of TrkB receptors specifically within the PVH, I selectively ablated TrkB receptors from PVH cells in the adult mouse brain.

Generation of mice with TrkB deletion in the PVH

In order to determine if TrkB receptors within the PVH play a role in energy homeostasis after development, I used the Cre-Lox system to selectively delete the TrkB coding sequence from cells within this nucleus in the adult mouse. The timing of the TrkB deletion was chosen due to BDNF-TrkB signaling being critical for growth and development of neurons (Hofer & Barde 1988, Liao GY 2015). Therefore, I deleted TrkB in the adult mouse to avoid interfering with these developmental processes and affect only the post-developmental functions of the receptor. Using mice homozygous for a floxed TrkB allele (fB/fB mice) at 8 weeks of age, I stereotaxically injected 0.2 - 0.3 µL of either the virus rAAV2/CMV-Cre-GFP (AAV-Cre) that expresses cytomegalovirus (CMV) promoter-driven Cre recombinase (Cre) and green fluorescent protein (GFP), or rAAV2/TRUFR-eGFP (AAV-GFP) that expresses CMV-driven GFP alone, into both hemispheres of the PVH.
Across the control and treatment groups, there was littermate matching with proportional representation; and initially, animals were similarly lean with body weights that were not significantly different from each other for both males and females (Table 1).

**Verification of TrkB deletion**

In AAV-Cre infected cells, virally encoded Cre began expressing after approximately one week, which translocated to the nucleus and performed recombination between LoxP sites flanking the TrkB coding sequence, removing it from the genome. To demonstrate that this process occurs in vivo, I injected a small set of fB/fB mice with the viruses, one virus per animal, and Juanji An performed in situ hybridization using radiolabeled mRNA probes against the coding sequence of TrkB (Figure 6). In AAV-GFP injected mice, the strength of the radioactive signal within the PVH was remarkably strong. However, in AAV-Cre injected mice, the signal specifically within the PVH was reduced essentially to background levels. These findings demonstrate that the TrkB coding sequence had been deleted, thus TrkB receptors were ablated as a result.

**Injection site analysis**

Following the completion of all experimental measurements, I analyzed brain sections containing the PVH for GFP expression in order to verify the accuracy of all injections. Given that unilateral manipulations of the PVH such as lesioning can be compensated for by the contralateral side (Leibowitz 1981), using a representation of specifically bilateral TrkB deletion to score these injections avoids this potential confound. Therefore, I quantified the infections by generating an overall bilateral PVH score for each female (Table 2, Table 3) and male (Table 4) animal. Using these overall scores, I classified each injection as either successful or unsuccessful by applying a score threshold in females that clearly separates clustered low (unsuccessful)
scores from the remaining higher (successful) scores (Figure 7). The overall scores for all males were above this threshold value. For other brain regions near the PVH and along the needle tract, I generated total scores that represent the strength of infection relative to the total size of the nucleus, regardless of laterality.

There was a non-trivial amount of infection along the needle tract across animals. However, the majority of these nuclei were reasonably represented across both successful and unsuccessful groups. Also, apart from one of females in the unsuccessful group that had a particularly strong needle tract infection, the majority of individual nuclei did not contain a considerable amount of infection, with the exception of a few small thalamic nuclei that more easily attain a higher score.

I also generated overall bilateral PVH scores for AAV-GFP injected female (Table 3, top) and male (Table 4, right) control mice. Two of the female controls had score values of zero, one of which was due to the infection being strictly unilateral. Therefore, to demonstrate that viral expression was present in this animal, I also generated ‘total PVH scores’ for all female controls (Table 3, bottom). However, the other zero-score female control displayed low expression generally, which was likely due to an issue of low injection volume. Despite this, the animal was retained within the study as a control due to the remaining aspects of the treatment that it shared with the rest of the animals in the study.
Figure 5: AAV-Cre injected into the PVH induces expression of Cre and GFP.

A: Diagram of a mouse brain coronal section through the central segment of the PVH (red fill), including a zoom of the hypothalamus. [Modified from Paxinos and Franklin, 2001]

B-D: Large-scan epifluorescence images of serial brain sections from a representative animal with a successful PVH injection.

B'-D': Flattened confocal stack images at higher magnification of the PVH from B, C, & D; respectively.

GFP expression is displayed in green, and the non-specific nuclear stain (DAPI) in blue.
Table 1: Initial body weights of male and female PVH-injected $fB/fB$ animals.

<table>
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<th>AAV-GFP</th>
<th>AAV-Cre</th>
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<td>21.6 ± 0.86g</td>
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Initial body weights measured immediately before stereotaxic injection surgery for male and female $fB/fB$ mice receiving injections of either AAV-GFP or AAV-Cre into the PVH.

Expressed as group averages ± SEM. Two-tailed t-test, by gender.
Figure 6: AAV-Cre potently reduces TrkB mRNA expression in fB/fB mice.

A,B: In situ hybridization using radioactively labeled probes against the TrkB receptor coding sequence in fB/fB animals injected with either AAV-GFP (A) or AAV-Cre (B). Arrows indicate the location of the PVH.

[I performed the injections, while the in situ hybridization was performed by Juanji An.]
Table 2: Infection scores for AAV-Cre injected female fB/fB mice.

<table>
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<th>Female AAV-Cre Bilateral PVH Scores &amp; Non-PVH Total Scores</th>
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<th>SUCCESSFUL</th>
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Infection scores for PVH subdivisions, non-PVH injection site nuclei, and needle tract nuclei; tabulated for each AAV-Cre injected female fB/fB mouse. The table includes calculated ‘overall bilateral PVH score’ values (below PVH subdivision analysis). Each column represents a separate animal.

Bilateral PVH scores denoted by the plus symbol (+), and total scores denoted by interpunct symbols (∗).
Table 3: PVH infection scores for AAV-GFP injected female \( fB/fB \) mice.

Infection scores for PVH subdivisions, tabulated for each AAV-GFP injected female \( fB/fB \) mouse, expressed as either bilateral scores (left block) or total scores (right block). The table includes calculated ‘overall bilateral PVH score’ values and ‘overall total PVH score’ values (bottom row). Each column represents a separate animal within a scoring block, and animals are listed in the same order within each block.

Bilateral scores denoted by the plus symbol (+), and total scores denoted by interpunct symbols (•).
Figure 7: Exclusion threshold for female AAV-Cre injected $fB/fB$ mice.

Bilateral PVH scores for all AAV-Cre injected female $fB/fB$ mice, with horizontal dashed line indicating the threshold value of 6, separating unsuccessful injection scores (dark red) clustered below the successful injection scores (red) above.
Table 4: Infection scores for AAV-Cre and AAV-GFP injected male fB/fB mice.

<table>
<thead>
<tr>
<th>Male Bilateral PVH Scores &amp; Non-PVH Total Scores</th>
<th>SUCCESSFUL AAV-Cre</th>
<th>AAV-GFP Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior Central PVH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dorsal:</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ventral:</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Central PVH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dorsomedial:</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>dorsolateral:</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>ventral:</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>posteriPVH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dorsal:</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ventral:</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>lateral:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall Bilateral PVH Score</td>
<td>12</td>
<td>14</td>
</tr>
</tbody>
</table>

| Non-PVH Hypothalamus                         |                    |                  |
| Anterior (AH):                               | •                  | •                |
| Dorsomedial (DMH):                           | •                  | •                |
| Ventromedial (VMH):                          | -                  | -                |
| Lateral (LH):                                | -                  | -                |
| Arcuate (ARC):                               | -                  | -                |
| Posterior (PH):                              | -                  | -                |
| Suprachiasmatic (SCN):                       | -                  | -                |
| Medial Preoptic (MPO):                       | -                  | -                |
| Median Preoptic (MnPO):                      | -                  | -                |
| Zona Incerta:                                | *                  | •                |
| Subincertal Nucleus:                         | -                  | -                |
| Bed Nucleus of Stria Terminalis:             | -                  | -                |
| Mediodorsal (MD):                            | •                  | •                |
| Paratentinal (PT):                           | •                  | •                |
| Submedius (Sub):                             | -                  | -                |
| Reuniens (Re):                               | •                  | •                |
| Anteromedial (AM):                           | •                  | •                |
| Paraventricular (PVT):                       | -                  | -                |
| Interanteromedial (IAM):                     | -                  | -                |
| Rhomboid (Rh):                               | -                  | -                |
| Central Medial (CM):                         | •                  | •                |
| Interanterodorsal (IAD):                     | -                  | -                |
| Ventromedial (VM):                           | -                  | -                |
| Habenula:                                    | •                  | •                |
| Hippocampus:                                 | -                  | -                |
| Cingulate/Motor Cortex:                      | -                  | -                |

Infection scores for PVH subdivisions, non-PVH injection site nuclei, and needle tract nuclei; tabulated for each AAV-Cre injected male fB/fB mouse. The table includes calculated ‘overall bilateral PVH score’ values (below PVH subdivision analysis). Each column represents a separate animal.

Bilateral PVH scores denoted by the plus symbol (+), and total scores denoted by the interpunct symbol (•).
**PVH TrkB deletion causes obesity**

For all male and female $fB/fB$ mice injected with virus into the PVH, I measured weekly body weight for a period of 10 weeks, beginning on the day of stereotaxic injection, immediately before surgery. At an early time point (2 weeks post-injection), and again at a late time point (9 weeks post-injection), I measured body composition and immediately transferred the mice into individual metabolic cages within the Comprehensive Lab Animal Monitoring System (CLAMS) unit. Oxygen consumption was measured and normalized to lean mass, and locomotor activity was also measured, over the course of 60 hours (three dark cycles and two light cycles). Following the CLAMS session, I transferred mice into individual home cages and measured daily food intake for seven days. Mice were then re-grouped into their original group-housing sets to minimize any stress-induced effects on body weight. A timeline of these experimental measurements is shown in Figure 8.

**Body Weight**

Female AAV-GFP injected $fB/fB$ control mice maintained a minimal amount of weight gain on chow diet, increasing body mass by an average of $3.4 \pm 0.18g$ over the course of 10 weeks. Successful AAV-Cre injected female $fB/fB$ mice gained significantly more weight than controls ($p < 0.0001$), increasing body mass by an average of $30.4 \pm 2.03g$ on the same diet and time frame. Unsuccessful AAV-Cre injected females, however, increased body mass by only $9.4 \pm 2.83g$, and were not significantly different than controls. These female values of calculated 10-week weight change for all three groups were analyzed by one-way ANOVA with Tukey post-tests. Male AAV-GFP injected $fB/fB$ control mice gained a moderate amount of weight on chow diet, increasing body mass by an
average of 13.0 ± 0.05g over the course of 10 weeks. Male AAV-Cre injected mice gained significantly more weight than controls (p = 0.0005), increasing body mass by an average of 26.5 ± 1.05g on the same diet and time frame. These male values of calculated weight change, composed of only two groups, were analyzed by one-tailed t-test. Analyzing body weight data across all time points revealed that successful AAV-Cre injected mice became significantly heavier than controls at 2 weeks post-injection in females (Figure 9A) and at 3 weeks post-injection in males (Figure 9B). However, unsuccessful females were not significantly different from controls at any time point; therefore, they were not considered beyond body weight measurements within this study.

Using the body weights of female AAV-Cre injected mice at 8 weeks post-injection, I performed a correlation analysis between body weight and overall bilateral PVH score by linear regression. The relationship between these two parameters was relatively linear, with a value for the coefficient of determination (r²) of 0.7315 (Figure 10). This post hoc analysis of the viral infection scoring suggests a level of accuracy that is sufficient to validate the use of a threshold for sorting animals into successful and unsuccessful categories.

Body Composition

Using NMR spectroscopy, I measured body composition as part of the preparations for metabolic cage analysis, due to the requirement of lean mass determination for normalization of data from metabolic measurements. I measured male mice at 9 weeks post-injection when AAV-Cre injected animals were significantly heavier than AAV-GFP injected controls. However, data gathered after an obesity phenotype is established cannot inform as to whether any metabolic differences found are causative or are a secondary effect of increased body
weight. Therefore, in the subsequently injected female mice, I additionally measured body composition at 2 weeks post-injection.

For females at 2 & 9 weeks and males at 9 weeks, fat and lean mass compartments in AAV-Cre injected animals were significantly higher than controls; and the increase in fat mass accounted for the majority of the overall increase in body weight (Figure 9C-E). Together with the magnitude of the body weight differences between groups, these findings reasonably classify this phenotype as obesity. However, at the 2 week time point in female mice, when the body weight difference was only beginning to emerge, the magnitude of the increases in fat and lean mass was considerably less than at 9 weeks. These findings are consistent with previously characterized obesity phenotypes, where increased fat mass also accounted for a majority of weight gain (An 2015, Dong 2012, Shah 2014).
Figure 8: Experimental timeline for PVH TrkB deletion.

Diagram of the experimental timeline for male and female $fB/fB$ mice receiving viral injections into the PVH. Shown are the periods for weekly body weight measurements represented by a grey block, body composition and energy expenditure (BC/EE) measurements by blue blocks, and food intake (FI) measurements by orange blocks, with the arrow indicating the age and relative time of the stereotaxic injection. Above each set of blue and orange blocks is information regarding which genders were measured at which time point.
Figure 9: PVH TrkB deletion causes obesity.

A,B: Histograms showing weekly body weight across time for female (A) and male (B) AAV-GFP and AAV-Cre injected fB/fB mice. Body weights of unsuccessful females are displayed in dark red, and those of successful females are displayed in bright red. Two-way ANOVA with repeated measures and Bonferroni post-tests.

C-E: Fat and lean mass compartments measured by NMR spectroscopy for females at 2 weeks (C), females at 9 weeks (D), and males at 9 weeks (E) post-injection. Two-way ANOVA with Bonferroni post-tests.

Error bars indicate standard error. (*, p<0.05; **, p<0.01; ***, p<0.001)
Figure 10: Correlation between body weight and PVH score in AAV-Cre injected female fB/fB mice.

Plot of body weight at 8 weeks post-injection versus overall bilateral PVH scores for AAV-Cre injected female fB/fB mice. Those previously classified as unsuccessful are shown in dark red, and those classified as successful are shown in bright red. Superimposed is the best-fit line (black) determined by linear regression.
Obesity caused by PVH TrkB deletion is due to hyperphagia

According to the tenets of energy homeostasis, weight gain is caused by an unbalanced increase in food intake (calories in), decrease in energy expenditure (calories out), or a combination of both (An 2015, Dong 2012, Ozek 2015, Ren 2012, Rezai-Zadeh 2014). In order to further characterize and determine the source of increased weight gain caused by PVH TrkB deletion, I also measured energy expenditure, locomotor activity, and food intake in PVH TrkB deletion animals. Together with body composition analysis, these measurements were performed as a set of procedures. Therefore, I measured energy expenditure and locomotor activity immediately after body composition at 9 weeks post-injection in male mice, and at 2 & 9 weeks post-injection in female mice. During the week following each round of these assays, I measured body weight and food intake daily; which therefore occurred at 10 weeks post-injection in males and at 3 & 10 weeks post-injection in females.

Energy expenditure and locomotor activity

Immediately following body composition measurements, I used the CLAMS unit to measure oxygen consumption (VO₂) as an indicator of energy expenditure, and locomotor activity. Respiratory exchange ratio (RER) was calculated as carbon dioxide production (VCO₂), which was also measured, divided by oxygen consumption (VCO₂/VO₂). RER is used as an indicator of the main fuel type an animal is predominantly utilizing for energy (carbohydrates versus fats).

In females, there were no significant differences in VO₂ or RER between groups during either the light or dark cycle at either time point (Figure 11A,B,D,E). These data indicate that no energy expenditure deficits are produced by PVH TrkB deletion in females; and basal fuel utilization was similar between groups despite substantially larger fat stores. At 9 weeks, when
female AAV-Cre mice were substantially heavier, locomotor activity was significantly reduced during the dark cycle, with a trend during the light cycle (Figure 11F). This reduction in locomotor activity is consistent with many studies where activity was measured after the establishment of a large increase in body weight and fat mass (An 2015, Bumaschny 2012, Dong 2012, Huo 2009), and is generally an effect of reduced mobility in heavier animals. However, at 2 weeks, when group body weights were only beginning to diverge, no significant differences in locomotor activity were found during either cycle (Figure 11C). Taken together, these data suggest that neither deficits in energy expenditure nor any effect of locomotion account for the phenotype of increased body weight produced by the ablation of TrkB receptors from the PVH.

It should be noted that, during the CLAMS session, recorded VO$_2$ and VCO$_2$ values at 3 weeks post-injection for one of the AAV-Cre injected females were substantially lower than for the values from the remaining AAV-Cre and also the AAV-GFP mice. Upon examining the cage, each of which should be airtight in order to precisely monitor changes in oxygen and carbon dioxide, I discovered that the edge of one corner had come unglued. This created an air leak that likely interfered with the accuracy of measurements, and thus the VO$_2$, VCO$_2$, and calculated RER data for this specific animal at this time point were suspect to outlier status. As statistical evidence, I performed Chauvenet’s criterion and Dixon’s Q test analyses, both of which indicated that these values were outliers. Therefore, at the 3 week time point in females, these data were removed, decreasing the n by 1 for AAV-Cre mice. However, infrared beam breaks were not affected by the air leak, thus locomotor activity data for this animal were retained.

In males, there were also no significant differences in oxygen consumption or respiratory exchange ratio between groups during either cycle (Figure 11G,H). As in females, these data
indicate that AAV-Cre mice do not exhibit deficits in energy expenditure, and that fuel utilization is similar between groups despite substantially larger fat stores. However, locomotor activity was also significantly reduced in male AAV-Cre injected mice during the dark cycle, with a trend during the light cycle (Figure 11I). However, since male mice were only measured at 9 weeks, there is insufficient data to conclude whether the locomotor reduction is a causative factor of the obesity in these animals.
**Female 2-Week Oxygen Consumption**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Light</th>
<th>Dark</th>
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- **AVG VO2 (mg O2/kg lean mass/hr)**

**Female 2-Week Respiratory Exchange**

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- **AVG RER (VCO2 : VO2)**

**Female 2-Week Locomotor Activity**

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- **X-Beam Breaks (x1000)**

**Female 9-Week Oxygen Consumption**

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- **AVG VO2 (mg O2/kg lean mass/hr)**

**Female 9-Week Respiratory Exchange**

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- **AVG RER (VCO2 : VO2)**

**Female 9-Week Locomotor Activity**

<table>
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- **X-Beam Breaks (x1000)**

**Male 9-Week Oxygen Consumption**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Light</th>
<th>Dark</th>
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<tbody>
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</table>

- **AVG VO2 (mg O2/kg lean mass/hr)**

**Male 9-Week Respiratory Exchange**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Light</th>
<th>Dark</th>
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</table>

- **AVG RER (VCO2 : VO2)**

**Male 9-Week Locomotor Activity**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Light</th>
<th>Dark</th>
</tr>
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</table>

- **X-Beam Breaks (x1000)**
Figure 11: PVH TrkB deletion does not cause reduced energy expenditure.

Oxygen consumption (A,D,G), respiratory exchange ratio (B,E,H) and locomotor activity (C,F,I) for females at 2 weeks (A-C), females at 9 weeks (D-F), and males at 9 weeks (G-I).

**A,D,G:** Lean-mass-normalized oxygen consumption rates averaged separately across light and dark cycle for PVH-injected fB/fB mice. Two-way ANOVA with repeated measures.

**B,E,H:** Respiratory exchange ratios averaged separately across the light and dark cycle for PVH-injected fB/fB mice. Two-way ANOVA with repeated measures.

**C,F,I:** Total X-beam breaks summated separately across the light and dark cycle for PVH-injected fB/fB mice. Two-way ANOVA with repeated measures (C,F,I) and Bonferroni post-tests (F,I).

Error bars indicate standard error. (*, p<0.05; **, p<0.01; ***, p<0.001)
**Food intake**

For AAV-Cre injected females, average daily food intake was significantly increased at both the early and late time points (Figure 12A). The magnitude of the increase at 3 weeks was strikingly high for such an early point. This strongly suggests that food intake is driving the increase in body weight, rather than being a consequence of increased body weight due to another cause. To further investigate this potentiality, I compared weight-normalized food intake (food intake / body weight) between groups using data from the 3 week time point when weight gain was only beginning to manifest, and found this measure to be significantly higher in AAV-Cre injected mice (Figure 12D). However, this difference is only meaningful if the relationship between food intake and body weight is not exponential; in which case, small incremental increases in body weight could account for increasingly larger elevations in food intake. Therefore, I plotted food intake as a function of body weight, and tested the proportionality of the relationship across both groups by linear regression. This analysis revealed that the relationship was linear across all animals, with an $r^2$ value of 0.9096 (Figure 12E). Since this relationship was linear, and normalized food intake was increased in AAV-Cre injected mice, these findings demonstrate that hyperphagia is at least partially driving the increase in body weight.

For males at 9 weeks, average daily food intake was not significantly different between groups (Figure 12B). This was likely due in part to the low n for this set of animals. However, two additional coincident factors were also present at this time point. (1) The body weights of AAV-Cre injected mice had essentially been exhibiting a plateau for approximately 3 weeks while control mice were still gaining weight; and (2) the individual ratios of weight gain to food intake over a set period, or feeding efficiency, were significantly reduced in AAV-Cre injected
males (Figure 12C). Given that food intake values were similar between groups despite significant body weight differences, it is likely that food intake became reduced as the consequence of a ceiling effect on body weight, bringing the food intake averages closer together. However, since food intake was not also measured prior to this time point in males, there is insufficient data to conclusively determine if this is the case.
Figure 12: PVH TrkB deletion causes hyperphagia.

A: Average daily food intake for female AAV-GFP and AAV-Cre injected fB/fB mice at 3 & 9 weeks post-injection. Two-way ANOVA with repeated measures and Bonferroni post-tests.

B,C: Daily food intake (A) and feeding efficiency (B) for male AAV-GFP and AAV-Cre injected fB/fB mice at 9 weeks post-injection. One-tailed t-test.

D: Food intake normalized to body weight for females at 3 weeks post-injection. One-tailed t-test.

E: Plot of daily food intake vs body weight for females at 3 weeks post-injection. Superimposed is the best-fit line (dark red) determined by linear regression.

Error bars indicate standard error. (*, p<0.05; **, p<0.01; ***, p<0.001)
Discussion

Brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB) have been implicated in the regulation of energy homeostasis, particularly within the brain. However, efforts to determine the homeostatic role of BDNF-TrkB signaling have been challenging due to the involvement of this signaling in a great number of basic biological functions, including a critical role in tissue and organ development, and cell survivability. However, populations of BDNF- and TrkB-expressing neurons have been discovered that regulate energy homeostasis, and the hypothalamus has been a region of intense focus for teasing out the role of BDNF-TrkB signaling in energy homeostasis regulation.

Previous findings of obesity in mice where TrkB was deleted in SIM1-expressing cells have implicated these receptors in the paraventricular nucleus of the hypothalamus (PVH), which is one of several locations that express SIM1. In the present study, I show that TrkB receptors specifically within the PVH are critical for the regulation of body weight. I achieve this by demonstrating a severe obesity phenotype in mice where the TrkB coding sequence was selectively deleted from cells within the PVH. This obesity appears to be self-limiting, as animal body weights plateau at what could either be a sufficient level of adiposity or a new body weight set point defended by the same systems of energy homeostasis. I also show that the obesity is neither caused by, nor associated with, reduced energy expenditure; and that hyperphagia is the sole driver of increased body weight in these animals. However, at the late time point, hyperphagia was only observable in females. Whereas males, which had exhibited a body weight plateau for ~3 weeks at the time of food intake measurements, were not eating significantly more than controls under these conditions.
The body weight set point hypothesis describes a general mechanism whereby changes in body weight are not necessarily a failure of energy homeostasis, but rather could be the result of brain-encoded body weight set-point modulation. Consistent with this hypothesis, the relatively sudden loss of TrkB receptors from the PVH drastically increases food intake within a short time frame, which drives weight gain. The plateau effect on body weight seen at later stages in these animals could be considered as the deceleration of weight gain as body weight approaches the new (higher) set-point. This would also explain the finding that food intake in male PVH TrkB deletion mice, measured after the establishment of a body weight plateau, was not significantly higher than controls since, according to the hypothesis, increased food intake would no longer be necessary to drive weight gain once the new set-point had been reached. The body weights of female PVH TrkB deletion mice were only just beginning to plateau when food intake was measured at 10 weeks post-injection. However, group average food intake for these mice was slightly reduced at 10 weeks compared to 3 weeks, suggesting that food intake may be on the decline. Therefore, males may have reached the new set-point sooner, possibly due to an increased baseline weight gain relative to females that also causes male controls to gain more weight than female controls. Nonetheless, male body weights reaching plateau earlier than females is sufficient to explain the disparity between male and female food intake at the late time point.

Based on the findings of these experiments, TrkB receptors within the PVH are critical for the suppression of food intake and the regulation of body weight, but are not involved in the regulation of energy expenditure. However, the circuitry of PVH$^{TrkB}$ neurons, through which these effects are mediated, have yet to be determined.
Neuronal tracing and retrograde confirmation of PVH$^{\text{TrkB}}$ neurons

TrkB receptors within the PVH regulate body weight by suppressing food intake, but do not regulate energy expenditure. However, the circuitry of these particular PVH neurons that mediates this effect remains unknown. Previous studies have identified many brain regions that receive projections from generic PVH neurons (Geerling 2010, Tóth 1999), while others have focused on projections from non-TrkB PVH neurons (An 2015, Garfield 2015, Sutton 2014, Weiss & Leibowitz 1985).

In order to determine the locations to which PVH$^{\text{TrkB}}$ neurons project, I used anterograde tracing to visualize brain regions containing axon terminals from these neurons. To do this, I delivered a virus into one hemisphere of the PVH that caused TrkB-expressing cells within the injection site to begin expressing the red fluorescent protein tdTomato, which distributed throughout these cells including cell bodies, axons, and axon terminals. I then compiled a list of the locations containing what appeared as terminal-like tdTomato signal (TLS), which indicated that a location may receive projections from PVH$^{\text{TrkB}}$ neurons. However, this method does not conclusively demonstrate the existence of synapses within identified regions. Therefore, in separate animals that globally express tdTomato in all TrkB-expressing cells, including PVH$^{\text{TrkB}}$ neurons, I injected a retrograde tracer (green Retrobeads™; GRBs) into one of the projection targets identified through anterograde tracing, using multiple animals to collectively cover all targets that are capable of clean injections. This GRB tracer can be taken up by axon terminals within the injection site and transported back to their respective cell bodies, including those presumably in the PVH. However, the physical size of these beads is sufficiently large that they are not taken up by damaged axons projecting to other targets through the
injection site or needle tract. I then analyzed the colocalization of GRB signal and tdTomato expression in PVH^{TrkB} neurons to confirm the existence of individual projections.
**Generation of PVH\textsuperscript{TrkB} projection reporter mice**

To generate a list of candidate brain regions that likely receive projections from PVH\textsuperscript{TrkB} neurons, I used the Cre-Lox system to express tdTomato within PVH\textsuperscript{TrkB} neuron cell bodies, axons, and axon terminals. Using male and female \textit{TrkB\textsuperscript{CreER/+}} mice at 8 weeks of age, I stereotaxically injected 0.2 - 0.3 µL of the Cre-dependent tdTomato-expressing virus rAAV2/CAG-FLEX-tdTomato (AAV-FLEX-tdT) into one hemisphere of the PVH. Data from projection studies of generic PVH neurons demonstrate that a portion of PVH projections remain within the same (ipsilateral) hemisphere, while others cross over to the opposite (contralateral) hemisphere via specific decussation sites (Geerling JC 2010, Tóth ZE 1999). These studies also show that, within a set of PVH neurons projecting to the same target, subsets of these neurons can project either ipsilaterally, contralaterally, or to both hemispheres via decussating colaterals. Therefore, delivering this virus unilaterally allows for the determination of projection lateralization, since a comparison can be made between the hemisphere of a brain region containing TLS and the hemisphere of the PVH that was injected.

Because neurons along the length of the needle tract may also become infected, false positives could be produced by tdTomato signal within axon terminals of non-PVH TrkB-expressing neurons along the needle tract. Therefore, I injected half of the mice using a vertical needle path (Figure 13A), and the other half using an angled needle path within the coronal plane, approaching from the lateral brain surface of the ipsilateral hemisphere (Figure 13B). Any brain regions containing tdTomato signal in successful vertically-injected animals, but not in successful angle-injected animals, or vice versa, are likely terminal fields for non-PVH needle tract TrkB neurons.
The coding sequence for tdTomato within the genome of the AAV-FLEX-tdT virus is located within a specific variant of “Double-floxed Inverted Open reading frame” (DIO) cassettes (known as FLEX), preventing expression until Cre recombinase (Cre) performs recombination to revert the sequence and allow expression. Therefore, within the injection site of TrkB<sup>CreER/+</sup> mice, only TrkB-expressing neurons are capable of disinhibiting viral tdTomato expression. The particular gene expressed by TrkB<sup>CreER/+</sup> mice is a fusion protein of Cre and an estrogen receptor that has been modified to respond only to exogenous tamoxifen. This protein is trapped in the cytosol until activated by tamoxifen to translocate to the nucleus, where Cre can then mediate recombination. Thus, tamoxifen must be administered to induce Cre-mediated disinhibition of tdTomato expression.

Following the stereotaxic injections, I allowed the animals to recover for 1 week before administering tamoxifen (2 mg/day I.P.) for 5 days. In order to ensure sufficient time for tamoxifen-induced Cre recombination, tdTomato expression, and trafficking of the fluorescent proteins to axon terminals; I allowed at least 3 weeks for these processes before collecting nervous tissue and performing histological analysis.

**Injection site analysis**

Using the virally-induced tdTomato expression within cell bodies, I analyzed the infection strength of each animal within the PVH and in other regions near the PVH and along the needle tract. Within the PVH, I generated scores for each subdivision in a manner similar to that in section 3.1; however, these scores only accounted for individual hemispheres with no averaging since the injections were delivered unilaterally. For one angled injection animal, there was a small amount of PVH infection on the contralateral side within two subdivisions. For this animal,
separate subdivision scores were generated for each hemisphere and reported together separated by a forward slash (/) (Table 5, top). Scores for non-PVH nuclei were also generated in a manner similar to that in section 3.1; however, only the volume of the region from one hemisphere was taken into account for this scoring (Table 5, bottom).
Figure 13: AAV-FLEX-tdT injections into the PVH.

A,B: Large-scan epifluorescence images of coronal brain sections containing the PVH from two representative TrkB<sup>CreER<sup>+</sup></sup> mice receiving either a vertical (A) or angled (B) AAV-FLEX-tdT injection into the PVH.

A’,B’: Flattened confocal stack images at higher magnification of the PVH in A (A’) and B (B’). PVH boundary outlined with a white dashed line.

tdTomato expression displayed in red, and the non-specific nuclear stain (DAPI) in blue.
Table 5: Infection scores for AAV-FLEX-tdT injected TrkB^{CreER^{+}} mice.

<table>
<thead>
<tr>
<th>AAV-FLEX-tdT Cell Bodies</th>
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<td>•••••••••</td>
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<td><strong>dorsolateral</strong></td>
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<td><strong>lateral</strong></td>
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Infection scores for PVH subdivisions, non-PVH injection site nuclei, and needle tract nuclei; tabulated for each AAV-FLEX-tdT injected TrkB^{CreER^{+}} mouse, and separated into vertical and angle injected animals. Each column represents a separate animal, and animals are listed in descending order of whole PVH infection from left to right separately within vertical and angled groupings.

Columns representing male mice are tinted blue, and females are tinted red.
Identification of probable \( \text{PVH}^{\text{TrkB}} \) projection targets

Using tdTomato expression within axon terminals of infected PVH\(^{\text{TrkB}} \) neurons, I analyzed the entire brain and the spinal cord to compile a list of regions containing terminal-like tdTomato signal (TLS). For each animal, I generated a score value for both hemispheres of every region containing TLS, based on a 5-point scoring method similar to that used in section 3.1, but only taking into account the total size of the nucleus within one hemisphere. I then implemented three sets of exclusion criteria to generate the final list of TLS-containing regions likely to be true PVH\(^{\text{TrkB}} \) projection targets. I first compared the lists of vertically injected animals to those of angle injected animals, and regions that only appeared in one set were excluded from the final list. I then used the list from one animal that received a shallow miss injection, and thus did not contain any infection within the PVH (Figure 14). All TLS found in the brain of this particular animal originated from TrkB-expressing neurons along the needle tract. Therefore, these regions were also excluded from the final list. However, this was only done for regions where the signal strength in the shallow miss animal was greater than or equal to that in all other animals. This was done to prevent false negatives in the event that a region simultaneously receives projections from PVH\(^{\text{TrkB}} \) neurons and TrkB-expression neurons along the needle tract. Finally, I examined the consistency of TLS-containing regions across animals, and any regions that were patently inconsistent relative to the PVH infections were removed. This inconsistency was determined as the apparent randomness of the presence or absence, and signal strength, of TLS within a region across animals that had nearly identical PVH infections. Of note, none of the regions excluded by these methods were known to receive projections from generic PVH neurons.
These sets of exclusions produced the final list of TLS regions that simply could not be excluded. I then examined this list in order to determine, when possible, the potential relationship between TLS and the infection within PVH subdivisions. This relationship analysis examined the trend in signal strength of TLS within a region across all animals and compared this trend to that of the infection within PVH subdivisions. Regions for which a relationship was discernable were classified as “relationship present,” and those for which a consistent relationship could not be detected were classified as “no direct relationship.” TLS scores for all regions on the final list were tabulated and reported, though regions within different classification groups were clustered separately in the tabulation (Table 6). I also generated a table aligning ipsilateral TLS scores with the respective PVH infection scores, with animals sorted based on total PVH infection, irrespective of injection angle (Table 7). Additionally, I compiled and tabulated the apparent relationships that classified regions as ‘relationship present’ (Table 8), and these regions for which a potential relationship could be established were considered as significantly more likely to be true projection targets than those classified as ‘no direct relationship.’ Additionally, no appreciable differences were found between genders.

The resulting sub-list of ‘relationship present’ regions consists of (in descending order of signal strength and occurrence): the median eminence (ME) & posterior pituitary (Figure 15A1-A4); nuclei of the dorsolateral pons (Figure 15B1-B4) including the lateral parabrachial nucleus (l-PBN), medial parabrachial nucleus (m-PBN), and locus coeruleus (LC); nuclei of the dorsomedial medulla (Figure 16A1-A3) including the nucleus of the solitary tract (or nucleus tractus solitarius; NTS) and dorsal motor nucleus of the vagus (DMV); and the ventrolateral part of the periaqueductal gray (vl-PAG) (Figure 16B1-B3). TLS in all regions except the NTS were found ipsilateral to the PVH injection site; while TLS within the NTS was found bilaterally, with
an ipsilateral dominance. The contralateral TLS was found to decussate at the level of central NTS within the commissural subnuclear compartment. Regions containing some amount of TLS that were not excluded, but for which a consistent patterned relationship with the PVH infection could not be determined were all within the hypothalamus. These regions consist of: the arcuate nucleus (ARC), dorsomedial nucleus (DMH), anterior hypothalamus (AH), ventromedial nucleus (VMH), and the area that occupies the spaces between the DMH & VMH and between the DMH and the third ventricle (internuclear space).

Of note, regions that did not contain TLS but are known to receive projections from generic PVH neurons include Barrington’s nucleus, the ventrolateral medulla, and the intermediolateral column of the spinal cord.
Figure 14: Shallow PVH miss of one AAV-FLEX-tdT injection.

A: Large-scan epifluorescence image of a coronal brain section containing the PVH from an angled-injection animal where no infection was found within the PVH due to a shallow injection (A).

B: Enlargement of the PVH region, cropped from the image in A, with PVH boundaries outlined in white dashed lines.

tdTomato expression displayed in red, and the non-specific nuclear stain (DAPI) in blue.
Table 6: Post-exclusion TLS scores for AAV-FLEX-tdT injected $TrkB^{CreER^{+}}$ mice.

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TLS scores for non-excluded brain regions, tabulated for each AAV-FLEX-tdT injected $TrkB^{CreER^{+}}$ mouse. Regions are grouped based on if a parallel patterned relationship between the PVH infection and TLS within the region was found. Each column pair (white & grey) represents a separate animal, and animals are listed in the same order as Table 4.

TLS scores ipsilateral to the PVH injection are tabulated in white subcolumns (labeled “I”), while scores for the contralateral side are tabulated in grey subcolumns (labeled “C”).
Table 7: Comparison of ipsilateral TLS scores with PVH infection.

<table>
<thead>
<tr>
<th>AAV-FLEX-tdT Cell Bodies</th>
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<th>Ipsilateral TLS Scores</th>
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<td><strong>VMH</strong>:</td>
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<td><strong>AH</strong>:</td>
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<tr>
<td><strong>DMH</strong>:</td>
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<tr>
<td><strong>Arcuate (ARC)</strong>:</td>
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</tbody>
</table>

Infection scores for PVH subdivisions, the AH, and DMH from table 4 (top) aligned with ipsilateral TLS scores from table 5 (bottom). Each column represents a separate animal, and all animals are listed in descending order of whole PVH infection from left to right, irrespective of injection angle. Ipsilateral TLS scores are also listed in descending order of whole group signal across animals from top to bottom, within each PVH relationship group.

Columns representing male mice are tinted blue, and females are tinted red.
Table 8: Apparent relationships between TLS signal and AAV-FLEX-tdT infection in the PVH.

<table>
<thead>
<tr>
<th>Anterior PVH &amp; Central PVH</th>
<th>Median Eminence (ME) &amp; Posterior Pituitary (PP) &lt;Bilateral&gt;</th>
</tr>
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<tbody>
<tr>
<td>Dorsomedial Central PVH &amp;/or Medial Posterior PVH</td>
<td>Dorsolateral Pons Group &lt;Ipsilateral&gt;</td>
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<td>Medial Parabrachial Nucleus (m-PBN) Lateral Parabrachial Nucleus (l-PBN) Locus Coeruleus (LC)</td>
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<td>Dorsomedial Central PVH &amp;/or Medial Posterior PVH</td>
<td>Dorsomedial Medulla Group</td>
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<td>Nucleus of the Solitary Tract (NTS) &lt;Bilateral&gt;</td>
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<td>Dorsal Motor Nucleus of the Vagus (DMV) &lt;Ipsilateral&gt;</td>
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<tr>
<td>Dorsomedial Central PVH &amp;/or Medial Posterior PVH</td>
<td>Ventrolateral Periaqueductal Gray (vl-PAG) &lt;Ipsilateral&gt;</td>
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Shown are PVH subdivisions (left) horizontally paired with likely projection targets (right), together depicting potential PVH subdivision-specific projections. These potential projections represent the apparent relationships between TLS found in the target region and the analysis of the viral infection within the PVH.
Median Eminence (ME) & Posterior Pituitary (PP):

Dorsolateral Pons Group:
Lateral Parabrachial Nucleus (LPB)
Medial Parabrachial Nucleus (MPB)
Locus Coeruleus (LC)
**Figure 15: TLS in the ME, PP, and Dorsolateral Pons Group.**

**Set A:** Median Eminence (ME) and Posterior Pituitary (PP)

A₁: Mouse brain atlas diagram showing the ME in red.

A₂: Flattened confocal stack image showing TLS within the ME.

A₃, A₄: Epifluorescence images showing TLS within the PP, with boundaries outlined.

**Set B:** Dorsolateral Pons Group (DLPG)

B₁: Mouse brain atlas diagram of DLPG showing the L-PBN in yellow, the superior cerebellar peduncle (scp) in grey, the M-PBN in green, and the LC in blue.

B₂: Large-scan epifluorescence image of a coronal brain section containing the DLPG with TLS in each region, superior cerebellar peduncle (scp) shown in black with white outline, and the DLPG region outlined in yellow.

B₃, B₄: Flattened confocal stack images at higher magnification showing TLS in the M-PBN and L-PBN (B₃), and the M-PBN and LC slightly more caudally (B₄); with boundaries of the scp, LC, and fourth ventricle (4V) outlined.

tdTomato expression displayed in red, and the non-specific nuclear stain (DAPI) in blue.
**Dorsomedial Medulla Group:**
Nucleus of the Solitary Tract (NTS)
Dorsal Motor Nucleus of the Vagus (10N; DMV)

**Ventrolateral Periaqueductal Gray (VLPAG; vl-PAG):**
Figure 16: TLS in the NTS, DMV, and vl-PAG.

**Set A:** Dorsomedial Medulla Group (DMMG)

A₁: Mouse brain atlas diagram of the DMMG showing the NTS in light purple and the DMV in dark purple.

A₂,A₃: Flattened confocal stack image showing TLS within the DMMG without DAPI (A₂), and with DAPI and boundaries outlined (A₃).

**Set B:** Ventrolateral Periaqueductal Gray (vl-PAG)

B₁: Mouse brain atlas diagram showing the vl-PAG in brown.

B₂,B₃: Flattened confocal stack images showing TLS in the vl-PAG from two separate animals.

tdTomato expression displayed in red, and the non-specific nuclear stain (DAPI) in blue.
Figure 17: Illustration of PVH\textsuperscript{TrkB} neuron projections.

Sagittal view diagram of the mouse brain, showing the PVH (white region) containing TrkB-expressing neurons depicted as filled with tdTomato protein (red circles with squiggle lines representing dendrites inside the PVH).

Also shown are tdTomato-filled axons (red lines) and terminals (red triangles at the ends of these lines). One set of axons is depicted as a single line projecting ventrally to and through the median eminence (ME; orange oval) and into the pituitary (PIT; black oval). The other set of axons is a common fiber bundle for descending projections, and is depicted as a triple line travelling caudally, with oscillatory movements in the dorsoventral (and also mediolateral) direction as it progresses.

In addition to the ME and PIT, the projection targets of the ventrolateral periaqueductal gray (vl-PAG; yellow oval), parabrachial nucleus (PBN; green oval), locus coeruleus (LC; blue circle), dorsal motor nucleus of the vagus (DMV; grey oval) and nucleus of the solitary tract (NTS; pink oval) are depicted as being innervated by PVH\textsuperscript{TrkB} neuron axons projecting through the common fiber bundle.

For both the hypothalamo-pituitary (HP) projections and the common fiber bundle, axon paths are depicted as seen in brain tissue sections. Significant lengths of the HP axons can be seen within a single brain section, since they travel nearly parallel to the coronal plane, and only a few sections are necessary to capture the entirety of their length. The common fiber bundle, however, travels orthogonally to the coronal plane. Their path was determined by tracking the changing location of the axonal signal cluster within the coronal plane as axons traveled caudally within the brain.
**Generation of PVH\(^{TrkB}\) projection confirmation mice**

Anterograde tracing of axonal projections provides evidence that a projection may exist; however, the methodology fails to demonstrate the existence of synapses within a location identified by the appearance of axon terminals. Retrograde tracing, on the other hand, utilizes compounds that are able to be taken up by axon terminals within an injection site, and then undergo retrograde transport back to the respective cell bodies, where they can be visualized by either immunohistochemical staining or through a pre-conjugated fluorophore.

In order to demonstrate that functional synapses exist within potential projection targets identified through anterograde tracing, I used green fluorophore-conjugated Retrobeads\(^{TM}\) (green retrobeads; GRBs) to label PVH neurons that project to each specific location. To visualize TrkB-expressing neurons within the PVH, I utilized a TrkB reporter mouse that simultaneously harbors the \(TrkB^{CreER/+}\) allele and a global Cre-dependent tdTomato allele (\(Ai9\)), or \(TrkB^{CreER/+};Ai9/+\) mice. Following tamoxifen administration and Cre recombination, all TrkB-expressing cells in these mice, including neurons within the PVH, begin to express tdTomato. Injecting GRBs into identified targets in these mice allows for colocalization analysis between GRB signal within the PVH and tdTomato signal in PVH\(^{TrkB}\) neurons. Performing these injections unilaterally would also provide confirmation on the lateralization of the projections, as an ipsilateral projection would result in GRB signal within the hemisphere of the PVH ipsilateral to the injection, or the contralateral hemisphere for contralateral projections. Therefore, in \(TrkB^{CreER/+};Ai9\) mice at 8 weeks of age, I unilaterally injected GRBs into one of the following identified targets, each in separate animals: the ventrolateral periaqueductal gray (vl-PAG), the lateral parabrachial nucleus (l-PBN), the locus coeruleus (LC), and the nucleus
of the solitary tract (NTS). Injections to the locus coeruleus were performed using an angled needle path within the coronal plane, approaching from the lateral brain surface of the contralateral hemisphere, in order to completely avoid GRB coverage of the PBN. All other injections were performed vertically. Following the stereotaxic injections, I allowed the animals to recover for 1 week before administering tamixofen (2 mg/day I.P.) for 5 days. In order to ensure sufficient time for Cre recombination, tdTomato expression, and trafficking of the GRBs; I allowed 3 weeks for these processes, or 4 weeks for NTS injected mice, before collecting brain tissue and performing histological analysis.

Retrotracer Exclusions

The median eminence (ME) is located at the ventral-most surface of the brain along the midline, and is essentially a very thin bridge of tissue that forms the ventral boundary of the third ventricle. The tissue is not of sufficient thickness to inject using available means of stereotaxic injection; therefore, it was excluded from this study.

Selectively injecting the dorsal motor nucleus of the vagus (DMV) is manifestly problematic, as it is enveloped by the dorsally situated NTS from its medial, lateral, and dorsal boundaries. This is the case throughout the majority of the rostrocaudal extent of both nuclei. Using standard stereotaxic methods, needle approach paths to the DMV that do not pass through the NTS exist only at the rostral-most end of these nuclei. At this location, the cross-sectional areas of both nuclei are much smaller, and the degree of TLS found here was considerably lower than in all other segments. Since injected material has a strong tendency to flow back up the needle tract, at least partially, DMV injections essentially always diffuse into the NTS, preventing the ability to conclusively confirm projections to this nucleus. Therefore, the DMV was excluded.
Selectively injecting the medial parabrachial nucleus (m-PBN) is reasonably unfeasible. The nucleus appears diagonally within the coronal plane in a dorsomedial to ventrolateral orientation. The l-PBN is oriented in a strictly parallel relationship to the m-PBN, situated directly dorsal and slightly lateral to the m-PBN, blanketing both it and the superior cerebellar peduncle fiber bundle that separates them. Given this layout, the m-PBN cannot be injected vertically without passing through the l-PBN. An angled approach to the m-PBN from the dorsolateral brain surface would also pass through the l-PBN along essentially its entire rostrocaudal and mediolateral lengths. Directly medial to the m-PBN is the LC, which also cannot be injected vertically due to proximity diffusion into the PBN near the LC or along the needle tract. However, the LC can be injected at an angle with an approach path from the contralateral brain surface. Theoretically, the m-PBN could also be injected in this manner, though it would have to precisely insert between the l-PBN & the LC, and also be of low volume to minimize the spread from diffusion. Given the considerable difficulty of injecting the m-PBN, and that TLS in this region was coincident with the l-PBN, the m-PBN was excluded from this study.
**Confirmation of PVH$^{TrkB}$ neuron projections**

Using the fluorescent signal of GRBs that remain within the injection site, I first verified that the injections landed sufficiently within the intended region, and also not within other regions where TLS was found. For successfully injected animals, I then analyzed the colocalization of retrogradely transported GRB signal with tdTomato expressed in the cell bodies of TrkB-expressing neurons within the PVH ($^{PVH}$TrkB neurons). To do this, I imaged GRB-positive segments of the PVH using scanning confocal microscopy, with a maximally closed pinhole. I captured individual confocal plane images over the entire 40 µm thickness of the tissue section within the z-dimension. Each individual confocal plane image was examined for the abundance of GRB-positive cells (GRB-cells), and additionally for the abundance of $^{PVH}$TrkB neurons containing at least one GRB cluster (colocalized cells). While technically only one colocalized cell is necessary to confirm that at least a weak projection exists given a clean specific injection, I quantified the abundance of both GRB-cells and colocalized cells within PVH subdivisions by generating scores in a manner identical to PVH infection scoring in anterograde tracing; and I tabulated the results (Table 9). Taking into account the coverage of the target nucleus by the GRB injection, these scores can be used as an indicator of projection strength. For all successfully injected animals, irrespective of injection site, no GRB signal was found within the anterior segment of the PVH. This is consistent with the apparent relationships between TLS and PVH infection detected through anterograde tracing.

For injections to the vl-PAG (Figure 18A1), the hemisphere of the PVH ipsilateral to the injection contained few GRB-positive neurons (GRB-neurons), which were found only within the ventral subdivision of central PVH and medial subdivision of posterior PVH (Figure 18A2).
The contralateral PVH contained only trivial amounts of GRB signal. All colocalized cells were found within the medial subdivision of posterior PVH. These findings are consistent with the proposed projection relationship from anterograde tracing. However, the adjacent zona incerta (ZI), which is situated slightly dorsolateral to the PVH, was rich with GRB signal, ipsilateral to the injection. Given that AAV-FLEX-tdT infection within ZI, when found, was typically coincident with infection in the dorsolateral subdivision of central PVH, TrkB projections from ZI may instead account for the apparent relationship between TLS in the vl-PAG and the GRB-signal lacking dorsolateral PVH subdivision.

For injections to the l-PBN (Figure 18B1,B2), the ipsilateral PVH was rich with GRB signal (Figure 18B3-B4'), while the contralateral side contained only a trivial amount. These injections produced the greatest number of GRB-neurons within the ipsilateral PVH. The vast majority of these cells were found within central PVH in all three subdivisions, with the remaining cells located in the medial subdivision of posterior PVH. Within central PVH, however, few colocalized cells were found in the ventral subdivision, and a majority in the dorsal part was found within the dorsomedial subdivision. In posterior PVH, all GRB-neurons were found in the medial subdivision, including a trivial number of colocalized cells. These findings are also consistent with the proposed projection relationship from anterograde tracing.

For injections to the LC (Figure 19A1,A2), essentially all GRB-neurons were found within the ipsilateral PVH (Figure 19A3-A5'), with a trivial number on the contralateral side. Central PVH contained the vast majority of GRB-neurons, with posterior PVH containing near-trivial numbers in the medial subdivision. Within central PVH, the vast majority of colocalized cells were found near the interface of the dorsal and ventral subdivisions, with the majority of these located within
the ventral subdivision. These finding are not entirely consistent with the proposed relationship from anterograde tracing, though this is likely due to the functionally arbitrary boundaries of the PVH subdivisions used for analysis. Since most of the colocalized cells were clustered across one of the boundaries, an anterograde tracing score for this overlapping region would need to be generated to more accurately parse the relationship.

For injections to the NTS (Figure 19B), both hemispheres of the PVH contained a similar number of GRB-neurons, though the overall amount of GRB signal was much stronger ipsilateral to the injection (Figure 19B'). Similar numbers of GRB-neurons were found in central and posterior PVH. The majority found in central PVH was located within the dorsomedial subdivision, with a minority in the ventral subdivision; and the majority found in posterior PVH was located within the medial subdivision. However, colocalized cells were found almost exclusively in posterior PVH, specifically in the more anterior portion but not in the more posterior portion; the majority of which were within the medial subdivision. These findings are consistent with the proposed projection relationship from anterograde tracing.
Table 9: Retrotracer tracer signal and colocalization analysis for GRB-injected $TrkB^{CreER/++;Ai9}$ mice.

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<th>Colocalization Analysis for Retrograde Projection Confirmation</th>
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Scores representing the total number of GRB-positive neurons [GRB(+)]) and colocalized tdTomato-expressing neurons positive for GRB signal [(+(+])] within subdivisions of the PVH ipsilateral and contralateral to the GRB injection site.
Ventrolateral Periaqueductal Gray (vl-PAG):

Lateral Parabrachial Nucleus (l-PBN):
**Figure 18: Confirmation of PVH\textsuperscript{TrkB} projections to vl-PAG and l-PBN.**

**Set A:** Images from a TrkB\textsuperscript{CreER\textsuperscript{+};Ai9} mouse with GRBs delivered to the vl-PAG.

A\textsubscript{1}: Large-scan image of the injection site brain section, with whole PAG and boundaries of the ventrolateral region outlined.

A\textsubscript{2}: Flattened confocal stack image of bilateral PVH, with yellow-outlined region highlighting GRB signal.

A\textsubscript{2'}: Single confocal plane image of the yellow-outlined region in A\textsubscript{2}, with arrows indicating colocalized cells.

**Set B:** Images from a TrkB\textsuperscript{CreER\textsuperscript{+};Ai9} mouse with GRBs delivered to the l-PBN.

B\textsubscript{1},B\textsubscript{2}: Large-scan images of injection site brain sections, with superior cerebellar peduncle (scp) highlighted in semi-transparent gray.

B\textsubscript{3}: Flattened confocal stack image of bilateral PVH.

B\textsubscript{4}: Higher magnification flattened confocal stack image of ipsilateral PVH, with yellow-outlined region highlighting majority of GRB signal.

B\textsubscript{4'}: Single confocal plane image from the yellow-outlined region in B\textsubscript{4}, with arrows indicating colocalized cells.

tdTomato expression displayed in red, GRB signal in green, and the non-specific nuclear stain (DAPI) in blue.
Locus Coeruleus (LC):

Nucleus of the Solitary Tract (NTS):
Figure 19: Confirmation of PVH$^{TrkB}$ projections to LC and NTS.

**Set A:** Images from a $TrkB^{CreER+}/;Ai9$ mouse with GRBs delivered to the LC.

A$_1$, A$_2$: Large-scan images of injection site brain sections, with tyrosine hydroxylase staining to indicate the location of the LC.

A$_3$: Flattened confocal stack image of bilateral PVH.

A$_4$, A$_5$: Separate confocal plane images from the same higher magnification image stack of ipsilateral PVH, with yellow-outlined regions highlighting GRB signal.

A$_4'$, A$_5'$: Zooms of the yellow-outlined regions in A$_4$ & A$_5$, respectively; with arrows indicating colocalized cells.

**Set B:** Images from a $TrkB^{CreER+}/;Ai9$ mouse with GRBs delivered to the NTS.

B$_1$: Cropped large-scan image of the injection site brain section, with tdTomato expression within diffuse TrkB neuron and astrocyte arborizations inside the NTS to aid visualization.

B$_2$: Flattened confocal stack image of bilateral PVH.

B$_3$: Single confocal plane image from the confocal image stack shown flattened in B$_2$, with yellow-outlined region highlighting majority of GRB signal bilaterally.

B$_3'$: Zoom of the yellow-outlined region in B$_3$, with arrows indicating colocalized cells.

tdTomato expression displayed in red, GRB signal in green, and the non-specific nuclear stain (DAPI) in blue, and tyrosine hydroxylase staining in magenta (A$_1$ and A$_2$ only).
Discussion

The vast majority of projections from generic, non-neuroendocrine neurons of the PVH have been shown to be descending connections to nuclei in the midbrain and brainstem (Geerling 2010, Tóth 1999). However, these studies were not exhaustive analyses of projections from the entire volume of the PVH, and therefore may have overlooked a non-trivial amount of ascending connections to the forebrain. Nevertheless, the projections from specific populations of PVH neurons, such as those expressing MC4R (PVH^MC4R neurons), have been shown to correspond with subsets of the known descending projections (Garfield 2015).

In the present study, I demonstrate anatomical projections of \textit{TrkB}-expressing neurons in the PVH (PVH^{TrkB} neurons) to the median eminence & posterior pituitary, periaqueductal gray, dorsolateral pons, and dorsomedial medulla. The strength of each projection varied between regions, as evidenced by both the differing amounts of terminal-like signal (TLS) found within each region through anterograde tracing, and the amounts of colocalized cells from retrograde tracing. These differences may indicate the relative importance of individual projections to the overall functions mediated by these neurons. In this case, the periaqueductal gray would appear to be of relative little importance, compared with the other regions, as it displayed considerably less of both indicators. In particular, the median eminence and nucleus of the solitary tract, which both receive bilateral projections with an ipsilateral dominance, contained the highest density of TLS, and the NTS contained the most colocalized cells when accounting for both hemispheres. However, the parabrachial nucleus been demonstrated to play a significant role in energy homeostasis; and PVH^{TrkB} neurons have a substantial projection to this location.
Many of the regions confirmed to receive projections from PVH\textsuperscript{TrkB} neurons have been shown to regulate energy homeostasis, such as the LC, NTS, and the PBN. Additionally, several specific projections from the PVH have also been shown to directly regulate energy homeostasis, such as neuroendocrine axes projecting to the ME and PP, and PVH\textsuperscript{MC\textsubscript{4}R} neurons projecting to the PBN. Despite these known possibilities, it remains unclear which specific projections of PVH\textsuperscript{TrkB} neurons mediate food intake suppression.
Projection-specific PVH $TrkB$ deletion

TrkB-expressing neurons within the PVH suppress food intake, and project to several other regions known to be involved in the regulation of energy homeostasis. However, the degree of involvement in the suppression of food intake contributed by each individual projection remains to be determined. Previous studies have shown that PVH neurons expressing the melanocortin 4 receptor ($MC4R$; $PVH^{MC4R}$ neurons), which are a separate population from $PVH^{TrkB}$ neurons (Figure 2), also suppress feeding; and do so by projecting to the parabrachial nucleus (Shah 2014). However, these neurons do not project to the NTS, but do project to regions that were not found to be terminal fields of $PVH^{TrkB}$ neurons including the rostral ventrolateral medulla and the intermediolateral cell column of the spinal cord (Garfield 2015, Shah 2014). Parvocellular oxytocin neurons of the PVH project to the parabrachial nucleus, where they have been shown to regulate fluid intake but not feeding (Ryan 2017); the NTS, where they likely supplement the satiety-related effects of circulating oxytocin released from magnocellular neurons that also acts in this region (Blevins 2004, Uchoa 2013); and the intermediolateral cell column of the spinal cord, where they regulate sympathetic activity and pain signals (Eliava 2016, Sawchenko & Swanson 1982, Sutton 2014). These findings include data that demonstrate the existence of two specific PVH projections that suppress appetite, and these projections target some of the same nuclei as $PVH^{TrkB}$ neurons. Given this overlap, it is likely that a projection from $PVH^{TrkB}$ neurons to at least one of these common targets mediates food intake suppression; however, it remains unclear which of the identified projections are involved in this effect.
Generation of mice with projection-specific TrkB deletion in the PVH

In order to separately determine the relative contribution of individual PVH\textsuperscript{TrkB} neuron projections, I selectively deleted \textit{TrkB} from the populations of PVH neurons that project to a specific target (projection-specific PVH \textit{TrkB} deletion). To do this, I used homozygous floxed \textit{TrkB} (\textit{fB/fB}) mice and a two-virus injection paradigm that combines the Cre-Lox and FLP-FRT systems, with one virus delivered bilaterally to the PVH and the other bilaterally to a single projection target of PVH\textsuperscript{TrkB} neurons confirmed by retrotracing (Figure 20). For the treatment group, I used the virus rAAV2/fDIO-mcherry2Acre (AAV-fDIO-Cre) for the PVH, which contains the coding sequence for Cre recombinase (Cre) within a flippase (FLP)-dependent adaptation of DIO (fDIO), thus preventing Cre expression until FLP-mediated recombination reverts the inverted cassette. For the control group, I used the virus rAAV2/fDIO-mCherry (AAV-fDIO-mCherry), which expresses only mCherry following FLP-mediated recombination. However, until PVH neurons infected by one of these viruses also express FLP, they are unable to express either Cre & mCherry or mCherry alone, respectively. To deliver FLP expression to the PVH, and to produce projection specificity, I used the retrograde virus CAV2/CMV-flpE-GFP (CAV-FLP) for injections into individual PVH\textsuperscript{TrkB} projection targets, in separate animals. The virus was taken up by axon terminals within the injection site and transported back to their respective cell bodies, including those in the PVH, which become infected by this virus. These neurons then began expressing FLP, which alone does not produce an effect in normal cells. However, within AAV-infected PVH neurons that project to the target, expressed FLP disinhibited the expression of Cre and mCherry. Since the \textit{fB} allele has only the \textit{TrkB} coding sequence floxed, performing this two-virus manipulation in mice homozygous for
this allele (fB/fB mice) causes the deletion of the coding sequence (CDS) from the TrkB gene, and only in PVH neurons that project to the target (PVH^{TrkB-ΔCDS→Target} mice).

For projection-specific TrkB deletions, I targeted PVH projections to the parabrachial nucleus (PVH^{TrkB→PBN}) and the nucleus of the solitary tract (PVH^{TrkB→NTS}), due to the strength of TLS found within these regions, and to their demonstrated involvement in energy homeostasis. Considering that similar results were found between male and female mice with whole PVH TrkB deletions, and that no gender differences were apparent for anterograde tracing, I performed PVH projection-specific TrkB deletions in female mice only. Therefore, in female fB/fB mice at 8 weeks of age, I stereotaxically injected 250 nL of either AAV-fDIO-Cre or AAV-fDIO-mCherry bilaterally into the PVH. During the same surgery, I also injected 500 nL of the retrovirus CAV-FLP bilaterally into the PBN (300 nL in l-PBN and 200 nL in m-PBN) to produce PVH^{TrkB-ΔCDS→PBN} animals; or 100 nL of an 80:20 mix of CAV-FLP to GRBs bilaterally into each injection site within the nucleus of the solitary tract to produce PVH^{TrkB-ΔCDS→NTS} animals. I delivered retrovirus to each hemisphere of the PBN at four sites: two separate depths from a more rostromedial set of coordinates, and two depths from a more caudolateral set of coordinates. Each of the four shallow depths bilaterally, targeting the l-PBN, received 150 nL of virus; and then each lower depth, targeting the m-PBN, received an additional 100 nL of virus. I injected the NTS at two AP levels, one targeting central NTS at the level of area postrema; and the other targeting caudal NTS, which was caudal to area postrema. I also included injections into the rostral NTS (r-NTS), though this was done in only a subset of animals, and they were essentially identical in phenotype to within-group animals that did not receive injections to r-NTS.
Beginning with pre-surgery weight on the day of stereotaxic injection, I measured the body weight of all experimental animals weekly for a period of 8-9 weeks. Following this period, I also measured body composition and daily food intake.

**Injection site analysis**

Following the completion of all experimental measurements, I performed histological analysis to verify the accuracy of all injections. For NTS injections, the sites of infusion were clearly visible due to the strong presence of GRBs that remain within the injection site. TH staining was performed on all NTS-containing sections to label noradrenergic NTS neurons and aid in determining the boundaries of the nucleus. Only small amounts of GRB signal was found outside of the NTS across animals; and all animals showed GRB signal within the NTS (Figure 21A-F).

For PBN injections, the sites of infusion were visible due to the presence of two specific markers. The combination of needle tract tissue damage and the presence of retrovirus, which can have a slight immunogenic effect, created a small region of minor tissue damage that only occurred at site of infusion where the damage and virus were coincident. The retrovirus also typically infected a small amount of neurons within the injection site, possibly interneurons, which began expression GFP in their cell bodies. Tyrosine hydroxylase (TH) staining was performed on all PBN-containing sections to label the locus coeruleus (LC) and ensure that virus was not also delivered to this region. Any animals that contained either of the two markers within the LC, or contained neither marker within the PBN of either hemisphere and additionally showed negative results in PVH analysis, were removed from the study. The majority of animals were positive for at least one marker within the PBN (Figure 22A,B).
For the PVH injection sites of PVH^TrkB-ΔCDS→NTS (Figure 21G) and PVH^TrkB-ΔCDS→PBN (Figure 22C) mice, the abundance of mCherry-expressing neurons (mCherry-neurons) was analyzed within the PVH and non-PVH nuclei within the injection site and along the needle tract. Only trivial or near-trivial numbers of mCherry-neurons were found in nuclei other than the PVH across all animals, with the exception of two PVH^TrkB-ΔCDS→PBN mice that were excluded for other reasons. The PVH of only one PVH^TrkB-ΔCDS→NTS mouse and two (separate) PVH^TrkB-ΔCDS→PBN mice lacked mCherry-neurons in the PVH. For all animals, the abundance of mCherry-neurons was scored within each PVH subdivision, separately for each hemisphere. These scores were tabulated, and a weighted score was calculated for each animal (Table 10, Table 11). The only scores that were used to calculate the weighted score were those from subdivisions that contained colocalized cells in the retrotracing study. The formula for calculating the weighted score was created using the retrotracing scores for the abundance of colocalized cells. Specifically, the proportionality between the scores for colocalized cell abundance was used to weight the mCherry-neuron abundance scores for each respective PVH subdivision.

For bilateral PVH→NTS projections, colocalized cells were found within the medial subdivision of posterior PVH (++ ipsilateral, ++ contralateral) and the dorsomedial subdivision of central PVH (+ ipsilateral, < + contralateral). Using a value of 0 for < + (near-trivial), summating the scores of both hemispheres for each subdivision produces values of 4 and 1, respectively; producing an overall total value of 5. Subdivision weighting factors were then calculated as the sum of subdivision scores divided by the overall total for all subdivisions: \( \frac{4}{5} \) for medial posterior PVH and \( \frac{1}{5} \) for dorsomedial central PVH. To calculate the weighted score for PVH^TrkB-ΔCDS→NTS
mice, the sum of mCherry-neuron abundance scores for both hemispheres of these subdivisions are multiplied by their respective subdivision weighting factor and then summated. Thus, the weighted score (WS) for these animals can be calculated by the formula:

$$WS = \frac{4}{5} \cdot \left( \text{sum of } PVH_{P,m} \text{ scores} \right) + \frac{1}{5} \cdot \left( \text{sum of } PVH_{C,dm} \text{ scores} \right)$$

Using these scores, the three animals with the lowest weighted score values were removed from the study. The score for one of these animals was zero, and the values for the other two scores were similar. The characteristics of this threshold were such that the lowest weighted score for retained animals was more than double the value of the highest weighted score for excluded animals.

For ipsilateral PVH→PBN projections, all colocalized cells were found in central PVH within the dorsomedial (++) and dorsolateral (+) subdivisions. Thus, the total for all subdivisions is 3; and the subdivision weighting factors are \(\frac{2}{3}\) for dorsomedial central PVH and \(\frac{1}{3}\) for dorsolateral central PVH. Thus, the weighted score (WS) for these animals can be calculated by the formula:

$$WS = \frac{2}{3} \cdot \left( \text{sum of } PVH_{C,dm} \text{ scores} \right) + \frac{1}{3} \cdot \left( \text{sum of } PVH_{C,dt} \text{ scores} \right)$$

Using these scores, the three animals with the lowest weighted score values were removed from the study. The characteristics of this threshold were such that all three excluded animals had score values of zero, with all retained animals having non-zero score values (lowest is 2.00).
Figure 20: Illustration of PVH\textsuperscript{TrkB} projection targeting.

Sagittal view diagram of the mouse brain; showing the PVH (blue), a generic PVH\textsuperscript{TrkB} neuron projection target (purple), generic non target regions (grey), and the posterior pituitary (tan). The brain is displayed in the state after viral infections and gene expression; with color-coded arrows illustrating the locations of specific viral delivery. Only one Flp-dependent virus from the red category (top left) was used for each animal.

All projections to the target region are depicted as populations of neuronal cell bodies, each with a bundle of axons (green filled circles and lines; indicating the expression of Flp), after retrograde trafficking and infection by a retrograde Flp virus injected into the target.

PVH neurons of any type that do not project to the target (Any) are depicted as a population of cell bodies with multiple bundles of axons (black filled circle and lines; indicating no expression from either virus) after infection by only a Flp-dependent virus injected into the PVH.

PVH neurons that do project to the target are depicted as separate populations of TrkB-expressing (TrkB) and non-TrkB-expressing (Non) cell bodies and a common bundle of axons (yellow filled circles and line; indicating the expression of both Flp [green] and Flp-dependent mCherry with or without Cre recombinase [red]) after infection by both a Flp-dependent virus injected into the PVH and a retrograde Flp virus injected into the target.

When these injections are performed in animals homozygous for floxed TrkB (\textit{fB/fB} mice), the TrkB coding sequence is deleted from all PVH neurons that project to the target, and any of these neurons that express TrkB will have their receptors ablated.
Table 10: mCherry-neuron abundance scores in PVH subdivisions for PVH→NTS TrkB deletions.

<table>
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<tr>
<th>PVH Scoring for PVH-&gt;NTS Projection-Specific TrkB Deletion</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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Scores representing the abundance of mCherry-expressing neurons within subdivisions of the PVH, including scores for both hemispheres. Weighted score values for each animal are reported below subdivision scores, and animals are sorted by this score in descending order left to right. Animals excluded for low weighted score value are displayed in red.
Table 11: mCherry-neuron abundance scores in PVH subdivisions for PVH→PBN TrkB deletions.

<table>
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<tr>
<th>PVH Scoring for PVH→PBN Projection-Specific TrkB Deletion</th>
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Scores representing the abundance of mCherry-expressing neurons within subdivisions of the PVH, including scores for both hemispheres. Weighted score values for each animal are reported below subdivision scores, and animals are sorted by this score in descending order left to right. Animals excluded for low weighted score value are displayed in red.
**PVH → NTS TrkB deletion has no effect on energy homeostasis**

Remarkably inconsistent with the results of whole PVH TrkB deletions, the body weights of PVH^{TrkB-ΔCDS} → NTS mice were nearly identical to controls (Figure 21H). Consequently, fat and lean masses were similar (Figure 21I); and 4-hour food intake was also not different (Figure 21J). Hourly food intake was measured in lieu of daily food intake due to the lack of a difference in body weight, and with the hypothesis that feeding dynamics may have been altered, but with no net change in total food intake. However, this was not the case, as even short-term feeding was similar between groups.

The high density of TLS found within the NTS suggests that this population of PVH^{TrkB} neurons mediates an important function. However, these negative findings demonstrate that the regulation of energy homeostasis is not directly mediated by these neurons. The NTS is known to also regulate respiratory, cardiovascular, and gustatory (taste) processes; and mediate many visceral reflexes within these systems, including cough and the baroreflex. PVH^{TrkB} neurons that project to the NTS may modulate one or more of these functions, none of which have been shown to produce weight gain when regulation is disrupted.
**Female PVH/NTS-Injected fB/fB**

**Body Weight (g)**

Weeks Post-Injection

- (n = 06) Control
- (n = 08) Cre

**Body Composition**

- Fat
- Lean

**4-Hour Feeding**

- Control (n = 05)
- Cre (n = 04)

*ns*
PVH \rightarrow PBN TrkB deletion recapitulates hyperphagic obesity

Consistent with the results of whole PVH TrkB deletion, PVH^{TrkB-ΔCDS}→PBN mice gained significantly more weight than controls (Figure 22D); and the increase in body weight was the result of increases in both fat and lean mass compartments, with fat mass accounting for a considerably larger portion of the body weight increase (Figure 22E). AAV-fDIO-Cre injected mice became significantly heavier than controls at 4 weeks post-injection, within approximately the same time frame as AAV-Cre injected mice; however, the overall effect was not as robust.

Also consistent with whole PVH TrkB deletion, average daily food intake was significantly increased in PVH^{TrkB-ΔCDS}→PBN animals (Figure 22F). Parallel to the difference in effect size for body weight between deletion paradigms, the effect on food intake was also not as robust, and likely for the same reasons.

Taken together, these data demonstrate that the projections of PVH^{TrkB} neurons to the PBN suppress food intake to maintain body weight. However, this manipulation produced only a partial phenotype; and the number of Cre-reported cells was significantly fewer than in whole PVH TrkB deletions. This may have been due to incomplete viral coverage of the PBN, PVH, or both; or due to a phenomenon where multiple projections mediate food intake suppression and whole PVH TrkB deletions disrupted all projections simultaneously. Thus, the full magnitude of the relative contribution from this projection remains unclear. To determine this magnitude, each projection would need to be evaluated for its involvement in food intake suppression.
Figure 22: PVH$^{TrkB-ΔCDS}$→PBN mice recapitulate PVH $TrkB$ deletion.

A,B: Large scan epifluorescence images of coronal brain sections containing the injection site from a representative animal, with tyrosine hydroxylase staining to indicate the location of the LC (magenta).

C: Flattened confocal stack image of the PVH from the same animal as A & B.

D-F: Histograms showing weekly body weight across time (D), body composition at 10 weeks post-injection (E), and food intake at 10 weeks (F). Two-way ANOVA with repeated measures and Bonferroni post-tests (D), two-way ANOVA with Bonferroni post-tests (E); one-tailed t-test (F).

GRB signal displayed in green, tyrosine hydroxylase staining in magenta (A & B only), and the non-specific nuclear stain (DAPI) in blue.

Error bars indicate standard error. (*, p<0.05; **, p<0.01; ***, p<0.001)
**Discussion**

In the present study, I tested the relative contribution of two projections from PVH\textsuperscript{TrkB} neurons to the regulation of food intake suppression. This was accomplished by targeting the deletion of *TrkB* to specific populations of PVH neurons that project to each target, separately, both of which are nuclei known to play critical roles in the regulation of food intake.

I show that *TrkB* deletion in NTS-projecting PVH neurons have no effect on the regulation of energy homeostasis. Considering that the NTS has been shown to be a critical regulator of food intake suppression and satiety, these data demonstrate that TrkB receptors on these neurons are dispensable for the regulation of food intake and body weight. However, the strength of TLS found within the NTS suggests that this projection serves an important function. This lack of an effect suggests that the important function is not energy homeostasis regulation. The NTS is also known to mediate taste processing, blood pressure through the baroreflex, and breathing; though none of these functions have shown phenotypes of weight gain when disrupted.

I also show that *TrkB* deletion in PBN-projecting PVH neurons recapitulates the weight gain, fat accumulation, and hyperphagia seen in whole PVH *TrkB* deletions. However, the effect sizes were not as large as in whole PVH deletions. The numbers of Cre-reported cells (mCherry-neurons for projection-specific deletions, and GFP-cells for whole PVH deletions) were also not as large as in whole PVH deletions. This was likely due in part to the deletion occurring in only a subset of all PBN-projecting PVH\textsuperscript{TrkB} neurons, caused by incomplete viral coverage of the PBN, PVH, or both nuclei. However, multiple projections from PVH\textsuperscript{TrkB} neurons exist, and neurons projecting to targets other than the PBN were neither labeled by this manipulation nor had *TrkB* deleted from their genome. It is possible that multiple projections of PVH\textsuperscript{TrkB} neurons...
regulate food intake suppression, each with a maximum effectiveness that corresponds to a portion of the effect mediated by the entire population.

Taken together, these findings indicate differential roles for separate projections from PVH^{TrkB} neurons, and demonstrate that PBN-projecting PVH^{TrkB} neurons regulate body weight by suppressing food intake.
CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTIONS
The first part of my thesis demonstrates that TrkB expression within the PVH is critical for food intake suppression and body weight regulation. The deletion of TrkB specifically from this nucleus in the adult brain produces severe obesity driven by hyperphagia, but does not affect energy expenditure. This is the first direct evidence to demonstrate that TrkB receptors in the PVH regulate energy homoeostasis. Previous studies have investigated the effects of TrkB deletion in many non-PVH nuclei, including other hypothalamic nuclei (Ozek 2015), while a recent study by our lab deleted this receptor from a set of brain regions that included the PVH (Figure 3), though this deletion occurred early in development and thus may have been confounded by developmental compensation. By selectively deleting TrkB from the PVH in the adult brain, these data also demonstrate a specifically post-developental role for these receptors.

In addition to neurons, however, hypothalamic nuclei including the PVH also contain astrocytes, for which a role in the regulation of energy homeostasis has recently been established (Buckman 2013, Fuente-Martín 2012). TrkB-expressing astrocytes have been found within the arcuate nucleus of the hypothalamus (Liao GY 2015), though it has yet to be determined whether PVH astrocytes express TrkB. Theoretically, if this is the case, the loss of TrkB from these cells may also be contributing to the severe weight gain seen in whole PVH TrkB deletions. Selective ablation of these receptors from PVH astrocytes could provide additional evidence for whether these non-neuronal cells play a role in PVH-mediated satiety.

The second part of my thesis demonstrates the locations to which TrkB-expressing neurons of the PVH (PVHTrkB neurons) project. The purpose of this study was ultimately to determine if a single projection, or multiple projections, contribute to the suppression of food intake mediated by the entire population of TrkB-expressing PVH cells. Many of the regions identified as
projection targets have well-established roles in the regulation of food intake suppression. In particular, the parabrachial nucleus (PBN) and the nucleus of the solitary tract (NTS) mediate complex functions that process information from both the periphery and other regions within the brain. PVH\textsuperscript{TrkB} neurons project heavily to the NTS, and strongly to the PBN. In addition, heavy bilateral projections to the median eminence (ME) and strong bilateral projections to the posterior pituitary (PP) were also found. These projections implicate neuroendocrine systems, as the projections follow the same path as the hypothalamic portion of neuroendocrine axes. A weak projection to the ventrolateral periaqueductal gray (vl-PAG) was also found; however, this projection is relatively minor compared to all other projections identified.

This study could be expanded into double retrotracer experiments, where the presence or absence of PVH\textsuperscript{TrkB} neuron collaterals could be determined. For these experiments, \textit{TrkB}\textsuperscript{CreER\textsuperscript{+};Ai\textsuperscript{9/+}} would again be used to report PVH\textsuperscript{TrkB} neurons, but two different retrotracers of different colors would be delivered into separate projection targets within the same animal. Colocalization analysis would be performed as in the second half of section 3.2, though with the addition of signal from the second retrotracer. TrkB-expressing neurons containing signal from both retrotracers would serve as confirmation that collaterals exist for the specific set of targets injected; whereas a lack of triple-positive cells would indicate that collaterals do not exist given sufficient coverage of the injection sites.

The third part of my thesis demonstrates the relative contributions of two separate PVH\textsuperscript{TrkB} neuron projections to the suppression of food intake. This was accomplished by selectively deleting \textit{TrkB} from individual populations of neurons that project to a specific target, performed in separate animals for different targets. I show that \textit{TrkB} deletion in NTS-projecting PVH
neurons does not affect food intake or body weight; and these findings demonstrate that $\text{PVH}^{\text{TrkB}} \rightarrow \text{NTS}$ projections are not involved in the regulation of energy homeostasis. However, the endogenous function of these projections remains unknown.

I also show that $\text{TrkB}$ deletion in PBN-projecting PVH neurons recapitulates the hyperphagic obesity phenotype seen in whole PVH $\text{TrkB}$ deletions, though to a lesser degree. This was likely due to the deletion of $\text{TrkB}$ within only a subset of all PBN-projecting PVH$^{\text{TrkB}}$ neurons caused by incomplete coverage of the PVH, PBN, or both nuclei. However, multiple projections exist for PVH$^{\text{TrkB}}$ neurons, and it is also likely that more than one projection is involved in the regulation of energy homeostasis. In this case, the maximum effectiveness of each projection would represent a portion of the total effect mediated by all PVH$^{\text{TrkB}}$ neurons. However, these data demonstrate that PVH$^{\text{TrkB}}$ neurons that project to the PBN suppress food intake and regulate body weight.

While not tested, there is the potential for the existence of axon collaterals, where single PVH$^{\text{TrkB}}$ neurons may project to more than one location. In this case, deleting $\text{TrkB}$ from PVH neurons that project to one target could produce effects mediated through a different target. However, based on the literature, the only PVH neurons that have been found to exhibit axon collaterals are neurons that project to the spinal cord. PVH$^{\text{TrkB}}$ neurons do not project to the spinal cord; therefore, it is unlikely that the projection-specific deletions were confounded by collaterals. Nonetheless, double retrograde tracing in TrkB-reporter mice can be used to determine if PVH$^{\text{TrkB}}$ neurons exhibit collaterals. However, the single retrograde tracing data suggests that collaterals to both the NTS and PBN likely do not exist, since colocalized cells were generally found within separate subdivisions of the PVH, and the subdivisions of
predominance for each region were patently different. Despite this limitation, these data
demonstrate that TrkB receptors on PVH neurons that project to the PBN are critical for the
suppression of food intake and the regulation of body weight.

The projection of PVHTrkB neurons to the median eminence was not targeted in this study
due to a lack of sufficient technological means necessary to perform the injection. However,
targeting this projection has a reasonably high likelihood of producing an effect on food intake,
as neuroendocrine axes such as the HPT axis have been shown to regulate energy homeostasis.

PVHTrkB neurons that project to the pituitary alongside these axes are likely either modulating the
activity or function of these axes, or they express a hormone that was not stained for in the
immunohistochemical staining assays. Therefore, targeting TrkB deletion to this projection could
provide evidence for hormonal actions in the suppression of food intake. Toward a potential
method for targeting this projection, it has been demonstrated that retrograde tracers injected into
the bloodstream can be taken up by axon terminals of neuroendocrine neurons projecting to the
ME and pituitary, since these terminals lie outside of the blood brain barrier (Biag 2012). If a
retrograde virus preparation was created that was capable of being taken up in this manner, the
projections of PVHTrkB neurons to the ME and pituitary could also be tested for their relative
contributions to the suppression of food intake.

Also, projections to the locus coeruleus (LC) were not targeted in this study. However, the
technology and technical methodology for targeting this projection are currently available,
though the level of complexity is significantly higher. These injections represent the next logical
step for this segment of my thesis. Targeting the deletion of TrkB to this projection would
provide further context to the downstream effectors of PVH^{TrkB} neurons, and could elucidate whether the LC plays a role in mediating the suppression of food intake from PVH^{TrkB} neurons.

Another approach for determining the role of TrkB receptors within specific PVH projections would be to perform rescue experiments. \textit{TrkB} would first be deleted from whole PVH in the same manner as section 3.1. However, a modified version of the two-virus projection-targeting manipulation from section 3.3 could then be used to re-express \textit{TrkB} within specific projections. This would require a similar flippase-expressing retrovirus, but also a novel flippase-dependent virus that expresses \textit{TrkB} driven specifically by the relatively large and complex \textit{TrkB} promoter. Using a non-specific promoter, which would generally classify the manipulation as overexpression, would cause \textit{TrkB} to become expressed by PVH neurons projecting to the target that did not previously express the receptor. This effect alone would be a considerable confound; as ectopic BDNF-TrkB signaling could potentially affect biological functions that it was previously not involved in. Therefore, using the endogenous \textit{TrkB} promoter to drive re-expression prevents this confound, and the re-expression levels and expression dynamics would be similar to before whole PVH deletion for the targeted PVH neurons.

Including PVH^{TrkB} neurons that project to the PBN, which have now been found to suppress food intake, the acute regulation of feeding can be measured in populations of neurons projecting to specific targets using projection-specific chemogenetics. For these experiments, an obligatory flippase-expressing retrovirus is required, but a novel flippase-dependent virus expressing either an excitatory or an inhibitory designer receptor would also be necessary. In this way, designer drugs can be administered peripherally while still maintaining projection specificity. For projection and cell-type specificity, this manipulation can be performed in
animals that express Cre recombinase in cells of interest. However, a different novel virus that expressed a designer receptor, but was both flippase- and Cre-dependent, would be required.

The immediate clinical relevance of this data comes from the findings in whole PVH \textit{TrkB} deletions that demonstrate a critical role for these specific receptors due to the magnitude of the phenotype when they are ablated. A potential clinical implementation could be a novel drug delivery mechanism to target a TrkB agonist to the PVH for hunger suppression. One strategy to accomplish this would utilize a blood brain barrier permeable TrkB agonist such as 7,8-dihydroxyflavone or derivatives. This molecule would be chemically conjugated to a second molecule or nanosphere that provides two functions: (1) it prevents the agonist from crossing the blood brain barrier, and (2) it behaves like a retrograde tracer, or is one, such that once it reaches the bloodstream, it can be taken up by axon terminals of neuroendocrine neurons and transported back to their respective cell bodies in the arcuate, supraoptic, and paraventricular nuclei of the hypothalamus. This phenomenon has been demonstrated in mammals using several different retrotracers. The particular chemical bond that joins the agonist to this molecule is designed in such a way that, once it reaches the cell body of the neuroendocrine neuron, a neuron-specific enzyme (or preferably a PVH neuron specific enzyme) catalyzes the cleavage of this bond, liberating the TrkB agonist. Since the selected TrkB agonist is blood brain barrier permeable, it should also be permeable to cell membranes. Thus, once liberated, the TrkB agonist can diffuse out of the cell and activate TrkB receptors in the near vicinity of the PVH, and possibly also the arcuate and supraoptic nucleus depending on the expression specificity of the liberating enzyme. Though the agonist can technically diffuse to any location within the brain, the concentration of agonist will always be highest at the site of liberation, which also happens to be the intended site of action.


