HORMONE REGULATION OF NPY NEURON ACTIVITY IN THE

ARCUATE NUCLEUS OF THE HYPOTHALAMUS

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DEDICATION

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HORMONE REGULATION OF NPY NEURON ACTIVITY IN THE ARCUATE NUCLEUS OF THE HYPOTHALAMUS

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Neuropeptide Y (NPY)-expressing neurons in the arcuate nucleus of the hypothalamus are part of a neuroendocrine feedback loop that regulates feeding behavior and glucose homeostasis. NPY neurons sense peripheral signals of energy stores, including the hormones leptin, insulin, and ghrelin, and integrate those signals with inputs from other brain regions including serotonergic neurons from the dorsal raphe. These inputs modify both long-term changes in gene transcription and acute changes in the electrical activity of these neurons, leading to a coordinated response to maintain energy and glucose homeostasis. While the regulation of transcriptional changes in NPY neurons is relatively well-understood, the

mechanisms by which insulin, leptin, ghrelin, and serotonin acutely modify the electrical activity of these neurons remains unclear. To determine ion channels that are coupled to hormone and neurotransmitter receptors present on NPY neurons, whole-cell patch clamp electrophysiology was performed on identified NPY neurons throughout the rostral-caudal extent of the arcuate nucleus of the hypothalamus. Leptin and insulin acutely hyperpolarized NPY neurons, and these effects were dependent upon opening of ATP-sensitive potassium (K_{ATP}) channels. In addition, serotonin and specific serotonin receptor-1b agonists acutely hyperpolarized NPY neurons. Conversely, the hormone ghrelin was found to acutely depolarize NPY neurons via a mixed cation conductance. Taken together, these studies demonstrate that at all levels of the arcuate nucleus, insulin, leptin, and serotonin acutely inhibit NPY neurons and that ghrelin acutely activates these neurons. Finally, a genetic model of NPY neuron-specific ablation of the intracellular phosphatidylinositol-3-kinase (PI3K) pathway was developed to assess the requirement of this cascade in mediating the acute effects of these hormones and the effects of NPY neurons on body weight and glucose homeostasis.

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PRIOR PUBLICATIONS

J.E. Janis, J.C. Barker, **C.S. Javadi**, I. Ducic, R. Hagan, B. Guyuron. A Review of Current Evidence in the Surgical Treatment of Migraine Headaches. Plast. Reconstr. Surg. 2014 Oct;134(4 Suppl 2):131S-41S.

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CHAPTER ONE
Introduction

To regulate energy balance, the central nervous system integrates homeostatic and hedonic needs to drive a coordinated response in peripheral metabolism and in motivated behavior. In particular, the hypothalamus receives signals of peripheral energy stores, including circulating metabolites and hormones, as well as neurotransmitters from other brain regions and from the peripheral nervous system. In response to these signals, the brain modifies feeding behavior and energy expenditure to promote homeostasis (Schwartz et al., 1999; Woods and Seeley, 2000).

Pro-opiomelanocortin (POMC)-expressing neurons and neuropeptide Y (NPY)-expressing neurons in the arcuate nucleus of hypothalamus are among the first-order neurons that integrate these peripheral signals and coordinate an appropriate response via projections to other brain regions. POMC and NPY neurons express leptin and insulin receptors, the activation of which alters the expression and release of neuropeptides involved in energy balance (Baskin et al., 1999; Mercer et al., 1996). POMC neurons release alpha-melanocyte stimulating hormone, which decreases food intake through activation of melanocortin-4 receptors (MC4Rs). As a counterbalance to POMC neurons, neighboring NPY neurons promote positive energy balance through the release of the orexigenic peptide NPY and

agouti-related protein (AgRP), which antagonizes MC4Rs. NPY neurons also directly inhibit POMC neurons via GABAergic synapses (Elmquist et al., 1999; 2005).

The precise contribution of NPY neurons to energy balance remains unclear. Experimental manipulations of these neurons and the neurotransmitters they release have produced varying effects on food intake, body weight, and glucose homeostasis. Early studies established the hypothesis that NPY neuronal activity is a determinant of feeding behavior. Overexpression or central administration of the AgRP peptide was shown to increase food intake and cause obesity (Ollmann et al., 1997). In fasted mice, NPY neurons were found to fire action potentials at an increased frequency (Takahashi and Cone, 2005). Abolishing GABA transmission from NPY neurons via targeted deletion of the vesicular GABA transporter *vgat* yielded lean mice resistant to diet-induced obesity (Tong et al., 2008). Using optogenetic techniques, Aponte and colleagues found that acute activation of ~800 NPY neurons is sufficient to evoke rapid feeding even in fed mice (Aponte et al., 2010). These results pointed to a crucial role for NPY neurons in feeding behavior.

However, mice lacking the NPY and AgRP peptides altogether display wild-type body weight responses to fasting, high fat diet, and exogenous leptin (Qian et al., 2002). Building upon these results, studies in which NPY neurons were ablated in adult mice found a reduction in food intake (Gropp et al., 2005; Luquet et al., 2005), even in mice lacking functional *npy* and *agrp* genes (Phillips and Palmiter, 2008) or in *agouti* mice, in which downstream melanocortin signaling is blocked by ectopic production of agouti protein (Wu

et al., 2008). These studies indicate that the hypophagia observed after ablation of NPY neurons does not depend on loss of inhibition or activation of the melanocortin pathway. Collectively, these results suggest that the neuronal activity of NPY neurons is more essential to feeding behavior than the NPY or AgRP peptides themselves and that other neural circuits may compensate for loss of NPY neurons during development. Determining the endogenous factors that modulate the acute neuronal activity of NPY neurons may provide insight into their role in energy balance and possibly direct the development of therapeutics for the treatment of diabetes and obesity.

Serotonin (5-hydroxytryptamine, 5-HT) has been known to play a role in the regulation of energy homeostasis since early observations that depletion of central serotonin stores promotes hyperphagia and obesity (Breisch et al., 1976; Garattini et al., 1986; Goudie et al., 1976). Compounds that increase central 5-HT bioavailability, such as the anti-obesity drug d-fenfluramine, or compounds that act as serotonin receptor agonists, such as meta-chlorophenylpiperazine (mCPP), reduce food intake and body weight (Copp et al., 1967; Samanin et al., 1980). More recent studies have found that serotonin modulates melanocortin pathways in the arcuate nucleus of the hypothalamus to inhibit feeding (Heisler et al., 2002; 2006; Lam et al., 2008). Identifying the specific mechanisms through which serotonin modifies NPY neuronal activity will likely provide additional clues to the role of NPY neurons in energy balance.

Until recently, electrophysiologic studies of NPY neurons have been limited by the lack of suitable reporters of NPY expression in hypothalamic slice preparations. These now-available reporter lines allow the direct assessment of the acute cellular responses of identified NPY neurons to the key hormones insulin and leptin and to the neurotransmitter serotonin.

CHAPTER TWO
Review of the Literature

CENTRAL REGULATION OF ENERGY HOMEOSTASIS

Early Studies of Hypothalamic Lesions

The hypothesis that the central nervous system and whole-body energy balance are linked was made as early as 1888. Sir Byrom Bramwell noted in a series of lectures in Edinburgh that:

> Tumours of the pituitary body are in many instances attended with an excessive development of the subcutaneous fat, and in some cases with the presence of sugar in the urine… Whether these symptoms are due to the fact that the pituitary body itself is diseased, or whether, as seems more likely, to the secondary results which tumours in this situation produce in the surrounding cerebral tissue, has not yet been decided (Bramwell, 1888).

In these patients with pituitary tumors, Bramwell and Fröhlich noted a syndrome of excessive subcutaneous fat and hypogonadism, now called Fröhlich syndrome. Fröhlich and Cushing hypothesized at the time that injury to, or insufficiency of, the pituitary was responsible (Crowe et al., 1910; Fröhlich, 1901), while Bramwell supported the view that damage to the overlying hypothalamus via mass effect was the cause of the adiposogenital syndrome. Bernhard Aschner later demonstrated in dogs that careful removal of the pituitary without damage to the surrounding hypothalamus did not result in obesity (Aschner, 1912).

In the century that followed, investigators studied ever more precise lesions of the hypothalamus, uncovering specific neural circuits in the hypothalamus that detect peripheral signals of energy balance and drive whole body metabolism and feeding behavior.

PERIPHERAL SIGNALS OF ENERGY BALANCE

Insulin

Insulin was discovered in 1921 by the Canadian surgeon Frederick Banting, his mentor, J.J.R Macleod, and his student, Charles Best, but it was not until the 1960s that insulin's effects on the brain were tested extensively. In 1965, injection of insulin into the cisterna magna of dogs was shown to decrease peripheral blood glucose, an effect that was abolished by vagotomy (Chowers et al., 1966). This experiment suggested that either increased glucose utilization by the CNS or insulin acting on insulin receptors in the CNS can stimulate insulin secretion by the pancreas via the parasympathetic nervous system. However, the hypothesis that insulin had direct effects on the brain was a subject of considerable controversy at the time (Goodner and Berrie, 1977; Margolis and Altszuler, 1968; Mellerup and Rafaelsen, 1969; Sloviter and Yamada, 1971; Szabo and Szabo, 1972), until investigators identified *bone fide* insulin receptors in the CNS of various species using radiolabeled insulin (Havrankova et al., 1978; Posner et al., 1974). In the rat, van Houton and colleagues localized these insulin binding sites to the circumventricular organs, the medial basal hypothalamus, and the paravagal region (van Houten et al., 1979). These early studies linked insulin with CNS regions associated with glucose monitoring, satiety, and gastrointestinal regulation (Baskin et al., 1987).

Subsequent studies provided evidence that insulin is not a regulator of glucose uptake within the CNS but instead acts as a neuromodulator (Woods et al., 1985). Cloning of glucose transporters in the 1980s revealed that the brain does not require insulin in order to take up glucose from the surrounding extracellular fluid. This insulin-independent glucose uptake ensures that in fasting conditions, the brain has preferential access to glucose (Birnbaum et al., 1986). Later studies elucidated the kinetics of peripheral insulin crossing the blood-brain barrier. Early reports found that peripheral and CSF insulin concentrations changed in a proportional manner, suggesting that CSF uptake occurs via passive diffusion (Woods and Porte, 1977). A subsequent observation, however, in the genetically obese Zucker (*fa/fa*) rat model found reduced uptake of insulin into the CSF from high levels in the plasma, suggesting that specific receptor-mediated uptake occurs at the blood-brain barrier (Stein et al., 1987). Schwartz and colleagues measured CSF insulin levels during a euglycemic, hyperinsulinemic clamp in dogs and found a delay in uptake that is atypical for passive diffusion alone. In the same report, they demonstrated that the penetration of proinsulin into CSF was reduced compared with that of insulin, consistent with the hypothesis that insulin uptake into the CSF occurs at least in part via specific transport (Schwartz et al., 1990).

Peripheral insulin sensitivity largely determines plasma and therefore CNS insulin levels. As such, insulin can signal not only total body fat stores, but also body fat distribution, as central adiposity is a key determinant of whole-body insulin sensitivity and plasma insulin levels

(Fujimoto et al., 1994; Kahn et al., 1993; Shulman, 2014). Further, plasma insulin shifts rapidly in response to food ingestion (Polonsky et al., 1988).

Central infusion studies of insulin provided more direct evidence of insulin's role as a signaling molecule. Infusion of insulin into the lateral ventricle in baboons was found to suppress food intake and body weight in a dose-dependent manner. The same investigators also elevated endogenous insulin levels by intravenous infusion of glucose at doses that would supply 25% and 50% of total calories; the subsequent hyperinsulinemia similarly suppressed food intake (Porte and Woods, 1981).

These findings suggested that insulin can act as a feedback signal to the CNS. However, in order to demonstrate a classical feedback loop, the converse experiment was required—demonstrating that hypothalamic-initiated parasympathetic activity can increase insulin secretion. Evidence for this arm of the feedback loop was provided from studies of genetically obese Zucker rats. The hyperinsulinemia of these animals was found to be abolished by atropine, suggesting that insulin release is in part mediated by the vagus nerve (Rohner-Jeanrenaud et al., 1983). Steffens tested this hypothesis by infusing norepinephrine directly into the lateral hypothalamic area and found increased insulin release from the pancreas (Steffens, 1981).

Several other studies elaborated on the link between hypothalamic nuclei and metabolism in the liver and adipose tissue (Shimazu, 1981). These studies revealed a counter-balancing

network of hypothalamic nuclei acting on peripheral tissues via autonomic fibers. Electrical stimulation of the acetylcholine-sensitive neurons in the lateral hypothalamic nucleus (LH) provoked liver glycogenesis by activation of glycogen synthetase via the vagus nerve; on the other hand, activation of the norepinephrine-sensitive neurons in the ventromedial hypothalamic nucleus (VMH) promoted liver glycogenolysis by activation of glycogen phosphorylase via splanchnic nerves. The VMH-sympathetic nervous system was also shown to modulate both triglyceride synthesis and breakdown in brown adipose tissue. Thus, hypothalamic centers can regulate carbohydrate, amino acid, and lipid metabolism via autonomic connections (Shimazu, 1981; Woods et al., 1985).

Other investigators examined the role of central insulin in the regulation of circadian rhythm. Insulin infusion in rats during the daytime was known to produce a meal pattern identical to that normally observed at night (Larue-Achagiotis and le Magnen, 1979). Sakaguchi, Takahashi, and Bray examined the effects of centrally administered insulin on hypothalamic centers regulating the circadian rhythm. They found that a diurnal rhythm exists for the sympathetic firing rate—sympathetic firing is highest at noon, when food intake is the lowest. Injection of insulin into the VMH produced a dose-dependent depression of sympathetic firing rate, greatest at nighttime. Injection into the suprachiasmatic nucleus (SCN), however, depressed firing during the day but increased firing at night. That is, the inverse relationship between sympathetic firing rate and food intake observed in basal conditions was attenuated by insulin (Sakaguchi and Bray, 1987; Sakaguchi et al., 1988).

These findings demonstrated that insulin can modify circadian rhythm at the level of the SCN.

Several investigators sought to reveal the mechanism by which central insulin can suppress food intake. One such hypothesis tested was that insulin acts to reduce expression of the gene encoding neuropeptide Y (NPY), a peptide known to be potent stimulator of feeding in the rat (Stanley et al., 1986). NPY is primarily produced by neurons in the hypothalamic arcuate nucleus (O'Donohue et al., 1985) and is released from terminals located in the paraventricular nucleus (Kalra et al., 1991). The arcuate nucleus is rich in insulin receptors (Baskin et al., 1988) and is particularly sensitive to the anorectic action of insulin (O'Donohue et al., 1985). The hypothesis that insulin suppresses *npy* gene expression was put forth after observations that hypoinsulinemic states (e.g., fasting and type I diabetes mellitus) are associated with an increase in the hypothalamic content of both NPY (Sahu et al., 1988; Williams et al., 1989) and its messenger RNA, preproNPY mRNA (White and Kershaw, 1990; White et al., 1990). Furthermore, systemic insulin therapy normalizes these parameters. Conversely, the hyperinsulinemic Zucker rat model has increased hypothalamic NPY protein (Higuchi et al., 1988) and mRNA (Beck et al., 1990; Sanacora et al., 1990) and is insensitive to the satiety action of central insulin (Ikeda et al., 1986). Schwartz and colleagues provided the first direct evidence of this hypothesis. They found that intracerebroventricular (i.c.v) administration of insulin in the arcuate nucleus suppressed preproNPY mRNA in lean rats but not in Zucker rats. This interaction likely plays a role in the hyperphagia and obesity of the Zucker model

and other diabetic states (Schwartz et al., 1992; 1991; Wang and Leibowitz, 1997) and points to a crucial role of NPY neurons in the central actions of insulin.

Leptin

Leptin is a peptide hormone secreted by white adipose tissue during periods of nutrient abundance (Zhang et al., 1994). Coleman and colleagues hypothesized the existence of such a circulating satiety factor during their study of two mutant mouse strains, *ob/ob* mice and *db/db* mice, both of which display hyperphagia, obesity, and hypogonadism (Coleman, 1973; Coleman and Hummel, 1969). Coleman performed parabiosis experiments in which he established cross-circulation between *ob/ob*, *db/db*, and wildtype mice, the results of which suggested the *ob* gene encoded a circulating factor that binds to a corresponding receptor mutated in *db/db* mice. This hypothesis was confirmed by the cloning of the *ob* gene product, leptin (Zhang et al., 1994), and its receptor, the *db* gene product LepR (Tartaglia et al., 1995).

At 16-kD, leptin is a much larger protein than insulin and was thought, like insulin, to cross the blood-brain barrier via saturable facilitated transport. Banks and Schwartz confirmed this hypothesis (Banks et al., 1996; Schwartz et al., 1996b), and Banks later found that this transport into the CNS was impaired in obesity (Banks et al., 1999).

Subsequent to their discovery, leptin receptors were localized to brain regions that are integral to body weight and food intake regulation. NPY neurons within the arcuate nucleus were found to coexpress leptin receptor mRNA (Mercer et al., 1996). Leptin deficiency in the

fasting state or in *ob/ob* or *db/db* mice dramatically raises preproNPY mRNA, while exogenous leptin administration had the opposite effect of decreasing arcuate preproNPY mRNA in *ob/ob* or in fasted mice (Ahima et al., 1996; Schwartz et al., 1996c; Stephens et al., 1995).

Leptin binding to the extracellular domain of its receptor, LepRb, induces the recruitment and activation of Janus kinase (JAK). In turn, JAK binds to and phosphorylates LepRb, which activates STAT3. Phosphorylated STAT3 then binds to the *pomc* and *agrp* promoters, enhancing POMC and inhibiting AgRP expression (Ernst et al., 2009; Mesaros et al., 2008; Vaisse et al., 1996).

The investigation of the neuroanatomy underlying leptin's effects on food intake revealed a fundamental neural circuit in the arcuate nucleus. Elias and colleagues demonstrated that arcuate NPY and POMC neurons innervate MCH and ORX neurons in the lateral hypothalamic area. They also found that leptin differentially regulates these two populations: in response to leptin, NPY neurons were SOCS-3(+) and Fos(-), suggesting they were inhibited, while POMC neurons were SOCS-3(+) and Fos(+), signifying activation (Elias et al., 1999).

Ghrelin

Ghrelin is a 28-amino acid octanoylated peptide hormone secreted by enteroendocrine cells of the gastric oxyntic mucosa (Kojima et al., 1999; Sakata et al., 2009). It is the only known

circulating hormone that directly stimulates appetite (Cummings, 2006; Nakazato et al., 2001; Wren et al., 2001). Ghrelin levels in the blood rise during food restriction, before meal initiation, and after acute or chronic stress (Cummings et al., 2001; Lutter et al., 2008a; 2008b). Physiologic or pharmacologic increases in ghrelin levels promote both homeostatic and hedonic feeding behaviors in rodents and in humans (Cummings, 2006; Karra et al., 2013; Nakazato et al., 2001; Perello and Zigman, 2012; Uchida et al., 2013; Wren et al., 2001). Increases in food intake are observed after acute or chronic administration of ghrelin centrally via intracerebroventricular microinjection or peripherally via intraperitoneal injection (Nakazato et al., 2001; Wang et al., 2002; Wren et al., 2001; Zigman et al., 2005). Ghrelin's effects occur via binding to its only known receptor, the growth hormone secretagogue receptor type 1a (GHSR) (Howard et al., 1996; Nakazato et al., 2001). GHSR is a 7-pass transmembrane protein coupled to the Gq subfamily of heterotrimeric GTP-binding proteins that activate phospholipase C which, in turn, induces release of intracellular calcium stores (Howard et al., 1996). In order to bind and activate the GHSR, ghrelin must undergo Ser^3 O-octanoylation, a post-translational modification catalyzed by the enzyme ghrelin O-acyl transferase (GOAT) (Yang et al., 2008).

Ghrelin has several additional actions in addition to its potent orexigenic effect. It is a potent growth hormone secretagogue (Kojima et al., 1999). Ghrelin also stimulates gastrointestinal motility and accelerates gastric emptying (Peeters, 2003). Des-acyl-ghrelin, the non-octanoylated form, can also modify food intake and glucose homeostasis independent of GHSR binding (Delhanty et al., 2012). Ghrelin has also been shown to minimize depressive-

like behavior during chronic social stress(Lutter et al., 2008b).

Ghrelin plays a significant role in the maintenance of euglycemia. Insulin-induced hypoglycemia raises gastric ghrelin transcript levels (Toshinai et al., 2001). Ghrelin release from cultured gastric ghrelin cells is potentiated by exposure to low-glucose media (Sakata et al., 2012). Exogenous administration of ghrelin in rodents increases blood glucose, lowers insulin levels, raises the levels of the counter-regulatory hormones glucagon and growth hormone, and diminishes insulin responses during glucose tolerance testing (Chuang et al., 2011b; Dezaki et al., 2004; 2007; Zhao et al., 2010). Conversely, blood glucose is lowered when ghrelin, GHSR, or GOAT is genetically deleted or pharmacologically blocked (Chuang et al., 2011b; Dezaki et al., 2007; 2006; Longo et al., 2008; Sun et al., 2006; Zigman et al., 2005). Ghrelin's major physiologic role in blood glucose regulation may be preventing life-threatening hypoglycemia during extreme caloric restriction (Goldstein et al., 2011). In GOAT-knockout and ghrelin-knockout mice exposed to seven days of 40% of their usual caloric intake, fasting blood glucose progressively declined to near-fatal levels (Li et al., 2012; Zhao et al., 2010).

The orexigenic and glucoregulatory effects of ghrelin are mediated by its direct engagement with neurons in the central nervous system. GHSRs are expressed in central nervous system nuclei (Cowley et al., 2003; Zigman et al., 2006), and several of these nuclei express c-fos after ghrelin administration (Faulconbridge et al., 2008; Lawrence et al., 2002; Nakazato et al., 2001).

Using a reactivatable GHSR-null mouse model that lacks GHSR expression except in cell populations engineered to express Cre recombinase (Zigman et al., 2005), Zigman and colleagues identified CNS sites that mediate subsets of ghrelin's actions on glucose control and feeding behavior. Selective restoration of GHSR expression in tyrosine hydroxylase-expressing catecholaminergic neurons partially restores exogenous ghrelin-induced food intake and completely restores the feeding response to chronic stress and conditioned place preference for high fat diet, a hedonic feeding behavior. However, selective catecholaminergic expression did not normalize fasting blood glucose (Chuang et al., 2011a). Conversely, selective expression in in hindbrain Phox2b-expressing sympathetic neurons is sufficient to normalize fasting blood glucose, but not ghrelin-induced feeding (Scott et al., 2012). These data demonstrate that the highly distributed neuronal network that integrates ghrelin signaling can be divided into discrete patterns of autonomic response with identified neuronal subpopulations.

Among the CNS sites which mediate ghrelin's actions on feeding and blood glucose, the AgRP-expressing neurons in the arcuate nucleus of the hypothalamus have been the most extensively studied. AgRP neurons are the predominant site of GHSR expression in the arcuate nucleus (Willesen et al., 1999; Zigman et al., 2006). Chemical ablation of the arcuate nucleus attenuates the orexigenic actions of centrally-administered ghrelin (Bagnasco et al., 2003; Tamura et al., 2002; Wren et al., 2001). Antagonists or antibodies against AgRP and NPY abolish the orexigenic response to administered ghrelin (Asakawa et al., 2001; Nakazato et al., 2001). AgRP-neuron specific deletion of *vgat*, which disrupts release of

GABA onto neighboring anorexigenic POMC neurons and other downstream neurons, attenuates administered ghrelin-induced feeding (Tong et al., 2008). Ghrelin or GHSR agonists induce c-fos expression within AgRP neurons, increase their transcription of the orexigenic peptides AgRP and NPY, and stimulate calcium influx into AgRP neurons (Cowley et al., 2003; Dickson and Luckman, 1997; Hewson and Dickson, 2000; Kamegai et al., 2000; Kohno et al., 2003; Lawrence et al., 2002; Nakazato et al., 2001; Seoane et al., 2003).

The functional significance of ghrelin acting through AgRP neurons was tested using the reactivatable GHSR allele, described above, crossed to a transgenic mouse line expressing a tamoxifen-inducible,AgRP-specific promotor driving Cre recombinase expression (AgRP-CreER^{T2}). The mice in this model are functionally GHSR-null until they are exposed to tamoxifen, at which point they re-express GHSR at endogenous levels only in AgRP neurons (Wang et al., 2014). The use of a tamoxifen-inducible AgRP promotor driving Cre recombinase expression avoids the "delta" phenomenon that has affected prior AgRP models, in which transient AgRP expression early in development would trigger Cre-mediated recombination throughout the brain rather than in selective adult AgRP neurons. These earlier models include the transgenic AgRP-Cre mice (Kaelin et al., 2004) and "knock-in" AgRP-IRES-Cre mice (Fukuda et al., 2008; Tong et al., 2008).

Using the AgRP-CreER^{T2} model, Wang and colleagues found that selective GHSR re-expression partially restores administered ghrelin-induced feeding and fully restores the

defense against hypoglycemia observed during both overnight fasting and longer-term caloric restriction (Wang et al., 2014). In a caloric restriction paradigm in which mice are provided access to only 40% of their usual calories for seven days, GHSR-null mice experience a pronounced drop in blood glucose compared to wild-type mice. These finding is also seen in ghrelin-knockout mice (Li et al., 2012) and GOAT-knockout mice (Zhao et al., 2010). AgRP-CreER^{T2} mice, however, do not experience this exacerbated fall in blood glucose, suggesting that ghrelin acting solely on AgRP neurons is sufficient to orchestrate the defense against severe hypoglycemia (Wang et al., 2014).

As had been reported previously (Chuang et al., 2011b), no differences in fasting insulin levels are observed among GHSR-null, AgRP-CreER^{T2}, and wild-type mice. The counter-regulatory hormone glucagon, however, is reduced during fasting conditions in GHSR-null mice compared to wild-type mice (Chuang et al., 2011b), and this lack of a glucagon response is thought to contribute to the inability to maintain blood glucose during fasting conditions. Upon selective AgRP-neuron GHSR restoration in AgRP-CreER^{T2} mice, fasting glucagon levels were normalized to wild-type levels, suggesting that AgRP neurons are sufficient to mediate the glucagon response to fasting (Wang et al., 2014).

AgRP neuron-selective GHSR expression also induced gluconeogenic gene expression in the liver (Wang et al., 2014). Transcript levels of glucose-6 phosphatase (G6p), phosphoenolpyruvate carboxykinase (Pepck), and hepatocyte nuclear factor 4α (Hnf4α) all were increased in tamoxifen-treated GHSR-null/AgRP- CreER^{T2} mice, compared to both

GHSR-null and wild-type littermates. These data suggest that ghrelin acts via AgRP neurons to stimulate hepatic gluconeogenesis to defend against severe caloric restriction (Chuang et al., 2011b; Wang et al., 2014).

Taken together, these studies demonstrate that AgRP neurons are sufficient to mediate the effects of ghrelin on blood glucose during acute and chronic caloric restriction, and that AgRP neurons play a large role in the effects of ghrelin on feeding behavior.

ROLE OF SEROTONIN IN ENERGY HOMEOSTASIS

A substantial number of studies demonstrate that serotonin plays an important role in the control of feeding behavior and produces effects on feeding similar to leptin. Drugs that enhance serotonergic transmission reduce food intake both in rodents (Blundell and Hill, 1988; Clifton et al., 1989; Grill et al., 1997; Kitchener and Dourish, 1994; Weiss et al., 1986) and in humans (Goodall and Silverstone, 1988; McGuirk and Silverstone, 1990; Sargent et al., 1997). Conversely, agents that antagonize serotonin receptors or decrease serotonin stores increase food intake (Breisch et al., 1976; Dourish et al., 1985; Fletcher, 1988; Garattini et al., 1986; Goudie et al., 1976). While serotonin in the periphery has substantial importance as a gut neurotransmitter (Simansky, 1996), peripheral serotonin does not cross the blood-brain barrier. Furthermore, central administration alone of agents that alter serotonin signaling recapitulate the effects on feeding seen with systemic administration.

Serotonergic neurons in the midbrain raphe nuclei project to multiple regions of the brain to engage a large repertoire of serotonin receptor subtypes, including at least 18 separate 5-HT receptor genes and their splice variants. Efforts to define the role of specific receptor subtypes in the regulation of feeding have relied on drugs with relatively poor selectivity and potential off-target effects. Nonetheless, these pharmacological studies suggest that 5-HTR_{1b} and 5-HTR_{2c} receptor subtypes are important in feeding. The 5-$HTR_{1b/2c}$ agonist mCPP or the 5-$HTR_{1a/1b}$ receptor agonist RU24969 reduce feeding in a similar manner to the serotonin reuptake inhibitor sertraline (Simansky and Vaidya, 1990).

Recent studies have revealed that serotonin terminals synapse with POMC and NPY neurons, which express the 5-HTR_{2c} and 5-HTR_{1b} receptors, respectively. 5-HT or 5-HTR_{1b} receptor agonists acutely inhibit NPY firing (Heisler et al., 2002; 2006). Mice lacking this receptor display an attenuated anorectic response to d-fenfluramine, but exhibit only modest increases in food intake and body weight (Lam et al., 2008). Mice lacking the 5-HTR_{2c} receptor also display an attenuated response to d-fenfluramine, but, unlike 5- HTR_{1b} null mice, develop a more severe hyperphagia in addition to obesity. Re-expression of 5-HTR_{2c} receptors solely in POMC neurons was sufficient to rescue these effects. Further studies are needed to dissect the precise contribution of 5-HTR_{1b} signaling in NPY neuronal activity and downstream melanocortin signaling.

A recent study by Perry and colleagues found that mice lacking *girk4*, a subunit of a potassium channel, have an increased food intake and obesity (Perry et al., 2008). This

channel, the G protein-gated inwardly rectifying K^+ (GIRK) channel, is a homo- and heterotetrameric complex formed by four related subunits, GIRK1-4, gated by Gβγ subunits of the Gi/o subclass of heterotrimeric G proteins (Karschin et al., 1996; Krapivinsky et al., 1995). GIRK channels mediate the slow inhibitory effect of neurotransmitters and hormones in the heart and nervous system (North, 1989) and contribute to the resting membrane potential of several neuronal populations, including mesolimbic dopamine neurons (Cruz et al., 2004) and hippocampal neurons (Lüscher et al., 1997).

A study in POMC neurons suggested that 5-HTR_{2c} agonists inhibit a $GABA_B$-activated GIRK current (Qiu et al., 2007). However, a follow-up study found that the 5-HTR_{2C} agonist mCPP depolarized POMC neurons via activation of TRPC channels and not via inhibition of GIRK channels (Sohn et al., 2011). The same authors, however, found a major role of the GIRK1 subunit in both constitutively active and $GABA_B$-activated GIRK channels in POMC neurons. They recorded from POMC neurons in *girk1* knockout mice and observed a relatively depolarized resting membrane potential and an impaired hyperpolarization to baclofen, a $GABA_B$ receptor agonist. As GIRK1 subunits alone cannot form a functional channel (Hedin et al., 1996; Kennedy et al., 1996; 1999; Krapivinsky et al., 1995; Ma et al., 2002), this result suggests that GIRK1 complexes with GIRK3 or GIRK4 to form the GIRK channel in POMC neurons. The requirement of GIRK channels in POMC neurons for the response to baclofen also suggests that GIRK channels may be the postsynaptic target of the GABAergic input to POMC neurons from adjacent NPY neurons (Cowley et al., 2001; Sohn et al., 2011; Tong et al., 2008). Whether GIRK channels are important regulators of the

resting membrane potential of NPY neurons or the response to $GABA_B$ activators remains to be determined.

REGULATION OF HYPOTHALAMIC NEURON ACTIVITY

Early studies in unidentified neurons in the hypothalamic arcuate nucleus found that leptin inhibited a population of glucose-responsive neurons via ATP-sensitive K^+ (K_{ATP}) channels (Spanswick et al., 1997). In a follow-up study, glucose-responsive neurons were also inhibited by insulin via K_{ATP} channels (Spanswick et al., 2000). Elias and colleagues found that leptin increased c-fos, an indirect marker of neuronal activity, in POMC neurons, but not in NPY neurons, while SOCS-3 mRNA, a transcript induced by leptin binding to its receptor, increased in both populations (Elias et al., 1999). These studies suggested that leptin directly inhibits some populations and directly activates other populations of neurons in the arcuate nucleus.

Subsequent studies used reporter strains or post-hoc analysis to better define these sub-populations. Using a POMC-GFP reporter, Cowley and colleagues showed that leptin activates POMC neurons via opening of a non-specific cation channel and via reduced inhibitory GABAergic transmission by NPY neurons onto POMC cells. Leptin also hyperpolarized non-GFP neurons in this study via K_{ATP} channels (Cowley et al., 2001). Later studies confirmed that leptin depolarizes, and insulin hyperpolarizes, POMC neurons (Choudhury et al., 2005; Hill et al., 2008; Williams et al., 2010). Using single-cell RT-PCR

to identify unlabeled arcuate neurons post-hoc, van den Top and colleagues showed that NPY neurons are hyperpolarized by leptin (van den Top et al., 2004). Using identified AgRP neurons, Könner and co-workers found that insulin also hyperpolarizes AgRP neurons and that this effect requires an intact insulin receptor in AgRP neurons (Könner et al., 2007). Taken together, these observations and others suggest that leptin and insulin inhibit NPY/AgRP neurons in the arcuate nucleus (Wang et al., 2008).

In contrast to the aforementioned studies, other groups have reported that NPY neurons do not respond acutely to leptin. Moreover, in contrast to published findings, NPY neurons were reported to be activated by insulin (Al-Qassab et al., 2009; Claret et al., 2007). Thus, considerable debate exists in the literature regarding the acute cellular responses of NPY neurons to leptin and insulin. One possible explanation for these discrepancies may be due to the inherent neuroanatomic complexity of NPY neurons. NPY neurons are distributed throughout the relatively large rostral-caudal extent of the arcuate nucleus (from -0.94 mm to -2.30 mm bregma in mice). Thus, it is entirely possible that different sub-populations of NPY neurons may have been targeted for recording in these studies. Alternatively, artifacts in fluorescent reporter strains would permit the targeting of non-NPY neurons in these studies.

Intracellular mechanisms

Recent evidence indicates that leptin's short- and long-term effects are mediated by distinct intracellular signaling pathways. Upon binding to leptin, the leptin receptor activates Janus kinase 2 (JAK2), which leads to activation of downstream pathways, including signal

transducer and activator of transcription 3 (STAT3), mitogen activated protein kinase (ERK1/2), and phosphatidylinositol 3-kinase (PI3K). Long-term changes in gene expression are thought to be mediated by the JAK-STAT pathway, while the PI3K pathway has been implicated in mediating leptin's acute effects. PI3Ks catalyze the phosphorylation of phosphatidylinositol 3-phosphate (PIP) to 3,4-bis- (PIP2) and 3,4,5-trisphosphate (PIP3) forms, which in turn bind to a wide variety of intracellular proteins (Cantley, 2002). PI3Ks exist as heterodimers of one p110 catalytic subunit (p110α, p110β, or p110δ) and one of five p85 regulatory subunits (Vanhaesebroeck et al., 2005). Leptin has been found to stimulate PI3K binding to insulin receptor substrate-1 and insulin receptor substrate-2, and to activate PI3K and its downstream target protein kinase B/Akt (Harvey et al., 2000; Zhao et al., 2000).

Niswender and co-workers found that intracerebroventricular (i.c.v.) infusion of the PI3K inhibitors wortmannin and LY294002 abolished the ability of both leptin and insulin to acutely reduce food intake (Niswender et al., 2001; 2003). Plum and colleagues, in a converse experiment, constitutively activated the PI3K pathway in POMC neurons by inactivating the PIP3 phosphatase, PTEN. Increased PI3K activity resulted in hyperphagia that persisted even with supplemental leptin at doses that normally induce anorexia. The authors also observed increased potassium channel activity in POMC cells lacking PTEN, which, by allowing intracellular potassium to exit the cell, hyperpolarized their membranes. This inhibition was reversible by the specific K_{ATP} channel blocker tolbutamide or the PI3K inhibitor LY294002 (Plum et al., 2006). These findings suggest that the acute actions of

leptin and insulin are dependent on the PI3K pathway, and that enhanced PI3K can inhibit hypothalamic neurons via K_{ATP} channels.

In POMC neurons, pharmacological disruption of PI3K signaling, via wortmannin and LY294002, or genetic disruption, via POMC-specific deletion of p85 regulatory subunits, blocked the acute electrophysiological effects of leptin and insulin in POMC neurons. Furthermore, with either pharmacological or genetic PI3K blockade, the acute effects of leptin on feeding and glucose homeostasis were abolished (Hill et al., 2008; 2009). Clearly, the importance of the PI3K pathway in POMC neurons has been uncovered. However, the role of PI3K in NPY neurons, the counterbalancing partner to POMC neurons in the melanocortin circuit, has yet to be conclusively determined.

A growing body of work has implicated leptin and insulin signaling in the hypothalamus in the regulation of peripheral glucose metabolism. Leptin-deficient rodents exhibit insulin resistance independent of effects on body weight (Zhang et al., 1994). Leptin administration in leptin-null (*ob/ob*) mice, at doses that do not alter body weight, normalizes serum glucose (Pelleymounter et al., 1995) and leads to a 40% greater reduction in serum glucose compared with pair-fed *ob/ob* mice (Schwartz et al., 1996a). Leptin treatment also improves the severe insulin resistance and diabetes characteristic of certain lipodystrophy syndromes both in mouse models of lipodystrophy (Shimomura et al., 1999) and in humans (Oral et al., 2002). Recombinant leptin, either alone or combined with low-dose insulin, was recently found to provide equivalent or superior glycemic stability than insulin monotherapy in non-obese

diabetic (NOD) mice, and trials of recombinant leptin in type 1 diabetes have showed promise (Wang et al., 2010; Yu et al., 2008). To address whether leptin's effects on insulin-target tissues are mediated indirectly through the brain or directly through leptin receptors in peripheral tissues, Coppari and colleagues used an adenoviral strategy to restore leptin receptors in leptin receptor-null mice selectively and unilatearlly in the arcuate nucleus and found decreased insulinemia and normalization of serum glucose (Coppari et al., 2005). However, the precise contribution of different neuronal sub-populations within the arcuate, including NPY neurons, to glucose metabolism has not been conclusively studied.

In the central nervous system, NPY alters glucose homeostasis by promoting insulin release. Leptin-null (*ob/ob*) mice, which have increased NPY expression due to a lack of an inhibitory input on NPY neurons, have increased serum insulin. Erickson and colleagues tested whether the hyperinsulinemia in leptin-null mice is due to their increased body weight or due, in part, to increased NPY expression, by generating leptin-null mice which also lack the *npy* gene (*ob/ob*; $npy^{-/-}$). These mice display normalized serum glucose and 50% less serum insulin compared to mice lacking leptin alone, suggesting that the elevated NPY levels in leptin-null mice contribute to their insulinemia (Erickson et al., 1996). Central (i.c.v.) administration of NPY induces a number of behavioral and metabolic changes, including hyperphagia (Clark et al., 1984), hypothermia (Esteban et al., 1989; Tachibana et al., 2006), hyperinsulinemia (Marks and Waite, 1996; Moltz and McDonald, 1985), hyperglucagonemia (Marks and Waite, 1996), hyperglycemia (Tachibana et al., 2006), decreased energy expenditure (Billington et al., 1991; Egawa et al., 1991; Hwa et al., 1999; Tachibana et al.,

2006), and increased glucose turnover (Marks and Waite, 1997). Notably, i.c.v. NPY increases the insulinemic response to intravenous glucose and attenuates the hypoglycemic response to intravenous insulin (Marks and Waite, 1996). Finally, i.c.v. administration of NPY over several days induces obesity-like hormonal and metabolic changes (hyperinsulinemia, hyperglycemia, and dyslipidemia) even when hyperphagia was prevented by pair-feeding (Raposinho et al., 2001; Vettor et al., 1994; Zarjevski et al., 1993). Together, these reports imply that NPY promotes insulin secretion and gluconeogenesis even in the absence of available food.

These two effects have been dissected apart in studies that have implicated melanocortin signaling in the regulation of peripheral insulin sensitivity and non-melanocortin signaling in the regulation of hepatic glucose production. Centrally administered α-MSH in rats enhances tyrosine phosphorylation of insulin receptor substrates, a marker of insulin sensitivity. Conversely, mice treated with the synthetic MC3R and MC4R antagonist SHU9119 have decreased insulin sensitivity. These effects are independent of any effects on body weight (Obici et al., 2001). However, i.c.v. SHU9119 does not affect the ability of hyperinsulinemia to inhibit hepatic glucose production, which suggests that melanocortin signaling is not responsible (Obici et al., 2002b).

Whether these melanocortin-independent effects on hepatic gluconeogenesis are downstream of hypothalamic neurons was investigated by antagonism of insulin signaling and by down-regulation of insulin receptor expression selectively in hypothalamic nuclei. I.C.V. infusion

of insulin can suppress glucose production by 40% in the presence of basal circulating insulin concentrations, whereas antagonism of insulin signaling or down-regulation of insulin receptor expression in hypothalamic nuclei considerably impairs the ability of circulating insulin to inhibit hepatic glucose production (Obici et al., 2002a; 2002b). van den Hoek and colleagues examined the effects of a continuous i.c.v. infusion of NPY on glucose flux during a hyperinsulinemic euglycemic clamp in mice. They found that the physiological action of insulin to downregulate hypothalamic NPY release is required for insulin's ability to suppress hepatic glucose production, whereas it is not mandatory for its ability to modulate glucose disposal or lipolysis (van den Hoek et al., 2004).

A study in which the insulin receptor was deleted solely in AgRP neurons (IRΔAgRP mice) confirms the unique role of NPY/AgRP neurons in hepatic gluconeogenesis. Peripheral insulin in IRΔAgRP mice failed to suppress hepatic glucose production. This effect was not observed in targeted deletions of the insulin receptor in POMC neurons (Könner et al., 2007). Thus, NPY neurons may be uniquely important among hypothalamic neurons in whole body glucose homeostasis.

Other studies have investigated the role of PI3K in mediating the effects of leptin and insulin in the arcuate nucleus to regulate glucose homeostasis. As mentioned, selective restoration of leptin receptors in the arcuate nucleus in leptin receptor-null rodents improves insulin sensitivity (Coppari et al., 2005; Morton et al., 2005). Importantly, this improvement in glucose homeostasis is attenuated by the PI3K inhibitor LY294002. Insulin sensitivity is also

improved by adenoviral-mediated expression in the arcuate nucleus of a constitutively active form of protein kinase B/Akt, a downstream target of PI3K (Morton et al., 2005). Other studies found the action of centrally administered insulin in arcuate neurons to acutely suppress hepatic glucose production is both PI3K- and K_{ATP} channel-dependent (Obici et al., 2002b; Pocai et al., 2005).

PI3Ks act in the hypothalamus as dimers of a catalytic subunit (p110α or p110β) and a p85 regulatory subunit. Selective deletion of either p110α or p110β catalytic subunit alone in NPY neurons fails to alter fasting glucose levels, glucose tolerance, or fasting insulin levels (Al-Qassab et al., 2009). However, the presence of one p110 isoform may be sufficient to compensate for the loss of the other. Prior studies have found a functional redundancy of p110 isoforms in other tissues (Brachmann et al., 2005; Chaussade et al., 2007). Therefore, deletion of both p110α and p110β isoforms is a more direct test of the PI3K-dependence of leptin and insulin responses in NPY neurons.

CHAPTER THREE
Methodology

EXPERIMENTAL ANIMALS

Animal husbandry

Adult male and female mice were housed in a light-controlled (12 hour, 12 hour off) and temperature-controlled environment at the UT Southwestern Animal Resource Center. All animals were under professional veterinary care and supervision consistent with the recommendations of the American Veterinary Medical Association. An anesthetic, chloral hydrate, was used for all surgical procedures at a dose sufficient to provide a deep surgical plane of anesthesia as demonstrated by a lack of response to tail or hind paw pinch. The animals and procedures used were approved by the UT Southwestern Institutional Animal Care and Use Committee under animal protocol number 2009-0287.

Mouse models

Several mouse lines were used during this research. NPY-GFP mice were obtained from Dr. Bradford Lowell. This line was generated using a large bacterial artificial chromosome sequence containing the NPY promoter driving expression of the human codon-corrected Renilla GFP. van den Pol and colleagues confirmed the fidelity of GFP expression in NPY neurons by demonstrating co-localization of GFP with antisera against NPY and AgRP. Additionally, GFP-positive neurons were harvested from the arcuate nucleus and their

expression of NPY mRNA was confirmed with single-cell RT-PCR (van den Pol et al., 2009).

To identify the subset of LepR-expressing NPY neurons, NPY-GFP reporter mice were crossed with a line expressing Cre recombinase under the control of the endogenous *lepr* promoter (LepRb-IRES-Cre mice) (DeFalco et al., 2001). The progeny were bred to homozygosity and then crossed to a Cre reporter line with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant, tdTomato ($STOP^{fl/fl}$-tdTomato). Together, this triple-allele line, NPY-GFP; LepR-Cre; $STOP^{fl/fl}$-tdTomato, allows the targeting of double-labeled yellow cells expressing both GFP and tdTomato. This line is also referred to as the NLT line in this paper.

To assess the role of PI3K signaling in NPY neurons, a knock-in line expressing Cre recombinase in AgRP neurons, AgRP-cre, (Xu et al., 2005) was crossed with line containing a loxP-flanked PI3K catalytic subunit, $p110\alpha^{fl/fl}$. The offspring were then crossed with a line containing a second loxP-flanked PI3K catalytic subunit, $p110\beta^{fl/fl}$. Finally, these mice were crossed to the tdTomato Cre reporter line described above ($STOP^{fl/fl}$-tdTomato) and bred to homozygosity to yield AgRP-cre; $p110\alpha^{fl/fl}$; $p110\beta^{fl/fl}$; tdTomato mice. This line is also referred to as the APT line in this thesis.

To test whether G protein-gated inwardly rectifying K^+ (GIRK) channels contribute to the resting membrane potential of NPY neurons and to the response to $5\text{-}HTR_{1b}$ agonists in NPY

neurons, NPY-GFP mice were crossed with *girk1* knock-out mice to generate NPY-GFP; girk1$^{-/-}$ mice.

ELECTROPHYSIOLOGY

Acute brain slice preparation

Whole cell patch-clamp recordings from neurons in hypothalamic slice preparations was performed as described by Hill and colleagues (Hill et al., 2008). Eight- to 12-week old mice were terminally anesthetized with 7% chloral hydrate (350 mg/kg intraperitoneal) and transcardially perfused with a modified ice-cold artificial cerebrospinal fluid (aCSF), described below, in which an equiosmolar amount of sucrose is substituted for NaCl. The mice were decapitated and the entire brain was removed. After removal, brains were immediately submerged in ice-cold, carbogen-saturated (95% O_2 and 5% CO_2) aCSF (126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 21.4 mM $NaHCO_3$, and 10 mM glucose). A brain block containing the hypothalamus was made, and 250 μM coronal sections were cut with a Leica VT1000S Vibratome . Slices then were incubated in carbogen-saturated aCSF at 32°C for at least 1 hour before cell recording. Slices were transferred to a submersion recording chamber mounted under an upright microscope and allowed to equilibrate for 10–20 minues prior to recording. The slices were then bathed in carbogen-saturated aCSF at 32°C at a flow rate of 2 mL/min.

Whole cell patch clamp electrophysiology

Epifluorescence was used to target fluorescent cells, after which the light source was switched to infrared differential interference contrast imaging for whole-cell recording on a

Zeiss Axioskop FS2 Plus equipped with a fixed stage and a Hamamatsu C2741-60 charged-coupled device camera. Electrophysiological signals were recorded using an Axopatch 700B amplifier (Axon Instruments), low-pass filtered at 2–5 kHz, digitized at 88 kHz (Neuro-corder; Cygnus Technology), stored on videotape, and analyzed on a PC with pCLAMP (Axon Instruments).

Recording electrodes were pulled from borosilicate glass capillaries of 1.65 mm outer diameter and 0.45 mm wall thickness (Garner Glass Co., Claremount, CA) to a resistance of 2.5–5 MΩ when filled with a K-gluconate internal solution (110–128 mM K-gluconate, 10 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM ethylene glycol tetraacetic acid (EGTA), 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, 2–5 mM (Mg^{2+})-ATP, and 0.3 mM (Na^+)-GTP, titrated to pH 7.3). The recording was continued if the seal resistance was 1-5 GΩ and the series resistance was less than 25 MΩ, uncompensated. Input resistance was assessed by measuring voltage deflection at the end of the response to a hyperpolarizing rectangular current pulse, e.g., 400–500 ms of –20 pA.

The following drugs were added to the aCSF for specific experiments: 6-cyano-7-nitroquinoxaline- 2,3-dione (CNQX, 10 μM, Tocris), DL-2-amino-5-phosphono-valeric acid (AP-5, 50 μM, Tocris), insulin (50 nM, Humulin-R 100 U/ml, Lilly), leptin (100 nM, provided by A.F. Parlow, Harbor-UCLA Medical Center, through the National Hormone and Peptide Program), LY294002 (10 μM, Calbiochem), tetrodotoxin (TTX, 2 mM, Tocris), paxilline (10 nM, Tocris), and wortmannin (10–100 nM; Alomone Laboratories),

Compounds dissolved in DMSO (wortmannin or LY294002) were added to aCSF with a final DMSO concentration of less than 0.1%. Solutions containing hormones or drugs were washed in for 3 to 6 minutes. For each recording, a neuron was considered activated or inhibited if the change in membrane potential with drug or hormone application was at least 3 mV in amplitude for several minutes and the effect was temporally associated with the drug reaching the recording chamber.

METABOLIC PHENOTYPING

Body weight, body composition, energy expenditure, locomotor activity, and insulin and glucose tolerance were compared between experimental AgRP-cre; $p110\alpha\beta^{fl/fl}$; $STOP^{fl/fl}$-tdTomato mice and control littermates negative for AgRP-cre. Two separate cohorts of animals were used to produce the metabolic data. To control for the possibility that changes in body weight or composition would contribute to energy expenditure measurements, metabolic assessment was performed on weight- and body composition-matched littermates.

Body Weight and Composition

Body composition, including fat mass, lean tissue mass, free water, and total body water, was assessed in *ad libitum* fed mice using a Minispec mq10 nuclear magnetic resonance spectrometer (Bruker Corp.).

Energy expenditure and locomotor activity

Mice were acclimated to the metabolic cages (TSE Systems Gmbh) for four days before measurements were taken. Mice then were analyzed for four days and were provided food *ad libitum* during this period. Energy expenditure was assessed by indirect calorimetry, and locomotor activity was measured by an infrared light beam detection system. These measurements were determined for both the 12-hour light and 12-hour dark cycles as well as for the entire 24-hour day and were averaged over the 4-day period of measurement. Data were collected using the TSE Labmaster software (TSE Systems Gmbh).

DATA ANALYSIS

Statistical analysis was completed using GraphPad 5 (Prism) and R (R Foundation). Data were evaluated using a Welch Two Sample t test with a p value of less than 0.05 considered significant. Data are presented as the mean ± SEM.

CHAPTER FOUR
Results

ELECTROPHYSIOLOGY

Characteristics of Recorded Cells

The characteristics and biophysical properties of cells recorded during electrophysiology experiments are listed in Table 1. Five groups are reported: NPY-GFP males (n = 150 cells), NPY-GFP; LepR-Cre; $STOP^{fl/fl}$-tdTomato males (referred to as NLT males, n = 81 cells), a pooled group containing NPY-GFP and NLT males (n = 231 cells), AgRP-cre; $p110\alpha^{fl/fl}$; $p110\beta^{fl/fl}$; tdTomato males (referred to APT males, n = 10 cells), and NPY-GFP; $girk1^{-/-}$ males (referred to as NG1 males, n = 39 cells).

Table 1. Biophysical properties of recorded cells.

	NPY-GFP males, n = 150	NPY-GFP; LepR-cre; $STOP^{fl/fl}$-tdTomato, n = 81	NPY-GFP + NPY-GFP; LepR-cre; $STOP^{fl/fl}$-tdTomato, n = 231	AgRP-cre; $p110\alpha\beta^{fl/fl}$, n = 10	NPY-GFP; $GIRK1^{KO}$, n = 39
Age (days)	56.1 ± 2.1 (126)	97.9 ± 3.7 (79)	72.2 ± 2.4 (205)	52.6 ± 2.7 (10)	68.5 ± 7 (35)
Whole cell capacitance (pF)	11.4 ± 0.3 (143)	11 ± 0.3 (78)	11.2 ± 0.2 (221)	11.6 ± 1 (10)	10.5 ± 0.4 (38)
Input resistance (MΩ)	10.2 ± 0.5 (143)	10.2 ± 0.7 (78)	10.2 ± 0.4 (221)	11.3 ± 2.1 (10)	10.6 ± 1.2 (38)
Opening membrane potential (mV)	-39 ± 0.5 (136)	-36.9 ± 0.8 (74)	-38.3 ± 0.4 (210)	-30.2 ± 9.5 (7)	-35.9 ± 2.5 (32)
Membrane potential after equilibration (mV)	-46.4 ± 1.1 (113)	-43.2 ± 1.4 (62)	-45.2 ± 0.9 (175)	-48.7 ± 3.6 (9)	-39.9 ± 1.7 (39)

The whole cell capacitance and input resistance of all NPY neurons were similar, indicating that the biophysical properties of cells across the reporter lines (NPY-GFP and NLT) and experimental lines (APT and NG1) were identical at baseline. Whole-cell capacitance is an indirect measurement of cell size. The input resistance of a neuron reflects the extent to which membrane channels are open. It is calculated by measuring the change in voltage associated with the injection of a current, divided by the input current. In particular, a greater input resistance implies a greater change in the membrane potential in response to a current; alternatively, in a cell with greater input resistance, less current is required to elicit the same depolarization. Of note, the input resistances in all lines were small relative to the GΩ seal resistances which lie in series to the input resistance. This relationship allows an accurate measurement of membrane potential in whole cell patch clamp recording.

Few published reports list the biophysical properties of AgRP neurons, but those that do list higher input resistances between 1 and 4 GΩ (Al-Qassab et al., 2009; Liu et al., 2012; Qiu et al., 2014; Tsaousidou et al., 2014). Interestingly, the input resistances in the NPY-GFP and NLT reporter lines and in the APT lines were identical, suggesting that at baseline, ablation of the PI3K pathway in AgRP neurons does not alter baseline excitability.

The cell capacitance and resting membrane potential was identical across all lines and is in agreement with other reports listing cell capacitances of 10-20 pF and resting membrane

potentials of between -40 and -60 mV (Al-Qassab et al., 2009; Liu et al., 2012; Sohn et al., 2011).

Most NPY neurons hyperpolarized gradually by 5 – 10 mV after break-in (bottom row, Table 1). As such, applications of drugs or of hormones to neurons were begun only after a 10-20 minute period of equilibration (see Methods).

Sohn and colleagues reported that POMC neurons in *girk1* knockout mice exhibited a significant depolarization of the resting membrane potential of ~ 6 mV (Sohn et al., 2011). In this study in NPY neurons, a significant difference in resting membrane potentials of wild type and of *girk1* knockout mice was also observed (Table 1). Wild type NPY-GFP and NLT cells had a resting membrane potential of -45.2 mV, while GIRK1 knockout NPY neurons were depolarized by ~ 5 mV with a mean of -39.9 mV ($p = 0.004$). In contrast, the difference in the input resistance of the two populations was not significant ($p = 0.768$).

NPY Neurons are Hyperpolarized by K_{ATP} Channel Activation

To test whether NPY neurons hyperpolarize with the opening of K_{ATP} channels, a specific K_{ATP} channel activator, diazoxide, was applied to four NPY-GFP and four NLT cells. Hyperpolarization of all eight cells occurred with a mean amplitude of -19.3 ± 2.6 mV. Current steps were obtained in control and in diazoxide-treated conditions of a representative recording (Figure 1a) and plotted on a current-voltage graph (Figure 1b).

Figure 1a. Current steps during baseline and diazoxide conditions.

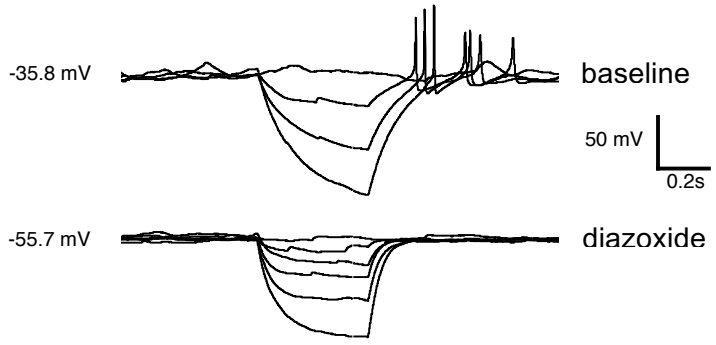
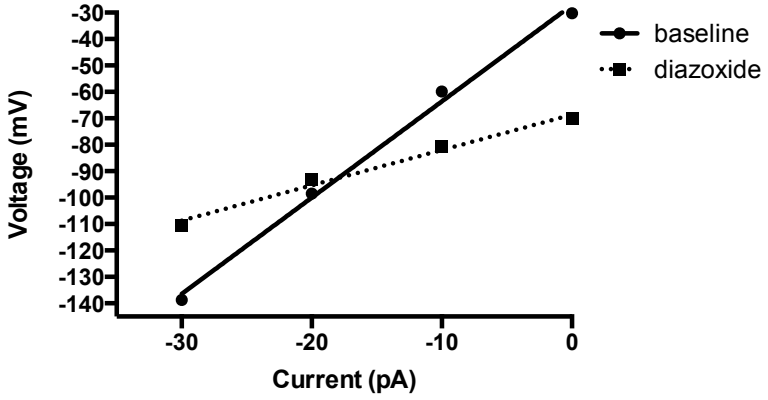


Figure 1b. Diazoxide hyperpolarizes NPY neurons via a K^+ conductance.

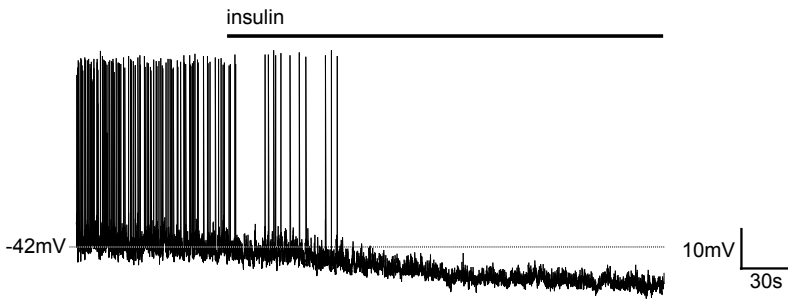


In the diazoxide condition, the cell resistance as measured by the slope of the current-voltage relationship decreased compared to baseline, indicating that diazoxide increased cell conductance. In addition, the intersection of the current-voltage relationships between the baseline and diazoxide conditions revealed a reversal potential of this conductance of approximately -95 mV, close to the calculated Nernst potential of potassium in these neurons (Appendix A).

Insulin Hyperpolarizes NPY Neurons via K_{ATP} Channels

Insulin (50 nM) was then applied to NPY-GFP males (n = 56) and to NLT males (n = 21) throughout the rostral-caudal axis of the arcuate nucleus. In NPY-GFP males, insulin was found to hyperpolarize 15 out of 56 cells, a response rate of 26.8%. The mean amplitude of hyperpolarization was -10.1 ± 0.5 mV. In NLT yellow males, zero out of 21 neurons were hyperpolarized by insulin. Taking both reporter lines together, insulin hyperpolarized 15 out of 77 neurons, a response rate of 19.5%. A representative trace of an insulin-induced hyperpolarization is shown in Figure 2a.

Figure 2a. Insulin hyperpolarizes NPY neurons.



Current or voltage steps were applied in baseline and insulin conditions (Figure 2b) and the resulting voltage deflections were plotted (Figure 2c). The slope of the insulin current-voltage relationship decreased compared to baseline indicating an increased conductance, and the intersection of the two plots revealed a reversal potential of the conductance of approximately -67 mV, closest to the Nernst potential of potassium.

Figure 2b. Current steps during baseline and insulin conditions.

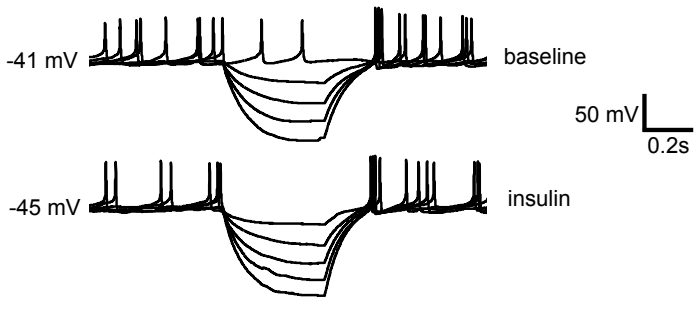
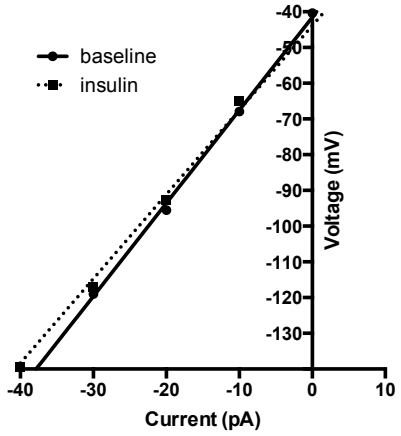
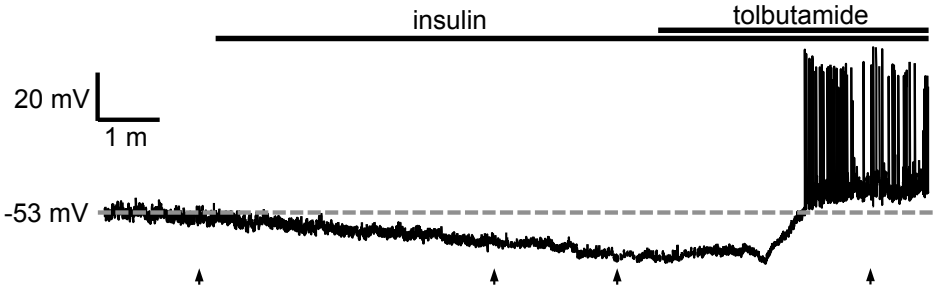


Figure 2c. Insulin hyperpolarizes NPY neurons via a K^+ conductance



To test whether the insulin-activated potassium conductance occurred through K_{ATP} channels, the specific K_{ATP} channel blocker tolbutamide was applied during an insulin-induced hyperpolarization. Tolbutamide rescued the insulin-induced hyperpolarization (Figure 2d).

Figure 2d. Insulin hyperpolarizes NPY neurons via a K_{ATP} channels.



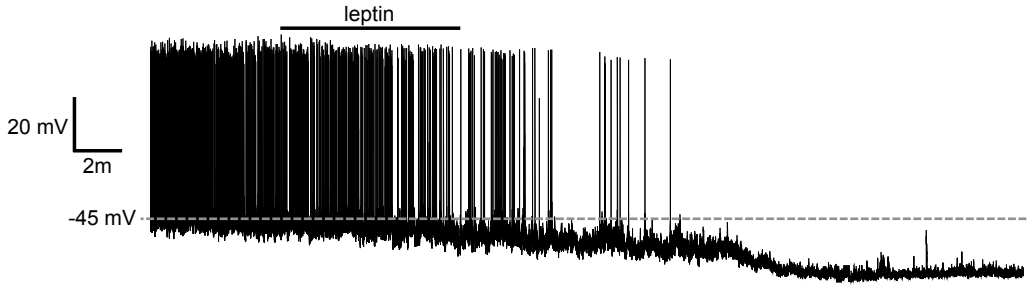
Acute Effects of Leptin, Ghrelin, and Glucagon

The above experimental approach was applied to determine the acute effects of leptin, ghrelin, and glucagon. Summary tables listing responses to each hormone by genotype and by rostral-caudal axis position are presented after the representative traces below.

Leptin Hyperpolarizes NPY Neurons via K_{ATP} Channels

Leptin (50 to 100 nM) was applied to NPY-GFP (n = 52) and to NLT males (n = 43) throughout the rostral-caudal axis of the arcuate nucleus. In NPY-GFP males, leptin was found to hyperpolarize 13 out of 52 cells, a response rate of 25%. The mean amplitude of responses was -6.6 ± 0.3 mV. In NLT yellow males, 16 out of 43 neurons were hyperpolarized by leptin (37.2%) with a mean amplitude of -12.4 ± 1.8 mV. Taking both reporter lines together, leptin hyperpolarized 29 out of 95 neurons (30.5%) with a mean amplitude of -9.8 ± 1.2 mV.

Figure 3a. Leptin hyperpolarizes NPY neurons.



A series of hyperpolarizing currents steps were applied in baseline and in leptin-treated conditions. The resulting voltage deflections are shown in Figure 3b and are plotted in Figure 3c.

Figure 3b. Current steps during baseline and leptin conditions.

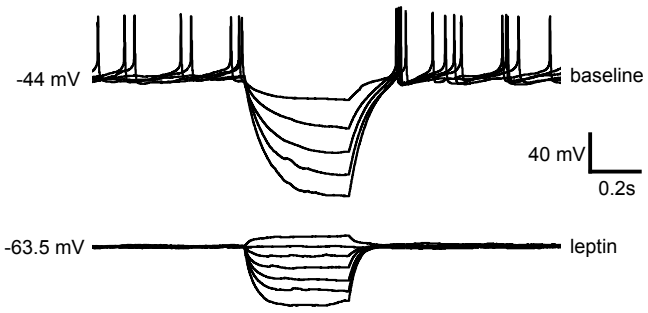
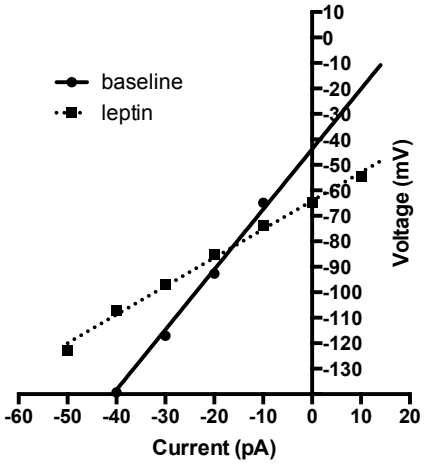
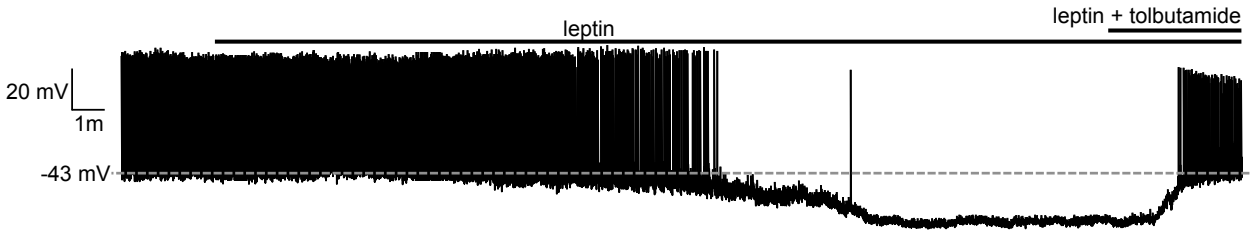


Figure 3c. Leptin hyperpolarizes NPY neurons via a K^+ conductance.



The intersection of the current-voltage relationships (Figure 3c) indicates a reversal potential of -82 mV for the leptin-activated current, suggesting that the leptin receptor is coupled to a potassium conductance.

Figure 3d. Leptin hyperpolarizes NPY neurons via a K_{ATP} channels.



Finally, the specific K_{ATP} channel blocker tolbutamide was sufficient to restore the resting membrane potential and action potential firing rate to baseline levels after a leptin-induced hyperpolarization (Figure 3d).

Ghrelin Depolarizes NPY Neurons via a Mixed Cation Conductance

Ghrelin was applied to NPY-GFP (n = 12) and to NLT cells (n = 6). This study determined the response rate in NPY neurons and the reversal potential of the ghrelin-coupled

conductance. In NPY-GFP males, ghrelin depolarized 4 out of 12 cells, a response rate of 33.3%. The mean amplitude of responses was 8.2 ± 1.6 mV. In NLT yellow males, 2 out of 6 neurons were depolarized by ghrelin (33.3%) with a mean amplitude of 8.5 ± 0.9 mV. Together, ghrelin activated 6 out of 18 neurons (33.3 %) with a mean amplitude of 8.3 ± 1 mV. A representative depolarization is shown in Figure 4a.

Figure 4a. Ghrelin depolarizes NPY neurons.

Current steps were applied during baseline and ghrelin-depolarized conditions, and the voltage responses are presented below (Figures 4b and 4c).

Figure 4b. Current steps during baseline and ghrelin conditions.

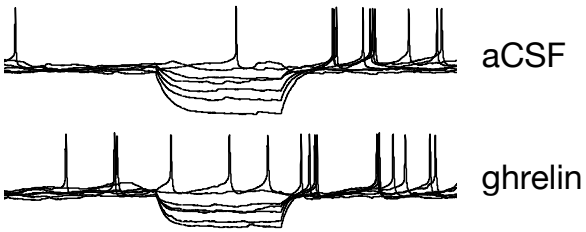
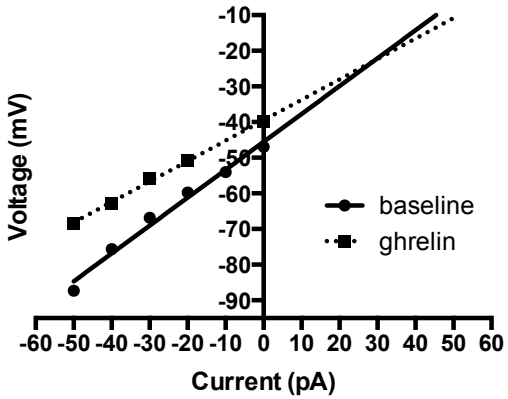


Figure 4c. Ghrelin depolarizes NPY neurons via a mixed cation conductance.

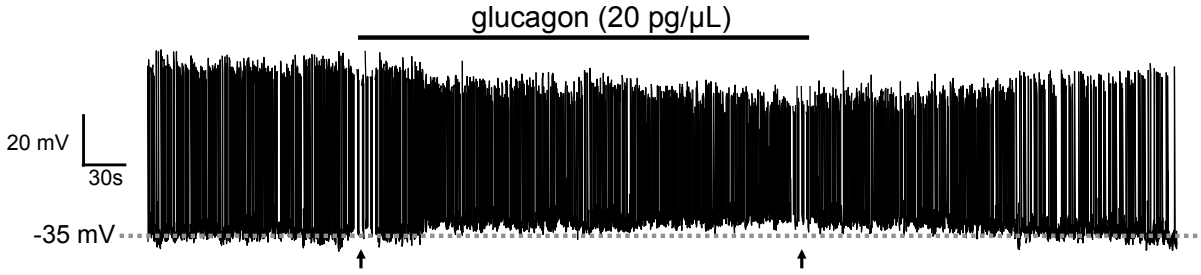


The slope of the ghrelin current-voltage plot is decreased compared to baseline, implying an increased cellular conductance and an opened channel. The intersection of the current-voltage plots indicates a reversal potential of the ghrelin-activated conductance of -24 mV. This voltage lies in between the Nernst potentials of the two principle intracellular and extracellular cations, Na^+ and K^+, suggesting a mixed cation conductance is opened.

Glucagon Depolarizes NPY Neurons via a Mixed Cation Conductance

Glucagon is 29-amino acid polypeptide secreted from pancreatic alpha cells. Early reports showed that glucagon crosses the blood-brain barrier, binds receptors in the rat hypothalamus (Hoosein and Gurd, 1984), and inhibits glucose-sensitive LHA neurons (Inokuchi et al., 1986). Recently, glucagon receptors were found to colocalized with AgRP/NPY neurons in rats (Mighiu et al., 2013). A pilot study was conducted to assess whether glucagon can alter the acute activity of NPY neurons in mice. Glucagon was applied to 5 NLT neurons. Two of 5 cells (40%) were depolarized by glucagon with a mean amplitude of 7 ± 0.8 mV (Figure 5a).

Figure 5a. Glucagon depolarizes NPY neurons.



Current steps were recorded (Figure 5b) as above and plotted (Figure 5c) to reveal a reversal potential of the glucagon-activated current of 19 mV, suggestive of a mixed cation conductance.

Figure 5b. Current steps during baseline and glucagon conditions.

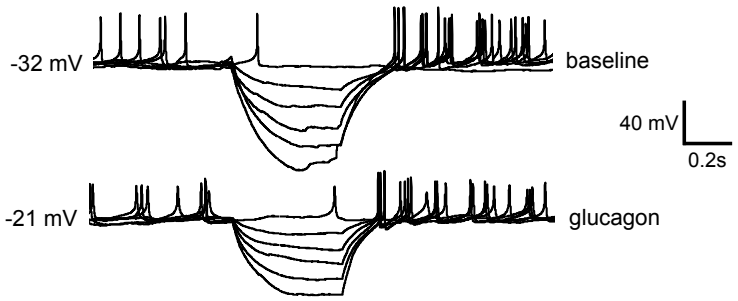
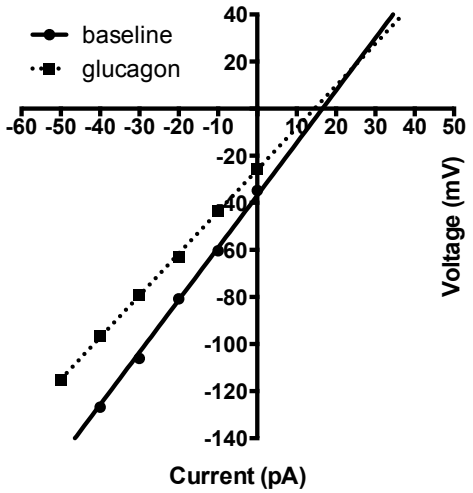


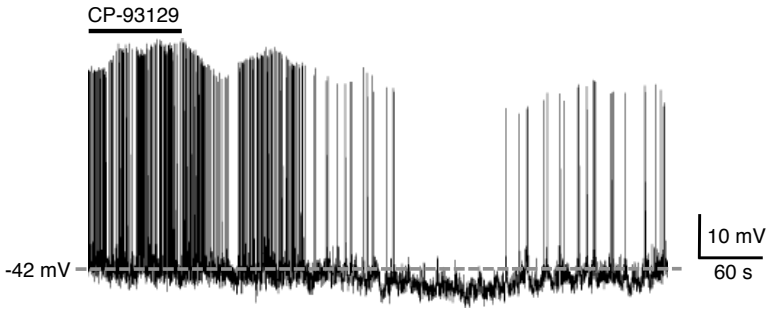
Figure 5c. Glucagon depolarizes NPY neurons via a mixed cation conductance.



Serotonin Receptor 1b Agonists Inhibit NPY Neurons

Serotonin (5-HT) and specific serotonin receptor 1b (5-HTR_{1b}) agonists (5-nonyloxytryptamine oxalate [5-NTO] and CP93129) were applied to NLT mice. Ten of 17 (58.8%) NLT cells were hyperpolarized by 5-NTO with a mean amplitude of -6.6 ± 1.4 mV. One out of seven (14.3%) NLT cells were hyperpolarized by CP93129 with a mean amplitude of -11.4 ± 0 mV. A representative trace of a CP93129-induced hyperpolarization is shown in Figure 6.

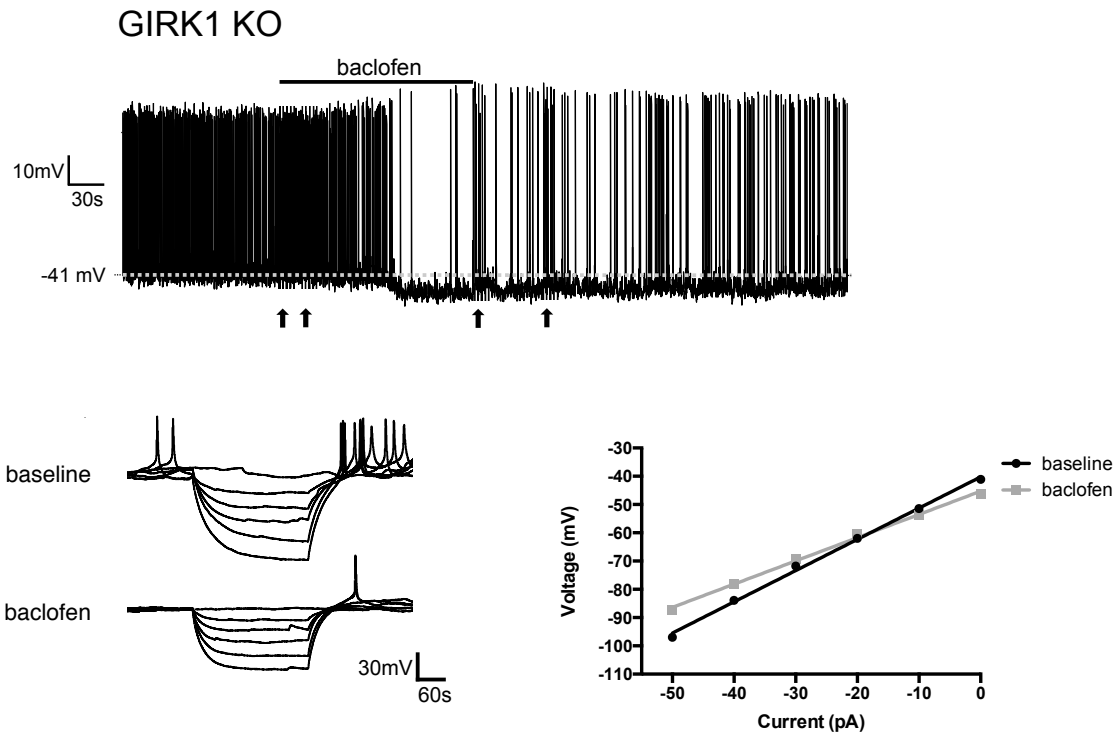
Figure 6. Serotonin Receptor 1b Agonists Inhibit NPY Neurons



GIRK1 Subunits Are Not Required for the $GABA_B$-Activated Current in NPY Neurons

A recent report found a major role of the GIRK1 subunit in both constitutively active and $GABA_B$-activated GIRK channels in POMC neurons (Sohn et al., 2011). They observed an impaired hyperpolarization to baclofen, a $GABA_B$ receptor agonist, in POMC neurons from *girk1* knockout mice. In this study, 10 of 15 (66.7%) NPY-GFP neurons in *girk1* knockout mice were hyperpolarized by baclofen with a mean amplitude of -7.3 ± 1.1 mV. A representative trace showing a baclofen-induced hyperpolarization is shown in Figure 7. Additionally, current steps in baseline and in baclofen-treated conditions are shown. These current steps were plotted and confirmed a baclofen-activated potassium conductance.

Figure 7. Baclofen hyperpolarizes *girk1*$^{-/-}$ NPY neurons via a K^+ conductance

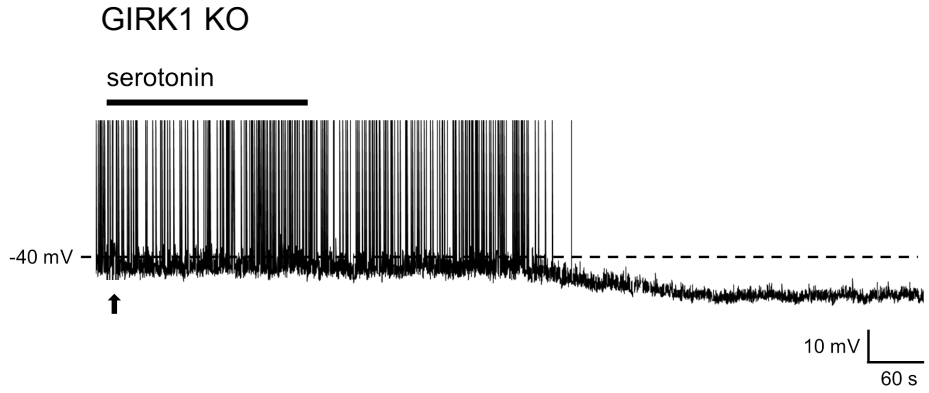


GIRK1 Subunits Are Not Required for the Serotonin-Activated Current in NPY Neurons

Since this study and others show that 5-HT and 5-HTR_{1b} agonists hyperpolarize NPY neurons via a K^+ conductance, the possibility that this K^+ conductance is mediated by GIRK channels was tested. 5-HT and 5-HTR_{1b} agonists were applied to NPY neurons in *girk1* knockout mice. Ten out of 20 NPY-GFP; *girk1*$^{-/-}$ cells (50%) were hyperpolarized by 5-HT. A representative trace showing a serotonin-induced hyperpolarization is shown in Figure 8. Specific 5-HTR_{1b} agonists also hyperpolarized NPY-GFP;*girk1*$^{-/-}$ cells. Two out of 7 (28.6%) were hyperpolarized by CP93129 (-12.8 ± 5 mV) and 1 out of 5 (20%) were hyperpolarized

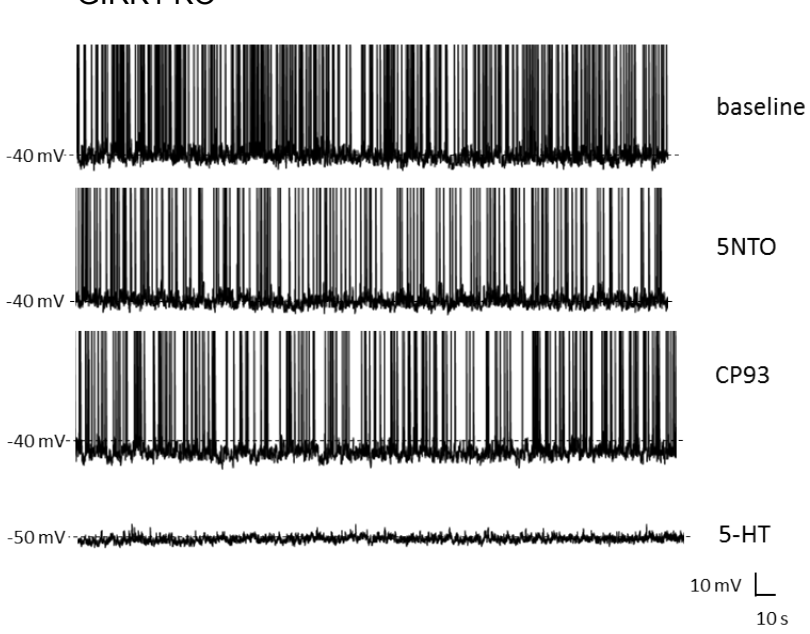
by 5-NTO (-5.4 mV). These responses suggest that GIRK1 is not required for the acute effect of 5-HT and 5-HTR_{1b} agonists on NPY neurons.

Figure 8. Serotonin hyperpolarizes *girk1*$^{-/-}$ NPY neurons



Additionally, serial applications of 5-HT and 5-HTR_{1b} agonists were performed, and a subset of cells were found that hyperpolarize to 5-HT but not to 5-HTR_{1b} agonists. Current clamp recordings from one representative cell are shown in Figure 9, in which serial application of 5-NTO and CP93129 did not alter the baseline resting membrane potential or action potential firing rate, but application of 5-HT hyperpolarized the cell and abolished action potential firing.

Figure 9. Response to 5-HT but not 5-HTR$_{1b}$ agonists in NPY neurons.

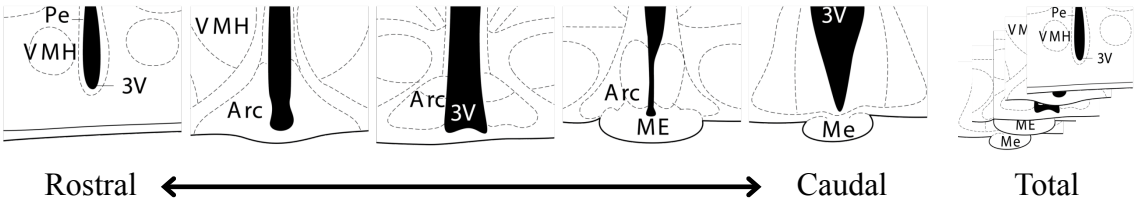


A summary of response rate and amplitude to each hormone and 5-HTR$_{1b}$ agonist applied is shown in Table 2. In Table 3, the response rate is further differentiated by position along the rostral-caudal axis of the arcuate nucleus divided into 5 sections.

Table 2. Summary of responses in NPY-GFP and NLT reporter mice.

	NPY-GFP males, n = 150		NPY-GFP; LepR-cre; $STOP^{fl/fl}$-tdTomato, n = 81		NPY-GFP + NPY-GFP; LepR-cre; $STOP^{fl/fl}$-tdTomato, n = 231	
Leptin	13 / 52 (25%)	-6.6 ± 0.3 mV	16 / 43 (37.2%)	-12.4 ± 1.8 mV	29 / 95 (30.5%)	-9.8 ± 1.2 mV
Insulin	15 / 56 (26.8%)	-10.1 ± 0.5 mV	0 / 21 (0%)		15 / 77 (19.5%)	-10.1 ± 2 mV
CP 93129	1 / 5 (20%)	-11.4 ± 0 mV	0 / 2 (0%)		1 / 7 (14.3%)	-11.4 ± 0 mV
5-NTO	9 / 16 (56.3%)	-6.7 ± 1.5 mV	1 / 1 (100%)	-5.4 ± 0 mV	10 / 17 (58.8%)	-6.6 ± 1.4 mV
Ghrelin	4 / 12 (33.3%)	8.2 ± 1.6 mV	2 / 6 (33.3%)	8.5 ± 0.9 mV	6 / 18 (33.3%)	8.3 ± 1 mV
Glucagon	2 / 5 (40%)	7 ± 0.8 mV	0 / 0 (0%)		2 / 5 (40%)	7 ± 0.8 mV

Table 3. Distribution of responses along the rostral-caudal axis of the arcuate nucleus.

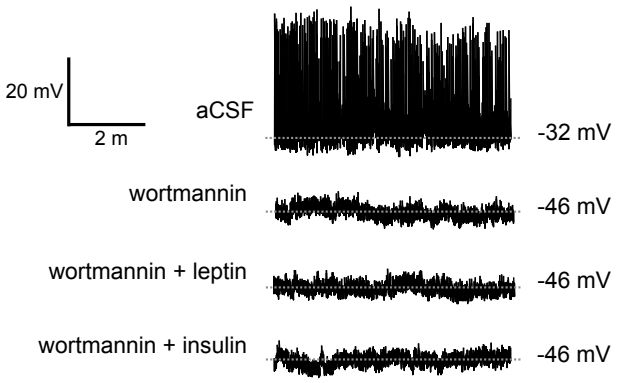


	1	2	3	4	5	**Total**
Leptin	5/9 (55.6%)	9/23 (39.1%)	5/20 (25%)	5/16 (31.3%)	5/18 (27.8%)	**29/95 (30.5%)**
Insulin	0/7 (0%)	3/17 (17.6%)	6/19 (31.6%)	0/11 (0%)	6/20 (30%)	**15/77 (19.5%)**
Leptin & insulin	0/4 (0%)	0/14 (0%)	1/15 (6.7%)	0/6 (0%)	3/17 (17.6%)	**4/59 (6.8%)**
5-HT_{1b} agonists	0/2 (0%)	4/6 (66.7%)	2/6 (33.3%)	0/1 (0%)	5/6 (83.3%)	**11/24 (45.8%)**
Ghrelin	1/2 (50%)	0/1 (0%)	3/8 (37.5%)	1/6 (16.7%)	0	**6/18 (33.3%)**
Glucagon	0	1/1 (100%)	1/4 (25%)	0	0	**2/5 (40%)**

Acute Effects of Leptin and Insulin May Require PI3K Signaling

To test whether the acute effects of leptin and insulin are mediated by the phosphoinositide 3-kinase (PI3K) pathway, both hormones were applied to cells that had been pre-treated with 100 nM wortmannin, a specific covalent inhibitor of PI3Ks. In the six applications of each hormone, zero responses were observed to either leptin or insulin. Given a 19.5% average response rate to insulin and a 30.5% response rate to leptin, at least 1 to 2 responses would be expected if wortmannin inhibition had no effect. However, the size of this experiment is underpowered to conclusively state PI3K inhibition is required. A representative trace of wortmannin pre-treatment and leptin and insulin administration is shown in Figure 4.

Figure 10. No response to leptin or to insulin with wortmannin pre-treatment.



Wortmannin is thought to be PI3K selective at a concentration ≤ 100 nM (Brown, 2007). However, to exclude the possibility of off-target inhibition with wortmannin or other PI3K inhibitors (e.g., LY294002), a genetic PI3K ablation model was developed, AgRP-cre; $p110\alpha^{fl/fl}$; $p110\beta^{fl/fl}$; tdTomato, referred to as the APT line. In this model, the two catalytic subunits of PI3Ks present in the hypothalamus are deleted specifically in AgRP neurons. Leptin and insulin were applied to APT mice to test for a response in the absence of PI3K

signaling. Four applications were recorded without a response. Additional recordings are necessary to confirm that PI3K inhibition abolishes the acute effects of these hormones. A representative trace of an APT cell is shown in Figure 5. Interestingly, the cell retains spontaneous action potential firing without PI3K signaling, which suggests that baseline firing does not require constitutive PI3K signaling. The addition of leptin to the recording chamber did not alter the resting membrane potential or the firing rate. A summary of leptin and insulin applications in cells that lack PI3K signaling is shown in Table 5.

Figure 11. Representative trace of an APT cell that is unresponsive to leptin.

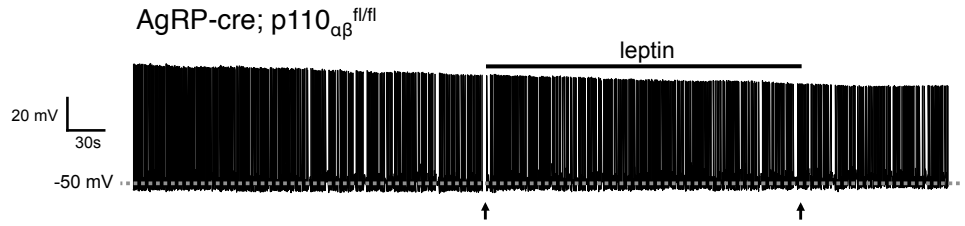


Table 4. Summary of responses to leptin and insulin in the absence of PI3K signaling.

	100nM wortmannin pre-treatment	AgRP-cre; $p110_{\alpha\beta}^{fl/fl}$
Leptin	0/6 (0%)	0/4 (0%)
Insulin	0/6 (0%)	0/4 (0%)

Testing for Post-Synaptic Actions

To confirm that the hormones and 5-HTR_{1b} agonists in this study exert their acute effects via direct post-synaptic action, a series of experiments blocking action potentials and fast synaptic transmission will be required. Initial results towards this aim are presented in Table 6 below. Insulin and leptin were applied to cells pretreated with a combination of three fast

synaptic transmission blockers: 10 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), 50 μM picrotoxin, and 50 μM AP-5 ((2R)-amino-5-phosphonopentanoate). CNQX is a competitive AMPA/kainate receptor antagonist; picrotoxin is a non-competitive $GABA_A$ receptor channel blocker; and AP-5 is a competitive NMDA receptor antagonist. As the acute actions of insulin and of leptin are thought to be post-synaptic effects of binding the insulin receptor and leptin receptor, respectively, this combination of blockers is not expected to block their acute effects. A complementary strategy would be the application of tetrodotoxin to block voltage-gated sodium channels and abolish all synaptic transmission.

Table 5. Responses to insulin and leptin after pretreatment with fast synaptic blockers

	CNQX (10 μM) + Picrotoxin (50 μM) + AP-5 (50 μM)
Leptin	0/4 (0%)
Insulin	0/4 (0%)

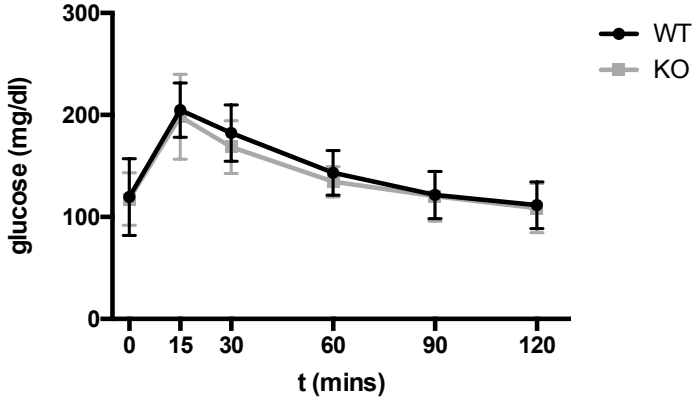
METABOLIC PHENOTYPING

Metabolic parameters were assessed in APT (AgRP-cre; $p110\alpha^{fl/fl}$; $p110\beta^{fl/fl}$; tdTomato) males including glucose and insulin tolerance, body composition, oxygen consumption (VO2), carbon dioxide production (VCO2), and heat production.

Glucose and insulin tolerance testing

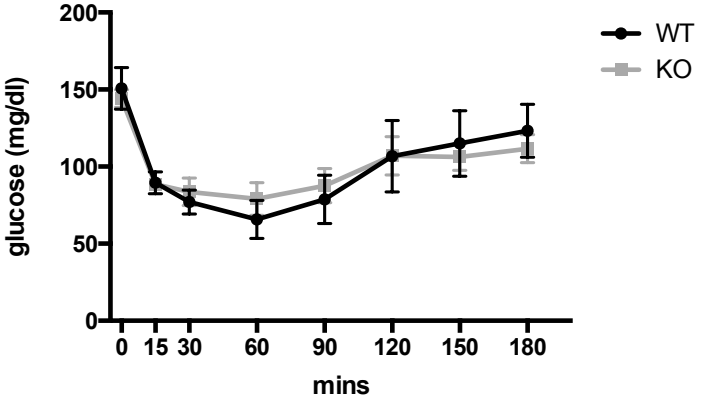
Glucose and insulin tolerance testing were performed on young mice and littermate controls with matched body weight to exclude the possibility that differences in body weight would interfere with glucose homeostasis. Glucose sensitivity was assessed by injecting 1 mg/kg of dextrose intraperitoneally in mice at 11 weeks of age. This glucose load was cleared equally fast by both APT and wildtype mice (Figure 12).

Figure 12. Glucose tolerance is unchanged between APT (KO) and wildtype (WT) mice



Next, insulin sensitivity was assessed by insulin tolerance testing. 1U/kg Humulin was injected intraperitoneally in APT and in wildtype mice at 13 weeks of age, and serial tail vein glucose measurements were recorded. This insulin bolus induced a robust decrease in blood glucose that was identical between APT and wildtype groups (Figure 13).

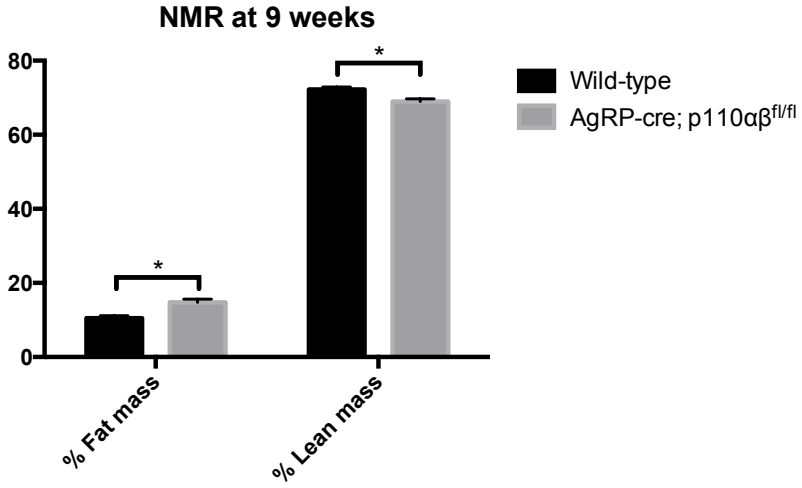
Figure 13. Insulin tolerance is unchanged between APT (KO) and wildtype (WT) mice



Body composition

Body composition was assessed in 9 week-old APT mice and in littermate controls with matched body weight. Fat mass, lean mass, free water, and total body water were measured by nuclear magnetic resonance spectroscopy. APT mice were found to have increased fat mass and decreased lean mass (Figure 14), $p = 0.03$.

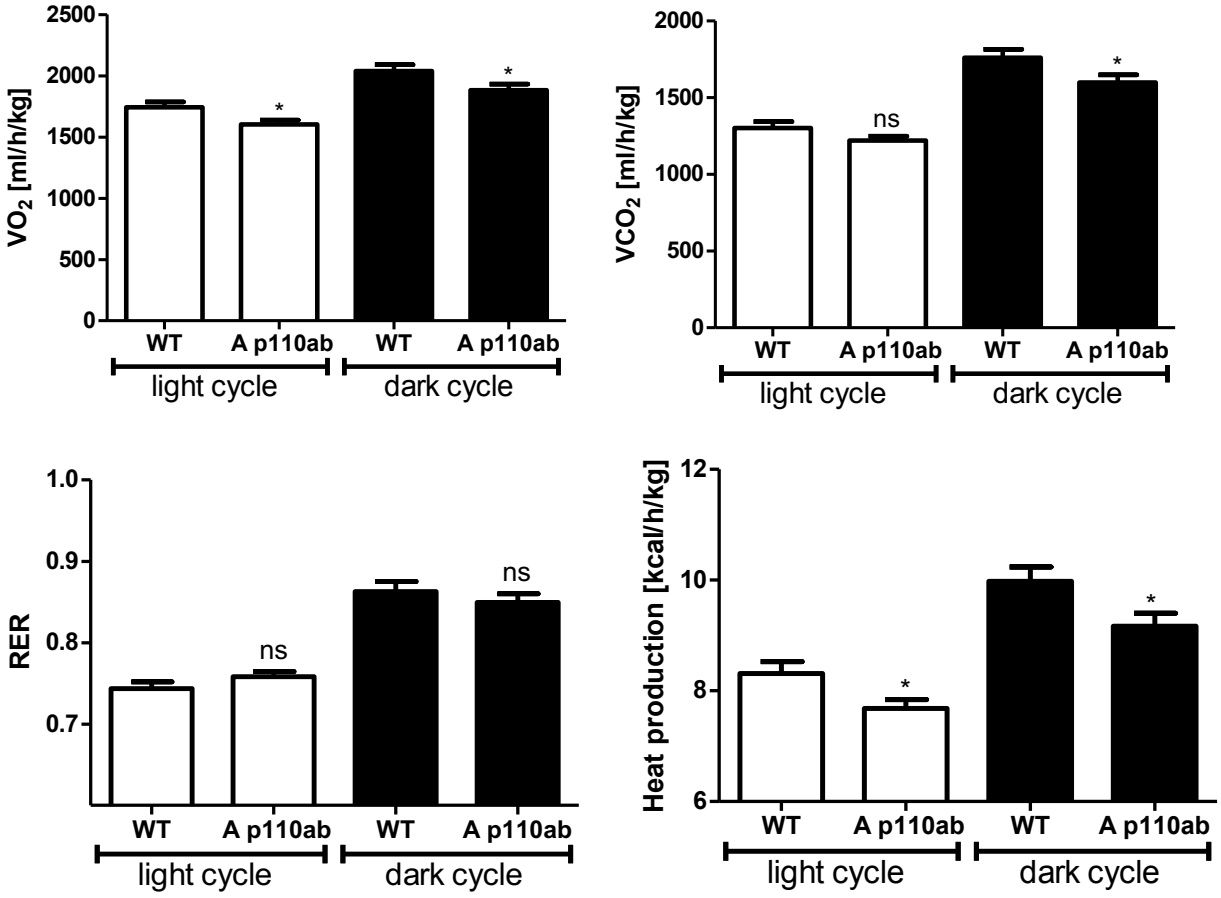
Figure 14. APT mice have decrease % lean mass and increased % fat mass



Energy expenditure and locomotor activity

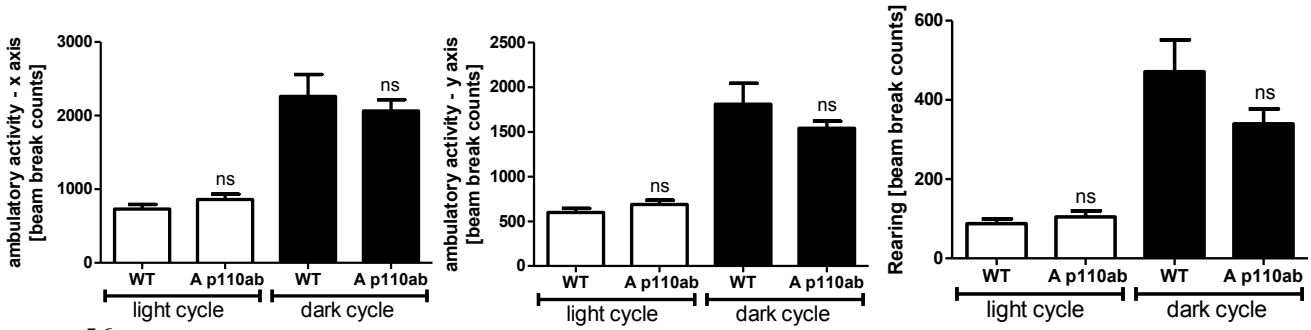
Metabolic efficiency in *ad libitum* fed APT and control littermates was assessed using indirect calorimetry. APT mice displayed significantly decreased oxygen consumption during both day and night cycles. Heat production was also significantly decreased in APT mice (Figure 15).

Figure 15. APT mice have decreased heat production and VO2



Locomotor activity during this experiment was not different between APT and wildtype littermates (Figure 16).

Figure 16. Locomotor activity is unchanged in APT mice



CHAPTER FIVE
Conclusions and Recommendations

NPY NEURONS ARE INHIBITED BY LEPTIN, INSULIN, AND SEROTONIN

This report is the first to systematically record the acute electrophysiological responses of NPY neurons throughout the rostral-caudal extent of the arcuate nucleus of the hypothalamus. In addition, validated fluorescent reporter lines, the NPY-GFP and AgRP-cre mice, were used to target NPY neurons in this study. These two features of the experimental design increase the likelihood that the sample of NPY neurons in this study represent the true population of arcuate NPY neurons.

The findings that insulin, leptin, and serotonin all inhibit NPY neurons via potassium channels is in agreement with some but not all published reports. In particular, van den Top and colleagues found that leptin induced hyperpolarizations in unlabeled arcuate neurons identified as NPY neurons *post hoc* by RT-PCR (van den Top et al., 2004). A paper from Jens Brüning's group used the AgRP-cre line as a reporter line to identify NPY neurons and reported that insulin hyperpolarizes arcuate NPY neurons (Könner et al., 2007). In contrast to these studies, a study using the AgRP-cre reporter line found that insulin depolarized 4 of 8 cells, while leptin had no effect on 11 cells (Claret et al., 2007). A follow-up study from the same group found that insulin depolarized 4 of 13 AgRP-cre cells while leptin had no effect in 7 cells (Al-Qassab et al., 2009).

In the present study using a much larger sample size, no NPY neurons exhibited the depolarizations in response to insulin as seen in the above two studies. Further, NPY neurons in this study were inhibited by leptin in 27.8% to 55.6% of neurons depending on their position along the rostral-caudal axis. One possible explanation for absence of leptin responses is the above reports is that the investigators did not record from sufficient numbers of cells to see an effect. If the investigators used hypothalamic slices from the caudal-most portion of the arcuate nucleus, they would have targeted neurons with the lowest response rate and potentially missed a more responsive population.

The insulin-induced depolarizations seen in the above studies may be explained by the observation that a cell targeted by the investigator may not necessarily be the cell attached to the recording pipette. The present study used a fluorescent dye (Alexa Fluor 350) in the pipette solution to allow the confirmation of cell targeting after the termination of the recording (see Methods). By observing co-localization of Alex Fluor 350 dye with GFP and/or tdTomato, we can be more certain the pipette was attached to the desired cell.

There are limitations of this study. One, the acute effects of hormones and serotonin in this study were not confirmed to be direct postsynaptic effects. In order to demonstrate direct action on NPY neurons, observing effects in the presence of synaptic blockers is required. Initial efforts toward this aim are presented in Table 5, but additional recordings are needed.

Second, while this report did not detect responses to insulin or to leptin during pharmacologic or genetic ablation of the PI3K pathway, more recordings are needed to draw this conclusion definitively. The APT mice (AgRP-cre; $p110\alpha^{fl/fl}$; $p110\beta^{fl/fl}$; tdTomato) generated in this study will be useful towards this goal as they eliminate the possibility of off-target effects of the pharmacologic PI3K inhibitors used in earlier studies.

Finally, this report confirms earlier work that activation of serotonin 1b receptors inhibits NPY neurons (Heisler et al., 2006) and demonstrates that NPY neurons throughout the arcuate nucleus can respond to 5-HT and 5-HTR_{1b} agonists. A subset of NPY neurons were also found that hyperpolarize to 5-HT but not to 5-HTR_{1b} agonists. This observation warrants investigation in future studies to test two possible hypotheses: 5-HT may have a direct postsynaptic effect on NPY neurons by binding to an unknown 5-HTR receptor on NPY neurons; or, 5-HT may be acting presynaptically on upstream neurons that in turn inhibit NPY neurons. These two hypotheses can be tested by recording from NPY neurons that have been pretreated with compounds that block voltage-gated Na^+ channels (e.g., tetrodotoxin) or with compounds that block fast synaptic transmission (e.g., CNQX, picrotoxin, and AP-5). Additionally, application of 5-HT or specific 5-HTR agonists to the available 5-HTR_{1b}-specific knockout mouse line in the presence of synaptic blockade would allow the detection of other serotonin subtypes that acutely modify NPY neuron activity.

This study also found that NPY neurons, like neighboring POMC neurons, do not require GIRK1 subunits for the acute effects of 5-HT and 5-HTR_{1b} agonists. As in POMC neurons,

knockout of *girk1* in NPY neurons also results in a significant depolarization of the baseline resting membrane potential. However, unlike in POMC neurons, knockout of *girk1* is insufficient to abolish the $GABA_B$-activated potassium current in NPY neurons. This finding suggests that NPY neurons have additional ion channels coupled to $GABA_B$ receptors. To test whether these channels are GIRK channels composed of other GIRK subunits, recordings can be made in an available mouse line in which all physiologic GIRK channels are deleted (NPY-GFP; $girk1^{-/-}$; $girk2^{-/-}$).

This study adds to literature on the acute effects of ghrelin. Previous reports in rats and in mice have shown that within NPY neurons, ghrelin or GHSR agonists induce c-fos expression, stimulate transcription of the neuropeptides AgRP and NPY, and increase calcium influx, all of which suggest that ghrelin activates NPY neurons (Dickson and Luckman, 1997; Hewson and Dickson, 2000; Kamegai et al., 2000; Kohno et al., 2003; Lawrence et al., 2002; Nakazato et al., 2001; Seoane et al., 2003). Further, one study has reported ghrelin-induced depolarizations in 9 NPY neurons (Cowley et al., 2003). The present report establishes a response rate from recordings of 18 NPY neurons and demonstrates that the ghrelin-induced depolarization occurs via a mixed cation conductance. Finally, this study reports for the first time that the counter-regulatory hormone glucagon acutely activates NPY neurons.

The findings of this study have implications for the design of therapeutics for diabetes mellitus and obesity. Despite intense interest, pharmacologic therapies for obesity remain

limited to three drugs with FDA approval. Two of these agents specifically target the brain, lorcaserin and phentermine/topiramate. Lorcaserin is a 5-HTR_{2c} agonist and reduces body weight largely by reductions in food intake both in humans and in rodents (Martin et al., 2011; Smith et al., 2010; Thomsen et al., 2008). Evidence from rodent studies implicates 5-HTR_{2c} signaling in POMC neurons in this effect (Xu et al., 2010; 2008). The present study suggests that 5-HTR_{1b} agonists may potentiate this effect by inhibiting NPY neurons and thus disinhibiting POMC neurons. 5-HTR_{1b} agonists may also have direct effects on food intake as well (Lam et al., 2008).

APPENDIX A
Calculated Nernst Potentials

Nernst potentials are shown for the major extracellular and intracellular ions present in the electrophysiology solutions used.

Ion	Extracellular concentration (aCSF)	Intracellular concentration (pipette solution)	Nernst potential
Na^+	148.6 mM	0.3 mM	+157 mV
K^+	2.5 mM	138 mM	-102 mV
Cl^-	135.7 mM	10.6 mM	-65 mV
Ca^{2+}	2.4 mM	0.3 mM	+26 mV

APPENDIX B
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