The Role of Lepr/Pirt Neurons in the Control of Reproductive Function by Leptin

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Abstract

Leptin, a fat cell-derived hormone, signals the repletion of fat (energy) stores, suppressing feeding and permitting energy expenditure on a host of processes, including reproduction. Leptin mediates these responses by binding and activating its cell surface receptor, LepRb (encoded by *Lepr*), on specialized populations of hypothalamic neurons. Each LepRb-expressing cell population identified to date mediates distinct aspects of leptin action. The Myers lab recently identified a new and presumably distinct population of hypothalamic LepRb neurons, marked by the expression of *Pirt* (LepRb^{Pirt} neurons). We used immunofluorescence to identify hypothalamic LepRb^{Pirt} cells, which reside in the arcuate nucleus (ARC) and require *Lepr* expression to respond to leptin. We also confirmed the lack of overlap between LepRb^{Pirt} neurons and other previously defined populations of ARC LepRb cell types, including POMC, AgRP, and KNDy neurons. Other members of the Myers lab showed that ARC *Pirt* neurons project into the median eminence and promote the secretion of luteinizing hormone (LH), prompting us to hypothesize that leptin acts on LepRb^{Pirt} neurons to support reproductive function, rather than controlling food intake or other aspects of energy balance. Indeed, ablating *Lepr* from *Pirt* neurons in mice did not alter feeding, but altered some parameters of reproductive function in females. ARC *Pirt* neurons fail to accumulate fluorogold from the circulation. Hence, median eminence-projecting ARC LepRb^{Pirt} neurons link leptin to the control of reproduction. Lastly, ARC LepRb^{Pirt} neurons presumably synapse on GnRH terminals or some other cell population in the hypothalamus/median eminence to modulate reproductive function.

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Figure Number	Person Responsible for the Illustrated Experiment
1	Alan Rupp
2	Andrew Cheng
3	Yi Wang
4	Yi Wang collected the samples, and the Diabetes Research Center Chemistry Laboratory at the University of Michigan ran the assays and produced the data.
5	a. The data collection steps up to the immunostaining were done by Yi Wang. Immunostaining and microscopy were performed by John Santinga. b. Abigail Tomlinson The figure was made by John Santinga.
6	John Santinga
7	John Santinga
8	John Santinga
9	Injections were performed by Abigail Tomlinson and John Santinga. The rest was done by John Santinga.

10	Abigail Tomlinson collected and analyzed the data and made the figure. John Santinga contributed to the collection of the data.
11	Abigail Tomlinson
12	Abigail Tomlinson collected and analyzed the data. John Santinga contributed to the analysis of the data.
13	Abigail Tomlinson collected the samples, and the Diabetes Research Center Chemistry Laboratory at the University of Michigan ran the assays and produced the data.

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Introduction

Scientific Background

The Hypothalamic Control of Energy Balance and Reproduction

The main focus of the Myers lab is to understand the neural mechanisms that control food intake and the maintenance of stable body weight. Defining the roles played by the hormone leptin figures prominently in research by the lab. Given the central role of leptin in the control of long-term food intake and energy balance, if we can better understand how the body responds to leptin, we can better equip ourselves to combat related health conditions in which feeding and body weight are dysregulated. Some of the relevant conditions include obesity, type 2 diabetes, polycystic ovary syndrome (PCOS), and other aspects of the metabolic syndrome (Azziz, 2018).

Hormones like leptin are chemicals released by organs that travel via the circulation to communicate with other parts of the body (Hiller-Sturmhöfel and Bartke, 1998). Leptin is produced and released by white adipose tissue in approximate proportion to the amount of triglyceride stored in the tissue (Friedman, 2011). Thus, leptin levels indicate the repletion of long-term metabolic fuel stores for the body (Friedman, 2011). This information helps the body to maintain homeostasis by setting the physiological state appropriate for fuel stores (Friedman, 2011).

Any process in the human body requires energy, which requires us to consistently consume and metabolize food so that we can obtain energy (Friedman, 2011). However, the rate of energy consumption and use are not always in line, and resultant deficits or buildups of energy stores are each deleterious to the human body (Friedman, 2011). As such, energy balance is an important facet of homeostatic regulation (Friedman, 2011). To maintain a healthy level of energy stores that can be accessed when called upon, chemical energy from food is stored in the bonds of triglyceride molecules, or fat, in adipocytes, the storage cells within adipose tissue (Friedman, 2011). To maintain constant fat stores in adipocytes, energy intake in the form of calories needs to closely match the number of calories expended (Friedman, 2011). Any difference between these parameters results in fat gain or loss. The body can sense rates of food intake, energy consumption, and energy storage, and can

modulate these factors according to the ideal homeostatic range (Friedman, 2011). Specific brain structures mediate this communication and control (Friedman, 2011).

The hypothalamus, a region of the brain that lies directly above the pituitary gland, controls many homeostatic processes by receiving neural and hormonal input from the body and modulating behavior, endocrine function, and autonomic nervous system tone appropriately for existing conditions (Williams et al., 2001). For instance, if information from hormones and other inputs indicates a deviation from the optimal homeostatic range, the hypothalamus releases hormones, changes autonomic nervous system tone, and modifies behaviors in an attempt to restore conditions to within the desired range (Williams et al., 2001).

A decrease in leptin indicates lower adipose stores (Park and Ahima, 2014). Less energy storage means that the body must acquire more energy and curtail the processes that require energy (Park and Ahima, 2014). For example, food intake will increase, and energy expenditure will decrease (Park and Ahima, 2014). Important, but nonessential, energy-demanding functions like reproduction and reproductive development will experience decreased activity (Park and Ahima, 2014). Conversely, if adipose stores increase (as in obesity), leptin increases, which tends to decrease the drive to eat and increase metabolic rate (Park and Ahima, 2014).

Leptin works via binding to a cell surface leptin receptor (LepRb) protein on specialized neurons in specific regions (called nuclei) of the hypothalamus (Park and Ahima, 2014). In the hypothalamus, each nucleus serves a specific set of functions (Williams et al., 2001). Even within a single nucleus, different types of neurons sense distinct sets of hormones and other signals, and play different roles in controlling homeostasis (Williams et al., 2001). The modulation of endocrine function represents a major mechanism by which the hypothalamus controls homeostasis (Williams et al., 2001). The endocrine system is a specialized intra-organismal communication network of organs (called glands) that send messenger molecules (called hormones) via the circulation to other organs with hormone-specific receptors to inform and modulate their activity (Hiller-Sturmhöfel and Bartke, 1998). Often, the receptor organs will signal back to those initiating glands as negative feedback (Hiller-Sturmhöfel and Bartke, 1998).

This self-regulation keeps these spatially distinct organs operating in concert and appropriately to maintain homeostasis (Hiller-Sturmhöfel and Bartke, 1998).

The endocrine system is integrated with the nervous system at the hypothalamus, which is considered the first step of an endocrine axis (Hiller-Sturmhöfel and Bartke, 1998). In one general model, hypothalamic neurons secrete so-called releasing hormones that travel via the pituitary portal circulation to act in the pituitary gland, promoting the release of so-called stimulating hormones (Hiller-Sturmhöfel and Bartke, 1998). These stimulating hormones are released into the circulation, which carries them to their target organs where they act to produce the hormones that control the function in question (Hiller-Sturmhöfel and Bartke, 1998). Often, the stimulating hormones and the hormones in question signal back to the hypothalamus, inhibiting production of the releasing hormone to ensure that hormone levels do not become too high; this represents an endocrine negative feedback loop (Hiller-Sturmhöfel and Bartke, 1998).

The Hypothalamic-Pituitary-Gonadal (HPG) Axis

The HPG axis controls the production of reproductive hormones and cells in both males (androgens and sperm, respectively) and females (estrogen, progesterone, and eggs, respectively) (Acevedo-Rodriguez et al., 2018). While the HPG axis is fairly static in males, producing testosterone and sperm at a continuous rate, the female reproductive axis is more complicated- producing estrogen and progesterone and causing the release of eggs (ovulation) in a cyclic manner (Acevedo-Rodriguez et al., 2018).

The cyclic control of the HPG axis in females allows for the development of mature eggs during the menstrual cycle (Acevedo-Rodriguez et al., 2018). The hypothalamus releases gonadotropin-releasing hormone (GnRH) into the pituitary portal circulation, which delivers GnRH to the anterior pituitary (the master endocrine gland) to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Acevedo-Rodriguez et al., 2018). These hormones will stimulate development of the reproductive organs, or gonads (Acevedo-Rodriguez et al., 2018). In females, the ovaries produce estrogen, which exerts negative feedback for most of the cycle, but switches to positive feedback when the egg is ready for ovulation (release into the fallopian tubes for

fertilization) (Silberstein and Merriam, 2000). Estrogen, LH, and FSH levels rise during the cycle, with estrogen levels peaking first, followed by FSH and LH peaks, and finally ovulation (Silberstein and Merriam, 2000). Progesterone cycles differently, maintaining low levels before and during ovulation and peaking after ovulation (Silberstein and Merriam, 2000).

Leptin is an important modulator of this overall cycle. Leptin communicates to the reproductive systems whether there exist sufficient energy stores to proceed with reproduction (Donato, Cravo, Frazão, Elias, 2011). This relationship is particularly relevant for females because their energetic cost of reproduction is high (Jasienska, 2020). The neural mechanisms by which leptin controls reproduction remain incompletely understood, however.

Critical Review of the Literature

Leptin and the Leptin Receptor

Our story begins in 1950, with a curious case of a colony of mice displaying autosomal recessive hyperphagia, reduced energy expenditure, and early-onset obesity (obese, or *"ob/ob,"* mice) (Jeet, 2001). In 1958, Hervey produced a similar obesity syndrome in mice by lesioning the ventromedial hypothalamus (Hervey, 1959). This suggested potential feedback control of an area of the nervous system known to regulate homeostasis (the hypothalamus) by the fat storage cells (adipocytes) (Jeet, 2001).

Roughly 40 years later, the *ob* gene mutation responsible for obesity in these mice was identified (Zhang et al., 1994). It turns out that this gene (*Lep*) encodes a hormone, named leptin, which serves as a fat cell-derived circulating factor that signals the status of energy stores to the hypothalamus to regulate energy expenditure, food intake, and body weight (Jeet, 2001). Increased fat storage in adipose tissue produces more leptin, which circulates to the hypothalamus—where it signals to decrease food intake and increase energy expenditure (Park and Ahima, 2014). Conversely, when adipocyte fat levels are suboptimal, less leptin circulates, and the opposite physiological effects are observed (Park and Ahima, 2014).

Another layer of complexity arose when experiments showing mice homozygous for a mutation in a different gene (*db; db/db* mice*)* presented with an obesity syndrome identical to that of *ob/ob* mice (Coleman and Hummel, 1969). It turned out that the *db* mutation mapped to the gene *(Lepr)* that encodes the cell surface receptor for leptin (LepRb) (Jeet, 2001). Hence, the hypothalamus requires leptin and *Lepr* to maintain a healthy range of energy usage and storage (Jeet, 2001).

Leptin binding to LepRb triggers intracellular signaling pathways to mediate the effects of the hormone (Pan and Myers, 2018). Leptin binding to the LepRb dimer causes activation of the associated JAK2 tyrosine kinase, which phosphorylates tyrosine residues on substrate proteins (including JAK2 and LepRb) (Pan and Myers, 2018). The phosphorylated tyrosine residues on the intracellular tail of LepRb recruit several signal transducers and activators of transcription (STAT) molecules, including STAT3 and STAT5 (Pan and Myers, 2018). This recruitment leads to STAT protein

phosphorylation (pSTAT), promoting STAT protein trafficking to the nucleus and transcriptional activation (Pan and Myers, 2018). In addition to providing a useful marker of LepRb signaling (by the detection of pSTAT3), STAT3 signaling plays a crucial role in leptin action (Bates and Myers, 2003).

After the initial discovery of leptin, it became clear that the hormone does more than just regulate food intake and energy expenditure. In 1996, Ahima et al., fasted mice (which reduces endogenous leptin) to provoke the fasting-induced suppression of the gonadal and thyroid endocrine axes (Ahima et al., 1996). They found that adding exogenous leptin blunted the fasting-induced changes in the gonadal and thyroid axes (Ahima et al., 1996). This suggests that low leptin informs the endocrine system how to respond to fasting, leading the researchers to conclude that falling leptin levels mediate the neuroendocrine "starvation response" (Ahima et al., 1996).

Leptin and the Control of Reproduction

This thesis focuses on the role of leptin in relation to its control of endocrine reproductive function. Thus, this account of the history of the neuroscientific research of leptin will turn to studies that investigate this particular axis. The link between fat storage and reproductive function is predictable, as Donato points out in a 2011 article, since significant energy reserves are required for reproductive development and its associated activities (Donato, Cravo, Frazão, Elias, 2011). Key evidence dating back to the 1950's revealed that both *ob/ob* mice and *db/db* mice are infertile, suggesting the necessity of leptin and *Lepr* for proper reproductive function (Donato, Cravo, Frazão, Elias, 2011).

A 1996 study by Chebab, et al., provides several useful insights into this phenomenon, particularly with the mice lacking leptin (Chebab et al., 1996). *Ob/ob* females have normal sexual development, but remain stuck in early puberty and fail to ovulate (Chebab et al., 1996). Indeed, reproductive hormones are reduced in *ob/ob* or *db/db* mice, consistent with a role for leptin in the control of the endocrine reproductive axis (Chebab et al., 1996). Interestingly, when ovaries of *ob/ob* or *db/db* mice are transplanted into lean mice, they are able to produce viable eggs, while transplantation of ovaries from normal mice into *ob/ob* or *db/db* mice fails to permit reproduction (Chebab et al., 1996). This suggests the reproductive deficit in *ob/ob* and *db/db* mice

lies in the endocrine reproductive axis, rather than in the gonad. On the other hand, administration of leptin to *ob/ob* mice restores fertility and induces early-onset puberty (Chebab et al., 1996).

Control of Neuroendocrine Reproductive Hormones by Leptin

More studies have been conducted to acquire a deeper understanding of the relationship between leptin signaling and the hormones that link the hypothalamus to the reproductive system. GnRH, the hypothalamic master hormone at the top of the HPG axis, is highly sensitive to energetic imbalance. A study by Nagatani et al. found that both food restriction and high-volume exercise suppresses GnRH secretion by the hypothalamus (Nagatani et al., 1998). Furthermore, according to the same study, GnRH pulsatility returned to normal levels within several hours of a relief of the energetic stressor (Nagatani et al., 1998).

Furthermore, Nagatani et. al., low leptin levels suppress LH levels and leptin administration restores LH levels in food-restricted animals (Nagatani et al., 1998). Lastly, leptin administration was found to prevent reduction in pulsatile LH secretion that normally occurs during fasting in mice (Nagatani et al., 1998). Pituitary gonadotrophs (cells that secrete LH and FSH) do not express *Lepr* (Donato et al., 2009), and the control of LH secretion by leptin appears to be mediated by leptin's control of GnRH secretion. GnRH neurons do not express LepRb or respond directly to leptin, however, suggesting that other leptin-responsive neurons must mediate the control of GnRH neurons by leptin (Donato, Cravo, Frazão, Elias, 2011). Thus, understanding the control of reproduction by leptin requires identifying the *Lepr* neurons that control GnRH neuron function.

Hypothalamic Circuits that Control Reproductive Function

The hypothalamus contains several regions, each containing neurons known to mediate different broad physiological processes. Within many of these regions there exist neural populations that contain LepRb and respond to leptin. Among these populations there should be specific subsets of cells that mediate leptin action on the gonadal axis. Deletion of *Lepr* from GABAergic neurons (neurons that release GABA, which generally serves as an inhibitory neurotransmitter) blocks reproduction,

suggesting that the LepRb neurons that control reproduction are GABAergic (Martin et al., 2014). A review compiled by Donato, et. al., describes the current state of knowledge in the field concerning hypothalamic regions associated with reproductive function and reproductive hormones (Donato, Cravo, Frazão, Elias, 2011). These regions include the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the ventromedial nucleus (VMN), and the ventral premammillary nucleus (PMv) (Donato, Cravo, Frazão, Elias, 2011). The PVN participates in the control of the gonadal and thyroid function, but contains no LepRb neurons and is glutamatergic (Donato, Cravo, Frazão, Elias, 2011). The VMN is known to have a high concentration of sex steroid receptors, and has been linked to female mouse sexual behavior, but VMN LepRb neurons are glutamatergic and control metabolic rate, not reproduction (Dhillon et al., 2006).

In contrast, Donato showed in 2011 that PMv fibers innervate GnRH cells, and also express sex steroid receptors (Donato J Jr, Cravo RM, Frazão R, Gautron et al., 2011). Indeed. PMV LepRb neurons appear to modulate GnRH release and reproductive function, although ablating *Lepr* from the PMv only slightly alters reproduction (Elias, 2014). Indeed, PMv LepRb neurons are glutamatergic (Vong et al., 2011). Hence, *Lepr* in other neurons must participate in reproductive control.

The ARC not only contains GABAergic neurons but is anatomically adjacent to the median eminence (ME), the brain structure at the base of hypothalamus that links the hypothalamus to the pituitary gland and endocrine control (Yin & Gore, 2010). GnRH axons project to the ME, where they synapse on the pituitary portal vasculature and release GnRH (Yin & Gore, 2010). The pituitary portal circulation carries GnRH to the anterior pituitary, where it acts on gonadotrophs to promote LH and FSH release (Yin & Gore, 2010).

Several major ARC neuronal subpopulations contain LepRb. These are each named by their expression of specific peptides: one population contains proopiomelanocortin (POMC); another contains agouti-related peptide (AgRP) and neuropeptide Y (NPY) (Donato, Cravo, Frazão, Elias, 2011); and a third population expresses *Kiss1*, which encodes kisspeptin, *Tac2* (which encodes neurokinin B (NKB or TAC2)), and *Pdyn* (which encodes dynorphin); these cells are called KNDy neurons

(Moore et al., 2018). The roles of these neuronal populations are well established with respect to leptin action and energy balance. POMC and AgRP neurons mediate some effects of leptin on food intake, body weight and glucose homeostasis (Rupp et al., 2018). Leptin inhibits orexigenic AgRP neurons while activating anorexigenic POMC neurons (Rau & Hentges, 2017).

On the other hand, KNDy neurons play well-known roles in the control of reproduction. KNDy neurons are the main pulse generator for GnRH neurons in the HPG axis (Loon, 2015). Only very few KNDy neurons contain LepRb, however, and KNDy neurons are not GABAergic (Moore et al., 2018). Indeed, deletion of *Lepr* from these cells produces no detectable phenotype (Donato, Cravo, Frazão, Elias, 2011). Thus, there must exist a GABAergic population of LepRb neurons that contributes to the control of reproduction by leptin.

Single-Cell Methods to Identify Hypothalamic Neuron Populations

The large number of hypothalamic cell types and their intermingled nature complicates the analysis of hypothalamic function, as does the existence of many as-yet uncharacterized hypothalamic neuron populations (Rupp et al., 2021). While hypothalamic cell populations were previously identified by trial and error, the rise of technologies such as single-cell RNA sequencing (scRNA-seq) combined with bioinformatic analysis has permitted the unbiased identification of cell types in complex tissues like the hypothalamus (Rupp et al., 2021).

The first sequencing of an entire transcriptome of single cells was completed by Eberwine et al., in 1992 using complementary DNA (cDNA) (Eberwine et al., 1992). This technology was later adapted to single-cell RNA sequencing. The first analysis of a single-cell transcriptome using next-generation sequencing was performed by Tang et. al., in 2009 (Tang et al., 2009). Even in this early study, the researchers demonstrated the vast data-generation capabilities of this new technology. Using RNA-Seq on a single mouse blastomere cell, they detected the expression of 75% more genes than previous techniques would have allowed and identified almost 2,000 previously unknown splice junctions. The scientists also found that multiple different transcript isoforms of a single gene could exist in the same cell (Tang et al., 2009). This important finding revealed the complexity of transcript variants produced by single cells, which was previously

unknowable using less precise approaches that could not delve into the inner workings of a single cell.

Current technology permits the simultaneous sequencing of many thousands of individual cells while maintaining information about the cell of origin for each molecule sequenced (Hwang et al., 2018). These techniques generally attach short DNA sequences (barcodes) to the nucleotides derived from a single cell prior to DNA sequencing (Getting Started: Single Cell 3' Gene Expression, 2021). For instance, the commonly-used 10x genomics platform uses a flow sorting technique to place a single cell in the same fluid droplet as unique barcode-containing primers for cDNA synthesis, so that all cDNAs derived from mRNA molecules from the single cell in the droplet can be identified *post hoc* by their barcode (Getting Started: Single Cell 3' Gene Expression, 2021).. Following next-generation sequencing of the cDNAs derived from many cells simultaneously, all of the sequences containing a single barcode can be clustered together informatically to identify the gene expression pattern of each single cell (Getting Started: Single Cell 3' Gene Expression, 2021).

Because of differences in gene expression (e.g., neurotransmitter- and receptor-encoding genes), the specific genes expressed in neurons drive neural function (Hwang et al., 2018). It follows that cells with related functions would have similar gene expression patterns, and new populations of neurons with unique functions could be identified by their corresponding unique expression pattern (Hwang et al., 2018). By subjecting large numbers of cells (including neurons) to scRNA-seq and comparing the expression of genes across all sequenced cells, populations of related cell types can be identified in an unbiased manner (Hwang et al., 2018). While the patterns of gene expression across multiple transcripts ultimately distinguish cells of one population from those of distinct populations, some of the genes that identify a given population are unique to that population (Hwang et al., 2018). Such population-specific genes can be employed as "markers" for the cell population that expresses them and can be utilized to permit the identification and manipulation of that cell population to the exclusion of other cell types (Hwang et al., 2018).

For tissues with fragile cells, like neurons, single-cell sequencing can best be accomplished by preparing nuclei from the cells and subjecting the nuclei to the same

process that would normally be applied to the whole cell (a process called single-nucleus RNA-seq (snRNA-seq) (Affinati et al., 2021). A great example of the usefulness of snRNA-seq in the field comes from my own lab—the Myers lab. In 2021 study members of the Myers lab investigated the VMN, a hypothalamic region known to have diverse functions using snRNA-seq (Affinati et al., 2021). However, the mapping of individual functions to specific cellular populations within the VMN remains in its infancy due to the lack of markers for truly distinct neuronal populations in the VMN (Affinati et al., 2021). This study identified 24 clusters of VMN neurons by subjecting mouse VMN tissue to snRNA-seq and clustering the nuclei based upon their gene expression patterns (Affinati et al., 2021).

To understand the mechanisms by which leptin mediates its effects, the Myers lab seeks to define the types of neurons that respond to leptin and to understand the function of each of these neuron types (Rupp et al., 2021). Neurons that directly respond to leptin must express *Lepr*, so we can refine our analysis of the single-cell dataset to look for cells that have high expression of *Lepr* (Rupp et al., 2021). Because *Lepr*-expressing cells are rare and because *Lepr* expression is low (and thus often not detected by snRNA-seq) even in leptin-responsive cells, snRNA-seq fails to detect *Lepr* in most relevant cells when analyzing all cells in a given tissue (such as the hypothalamus) (Rupp et al., 2021). Thus, to identify and study *Lepr*-expressing cells specifically, the Myers lab used mice in which the nuclei of LepRb neurons were labeled with a fluorescent reporter (Rupp et al., 2021). The lab dissected the hypothalamus and used cell sorting to isolate the nuclei from LepRb neurons that contained fluorescently-labeled nuclei; they then subjected the nuclei to snRNA-seq and unbiased clustering analysis (Rupp et al., 2021).

Mouse Models to Study LepRb Neurons

Once the Myers lab has identified a novel *Lepr*-expressing cell type for study and has defined a marker gene that permits its identification and manipulation, we investigate each relevant *Lepr*-expressing neuron population by using mouse *(Mus musculus)* genetic models (“Genetics for Molecular Neuroscience: Part 1,” 2022). Mice are ideal for studying mammalian neuroscience because they share a large portion of their genomes and most brain structures with humans and other mammals (“Genetics

for Molecular Neuroscience: Part 1," 2022). Mice also have a short generation time, and tools for genetic manipulation are most highly developed in mice, making them ideal for genetic studies ("Genetics for Molecular Neuroscience: Part 1," 2022).

The endocrine control of reproduction in mice is also similar to that in humans. Although female mice do not shed their uterine lining at the end of their cycle, their estrus cycle otherwise mirrors that of humans (Cora et al., 2015). Another reproductive difference is the vagina is closed in pre-pubescent mice (Cora et al., 2015). Vaginal opening (VO) is an early indicator of early pubertal onset (Cora et al., 2015). The next important step in female reproductive development is first estrus (VE); similar to menarche in humans, this is simply the first time the mouse enters the estrus stage of the cycle (Cora et al., 2015). The estrus cycle in mice is often monitored by morphologic changes in the cell types found in the vaginal cavity (Cora et al., 2015). The four stages of estrus are each marked by a specific combination of cell types (Cora et al., 2015). Stage 1 is proestrus, and has mainly nucleated epithelial cells and some cornified epithelial cells (Cora et al., 2015). Stage 2 is estrus, during which a spike in LH induces ovulation—the release of a mature egg for fertilization (Cora et al., 2015). This stage has mainly cornified epithelial cells and some nucleated epithelial cells (Cora et al., 2015). Stage 3 is metestrus, and has all three cell types (the previous two, and leukocytes) (Cora et al., 2015). Stage 4 is diestrus, and has mainly leukocytes, and small amounts of the cornified epithelial cells and nucleated epithelial cells (Cora et al., 2015).

Broadly, if we produce a mouse which lacks a particular gene, either completely or specifically in an area of interest, then we can track divergences from typical mouse physiology (Rupp et al., 2018). For the purposes of studying leptin signaling, the gene to be knocked out is *Lepr*. However, if *Lepr* is knocked out in every cell of the body, we cannot draw any conclusions about the function of a particular subregion with respect to leptin signaling.

Therefore, we need a gene editing system that can knock out a particular gene only in a specific region or cell type. That technology was pioneered in 1987 by Sauer and Henderson, who translated a viral genetic editing system utilizing Cre recombinase and LoxP sites to mammalian cells (Sauer and Henderson, 1988). Cre is a recombinase

protein that recombines a specific pair of identical DNA segments, called LoxP sites, so that they merge and delete any DNA that initially intervened between the LoxP sites (Song and Palmiter, 2018). The scientists were able to genetically insert a promoter sequence plus the Cre coding sequence, along with LoxP sites (at a different location) —first in yeast cells, and then in mammalian cells (Sauer and Henderson, 1988). By inserting two LoxP sites on either end of an intervening DNA sequence, they were also able to confirm that Cre successfully recombined the LoxP sites (Sauer and Henderson, 1988). As mentioned in their landmark article, this so-called Cre-Lox technology has major implications for the targeted study of genes in eukaryotes (Sauer and Henderson, 1988).

Furthermore, as we have learned from the discussion of next-generation sequencing methods, many cell populations can be identified by their expression of unique marker genes (Song and Palmiter, 2018). It follows that a genetically modified mouse that has Cre expression driven by a cell type-specific marker gene will exhibit recombination of LoxP sites only in the cell population identified by the marker gene (Song and Palmiter, 2018). If we place the LoxP sites on either side of *Lepr*, then *Lepr* will only be knocked out in the Cre-expressing cell population of interest (Rupp et al., 2018). This mouse would then be an ideal study animal; over its lifetime, one could take body weight, food intake, energy expenditure, body composition, blood glucose, and many other measurements, and compare the data to that from a wild type mouse (Rupp et al., 2018). The first such cell-type-specific gene knockout using the Cre-Lox system in mouse neuronal tissue was done in 1996 by Tsien and colleagues (Tsien et al., 1996).

Another critical group of proteins for animal studies in neuroscience is fluorescent proteins. The first fluorescent protein was discovered by Shimomora in 1962, when he isolated a protein that glowed bright green under UV light; this protein was named Green Fluorescent Protein (GFP) (The Royal Swedish Academy of Sciences, 2008). Up until the 1990's, neuroscientists did not have a method to easily and systematically visualize genetically-identified subsets of neurons in a tissue section (The Royal Swedish Academy of Sciences, 2008). This changed in 1994, when Martin Chalfie demonstrated that GFP could serve as a visual (colorful) tag (Chalfie et al., 1994). In one experiment in his study, for example, he inserted the gene for GFP into

the genome of a transparent roundworm species (Chalfie et al., 1994). He was able to selectively express this protein in certain cells, which then glowed green under the fluorescence microscope (Chalfie et al., 1994).

GFP expression is now frequently used to tag neurons of interest in mouse models in most neuroscience labs, including the Myers lab. This can be done by driving GFP expression from a promoter sequence for a marker gene of a neuronal population of interest, or by using a marker gene to express Cre recombinase in the presence of a Cre-inducible GFP transgene that is driven by a ubiquitous promoter ("Genetics for Molecular Neuroscience: Part 2," 2022). GFP-containing cells can often be directly visualized under a fluorescence microscope ("Genetics for Molecular Neuroscience: Part 2," 2022). In cases where GFP expression is too low for direct detection the signal can be enhanced in fixed tissue by immunofluorescence detection using anti-GFP antibodies ("Genetics for Molecular Neuroscience: Part 2," 2022).

To detect GFP-containing cells by immunofluorescence, tissue sections are typically obtained by first performing a non-survival procedure called a perfusion (Tu et al., 2021). In this step, the mouse brain tissue, and thus, all the proteins expressed in those cells, are fixed in place by formalin, a cross-linking agent (Tu et al., 2021). The brain is then extracted from the skull and sliced into very thin coronal sections using a mechanical sectioning device called a microtome (Tu et al., 2021). This produces a series of tissue slices each containing a spatially conserved array of neurons from the mouse brain (Tu et al., 2021). These sections can then be stained for the fluorescent proteins that are expressed in the cells (Tu et al., 2021). This usually consists of a two-day process where antibodies that actually confer fluorescence anneal to the endogenous protein (Tu et al., 2021). Then the stained sections are mounted on slides for visualization by a fluorescence microscope (Tu et al., 2021).

Viral Genetic Systems to Study Neurons

Viruses represent another powerful tool for the extrapolation of neuron function because of their ability to infect cells (Pierre-Yves, 2020). Viruses require a host organism to reproduce, so they have developed a highly efficient way to gain control of cellular machinery (Pierre-Yves, 2020). Broadly speaking, viruses will attach to the cell via a cell-surface receptor and inject their genome into the cytoplasm (Pierre-Yves,

2020). The host cell machinery will then replicate, transcribe, and translate that genome, which will produce more viral particles (Pierre-Yves, 2020). The new viral particles are then released from the infected cell by a regulated process, such as via budding from the dendrites or axon terminals, or by causing cell lysis (Pierre-Yves, 2020). Modern neuroscientists will inject a genetically engineered virus into the brains of mice to modulate the activity of particular neurons (Nectow and Nestler, 2020).

Most viruses only infect specific cell types based on the expression of a cognate cell surface receptor for the specific virus (Nectow and Nestler, 2020). For instance, rabies virus only infects neurons (Nectow and Nestler, 2020). Although some types of viruses are less selective in responding to the cell types that they infect, practically speaking, these can still be used in a cell type-dependent manner by incorporating a genetic payload into these viruses that is expressed in a Cre-dependent manner (Nectow and Nestler, 2020).

A host of viral reagents used by the Myers lab (and other labs) can be used to establish the upstream and downstream connections of specific viruses. While some of these viruses (e.g., rabies virus) can be used to jump synapses and mark neurons that lie upstream or downstream of specific neuronal cell types, a simpler system traces the projections of a specific cell type by expressing a synaptophysin-fluorescent protein fusion protein in the cell (Haggerty et al., 2019). This system is often packaged into a pleiotropic virus (for instance, an adeno-associated virus; AAV) that mediates the Cre-dependent (by means of a Cre-dependent DIO element) expression of its genetic cargo (Haggerty et al., 2019). Thus, although all cells in an area that receives the virus are infected, only the Cre-expressing cells produce the synaptophysin-fluorescent fusion protein (Haggerty et al., 2019). Since synaptophysin is trafficked to the axon terminal, the detection of fluorescence in the brain identifies the areas that receive projections from the AAV-DIO-synaptophysin-fluorescent protein infected Cre-expressing cells (Haggerty et al., 2019).

Other proteins can also be expressed in specific neuron types by means of Cre-dependent AAVs, and many of these can be used to perturb neuron activity to determine function (Roth, 2016). Chemogenetic cargos, such as designer receptors exclusively activated by designer drugs (DREADDs) can be used to increase (activating

DREADD; hM3Dq) or decrease (inhibitory DREADD; hM4Di) neuron activity in response to an otherwise inert ligand (CNO, in the case of DREADDs) (Roth, 2016). The same principle is used in optogenetics, except in this case the viral cargo permits the modulation of neuron activity in response to light of a certain wavelength (Roth, 2016).

Goals of the Thesis

Obesity is a crippling public health problem worldwide. Not only does obesity severely impact one's lifestyle, but it also has many comorbidities, such as diabetes, cardiovascular disease, and impaired fertility (Apovian, 2016). Leptin signals to the hypothalamus to induce physiological adjustments based on the repletion of adipose tissue fat stores (Jeet et al., 2016). If we can identify cell populations that mediate leptin's control of food intake, energy balance, and fertility we can target these cell types with drug therapies to mitigate obesity and its deleterious effects.

My lab recently performed a snRNA-seq analysis of *Lepr*-expressing mouse hypothalamic cells. In addition to identifying previously described populations of hypothalamic LepRb neurons, this analysis revealed the existence of several new populations of hypothalamic LepRb neurons, including a population marked by the expression of *Pirt* (LepRb^{Pirt} neurons) (Figure 1). Because the most prominent population of hypothalamic *Pirt* neurons resides in the ARC, which is rich in LepRb neurons, **we hypothesized that ARC *Pirt* neurons represent LepRb^{Pirt} cells**.

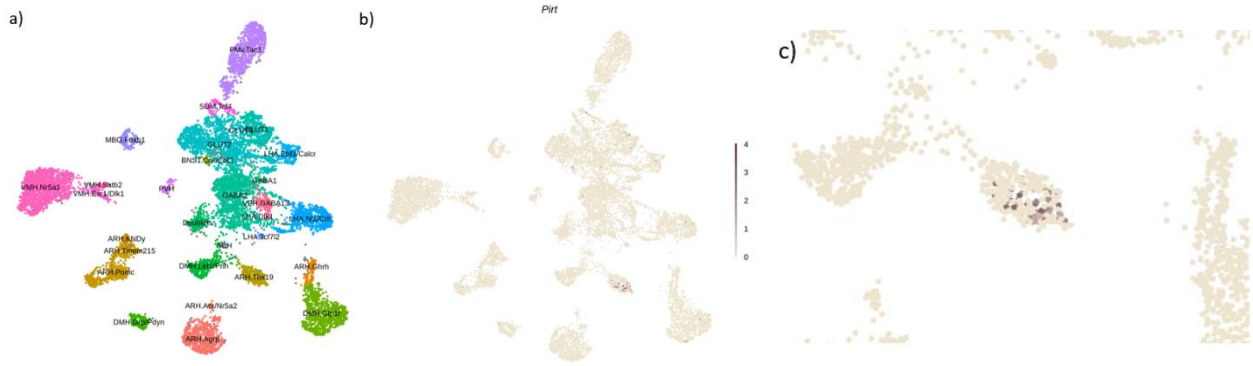


Figure 1: Identification of Hypothalamic LeprRb Neuron Populations by snRNA-seq. (a) Clustering of neurons based on differential gene expression analysis. Each cluster is color-coded and named by predicted spatial location and a unique marker gene, where possible. (b) The same neuron clusters as in (a) showing the distribution of *Pirt* expression. Darker color indicates higher *Pirt* expression. (c) Zoom of (b), showing the ARH.Tbx19 cluster, in which *Pirt* expression is concentrated. This cluster represents the LepRb^{Pirt} neurons referred to in this study.

Because tracing from ARC *Pirt* neurons using AAV-DIO-synaptophysin-mCherry showed that most of the projections from these neurons target the median eminence

(Figure 2), **we further hypothesized that ARC LepRb^{Pirt} neurons control endocrine function, rather than food intake and energy balance, in response to leptin**. Because our snRNA-seq analysis suggested that LepRb^{Pirt} neurons are GABAergic and because leptin promotes the activity of most endocrine axes (e.g., the thyroid and reproductive axes), we initially hypothesized that leptin would inhibit LepRb^{Pirt} neurons, thereby disinhibiting and promoting the activity of thyroid and reproductive function.

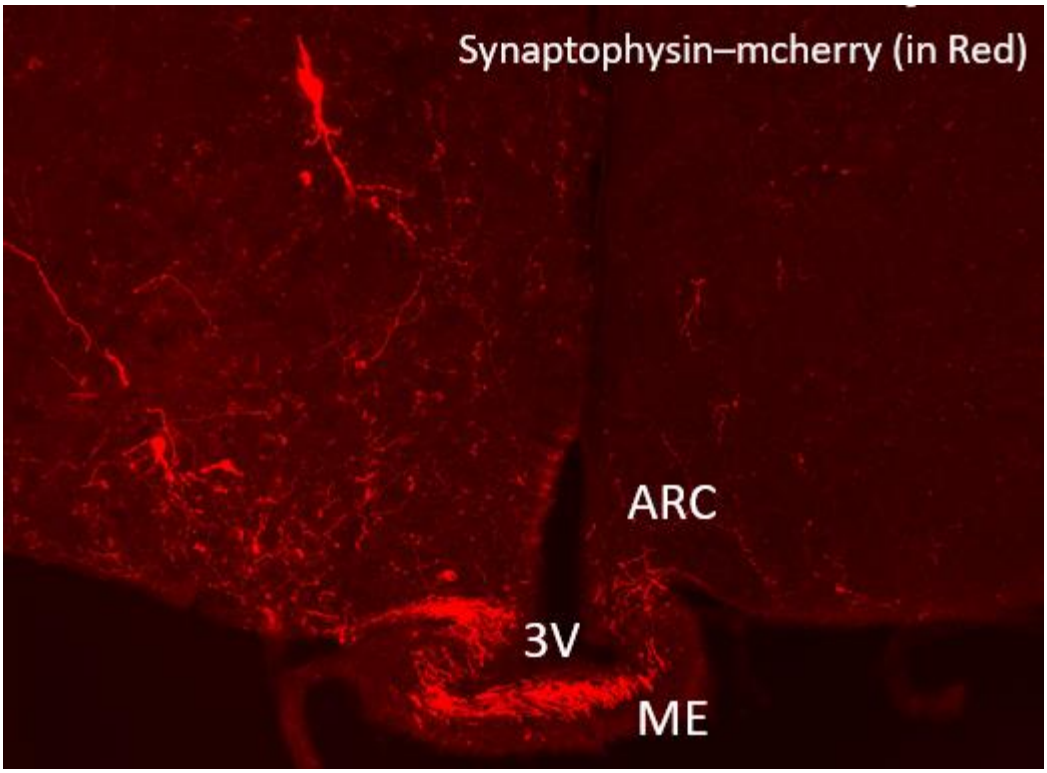


Figure 2: Trace of Cre-dependent AAV-DIO-synaptophysin-mCherry virus in *Pirt*^{Cre} mouse. *Pirt*^{Cre} mice (transgenic mice that express Cre only in ARC *Pirt* neurons) were injected in the ARC with Ad-iN-Synaptophysin-mCherry to mediate the Cre-dependent expression of the synaptically-targeted Synaptophysin-mCherry fusion protein in ARC *Pirt* neurons. Following perfusion, brains were sectioned and stained for mCherry. Shown is a representative image of mCherry immunoreactivity in the ARC and median eminence of one such animal. Image was acquired at 10x magnification. Location of the ARC, median eminence (ME) and third cerebral ventricle (3V) are indicated.

The Myers Lab subsequently found that the DREADD hM3Dq-mediated activation of ARC *Pirt* neurons (Figure 3) increased circulating LH, but did not alter blood levels of ACTH, TSH, or GH (Figure 4). **Therefore, we modified our model, hypothesizing that leptin activates LepRb^{Pirt} cells to increase the activity of the HPG axis, and therefore supporting reproduction.**

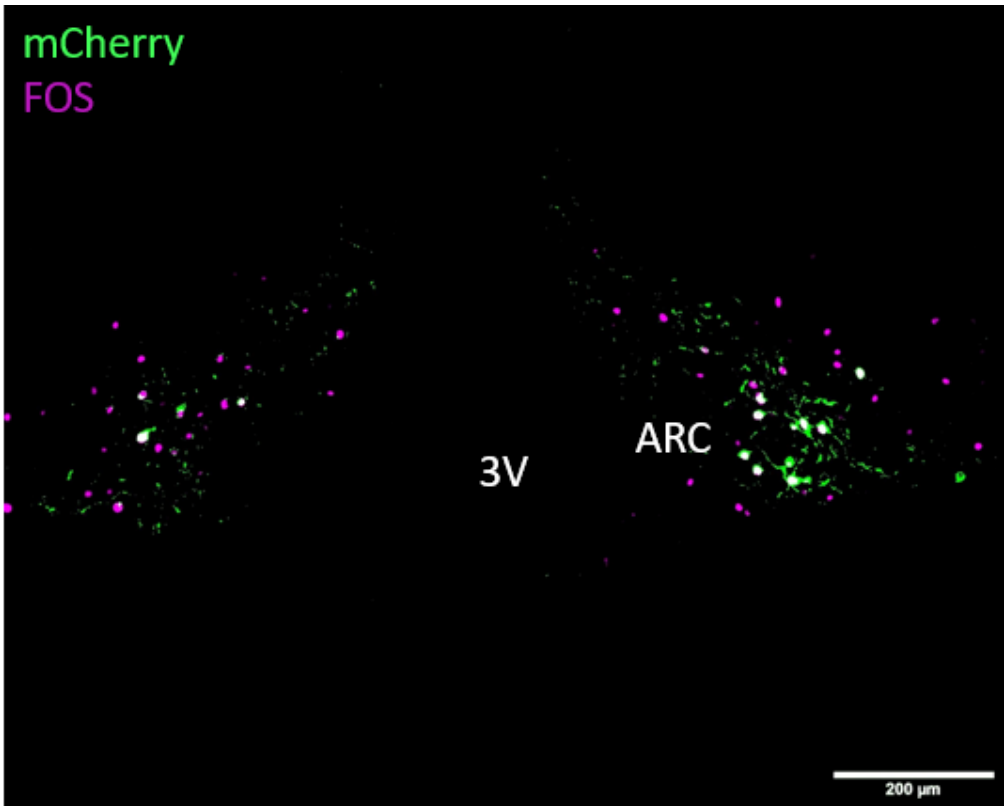


Figure 3: hM3Dq DREADD-Mediated Activation of ARC *Pirt* Neurons. *Pirt*^{Cre} mice were injected in the ARC with AAV-DIO-hM3Dq-mCherry to mediate the Cre-dependent expression of hM3Dq DREADD-mCherry in ARC *Pirt* neurons. Following recovery from surgery, the mice were injected with the DREADD ligand, CNO, to activate the *Pirt* neurons, and mice were perfused. Brains were sectioned and stained for mCherry (green) and a marker of neuronal activation, FOS (magenta). Shown is an image of the ARC of a representative animal.

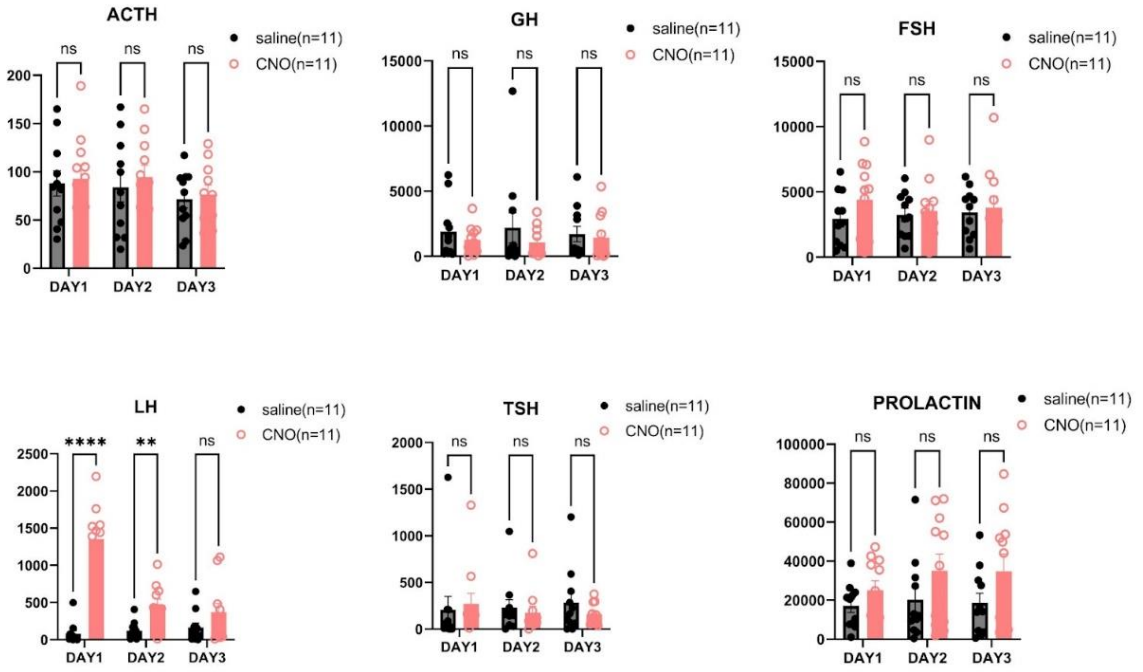


Figure 4: Circulating Pituitary Hormone Concentrations following hM3Dq DREADD-mediated Activation of ARC *Pirt* Neurons. *Pirt*^{Cre} mice were injected in the ARC with AAV-DIO-hM3Dq-mCherry to mediate the Cre-dependent expression of hM3Dq DREADD-mCherry in ARC *Pirt* neurons. Following recovery from surgery, the mice were injected with the DREADD ligand, CNO, or saline control twice per day for three days. Blood samples were taken for hormone measurements 30 minutes after the morning injection on each day and blood concentrations of the indicated hormones were determined. Shown are hormone concentrations for each day, plotted as mean -/+ SEM. **p<0.01, ****p<0.0001 by Student's t-test. ns= not significant (p>0.05).

In this model, leptin from adipose tissue that is replete with triglyceride travels via blood vessels, crosses the blood-brain barrier, and activates LepRb on ARC *Pirt* neurons, which would promote the release of GnRH into the median eminence, thus enhancing LH release and activating the HPG axis. This model suggests that leptin action via LepRb^{Pirt} neurons regulates endocrine reproductive function. This model also makes sense in terms of energy balance: when fat stores are high, there is available energy for reproductive function, so leptin should stimulate the HPG axis.

To test these hypotheses, we used Cre-Lox systems to identify *Pirt* neurons and to perturb *Lepr* expression in *Pirt* neurons to learn about LepRb^{Pirt} cells and their roles in leptin action. Specifically, we used immunofluorescence to define the overlap between

leptin-induced phosphorylation of STAT3 (pSTAT3; a marker of cell-autonomous leptin action) and the *Pirt*^{Cre}-dependent expression of GFP in *Pirt* neurons. We also examined the potential overlap of ARC *Pirt* cells with other types of ARC neurons (including those that contain POMC, NPY, or NKB) and examined the potential exposure of *Pirt* neurons to the circulation (e.g., the pituitary portal system) by examining their potential uptake of a fluorescent tracer from the bloodstream.

Finally, we crossed the *Pirt*^{Cre} mouse line onto *Lepr*^{Flox} mouse line (transgenic mice with LoxP sites flanking the *Lepr* gene) to generate *Pirt*^{Cre};*Lepr*^{Flox} mice that lack *Lepr* specifically in *Pirt* neurons (Lepr^{Pirt}KO mice). We monitored parameters of leptin function in these mice, including food intake, body weight, hormone levels, reproductive function, and glucose homeostasis (among others), providing insight into the roles of LepRb^{Pirt} cells in leptin action.

Materials and Methods

Animals

All mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. The mice were provided with *ad libitum* access to normal chow diet (Purina Lab Diet 5001) (except as noted below) and water in temperature-controlled (25°C) rooms on a 12-hour light-dark cycle with daily health status checks. All procedures involving animals were performed in accordance with and with the approval of the University of Michigan Institutional Animal Care and Use Committee: protocol 11066 (Myers, PI).

The *Pirt*^{Cre} line was obtained from the Dong lab at Johns Hopkins University (Kim et al., 2016). *Lepr*^{Flox} (McMinn et al., 2004) and the Cre-inducible reporter allele *Rosa26*$^{L10\text{-}GFP}$ (Krashes et al., 2014) were described previously and are standardly propagated in the Myers lab. We crossed *Rosa26*$^{L10\text{-}GFP}$ onto the *Rosa26*$^{L10\text{-}GFP}$ background to generate *Pirt*^{Cre};*Rosa26*$^{L10\text{-}GFP/L10\text{-}GFP}$ (Pirt$^{L10\text{-}GFP}$) reporter mice expressing GFP in *Pirt* neurons. We further crossed *Pirt*^{Cre} and Pirt$^{L10\text{-}GFP}$ mice onto the *Pirt*^{Cre} background, to generate *Pirt*$^{Cre/+}$;*Lepr*$^{Flox/Flox}$ and *Pirt*^{Cre};*Rosa26*$^{L10\text{-}GFP/L10\text{-}GFP}$;*Lepr*$^{Flox/Flox}$ (Lepr$^{Pirt\text{-}GFP}$KO) mice, respectively. *Pirt*$^{Cre/+}$;*Lepr*$^{Flox/Flox}$ mice were intercrossed to generate mice for longitudinal study, generating *Pirt*$^{Cre/Cre}$;*Lepr*$^{Flox/Flox}$ and *Pirt*$^{Cre/+}$;*Lepr*$^{Flox/Flox}$ mice predicted to lack *Lepr* in *Pirt* neurons (collectively, Lepr^{Pirt}KO mice) and *Pirt*$^{+/+}$;*Lepr*$^{Flox/Flox}$ controls.

Longitudinal Study

We studied Lepr^{Pirt}KO mice and their controls longitudinally. Body weight was measured weekly from the time of weaning. Blood glucose was measured biweekly. Body composition (body weight, fat mass, and lean mass) was measured once at the end of the study using an EchoMRI^{TM} instrument.

Females were monitored for vaginal opening (VO). Once this occurred, females were monitored daily for estrus by obtaining a cell sample from a swab of the vaginal cavity. Visualization of the samples under a standard light microscope allowed characterization of cell types. Based on the type of cell present in the vaginal cavity, we recorded daily what stage of estrus the mice were in. First Estrus (VE), which is the first

time an animal displays estrus cytology, was another pubertal marker we recorded. Each mouse was euthanized when they reached 80 days of age.

Fluorogold Tracer Injection

Our procedure for retrograde labeling of neurons that contact the circulation via fluorogold (FG) tracer injection (Schmued, 1990) was based on the protocol used by Faouzi et al. (Faouzi et al., 2007) with several important modifications. We subcutaneously injected FG into the abdomen, rather than the tail vein, of $Pirt^{L10\text{-}GFP}$ mice. The doses were 22 units of either 0.04% or 0.2% FG. We perfused 5 days after injection.

Immunostaining

$Pirt^{L10\text{-}GFP}$ mice intended for pSTAT3 staining were fasted for 12 hours to decrease endogenous leptin and then injected with leptin (5 mg/kg) 1 hour prior to euthanasia. Mice were euthanized with isoflurane and then perfused with phosphate buffered saline (PBS) for five minutes followed by an additional five minutes of 10% formalin. Brains were then removed and post-fixed in 10% formalin overnight at room temperature before being transferred to 30% sucrose. Brains were then sectioned as 30 μm thick free-floating sections and stained. Sections were treated sequentially with 1% hydrogen peroxide/1% sodium hydroxide; 0.3% glycine; and 0.04% sodium dodecyl sulfate before being rinsed in 0.3% phosphate buffered triton (PBT) and incubated in blocking solution (PBT containing 3% donkey serum (Fisher Scientific)). Sections were then incubated in PBT containing anti-GFP (chicken; 1:1000, #1020, Aves Laboratories) and rabbit anti-pSTAT3 (rabbit; 1:500, #9145, Cell Signaling) overnight at room temperature. The sections were then washed and incubated in PBT with fluorescent secondary antibodies: species-specific Alexa Fluor-488 or -568-conjugated secondary antibodies (Invitrogen A-11039 or A-11011, 1:250). Images were collected on an Olympus BX51 microscope. Images were background subtracted and enhanced by shrinking the range of brightness and contrast in ImageJ.

To determine the potential colocalization of NPY, POMC, NKB/TAC2, or FG with ARC *Pirt* neurons, $Pirt^{L10\text{-}GFP}$ mice were euthanized with isoflurane and then perfused with PBS for five minutes followed by an additional five minutes of 10% formalin. Brains were then removed and post-fixed in 10% formalin overnight hours at room

temperature, before being dehydrated in 30% sucrose. Brains were then sectioned as 30 μm free-floating sections. Sections were treated sequentially with a neutralization buffer for reactive oxygen species (0.3% Sodium Hydroxide and 1% Hydrogen Peroxide in PBS) and blocking solution (3% donkey serum, Fisher Scientific; in 0.3% PBT). Sections were then incubated in PBT containing anti-GFP (Chicken; 1:1000, #1020, Aves Laboratories) and anti-NPY (rabbit; 1:1000, 22940, Immunostar), anti-POMC (rabbit; 1:1000, H-029-30, Phoenix Pharmaceuticals Inc.), anti-NKB/TAC2 (rabbit; 1:1000, NB300-102, Novus Biologicals), or anti-fluorogold (rabbit; 1:1000; Fluorochrome). After incubation overnight at room temperature, sections were washed and incubated in PBT containing two fluorescent secondary antibodies: Alexa goat anti-chicken-488 (1:500, A11039, Invitrogen) for GFP and Alexa donkey anti-rabbit-647 (1:500, A-31573, Invitrogen). Images were collected on an Olympus BX51 microscope. Images were background subtracted and enhanced by shrinking the range of brightness and contrast in ImageJ.

Hormone Collection

At the end of the longitudinal study the Lepr^{Pirt}KO and control mice were euthanized via decapitation under anesthesia. During this process, we collected trunk blood serum and extracted the pituitary for hormone analysis. For the hormone analysis, we isolated the proteins from the blood using centrifugation and sent the blood serum proteins and pituitary to the Chemistry Laboratory of the Michigan Diabetes Research Center. They performed multiple assays (Pituitary Multiplex ELISA, Leptin ELISA, and Insulin ELISA) and returned to us the relative concentrations of leptin, insulin, adrenocorticotropic hormone (ACTH), FSH, growth hormone (GH), prolactin, thyroid-releasing hormone (TSH), and LH.

Statistical Analysis.

For the experiments where we measured a quantitative variable in experimental and control groups, we generated two normally distributed samples of data. To see if there was a statistically significant difference in the value of a particular variable, we performed a Student's t-test where significance was defined as $p<0.05$.

Results

LepRb^{Pirt} neurons reside in the ARC

To determine the distribution of LepRb^{Pirt} neurons throughout the hypothalamus, we treated Pirt$^{L10\text{-}GFP}$ and Lepr$^{Pirt\text{-}GFP}$KO mice with leptin (5mg/kg, 2 hours), prepared their brains for immunofluorescence, and examined the potential colocalization of pSTAT3-immunoreactivity (-IR) and GFP-IR (Figure 5). We observed leptin-stimulated pSTAT3-IR in hypothalamic regions known to contain LepRb neurons and found GFP-IR in the lateral ARC, as well as in a few neurons scattered in other parts of the hypothalamus. We found ARC GFP-IR neurons from leptin-treated Pirt$^{L10\text{-}GFP}$ mice contained pSTAT3-IR, while no pSTAT3-IR colocalized with GFP-IR in Lepr$^{Pirt\text{-}GFP}$KO mice (Figure 5). For the representative Pirt$^{L10\text{-}GFP}$ mouse brain slice shown in Figure 5, 20 of the 37 GFP-IR neurons (roughly 54%) in the ARC also contained pSTAT3-IR. In contrast, the representative Lepr$^{Pirt\text{-}GFP}$KO mouse brain slice shown in Figure 5 demonstrated pSTAT3-IR in 0 of the 55 GFP-IR neurons (0%) in the ARC. Colocalization of pSTAT3-IR with GFP-IR in the ARC of Pirt$^{L10\text{-}GFP}$ mice suggests that most ARC *Pirt* neurons express *Lepr* and represent LepRb^{Pirt} cells. The lack of pSTAT3-IR in GFP-IR neurons in Lepr$^{Pirt\text{-}GFP}$KO mice suggests that we successfully ablated *Lepr* in *Pirt* neurons, demonstrating the validity of the Lepr^{Pirt}KO model.

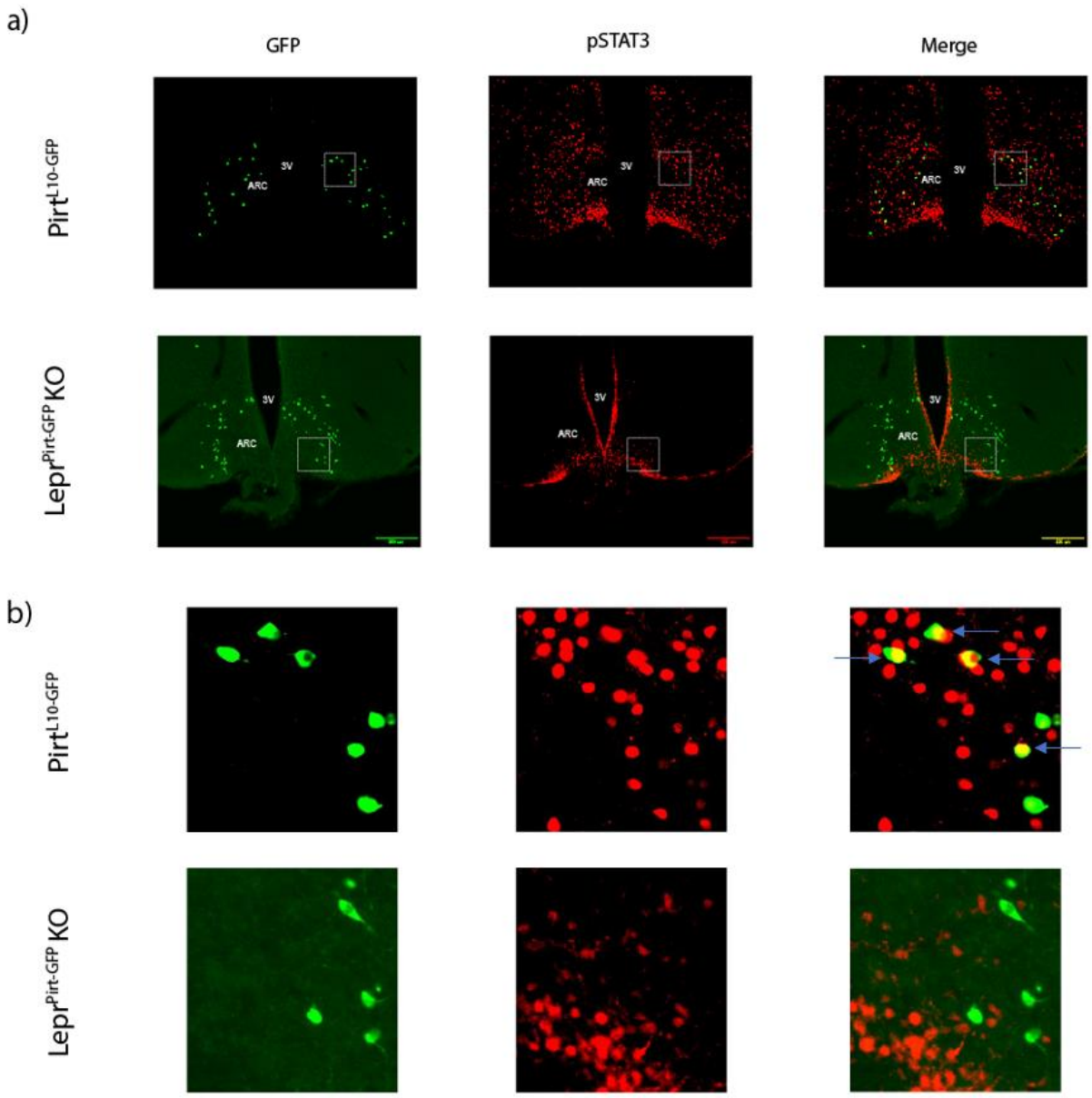


Figure 5: Colocalization of Leptin-Stimulated pSTAT3-IR and GFP-IR in the ARC of Pirt$^{L10\text{-}GFP}$ mice, but not in Lepr$^{L10\text{-}GFP}$KO mice. Pirt$^{L10\text{-}GFP}$ and Lepr$^{L10\text{-}GFP}$KO mice were treated with leptin (5mg/kg, 2 hours), and their brains were processed for the immunofluorescent detection of GFP-IR (left panels, green), pSTAT3-IR (middle panels, red). Merged images are shown on the right. (a) Shown are representative images of ARC-containing sections from Pirt$^{L10\text{-}GFP}$ (top panels) and Lepr$^{L10\text{-}GFP}$KO (bottom panels) mice. (b) Zoomed images corresponding to the boxed sections of the panels in (a). Blue arrows indicate colocalization of GFP-IR and pSTAT3-IR in a cell body.

No colocalization of LepRb with NPY, POMC, or KNDy neurons

To assess the potential overlap between *Pirt* and AgRP neurons in the ARC, we prepared the brains of Pirt$^{L10\text{-}GFP}$ mice for immunofluorescence and looked for colocalization of GFP-IR (which identifies *Pirt* neurons) and NPY-IR (which identifies AgRP neurons in the ARC). We found both GFP-IR and NPY-IR in the ARC, but not within the same cells, which suggests that *Pirt* and AgRP neurons represent distinct cell populations (Figure 6).

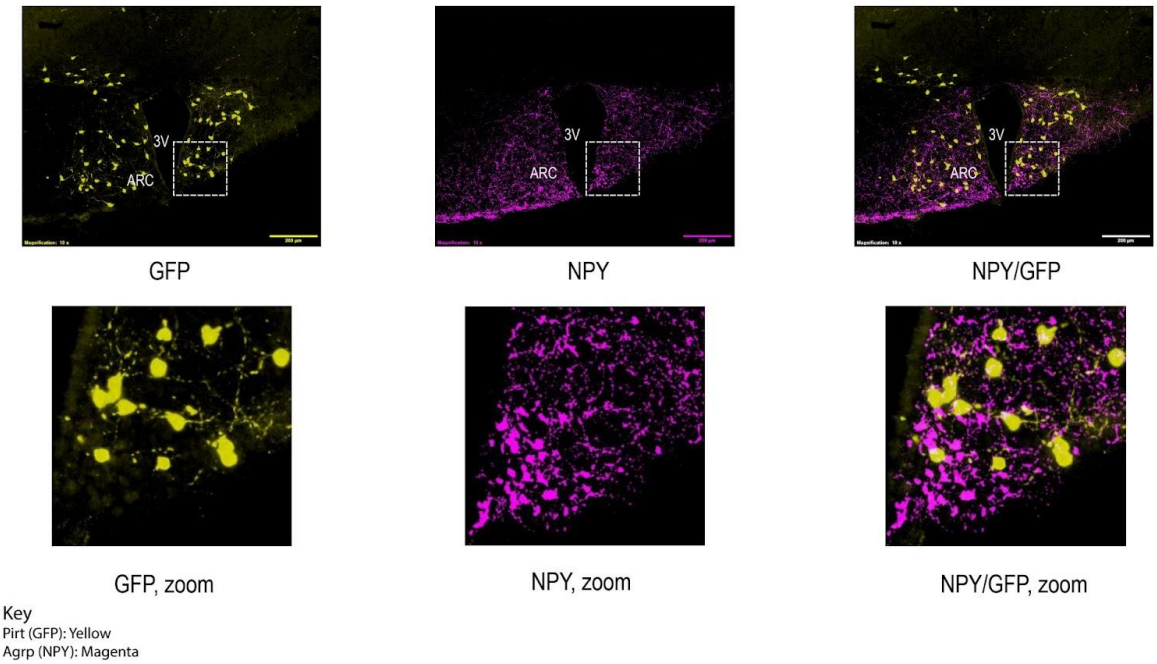


Figure 6: No colocalization of *Pirt* and AgRP neurons. Brains of Pirt$^{L10\text{-}GFP}$ mice were processed for the immunofluorescent detection of GFP to indicate *Pirt* neurons (left panels, yellow) and NPY to indicate AgRP neurons (middle panels, magenta). Merged images are shown on the right. Shown in the top panels are representative images of ARC-containing sections from a representative Pirt$^{L10\text{-}GFP}$ mouse, and in the bottom panels are zoomed images corresponding to the boxed sections in the top panels.

To assess potential overlap between *Pirt* neurons and POMC neurons in the ARC, we prepared the brains of Pirt$^{L10\text{-}GFP}$ mice for immunofluorescence and examined the potential colocalization of GFP-IR and POMC-IR (Figure 7). We observed both GFP-IR and POMC-IR neurons in the ARC, but not within the same cells. This suggests that *Pirt* and POMC neurons represent distinct cell populations.

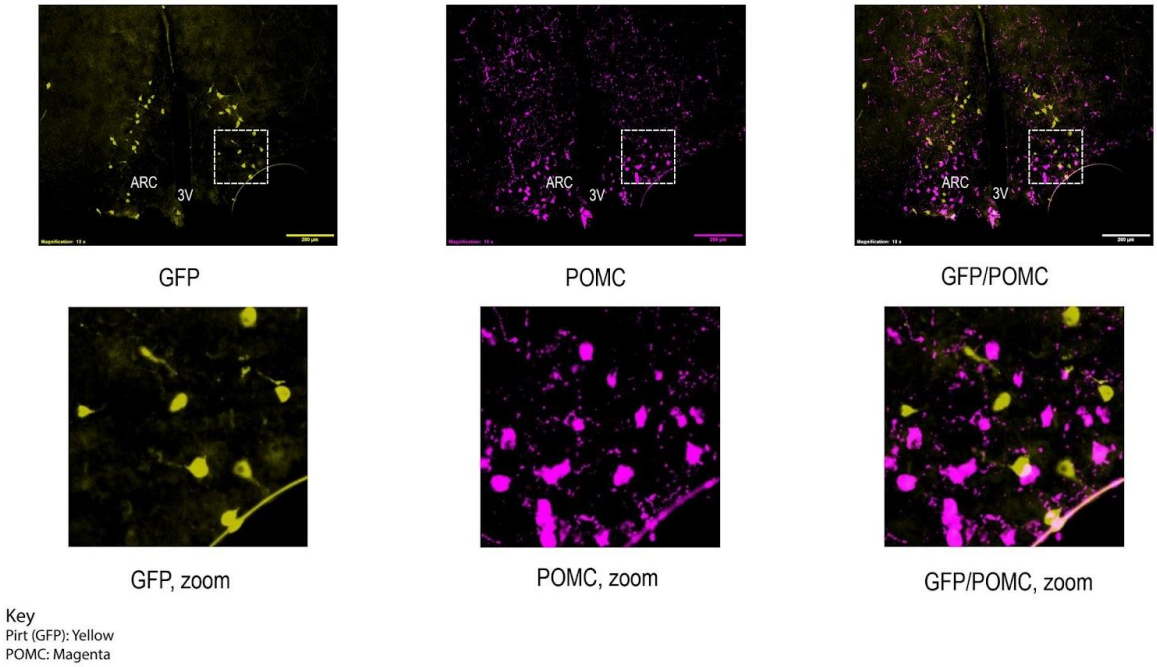


Figure 7: No colocalization of *Pirt* and Pomc neurons. Brains of Pirt$^{L10\text{-}GFP}$ mice were processed for the immunofluorescent detection of GFP to indicate *Pirt* neurons (left panels, yellow) and POMC (middle panels, magenta). Merged images are shown on the right. Shown in the top panels are representative images of ARC-containing sections from a representative Pirt$^{L10\text{-}GFP}$ mouse, and in the bottom panels are zoomed images corresponding to the boxed sections in the top panels.

To assess the potential overlap between *Pirt* neurons and KNDy neurons in the ARC, we prepared the brains of Pirt$^{L10\text{-}GFP}$ mice for immunofluorescence and looked for colocalization of GFP-IR and TAC2-IR (a marker of KNDy neurons) (Figure 8). We found both GFP-IR and TAC2-IR cells in the ARC, but not within the same cells. This finding suggests that *Pirt* and KNDy neurons represent distinct cell populations. Taken together, the lack of colocalization of NPY, POMC, and TAC2 with GFP in the ARC of Pirt$^{L10\text{-}GFP}$ mice reveals that *Pirt* neurons are distinct from other leptin-responsive ARC neuron populations.

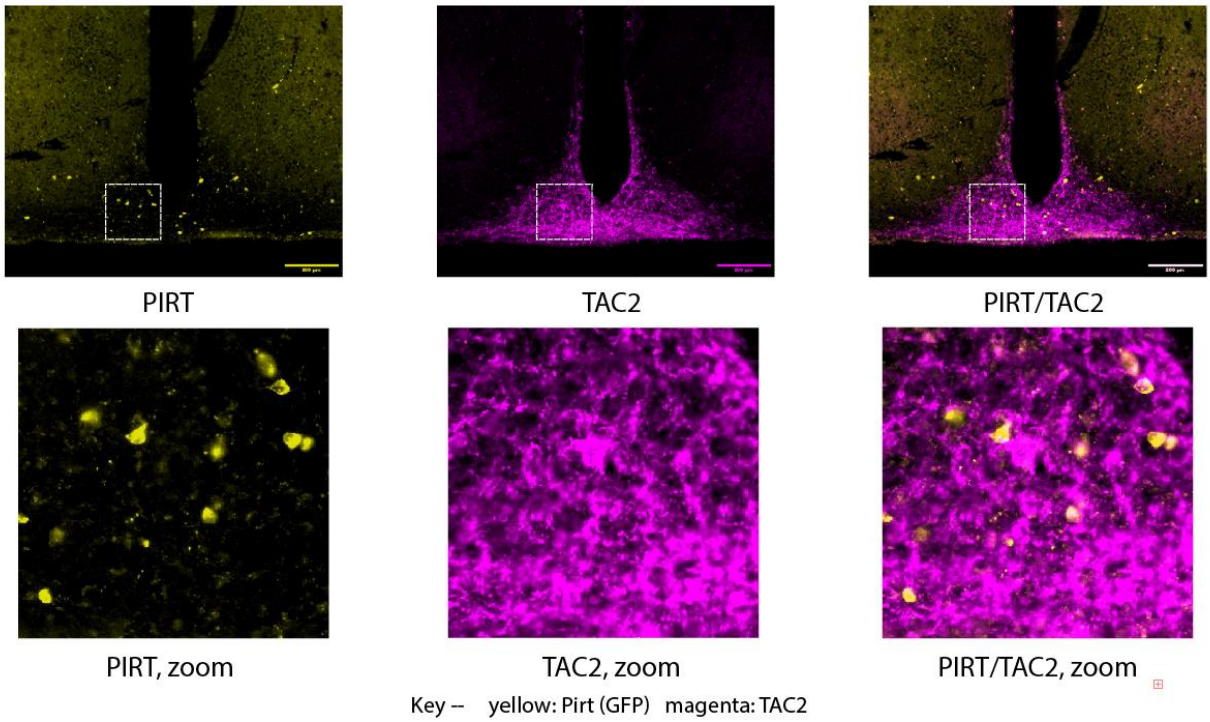


Figure 8: No colocalization of *Pirt* and KNDy neurons. Brains of Pirt$^{L10\text{-}GFP}$ mice were processed for the immunofluorescent detection of GFP to indicate *Pirt* neurons (left panels, yellow) and TAC2 to indicate KNDy neurons (middle panels, magenta). Merged images are shown on the right. Shown in the top panels are representative images of ARC-containing sections from a representative Pirt$^{L10\text{-}GFP}$ mouse, and in the bottom panels are zoomed images corresponding to the boxed sections in the top panels.

No uptake of circulating FG into ARC Pirt neurons

To see if ARC *Pirt* neurons project directly to the vasculature, we injected a retrograde tracer, fluorogold (FG), into the bloodstream of Pirt$^{L10\text{-}GFP}$ mice. We then prepared the mouse brains for immunofluorescence and looked for colocalization of GFP-IR and FG-IR (Figure 9). While we observed the expected distribution of GFP-IR *Pirt* neurons in the ARC, we observed little FG-IR, and no colocalization (Figure 9). Because the PVH contains ME-projecting neurons that synapse on the circulation, we also assessed FG-IR in the PVH as a control for FG labelling of endocrine neurons, which revealed many FG-IR neurons (Figure 9, right). These findings suggest that *Pirt* neurons do not project directly onto the vasculature.

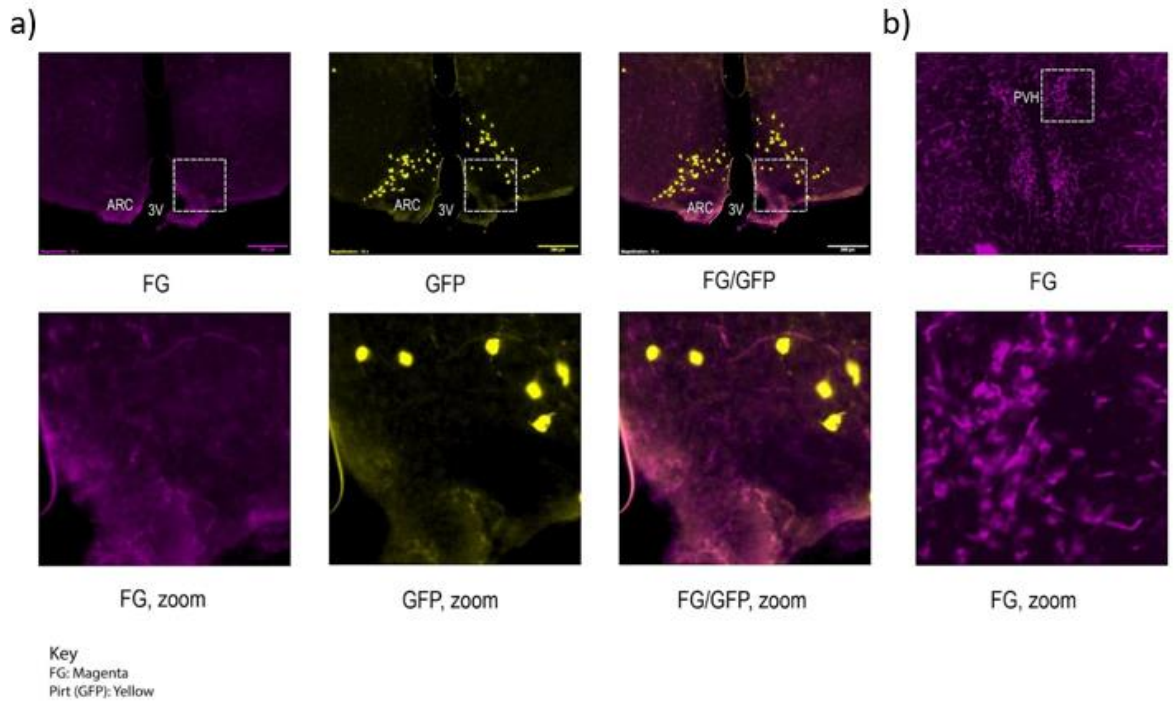
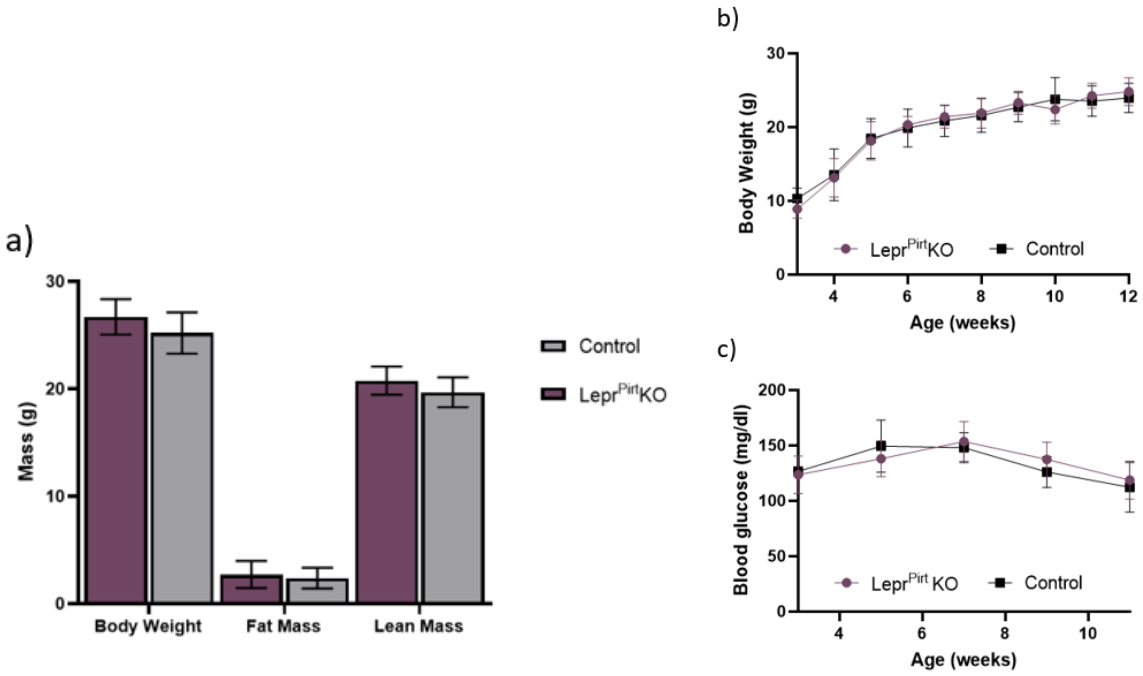


Figure 9: No colocalization of *Pirt* and FG-containing neurons. FG was injected peripherally into $Pirt^{L10\text{-}GFP}$ mice. After 5 days, the mice were perfused, and their brains were processed for the immunofluorescent detection of antibodies. Shown the top panels of (a) are images from the ARC of the immunofluorescent staining for GFP to indicate *Pirt* neurons (left panels, yellow) and FG (middle panels, magenta) in a representative $Pirt^{L10\text{-}GFP}$ mouse. Merged images are shown to the right. Shown in the top panel of (b) is an image from the PVH from the same brain as in (a), stained for FG (magenta). The presence of cell bodies containing FG in this region indicates successful injection and retrograde tracing. Shown in the bottom panels of (a) and (b) are zoomed images corresponding to the boxed panels in the top panels.

No differences in metabolic parameters between control and $Lepr^{Pirt}KO$ mice

Although we hypothesize that $LepRb^{Pirt}$ neurons mediate reproductive function, we need to rule out other possible roles in leptin action. Hence, we performed a longitudinal study comparing metabolic parameters in $Lepr^{Pirt}KO$ and control mice. Note that while the animal studies were on the Cre-inducible L10-GFP background (i.e., $Pirt^{L10\text{-}GFP}KO$ and $Pirt^{L10\text{-}GFP}$ mice), since we did not assess or otherwise utilize the L10-GFP in these animals, we will refer to them as $Lepr^{Pirt}KO$ and control mice for the sake of simplicity.

Body weight was measured weekly and blood glucose was measured biweekly. Body composition (body weight, fat mass, and lean mass) was also measured once at the end of the study. Analysis of the longitudinal data consisted of averaging and then comparing our measurements for the Lepr^{Pirt}KO and control mice. A statistically significant difference was not found between the Lepr^{Pirt}KO and control mice with respect to body weight, blood glucose, and body composition for either males or females (Figure 10).



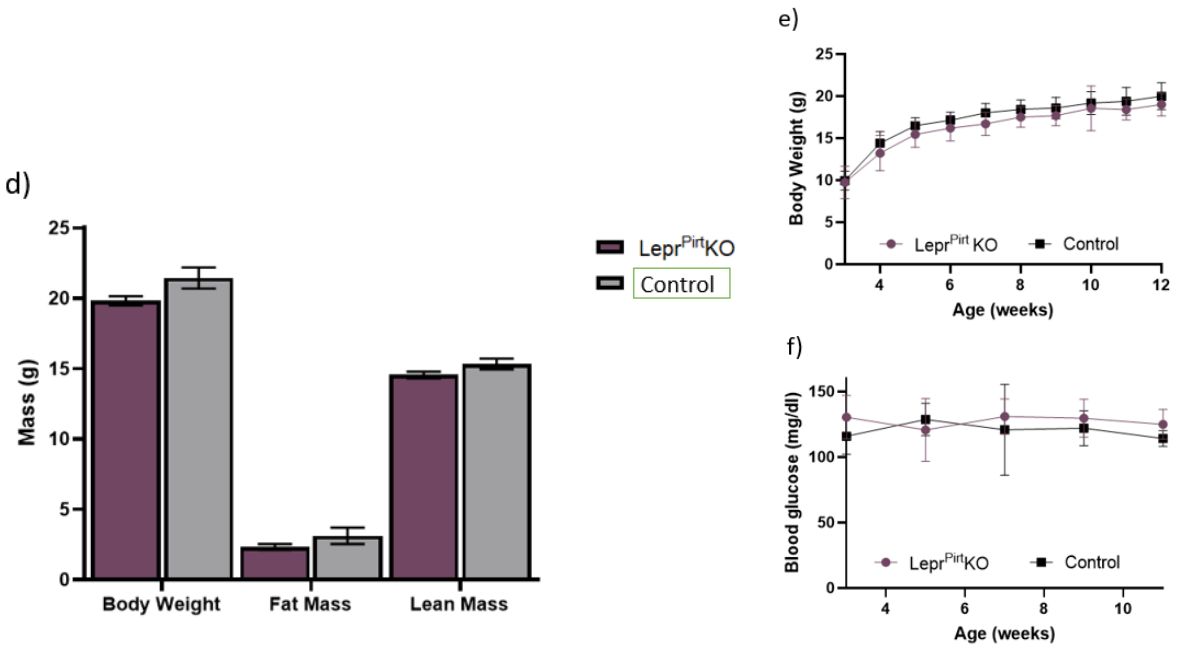


Figure 10: No significant difference in longitudinal measurements for Lepr^{Pirt}KO and control mice. The WT control group consists of Pirt$^{L10\text{-}GFP}$ mice, which are phenotypically wild-type. Shown in (a) is a bar graph comparing male Lepr^{Pirt}KO and control mice with respect to body weight, fat mass, and lean mass. Shown in (b) and (c) are line graphs comparing male Lepr^{Pirt}KO and control mice with respect to body weight and blood glucose, respectively. Shown in (d) is a bar graph comparing female Lepr^{Pirt}KO and control mice with respect to body weight, fat mass, and lean mass. Shown in (e) and (f) are line graphs comparing female Lepr^{Pirt}KO and control mice with respect to body weight and blood glucose, respectively. All results are shown as mean -/+ SEM. Sample sizes are male Lepr^{Pirt}KO: n=20, male control: n=9, female Lepr^{Pirt}KO: n=21, female control: n=10. For all comparisons: $p>0.05$ by Student's t-test.

Changes in reproductive parameters in the Lepr^{Pirt}KO mice

The rates at which mice reach pubertal milestones such as VO and VE serve as indicators of reproductive function. During the longitudinal study, we also monitored the mice for the pubertal markers with hopes of ascertaining whether there was a temporal difference in the onset of pubertal markers for the Lepr^{Pirt}KO and control mice. We hypothesized that VO and VE would be reached sooner in control mice. Female Lepr^{Pirt}KO and control mice were monitored daily for VO. Once VO occurred, the mice were then monitored for VE. We found that Lepr^{Pirt}KO mice exhibited VO (Figure 11a) and VE (Figure 11b) at younger ages than control mice.

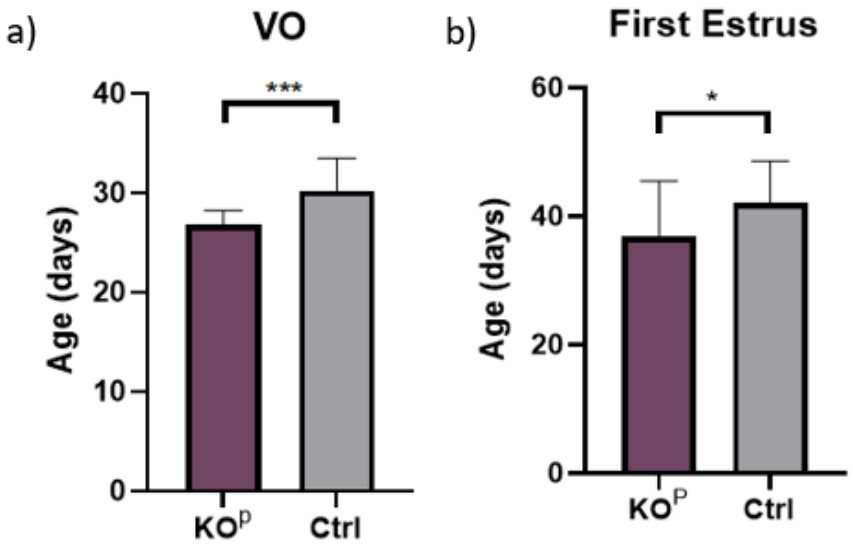


Figure 11: Lepr^{Pirt}KO (Lepr$^{Pirt\text{-}GFP}$KO; KO^{P}) mice reach pubertal milestones faster than control (Pirt$^{L10\text{-}GFP}$; Ctrl) mice. Female KO^{P} and Ctrl mice were monitored daily for VO and VE. (a) Bar graph showing the time taken for KO^{P} and Ctrl mice to undergo vaginal opening (VO). (b) Bar graph showing the time taken for KO^{P} and Ctrl mice to exhibit first estrus (VE). Data are plotted as mean -/+ SEM; *p<0.05, ***p<0.001 by Student's t-test.

Our current hypothesis suggests that Lepr^{Pirt}KO neurons control leptin-mediated effects on reproduction via stimulation of GnRH release and enhancement of progression through the estrous cycle. Thus, our hypothesis predicts decreased time spent in estrus and increased time spent in diestrus for Lepr^{Pirt}KO compared to control mice. To test our hypothesis, we tracked estrous cycling in the female Lepr^{Pirt}KO and control mice in our longitudinal study by examining cytology in the vaginal cavity. At the end of the study, we averaged the time spent in each stage for Lepr^{Pirt}KO and control mice. While we observed no statistically significant changes in time spent at each state, Lepr^{Pirt}KO mice tended to spend less time in estrus (p=0.1) and more time in diestrus (p=0.06) (Figure 12).

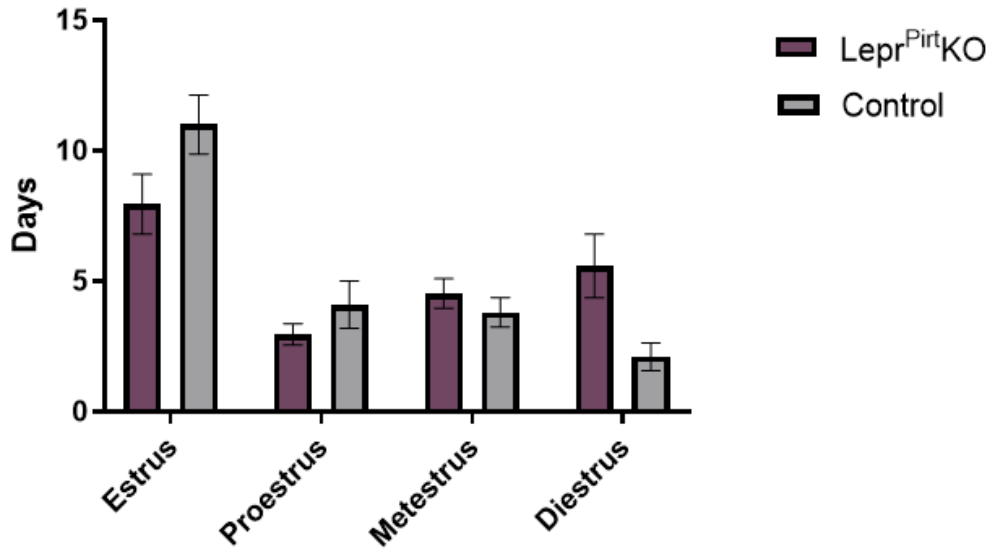


Figure 12: Time spent in each stage of the estrus cycle for $Lepr^{Pirt}KO$ ($Lepr^{Pirt\text{-}GFP}KO$) and control ($Pirt^{L10\text{-}GFP}$) mice. Animals were monitored daily for what stage of estrous they were in for 3 weeks from the time of estrus onset. Shown is a bar graph comparing $Lepr^{Pirt}KO$ and control mice for the number of days spent at each stage of estrous. Data are shown as mean -/+ SEM; n=21 for $Lepr^{Pirt}KO$ and n=10 for control. A Student's t-test comparing $Lepr^{Pirt}KO$ and control groups for each stage was performed where significance was defined as $p<0.05$. No significant differences were found.

No hormonal differences between control and KO mice

Our hypotheses suggest that $LepRb^{Pirt}$ cells modulate reproduction by altering the secretion of GnRH and thus pituitary-derived LH. To assess the effect of leptin input on $LepRb^{Pirt}$ cells on hormone concentrations, we obtained blood from $Lepr^{Pirt}KO$ and control mice for hormone measurements at the time of euthanasia. We assessed concentrations of leptin and insulin to determine any potential metabolic alterations and assessed pituitary hormone concentrations to determine any endocrine effects. A comparison of $Lepr^{Pirt}KO$ and control mice did not reveal a significant difference in concentration for any of the hormones (Figure 13).

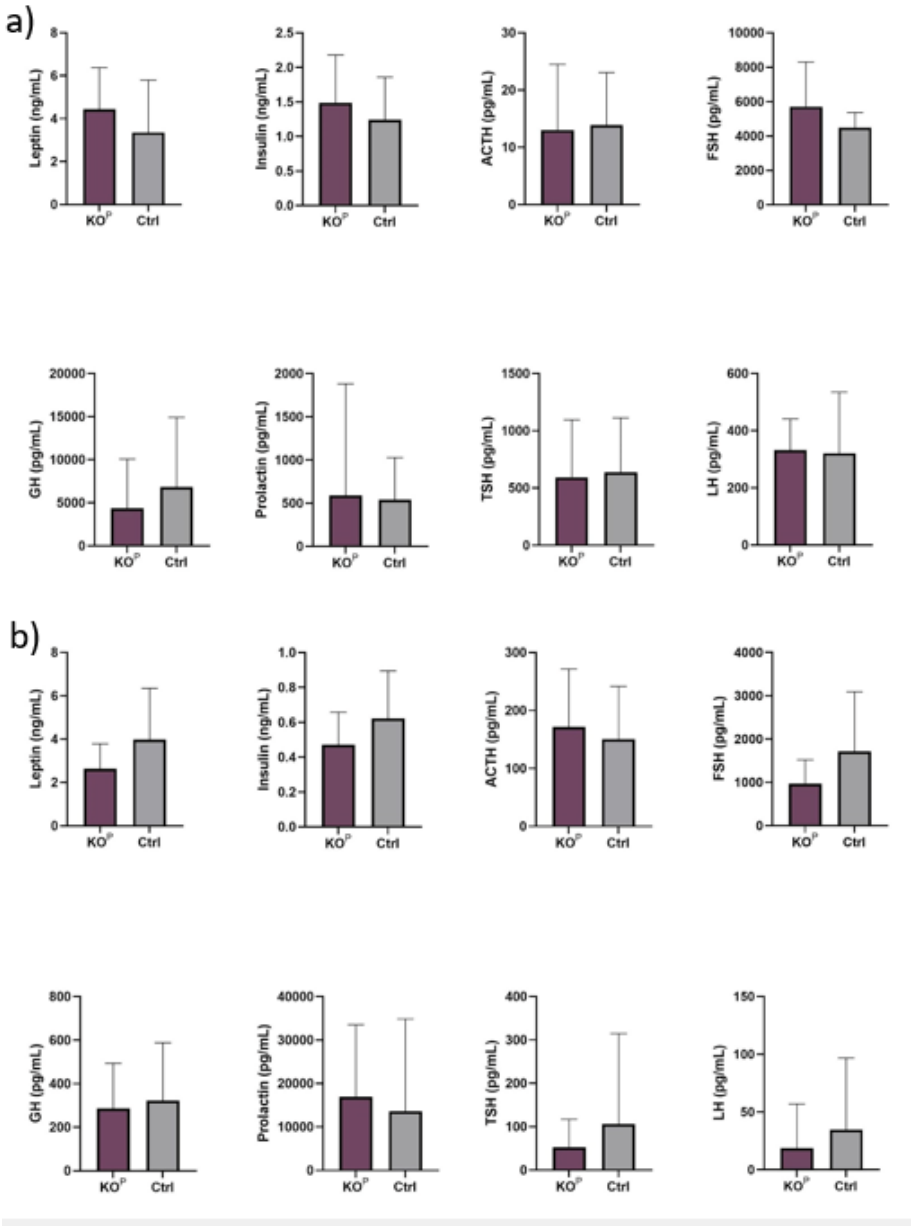


Figure 13: No significant differences in hormone concentrations for KO^{P} (Lepr$^{Pirt\text{-}GFP}$KO) vs Ctrl (Pirt$^{L10\text{-}GFP}$) mice. Shown are bar graphs comparing the concentration of specific hormones between KO^{P} and Ctrl males (a) and (b) females. The assayed concentrations of the indicated hormones are plotted as mean -/+ SEM. Sample sizes are male Lepr^{Pirt}KO: n=20, male control: n=9, female Lepr^{Pirt}KO: n=21, female control: n=10. Student's t-test comparing KO^{P} and Ctrl groups was performed where significance was defined as $p<0.05$. No significant differences were found.

Discussion

This study yielded varied results towards the understanding of the role of *Pirt* neurons in leptin-mediated reproductive functioning. We have shown that *Pirt* neurons express *Lepr*. We then showed that these LepRb^{Pirt} neurons are distinct from other *Lepr*-expressing ARC neuron populations. Using the retrograde tracer FG, we determined that although LepRb^{Pirt} neurons project into the median eminence, they do not project onto the pituitary portal system or other elements of the circulatory system. From our longitudinal studies, we found that knocking out *Lepr* in *Pirt* cells has no effect on body weight, blood glucose or body composition, but does alter reproductive parameters, consistent with the notion that LepRb^{Pirt} neurons link energy stores and reproductive function. Further investigation is required to understand the specific steps by which this effect is mediated.

The immunohistochemical studies were an important step to demonstrate that LepRb^{Pirt} cells represent a discrete population of ARC LepRb neurons. Because LepRb^{Pirt} neurons were predicted to represent a novel population of LepRb neurons by snRNA-seq, it was important to identify these cells and their location (we found that they reside in the ARC) and to demonstrate that they are distinct from other previously-described sets of LepRb^{Pirt} neurons, including AgRP, POMC, and KNDy cells. Luckily there are antibodies available to permit the immunofluorescent detection of each of those populations. Furthermore, we have a transgenic mouse line, Pirt$^{L10\text{-}GFP}$, that expresses GFP in only LepRb^{Pirt} cells, which means we can use a GFP antibody to identify *Pirt*-expressing cells. Therefore, by staining for GFP and one of the comparison neuron populations on the same mouse brain section, we can look for overlap of the fluorescent markers. Since there was no colocalization in any of the cases, we can conclude that LepRb^{Pirt} neurons comprise a novel and distinct population of ARC LepRb neurons. It follows that these cells may mediate unique roles in leptin action.

From the FG experiment we hoped to understand whether LepRb^{Pirt} neurons, which project into the median eminence, synapse on the pituitary portal circulation. If FG was found in the cell bodies of ARC *Pirt* neurons, that would suggest that LepRb^{Pirt} neurons synapse directly on blood vessels like those of the pituitary portal system. If FG was not found, LepRb^{Pirt} neurons must instead synapse onto other structures in the

median eminence to control the release of GnRH or other factors into the pituitary portal system to control LH secretion. We found the latter.

A logical population of neurons that may serve as the intermediary between LepRb^{Pirt} neurons and the pituitary would be GnRH neurons. Since LepRb^{Pirt} neurons do not express *Gnrh*, LepRb^{Pirt} neurons cannot directly release GnRH into the portal system. Therefore, LepRb^{Pirt} neurons could synapse onto GnRH nerve terminals in the median eminence en route from the GnRH cell soma to their respective synapses on the pituitary portal circulation. The next step is to identify the specific cells or processes that LepRb^{Pirt} cells project to. One experiment to test this would be to perform anterograde tracing of LepRb^{Pirt} cells in mice, and view this tracing with sagittal sections of the brain. Sagittal sections, compared to the typically done coronal sections, would allow us to more clearly see where the axons of LepRb^{Pirt} neurons terminate within the median eminence, enabling us to determine their potential proximity to GnRH nerve terminals.

The longitudinal study revealed that knocking out *Lepr* in LepRb^{Pirt} neurons had no significant effect on body weight, blood glucose, and body composition. This supports the hypothesis that LepRb^{Pirt} neurons mediate non-metabolic responses to leptin, such as reproduction or other endocrine functions.

The vaginal cytology data provide an interesting array of results. On the one hand, the finding that Lepr^{Pirt}KO mice tend to spend more time in the diestrus and less time in estrus supports our theory that leptin stimulates reproduction via LepRb^{Pirt} neurons. Because our results did not achieve statistical significance, it will be important to study additional animals to determine whether increasing sample size might produce a significant result. Although LepRb^{Pirt} neurons produce GABA, which tends to inhibit downstream neurons, GABA actually excites GnRH neurons in many cases (Watanabe et al., 2014), suggesting that the leptin-mediated activation of LepRb^{Pirt} neurons would increase GnRH secretion were they to synapse on GnRH terminals.

On the other hand, we found that Lepr^{Pirt}KO mice entered VO and VE earlier than control mice. Since puberty and reproductive development are energetically expensive processes, our hypothesis would predict that decreased leptin signaling delays VO and VE. While we do not understand this potentially contradictory result, we have begun to

repeat this study in a separate cohort of animals; this result did not repeat, suggesting that this initial finding may have been specious.

While our hypothesis predicts possible decreases in LH secretion in Lepr^{Pirt}KO mice, we did not find such differences (or any others) from the hormone analysis. However, upon further consideration, this result could have just been a product of the experimental procedure and does not necessarily discount our hypothetical model. As mentioned in the Results, the frequency and magnitude of LH pulses depend on the stage of the estrous cycle. Since we did not account for the cycle stage during hormone collection, additional variability in hormone concentration was introduced into our results. Additionally, we don't know for each animal whether the measurement corresponds to a peak or trough in hormone secretion, which adds additional variability to this analysis. We plan to repeat our analysis using cycle-controlled animals and repeated sampling to determine pulse amplitude and frequency for each animal.

In summary, our overall hypothesis was generally supported by our data, although some revision of our mechanistic understanding of the system was required. We found evidence that LepRb^{Pirt} neurons are a unique population of cells that link leptin with the endocrine-mediated modulation of reproduction. Furthermore, we found evidence that LepRb^{Pirt} neurons mediate this effect by stimulating GnRH secretion in response to leptin, rather than inhibiting GnRH. However, our hormone analysis gave no indication that LepRb^{Pirt} neurons modulate LH or FSH. LepRb^{Pirt} neurons were also shown to only have a minor effect on estrus cycling. As previously mentioned in this section, the hormone analysis and longitudinal cytology study that produced these results potentially have experimental flaws that affected the quality of the data. Therefore, we plan to repeat these experiments. Nevertheless, LepRb^{Pirt} neurons represent a promising area of study as a potential link between leptin signaling and reproductive development.

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