IMPLICATIONS OF NEUROPEPTIDE Y INDUCTION IN THE DORSOMEDIAL HYPOTHALAMUS

FOR HYPERPHAGIA AND OBESITY

by

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

αMSH	alpha melanocyte-stimulating hormone
ACTH	adrenocorticotrophin
Acvr1c	activin A receptor, type 1C
AgRP	agouti related peptide
ARH	arcuate nucleus of the hypothalamus
AVPV	anteroventro paravntricular nucleus
BA	barrington's nucleus
BAT	brown adipose tissue
BBB	blood brain barrier
BDA	biotinylated dextran amine
BMI	bicuculline methiodide
CART	cocaine- amphetamine-regulated transcript
CCK	cholecytokinin
CD	normal chow diet
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha
CRH	corticotrophin releasing hormone
DASP	anti-dopamine β-hydroxylase conjugated to saporin
DBH	dopamine-β-hydroxylase
DIO	diet induced obese
DMH	dorsomedial hypothalamus
DMHc	the compact zone of the DMH
DMHnc	the non-compact zone of the DMH
E	epinephrine
FACS	fluorescent activated cell sorting
FG	fluorogold

FOXA1	forkhead box A1
FOXO1	Forkhead box protein O1
GABA	ϒ-aminobutyric acid
GAD	glutamic acid decarboxylase
GALP	galanin-like peptide
GHSR	growth hormone secretagogue receptor
GLP-1	glucagon like peptide-1
GnIH	gonadotropin-inhibitory hormone
GnRH	gonadotropin releasing hormone
HFD	high fat diet
HPA	hypothalamic-pituitary-adrenocortical
HPG	hypothalamic-pituitary-gonadal
HPT	hypothalamic-pituitary-thyroid
HrGFP	humanized renilla reniformis green fluorescent protein
LC	locus ceorulous
LH	lateral hypothalamus
LPO	lateral preoptic nucleus
LPB	lateral parabrachial nucleus
LS	lateral septal nucleus
MC4R	melanocortin-4 receptor
MCH	melanin-concentrating hormone
MP	medial parvicellular
MTII	melanotan II
MPO	median preoptic
NE	norepinephrine
NIRKO	neuronal specific insulin receptor knockout
NOS	nitric oxide synthesizing

NPY	neuropeptide Y
NTS	nucleus of the solitary tract
ObRb	long form leptin receptor
OLETF	Otsuka Long-Evans Tokushima Fatty
OX_1R	orexin-1 receptor
PAG	periaqueductal grey
PB	parabrachial nucleus
PFA	perifornical area
PGC-1α	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
Pitx2	paired-like homeodomaine transcription factor 2
PM	posterior magnicellular
POA	preoptic area
POMC	pro-opiomelanocortin
PS	parastrial nucleus
PSCH	preoptic suprachiasmatic nucleus
PSTAT3	phosphorylated signal transducer and activator of transcription 3
PVH	paraventricular nucleus
PYY	peptide tyrosine tyrosine
RFRP	RFamide-related peptides
rRPa	rostral raphe pallidus
SCN	suprachiasmatic nucleus
Stap2	signal transducing adaptor family member 2
SUM	supramammilary nucleus
TIDA	tuberoinfundibular dopamine
TPH	tryptophan hydroxylase
TRH	thyrotrophin releasing hormone
UCP-1	uncoupling protein 1

VGAT	vesicular GABA transpoter
VLM	verntrolatenal medulla
VMH	ventromedial hypothalamus
VS	ventral subiculum
VTA	ventrotagmental area
WAT	white adipose tissue

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ABSTRACT

While the prevalence of obesity is increasing, the treatment of obesity has been disappointing for researchers, because the mechanisms that regulate body weight homeostasis and adiposity are incompletely understood. Food intake is tightly regulated by external factors such as food availability and palatability, as well as by internal factors including the hormonal status related to energy homeostasis. These circulating factors communicate with a complex network of feeding neurocircuitry to maintain body weight homeostasis.

Interactions between circulating hormones and the neurons in the arcuate nucleus of the hypothalamus (ARH) have been extensively characterized. Neuropeptide Y (NPY) neurons in the ARH are particularly important because they stimulate food intake and promote positive energy balance. In specific physiological conditions such as lactation and obesity, NPY neurons in the dorsomedial hypothalamus (DMH) appear to play a critical role in feeding behavior and energy expenditure. However, DMH-NPY neurons have not received much attention until recent years. Emerging evidence suggests that DMH-NPY neurons are highly implicated in the development of obesity by promoting energy intake over energy expenditure. The exact mechanisms by which DMH-NPY neurons regulate these behaviors are still poorly understood.

The goal of this thesis is to expand the neuroanatomical knowledge of DMH-NPY neurons that are activated during specific conditions, including development, lactation and obesity, to better understand the mechanisms of these neurons in feeding behavior.

Chapter 1 provides a review of the current knowledge of hypothalamic regulation of body weight homeostasis with particular emphasis on the anatomy and functions of the DMH neurons.

Chapter 2 describes the results of microarray gene analysis presenting alternate phenotypes of DMH-NPY neurons isolated from developing mice.

Chapter 3 illustrates biotinylated dextran amine (BDA)-labeled DMH-NPY neuronal projections in the lactation and diet-induced obese (DIO) mouse model with a particular focus on hypothalamic projections in the areas highly implicated in the regulation of feeding behavior and energy expenditure.

Chapter 4 focuses on the characterization of DMH-NPY induction in the diet induced obesity (DIO) mouse model. A surprising finding from this study is that leptin may regulate DMH-NPY neuronal activity in obesity. CART co-expression in DMH-NPY neurons also raises several questions regarding the role of leptin and CART in DIO conditions.

Finally, chapter 5 integrates findings from three different DMH-NPY induction models and discusses different mechanisms leading to the activation of DMH-NPY neurons which results in hyperphagic behavior. In summary, these findings contribute to the understanding of neural adaptations occurring during specific physiological states that favor excess energy intake and some of the neural pathways that may account for hyperphagia.

Chapter 1

INTRODUCTION

BACKGROUND

Obesity (body mass index (BMI) >30 kg m^{-2}) is a major health issue and affects more than 300 million people worldwide (Kelly et al., 2008). In the United States alone, about one-third of adults are obese and approximately 17% of children and adolescents are considered obese (Baskin et al., 2005). Obesity is not simply a cosmetic problem for the affected people, but it leads to many life-threatening health complications (Daniels, 2009; Tsiros et al., 2009), such as type II diabetes, hypertension, dyslipidemia, arterioscleroris and even cancer.

The origin of the obesity epidemic is a complex matter. Easy access to a calorically dense diet and a sedentary lifestyle are considered the major causes of the obesity epidemic in modern society (Bloom et al., 2008). Genetic mutations disrupt the normal energy balance and cause obesity in humans and animals (Friedman, 2009b). However, only a small percentage of morbid obesity is due to monogenic mutation; therefore, a combination of genes and environments is thought to cause weight variation in humans (Friedman, 2009b). Numerous studies demonstrated that both maternal (gestational obesity and diabetes) and early postnatal (diet and energy availability) environments have a significant influence on body weight, metabolism and behavior in adulthood (Grove and Smith, 2003; Sullivan et al., 2011).

Although weight control programs aimed at reducing the caloric intake promise weight loss in obese individuals, weight loss is temporary and cannot be easily maintained. Only a few drugs are currently approved for obesity treatment, and they are limited by their side effects and lack of efficacy (Velloso and Schwartz, 2011). The most effective therapy is bariatric surgery to modify the anatomy of the gastrointestinal tract,

thereby reducing food intake and/or absorption (Friedman, 2009b). However, this procedure is reserved for the severely obese populations, due to the risk factors and high cost.

The complex nature of neurocircuitry involved in energy homeostasis explains why obesity is so challenging to treat. Despite the variability in daily energy intake and expenditure, our brain maintains body weight in a stable range, known as the "set-point"(Farias et al., 2011). For example, overfeeding of humans and rodents results in compensatory reduction in food intake and increase in energy expenditure which returns them to their original body weight (Levin, 2010). However, the body is more efficient at protecting against negative energy balance during food deprivation compared to conditions of weight gain with overfeeding (Farias et al., 2011). Obese rodents maintain their elevated body weight set point, even after a long term calorie restriction (Levin and Dunn-Meynell, 2002). Therefore, the neural mechanisms defending the higher “set-point” in obese individuals may be the main reason why pharmacological and behavioral interventions have little success in producing long term weight loss. In addition to homeostatic feeding regulation, hedonic contribution to highly palatable diet consumption and habitual overeating adds a complexity to the central regulation of appetite and body weight. Increasing our knowledge of the central mechanisms maintaining energy homeostasis is critical for the development of effective obesity treatments in the future.

OVERVIEW OF HYPOTHALAMIC REGULATION OF BODY WEIGHT HOMEOSTASIS

Body weight homeostasis

Obesity is a state of positive energy balance and excessive energy intake over energy expenditure leads to the accumulation of excess body fat and weight gain in humans. To regulate the balance between energy intake and energy expenditure, the brain senses and integrates hormonal, nutrient and neural signals generated from peripheral sensors and organs. When energy intake exceeds energy utilization, negative feedback mechanisms in the brain lead to the inhibition of food intake and hepatic glucose production, and an increase in energy expenditure and fat storage (Sanchez-Lasheras et al., 2010). The most well known adiposity signals generated in the periphery are leptin and insulin. In times of food abundance and ample fat stores, leptin and insulin levels increase and provide a negative feedback on neural circuits that govern food intake (Morton et al., 2006; Sanchez-Lasheras et al., 2010). In contrast, nutrient deficiency signals result in feeding stimulation and mobilization of energy stores from adipose tissue and liver (Sanchez-Lasheras et al., 2010; Seeley and Woods, 2003). Various other factors such as ghrelin, cholecytokinin (CCK), and glucagon like peptide-1 (GLP-1), and other metabolic substrates are also produced in the periphery to signal the brain via autonomic afferents and by being transported across the blood–brain barrier (BBB) to modulate feeding behavior (Zac-Varghese et al., 2010). These peripheral signals are integrated by a distributed network of "metabolic sensing" neurons located in key areas of the hypothalamus and brainstem.

Hypothalamic feeding neurocircitry

The brief history of hypothalamic regulation of body weight

Early lesion studies demonstrated the important role of hypothalamus in body weight homeostasis. Ventromedial hypothalamus (VMH) lesions lead to hyperphagia and obesity in rats, placing the VMH as the major "satiety center" (Debons et al., 1977). Contrarily, lesioning of the lateral hypothalamus (LH) causes a reduction in food intake and weight loss, indicating that the LH is primarily involved in the stimulation of food intake (Anand and Brobeck, 1951). This dual center theory of feeding behavior was the first popular theory describing the hypothalamic regulation of food intake for several decades. However, the fundamental problem with electrolytic lesion studies is that both cell bodies and fibers are destroyed, making it impossible to distinguish whether the behavioral effects are attributable to loss of cell bodies or fibers of passage.

For years, many scientists believed the existence of circulating factors that signal the brain to control food intake. The arcuate nucleus of the hypothalamus (ARH) is considered a part of circumventricular organs lacking BBB, allowing circulating factors to have a direct access to the nucleus. ARH lesions using monosodium glutamate (MSG) result in endocrine deficits; reduced reproductive capacity, retarded growth, and obesity (Debons et al., 1962; Holzwarth-McBride et al., 1976; Olney, 1969), suggesting that the ARH is the key site in the hypothalamus influenced by peripheral signals.

The introduction of new molecular and genetic tools allowed more precise way to study the hypothalamic functions and the genes involved in the regulation of food intake and energy expenditure. The cloning of leptin in 1994 was a critically important discovery in the field that substantially extended our understanding of the hypothalamic

control of body weight homeostasis (Zhang et al., 1994). Studies in genetically modified mice demonstrated that leptin action in the brain is sufficient to regulate body weight, feeding, energy expenditure, and glucose metabolism (Gautron and Elmquist, 2011). In the hypothalamus, the ARH is the most recognized area for leptin action in inhibiting food intake.

The arcuate nucleus: the primary sensor for peripheral signals

The most well characterized system influenced by circulating leptin levels is the orexigenic and anorexigenic neurons in the ARH. Neuropeptide Y (NPY) and agouti related peptide (AgRP) are potent orexigenic neuropeptides, and they are co-expressed in the same neurons directly inhibited by leptin through a long form of the leptin receptor (ObRb) (Hakansson et al., 1996). On the other hand, the neurons that produce anorexigenic neuropeptides, pro-opiomelanocortin (POMC) and cocaine- amphetamine-regulated transcript (CART), are directly stimulated by leptin (Cowley et al., 2001; Elias et al., 2001). POMC/CART neurons are inhibited by neighboring NPY/AgRP neurons that also produce an inhibitory neurotransmitter, GABA (ɣ-aminobutyric acid) (Cowley et al., 2001; Parton et al., 2007; Tong et al., 2008b), indicating reciprocal interactions between these two neurons. In addition to responding to leptin, NPY/AgRP and POMC/CART neurons are also directly regulated by other circulating factors including insulin and ghrelin that have potent effects on feeding behavior. Therefore, these ARH neurons are very sensitive to the changes in nutritional/metabolic state of the animal. During calorie deprivation such as fasting, the suppression of leptin/insulin production and the rise in ghrelin levels in the blood stimulates food intake by increasing NPY/AgRP and decreasing POMC/CART expression (Marks et al., 1992; Smith, 1993). Diet induced

obese (DIO) animals, which have high levels of leptin and insulin, are unable to properly regulate body weight since they develop leptin and insulin resistance in the ARH neurons (Lin et al., 2000; Munzberg et al., 2004). The importance of the ARH in feeding regulation is also supported by the fact that both neuronal subsets relay the metabolic signals to other hypothalamic areas involved in feeding regulation including the paraventricular nucleus (PVH), the lateral hypothalamic area (LHA) and the dorsomedial hypothalamus (DMH).

The paraventricular nucleus: the site of integration

The PVH is in a strategic position to coordinate the activities in the autonomic and endocrine system by receiving inputs from a wide variety of hypothalamic regions including the ARH and DMH as well as the brainstem. The neurons in the PVH then send projections to sympathetic and parasympathetic preganglionic nuclei, as well as to the median eminence and posterior pituitary (Engelmann et al., 2004; Hallbeck et al., 2001; Sawchenko and Swanson, 1982a). The PVH plays essential roles in many physiological processes including feeding regulation, stress response, cardiovascular regulation and thermoregulation (Engelmann et al., 2004; Pyner, 2009; Simpson et al., 2009). The PVH contains corticotrophin releasing hormone (CRH) and thyrotrophin releasing hormone (TRH) neurons which are known to play important roles in energy homesostasis by activating hypothalamic-pituitary-adrenocortical (HPA) and hypothalamic-pituitary-thyroid (HPT) axis, respectively (Nillni; Papadimitriou and Priftis, 2009). Magnocellular neurosecretory cells release oxytocin and vasopressin from the posterior pituitary in response to specific physiological demands such as lactation and dehydration (Buijs, 1990).

As implied earlier, NPY/AgRP and POMC/CART neurons in the ARH modulate the activity of PVH neurons to regulate food intake and energy expenditure (Bai et al., 1985). The CRH and TRH neurons are directly regulated by the inputs from the ARH neurons (Legradi and Lechan, 1998; Li et al., 2000). In the fasting condition, increased NPY/AgRP and decreased POMC neuronal inputs to the PVH leads to the inhibition CRH and TRH functions which contributes to the stimulation of food intake (Fekete et al., 2001; Fuzesi et al., 2007). The PVH also receives fiber terminals containing appetite regulating neurotransmitters from other areas including the brainstem and DMH (Sawchenko et al., 1985; Thompson et al., 1996). The catecholaminergic projections from the brainstem appear to play major roles in the relay of visceral sensory information to the PVH. These catecholamine neurons also co-express NPY and CART and project to the CRH, TRH and oxytocin neurons in the PVH (Fekete et al., 2005; Parker and Crowley, 1993; Wittmann et al., 2002, 2005). The PVH is the major hypothalamic target of DMH neurons (Thompson et al., 1996). A previous study demonstrated that activation of specific neuroendocrine and autonomic elements of the PVH is triggered by leptin-activated afferents arising in the DMH (Elmquist et al., 1998). Therefore, DMH neurons serve as a relay center for the central actions of leptin on the TRH neurons in the PVH (Mihaly et al., 2001).

The dorsomedial hypothalamus: a relay station between the ARH and PVH

The DMH has long been implicated in the control of ingestion and body weight. Early studies showed that DMH lesions resulted in rats that were hypophagic and hypodipsic, and had reduced ponderal and linear growth (Bellinger and Bernardis, 2002), suggesting that the DMH is an additional ‘feeding center’ in the hypothalamus. The

DMH receives strong inputs from the neurons in the ARH and sends dense projections to the PVH (Bai et al., 1985; Thompson et al., 1996), placing the DMH as a relay station between the ARH and PVH. The DMH contains neuronal populations that are implicated in the regulation of energy homeostasis (Bernardis and Bellinger, 1998). Although the neuronal phenotypes are not extensively characterized, DMH neurons are regulated by the feeding signals generated in the ARH via NPY receptors and melanocortin receptors. The DMH also contains numerous leptin receptor expressing neurons (Elias et al., 2000) which send direct projections to the areas that are implicated in feeding regulation and brown adipose tissue (BAT) thermogenesis (Elmquist et al., 1998; Zhang et al., 2011b). The DMH has been known to play a crucial role in thermoregulation as a relay station between the preoptic area and brainstem neurons (Morrison and Nakamura, 2011). A recent study identified the DMH as a site of leptin action in the regulation of sympathetic nerve activity and BAT thermogenesis during diet-induced obese condition in mice (Enriori et al., 2011). The DMH also expresses a moderate level of ghrelin receptor, growth hormone secretagogue receptor (GHSR) (Olszewski et al., 2008), and peripheral injection of ghrelin induces c-fos expression in the DMH (Kobelt et al., 2008), suggesting that the DMH is an important site of ghrelin action.

The DMH also contains neurons expressing NPY. DMH-NPY expression is significantly up-regulated during hyperphagic conditions such as lactation and obesity in rodents (Guan et al., 1998a; Kesterson et al., 1997; Smith, 1993). The models of chronic negative energy balance such as intense exercise and chronic food restriction also show increased DMH-NPY expression (Bi et al., 2003; Lewis et al., 1993), suggesting that DMH-NPY induction is one of the rewiring mechanisms responsible for protecting body

weight during negative energy balance. Knocking down NPY expression in the DMH results in body weight loss and the reversal of obese phenotypes in diet induced obese rats (Chao et al., 2011), confirming the importance of DMH-NPY neurons in the central mechanism of obesity.

The lateral hypothalamus: the feeding center

The LH comprises a large, diffuse population of neurons which express orexin and melanin-concentrating hormone (MCH), neuropeptides that are known to stimulate food intake (Bittencourt et al., 1992; Sakurai et al., 1998). Intracerebroventricular (i.c.v.) injections of orexin and MCH increase feeding, and orexin mRNA is increased after fasting (Willie et al., 2001). MCH knockout mice have a reduced body weight and lean phenotype that is coupled to hypophagia and increased metabolic rate, while overexpression of MCH results in obese phenotype (Shimada et al., 1998). Orexin knockout mice and transgenic mice lacking orexin neurons exhibit hypophagia (Willie et al., 2001). NPY injection into the perifornical area (PFA) of the LH elicits a robust increase in food intake in rats (Stanley et al., 1993), suggesting that NPY may stimulate orexin and MCH populations. Indeed, NPY/AgRP fiber terminals from the ARH are located in close appositions to the orexin and MCH neurons (Broberger et al., 1998; Horvath et al., 1999).

In addition to the role in feeding stimulation, orexin neurons have been implicated in a variety of homeostatic functions including arousal, reward seeking behavior, sleep/wake cycle and thermogenesis via wide projections within the hypothalamus, brainstem and other areas. The LH also contains leptin receptor expressing neurons which modulate orexin neuronal activity via a direct projection and also send projections

to the ventro tagmental area (VTA) involved in reward seeking behavior (Leinninger et al., 2009). Interestingly, CART is co-expressed in the MCH neurons (Broberger, 1999; Vrang et al., 1999), although functional significance of CART and MCH co-expression in the same neuron is unknown.

The ventromedial hypothalamus: the satiety center

The VMH has long been considered as a major satiety center in the hypothalamus (Elmquist et al., 1999). VMH lesions mainly affect the sympathetic nervous system resulting in increased fat mass, and induction of hyperinsulinemia (Bernardis and Frohman, 1971; Niijima et al., 1984). Therefore, the VMH plays an important role in energy homeostasis by modulating autonomic nervous system activity. Steroidogenic factor 1(SF-1), a nuclear receptor expressed only in the VMH, is required for VMH development and normal regulation of energy balance (Ikeda et al., 1995). SF1 knockout mice exhibit severe obesity due mainly to decreased physical activity that indicates abnormal development of the VMH (Majdic et al., 2002). Moreover, mice lacking leptin receptor in the SF1 neurons have increased adiposity and body weight, suggesting that SF-1 neurons in the VMH are a major target of leptin.

Peripheral signals and brain interaction

Leptin as an adipostat

Ob gene encodes the hormone leptin which is produced and secreted by the white adipose tissue (WAT). The circulating level of leptin reflects the body fat mass. Animals lacking the hormone (*ob/ob* mice) or its receptor (*db/db* mice or obese Zucker rats) develop hyperphagia, extreme obesity, diabetes and other neuroendocrine anomalies (Chua et al., 1996; Farooqi et al., 2007; Zhang et al., 1994). Leptin administration to

ob/ob mice reverses the obesity and other associated metabolic disorders by decreasing food intake and increasing energy expenditure. Rare null mutation in the leptin gene also causes morbid obesity in humans that is rescued by leptin replacement therapy (Farooqi et al., 1999; Montague et al., 1997). However, initial enthusiasm about leptin as a potential monotherapy for obesity has diminished as leptin levels are positively correlated with body weight and adiposity, suggesting a disconnect between leptin and the brain develops with obesity. Furthermore, exogenous leptin treatment does not reverse the obesity and other metabolic disorders in most human obese subjects (Heymsfield et al., 1999). Therefore, leptin resistance is postulated as one of the underlying mechanisms of obesity development. The effect of leptin goes beyond the regulation of food intake and the range of its effects is remarkably wide and complex. Leptin receptors are expressed in many other brain sites that are involved in the regulation of feeding behavior, reproduction, and other neuroendocrine functions (Elias et al., 2000).

Insulin

In addition to its role in glucose homeostasis, the pancreatic hormone insulin also reflects adipose stores. In the periphery, insulin acts as anabolic hormone, promoting energy storage, glucose uptake, and lipogenesis. In contrast, i.c.v. infusion of insulin reduces food intake and body weight (Woods et al., 1979). Moreover, insulin signaling in the brain prevents glucose production by the liver through neuronal signals from the hypothalamus (Marks and Waite, 1997). Similar to leptin receptors, insulin receptors are expressed in the key areas of the hypothalamus controlling food intake (Marks et al., 1990). Neuronal specific insulin receptor knockout (NIRKO) mice develop diet-sensitive obesity with increases in body fat and plasma leptin levels, confirming that insulin

signaling in the CNS plays an important role in regulation of energy homeostasis (Bruning et al., 2000). Insulin shares its inhibitory role in food intake with leptin via a common set of neurons in the ARH (Porte et al., 2005). Evidence also suggests a cross talk between leptin and insulin action on these neurons via cell signaling pathways (Porte et al., 2005). NPY expression is suppressed by insulin in the ARH neurons, while POMC expression is increased (Benoit et al., 2002; Morton and Schwartz, 2001). Central insulin administration reduces NPY mRNA expression in the ARH of food deprived lean rats, but not in obese rats (Schwartz et al., 1991), suggesting that impaired insulin signaling in NPY neurons is one of the contributing factors in the development of obesity (Das et al., 2011).

Ghrelin and other gut peptides

The only known example of a peripheral hormone that has orexigenic property is ghrelin. Ghrelin is secreted from the stomach and plasma ghrelin levels are influeneced by acute and chronic changes in nutritional state (Horvath et al., 2001). For example, circulating ghrelin levels are increased by fasting, but they are low in obese people compared to normal lean subjects (Tschop et al., 2001). Exogenous ghrelin induces adiposity in rodents by stimulating food intake, as well as a reduction in fat utilization (Tschop et al., 2000). Similar to leptin, ghrelin is involved in the central regulation of energy balance. Ghrelin is the only known circulating factor that stimulates NPY/AgRP neurons in the ARH via GH secretagogue receptor 1a (GHGR-1a) (Kamegai et al., 2001). Ghrelin also communicates with other hypothalamic nuclei, such as the DMH and LH, either indirectly through NPY/AgRP neurons or directly via its own receptor (Kobelt et al., 2008; Toshinai et al., 2003).

There is a vast array of hormones secreted from the gastrointestinal tract that have anorectic effects (Zac-Varghese et al., 2010). These include cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide tyrosine tyrosine (PYY). CCK is the gut hormone released rapidly post-prandially to inhibit food intake and delay gastric emptying. CCK administration to humans and animals inhibits food intake by reducing meal size and duration. These inhibitory effects of CCK on food intake are known to be mediated by binding to CCK receptors on the vagus nerve. GLP-1 is also considered a satiety signal since circulating GLP-1 rise following food intake, but becomes low in the fasted state (Zac-Varghese et al., 2010). GLP-1 receptors are located in the brain areas involved in appetite regulation (Kieffer and Habener, 1999). Similarly, PYY is released into the circulation post-prandially and is reduced by fasting. PYY exerts its effects through NPY family receptors, preferentially Y2 receptor found on ARH-NPY neurons (Keire et al., 2000). Since Y2 receptors are autoinhibitory presynaptic receptors, the mechanism of PYY on food intake may involve reduced NPY signaling.

Neuropeptides involved in appetite regulation

NPY

NPY is one of the most abundant and widely distributed neurotransmitters in the brain. NPY and NPY Y receptors (Y1, Y2, Y4, Y5, and in mice Y6) are strongly implicated in appetite regulation in addition to cardiovascular regulation, modulation of neuroendocrine systems, stress, seizure, and cognition (Beck, 2006; Nguyen et al., 2010). The orexigenic effects of NPY are mainly mediated through Y1 and Y5 receptor subtypes which are highly expressed in the PVH (Wolak et al., 2003). NPY injection into the brain induces a robust feeding response (Clark et al., 1984), which is blocked by central

infusion of NPY antibody and NPY antisense oligodeoxynucleotides (Hulsey et al., 1995; Shibasaki et al., 1993). Despite the pivotal role in feeding stimulation, NPY knockout mice have normal food intake, body weight, and adiposity (Erickson et al., 1996), suggesting a compensatory mechanism during development to replenish the orexigenic system in the brain. However, these mice have abnormal refeeding response to fasting compared to wild type control (Bannon et al., 2000). Moreover, NPY knockout mice crossed with *ob/ob* mice show a significant reduction in food intake and increased energy expenditure, suggesting an important role of NPY in appetite regulation and energy expenditure.

While NPY is abundantly expressed throughout the brain, NPY is most highly expressed in the ARH. Increased ARH-NPY expression following acute food deprivation is one of the main mechanisms of refeeding response (Beck, 2006). Chronic food restriction also reduces body weight and circulating leptin levels, resulting in ARH-NPY up-regulation. In *ob/ob* mice lacking endogenous leptin, increased ARH-NPY mRNA levels are normalized by systemic leptin injection (Schwartz et al., 1996). Surprisingly, ARH-NPY mRNA levels are decreased in other genetically obese models and diet-induced obesity (Guan et al., 1998a; Kesterson et al., 1997), suggesting the presence of additional orexigenic drive elsewhere in the brain.

NPY expression is also elevated in the DMH during chronic hyperphagic conditions such as development, lactation and obesity (Guan et al., 1998a; Kesterson et al., 1997; Li et al., 1998a). DMH-NPY expression is not influenced by acute food restriction, but is induced after chronic food restriction (Bi et al., 2003). Less is known about DMH-NPY neuronal regulation and functions. A previous study demonstrated that NPY and leptin receptor expressing neurons are not co-localized in the DMH, suggesting

that DMH-NPY neurons are not directly regulated by leptin (Bi et al., 2003). Nonetheless, NPY induction in the DMH appears to be a major contributing factor for the hyperphagic behavior in these models.

AgRP

Since a single injection can affect food intake more than 24 hours, AgRP is considered one of the most potent orexigenic peptides in the brain (Hagan et al., 2000). CNS administration of AgRP stimulates food intake and decreases energy expenditure (Rossi et al., 1998; Small et al., 2003). AgRP levels are elevated in obese and diabetic animals and transgenic mice overexpressing AgRP are hyperphagic and obese (Kesterson et al., 1997; Stutz et al., 2005). AgRP can increase body weight in the absence of hyperphagia, suggesting that alteration of energy expenditure may be an important mechanism by which AgRP regulates body weight. Similar to NPY knockout mice, AgRP gene knock out mice exhibit normal feeding behavior with no changes in body weight (Qian et al., 2002). NPY/AgRP double knockout mice also do not show any feeding abnormalities expected with the loss of orexigenic drive (Qian et al., 2002). However, ablation of NPY/AgRP neurons in adult mice causes a rapid starvation, while neonatal ablation does not affect the feeding behavior, confirming the existence of compensatory mechanisms during the developmental period (Luquet et al., 2005). Similar to the regulation of NPY gene expression, AgRP expression is regulated by multiple hormonal signals including leptin, insulin, and ghrelin, and it is upregulated in many physiological conditions that favors energy intake. AgRP acts as an inverse agonist for the constitutively active brain melanocortin-4 receptor (MC4R) and serves to antagonize

the inhibitory effect of the POMC gene product, α-melanocyte stimulating hormone (α-MSH) on food intake (Haskell-Luevano and Monck, 2001).

POMC

POMC gene products are post-translationally cleaved to generate a range of bioactive peptides, including adrenocorticotrophin (ACTH), β-endorphin, α-, β-, and γ-melanocyte stimulating hormones (MSH) (Pritchard et al., 2002). Among them, α-MSH release from the POMC neurons exerts a tonic inhibitory effect on food intake via melanocortin receptors, MC3R and MC4R (Ellacott and Cone, 2004). Consistent with the inhibitory role on feeding behavior, POMC and MC4R knockout mice develop obesity characterized by hyperphagia, increased linear growth, and metabolic defects (Huszar et al., 1997; Yaswen et al., 1999). Similarly, humans with defects in the MC4R gene develop severe early onset obesity (Farooqi et al., 2000). This is different from NPY and AgRP genetic knockout models which have a minor phenotype in feeding behavior, suggesting a pivotal role of the melanocortin system in body weight homeostasis. There are only two POMC neuronal populations in the brain: one located in the ARH and the other in the nucleus of the solitary tract (NTS). Although the role of the POMC network in the hindbrain has been appreciated only recently, POMC neurons in the ARH have received the most attention in the field since they are regulated by leptin, insulin and glucose (Belgardt et al., 2009). Melanocortin terminals from the ARH and brainstem are widely distributed in the brain, but are particularly dense in regions of the hypothalamus that are known to control feeding behavior, such as the PVN, DMH and LH (Cowley et al., 1999).

CART

CART is the third most abundantly expressed mRNA in the hypothalamus (Vrang, 2006). In 1998, a study by Kristensen et al. first demonstrated that central CART injection potently inhibited food intake and CART is a new anorexigenic neuropeptide (Kristensen et al., 1998). Additionally, CART is co-expressed in over 90% of the POMC neurons in the ARH. Similar to POMC gene expression, CART expression in the ARH is positively regulated by leptin and decreases during fasting or in ob/ob mice (Kristensen et al., 1998). CART is also expressed in the areas of brainstem and spinal cord that are involved in autonomic regulation (Dun et al., 2000; Koylu et al., 1998). Interestingly, CART is also co-expressed with MCH in the LH, raising the possibility that it may regulate feeding behavior in a number of ways (Broberger, 1999; Menyhert et al., 2007; Vrang et al., 1999). Recent studies have shown that CART injection into discrete hypothalamic nuclei, including the ARH, PVH, and DMH, elicits a significant increase in feeding (Abbott et al., 2001; Hou et al.), suggesting a role of CART as an orexigenic peptide. In fact, the animals receiving central CART injection develop behavioral and motor abnormalities which affect feeding behavior; therefore, inhibition of food intake may not be the direct effect of CART injection. CART has also been strongly associated with BAT thermogenesis as CART injection up-regulates BAT uncoupling protein (UCP)-1 mRNA (Kong et al., 2003). Although CART fiber terminals are widespread in the brain, CART projections to the PVH have been intensely studied. CART projections from the ARH and brainstem play roles in the regulation of the TRH and CRH neurons in the PVH (Fekete et al., 2000; Fekete et al., 2005; Wittmann et al., 2005), suggesting potential pathways of CART action in food intake and BAT thermogenesis. Although

much evidence support that CART peptides act through G-protein signaling pathways, CART receptor has not been identified.

Orexin

Orexin plays key roles in the regulation of wakefulness, feeding, reward, autonomic functions and energy homeostasis. Orexin neurons are exclusively located within and around the LH and DMH (Nambu et al., 1999) and have a wide projection to the brain including the DMH, ARH, PVH and brainstem. Orexin mRNA expression is sensitive to nutritional states and is increased upon fasting or insulin-induced hypoglycemia in the LH area (Moriguchi et al., 1999). The excitability of orexin neurons is known to be modulated by leptin, glucose, and ghrelin (Tsuneki et al., 2010). However, leptin receptor expressing neurons in the LH are distinct from neighboring orexin cells. A recent study reported that leptin regulates orexin neuronal activity via direct projections from leptin receptor expressing cells within the LH (Louis et al., 2010). Orexin neurons receive innervations from NPY, AgRP and α-MSH immunoreactive fibers, suggesting another pathway for leptin to regulate orexin neurons (Elias et al., 1998). The actions of orexins are mediated by two G protein-coupled receptors, orexin-1 receptor (OX_1R) and orexin-2 receptor (OX_2R), which are differentially distributed throughout the brain (Sakurai, 2007).

MCH

MCH is exclusively expressed in the LH and zona incerta whereas its receptor, MCH1R, is widely expressed in central and peripheral tissues, suggesting a wide range of physiological functions. As discussed earlier, MCH injection into the LH increases food intake, while mice lacking MCH are hypophagic and lean (Qu et al., 1996; Shimada et

al., 1998). MCH1R KO mice are hyperphagic, but hyperactive, and lean (Marsh et al., 2002), confirming that MCH signaling is important for the normal regulation of energy balance. Similar to orexin neurons, the activity of MCH neurons is sensitive to the blood glucose level and is inhibited by leptin (Guyon et al., 2009). MCH is up-regulated in *ob/ob* mice and MCH receptor expression is increased by fasting or genetic leptin deficiency (Kokkotou et al., 2001; Tritos et al., 2001). However, MCH neurons do not express leptin receptors and are not directly innervated by local leptin receptor expressing neurons in the LH (Louis et al., 2010), suggesting that ARH-LH projections may relay leptin signals to modulate MCH neurons. Although both orexin and MCH have been considered orexigenic neuorpeptides, they have a differential role in sleep/wake cycle as the discharge profile of MCH neurons (sleep-on/wake-off) is reciprocal to the orexin neurons (Hassani et al., 2009). The lack of co-localization between MCH neurons and the neighboring orexin neurons suggests that they affect feeding behavior and other various functions through distinct neuronal pathways.

THE DORSOMEDIAL HYPOTHLAMUS

Although the DMH has been implicated in the regulation of many behavioral and physiological functions, including ingestive behavior, reproduction, stress, circadian rhythms, and thermogenesis (Bernardis and Bellinger, 1998; Dimicco and Zaretsky, 2007; Smith and Grove, 2002), DMH neuronal phenotypes and functions are largely unexplored. In this section, the current knowledge of DMH anatomy and functions will be extensively discussed to aid our understanding of this nucleus and its potential contribution in obesity.

Neuroanatomy of the DMH

Afferent projections to the DMH

Using retrograde tracer fluorogold (FG) injection into the DMH, Thompson and Swanson demonstrated that the majority of inputs to the DMH arise in the hypothalamus (Thompson and Swanson, 1998). With a few exceptions, each major nucleus of the hypothalamus provides inputs to the DMH. The preoptic area (POA) contains the largest number of FG labeled neurons in the hypothalamus projecting to the DMH. The POA is the location of the temperature sensitive neurons that receive and integrate peripheral inputs derived from sensory receptors (Nakamura and Morrison, 2008). Therefore, the DMH is considered downstream of the POA in the central pathway for thermoregulation (Dimicco and Zaretsky, 2007). Caudal to the POA, the DMH also receives significant inputs from the PVH and the suprachiasmatic nucleus (SCN). Recent studies strongly suggest that the DMH is critical for the expression of several SCN-driven circadian rhythms affecting locomotor activity, feeding, corticosteroid secretion and the sleep-wake cycle (Chou et al., 2003; Fuller et al., 2008; Gooley et al., 2006).

The ARH also sends major inputs to the DMH and relays peripheral signals to the DMH to modulate ingestive behavior and energy expenditure (Bai et al., 1985; Bouret et al., 2004a). The ARH establishes its projections to the DMH during the second postnatal week which is a critical period for the wiring of the brain. *Ob/ob* mice which lack the leptin surge during this critical period become morbidly obese and hyperphagic, and exhibit a permanent defect in ARH-DMH pathways (Bouret et al., 2004b), which may disrupt the normal feeding regulation and lead to the obesity.

In addition to ARH-DMH pathway, the DMH receives information about the metabolic state via brainstem projections. Inputs to the DMH from the brainstem arise primarily from the periaquductal grey (PAG), parabrachial nucleus (PB), and verntrolatenal medulla (VLM) which receive major inputs from the viscerosensory region of the NTS. The lesion studies demonstrated that the DMH plays a role in sensing feeding related signals from the periphery such as CCK (Bellinger, 1987). Peripheral injection of CCK induces c-fos expression in the DMH and microinjection of CCK into the DMH suppresses food intake in rats, suggesting that the DMH may receive this satiety signal via either a direct contact or brainstem-DMH pathway (Kobelt et al., 2006). Sawchenko and Swanson showed that dopamine-β-hydroxylase (DBH) containing catecholaminergic neurons in the NTS and VLM send a major input to the DMH (Sawchenko and Swanson, 1982b). NPY is also extensively co-expressed in these catecholaminergic neurons and NPY levels in the DMH are decreased by the brainstem transections. In lactation, the suckling stimulus is known to activate neurons in the PAG, PB and VLM which are anatomically connected to where DMH-NPY neurons are located (Chen and Smith, 2003). Therefore, these brainstem neurons are the potential candidates for mediating the activation of DMH-NPY neurons during lactation.

Efferent projections of the DMH

Anterograde tracer PHAL injections to the DMH revealed that DMH projections are largely intrahypothalamic, with smaller projections to the brainstem and telencephalon (ter Horst and Luiten, 1986; Thompson et al., 1996). Within the hypothalamus, one of the most densely innervated areas is the PVH. DMH projections are distributed throughout the parvicellular divisions of the PVH, which generates

descending projections to the brainstem and spinal cord for autonomic and sympathetic regulation. Compared to the parvicellular region, DMH projections to the magnocellular region of the PVH are less abundant. There are a few identified cell types in the DMH that send direct projections to the PVH. FG injection into the PVH during lactation revealed that lactation-induced DMH-NPY neurons project to the PVH (Li et al., 1998b), suggesting that metabolic adaptations during negative energy balance utilize DMH-PVH pathway. Using a combined retrograde transport-immunohistochemical method, galanin neurons in the DMH were identified to be the major galanin inputs to the PVH (Levin et al., 1987). More recent studies demonstrated the efferent projections of leptin receptor expressing neurons in the DMH using lepRb-specific cre recombinase to drive the cre-inducible expression of enhanced green fluorescent protein (EGFP) (Gautron et al., 2010; Zhang et al., 2011b). These leptin responsive neurons project to the PVH, ARH, POA, and brainstem nuclei which are important for the control of many physiological parameters such as corticosterone secretion, body temperature, and arousal (Gautron et al., 2010).

DMH fibers also provide a dense input to the LH. Although the fibers appear to exit the nucleus laterally and travel through the LH area, there are moderate terminal fields in the LH. The LH has been historically considered a major feeding center and contains orexigenic and leptin responsive neuronal populations. The DMH projections to the specific cell types in the LH have not been well characterized.

Continuing rostrally, the DMH projections are concentrated in the preoptic regions including parastrial nucleus (PS), preoptic suprachiasmatic nucleus (PSCH), medial preoptic (MPO), and anteroventro paravntricular nucleus (AVPV). The preoptic

region contains hormonal and neural feedback sites that control the reproductive function of the animal. Gonadotropin releasing hormone (GnRH) neurons are located in the preoptic area and neuroendocrine regulation of GnRH neurons has been extensively studied (Smith et al., 2010). GnRH neurons are innervated by numerous neuropeptidergic terminals including kisspeptin, NPY, MCH, and orexin. Evidence suggest that orexin and RFamide-related peptides 3 (RFRP-3) neurons in the DMH project to the preoptic area and regulate the reproductive axis (Backholer et al., 2009; Kriegsfeld et al.; Qi et al., 2009).

In the brainstem, the PAG receives the largest descending inputs from the DMH. Both the DMH and PAG are known to play a critical role in cardiovascular regulation. Evidence suggests that increased sympathetic responses evoked from the PAG require the neuronal activation in the DMH (de Menezes et al., 2009). The DMH sends a significant projection to Barrington's nucleus and the surrounding pontine central gray caudal to the PAG, and a few axons to the parabrachial nucleus (Thompson et al., 1996). Significant projections from the DMH were also reported in the rostral raphe pallidus (rRPa) which plays a key role in regulating BAT thermogenesis.

Overview of DMH functions in energy homeostasis

DMH in ingestive behavior

In 1943, Brugger reported that electrical stimulation of the area corresponding to the DMH causes a voracious drive to eat in cats, first demonstrating the potential role of the DMH in body weight energy homeostasis. A decade later, Larsson also demonstrated that stimulation of the DMH produces hyperphagic behavior in sheep (Larsson, 1954). These findings led to subsequent DMH lesion studies which revealed the role of the

DMH in feeding behavior and body weight regulation. In 1970, Bernadis reported that DMH lesioned rats are hypophagic and hypodipsic, but have normal percentage of fat and lean body mass when compared to control animals (Bernardis, 1970). DMH lesioned rats have a lower body weight set point, but they were still capable of regulating their body weight at the new set point with normal body composition (Bellinger et al., 1979). DMH lesioned rats challenged with high fat diet do not become as obese as sham operated rats (Bernardis and Bellinger, 1986, 1991; Bernardis et al., 1980). This finding suggests that DMH lesions offer some protection against the development of obesity and neurons residing in the DMH may be important for the development of diet induced obesity. This hypothesis is supported by the fact that high fat diet fed C57BL/6 mice show enhanced c-fos expression in the DMH in addition to LHA and PFA (Lin and Huang, 1999). In 1998, Guan et al. demonstrated that mice maintained with a prolonged high fat diet treatment show a profound increase in NPY expression in the DMH (Guan et al., 1998a), identifying a potential player in the DMH responsible for obesity phenotypes. Since then, NPY induction in the DMH has been reported in many obese animal models and also the models of negative energy balance that are associated with the hyperphagic behavior (Guan et al., 1998a; Guan et al., 1998b; Kesterson et al., 1997; Li et al., 1998a).

Our group demonstrated the role of DMH-NPY expression in feeding behavior by injecting melanocortin receptor agonist MTII to the DMH in the lactating model (Chen et al., 2004). Activating melnocortin signaling pathway in the DMH causes a significant reduction in body weight and food intake, and DMH-NPY expression in lactating rats. Since DMH neurons are heavily innervated by AgRP and α-MSH fibers from the ARH, it is reasonable to hypothesize that that reduced melanocortin inputs from the ARH during

lactation may permit the activation of DMH-NPY neurons. A site specific injection of galanin-like peptide (GALP) into the DMH stimulates feeding and i.c.v. GALP injection increases c-Fos expression specifically in the NPY-containing neurons in the DMH, but not in ARH-NPY neurons (Kuramochi et al., 2006), linking the hyperphagic behavior and DMH-NPY neurons. Central injection of resistin, an adipose hormone, in mice increases DMH-NPY expression which appears to mediate the effect of central resistin on hepatic insulin resistance (Singhal et al., 2007), linking DMH-NPY induction with the development of metabolic syndrome. More recently, the reversal of obese phenotypes by knocking down DMH-NPY expression in DIO rats further provides evidence for the importance of DMH-NPY neurons in feeding behavior and body weight regulation (Chao et al., 2011).

DMH in thermoregulation

The early studies showed that DMH lesions are associated with a significant reduction in BAT weight (Bernardis and Luboshitzky, 1983). Later, more direct evidence between the DMH and thermogenesis was presented by a study in which electrical stimulation of the DMH increased BAT temperature (Freeman and Wellman, 1987). Consistent with the proposed role in thermogenesis, administration of endotoxin or cold exposure increase c-fos expression in the DMH (Cano et al., 2003; Elmquist et al., 1996). Anatomical tracing studies also provided supporting evidence that the DMH is an important part of thermoregulatory pathways in the CNS. The neurons in the DMH are labeled with pseudorabies virus (PRV) by trans-synaptic infection originating from the BAT (Bamshad et al., 1999; Oldfield et al., 2002). Functionally, disinhibition of neurons in the DMH by local microinjection of the $GABA_A$ receptor anagonist, bicuculline

methiodide (BMI), markedly elevated BAT and core body temperature in rats (Zaretskaia et al., 2002). The brainstem pathway mediating the DMH evoked sympathetic activity involves the rRPa which is an important location of sympathetic premotor neurons innervating BAT (Bamshad et al., 1999; Cano et al., 2003; Oldfield et al., 2002). Cao et al. showed that BAT thermogenesis elicited by BMI microinjection into the DMH is abolished by microinjection of the $GABA_A$ receptor agonist, muscimol, into the rRPa (Cao et al., 2004), implying that activation of neurons in the rRPa is a critical component for BAT regulation. In addition to the efferent pathways involved in thermoregulation, the neurons in the DMH are known to receive afferent inputs from the POA, another important region involved in the control of body temperature. DMH neurons retrogradely labeled from the rRPa receive GABAergic projections from the POA, indicating that the POA is the key source of tonic inhibitory inputs to the DMH neurons (Nakamura et al., 2005). Leptin is also known to increase body temperature in rodents (Stehling et al., 1996; Stehling et al., 1997). Leptin receptors are abundantly expressed in the DMH (Elias et al., 2000) and systemic administration of leptin results in c-fos expression in the DMH (Elias et al., 2000), suggesting that the DMH may be an important site of leptin's action on thermogenesis. A recent study with the combined transgenic and anatomical tracing approaches confirmed that leptin receptor expressing neurons in the DMH make direct projections to the rRPa (Zhang et al., 2011b). Additionally, leptin administration into the DMH increases markers of sympathetic nerve activity in DIO mice even though these mice are resistant to the anorectic action of leptin (Enriori et al., 2011). A recent study by Chao et al. also demonstrated that DMH-NPY neurons may be involved in the regulation of BAT differentiation and thermogenesis (Chao et al., 2011). Knocking down NPY gene

expression in the DMH increases BAT activity and promotes the development of brown adipocytes in inguinal white adipose tissue. Our group recently discovered that DMH-NPY neurons do not send direct projections to the rRPa and may regulate BAT activity through alternate projections to the PVH and LH (unpublished data).

DMH in circadian rhythms

Excitotoxic lesions of the DMH cause near total loss of the circadian rhythms of sleep–wakefulness, feeding, locomotor activity and corticosteroid secretion in rats (Chou et al., 2003). Subsequent studies revealed that a food-entrainable clock may be located in the DMH (Gooley et al., 2006). When food is only available during the middle of the light period, the timing of greatest c-fos expression in the DMH shifts to the day (Angeles-Castellanos et al., 2004). C-fos expression and clock gene expression in the DMH are largely restricted to cells in the compact zone (Angeles-Castellanos et al., 2004; Mieda et al., 2006). In 2008, Fuller et al. reported a study using mice with targeted disruption of the clock gene *Bmal1* to investigate the cellular basis for entrainment of circadian rhythms by food cue (Fuller et al., 2008). Injection of a viral vector containing the *Bmal1* gene into the suprachiasmatic nuclei of the hypothalamus restores light-entrainable, but not food-entrainable, circadian rhythms. In contrast, restoration of the *Bmal1* gene in the DMH restores the ability of animals to entrain to food but not to light. These results suggest that the DMH contains a *Bmal1*-based oscillator that can drive food entrainment of circadian rhythms. Contrarily, Moriya et al reported that DMH lesions in mice do not affect the food-anticipatory circadian rhythms of behavior, temperature or clock gene expression (Moriya et al., 2009). Therefore, the role of the DMH as a putative

food-entrainable oscillator critical for food-anticipatory behavioral and temperature rhythms is still highly controversial.

DMH in reproduction

The DMH contains neurons projecting to the GnRH neurons that regulate the ovulatory cycle (Hahn and Coen, 2006). The retrograde labeling from the site of GnRH neurons predominates in the non-compact zone of the DMH. A novel negative regulator of the hypothalamic-pituitary-gonadal (HPG) axis known as gonadotropin-inhibitory hormone (GnIH) was recently discovered in quail (Tsutsui et al., 2000), and orthologous neuropeptides known as RFamide-related peptides (RFRPs) have also been identified in rodents and primates (Kriegsfeld et al., 2010). The DMH is the main site of RFRP expressing cells in the brain (Rizwan et al., 2009 ; Ukena and Tsutsui, 2001). RFRP neurons project to hypothalamic regions and cells involved in regulation of energy balance and reproduction (Qi et al., 2009). RFRP neurons have direct inhibitory actions on GnRH neurons, as shown by electrophysiological recordings and anatomical labeling of RFRP-immunoreactive neuronal contacts on GnRH neurons (Johnson et al., 2007; Wu et al., 2009a).

Leptin is essential for normal reproductive function (Brann et al., 2002) and leptin action on the reproductive axis may be at least partially mediated by leptin responsive CART neurons in the DMH. When FG is injected to the areas containing GnRH cell bodies, FG labeled neurons were detected in various hypothalamic nuclei, including the DMH (Rondini et al., 2004). Dual-label immunohistochemistry/in situ hybridization revealed that some of the FG labeled neurons also express CART mRNA in the DMH. CART neurons in the DMH have been shown to express leptin receptors and CART

expression is significantly reduced in the DMH in ob/ob mice that exhibit infertility (Elias et al., 2001; Kristensen et al., 1998). Leptin treatment corrects the infertility as well as CART expression in the DMH, suggesting that CART is one of the downstream mediators of leptin action on GnRH release.

Several studies suggested that orexin in the DMH is also involved in the regulation of GnRH and LH secretion. Orexin expressing cells in the DMH area send a direct projection to the GnRH cells (Campbell et al., 2003a; Iqbal et al., 2001). Central MTII injection increases orexin expression in the DMH, suggesting that melanocortin system participates in the regulation of GnRH neuronal functions at least in part by activating orexin neurons in the DMH (Backholer et al., 2009).

Neurotransmitters of the DMH implicated in energy homesostasis

NPY in the DMH

There are two anatomically distinct NPY populations in the DMH. Neurons in the compact zone of the DMH (DMHc) constitutively express NPY in rats. To date, the rat is the only species that has been identified to have this population of NPY neurons. However, NPY neurons located in the non-compact area of the DMH (DMHnc) express NPY only during specific hyperphagic conditions. During development, NPY mRNA is expressed in both subdivisions of the DMH in rats. NPY expression in the non-compact zone diminishes after the developmental period, but the compact zone NPY expression remains throughout the lifespan of the animal (Grove et al., 2001). Other neuropeptide expression of DMHnc-NPY neurons during normal conditions is unknown.

Mice also transiently express NPY in the non-compact zone during development. However, unlike rats, NPY expression is absent in the DMHc in mice. This discrepancy

between mice and rats is not understood. DMHc-NPY expression has been implicated in feeding behavior and energy expenditure in rats (Chao et al., 2011; Yang et al., 2009). However, the absence of DMHc-NPY expressing neurons in mice suggests that they are not required to maintain normal feeding behavior in mice. NPY expression in the DMHnc is clearly induced in obese mice models. Therefore, these neurons express NPY only during a time period of excess food consumption is desired. DMHnc-NPY induction in chronic hyperphagic models is a focus of this thesis and will be extensively discussed later in this chapter.

CART in the DMH

The DMH also contains CART expressing neurons. Leptin is a likely candidate for regulating DMH-CART neurons since many CART neurons express ObRb mRNA and they express SOC3 mRNA after leptin administration (Elias et al., 2001). Furthermore, *ob/ob* mice exhibit reduced DMH-CART expression which is restored by leptin treatment (Kristensen et al., 1998). However, the role of these leptin responsive DMH-CART neurons is not clear. Since CART expressing neurons in the ARH regulate food intake and BAT thermogenesis, DMH-CART neurons may also serve similar functions under the direct regulation of leptin. For example, DMH-CART up-regulation in DIO mice is mediated by hyperleptinemia (Yu et al., 2008) and may be responsible for increased sympathetic activity and BAT thermogenesis during DIO condition (Enriori et al., 2011). Together with NPY induction, CART up-regulation in the DMH in the DIO model is strongly implicated in the development of obesity phenotypes and will be extensively discussed in the chapter 4 of this thesis.

Orexin in the DMH

Orexin neurons are also distributed in the DMH and they send projections to the medulla neurons that are involved in autonomic functions (Harrison et al., 1999; Nambu et al., 1999). Glucoprivic response after giving 2-Deoxy-D-glucose (2DG) induces dual labeled cells for orexin-A and c-fos in the DMH, suggesting that orexin neurons in the DMH might be mediating the adaptive process to glucopenia (Briski and Sylvester, 2001). Other evidence exists that activation of orexin neurons in the DMH by melanocortin agonist MTII treatment may have the stimulatory effect on the reproductive axis (Backholer et al., 2009).

GABA in the DMH

GABA is co-expressed in NPY/AgRP neurons and GABA transmission plays an important role in the regulation of POMC neurons in the ARH (Tong et al., 2008b). The DMH contains one of the most prominent GABAergic cell groups of the hypothalamus (Ferraguti et al., 1990; Okamura et al., 1990). However, the phenotype of these GABAergic cells in the DMH has not been characterized. As a part of this thesis, we will address the question if NPY neurons in the DMH also express GABAergic neuronal markers, glutamic acid decarboxylase (Gad) 65 and 67 during chronic hyperphagic conditions.

RFRP-3, Opioids and Galanin in the DMH

RFRP-3 neurons in the DMH negatively regulate the reproductive axis and project to the GnRH cells in mice and rats (Kriegsfeld et al., 2006). RFRP3 neurons also have a stimulatory effect on food intake and project to the orexigenic neuronal network in the hypothalamus (Qi et al., 2009). The DMH also contains cell bodies and/or fiber tracts of a number of opioids including enkaphalin, β-endorphin, dynorphin and others. The

DMH has been postulated to be a site of opioid pathways that inhibits a satiety system with a resultant increase in ingestion (Bellinger et al., 1983). Galanin has been implicated in the regulation of food intake, metabolism, and reproduction under the control of leptin. Galanin is expressed in the DMH, but do not appear to express leptin receptor (Cheung et al., 2001).

NPY NEURONS IN THE DMHnc

NPY induction in the DMHnc: a potential mechanism for obesity syndrome

Hyperphagia is the common phenotype in all animal models of DMHnc-NPY induction. These animal models include development, lactation, diet-induced obesity, and genetic obesity. Interestingly, obesity is not a pre-requisite for DMHnc-NPY induction. For example, lactation also leads to a significant weight gain and hyperphagia, but many physiological parameters are clearly distinct from animals with the obesity syndrome. Therefore, DMHnc-NPY induction may be a defense mechanism to prevent negative energy balance rather than simply the cause of obesity. In this section, I will discuss the different animal models expressing NPY in the DMHnc.

Transient NPY expression during development

In addition to NPY expression in the ARH, NPY mRNA is transiently expressed in other hypothalamic regions, including the DMH, PFR, PVH and LHA, during postnatal development in the rat and mouse (unpublished observation) (Grove et al., 2001; Grove and Smith, 2003; Singer et al., 2000). NPY mRNA expression level is relatively low at the postnatal day 2 (P2) and peaks around P16 in all regions. However,

NPY mRNA expression in all the areas except the ARH disappears before puberty. In the DMH, relatively high levels of NPY mRNA expression are detected in both compact and non-compact subdivision in rats. As mentioned earlier, this NPY population in the compact zone is absent in developing mice, while non-compact NPY mRNA expression is clearly induced during the developmental period. The physiological significance of this transient DMHnc-NPY mRNA expression during development is not well understood. Based on the peak expression around P16, DMHnc-NPY mRNA expression may reflect the drive to eat solid food, possibly due to the change in dietary needs from milk to solid food (Grove and Smith, 2003). DMH-NPY mRNA expression is reduced by maternal deprivation (36 h) at P10–11 or P15–16 and this decrease in NPY expression within the DMH is largely limited to the non-compact zone (dorsal and ventral divisions), with little change in the compact zone (Grove et al., 2001). This finding strongly supports that there are two functionally separate populations of NPY neurons and DMHnc-NPY neurons are particularly sensitive to the nutritional availability during development. These NPY neurons may be regulated by leptin surge during the first two weeks of postnatal period (Grove et al., 2003). As a part of this thesis, I will address the question regarding the expression of leptin receptors in DMHnc-NPY neurons during postnatal development.

NPY induction in the DMHnc during Lactation

Lactation is a state of negative energy balance due to the energy drainage from constant milk production and is characterized by various alterations in the dam that allow her to adapt to this demanding condition. These adaptations include the cessation of reproductive cyclicity (Brogan et al., 1999; Fox and Smith, 1984), large increases in food and water intake (Malabu et al., 1994; Pickavance et al., 1996), induction of maternal

behavior (Bridges, 1994), and increases in serum oxytocin and prolactin levels (Buhimschi, 2004). One of the alterations in the brain of lactating rats is a dramatic induction of NPY mRNA expression in the DMHnc (Li et al., 1998a, b, 1999a; Smith, 1993). These NPY neurons in the DMHnc are activated by the suckling stimulus from the pups. NPY expression is activated rapidly after the onset of suckling (within 3h) and is quickly diminished after the removal of the suckling stimulus (Li et al., 1998a). Lactating rats express NPY in both subdivisions of the DMH; however, NPY expression in the compact zone does not appear to be altered by the suckling stimulus. The mechanism of this suckling induced NPY expression in the DMHnc is still not clear. C-fos, a marker for neuronal activation, coupled with neuronal tract tracing techniques, was used to identify the brain regions that are activated by suckling stimulus and project to the DMH neurons in lactating rats (Chen and Smith, 2003; Li et al., 1999a). These areas include the POA, lateral septal nucleus (LS), ventral subiculum(VS), and supramammilary nucleus (SUM), and brainstem regions, including the lateral parabrachial nucleus (LPB), PAG, and VLM (Chen and Smith, 2003). Suckling induced hyperprolactinemia could be responsible for the activation of DMHnc-NPY neurons. Inhibition of suckling induced prolactin secretion greatly attenuates the increase in NPY expression in the DMHnc, but not in the ARH, suggesting a role for prolactin in the DMHnc-NPY neuronal activation (Chen and Smith, 2004). Consistent with these data, DMHnc-NPY neurons express prolactin receptors, while ARH-NPY neurons do not.

Since DMHnc-NPY induction is observed in many animal models of melanocortin signaling defects, reduced melanocortin inputs from the ARH during lactation may be the key to the activation of DMHnc-NPY neurons. The site specific

injection of MTII into the DMH reduces food intake and body weight, and increased UCP1 expression in lactating rats (Chen et al., 2004). Moreover, DMHnc-NPY expression was reduced by about 70% compared to the control, suggesting that activating the melanocortin receptors inhibits DMHnc-NPY neurons. Since MC4Rs are not expressed in the DMH-NPY neurons, this melanocortin action may be mediated by stimulating GABAergic presynaptic terminals near DMHnc-NPY neurons. However, it is possible that MTII effect on NPY gene expression may be secondary to the changes in food intake after the administration of this anorectic drug. The efferent projections of the DMH are well characterized in rats, but the specific projections of DMH-NPY neuronal populations are not known. In this thesis, I intend to characterize the projections of DMHc vs. DMHnc-NPY neurons to further understand the mechanisms of these neuronal actions on feeding behavior.

NPY induction in diet-induced obesity

The health complications developed by high fat diet fed animal models resemble human metabolic syndrome including obesity and insulin resistance. Interestingly, mice fed with the high fat diet for 15 weeks show a significant increase in c-fos immunoreactivity in the DMH, suggesting a neural system in the DMH induced by high fat diet (Lin and Huang, 1999). Guan et al. reported that NPY expression is induced in the DMHnc after 24 weeks of high fat diet treatment in mice (Guan et al., 1998a), while NPY expression in the ARH is noticeably decreased. DMHnc-NPY induction observed in DIO mice is reversed after switching to the normal chow diet for several weeks. Since NPY expression is supressed in the ARH with prolonged high fat diet treatment, DMHnc-NPY expression may be the major drive for hyperphagia during DIO condition. However, the

exact timing of DMH-NPY induction has not been characterized in DIO mice and will be addressed as a study aim in this thesis.

NPY induction in genetic models of obesity

Induction of NPY in the DMHnc has been reported in numerous genetic models of obesity. Kesterson et al reported NPY induction in the DMHnc in two mice models of melanocortinergic obese syndroms, lethal yellow (A^y) and MC4-R knockout (MC4-RKO) (Kesterson et al., 1997). However, the absence of NPY gene expression in the DMH in young A^Y and MC4-RKO animals indicated that abrogation of melanocortin signaling alone is not sufficient for the induction of this change. The increase in NPY gene expression in the DMHnc reflects the obese state rather than the cause of obesity. Nonetheless, this study strongly suggests that activation of NPY expression in DMH neurons is correlated with a loss of MC4-R activity.

The tubby mouse is one of the established genetic models for obesity and is characterized with maturity onset obesity and increased food intake. Induction of NPY mRNA was also observed in the DMHnc of the tubby mice in an age and body mass dependent manner (Guan et al., 1998b). NPY induction in the DMHnc again coincided with the decrease in POMC level in the ARH in tubby mice, supporting the view that the changes in melanocortin tone in the brain may contribute to the induction of NPY in the DMHnc.

Brown adipose tissue deficient (UCP-DTA) mice develop obesity as a result of both decreased energy expenditure and hyperphagia. Similar to the other obese models, NPY induction in the DMHnc is also seen in the UCP-DTA mice and is accompanied by similar changes in ARH gene expression (Tritos et al., 1998).

Mecp2 gene mutation is the cause of the Rett Syndrome in human and leads to various other social, behavioral, and neuropsychiatric conditions. Mice with Mecp2 conditional knock out (CKO) in Sim1 expressing neurons exhibit normal activity and basal metabolic rate, but are hyperphagic and overweight (Fyffe et al., 2008). Interestingly, Mecp2 CKO mice also express high levels of NPY in the DMHnc which is linked to abnormal MC4R signaling in this model.

Interestingly, *ob/ob* mice do not display any detectable NPY expression in the DMH despite their morbid obesity and hyperphagia (Kesterson et al., 1997). In contrast to other obese models, ob/ob mice exhibit increased NPY expression in the ARH instead of the "ectopic" NPY expression in the DMHnc, implying that NPY overexpression in the ARH alone may be sufficient to increase food intake and sustain the obesity in ob/ob mice.

Obese Zucker rats have mutations in the leptin receptor gene and are widely used animal model of genetic obesity. They develop severe obesity associated with hyperphagia, defective non-shivering thermogenesis and preferential fat storage in adipose tissue (Chua et al., 1996). Unlike the mouse model of endogenous leptin deficiency, Zucker rats showed a significant increase in NPY concentrations in several regions in the hypothalamus including the DMH (McKibbin et al., 1991).

Bi et al. demonstrated that NPY gene expression is greatly increased in the DMH in OLETF (Otsuka Long-Evans Tokushima Fatty) rats lacking cholecystokinin (CCK)-1 receptor (Bi et al., 2001). Young preobese OLETF rats have increased DMH-NPY expression prior to developing obesity; therefore, elevated levels of NPY expression in the young OLETF rats may contribute to hyperphagia and obesity later in life. A major

difference between OLETF rats and other genetic obese models in mice is that a different population of NPY neurons is activated within the DMH. While NPY induction is observed in the DMHnc in obese mice models, NPY up-regulation is only limited to the DMHc in OLETF rats. Using viral mediated gene knock down approach, Yang et al. (Yang et al., 2009) demonstrated that NPY knock down in the DMHc ameliorates the hyperphagia and obesity related metabolic symptoms in OLETF rats, confirming that DMHc-NPY expression is indeed the cause of the obesity syndrome in OLETF rats. Furthermore, a recent study by the same group using a similar strategy demonstrated that DMH-NPY knock down provides protection against the development of obesity in DIO rats (Chao et al., 2011). The caveat to this study is that DMHc vs. DMHnc-NPY expression is not well characterized in DIO rats; therefore, it is difficult to conclude that antiobesity effects following the knock down are solely due to the loss of DMHc-NPY expression. However, a novel finding of this study is that NPY knock down causes the development of brown adipocytes in inguinal white adipose tissue through the activation of sympathetic nervous system. This finding strongly suggests a mechanism by which DMH-NPY expression inhibits sympathetic activation and contributes to the development of obesity.

Chronic caloric restriction and intense exercise model

While ARH-NPY expression is up-regulated by fasting, DMH-NPY expression is not altered by acute food deprivation (Bi et al., 2003). However, NPY up-regulation is observed in the DMHc after chronic food restriction. Similar to food restriction, long term intense exercise also increases NPY concentration in the DMH in rat (Lewis et al., 1993). These findings indicate that short term calorie deficit can be corrected by NPY up-

regulation in the ARH while a long term negative energy balance requires additional orexigenic drive. The contribution of NPY induction specifically in the DMHnc in these two models has not been discussed in the literature.

CONCLUSION

The ARH has received the most attention in the field and numerous discoveries from this 'arcuate-centric research' have further enhanced our understanding of the hypothalamic circuit in feeding regulation. Evidence from last 70 years of research on the DMH suggests that the DMH is one of the most influential hypothalamic nuclei involved in various aspects of energy homeostasis including ingestive behavior and thermogenesis. Despite the functional implications in feeding regulation, the exact role and contribution of the DMH neurons in obesity still remain elusive. DMHnc-NPY system is induced under both chronic hyperphagic (energy excess) and chronic negative energy balance (energy deficit), suggesting different inputs driving DMH-NPY expression. Evidence from animal models suggests that DMHnc-NPY expression plays a pivotal role in sustaining elevated body weight in obesity. Therefore, this NPY induction may be one of the neural adaptations responsible for hyperphagia typically observed in obese human. Many questions including the regulation of DMHnc-NPY induction, downstream effectors of this neuronal activation, and the alternate phenotypes of the neurons still remain to be answered. The main purpose of this thesis is to characterize the phenotypes of DMHnc-NPY neurons and the anatomy of the neuronal projections during hyperphagic/ obese conditions to better understand the role of DMHnc-NPY induction in obesity.

AIMS OF DISSERTATION AND APPROACH

Specific aim 1: Characterize the alternate phenotypes of DMH-NPY neurons during postnatal development (chapter 2)

***Hypothesis*:** DMH-NPY neurons are regulated differently than ARH-NPY neurons.

Approach: Microarray gene expression comparison of DMH and ARH-NPY neurons isolated from P15 NPY-hrGFP mice.

The specific aim 2: Characterize the efferent projections of DMHnc-NPY neurons activated during lactation and DIO (chapter 3)

***Hypothesis*:** DMH-NPY neurons project to the areas that are involved in the regulation of food intake and energy expenditure.

Approach: Anterograde tracer, biotinylated dextran amine (BDA), was streotaxically injected into the DMH in lactating rats and DIO mice. Immunohistochemical detection of BDA and NPY was used to trace DMH-NPY neuronal projections in the target areas.

The specific aim 3: Characterize the timing and mechanism of DMH-NPY and CART induction in DIO mice (chapter 4)

***Hypothesis*:** DMH-NPY and CART induction are not the cause, but are instead the consequence of obesity

***Approach*:** *In situ* hybridization and fluorescent immunohistochemistry was used to determine the timing of induction of DMH-NPY expression during the development of the DIO conditions in mice**.**

Chapter 2

Differential gene expression between neuropeptide Y expressing neurons of the dorsomedial nucleus of the hypothalamus and the arcuate nucleus: Microarray analysis study

Draper S., Kirigiti M., Glavas M., Grayson B., Chong C. N., Jiang B., Smith M. S., Zeltser L. M., Grove K. L. (2010) Brain Res. 1350, 139–150

ABSTRACT

The Dorsomedial Nucleus of the Hypothalamus (DMH) is known to play important roles in ingestive behavior and body weight homeostasis. The DMH contains neurons expressing Neuropeptide Y (NPY) during specific physiological conditions of hyperphagia and obesity, however, the role of DMH-NPY neurons has yet to be characterized. In contrast to the DMH-NPY neurons, NPY expressing neurons have been best characterized in the Arcuate Nucleus of the Hypothalamus (ARH). The purpose of this study is to characterize the chemical phenotype of DMH-NPY neurons by comparing the gene expression profiles of NPY neurons in the DMH and ARH isolated from postnatal NPY-hrGFP mice by microarray analysis. Twenty genes were differentially expressed in the DMH-NPY neurons compared to the ARH. Among them, there were several transcriptional factors that play important roles in the regulation of energy balance. DMH-NPY neurons expressed Glutamic Acid Decarboxylase (GAD) 65 and 67, suggesting that they may be GABAergic, similar to ARH-NPY neurons. While ARH-NPY neurons expressed leptin receptor (ObRb) and displayed the activation of STAT3 in response to leptin administration, DMH-NPY neurons showed neither. These findings strongly suggest that DMH-NPY neurons could play a distinct role in the control of energy homeostasis and are differentially regulated from ARH-NPY neurons through afferent inputs and transcriptional regulators.

INTRODUCTION

The dorsomedial nucleus of the hypothalamus (DMH) is one of several hypothalamic nuclei involved in the control of ingestive behavior and body weight

homeostasis (Bellinger and Bernardis, 2002; Bernardis and Bellinger, 1998). A seminal study by Larsson demonstrated that an electrical stimulation of the DMH causes a voracious drive to eat in sheep, suggesting that the primary output of the DMH is an orexigenic drive (Larsson, 1954). In 1970, Bernardis et al. (Bernardis, 1970) supported this conclusion with the demonstration that DMH lesioned rats were hypophagic, hypodipsic, and growth retarded. They have subsequently shown that DMH lesioned rats provide some protection against the development of diet induced obesity (DIO) when fed with a high fat diet (Bernardis and Bellinger, 1986). The importance of the DMH in the body weight homeostasis is further supported by the finding that the DMH receives major projections from the arcuate nucleus of hypothalamus (ARH), a key hypothalamic site for sensing peripheral signals, and has a major efferent projection to the paraventricular nucleus of hypothalamus (PVH) (Bouret et al., 2004a; Cone et al., 2001; Gao and Horvath, 2008; Grove and Smith, 2003; Schwartz et al., 2000; Thompson et al., 1996; Thompson and Swanson, 1998). The DMH has been also shown to play an important role in thermoregulation (Dimicco and Zaretsky, 2007). The electrical and chemical stimulation of the DMH increases brown adipose tissue (BAT) temperature (Freeman and Wellman, 1987; Zaretskaia et al., 2002). The DMH projects to the brainstem, the site of sympathetic neurons that innervate BAT (Cao et al., 2004). Thus, the DMH provides inputs to both neuroendocrine and autonomic circuits involved in the maintenance of energy homeostasis.

While the most characterized population of Neuropeptide Y (NPY) neurons in the hypothalamus is the ARH, the DMH also contains NPY expressing neurons during specific physiological states. The role of the DMH-NPY neurons is unknown, but the

level of NPY expression in the DMH appears to reflect the changes in energy status (Li et al., 1998a, b). NPY expression is high in the DMH during the postnatal period when the animals require high energy intake for the rapid growth (Grove et al., 2003; Grove et al., 2001; Grove and Smith, 2003). However, NPY expression in the DMH gradually declines after the second postnatal week and levels remain low in the normal adult rodents. Interestingly, several studies have shown that a high level of NPY expression is induced in specific regions of the DMH during lactation and in obesities (Guan et al., 1998a; Guan et al., 1998b; Kesterson et al., 1997; Li et al., 1998a, b), suggesting that DMH-NPY induction may contribute to the hyperphagic behavior. Our group has shown that a decrease in NPY expression in the DMH is correlated with a decrease in food intake and increase in BAT thermogenesis in lactating rats (Chen et al., 2004). Furthermore, Yang el al. recently reported that AAV mediated knockdown of NPY expression in the DMH ameliorated the hyperphagia and obesity of OLEFT rats (Yang et al., 2009). It is important to note that there are subregions of the DMH where NPY expression is differentially regulated, and that there are species differences in the expression of NPY within the DMH. While it is suggested that NPY expression in both subdivisions may play a role in modulating food intake and energy balance, the exact physiological role of DMH-NPY neurons is still unknown.

Unlike the DMH, the anatomy and function of the NPY system in the ARH in relation to energy homeostasis are well characterized (Cone et al., 2001; Schwartz et al., 2000). The ARH contains two major neuronal populations that play opposite roles in the control of food intake. Neurons co-expressing NPY and agouti-related peptide (*AgRP*) are known to stimulate feeding, whereas neurons expressing proopiomelanocortin (*Pomc*)

inhibit feeding. NPY and POMC neurons express the long form of the leptin receptor (*ObRb*) and are regulated by leptin (Cowley et al., 2001; Elias et al., 1999; Elmquist et al., 1999). ARH-NPY neurons also produce the inhibitory neurotransmitter Gamma-Aminobutyric acid(GABA), which plays a significant role in the function of these neurons (Tong et al., 2008a).

At the present time, there is no information available about the phenotype of DMH-NPY neurons in regards to the regulation of energy homeostasis. Therefore, this study is designed to compare the gene expression profile of purified DMH-NPY neurons to ARH-NPY neurons from postnatal NPY-hrGFP mice by using cDNA microarray analysis. We first looked for the genes that are differentially expressed in the DMH-NPY to identify additional DMH-NPY neuronal markers. Second, we compared the expression of GABAergic markers and *ObRb* between ARH and DMH-NPY neurons.

MATERIALS AND METHODS

Animals

NPY hrGFP mice on a C57BL6 background were purchased from Jackson Laboratory (Bar Harbor, Maine). These mice express humanized renilla reniformis Green Fluorescent Protein (hrGFP) under the control of the mouse NPY promoter. Animals were housed under 12hr of light/12hr of dark cycle per day. They were fed standard chow diet (Purina lab chow #5001) and had free access to water. All animal procedures were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

Microdissection and cell dissociation of ARH and DMH for FACS (Fluorescent

activated cell sorting)

Hemizygous NPY-hrGFP male mice were bred to C57BL/6 female mice (purchased from Jackson laboratory). The litters were normally born and the litter size was adjusted to 10. The pups were toe clipped for identification and genotyping. Postnatal day 16(P16) was chosen since the DMH express high levels of NPY-hrGFP positive cells. At P16, 18 GFP positive mice pups were killed by decapitation and the brains were quickly removed for microdissection in a petri dish containing ice cold Krebs buffer pH 7.4(126mM NaCl, 2.5mM KCl, 1.2mM $MgCl_2$-$6H_20$, 1.2mM NaH_2PO_4- H_20, 2.4mM $CaCl_2$-2 H_20, 21.4mM $NaHCO_3$, 11.1mM glucose). Coronal hypothalamic sections (300μm) were cut in a vibrating microtome (Leica VS 1000, Leica Microsystems, Inc., Bannockburn, IL). The sections containing ARH and DMH were identified and dissected under an inverted microscope.

For the microarray, three independent ARH and DMH pools were obtained, with each pool containing the tissue from 6 animals. The tissues were dissociated using the Papain Dissociation System (Worthington Biochemical Corporation, Lakewood, NJ). Briefly, the samples were placed into Papain/DNase for 1hour at 37°C and dissociated by a gentle trituration using a pipette. The NP-GFP+ cells were collected by FACS. The sorting was done on BD Aria II cell sorter (Becton Dickinson, NJ). A 488 nm laser was used to excite the GFP and the emission was measured in the FITC channel (530 nm). Non-fluorescent cells obtained from wild-type mice were used to gate on forward scatter (FSC) versus side scatter (SSC) and set the threshold for fluorescence. We collected approximately 8000 GFP positive cells from the ARH and 10000 cells from the DMH by FACS, for each pooled sample.

RNA isolation

After FACS sorting, the cells were collected in RNA extraction buffer (Arcturus Bioscience, Mountain View, CA) and stored at -80°C until RNA extraction. RNA was extracted using the PicoPure RNA isolation kit (Arcturus Bioscience) according to the manufacturer's protocol. An average of 11ng of total RNA was isolated from each pooled sample. The RNA quality and relative concentration were confirmed on the Picochip using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA)

Microarray procedures

Microarray assays were performed in the Affymetrix Microarray Core (Affymetrix, Inc., Santa Clara, Ca), a unit of the OHSU Gene Microarray Shared Resource. Briefly, total RNA was reverse-transcribed with an oligo-dT primer and double stranded cDNA is generated. The cDNA served as a template for the *in vitro* transcription (IVT) reaction that produced amplified amounts of biotin-labeled antisense mRNA. This biotinylated RNA is referred to as labeled cRNA. The samples were prepared using NuGen Ovation Biotin RNA Amplication and Labeling System_V2 (NuGEN Technologies, Inc. San Carlos, CA). Each sample target was hybridized to a Mouse Genome 430 2.0 GeneChip array. Imaging processing and expression analysis were performed using Affymetrix GCOS v. 1.4.036 software.

Microarray Analysis

A GCOS absolute expression analysis was performed for each GeneChip genome array hybridization. Following the initial analysis, the absolute analyses were rerun using global scaling to an average target intensity of *350*. The scaling allows for the direct

comparison of hybridization values from the different targets analyzed in this project (and with any additional GeneChip sample assays that are run using the same array type). For each analysis, scaled or unscaled, the AMC Project Report Ver. 12 (06/27/07) – GCOS parameters α1 and α2 were set to 0.05 and 0.065, respectively. These parameters set the point at which a probe set is called present (P), marginal (M), or undetectable (A). This call is based on the Detection p-value of the probe set as determined by the software.

Analysis of gene expression

For the microarray data analysis, normalized expression values by their Affymetrix identifier were imported into the online software server Genesifter (Seattle, WA). For the microarray comparisons multiple t-tests were used to identify genes with at least a 2-fold difference in gene expression (with Benjamini and Hockberg correction; $p < 0.05$) and at least an expression level of 200 and samples from at least one of the groups had to have a 100% present call (3 out of 3) according to Affymetric MAS 5.0.

RT-PCR

A new set of isolated DMH and ARH-NPY samples were collected for confirming gene expression. The samples were collected and RNA obtained as described above and complementary DNA was synthesized from 80-150ng total RNA using M-MLV Reverse Transcriptase (Fisher Scientific, Pittsburgh, PA). The following primer pairs were designed to amplify *Npy*: Forward 5′-GCTAGGTAACAAGCGAATGGGG-3′; Reverse 5′-CACATGGAAGGGTCTTCAAGC-3′, *AgRP*: Forward 5′-GGCCTCAAGAAGACAACTGC-3′; Reverse 5′-TGCGACTACAGAGGTTCGTG-3′, *Pomc*: Forward 5′-GAAGATGCCGAGATTCTGCT-3′; Reverse 5′-GTACTTCCGGGGGTTTTCAG, *Cyclophlin B*: Forward 5′-

CAAGACTGAGTGGCTGGATGG; Reverse 5′-ACTTGAAGGGGAATGAGGAAAATA-3′, *Bahl2*: Forward 5′-ACCCATCCACCCACACATAC; Reverse 5′-ATCACCCTCCTCTGCTCTGA, *Foxa1*: Forward 5′-AAACCGGTTATGCACATTGG; Reverse 5′-GCAAGAACTAAAATGGCCACA, *Pgc -1α*: Forward 5′-GGAGCCGTGACCACTGACA; Reverse 5′-TGGTTTGCTGCATGGTTCTG. PCR was performed using the platinum PCR supermix (InVitrogen, Carlsbad, CA) according to the manufacturer's instruction.

In situ hybridization

PCR primers to amplify riboprobes for *Pgc-1α* and *Foxa1* were designed using NIH BLAST. PCR products were ligated in pGEMT vector and amplified using JM109 competent cells. Plasmids were harvested and a sample sequenced to verify identity. Plasmids were linearized using appropriate enzymes. cDNA in which 50% of the UTP is radioactive was ^{33}P labeled. After sectioning in a cryostat, brain sections were fixed in 4% paraformaldehyde and then subjected to a series of washes resulting in dehydration, delipidation, and rehydration. The sections were exposed to the labeled probes overnight in a moist chamber at 55°C. After hybridization, the slides were washed in 4× SSC, then incubated in RNase A at 37°C, and in 0.1 × SSC at 60°C. Slides were then dehydrated through a graded ethanol series and dried. For visualization of the probe, labeled sections were exposed to film (Biomax MR, Kodak) overnight.

Fluorescent in situ hybridization (FISH)

P15 NPY-hrGFP mice were anesthetized and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in phosphate buffer. Brains were post-fixed at

4°C overnight and cryoprotected with 30% sucrose for 48 hours. Tissue was embedded in O.C.T (Tissue Tek) and frozen at -80°C. 10μm-thick coronal sections were collected on Superfrost Plus slides (Fisher). GFP fluorescence is lost during the high temperature in situ hybridization step; thus, we pre-imaged direct GFP fluorescence in conjunction with DRAQ5 nuclear stain (Cell Signaling Technology, Beverly MA). Following FISH, the tissue was re-stained with DRAQ5 and imaged. Using Photoshop, pre- and post-FISH images were aligned using the common DRAQ5 stain as a reference to generate a composite image (Padilla, et al submitted). Frozen sections were processed as described in the TSA Plus Cy3 System manual (Perkin Elmer), using *Foxa1* riboprobes labeled with digoxigenin (1:100 dilution) (Roche, Indianapolis, IN). Following the hybridization step, slides were washed and incubated overnight at 4°C with HRP-conjugated anti-digoxigenin (1:500) as described in the Perkin Elmer manual. The subsequent steps were modified as described in (Breininger and Baskin, 2000). Briefly, the slides were washed with TNT buffer for 3 times for 5 minutes each at a room temperature. Biotinyl tyramide diluted 1:50 in the amplification diluent was applied to the slides (renaissance TSA Biotin System; Perkin Elmer NEL 700A). After incubation for 10 min at RT, the slides were washed 3 times in TNT buffer for 5 min each at RT. The biotin deposited after TSA amplification was further amplified by ELF using the ELF 97 mRNA In Situ Hybridization Kit #2 (E-6604 Molecular Probes). Tissue sections were incubated in blocking reagent provided with the ELF kit for 30 minutes in a humidifying chamber. Alkaline phosphatase conjugate (1:50 in block reagent) and DRAQ5 (1:1000) was then applied to the sample for 1 hr. Slides were then washed 3 times, for 5minutes each time, with 1× wash buffer. Substrate working solution was then added to the samples for 10

min. The ELF precipitate deposited as a result of the enzyme activity emits a fluorescent product at 530nm. The post-FISH images of *Foxa1* expression were then aligned with the pre-images of NPY-GFP using DRAQ5 stain as a reference to create the composite image of *foxa1*, NPY-GFP, and DRAQ5 stain.

Immunohistochemistry

P 15 NPY-hrGFP mice were overdosed with pentobarbital (125 mg/kg body weight, i.p) and perfused with 0.9% ice cold saline solution and then 10% neutral buffered formalin (Fisher Scientific) for FOXA1 antibody staining or 4% paraformaldehyde containing borate buffer (pH 9.5) for PGC-1α antibody. The brains were post-fixed in the same fixatives overnight and transferred to 25% sucrose buffer solution. The brains were then frozen and sectioned at 25 μm on a microtome. The tissue sections were washed in 0.05M potassium phosphate-buffered saline (KPBS) several times and pre-incubated in blocking buffer (KPBS+0.4% triton X + 2% normal donkey serum) for 30 min before incubating in rabbit anti-FOXA 1, 1:400 (sc-22841, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-PGC-1α, 1:24000 (sc-13067, Santa Cruz Biotechnology) containing blocking buffer for 48 hrs at 4°C (Puigserver et al., 1999; Wolf et al., 2007). Following washes in KPBS, tissue sections were incubated for 1 hr in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), then subsequently washed and incubated in avidin-biotin (A/B) solution (Vectastain elite ABC, Vector laboratories, Burlingame, CA) for 30 min. The signals were further amplified using tyramide signal amplification-indirect kit (NEN Life Sciences products, Boston, MA). The signals were visualized using Alexa 568 conjugated to streptavidin (Molecular Probes, Eugene, OR)

For p-STAT 3 immunohistochemistry (Enriori et al., 2007), NPY-hrGFP pups were sham injected for 3 days with saline, then on P16 injected with either saline or 3mg/kg leptin (PeproTech, Rocky Hill, NJ) in the morning. They were kept away from the dams on a heating pad for 45 min, then perfused with 4% paraformaldehyde in borate buffer and processed as above. After sectioning, the tissue sections were incubated in 1:250 rabbit anti-p-STAT 3 (Cell Signaling, Danvers, MA) for 60 min at room temperature and then overnight at 4°C. After adding Biotinylated donkey anti- rabbit antibody and A/B solution, tissue sections were incubated with nickle DAB solution (1.25g nickle sulfate, 0.04g DAB, 50 ml of 150 mM Sodium Acetate, 41.5 ul of 3% hydrogen peroxide) for 5-30 min. Following the DAB reaction, the sections were incubated in rabbit anti hrGFP, 1:5000 (Stratagene, La Jolla, CA) overnight and Donkey anti rabbit Alexa 499, 1:200 for visualization.

Analysis of data

For the analysis of NPY and PGC-1α colocalization, 3 sections per animal from 3 different animals were selected for imaging using a Leica SP5 AOBS confocal microscope. 40 X images of NPY-GFP (488 nm Ar laser) and PGC-1α-immunoreactivity (IR) (561 nm DPSS laser) were taken at 1μm intervals through the ARH and DMH of each section. The image stacks were analyzed using Metamorph software by compiling a maximum projection of 10μm thickness, and manually counting how many total NPY-GFP neurons contained PGC-1α-IR. T-test ($p<0.05$) was used to determine significant difference between groups.

For the leptin-induced pSTAT3-IR in NPY neurons, 3 sections per animal from 3 different animals were selected for the analysis. Sections were visualized with a 20×

objective using a Nikon Eclipse E800 microscope. The area of interest was simultaneously illuminated with fluorescence to visualize the hrGFP and brightfield light to visualize the p-STAT3-IR. By quickly flipping back and forth between the fluorescence and the brightfield illumination, the total number of NPY-GFP neurons as well as the number of NPY neurons containing pSTAT3-IR was counted for each section. T-test ($p<0.05$) was used to determine significant difference between groups.

RESULTS

The location of the cuts used to microdissect the tissue containing ARH and DMH-NPY neurons in the mouse hypothalamus is shown in Figure 1.A. Since the DMH and ARH are in close proximity, the purity of the DMH versus ARH isolations was assessed by performing RT-PCR for the ARH-NPY-specific marker *AgRP* expression (Figure 1.B). As expected, GFP positive cells from ARH samples expressed *AgRP* mRNA, while GFP positive cells sorted from the DMH did not. Furthermore, the absence of *Pomc* transcripts in both ARH and DMH-NPY samples confirmed that the FACS samples contain a majority of GFP positive cells. However, Melanin-concentrating hormone (*Mch*) mRNAs was occasionally detected in the DMH-NPY samples (not shown). MCH is highly expressed in the lateral hypothalamus and the DMH (Bittencourt et al., 1992), indicating there is likely a low level of contamination by non-GFP expressing cells. Double-label immunohistochemistry was used to demonstrate that MCH immunoreactivity was never present in GFP positive neurons or fibers (not shown).

DMH-NPY neuron enriched genes

Using the high stringent analysis (Benjamini and Hockberg corrected p-value over 0.05), 20 DMH-NPY enriched genes and 82 ARH-NPY enriched genes were identified. The scatter plot shows the distribution of these differentially expressed genes and common genes between DMH and ARH-NPY neurons (Fig. 2-1C). The list of the 20 genes enriched in the DMH is shown in Table 2-1. Of particular interest were genes involved in transcriptional regulations. BarH-like *2*, a mammalian homologue of Drosophila BarH protein, shows the highest upregulation (6.4 fold) in the DMH-NPY. During embryonic development, BarH/Barhl is expressed primarily in the central nervous system where it plays an essential role in decisions of cell fate, migration and survival (Reig et al., 2007). Forkhead box A1 (*Foxa1*, also known as hepatocyte nuclear factor 3 alpha) is 5 fold higher in DMH-NPY than ARH-NPY. *Foxa1* is a member of the Forkhead/winged helix transcription factors and required for the regulation of genes essential for glucose homeostasis in many tissues including pancreas and liver (Kaestner, 2000). Paired-like homeodomain transcription factor 2 (*Pitx2*) is expressed 4.64 fold higher in the DMH-NPY neurons. *Pitx2* is expressed during mouse development in many tissues, including brain and plays a role in modulating the basal and hormonally regulated activity of prolactin (Quentien et al., 2006). Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*Pgc-1α*) is upregulated by 2.14 fold. *Pgc1-α* is a transcription co-factor involved in adaptive thermogenesis and glucose metabolism (Puigserver and Spiegelman, 2003).

Using a more moderate stringent analysis (raw p-value above 0.01, without the Benjamini and Hockberg correction), an additional 21 DMH-NPY neuron enriched genes were identified (Supplemental Table 2-1). Of particular interest, signal transducing

adaptor family member 2 (*Stap2*) is the cytosolic protein modulating signal transduction pathways. *Stap2* is upregulated by 3.29 and is also known to modulate STAT3 activity (Ikeda et al., 2009). Eph receptor A3, an important player in axon guidance during development, is also upregulated. CCAAT/enhancer binding protein (C/EBP), alpha (*Cebpa*) is a transcription factor and is known to bind to the promoter and modulate the expression of leptin (Hwang et al., 1996). The complete list of the genes is found in the supplemental material (Supplemental Table 2-1).

ARH-NPY neuron enriched genes

There was a relatively large list of genes that were enriched in the ARH-NPY neurons compared to DMH-NPY neurons (Supplemental Table 2-2). Most notable was the expression of Activin A receptor, type IC (*Acvr1c*) which was 32.4 higher in ARH-NPY neurons. *Acvr 1c* is a Type I Receptor Serine/Threonine Kinase for the TGFβ superfamily and was previously shown to be expressed in the ARH in rat brains (Tsuchida et al., 1996). *Acvr 1c* is known to be involved in apoptosis and respond to dietary excess, glucose and insulin stimulus (Andersson et al., 2008; Bertolino et al., 2008; Kim et al., 2004). *AgRP* gene expression, which is exclusively expressed in ARH-NPY neurons (Morton and Schwartz, 2001), was 9.38 fold higher in ARH versus DMH samples.

RT-PCR confirmation of microarray results

RT-PCR was used to verify the differential expression of *Foxa1* and *Pgc-1α* (Fig. 2-2A). The DMH-NPY samples clearly showed the expression of *Foxa1*. However, ARH-NPY samples did not show the expression indicating that the level of the gene in

the samples must be below the detection level. *Pgc-1α* was expressed in both samples, but was qualitatively higher in the DMH versus ARH-NPY neurons.

Localization of PGC-1α and Foxa1 mRNAs and proteins in DMH-NPY neurons

Pgc-1α mRNA expression was localized in the DMH by in situ hybridization using ^{33}P labeled riboprobes in P16 NPY-hrGFP mice (Fig. 2-2B). *Foxa 1* mRNA expression was detected in the ARH and DMH by a fluorescent in situ hybridization (Fig. 2-2C). In the ARH, *Foxa 1* mRNA was highly expressed in the dorsolateral area where NPY expressing neurons are scarce. *Foxa 1* mRNA expression was broadly expressed in the DMH, but there was little colocalization between *Foxa1* and NPY expressing neurons. PGC-1α and FOXA1 proteins were also localized in the DMH-NPY neurons by immunohistochemistry in P16 NPY-GFP mice. While high levels of PGC-1α immunoreactivity were detected in both the ARH and DMH (Fig. 2-3A), there was a significantly greater colocalization of PGC-1α in NPY-GFP cells in the DMH (Fig. 2-3B). Confocal analysis showed that 65% of total DMH-NPY neurons contained PGC-1α immunoreactivity compared to 35% in the ARH (Fig. 2-3C), Similarly, FOXA1 immunoreactivity was detected in both the ARH and DMH (Fig. 2-4A). However, only small population of GFP positive neurons in the DMH were co-localized with FOXA1 (Fig. 2-4B), with little colocalization in the ARH. FOXA1 immunoreactivity was highly concentrated in the dorsolateral area of ARH, recapitulating the mRNA distribution by in situ hybridization.

ObRb expression in the DMH vs. ARH-NPY neurons

Leptin is known to signal through *ObRb* to regulate gene transcription. It has been already shown that *ObRb* mRNA is expressed in the ARH-NPY neurons (Hakansson et al., 1996). While the initial Affymetrix chip analysis did not find a differential expression between the ARH and DMH-NPY neurons, the probe sets on these chips do not distinguish the different isoforms of the leptin receptor. Therefore, we used RT-PCR to compare *ObRb* expression in DMH and ARH-NPY neurons (Fig. 2-5C). Using this technique, *ObRb* was readily detected in ARH-NPY neurons but was not detected in DMH-NPY neurons. To compare leptin responsiveness in the two populations of NPY neurons, we injected P15 NPY-hrGFP mice with 3mg/kg leptin and observed p-Stat3 immuno-reactivity in the ARH and DMH (Fig. 2-5A). While 70% of NPY positive cells expressed p-Stat3-IR in the ARH, there was only 3-4% of NPY-GFP positive cells in the DMH were p-Stat3 positive (Fig. 2-5B). These results suggest that leptin may not directly regulate DMH-NPY neurons at P15.

GAD 65 and 67 expression in the DMH vs. ARH-NPY neurons

There is an abundance of data showing that ARH-NPY neurons are GABAergic. The microarray data indicated that DMH-NPY neurons express GABA synthesizing enzymes, Glutamic acid decarboxylase (GAD) 65 and 67, at a lower level than ARH-NPY neurons. However, the RT-PCR data showed that DMH-NPY neurons express detectable levels of Gad 65 or 67, suggesting that they may also be GABAergic (Fig. 2-6).

DISCUSSION

In this study, the microarray analyses have identified genes that are differentially expressed in DMH-NPY neurons compared to ARH-NPY neurons during the second postnatal week in mice. The role of ARH-NPY neurons in the regulation of energy balance and the phenotype of these neurons has been well characterized. The NPY expression in the ARH is evident from the late gestational period throughout life, with relatively modest changes occurring in response to metabolic changes such as fasting, diet induced obesity, and lactation(Grove et al., 2001; Guan et al., 1998a; Li et al., 1998a, b). However, the expression of NPY in the DMH appears to be dependent on specific physiological conditions. Strong NPY expression in the DMH is only observed in hyperphagic animal models such as neonates, lactating dams, and obesity. Recent studies demonstrated that the reduction of DMH-NPY expression causes a significant decrease in food intake and body weight, suggesting the role of DMH-NPY neurons as a major orexigenic signal in chronic hyperphagic conditions (Chen et al., 2004; Yang et al., 2009). Therefore, it is reasonable to speculate that DMH-NPY neurons are differentially regulated by afferent signals, and reflected in the activation of distinct transcriptional signals. The major goal of this study was to identify genes expressed in ARH and DMH-NPY neurons that may be responsible for their differential regulation during development.

The analysis of gene expression by microarray and PCR showed that *Pgc-1α* and *Foxa1* mRNA were expressed at higher levels in DMH-NPY neurons in comparison to ARH-NPY neurons. In agreement with the mRNA data, PGC-1α protein expression was observed in the DMH and was colocalized in a large percentage of DMH-NPY neurons.

In contrast to the PCR data, *Foxa 1* mRNA expression was not localized in the NPY neurons in the DMH using the fluorescent in situ hybridization. This discrepancy might be due to the low sensitivity of FISH assay. However, it is recognized that the PCR represent a highly amplified product of the gene. FOXA 1 immunoreacity was detected in a small number of DMH-NPY neurons, supporting the microarray and PCR results. Evidence suggests that *Foxa 1* is important for food intake since *Foxa1* null mutants exhibit progressive starvation, persistent hypoglycemia, hypotriglyceridemia and neonatal mortality between day 2 and 14 (Shih et al., 1999). Moreover, it is possible that *Foxa1* might be involved in the regulation of NPY since NPY expression is reduced in the pancreas and gut of *Foxa1* -/- mice. However, the expression of NPY in the brain was normal in the hypothalamus in this study. According to our results, it is unlikely that *Foxa 1* is a critical differentiated regulator between ARH and DMH-NPY neurons because of the low colocalization.

PGC-1α is a transcription cofactor that interacts with numerous transcription factors that are involved in adaptive thermogenesis and glucose metabolism (Liang and Ward, 2006). Null mutation for PGC-1α results in resistance to diet-induced obesity and reduced thermogenic capacity (Lin et al., 2004). These mice have an abnormal morphology in BAT and brain, and become profoundly hyperactive. Another study has also revealed that PGC-1α null mutation eventually leads to abnormal weight control, one of many metabolic disorders caused by the mutation (Leone et al., 2005)**.** *Pgc-1α* mRNA expression is high during development and is mainly localized in GABAergic neurons throughout the brain (Cowell et al., 2007). This is interesting because DMH-NPY expression also reaches the peak at P14 (Grove and Smith, 2003) and the present study

revealed that GABAergic makers are present in DMH-NPY neurons. This upregulation of PGC-1α could potentially play an important role in gene transcription for the increased mitochondrial activity and synaptogenesis during this time of substantial metabolic changes. In fact, there are several binding sites for transcription factors that are associated with PGC-1α in the promoter region of the *NPY* gene. *NPY* gene transcription in the ARH is regulated by forkhead box O1 (FOXO1) which is known to interact with PGC-1α (Puigserver et al., 2003). However, it is not known whether FOXO1 action on *Npy* gene transcription is dependent on PGC1-α in the ARH. The present study showed that PGC-1α expression is significantly higher in the DMH-NPY than ARH-NPY neurons. PGC1-α might possibly interact with another transcription factor to regulate *Npy* gene transcription in the DMH. While both PGC-1α and FOXA 1 are clearly associated with energy homeostasis, it remains to be determined if they are involved in the activation of DMH-NPY neurons in different models of obesity or hyperphagia.

As mentioned above, the afferent factors regulating ARH-NPY neurons are well characterized. It has been shown that the ARH-NPY neurons express the signaling form of leptin receptor, *ObRb*, and therefore they are under the direct regulation of leptin (Hakansson et al., 1996). ARH-NPY neurons are hyperpolarized by leptin treatment and *Npy* gene expression is decreased as leptin levels increase (Cowley et al., 2001). Therefore, leptin promotes anorexigenic behavior by inhibiting the orexigenic drive by NPY. The DMH is another site of leptin action in the hypothalamus as evidenced by abundant *ObRb* mRNA expression and p-Stat3-IR after leptin treatment (Scott et al., 2009).

Our results showed that *ObRb* mRNA expression was not detected in DMH-NPY neurons and only a small population of DMH-NPY neurons was p-Stat3-IR in response to leptin treatment. Therefore, it is highly unlikely that leptin is directly involved in the DMH-NPY induction during the postnatal period. This finding was surprising because DMH-NPY neurons project to the PVH and are believed to play a role in modulating neuroendocrine and autonomic output from the PVH in lactating rats (Li et al., 1998b). In fact, many of DMH neurons projecting the PVH are leptin targets. Intravenous leptin treatment activated c-Fos staining in many neurons in the DMH that project to the PVH(Elmquist et al., 1998). Our results strongly suggest that DMH-NPY neurons may be involved in modulating energy balance via different hormonal or neural inputs. However, it is possible that leptin can influence DMH-NPY neurons indirectly through other leptin responding neurons in the DMH.

The role of GABA in regard to energy homeostasis has been overlooked due to the abundant expression of these fast acting neurotransmitters in the brain. However, a recent study showed that mice became resistant to DIO when GABA release is inhibited by disruption of vesicular GABA transporter(VGAT) in NPY/AgRP neurons(Tong et al., 2008a). This effect is presumably due to the loss of inhibitory input to the local POMC cells, thus enhancing the neural activity. Another recent study demonstrated that GABA inputs from AgRP neurons to the Parabrachial nucleus (PBN) is also crucial for maintaining feeding (Wu et al., 2009b). Ablation of AgRP neurons in adult mice led to starvation and injection of $GABA_A$ receptor agonist into the PBN provided a protection against starvation phenotype. Therefore it is clear that GABA release from ARH-NPY/AgRP neurons is important for energy balance.

The DMH has been reported to contain GABAergic cell groups, as indicated by widespread *Gad* 65 and 67 mRNA expression in this area (Okamura et al., 1990). The highest density of *Gad* 65 and 67 mRNA containing neurons was concentrated in the dorsal and ventral area of the DMH. Some GABAergic input to the PVH originates from the DMH and may be involved in neuroendocrine and autonomic regulation (Boudaba et al., 1996). Our results showed that DMH-NPY neurons derived from the non-compact zone express GABAergic markers, *Gad* 65 and 67. Therefore, these cell groups could potentially innervate the PVH and release GABA to participate in the regulation of energy balance. However, further studies are required to characterize the targets of these GABAergic neurons and the role that they play in energy homeostasis.

In conclusion, we have identified DMH-NPY enriched genes during postnatal development in mice, when NPY expression is maximal in the DMH. It should be noted that the expression pattern of these genes may be specific to the postnatal period. It remains to be determined if these differentiated genes are expressed in DMH-NPY in other chronic hyperphagic models such as lactation and DIO. This study also suggests that DMH-NPY neurons may share some properties with ARH-NPY neurons, but could be differentially regulated through other hormonal or neuronal inputs.

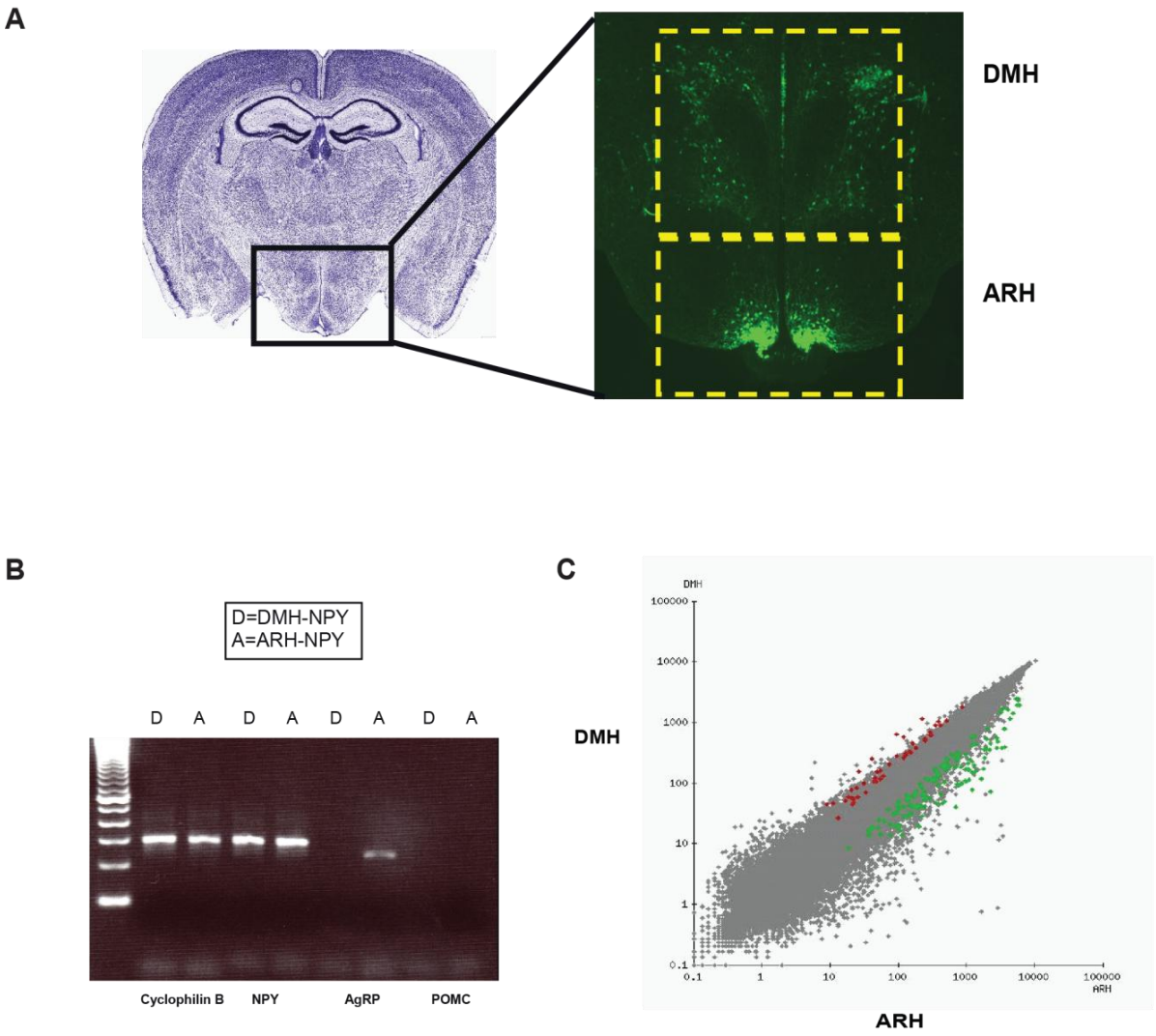

Figure 2-1. Microdissection of the DMH and ARH from P16-17 NPY-hrGFP mice.
A. Nissl staining of a coronal section. The inserted box shows the location of the DMH and ARH. The right panel illustrates the microdissection technique to obtain isolated DMH and ARH sections from P16-17 NPY-hrGFP mice. B. RT-PCR for Npy, AgRP and Pomc transcript in DMH and ARH-NPY neurons isolated from P16-17 NPY-hrGFP mice. Cyclophilin B was used as an internal control. C. Scatter plot showing DMH-NPY enriched genes (red) and ARH-NPY enriched genes (green) by high stringent analysis. The scale is arbitrary and indicates the level of gene expression in the ARH vs. DMH. The grey dots represent transcripts that are not significantly different between ARH and DMH samples.

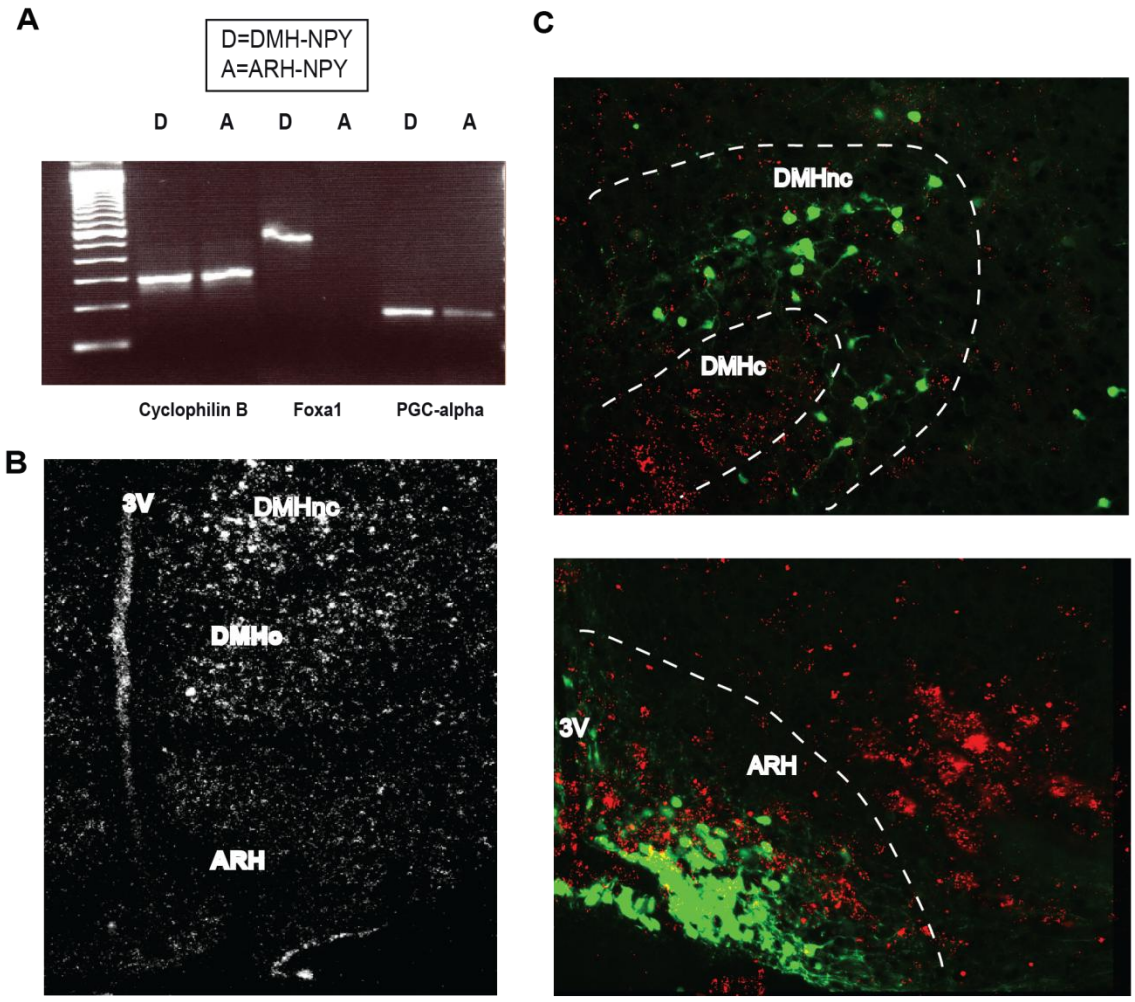

Figure 2-2. Verification of microarray analysis.
A. RT-PCR for Foxa1 and Pgc-1α mRNA expression in the DMH and ARH-NPY neurons isolated from P16-17 NPY-hrGFP mice. Cyclophilin B was used an internal control. B. Dark field photomicrograph showing Pgc-1 mRNA localization in the DMH from P16 NPY-hrGFP mouse. C. Fluorescent in situ hybridization for Foxa1 (red) in the DMH (upper) and ARH (lower) of P16 NPY-hrGFP mouse (green). DMHnc: DMH non-compact zone, DMHc: DMH compact zone

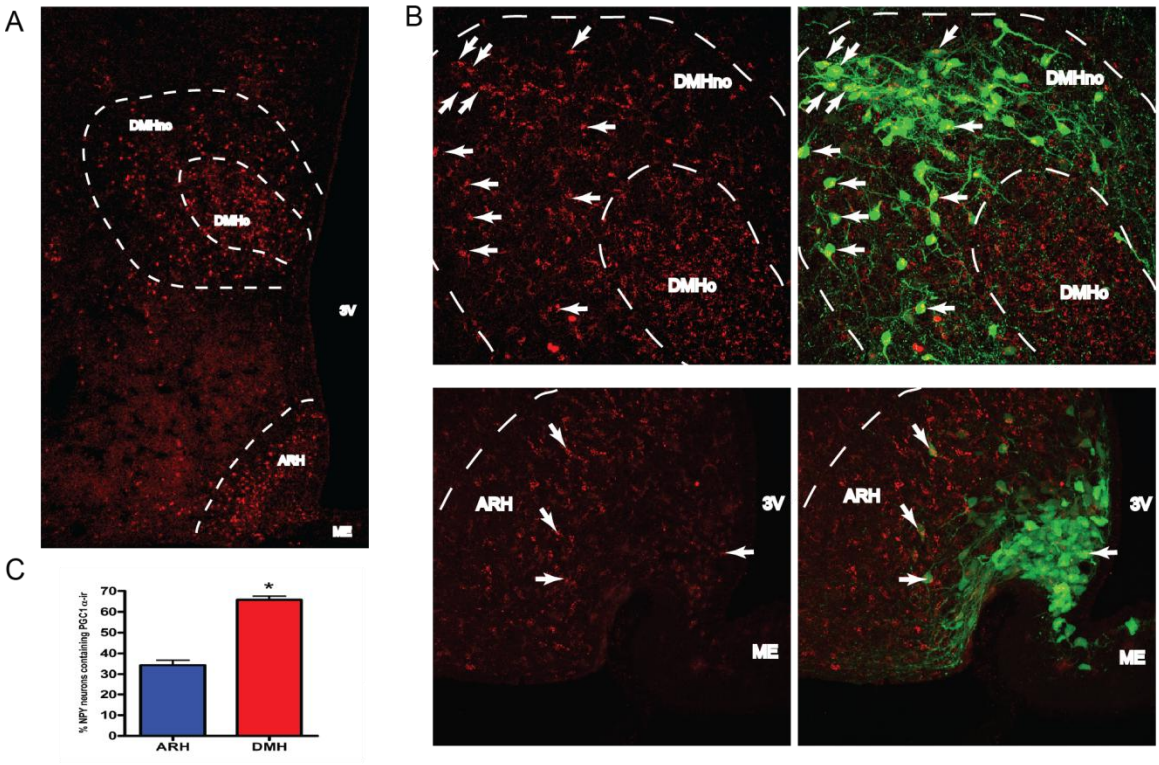

Figure 2-3. Immunohistochemistry for PGC-1α in P16 NPY-hrGFP mouse.
A. 4x objective images of PGC-1α staining in the DMH and ARH. B. Upper panels show 20x confocal images of PGC-1α (red) and overlay image with NPY-GFP (green) in the DMH. Lower panels show 20x confocal images of PGC-1α (red) and overlay image with NPY-GFP (green) in the ARH. The arrows indicate co-localization. C. The statistical analyses showed that 65% of DMH-NPY neurons were co-localized with PGC-1α while only 35% of ARH-NPY neurons were co-localized. (n=3 for each group, $P < 0.05$ by t-test) DMHnc: DMH non-compact zone, DMHc: DMH compact zone, ME: median eminence.

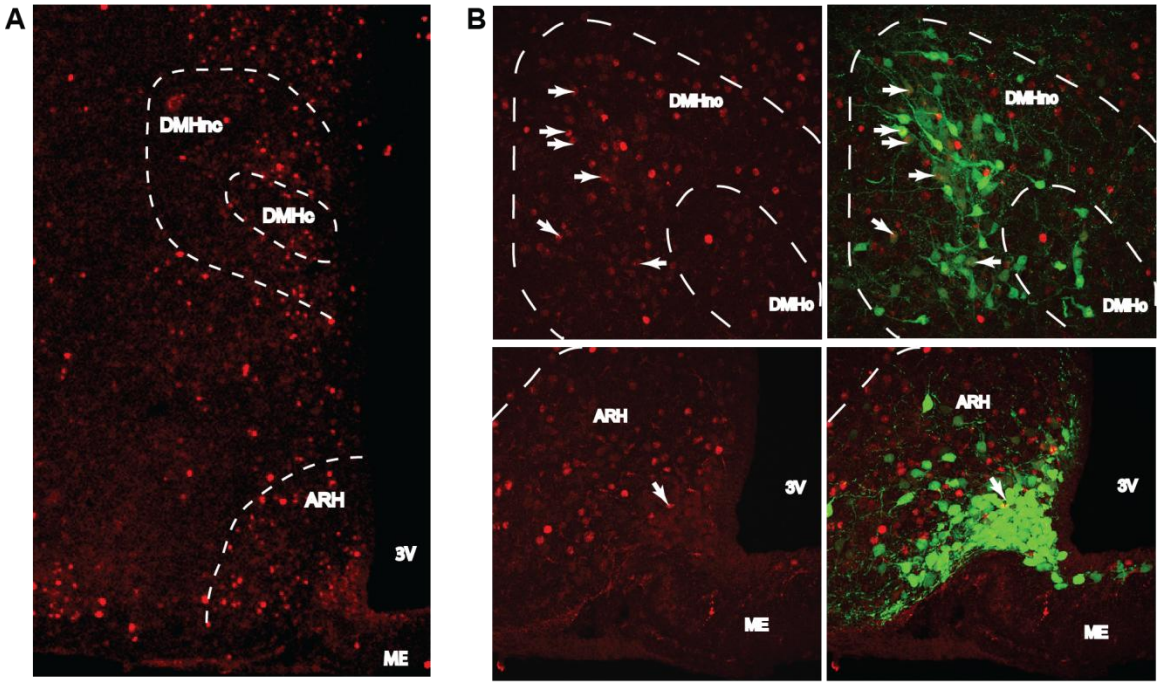

Figure 2-4. Immunohistochemistry for FOXA 1 in P16 NPY-hrGFP mouse.
A. 4x objective images of FOXA1 staining in the DMH and ARH. B. Upper panels show 20x confocal images of FOXA1 (red) and overlay image with NPY-GFP (green) in the DMH. Lower panels show 20x confocal images of FOXA1 (red) and overlay with NPY-GFP (green) in the ARH. The arrows indicate co-localization. DMHnc: DMH non-compact zone, DMHc: DMH compact zone, ME: median eminence.

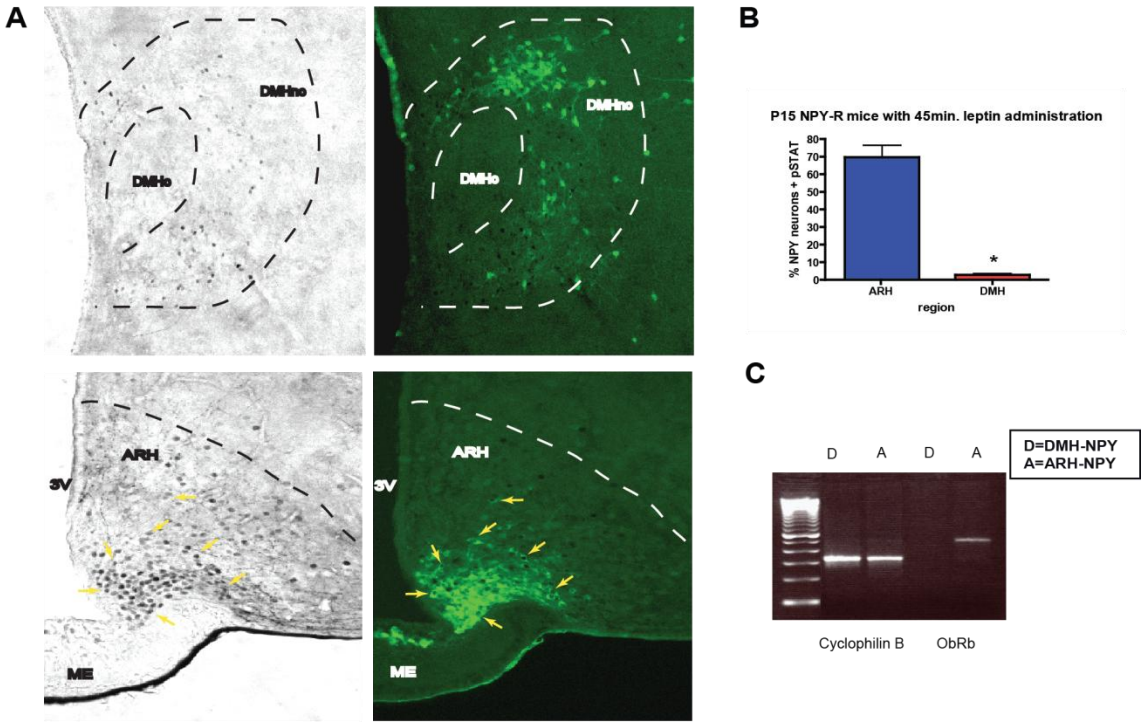

Figure 2-5. Leptin signaling in DMH and ARH-NPY neurons.
A. p-STAT3 activation in the DMH (upper panels) and ARH (lower panels) 45min after i.p. 3mg/kg leptin administration in P15 NPY-hrGFP mice. NPY neurons are shown as green and p-STAT3-IR are black nuclear staining. The arrows indicate NPY neurons containing p-STAT3 activation. B. The bar graph shows that only 3-4% NPY positive neurons in the DMH contained pStat3 activation compared to near 70% ARH-NPY neurons (n=3 for each group, p=0.0007 by unpaired t-test). C. RT-PCR for ObRb mRNA expression in the DMH and ARH-NPY neurons isolated from P16-17 NPY-hrGFP mice.

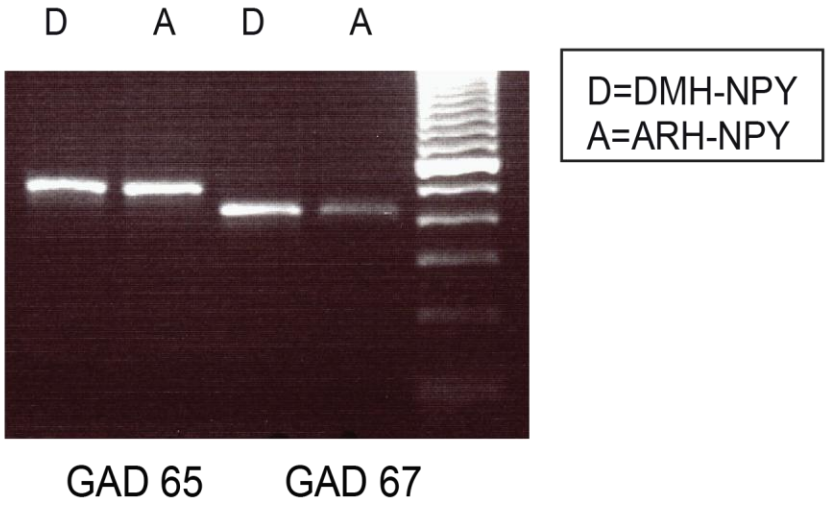

Figure 2-6. Gad 65 and 67 mRNA expressions in the DMH and ARH-NPY neurons. DMH and ARH-NPY samples were isolated from P16-17 NPY-hrGFP mice.

Table 2-1. DMH-NPY neuron enriched genes: High stringent Analysis

Gene Name	Accession number	DMH/ARHRatio[a]
Barh like 2	BB543853	6.5
Riken cDNA A430071A18	BB205989	6.0
Forkhead box A1	NM_008259	5.2
Paired-like homeodomain transcription factor Munc 30	U80011	4.6
Acyl-malonyl condensing enzyme 1	NM_019871	2.9
Outer dense fiber of sperm tails 2-like	AK016726	2.7
G protein-coupled receptor 177	BC018381	2.6
RIKEN cDNA 5530401A14	AK017430	2.4
Rhabdoid tumor deletion region gene 1	AK017008	2.3
Mdm2, transformed 3T3 cell double minute p53 binding protein	NM_134092	2.2
canopy 1 homolog	BB131676	2.2
Outer dense fiber of sperm tails 2-like	AK016726	2.2
Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	BB745167	2.1
Tubulin tyrosine ligase-like family, member 6	AI429241	2.1
Transcribed locus	BB466155	2.1
Tubulin tyrosine ligase-like family, member 9 (Ttll9), transcript variant 2	AK015740	2.1
AT-hook transcription factor	BB014626	2.0
Homeodomain only protein	BC024546	2.0
Transcribed locus	AV118079	2.0
Family with sequence similarity 13, member A	BB745929	2.0

[a] Affymetrix gene chip analysis was performed on DMH-NPY (n=3) and ARH-NPY(n=3) mRNA samples. Each pool contained tissue from 6 animals. Genes that didn't meet the quality cut off for expression level and variability were eliminated from the analysis. Only genes with a greater than 2 fold difference between the DMH and ARH samples were included.

Supplemental table 2-1. DMH-NPY neuron enriched genes: moderate stringent analysis

Gene Name	Accession number	DMH/ARH ratio
Signal transducing adaptor family member 2	BC026642	3
Eph-related receptor tyrosine kinase (Mek4) secreted	BB292785	3
CCAAT/enhancer binding protein , alpha	BC011118	2.7
Interleukin 33 (Il33)	NM_133775	2.7
Tektin 3	AK016718	2.6
Transcribed locus	AW209204	2.5
Carcinoembryonic antigen-related cell adhesion molecule 2	BC024320	2.5
RIKEN cDNA 4930534B04 gene	BE980134	2.4
Transcribed locus	BE687142	2.4
Cytochrome P450, family 2, subfamily s, polypeptide 1	AK004699	2.3
Transcriptional regulator, SIN3A (yeast) (Sin3a), transcript variant 1	NM_011378	2.3
Transcribed locus	BI737125	2.2
MKIAA0168 protein	AK018504	2.1
CDNA clone IMAGE:9053327	BB449058	2.1
PH domain-containing adaptor PHAD47	NM_031257	2.1
Transforming growth factor, beta receptor II (Tgfbr2), transcript variant 2	BG793483	2.1
PREDICTED: Mus musculus hypothetical protein LOC100042390	BF658932	2.1
Transcribed locus	BM119567	2.1
immunoglobulin superfamily, DCC subclass, member 3	BG093601	2.0
Par-6 (partitioning defective 6) homolog beta (C. elegans)	BE953582	2.0
Myeloblastosis oncogene	BC011513	2

Supplemental table 2-2. ARH-NPY neuron enriched genes: High stringent analysis

Gene Name	Accession number	ARH/DMH Ratio
Activin A receptor, type IC	BB432539	32.41
Protocadherin 20	BC024927	17.38
Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	U65091	16.05
Type II deiodinase	NM_010050	14.21
Homeobox protein SIX6	AF050130	10.62
Transcribed locus	BM899593	10.44
Transcribed locus	AK014624	9.82
Crystallin	NM_016669	9.77
Rho GTPase activating protein 6 , transcript variant 1	NM_009707	9.74
Frizzled homolog 5 (Drosophila) , transcript variant 1	BB795235	9.56
Agouti related protein	NM_007427	9.38
Transcribed locus	AW456568	9.23
DEP domain containing 6	BB324973	8.77
Zinc finger, DHHC containing 14	BB544336	8.76
Insulin receptor substrate 4	BB295945	8.71
Claudin 10, transcript variant 1	BC021770	8.69
Oligodendrocytic myelin paranodal and inner loop protein	AW490730	8.6
Rho GTPase activating protein 6	AF177664	8.14
Six6 opposite strand transcript 1	AK015397	8.13
Protein tyrosine phosphatase, receptor type, K	AV174021	8
Myelin oligodendrocyte glycoprotein	U64572	7.6
Myelin and lymphocyte protein, T-cell differentiation protein	AK019046	7.53
DEP domain containing 6 , transcript variant 1	AI957118	7.21
Transmembrane protein 132C	AV305379	6.8
Serine (or cysteine) peptidase inhibitor, clade A, member 3K	NM_009252	6.79
Aldehyde dehydrogenase family 1, subfamily A1	NM_013467	6.47
Proteolipid protein (myelin) 1	M14674	6.43
NEW1 domain containing protein	BB318221	6.4
Putative serine protease 35	BB042892	6.07
Myelin basic protein	NM_010777	5.94
Kainate receptor GluR7 3 subunit	BM899529	5.86
Aldolase C, fructose-bisphosphate	BM941201	5.19
Synaptotagmin-like 2 , transcript variant 3	NM_031394	5.12
Riken A230069A22 gene	AV327597	5.07
Thyroid hormone responsive SPOT14 homolog	BC009165	4.94
RIKEN cDNA 1500001A10 gene	BQ176215	4.79
Transcribed locus	AV328325	4.74
Transmembrane protein 176A , transcript variant 1	BC006049	4.73
UDP galactosyltransferase 8A	BC016885	4.6
Collagen, type XXIII, alpha 1	AI429655	4.57

Supplemental table 2-continued

Gene Name	Accession number	ARH/DMH Ratio
Leucine rich repeat containing 4	BB332932	4.11
Pleiotrophin	BF178348	3.87
Transcribed locus	BB761376	3.86
Transcribed locus	AV228812	3.64
Alpha-2-macroglobulin	BB185854	3.58
R-spondin 3 homolog	BG072958	3.46
Regulator of G-protein signaling 9-2 isoform	NM_011268	3.35
Slac2-c mRNA for Slp homologue lacking C2 domains-c	BB429683	3.19
CD38 antigen	BB256012	3.16
Sodium channel, voltage-gated, type IX, alpha	AI549833	3.08
RIKEN cDNA 2700045P11	BB426857	3.08
RIKEN cDNA 4933428G20	NM_021493	3.05
Melanoma cell adhesion molecule	NM_023061	2.98
Ubiquitin family domain containing 1	BG798405	2.98
Complement component 4B	NM_009780	2.89
Transcribed locus	AI467657	2.83
Ephrin A5 , transcript variant 2	BQ173967	2.81
Sh3 domain YSC-like 1	NM_013709	2.76
Zinc finger homeobox 3	BB704254	2.73
OCIA domain containing 2	BM937735	2.66
Adenylate cyclase 2	AV025455	2.63
Leucine rich repeat protein 1, neuronal	NM_008516	2.61
G protein-coupled receptor GPR62	BE955672	2.56
Claudin 11	NM_008770	2.54
F-box protein 2	BB311718	2.47
Transcribed locus	AV232123	2.45
ATP-grasp domain containing 1	BB356222	2.39
Progestin and adipoQ receptor family member VIII	AV328983	2.31
Neuropeptide Y	NM_023456	2.3
5-nucleotidase domain containing 2	BC011230	2.27
Strain BALB/c stearoyl-coenzyme A desaturase 1	BC007474	2.25
Rho-guanine nucleotide exchange factor	BG069493	2.24
ADAMTS-like 1	AV380797	2.23
Guanine nucleotide binding protein, alpha 14	AV101562	2.22
FXYD domain-containing ion transport regulator 2	AV002675	2.21
Follistatin-like 1	BI452727	2.2
RIKEN cDNA 1500009L16	AV216011	2.18
Vezatin isoform 0.8	AV274318	2.11
Solute carrier family 44, member 1	BC025941	2.09
Platelet-derived growth factor, C polypeptide	NM_019971	2.08
Spondin 1, (f-spondin) extracellular matrix protein	BC020531	2.07
SH3-domain GRB2-like endophilin B2	AI481067	2.06

Chapter 3

Efferent projections of NPY expressing neurons of the dorsomedial hypothalamus in chronic hyperphagic models

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ABSTRACT

The dorsomedial hypothalamus (DMH) has long been implicated in feeding behavior and thermogenesis. The DMH contains orexigenic neuropeptide Y (NPY) neurons, but the role of these neurons in the control of energy homeostasis is not well understood. NPY expression in the DMH is low under normal conditions in adult rodents, but is significantly increased during chronic hyperphagic conditions such as lactation and diet-induced obesity (DIO). To better understand the role of DMH-NPY neurons in hypothalamic feeding neurocircitry, we characterized the efferent projections of DMH-NPY neurons by injecting anterograde tracer biotinylated dextran amine (BDA) into the DMH in lactating rats and DIO mice. BDA and NPY co-labeled fibers were mainly limited to the hypothalamic areas including paraventricular nucleus of the hypothalamus (PVH), lateral hypothalamus/perifornical area (LH/PFA), and anteroventro periventricular nucleus (AVPV). Specifically in lactating rats, BDA and NPY co-labeled axonal swellings were in close apposition to CART expressing neurons in the PVH and AVPV. Although the DMH neurons are known to project to the rostral raphe pallidus (rRPa), a major thermoregulatory site in the brainstem, none of these projections contained NPY immunoreactivity. Instead, the majority of BDA-labeled fibers in the rRPa were orexinergic. Furthermore, DMH-NPY projections were not observed within the nucleus of solitary tract (NTS), another brainstem site critical for the regulation of sympathetic outflow. The present data suggest that NPY expression in the DMH during chronic hyperphagic conditions play important roles in feeding behavior and thermogenesis by modulating neuronal functions within the hypothalamus, but not in the brainstem.

INTRODUCTION

Neuropeptide Y (NPY) is a potent orexigenic neuropeptide and is widely expressed in the brain (Beck, 2006; Chee and Colmers, 2008). In the hypothalamus, NPY expressing neurons are primarily localized in the arcuate nucleus of the hypothalamus (ARH), a key site for the integration of hormonal and nutritional signals from the periphery (Gehlert et al., 1987; Morris, 1989). NPY neurons are also located in the dorsomedial hypothalamus (DMH), another important component of the hypothalamic neurocircuitry involved in ingestive behavior and thermoregulation (Bellinger and Bernardis, 2002; Dimicco and Zaretsky, 2007). Lesion studies in rats demonstrated that the DMH is critical for maintaining normal food intake and body weight (Bellinger and Bernardis, 2002; Bellinger et al., 1979; Bellinger et al., 1986), and that these animals are resistant to diet-induced obesity (DIO) (Bernardis and Bellinger, 1986, 1991). DMH neurons also regulate brown adipose tissue (BAT) activity and temperature via direct projections to the rostral raphe pallidus (rRPa), an important thermoregulatory site in the brainstem (Cano et al., 2003; Cao et al., 2004; Cao and Morrison, 2006; Oldfield et al., 2002). Several recent studies have also emphasized the importance of the DMH neurons in the regulation of energy homeostasis (Chao et al., 2011; Enriori et al., 2011; Tupone et al., 2011; Yang et al., 2009; Zhang et al., 2011b). In particular, NPY neurons in the DMH have been associated with hyperphagia and suppression of BAT thermogenic capacity in several rodent models (Chao et al., 2011; Chen et al., 2004; Guan et al., 1998a; Kesterson et al., 1997; Yang et al., 2009).

There are some key species differences in the expression of NPY within the DMH. NPY is constitutively expressed in the compact subdivision of the DMH (DMHc)

in intact rats, while no expression is detected in the DMHc in intact mice (Bi et al., 2003; Guan et al., 1998a; Li et al., 1998a). However, both rats and mice exhibit a transient expression of NPY in a separate population of neurons in the non-compact subdivision (DMHnc) during specific physiological conditions, including during early postnatal development, lactation, and obesity (Grove et al., 2001; Grove and Smith, 2003; Guan et al., 1998a; Guan et al., 1998b; Kesterson et al., 1997; Li et al., 1998a; Smith and Grove, 2002). The common link between these conditions is hyperphagia.

Lactation is a state of negative energy balance due to the energy drain from suckling and milk production. To compensate for this energy drain, lactating animals exhibit several adaptive physiological responses, including hyperphagia, energy conservation by reducing heat production, and suppression of cyclic reproductive function (Brogan et al., 1999; Wade and Schneider, 1992; Wade et al., 1996). Hyperphagic behavior in lactation is supported by an increase in NPY and agouti-related protein (AgRP) mRNA expression in the ARH (Li et al., 1998a, b). In addition, NPY mRNA expression is increased in the DMHnc, which is also believed to play a crucial role in the physiological adaptations occurring during lactation (Chen and Smith, 2004; Chen et al., 2004; Smith and Grove, 2002; Smith et al., 2010).

NPY mRNA induction in the DMH is also reported in several obese mouse models including diet-induced obese, lethal yellow (A^y), MC4-R knockout (MC4-RKO), brown adipose tissue deficient (UCP-DTA) , and tubby mice (Guan et al., 1998a; Guan et al., 1998b; Kesterson et al., 1997; Tritos et al., 1998). Guan et al. first reported that NPY mRNA expression is induced in the DMH after 24 weeks of high fat diet treatment in mice (Guan et al., 1998a), while NPY expression in the ARH is noticeably decreased,

suggesting that DMH-NPY induction plays a major role in hyperphagic behavior observed in obese mice.

The DMH neurons project to many hypothalamic areas, including the paraventricular nucleus (PVH) and lateral hypothalamus (LH) (Thompson and Swanson, 1998), where hypothalamic and brainstem signals are integrated to modulate feeding behavior and sympathetic outflow (Bai et al., 1985; Broberger et al., 1998; Legradi and Lechan, 1999; Li et al., 1998b). Although DMH-NPY mRNA induction is closely linked to hyperphagic behavior, there is little evidence of a functional connection between DMH-NPY neurons and these feeding regulatory sites in the brain. To better understand the role of DMH-NPY induction in chronic hyperphagic models, we injected the anterograde tracer, biotinylated dextran amine (BDA), into the DMH to identify the potential targets of DMH-NPY projections in the hypothalamus and brainstem of lactating rats and DIO mice.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee. *Rat studies:* Pregnant or cycling female Wistar rats (220g) were purchased from Simonson Laboratories (Gilroy, CA). The animals were housed individually and maintained under a 12 hr light/dark cycle (lights on at 7:00 A.M.) and constant temperature (23 ± 2°C). Food and water were provided *ad libitum*. The pregnant rats were checked for the birth of the pups every morning; the day of delivery was considered day 0 postpartum, and litters were adjusted to eight pups on day 2. *Mouse study:* 4 weeks old C57BL6 male mice were purchased from the Jackson

laboratory (Bar Harbor, Maine, USA). To generate DIO mice, 5 week-old C57BL6 mice were fed a 60% high fat diet (Research Diets, NJ, USA, Cat# D12492) or normal chow diet (Purina lab chow #5001) for 20 weeks. Five mice were group-housed in the same cage and maintained under a 12 hr light/dark cycle (lights on at 7:00 A.M.) and constant temperature (23 ± 2°C). Food and water were provided *ad libitum*.

Stereotaxic surgery for BDA injection

Lactating rats

On postpartum day 6 or 7, the lactating rats were separated from the pups and anesthetized with 3% isoflurane. The rats (both lactating and cycling) were placed in a stereotaxic apparatus and maintained under 2-2.5% isoflurane mixed with oxygen for the entire duration of the surgery. A small hole was drilled into the skull under aseptic conditions. A glass micropipette (20μm tip diameter) connected to an air pressure injector system was positioned via the stereotaxic manipulator. 60nls of the anterograde tracer biotin dextran amine (BDA; 5% in water; Molecular Probes, Eugene, OR; 10,000 MW; cat.# D1956)(Reiner et al., 2000) was inserted into the area surrounding the compact zone of DMH (coordinates : 3.3 mm caudal, 0.5 mm lateral to bregma, and 8.4 mm ventral to the dura), according to the atlas of Paxinos and Watson. This is the area containing a high density of suckling-activated NPY neurons. For the cycling rats, which have no activated NPY neurons in the DMHnc, BDA injection was targeted for the NPY neurons in the DMHc (coordinates: 3.3 mm caudal, 0.4 mm lateral to bregma, and 8.4 mm ventral to the dura). After injection, the micropipette was removed and the incision was closed with surgical staples. Rats were monitored during the recovery period, and the pups were returned to the dams immediately after the recovery. The rats were monitored for the

normal food intake and weight recovery for 7 days before sacrificing for the following experiments. As a comparison to lactating rats (with NPY expression in both DMHnc and DMHc), we also investigated DMH projections in virgin rats (which have expression only in the DMHc). The virgin rats were studied during random stages of the estrous cycle.

DIO mice

On the day of surgery, HFD or CD mice were anesthetized with tribromoethanol (20 mg/kg body weight, i.p). The mice were placed in a stereotaxic apparatus and a small hole was drilled into the skull under aseptic conditions. A glass micropipette (20 µm tip diameter) connected to an air pressure injector system was positioned via the stereotaxic manipulator. 40 nls of BDA was injected into the area surrounding the compact zone of DMH [coordinates: 1.2 mm caudal, 0.2 mm lateral to bregma, and 5.15 mm ventral to the dura, according to the mouse brain atlas of Paxinos and Franklin (second edition, 2001)]. After injection, the micropipette was removed and the incision was closed with surgical staples. The mice were monitored for the normal food intake and weight recovery for 3 days before sacrificing for the BDA visualization.

Tissue preparation

The animals were anesthetized with tribromoethanol (20 mg/100g body weight, i.p.) and perfused transcardially with 150 ml of 0.9 % in saline, followed by 300 ml of 4% -paraformaldehyde, pH 7.4. The brain was removed and post-fixed overnight in 4% paraformaldehyde. The following day, the brains were transferred and stored in 25% sucrose solution containing 0.05 M potassium PBS (KPBS) until frozen in dry ice. The

frozen brains were cut on a sliding microtome in 25 µm sections and collected and stored in cryoprotectant at –20°C until use.

Immunohistochemistry for BDA and NPY

To visualize the BDA injection site, the sections containing the DMH were washed in 0.05 M potassium PBS (KPBS) and incubated in 1:200 Streptavidin Alexa Fluor 568 conjugate (Invitrogen, Cat # S11226) in KPBS for 4 hrs. The sections were then rinsed in KPBS and incubated in Hoechst nuclear staining solution (Molecular probes, Cat# H1398) for 5min in room temperature to identify the morphological limits of the DMH. BDA injection sites were determined under fluorescent microscopy.

To visualize BDA and NPY co-localized fibers, a one in six series of 25 µm sections were rinsed in KPBS, followed by blocking solution containing 0.4% Triton-X and 2% normal donkey serum in KPBS (KPBS-TX-NDS) for 30 min at room temperature. Sections were then incubated in 1:3000 sheep anti NPY antibody (Chemicon; Cat # AB1583) in KPBS-TX-NDS for 48 hr at 4º C. After incubation, the tissue was rinsed in KPBS and incubated in 1:1000 Alexa Fluor 488 donkey anti-sheep (Invitrogen ; Cat# A11015) and 1:200 Streptavidin Alexa Fluor 568 in KPBS containing 0.4% Triton-X (KPBS-TX) for 4 hr at room temperature. The tissue sections were then mounted on gelatin-coated glass slides and coverslipped with SlowFade Gold antifade reagents (Invitrogen; Cat# S36936).

Triple-label Immunohistochemistry

PVH : To visualize BDA/NPY co-localized axonal swellings in relation to CART neurons, tissue sections were incubated in NPY antibody (described above) and 1:5000 rabbit anti CART (Phoenix pharmaceuticals; Cat# CA H-003-62) in KPBS-TX-NDS for

48 hrs at 4º C. The tissues were then rinsed and incubated in 1:1000 Alexa Fluor 488 donkey anti-rabbit (Invitrogen; Cat# A21206,) 1:200 Alexa Fluor 568 Streptavidin for BDA detection and 1:1000 Dylight 649 donkey anti-sheep (Jackson ImmunoResearch; Cat# 713-496-147) for 4 hrs at room temperature. After rinsing in KPBS, the tissue sections were mounted on gelatin-coated glass slides and coverslipped with SlowFade Gold antifade reagents.

AVPV: To visualize BDA/NPY fibers with GnRH neurons, tissue sections were incubated in 1:5000 mouse anti GnRH (HU4H, Dr. Henryk Urbanski, OHSU, OR) and NPY antibody containing KPBS-TX-NDS solution for 48hrs. GnRH, BDA and NPY were visualized with 1:1000 Alexa Fluor 488 donkey anti-mouse, 1:200 Alexa Fluor 568 Streptavidin and 1:1000 Dylight 649 donkey anti-sheep. BDA, NPY, and CART triple label immunostaining was performed as described in the PVH.

rRPa: Tissue sections were incubated with 1:2000 goat anti orexin antibody for 48 hrs and BDA/orexin colocalized fibers were visualized by 1: 1000 Alexa Fluor 488 donkey anti goat and 1:200 Alexa fluor 568 streptavidin treatment. For triple labeling of BDA, orexin and TPH, tissue sections were incubated in 1:2000 goat orexin antibody and 1:1000 mouse anti TPH (Sigma, St Louis, MO, USA; Cat# T0678,) KPBS-TX-NDS solution. 1:1000 Alexa Fluor 488 donkey anti-mouse, 1:200 Alexa Fluor 568 Streptavidin and 1:1000 Cy5 donkey anti-goat (Jackson ImmunoResearch; Cat# 705-176-147)

Confocal microscopy

Confocal laser microscopy was used to analyze the double- and triple-label IF images for BDA/NPY colocalization and close appositions as described by our laboratory

(Campbell et al., 2003b; Glavas et al., 2008). The TSC SP confocal system (Leica Corp., Germany) was used scan the images, consisting of a RBE inverted microscope (Leica Corp., an Ar laser-producing light at 488 nm (for visualization of FITC), a Kr laser-producing light at 568 nm (for visualizing TRITC), and a HeNe laser-producing light at 647 nm (for visualization of Cy5). Various objectives (25X, numerical aperture 0.75 and 40X, numerical aperture 1.25) were used to scan and capture images. In order to detect BDA/NPYco-localization, each brain area (two medial sections per animal) was divided into smaller areas using 2x optical zoom at 40X magnification. Each image was scanned at 1µm intervals. For each experiment, fluorophore signals were checked individually for bleed-through to the apposing detector. Bleed-through was eliminated by adjusting laser intensity and the width of the detector window. To assess colocalization of BDA/NPY signals in close apposition to a cell body, a sequential series of optical sections, 0.5 µm intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. The signals were obtained for each fluorophore on one series of optical sections and stored separately as a series of 512 × 512 pixel images. The stacks of individual optical slices were analyzed using the Image J software to determine co-localization and close appositions. The confocal images are presented as projections of stacks of optical images or as individual slices, as indicated. The brightness and contrast of the images were adjusted in Photoshop to match microscope visualization (Adobe Systems Inc., San Jose, CA).

RESULTS

BDA labeling of DMH projections in rats and mice

In order to trace DMH-NPY neuronal projections, BDA was streotaxically injected into the DMH in lactating and cycling rats. DMH neuronal projections labeled with BDA closely resembled the pattern of PHA-L labeled DMH projections in the rat reported by Thompson and Swanson (1998). The BDA-labeled fiber projections were mainly restricted to the hypothalamus, with limited projections to the brainstem. The fiber projection pattern was indistinguishable between lactating and cycling rats. We also injected BDA into the DMH of mice fed with either high fat diet (DIO) or normal chow diet (CD) for 20 weeks. BDA-labeled fiber projections in mice were similar to DMH projections in rats. As illustrated in Fig 3-1A,B (rats), and Fig 3-2A,B (mice), BDA injection sites were included in the areas where the most NPY induction is observed during lactation and DIO condition. In the present study, all the BDA injections within the DMH border also showed a similar fiber distribution in major target areas, including the AVPV, PVH, and LH in the hypothalamus, and the rRP in the brainstem as illustrated in Fig. 3-1C-F (rats) and Fig. 3-2C-F (mice). In the ascending pathways, most BDA labeled fibers are found in the PVH, perifornical area (PFA) in the LH, and preoptic areas including parastrial nucleus (PS), lateral preoptic (LPO) and anteroventro periventricular nucleus (AVPV). Caudal to the DMH, numerous BDA labeled fibers were also detected in supramamilary nucleus (SUM), ventral tegmental area (VTA), ventrolateral periaqueductal grey (VLPAG), barrington's nucleus (BA), locus ceoruleous (LC) and lateral parabrachial nucleus (LPB). A small number of BDA labeled fibers were also observed in caudal brainstem areas including the rRPa, raphe magnus (RM), nucleus of the solitary tract (NTS), and ventral lateral medulla (VLM).

DMH-NPY neurons project to the PVH in lactating rats

To identify BDA/NPY co-localized fibers, each PVH section (two medial PVH sections per animal) was scanned at 1μm intervals using a confocal laser microscope (Fig. 3-3A). BDA/NPY co-localized fibers (total 8-10 fibers/animal) were detected in the PVH (Fig. 3-3B-D), primarily located in the medial parvicellular and posterior magnocellular areas in lactating rats (n=4) (Fig. 3-3E). In lactating animals, it is likely that BDA labeled NPY neurons in both compact (DMHc) and non-compact (DMHnc) subdivision. No BDA/NPY co-localized fibers were detected in the PVH when the BDA was injected into the DMH border or slightly outside of the DMH in lactating rats. While cycling female rats express a low level of NPY mRNA in the DMHc with no expression within the DMHnc, the pattern of BDA/NPY co-localized fibers (total 2-4/ animal, n=3) within the PVH was similar to that observed in the lactating rat (Fig. 3-3F).

CART-expressing neurons are located in the parvicellular area where they are often co-localized with thyrotropin-releasing hormone (TRH) neurons involved in the regulation of energy homeostasis by the activation of the hypothalamic-pituitary-thyroid axis (Elias et al., 2001; Lechan and Fekete, 2006; Silva, 1995). Next, we determined the spatial relationship between DMH-NPY projections and CART-immunoreactive (ir) neurons in the PVH using single optical slice analysis. Triple-label immunostaining for BDA/NPY/CART revealed that DMH-NPY projections are in close apposition to CART-ir neurons in the parvicellular area of the PVH in lactating rats (Fig. 3-4A-D). Single optical slices at 0.5 μm increments at the level of a CART cell body showed that the BDA/NPY-positive axonal swelling makes a close apposition with the CART cell (Fig. 3-4D-1~3).

DMH-NPY neurons project to the LH in lactating rats

Using the same confocal analysis method (2 sections/animal), BDA/NPY co-localized fibers (total 5-6/animal) were observed in the LH of lactating rats (n=4), mainly concentrated in the perifonical area (Fig. 3-5). While BDA/NPY fibers were readily detected in all of the lactating rats, the occurrence of co-labeled fibers in the virgin cycling rats were scarce (total 0-2/animal, n=3).

DMH-NPY neurons project to the AVPV in lactating rats

NPY is also one of the essential players in modulating reproductive function and NPY inhibits GnRH neuronal activity in lactation (Smith et al., 2010). Although BDA containing fibers were observed in close apposition to GnRH neurons, none of them contained NPY immunoreactivity (data not shown; n=4). However, the AVPV, which is known to have strong connections with the GnRH axis, did contain BDA/NPY co-localized fibers in lactating rats (Fig. 3-6). In contrast, BDA/NPY fibers were not present in cycling rats (n=3). On the other hand, BDA/NPY containing axonal swellings were detected in a close apposition to CART expressing neurons in the AVPV in lactating rats (Fig. 3-6).

DMH-NPY neurons do not project to the rRPa in lactating rats

While DMH projections to the brainstem were sparse relative to the hypothalamus, we did detect significant BDA positive fibers in a few key areas in the regulation of sympathetic outflow. One such area with readily detectable BDA-labeled fibers was in the rRPa. However, while NPY positive fibers were abundant within the rRPa, no BDA/NPY co-localization was observed in this region of either lactating or cycling rats (Fig 3-7 A,B). Instead, all of the BDA- labeled fibers projecting to the rRPa were double-labeled for orexin (Fig. 3-7 C-F). Additionally, we found BDA /orexin co-

localized axonal swellings in close apposition to the neurons expressing tryptophan hydroxylase (TPH), a serotonergic cell marker (Fig. 3-7 F-1~3). Furthermore, no BDA/NPY co-localized fibers were observed in other brainstem regions including the NTS, VLM and LPB.

DMH-NPY neuronal projections in DIO mice

In contrast to the rat, NPY neurons are limited to the DMHnc in the DIO mouse. In spite of this, BDA/NPY projections were similar between DIO mouse and the lactating rat. Most notably, BDA fibers containing NPY immunoreactivity were readily detectable in the parvicellular area of the PVH (Fig. 3-8) and PFA of the LH in DIO mice (two sections for each area, n=4). In the brainstem, consistent with the findings in lactating rats, BDA and NPY were not co-localized in the rRPa in DIO mice. Similar to the rat, BDA labeled fibers contained orexin immunoreactivity in the rRPa (Fig. 3-9). Importantly, no BDA/NPY co-localized fibers were observed in the same regions in CD mice (n=2), which do not have detectable NPY mRNA expression in the DMH.

DISCUSSION

The present study characterizes the efferent projections of NPY expressing neurons in the DMH in chronic hyperphagic models, the lactating rat and diet-induced obese mouse, using BDA anterograde tracing method. The robust DMH-NPY projections were to the hypothalamic regions involved in the regulation of food intake and autonomic function, including the PVH and LH. We also demonstrated interactions between DMH-NPY projections and CART neurons in the hypothalamic target areas. Contrarily, DMH-NPY projections were not observed in the brainstem areas implicated in energy

homeostasis, including the rRPa and NTS. Instead, the majority of BDA fibers in the rRPa contained orexin immunoreactivity, suggesting that orexin neurons in the DMH play a role in the regulation of BAT thermogenesis.

Technical considerations

NPY is expressed in two subregions of the DMH, the DMHc and DMHnc, depending on the species. NPY is constitutively expressed in the DMHc in intact rats. However, there is no evidence of DMHc-NPY expression in other species including the mouse and non-human primate either, during development or in adults (Grayson et al., 2006), suggesting that DMHc-NPY expression is unique to the rats. The absence of NPY expression in the DMHc suggests that DMHc-NPY neurons are not required for normal energy balance in mice. On the other hand, both rats and mice exhibit a transient expression of NPY in the DMHnc during postnatal development, lactation, and obesity, all of which are characterized by hyperphagia, suggesting that DMHnc-NPY neurons have similar functions in both species.

The DMH is closely surrounded by other hypothalamic regions including the PVH, LHA, VMH and ARH, making the DMH especially difficult to make accurate tracer injections without diffusing into neighboring regions. Thus, we injected a small amount of BDA tracer into the DMH to limit the spread, and specifically to avoid the spread to the ARH which also contains NPY neurons. Since no BDA contamination was observed in the ARH, DMH-NPY neurons are likely to be the only sources of NPY fiber projections labeled with BDA. In spite of the small injection volumes, we were unable to distinguish between projections of the two subregions of the DMH since the tracer injections directed at the DMHnc could diffuse into the neighboring DMHc. In an attempt

to distinguish between the DMHc and DMHnc projections, we instead used a combination of models: a) lactating rats that have NPY expression in both DMH subregions, b) virgin rats that have NPY expression specifically in the DMHc, c) DIO mice that have NPY expression specifically in the DMHnc, and d) CD mice that have no NPY expression in the DMH.

Another limitation of these studies is that NPY cells with the DMHnc are scattered throughout the rostral to caudal extent of the nucleus. Therefore, it is likely that the amount of BDA volumes used in this study only labeled a small percentage of NPY neurons in the DMH in each animal. This sparse labeling greatly limits our ability to quantify the density of projections. Due to the technical limitations with the antibody staining, we were also unable to count the number of DMH-NPY neurons labeled with BDA. While we observed some differences in the projections between the models, it is not clear whether this is due to a true difference in projections or due to the number of NPY neurons labeled in each case. Despite these variations, all animals with injection sites within the borders of the DMH showed a similar BDA/NPY co-localized fiber projection pattern.

In the present study, a single optical slice analysis was used to characterize the spatial relationship between BDA/NPY-containing axonal swellings and CART neurons. Although we often detected a close apposition between them in the lactating animals, it is difficult to make any quantifiable conclusions from this observation due to the small number of DMH-NPY projections labeled with BDA. A close apposition within 0.5 μm distance suggests a potential synaptic contact, but it is also not possible from this technique to determine the precise anatomical structure of close appositions without

electron microscopy analysis. However, previous studies which imaged close appositions of peptidergic fibers on a cell soma using confocal microscopy found evidence that the peptidergic appositions were indeed synaptic in nature (Kiyoshi et al., 1998; Takenoya et al., 2006). Furthermore, lack of synapse contacts does not rule out the possibility of volume transmission of NPY over considerable distances to reach the targets (Agnati et al., 2010).

The PVH is the major target for DMH-NPY neurons

According to the previous report (Swanson and Kuypers, 1980), the DMH projects heavily to the parvicellular region of the PVH, which contains neurons that project to sympathetic and parasympathetic preganglionic nuclei, as well as to the median eminence and posterior pituitary (Engelmann et al., 2004; Hallbeck et al., 2001; Sawchenko and Swanson, 1982a). In the present study, BDA fibers containing NPY immunoreactivity were observed in the parvicellular and, although less dense, the magnocellular areas of the PVH in both lactating rats and DIO mice.

TRH neurons are one of the important players in the regulation of food intake and autonomic function in the parvicellular area (Lechan and Fekete, 2006). In the fasting condition, increased NPY inputs from the ARH inhibit TRH production and stimulate food intake (Fekete and Lechan, 2007). Despite high levels of NPY expression in the ARH, lactation is associated with increased TRH expression in the PVH which stimulates prolactin secretion (Sanchez et al., 2007). In addition to its main role in milk production, prolactin has an orexigenic effect and is involved in hyperphagia associated with lactation (Chen and Smith, 2004; Woodside, 2007). CART is also expressed in the parvicellular area (Elias et al., 2001; Fekete et al., 2000), and is linked to the inhibition of TRH

induced prolactin release from the anterior pituitary gland (Fekete and Lechan, 2006). Therefore, an inhibition of CART expression controlled by DMH-NPY neurons could potentially contribute to the increase in prolactin secretion and hyperphagia during lactation. In support of this hypothesis, we demonstrated that DMH-NPY fibers are in close apposition to CART expressing neurons in the PVH in lactating rats. CART expression in the PVH is also implicated in regulation of BAT thermogenesis (Kong et al., 2003; Lechan and Fekete, 2006). Icv injection of CART up-regulates BAT uncoupling protein-1 (UCP-1) mRNA in rats (Wang et al., 2000) and CART mRNA expression in the PVH is increased during cold exposure in lactating rats (Sanchez et al., 2007). Therefore, increased DMH-NPY inputs to suppress CART neurons may also provide some explanation for the suppression of BAT activity during lactation.

The LH is a potential mediator of hyperphagia

Although NPY in the PVH coordinates various aspects of energy metabolism in addition to controlling appetite, NPY in the LH is a potent stimulator of ingestive behavior but may have less of a direct impact on other metabolic parameters (Currie and Coscina, 1995). DMH-NPY neurons project to the PFA of the LH in both lactating rats and DIO mice, suggesting that NPY neurons in the DMHnc are the main sources of DMH-LH projections and may contribute to the hyperphagic behavior. In support of this, BDA/NPY co-localized fibers from the DMHc were scarce in the LH in cycling rats.

The NPY effect on food intake may be mediated by the orexigenic neuropeptides, melanin concentrating hormone (MCH) and orexin, in the LH. Expression of MCH and orexin is significantly increased in the LH during lactation (Sun et al., 2003; Sun et al., 2004), suggesting a potential role in the hyperphagia. However, whether NPY plays any

role in increasing MCH and orexin expression during lactation is unknown. Although some studies have suggested direct actions of NPY on these neurons (Broberger et al., 1998; Horvath et al., 1999), more recent studies have indicated that this may be indirect through modulation of presynaptic inputs into the MCH and orexin neurons. NPY Y-R1 is not expressed in MCH and orexin neurons, but it was detected in nitric oxide synthesizing (NOS) neurons which also stimulate food intake (Fetissov et al., 2003; Morley and Flood, 1991). This is supported by the finding that NPY injection into the LH did not significantly stimulate c-Fos in either MCH or orexin neurons, although c-Fos expression was observed in a population of neurons with unknown phenotype (Campbell et al., 2003b). Therefore, NPY effect on food intake may be mediated by other unidentified cell types in the LH.

Potential role of DMH-NPY neurons in suppression of reproduction

The increase in hypothalamic NPY activity during lactation may be a key element in linking changes in food intake to the suppression of GnRH neuronal activity. NPY fibers from the ARH make direct contacts with GnRH neurons and act as an inhibitory signal to GnRH/LH secretion during lactation where estrogen levels are very low and NPY is chronically elevated (Smith et al., 2010). The NPY effect on GnRH neurons is directly mediated by NPY Y5 receptors and/or an indirect modulation via NPY Y1 receptors (Campbell et al., 2001; Li et al., 1999b). In the present study, we failed to observe DMH-NPY terminals derived from the DMH on GnRH cell bodies. Therefore, it is likely that DMH-NPY neuronal projections may affect other neuronal functions that are directly linked to the regulation of GnRH neurons.

AVPV-CART neurons send projections to the area where GnRH neurons are located and CART fibers make close contacts with GnRH neurons (Leslie et al., 2001; Rondini et al., 2004). CART increases GnRH pulse amplitude in cycling females and decrease GnRH pulse intervals in prepubertal rats (Lebrethon et al., 2000; Parent et al., 2000). In the present study, we demonstrated that DMH-NPY fiber terminals are in close apposition to the CART neurons within the AVPV in lactating rats. Furthermore, CART neurons in the AVPV appear to be inhibited in lactating rats (unpublished observation). Our group has recently reported that kisspeptin neurons in the AVPV are inhibited during lactation (True et al., 2011). Since kisspeptin in the AVPV is stimulatory to GnRH functions (Kinoshita et al., 2005; Smith et al., 2006; Smith et al., 2005), suppression of kisspeptin release may contribute the suppression of reproductive function during lactation. Therefore, our studies identify DMH-NPY neurons as candidates for inhibiting CART neurons during lactation, resulting in suppressed GnRH activity.

Projections of DMH-orexin neurons involved in BAT thermoregulation

Since the DMH contains neurons that are synaptically linked to the premotor neurons involved in BAT regulation (Bamshad et al., 1999; Oldfield et al., 2002), NPY neurons in the DMH might be responsible for the alteration of BAT energy expenditure in lactation and obesity. One of the critical brainstem areas involved in the sympathetic regulation of BAT is the rRPa which has been shown to contain dense NPY terminals. However, our data showed that NPY neurons from the DMH do not project to the rRPa in either the lactating rat or DIO mouse. Instead, NPY terminals in the rRPa are exclusively derived from the brainstem catecholamine neurons (unpublished observation). It is well established that orexin is also involved in the modulation of sympathetic nerve activity

and BAT thermogenesis (Date et al., 1999; Szekely et al., 2002; Tupone et al., 2011). Furthermore, orexin administration to the fourth ventricle activates c-Fos expression in the rRPa and orexin fibers are detected in close apposition with BAT projecting neurons in the rRPa (Berthoud et al., 2005; Tupone et al., 2011; Zheng et al., 2005). Indeed, we demonstrated that the BDA fibers within the rRPa were co-labeled with orexin and that orexin-BDA fibers make close apposition to serotonin neurons. The serotonin neurons in this region have been reported to be important for the regulation of BAT thermogenesis (Madden and Morrison, 2010; Tupone et al., 2011).

Functional significance and summary

NPY induction in the DMHnc in both rats and mice during hyperphagic conditions suggests a key role in the stimulation of food intake. In a recent study, AAV mediated RNA interference was used to specifically knock down DMH-NPY expression in DIO rats which reduced food intake and induced a protection from HFD -induced obesity (Chao et al., 2011), suggesting that NPY expression in the DMH may be partially responsible for the development of obesity. Additionally, these rats exhibited increased BAT sympathetic activity and BAT differentiation. These findings strongly imply that DMH-NPY expression is involved in an inhibitory regulation of BAT thermogenesis as well as the stimulation of food intake. However, the mechanisms by which DMH-NPY neurons regulate feeding behavior and BAT thermogenesis are not well understood. In the current study, we identified DMH-NPY projections to the PVH and LH as potential pathways for the hyperphagia and BAT thermogenesis in lactation and diet-induced obesity. NPY neurons in the DMHc are clearly not the major orexigenic inputs to these hypothalamic targets in normal conditions. Our data suggest that hormonal/neural

adaptations during physiological conditions requiring energy conservation activate additional NPY neuronal populations in the DMH and increase DMH-NPY inputs to the PVH and LH, contributing to the hyperphagia and reduced BAT thermogenesis. Although we provided evidence for possible interactions between DMH-NPY projections and CART neurons, additional studies are needed to confirm the functional connection between two neurons.

In summary, we characterized the distribution of DMH-NPY neuronal projections in the brain areas involved in the regulation of food intake and BAT thermogenesis in two chronic hyperphagic models. Comparison of DMH-NPY projections from two hyperphagic models clearly suggests that DMH-NPY induction plays a critical role in promoting hyperphagia, reduced BAT thermogenesis, and suppression of reproduction via hypothalamic projections, but not the brainstem (Fig. 3-10). The current findings provide a neuroanatomical framework to understand the potential roles and mechanism of DMH-NPY neurons.

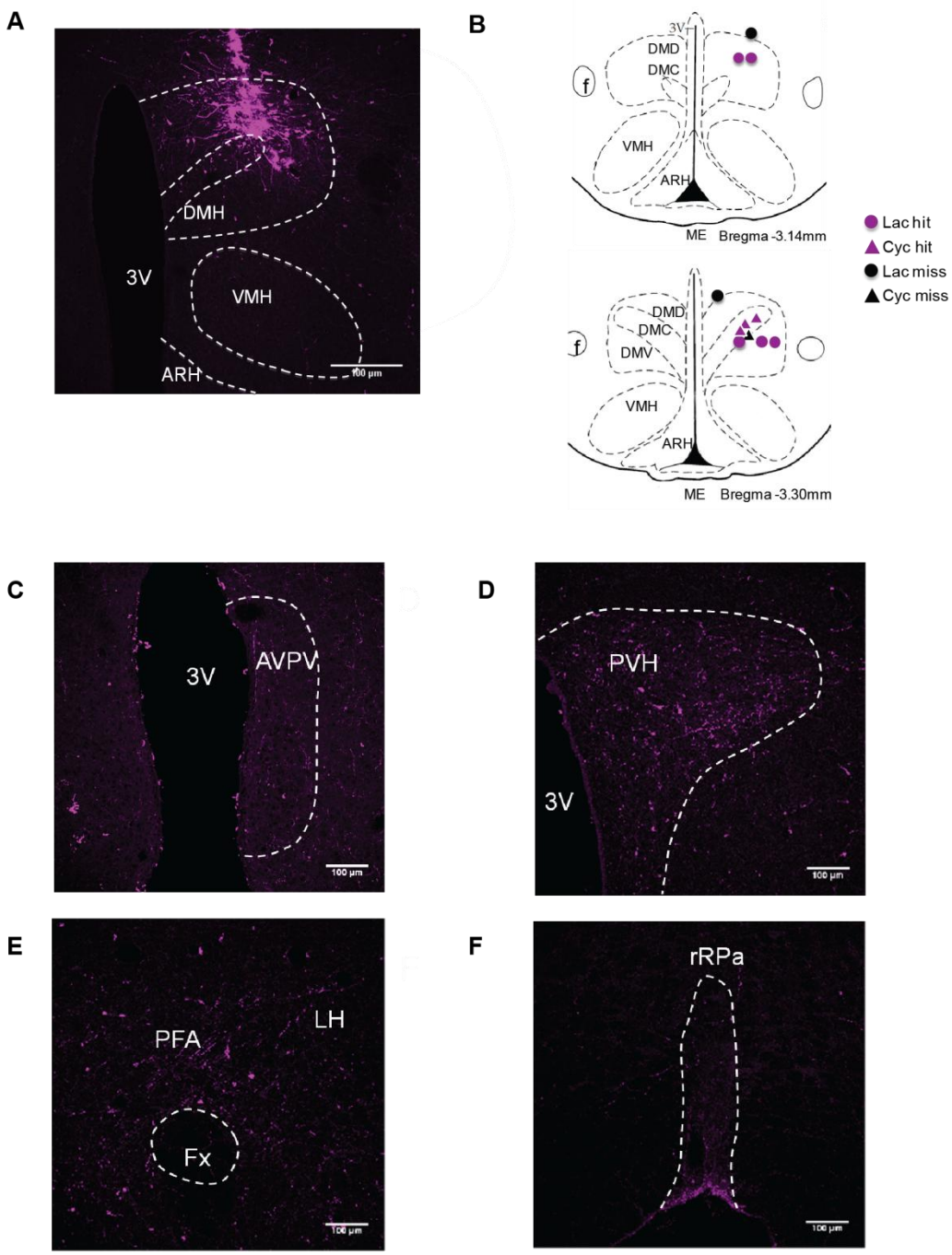

Figure 3-1. BDA injection in the DMH of lactating rats.
(A) Injection site in the DMH in 10x confocal image. BDA (magenta) was visualized 7 days after injection using streptavidin amplification method. (B)The mapping of BDA injection sites in rostral (upper) and caudal (lower) DMH. Lactating rats (circle) and cycling rats (triangles) hit and miss cases are illustrated. BDA fiber distribution in the AVPV (C), PVH (D), LH/PFA (E), and rRPa (F) are illustrated in 20x confocal images.

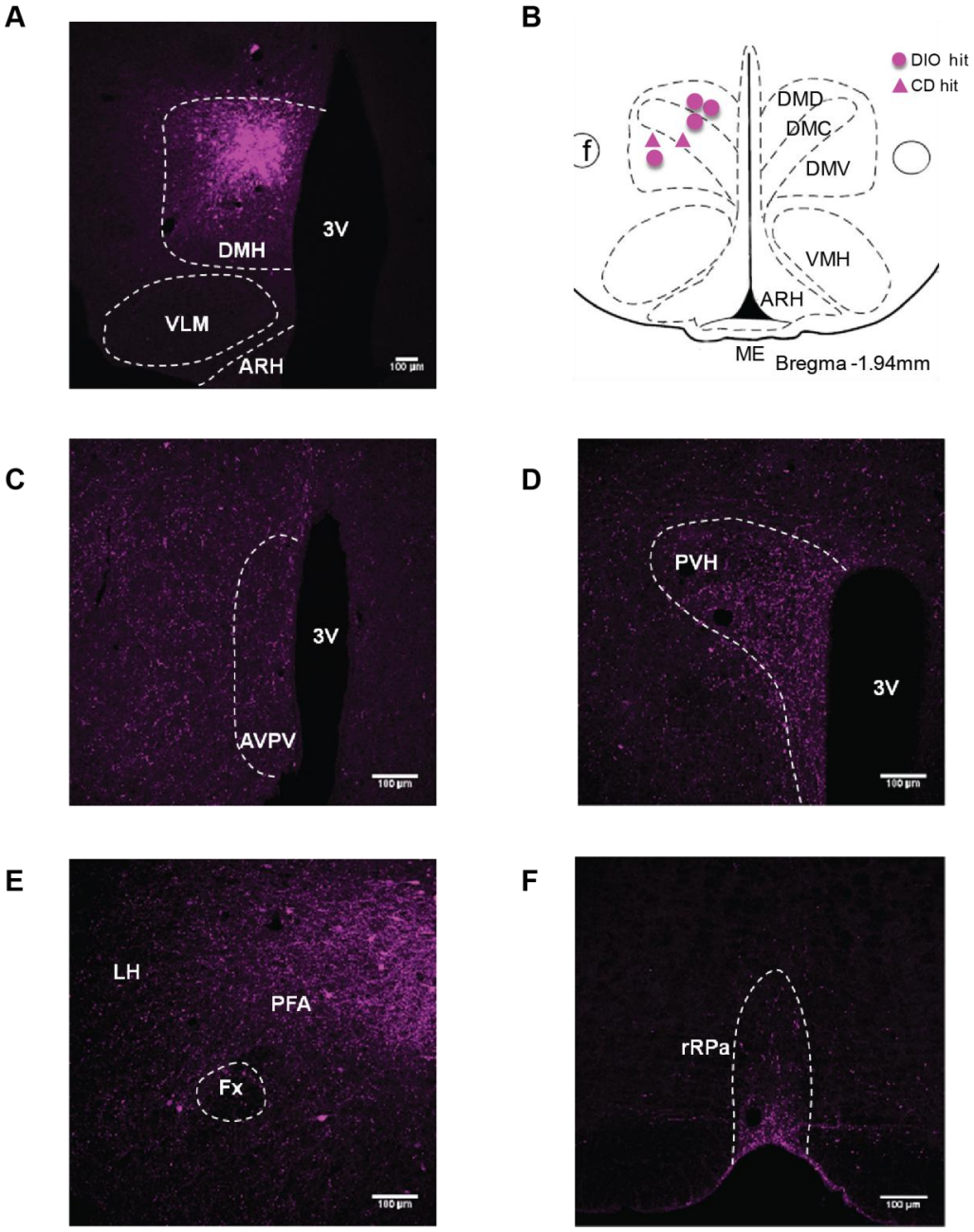

Figure 3-2. BDA injection in the DMH of DIO mice.
(A) Injection site in the DMH in 10x confocal image. BDA (magenta) was visualized 3 days after injection. (B) The mapping of BDA injection sites. The circles indicate the hit cases in DIO group and triangles indicate CD mice. BDA labeled fibers were mainly distributed in the AVPV (C), PVH (D), LH (E) in the hypothalamus, and rRPa (F) in the brainstem illustrated in 20x confocal images.

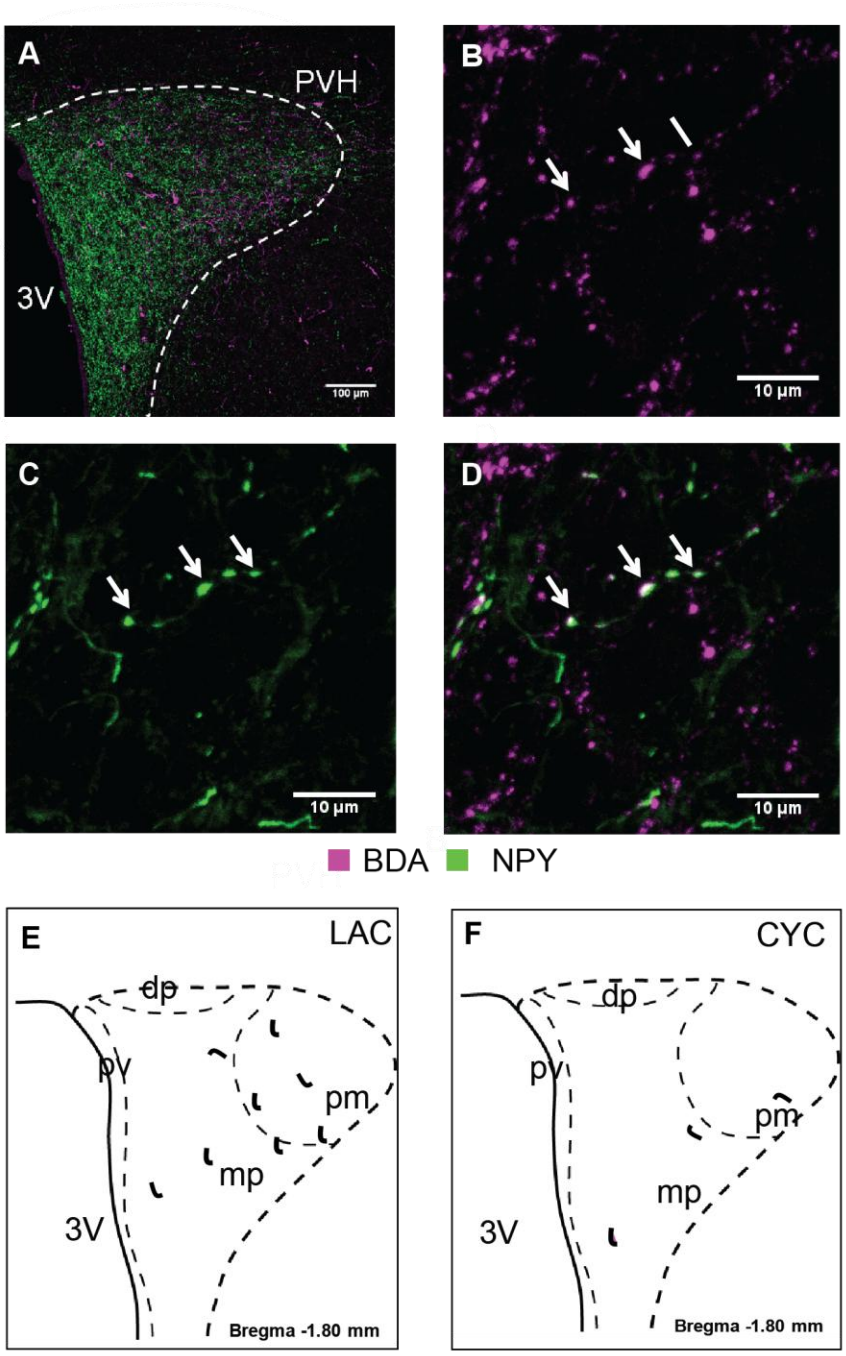

Figure 3-3. DMH-NPY projections to the PVH in lactating rats.
(A) 20x confocal image showing BDA (magenta) and NPY (green) fiber distribution in the PVH. (B) BDA fiber distribution in 40x confocal image. Arrows indicate BDA/ NPY co-localized fiber. (C) NPY fiber distribution in 40x confocal image. Arrows indicate BDA/NPY co-localized fiber. (D) Overlay of 40x confocal images showing BDA/NPY co-localized fiber. Schematic drawings show the distribution of BDA fibers co-localized with NPY in the PVH of lactating (E) and cycling (F) rat.

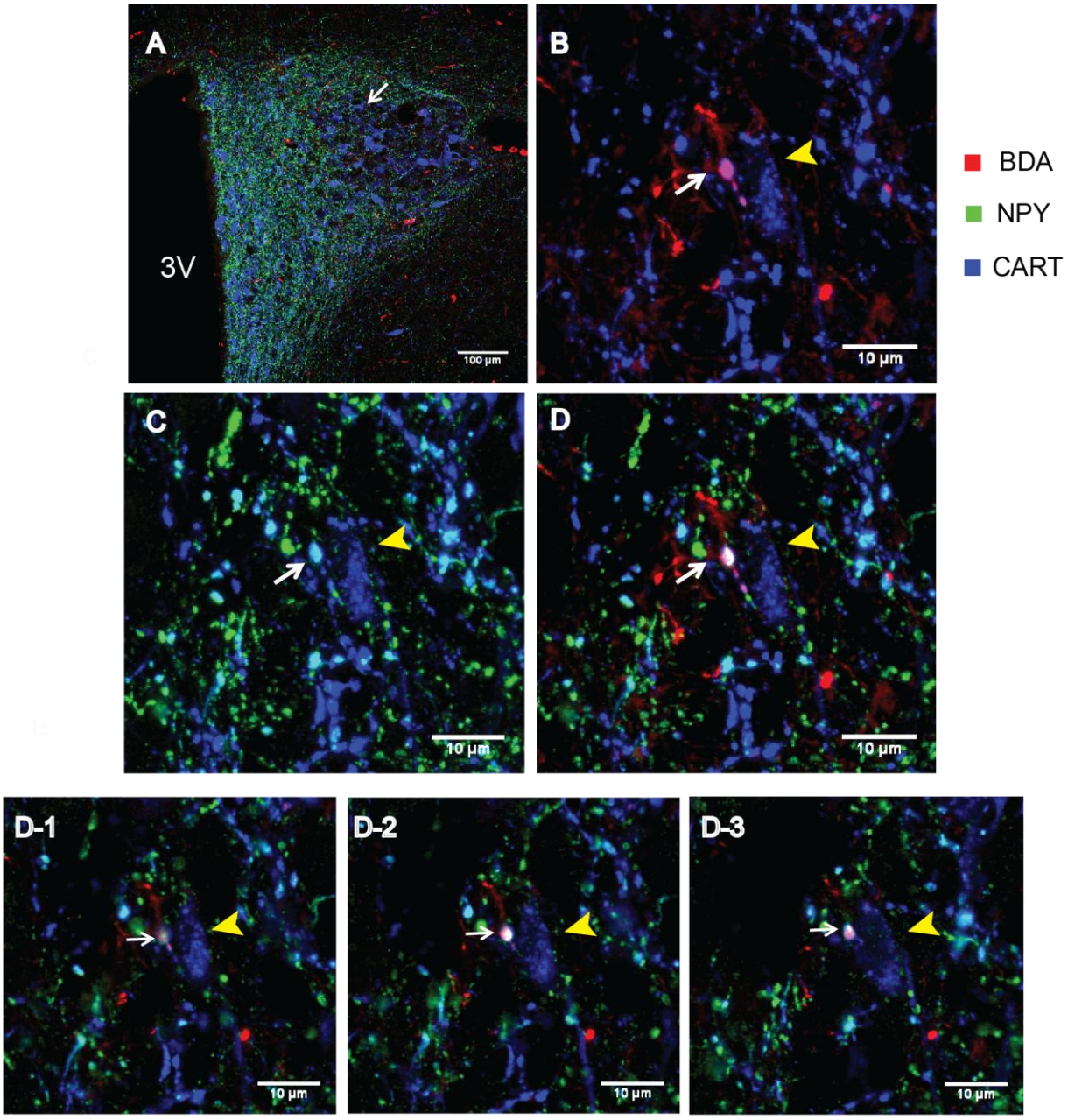

Figure 3-4. DMH-NPY projections in close apposition with CART neurons in the PVH of lactating rats.

(A) 20x confocal image showing triple label immunostaining for NPY (green), BDA (red), and CART (blue) in the PVH of a lactating rat. The arrow indicates a fiber containing NPY and BDA near a CART cell body. (B) 40x confocal image showing BDA-containing axonal swelling(white arrow) in close apposition to a CART cell (yellow arrowhead). (C) 40x confocal image showing NPY-containing axonal swelling in close apposition to a CART cell. (D) Overlay image for BDA and NPY co-localized fiber in close apposition to a CART cell. (D-1~3) Stacks of optical slices (3 µm total at 0.5µm increments) of a CART cell body in close contacts with BDA and NPY-containing axonal swelling. Z-coordinates are indicated in single optical slices relative to a reference point, z = 0 µm.

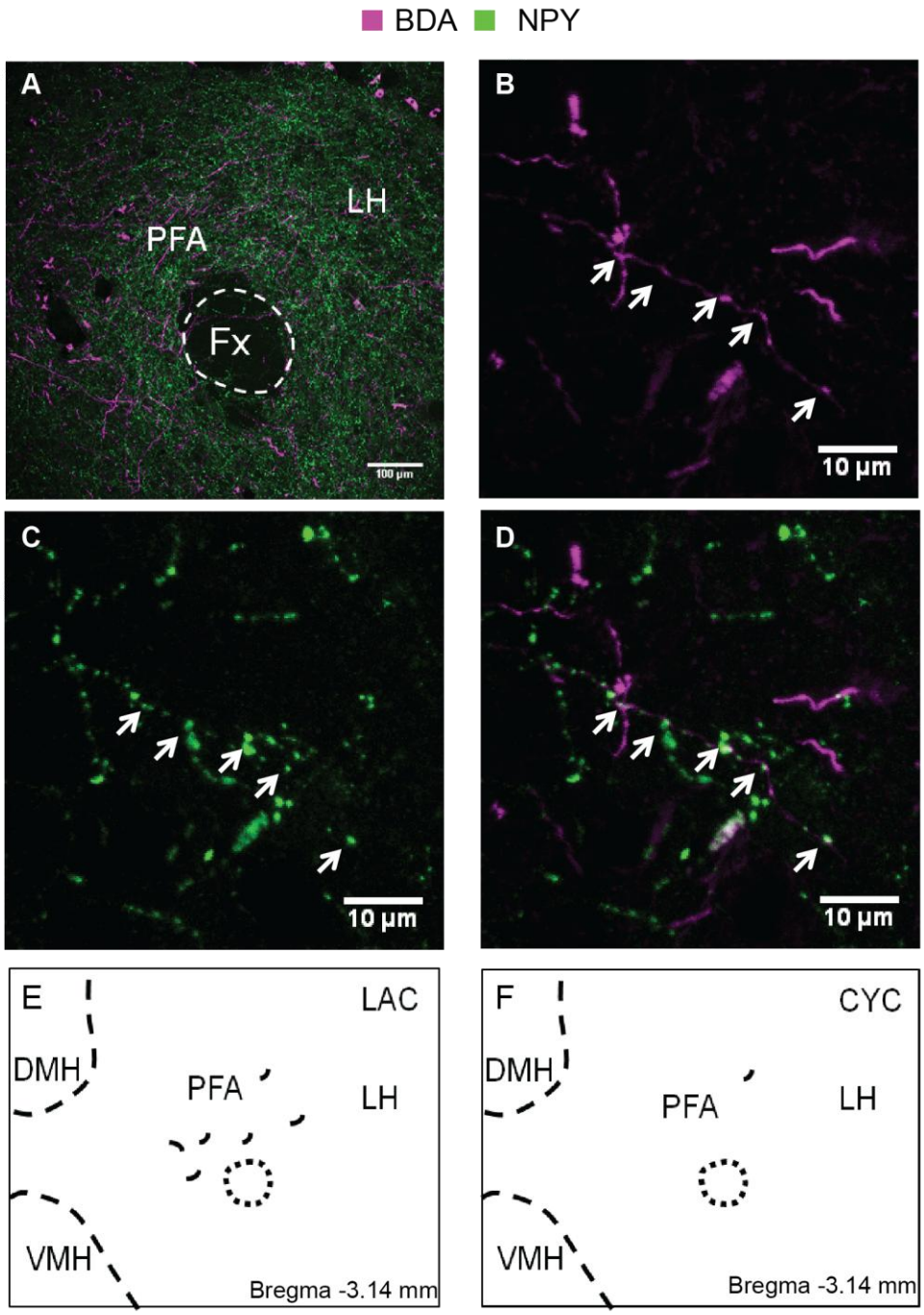

Figure 3-5. DMH-NPY projections to the LH in lactating rats.
(A) 20x confocal image showing BDA (magenta) and NPY (green) fiber distribution in the LH. (B) BDA fiber distribution in 40x confocal image. Arrows indicate the co-localized fiber. (C) NPY fiber distribution in 40x confocal image. (D) Overlay 40x confocal image showing BDA/NPY co-localized fiber indicated by arrows. Schematic drawing showing the distribution of BDA fibers co-localized with NPY in the LH of lactating (E) and cycling (F) rats.

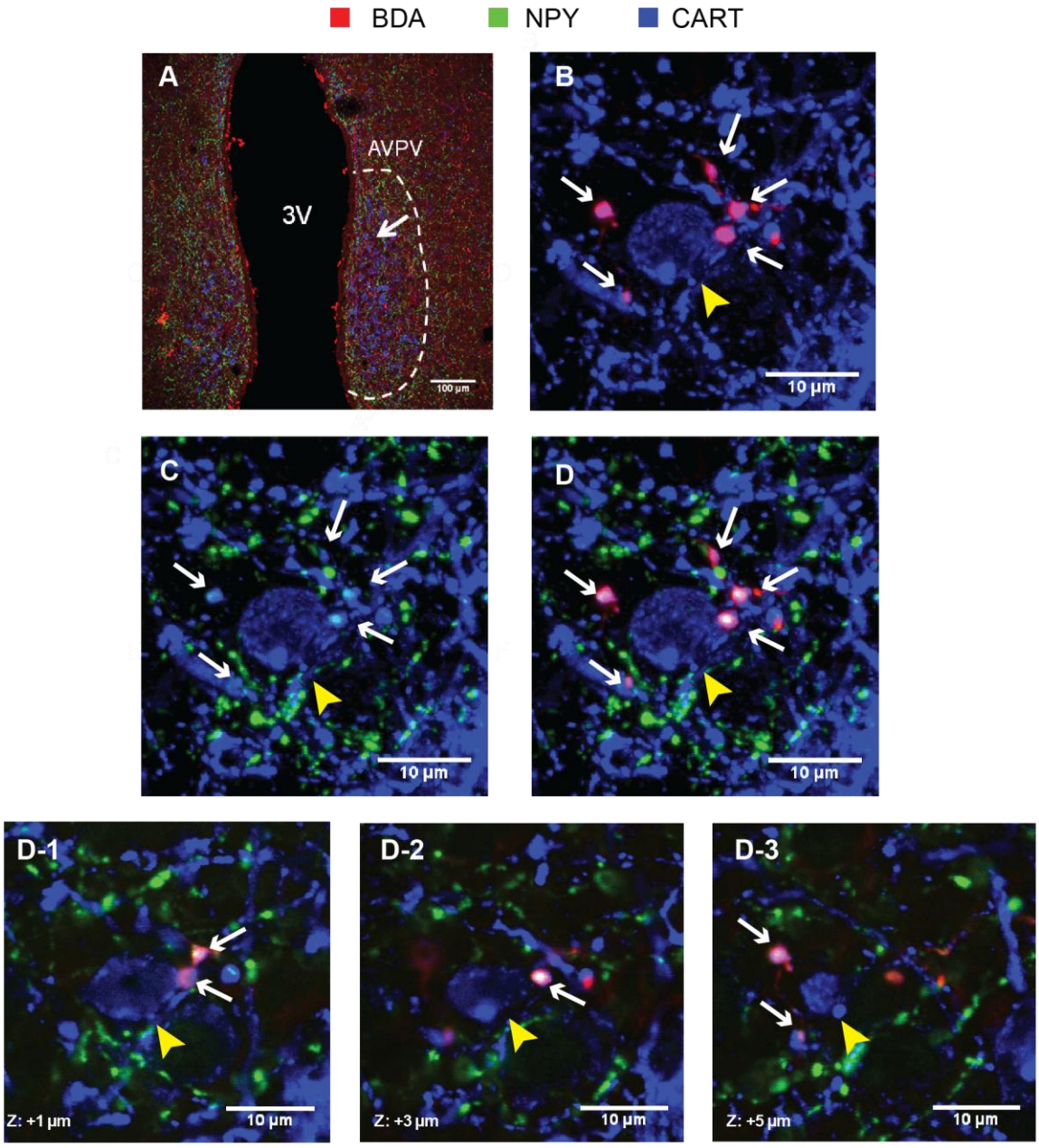

Figure 3-6. DMH-NPY fibers in close apposition to CART neurons in the AVPV of lactating rats.

(A) 20x confocal image showing triple label immunostaining for NPY (green), BDA (red), and CART (blue) in the AVPV of a lactating rat. The arrow indicates a fiber containing immunoreactivity for NPY and BDA near a CART neuron. (B) 40x confocal image showing BDA containing axonal swellings (white arrows) in close apposition to a CART neuron (yellow arrowhead). (C) 40x confocal image showing NPY-containing axonal swellings (white arrows) in close apposition to a CART neuron. (D) Overlay image for BDA/ NPY-containing axonal swellings in close apposition to a CART neuron. (D-1~3) Analysis of single optical slice (4 µm total, 0.5 µm increments) reveals a close apposition.

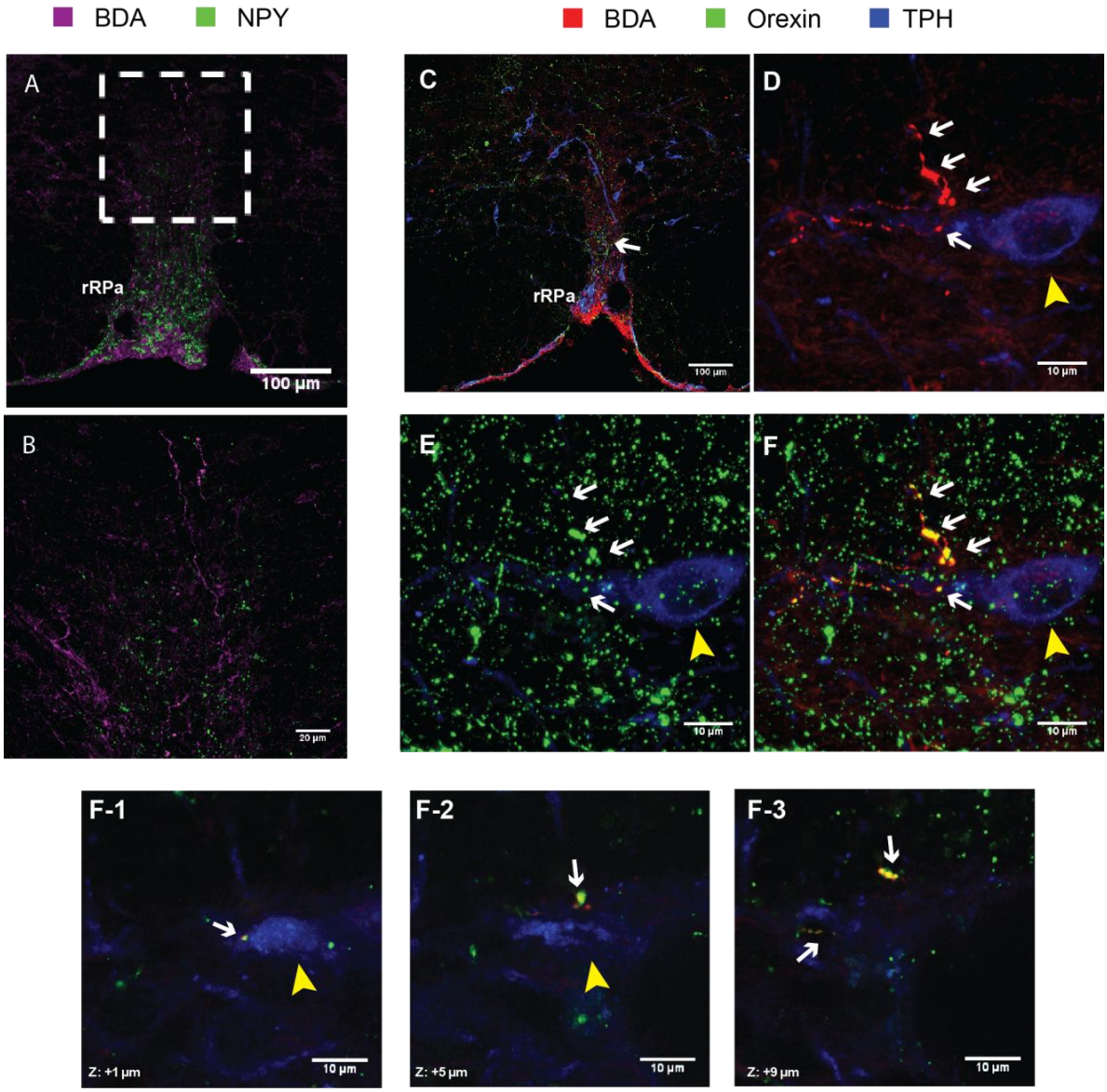

Figure 3-7. DMH-NPY neurons do not project to the rRPa, but DMH-orexin neurons project to serotonin neurons in the rRPa in lactating rats.
(A) 20x confocal image showing BDA (magenta) and NPY(green) fiber distribution in the rRPa in a lactating rat. (B) A magnified image of dotted area in (A) showing no co-localization for BDA and NPY fibers. (C) 20x confocal image showing triple label immunostaining for orexin (green), BDA (red), and TPH (blue) in the rRPa. The arrow indicates a fiber containg orexin and BDA near a TPH expressing neuron. (D) 40x confocal image showing a BDA-containing axonal swellings (white arrows) in close apposition to a TPH neuron (yellow arrowhead). (E) 40x confocal image showing a orexin fiber in close apposition to a TPH neuron. (F) Overlay image for a BDA/orexin co-localized fiber in close apposition to a TPH neuron. (F-1~3) Analysis of single optical slice (8 µm total, 1 µm increments) confirms that BDA/orexin-containing axonal swellings are in close apposition to a TPH neuron.

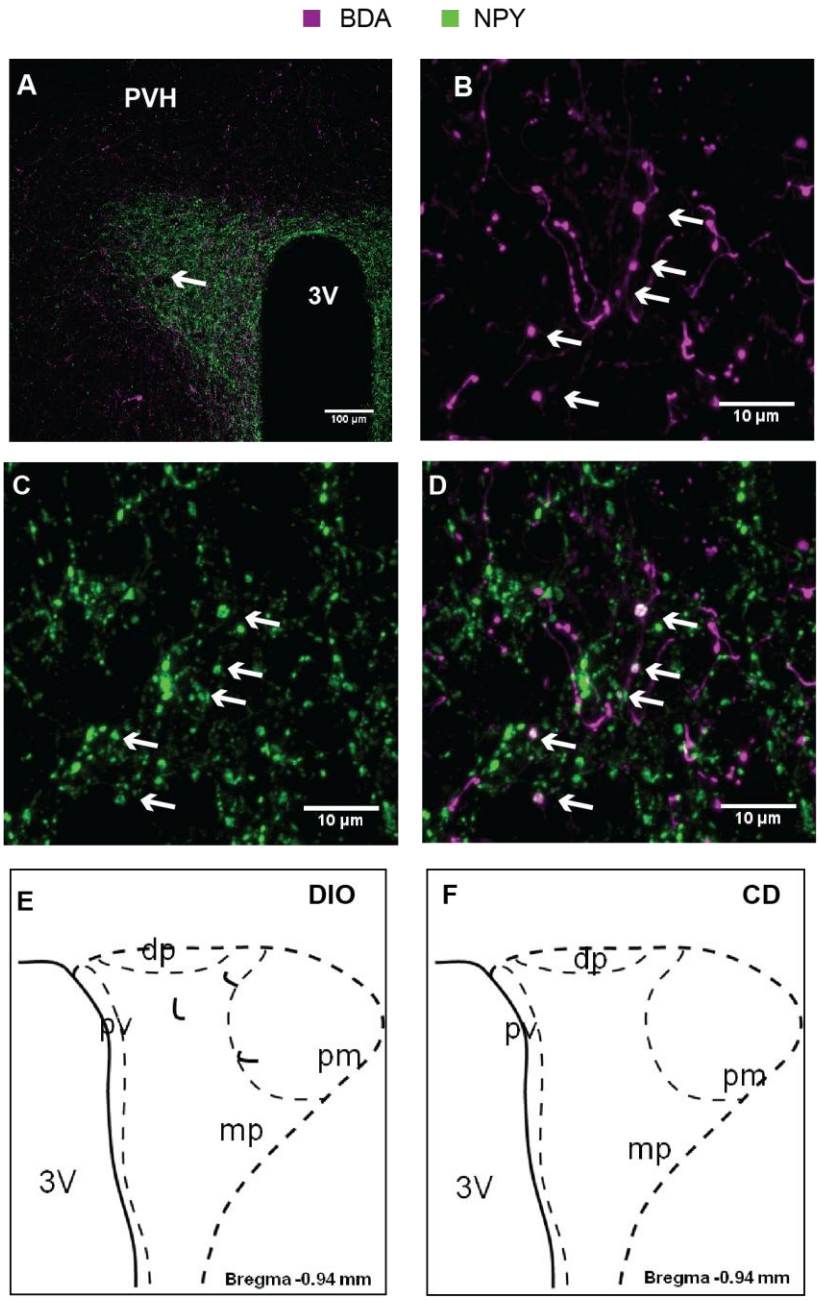

Figure 3-8. DMH-NPY projections to the PVH in DIO mice.
(A) 20x confocal image showing BDA (magenta) and NPY (green) immunostaining in the PVH. An arrow indicates the location of BDA/NPY co-localized fiber. (B) 40x confocal image showing BDA fibers in the PVH. (C) 40x confocal image showing NPY fibers in the PVH. (D) Overlay 40x confocal image showing BDA/NPY co-localized fiber in the PVH. Arrows indicate the location of co-localization. Schematic drawings show the distribution of BDA fibers co-localized with NPY in the PVH of a DIO mouse (E) and no BDA/NPY co-localized fibers in a CD mouse (F).

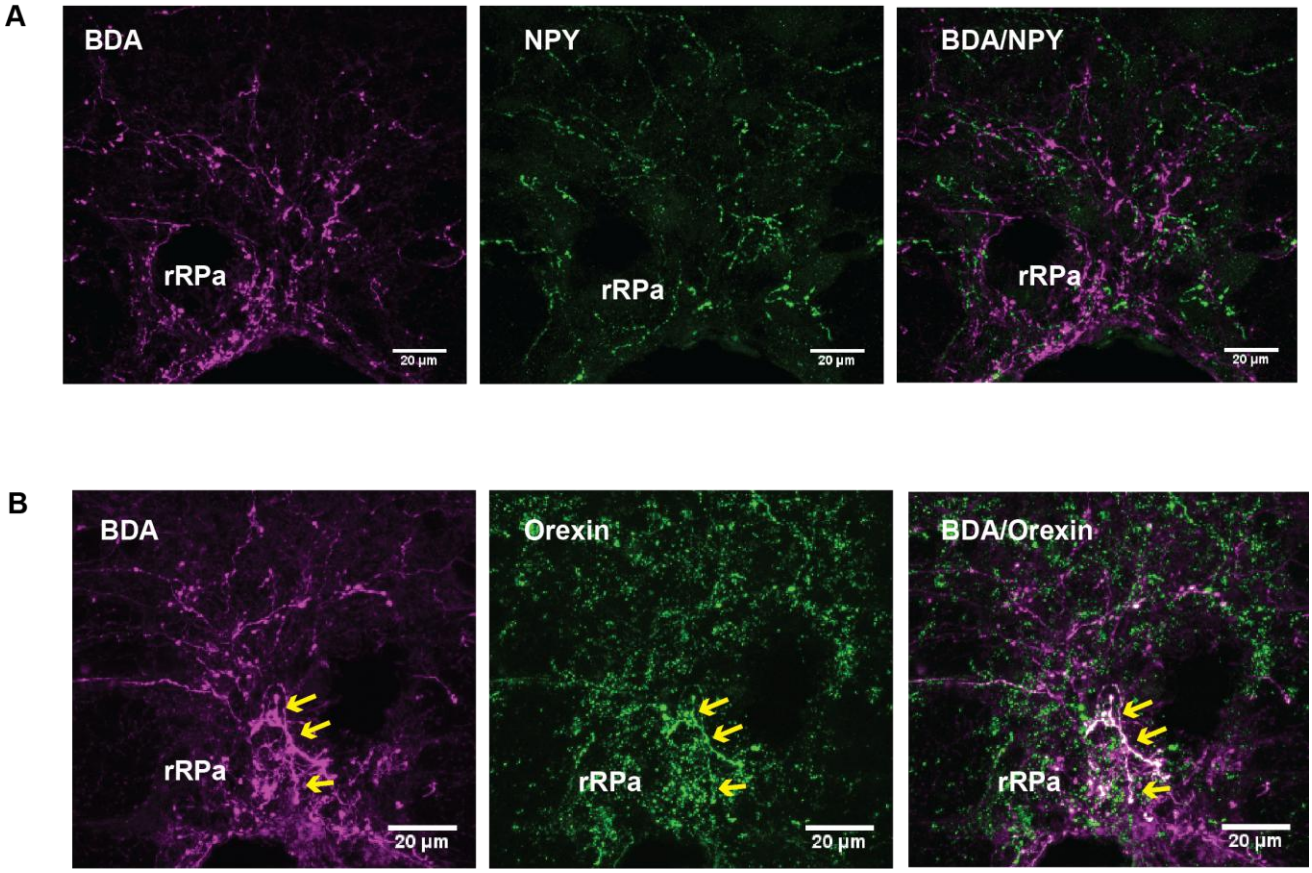

Figure 3-9. DMH-NPY neurons do not project to the rRPa, but DMH-orexin neurons project to the rRPa in DIO mice.
(A) 20x confocal images showing BDA (magenta, left) , NPY(green, middle) and BDA/NPY (right) fiber distribution in the rRPa in a DIO mouse. No BDA/NPY co-localization is observed.
(B) 20x confocal images showing BDA (magenta, left) , orexin (green, middle) and BDA/orexin (right) fiber distribution in the rRPa in a DIO mouse. Yellow arros indicate BDA/orexin co-localization.

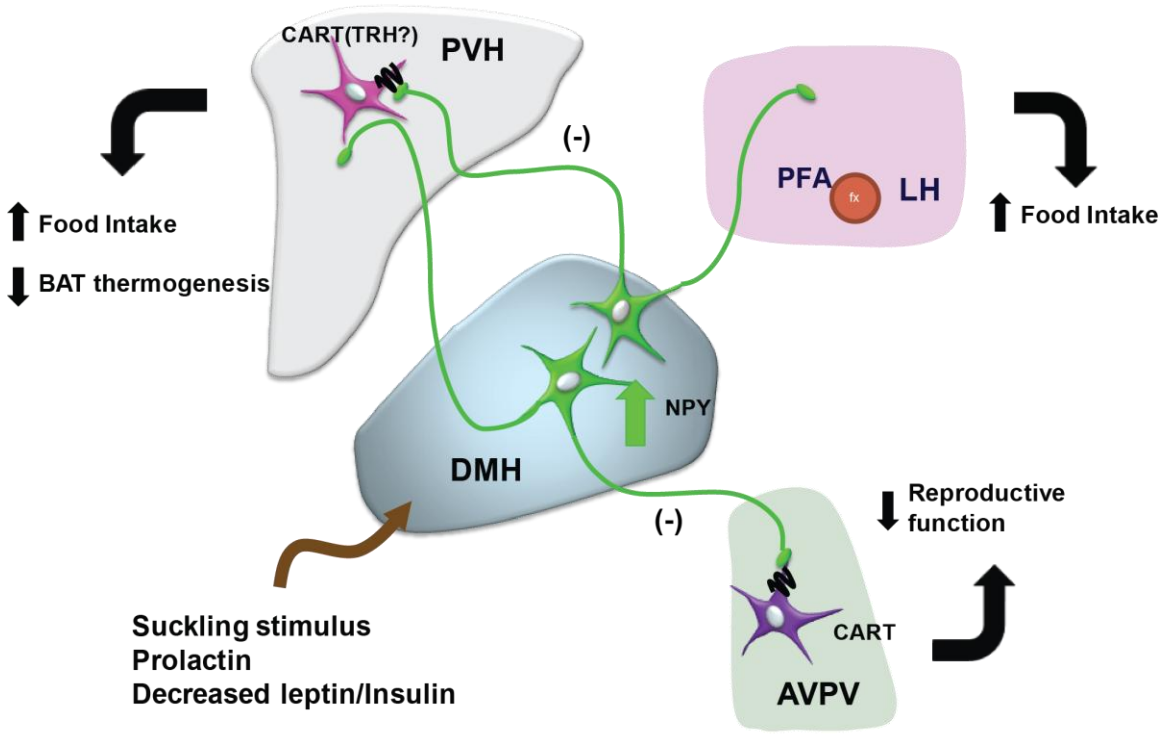

Figure 3-10. A schematic of DMH-NPY projections activated during lactation and the potential effects on food intake, BAT thermogenesis, and reproduction.

Chapter 4

NPY and CART co-expressing neurons in the dorsomedial hypothalamus are sensitive to leptin in diet-induced obese mice

Shin J Lee, Melissa Kirigiti, Sarah R Linsley, Alberto Loche, M Susan Smith, and Kevin L Grove

Abstract

NPY neurons in the DMH have been implicated in the regulation of feeding behavior and brown adipose tissue (BAT) thermogenesis. DMH-NPY mRNA expression is absent during normal conditions in mice, but is induced in diet-induced obese (DIO) mice. Interestingly, increased CART mRNA expression has been reported in the same region of the DMH in DIO mice, suggesting that NPY and CART together may serve an important function during DIO development. To characterize DMH-NPY and CART expression during DIO development, C57BL/6 male mice were fed either high fat diet (HFD) or normal chow diet (CD) for 2, 10, 16 and 20 weeks. Using in situ hybridization, we demonstrated that DMH-CART mRNA expression was significantly up-regulated by 10 weeks in HFD fed mice, followed by a significant NPY mRNA induction in the same region in the DMH between 16 to 20 weeks. DMH-NPY mRNA expression was decreased in response to fasting in DIO mice, while DMH-CART mRNA was not affected. Double label in-situ hybridization revealed that NPY and CART mRNAs are co-localized in the same neurons in the DMH of DIO mice. In addition, we characterized DMH neuronal projections containing NPY/CART immunoreactivity which were distinguishable from the projections from other NPY/CART expressing neuronal populations in the brainstem. Leptin administration activated p-STAT3 and c-fos expression in DMH-NPY/CART neurons, suggesting a role of leptin in the regulation of NPY/CART co-expressing neurons in DIO mice. The present study provides neuroanatomical evidence of NPY/CART co-expressing neurons in the DMH under the direct regulation of leptin in DIO mice.

INTRODUCTION

Obesity is a serious health issue that often leads to life threatening diseases such as type II diabetes and cardiovascular diseases and affects more than 300 million people worldwide (Daniels, 2009; Kelly et al., 2008; Tsiros et al., 2009). One of the key players in the central regulation of body weight homeostasis is leptin, a hormone secreted by fat cells, which signals the brain the status of body energy stores, inhibits food intake, and promotes energy expenditure (Friedman, 2009a). However, leptin treatment is not effective in the majority of obese humans and animals who exhibit high levels of circulating leptin, to which they are apparently resistant (Halaas et al., 1997; Heymsfield et al., 1999). Therefore, leptin resistance is emerging as a permissive condition for obesity (Friedman, 2009a), allowing the hyperphagic behavior and decreased energy expenditure.

A complicated network of brain and peripheral interactions maintains a consistent body weight and prevents any disruption in energy balance. The arcuate nucleus of the hypothalamus (ARH) receives peripheral feedback signals, and modulates feeding behavior and energy expenditure according to the nutritional state of the animal (Pandit et al., 2011). The ARH contains at least two major neuronal populations that relay metabolic signals to other brain areas implicated in the regulation of body weight homesostasis. Neuropeptide Y (NPY)/agouti related peptide (AgRP) neurons promote feeding behavior and are inhibited by leptin, while pro-opiomelanocortin (POMC)/cocaine- amphetamine-regulated transcript (CART) neurons produce anorexigenic peptides under direct stimulation of leptin (Ahima et al., 1996; Cowley et al., 2001; Schwartz et al., 1997; Spanswick et al., 1997; Stephens et al., 1995).

The dorsomedial hypothalamus (DMH) has been highlighted as an important feeding center ever since DMH lesions were demonstrated to cause hypophagia and weight reduction (Bellinger and Bernardis, 2002). When DMH lesioned rats are fed a palatable diet, they do not become as obese as the control rats (Bernardis and Bellinger, 1986, 1991), suggesting that DMH lesions provide some protection against diet-induced obesity (DIO). DMH neurons may play an important role in maintaining normal energy balance by relaying the ARH inputs to the PVH where the feeding related signals are integrated (Bai et al., 1985; ter Horst and Luiten, 1986; Thompson et al., 1996). However, the phenotypes of DMH neurons involved in energy homeostasis have not been extensively characterized.

Similar to the ARH, the DMH contain NPY-expressing neurons that are associated with food intake and energy expenditure (Bernardis and Bellinger, 1998). However, while NPY mRNA is constitutively expressed in the ARH, it is expressed in the DMH in mice only during chronic hyperphagic conditions such as postnatal development, lactation, and obesity (Grove et al., 2001; Guan et al., 1998a; Guan et al., 1998b; Kesterson et al., 1997; Li et al., 1998a; Tritos et al., 1998). In 1998, Guan et al. discovered that NPY mRNA expression is induced in the DMH in DIO mice (Guan et al., 1998a). One of the main characteristics of DIO model is high levels of circulating leptin, which contributes to the state of leptin resistance and obesity (Lin et al., 2000; Munzberg et al., 2004). DMH-NPY mRNA induction is also observed in genetically obese mice such as melanocortin receptor 4 (MC4R) KO mice and agouti mice that are characterized by hyperleptinemia (Kesterson et al., 1997), suggesting that DMH-NPY induction is strongly associated with hyperleptinemia. In support of this hypothesis, genetically leptin

deficient *ob/ob* mice or *db/db* mice with leptin receptor mutation do not express NPY in the DMH despite their morbid obesity and hyerphagia (Kesterson et al., 1997). Nonetheless, the direct effect of leptin on DMH-NPY neurons is unknown.

CART-expressing neurons in the DMH are under the direct regulation of circulating leptin (Elias et al., 2001); however, the role of DMH-CART neurons in feeding regulation is poorly understood. A recent study showed that CART mRNA expression is up-regulated in the DMH in DIO mice (Yu et al., 2008), suggesting that DMH-CART neurons play a critical role in obesity development. CART was discovered as an anorectic peptide since intracerebroventricular (ICV) injection of CART peptides decreases food intake and an antiserum against CART increases feeding in normal rats (Kristensen et al., 1998). CART-expressing neurons in the ARH are positively regulated by leptin, and fasting, which is associated with a reduction in circulating leptin, significantly reduces CART mRNA expression in the ARH. Furthermore, reduced CART expression in the ARH and DMH in the *ob/ob* mouse can be normalized by exogenous leptin treatment, supporting the anorectic role of CART regulated by leptin. However, several studies have demonstrated that CART is not a simple anorectic agent and may play an opposite role in feeding behavior. For example, CART injection into specific hypothalamic nuclei including the DMH and PVH increases food intake (Abbott et al., 2001; Hou et al., 2010). Overexpression of CART in the PVH using viral mediated gene delivery also increases food intake and weight gain in rats (Smith et al., 2008), suggesting that CART has dual properties in feeding regulation depending on the site of its action.

To better understand the role of NPY and CART up-regulation in the DMH in DIO mice, we designed a time course study to examine NPY and CART expression in the

DMH during the development of DIO. We discovered that NPY and CART are co-localized in the same neurons in the DMH in DIO mice after 20 week HFD treatment. Additionally, combined with the anterograde tracer biotinylated dextran amine (BDA), we confirmed the presence of NPY/CART co-labeled fibers in the hypothalamic targets implicated in feeding behavior and BAT thermogenesis. We also investigated the direct role of leptin on NPY/CART neurons in the DMH by intraperitoneal injection of leptin in DIO mice.

MATERIALS AND METHODS

Animals

4 weeks old C57BL6 male mice were purchased from the Jackson laboratory (Bar Harbor, Maine, USA) for in situ hybridization experiments. For immunohistochemical experiments, hemizygous NPY-hrGFP male mice on a C57BL6 background were purchased from the Jackson Laboratory (stock # 006417) for breeding. These mice express humanized renilla reniformis Green Fluorescent Protein (hrGFP) under the control of the mouse NPY promoter. NPY-hrGFP male mice were bred to C57BL/6 female mice (purchased from Jackson laboratory) to generate the experimental animals. The litters were normally born and the pups were toe-clipped for identification and genotyping. Five mice were group-housed in the same cage and maintained under a 12 hr light/dark cycle (lights on at 7:00 A.M.) and constant temperature (23 ± 2°C). Food and water were provided *ad libitum*. All animal procedures were approved by the Oregon National primate Research Center Institutional Animal Care and Use Committee.

Diet treatment

To generate DIO mice, 5 week-old C57BL6 mice were fed a 60% high fat diet (HFD; Research Diets, NJ, USA, Cat# D12492) or normal chow diet (CD; Purina lab chow #5001) for 2, 10, 16 and 20 weeks (n=5 for each group). A cohert of the 20 week HFD animals were fasted 24 hours prior to sacrifice. C57BL6 mice were chosen because they have been widely used and known to be more susceptible to DIO compared to other strains of mice (Surwit et al., 1988). The same diet treatment regime was used for the study with NPY-hrGFP mice. Animals were sacrificed in the morning at the end of diet treatment and food was removed from the cage 3 hours prior to the tissue collection. HFD and CD mice brains were collected fresh frozen for in-situ hybridization experiments and trunk bloods were collected for glucose and hormonal assay. Brown adipose tissue (BAT) was collected for the measurement of UCP1. The brains from NPY-hrGFP mice were collected after transcardial perfusion with 4% paraformaldehyde containing sodium phosphate buffer (pH 7.4).

Glucose, leptin and insulin measurements

For the glucose measurements, 10 µl of trunk blood was applied to the glucose strips (Roche, Cat # 12030381001). For leptin and insulin measurements, the serum was separated from collected trunk blood samples and aliquoted for radioimmunoassay (RIA). Leptin and insulin RIAs were performed by the Oregon National Primate Research Center Endocrine Service Lab using the leptin (Linco Research, Inc., St. Charles, MO, USA, Cat # ML-82K) and insulin (Linco Research, Inc., Cat# RI-13K) RIA kit with a lower detection threshold of 0.5 ng/ml.

Real time PCR

Real-time PCR was performed to quantify mRNA levels of uncoupling protein 1 (UCP1) and β3-adrenergic receptors (Adrb3). 18S RNA was used as the internal control. BAT RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, Cat# 15596-026) and purified using an affinity resin column (QIAGEN, Valencia, CA). RNA samples were prepared for real-time PCR by random-primed reverse transcription reaction using random hexamer primers (Promega, Madison, WI) and 1 μg of RNA. The reverse transcription reaction was then diluted 1:50 for PCR analysis. Reactions were conducted in triplicate for increased accuracy. Ten microliters of reaction mixture contained 5 μl TaqMan Universal PCR Master Mix, 300 nM specific target gene primers, 80 nM 18 S RNA gene primers, 250 nM specific probes, and 2 μl cDNA. The amplification was performed as follows: 2 min at 50 C, 10 min at 95 C, then 40 cycles each at 95 C for 15 sec and 60 C for 60 sec in the ABI/Prism 7700 Sequences Detector System (Applied Biosystems, Foster City, CA). After PCR was completed, baseline and threshold values were set to optimize the amplification plot, and the data were exported to an Excel spreadsheet. Standard curves were drawn on the basis of the log of the input RNA *vs.* the critical threshold cycle, which is the cycle in which the fluorescence of the sample was greater than the threshold of baseline fluorescence. These standard curves allowed for the critical threshold values to be converted to relative RNA concentrations for each sample. 18S RNA amplifications were conducted with the Pre-Developed TaqMan Assay Reagent (Applied Biosystems) and other primers and probes were designed using the Primer Express software from Applied Biosystems. The sequences are as follows: UCP1 forward TCCCTCAGGATTGGCCTCTAC, reverse

GTCATCAAGCCAGCCGAGAT, and probe, CGCCTGCCTCTTTGGGAAGCAA; Adrb3 forward, CCGCAGCTGACTTGGTAGTG, reverseCCACAGTTCGCAACCAG TTTC, and probe CTGACTGGCCATTGGCCCTTGG

In- situ hybridization and analysis

The fresh frozen brains (n=5 per group) were sectioned (20 μm) on a cryostat in a one-in-three coronal series through the caudal hypothalamus containing the DMH and ARH. For NPY antisense probe, the plasmid (obtained from Dr. S. L. Sabol, National Institutes of Health, Bethesda, MD) contained 511 bp NPY cDNA, and a cRNA probe was transcribed using T_3 RNA polymerase in which 25% of the uridine 5-triphosphate was ^{35}S-labeled (PerkinElmer, Wellesley, MA). For CART antisense probe, the plasmid was obtained from Dr. Joel Elmquist (Elias et al., 2001) and the ^{35}S-labeled cRNA probes were generated with the T_3 RNA polymerase. Brain sections were fixed, dehydrated, delipidated, rehydrated, and then air dried, as previously described (Li et al., 1998a). The sections were exposed to the labeled NPY or CART probe overnight in a humidified chamber at 55 C. After incubation, the slides were washed, dehydrated, and dried. The slides were then exposed to Kodak biomax film (Sigma-Aldrich, St. Louis, MO, CAT# Z350370) overnight and then developed. For the analysis, DMH and ARH film images were captured using a CoolSNAP charge-coupled camera (Photometrics, Tucson, AZ) and analyzed using the MetaMorph Imaging system (Universal Imaging Corp., West Chester, PA). The images were analyzed using a sampling box that encompassed the entire region of interest and measured as the integrated intensity. Background labeling, determined using the same sampling box over an adjacent region that contained no NPY or CART mRNA expression, was subtracted from this measurement.

Double label in-situ hybridization and analysis

Simultaneous visualization of NPY and CART mRNA in the DIO mice brain (n = 4) was performed as previously reported (Scarlett et al., 2007), with slight modifications. Coronal sections (20 μm) were cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Antisense ^{33}P-labeled rat CART riboprobe and antisense digoxigenin-labeled rat NPY riboprobe were denatured, dissolved in hybridization buffer along with tRNA (1.7 mg/ml), and applied to slides. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55 ºC. The following day, slides were treated with RNase A and washed under conditions of increasing stringency. The sections were incubated in blocking buffer and then in Tris buffer containing antidigoxigenin fragments conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN), diluted 1:250, for 3 h at room temperature. NPY cells were visualized with Vector Red substrate (Vector Laboratories, Cat# SK-5100) according to the manufacturer's protocol. Slides were dipped in 100% ethanol, air dried, and then dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed 7 d later and coverslipped. NPY (red) and CART (black silver grain) mRNA-containing cells from both left and right side of the DMH were identified and counted under bright field microscope (3 sections/animal, n=3).

Immunohistrochemistry

1. CART in NPY-GFP mice

NPY-hrGFP mice (n=3 per group) were anesthetized with tribromoethanol (20 mg/kg body weight, i.p) and perfused with 0.9% ice cold saline solution and then 4% paraformaldehyde containing sodium phosphate buffer (pH 7.4). The brains were post-

fixed in the paraformaldehyde overnight and transferred to 25% sucrose buffer solution. The brains were then frozen and sectioned at 25 µm on a microtome. The tissue sections were washed in 0.05M potassium phosphate-buffered saline (KPBS) several times and pre-incubated in blocking buffer (KPBS+0.4% triton X + 2% normal donkey serum) for 30 min before incubating in 1:5000 rabbit anti-CART (Phoenix pharmaceuticals, INC, Burlingame; Cat# CA H-003-62) containing blocking buffer for 48 hrs at 4°C. Following washes in KPBS, tissue sections were incubated for 1 hr in 1:1000 Alexa Fluor 568 donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA; CAT# A10042) at room temperature, then subsequently washed and mounted on gelatin-coated glass slides and coverslipped with SlowFade Gold antifade reagents (Invitrogen; Cat# S36936).

2. BDA/NPY/CART

To visualize DMH-NPY/CART fiber projections in biotinylated dextran amine (BDA) injected DIO mice (described in the materials and methods in chapter 3, n=4), a one in six series of 25 µm sections were rinsed in KPBS, followed by blocking solution containing 0.4% Triton-X and 2% normal donkey serum in KPBS (KPBS-TX-NDS) for 30 min at room temperature. Sections were then incubated in 1:3000 sheep anti-NPY antibody (Chemicon, Temecula, CA; Cat#AB1583) and 1:5000 rabbit anti-CART antibody (Phoenix pharmaceuticals, INC.) in KPBS-TX-NDS for 48 hr at 4º C. After incubation, the tissue was rinsed in KPBS and incubated in 1:1000 Alexa Fluor 488 donkey anti-sheep (Invitrogen ; Cat# A11015) ,1:200 Streptavidin Alexa Fluor 568 in KPBS, and 1:1000 Alexa Fluor 647 donkey anti-rabbit, containing 0.4% Triton-X(KPBS-TX) for 4 hr at room temperature. The tissue sections were then mounted on gelatin-

coated glass slides and coverslipped with SlowFade Gold antifade reagents (Invitrogen; Cat# S36936).

3. NPY/CART /TH

To visualize NPY/CART co-expression in catecholamine neurons in the brainstem in DIO and CD mice, NPY-hrGFP mouse brainstem sections were incubated in 1:5000 rabbit anti-CART antibody and 1:1000 mouse anti-tyrosine hydroxylase (Immunostar, Hudson, WI; Cat# 22941) antibody containing blocking buffer for 48hrs at 4°C. Following washes in KPBS, tissue sections were incubated for 1 hr in 1:1000 Alexa Fluor 568 donkey anti-rabbit antibody and 1:1000 Alexa Fluor 647 donkey anti-mouse antibody (Invitrogen, Cat# A31571) at room temperature.

Confocal microscopy

Confocal laser microscopy was used to analyze the triple-label immunostained images for colocalization. The TSC SP confocal system (Leica Corp., Germany), consisting of a RBE inverted microscope (Leica Corp., an Ar laser-producing light at 488 nm (for visualization of FITC), a Kr laser-producing light at 568 nm (for visualizing TRITC), and a HeNe laser-producing light at 647 nm (for visualization of Cy5), was used to scan the images. Various objectives (×25, numerical aperture 0.75 and ×40, numerical aperture 1.25) were used to scan and capture images. For each experiment, fluorophore signals were checked individually for bleed-through to the apposing detector. All bleed-through was eliminated by adjusting laser intensity and detector window width. To assess colocalization of two signals, a series of continuous optical sections, 0.5 μm intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. The signals were obtained for each fluorophore on one series of optical sections and stored

separately as a series of 512 × 512 pixel images. The stacks of individual optical slices were analyzed using the Image J software to determine co-localization and contacts. The confocal images are presented as projections of stacks of optical images or as individual slices, as indicated. The brightness and contrast of the images were adjusted in Photoshop to match microscope visualization (Adobe Systems Inc., San Jose, CA).

Leptin injection, pSTAT3, and c-fos Immunohistochemistry

1. Leptin injection

Leptin responsiveness was assessed by quantifying phosphorylated signal transducer and activator of transcription-3 (pSTAT3)-immunoreactive (ir) cells in response to leptin in 20 wks HFD vs. CD diet fed NPY-hrGFP mice. Animals were sham injected for 7 days before testing and fasted from 0700 to 1100 hr on testing day. Mice were injected ip with saline or leptin (Preprotech, Cat# 450-31, 2 μg leptin/ body weight in grams, i.p.) at 1100 hr. After 45 min, mice were sedated with tribromoethanol and perfused transcardially with saline followed by ice-cold, borate-buffered 4% paraformaldehyde (pH 9.5). Perfused brains were sectioned (25 μm) on a microtome in a one-in-six coronal series through the hypothalamus.

2. P-STAT3

pSTAT3 immunohistochemistry was performed as previously described (Draper et al., 2010), on every third section, using 1:250 rabbit anti-pSTAT3 antibody (Cell Signaling Technology, Inc., Danvers, MA; Catalog # 9145). For the leptin-induced pSTAT3-IR in NPY neurons, 3 sections per animal from 3 different animals were selected for the analysis. Sections were visualized with a 20x objective using a Nikon Eclipse E800 microscope. The area of interest was simultaneously illuminated with

fluorescence to visualize the hrGFP and brightfield light to visualize the p-STAT3-IR. The total number of NPY-GFP neurons as well as the number of NPY neurons containing pSTAT3-IR was counted for each section (three sections per animal). T-test ($p<0.05$) was used to determine significant difference between groups.

3. c-fos

For c-fos immunohistochenistry, sections were then incubated with rabbit anti-c-Fos antibody (1:20,000; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer for 48 h. After incubation, the tissue was rinsed in KPBS, incubated in biotinylated donkey antirabbit IgG (1:600; Jackson ImmunoResearch, West Grove, PA) in KPBS with 0.4% Triton X-100 for 1 h and then washed and incubated in avidin-biotin solution (Vectastain; Vector Laboratories, Burlingame, CA) for 1 h. c-Fos immunoreactivity was visualized with 3,3′-diaminobenzidine enhanced with nickel chloride. Tissue sections were mounted on gelatin-coated glass slides, and coverslipped. 2 sections per animal from 3 different animals were selected for the analysis. Sections were visualized with a 20x objective using a Nikon Eclipse E800 microscope. The area of interest was simultaneously illuminated with fluorescence to visualize the hrGFP and brightfield light to visualize the c-fos. The total number of NPY-GFP neurons as well as the number of NPY neurons containing c-fos was counted for each section. T-test ($p<0.05$) was used to determine significant difference between groups.

RESULTS

Characterization of DIO mice

Mice fed a HFD (60% fat) exhibited rapid weight gain during the first 10 weeks of diet treatment and were 33% heavier than CD mice by 10 weeks (Fig. 4-1A). After 10

weeks, HFD mice exhibited steady weight gain and were 38% heavier than CD mice at 20 weeks. Serum leptin levels were significantly increased in HFD mice compared to CD mice by 2 weeks and were further increased in HFD mice after 10 weeks (Fig. 4-1B), at which time steady state levels were achieved. While 20 week CD mice exhibited a significant decrease in serum leptin levels after 24 hour fasting, no significant decrease in leptin levels was observed in HFD mice after fasting. A significant increase in serum insulin levels was first detected at 10 weeks in HFD mice compared to the CD mice (Fig. 4-1C), and HFD mice developed more severe hyperinsulinemia with prolonged HFD treatment. Fasting caused a significant decrease in insulin levels in both group, although the HFD animals retained elevated levels relative to the fasted CD mice. Blood glucose levels were significantly higher in HFD mice compared to CD mice starting at 2 weeks and hyperglycemia in HFD mice was further exaggerated at later time points (Fig. 4-1D). Fasting caused a significant decrease in glucose levels in both HFD and CD mice, although fasting glucose levels in HFD mice were significantly higher than CD mice. Uncoupling protein 1 (UCP1) and β-adrenergic receptor 3 (adrb3) expression in BAT may provide indirect indications of the relative sympathetic activity. Although there was no statistical difference between HFD and CD mice in either UCP1 or adrb3 levels, a significant increase in these UCP1 gene expressions was detected in HFD mice at 16 weeks compared to 2 weeks on HFD (Fig. 4-1E), and in adrb3 expression at 20 weeks compare to 2 weeks (Fig. 4-1F). Fasting caused a significant decrease in UCP1 expression in both HFD and CD mice, while adrb3 expression was decreased in CD mice, but was increased in HFD mice.

Time course of NPY induction in the DMH

Using in-situ hybridization, we demonstrated the time course of NPY mRNA induction in the DMH during DIO development. Consistent with a previous report (Guan et al., 1998a), there was no visible NPY mRNA expression in the DMH of CD mice at any time point. DMH-NPY mRNA level in 2 week HFD mice was similar to that in CD mice (Fig. 4-2A and 4-2B). After 10weeks, NPY induction in the DMH was apparent in HFD mice and NPY mRNA level was further significantly increased with 20 week HFD treatment. In contrast, ARH-NPY mRNA level was not different between HFD and CD mice at any time point (Fig. 4-2C). At 20 weeks, 24 hour fasting led to a significant reduction in DMH-NPY expression only in HFD mice (Fig. 4-2B), but a significant increase in ARH-NPY expression in both HFD and CD mice (Fig. 4-2C).

Time course of CART up-regulation in the DMH

Using tissue sections from the same cohort of animals, we performed in situ hybridization for CART mRNA and measured the changes in CART expression in the DMH and ARH during DIO development. In CD mice, the basal expression of CART mRNA was low in the DMH at 2 weeks and remained unchanged at all time points. At 2 weeks on HFD, CART expression in the DMH was not different from that in the CD mice (Fig. 4-3A and 4-3B). CART expression was significantly increased in HFD mice at 10 weeks and remained elevated at later time points. ARH-CART mRNA expression remained unchanged regardless of diet and time (Fig. 4-3C). 24 hour fasting did not affect CART mRNA level in the DMH (Fig. 4-3B) or the ARH (Fig. 4-3C) in 20 week HFD or CD mice. Although fasting decreased leptin levels in CD mice, it did not reduce basal CART expression in the DMH.

NPY and CART are co-expressed in the DMH of DIO mice

We also performed immunohistochemistry for CART peptide in NPY-hrGFP mice fed either CD or HFD for 2 and 20 weeks. At 2 weeks, both CART-and NPY-hrGFP -immunoreactive (ir) cell bodies were not detectable in the DMH in either the HFD or CD mice (data not shown). However, at 20 weeks, both CART-and NPY-hrGFP –ir cell bodies were readily detectable in the DMH in HFD mice, consistent with the mRNA data. Surprisingly, numerous NPY/CART co-labeled cells were detected in the ventral and dorsal portions of the DMH in 20 week HFD mice (Fig. 4-4), while no such co-localization was observed in CD mice (data not shown). While nearly all of the NPY-hrGFP cells were co-labeled with CART, there were also a large number of CART neurons that did not co-label with NPY-hrGFP. To confirm the co-localization of CART and NPY, double labeled in-situ hybridization was performed on tissue obtained from 24 week HFD fed mice. While all NPY mRNA labeled cells were co-labeled with CART mRNA (Fig. 4-5A), only 12 ±3 % of total CART mRNA expressing neurons (n=3) expressed NPY mRNA. Additionally, a few NPY/CART mRNA co-localized cells were also observed in the LH (data not shown). Consistent with the previous reports, there were no NPY/CART co-localized cells in the ARH (Fig. 4-5B). NPY mRNA expression was observed in the ventromedial portion in the ARH, while CART expression was concentrated in the dorsolateral portion.

DMH-NPY/CART projections in DIO mice

We previously injected anterograde tracer BDA into the DMH of 20 week HFD fed mice to characterize DMH-NPY projections (see Chapter 3). DMH-NPY fiber projections were mainly observed in the hypothalamic nuclei including the PVH and LH. In the present study, triple-lable immunostaining was used in this tissue to confirm the

presence of CART immunoreactivity in BDA/NPY co-labled fibers (DMH derived) within the PVH (Fig. 4-6A-C) and LH. Consistent with lack of DMH-NPY projections to the brainstem areas, BDA/NPY/CART triple-labeled fibers were absent in the rRPa, a major thermoregulatory site in the brainstem, in DIO mice (Fig. 4-6 D-F). However, surprisingly, NPY/CART double-labeled fibers were observed in the rRPa (Fig. 4-6F).

Using triple-label immunostaining, we determined that other NPY/CART co-expressing neurons were located in the adrenergic C1 region of rostral ventrolateral medulla (VLM) and they also contained TH immunoreactivity in both HFD and CD mice (Fig. 4-7). Double-label in-situ hybridization confirmed that NPY and CART mRNA are co-expressed in the VLM as well as the nucleus of solitary tract (NTS) (Fig. 4-7E). Using triple-label immunostaining for NPY, CART and TH, we also confirmed brainstem derived NPY/ CART fibers in the PVH in both HFD and CD mice (data not shown).

NPY/CART neurons in the DMH are leptin sensitive in DIO mice

Since DMH-CART neurons express a signaling form of leptin receptor *ObRb* (Elias et al., 2001), we investigated the presence of leptin signaling in DMH-NPY /CART co-expressing neurons in DIO mice. We injected leptin i.p (2 µg/g) and visualized p-STAT3 and c-fos expression in DMH-NPY cells using NPY-hrGFP mice fed with either HFD or CD for 20 weeks. The number of ARH-NPY neurons co-localized with p-STAT3 were significantly higher in CD mice compared to that of HFD mice (Fig. 4-8), confirming the presence of leptin resistance. Consistent with previous reports (Enriori et al., 2007; Enriori et al., 2011; Glavas et al., 2010; Munzberg et al., 2004), pSTAT3 staining was abundant in the DMH of both CD and HFD mice after leptin treatment. In HFD mice, 64 ± 2 % of NPY-GFP neurons contained p-STAT3 activation in the DMH

(Fig. 4-9). Since NPY-GFP cells are undetectable in CD mice, no co-localization between NPY-GFP and p-STAT3 activation was observed.

Similar to p-STAT 3 response, leptin stimulated c-fos expression was higher in the ARH of the CD mice compared to that of HFD mice (Fig. 4-10). However, there were few c-fos expressing ARH-NPY cells in either CD or HFD mice, consistent with the fact that ARH-NPY neurons are inhibited by leptin. C-fos expression was comparable in the DMH between CD and HFD mice. In HFD mice, 47 ± 6 % of NPY-GFP neurons (n=4) contained c-fos expression in the DMH (Fig. 4-11). Since NPY-GFP cells are undetectable in CD mice, no co-localization was observed. This is the first study demonstrating that NPY neurons in the DMH are under the direct regulation of leptin in DIO condition.

DISCUSSION

Induction of NPY in the DMH has been reported in several rodent models of obesity and is associated with hyperphagic behavior (Guan et al., 1998a; Guan et al., 1998b; Kesterson et al., 1997; Tritos et al., 1998). To further characterize DMH-NPY induction in obesity, we investigated the time course of NPY mRNA induction during the development of DIO in mice. Unexpectedly, NPY mRNA was not fully induced in the DMH until 20 weeks after initiating HFD treatment. Concurrently, we observed a significant increase in CART mRNA expression in the DMH in parallel with leptin levels under the same HFD treatment regime. Furthermore, all NPY neurons in the DMH in the HFD mice were co-labeled with CART, although only small percentage of total CART neurons co-expresses NPY. These NPY/CART expressing neurons were activated by

peripheral leptin treatment, indicating that they express functional leptin receptors in DIO condition.

The present findings imply that DMH-NPY induction may not be the cause of obesity, but the drive for overeating in the later stages of obesity. High-fat diets generally promote hyperphagic behavior, although hyperphagia is not always necessary for obesity (Oscai et al., 1987; Wade, 1982). However, this initial hyperphagic response to a HFD is only transient and the food intake is gradually normalized to the control level, due to the rise in leptin levels, followed by a reduction in orexigenic neuropeptide expression in the ARH (Ziotopoulou et al., 2000). Despite high leptin levels, prolonged HFD consumption leads to a greater deposition of lipid which results in substantial weight gain in mice without increasing food intake. Therefore, high fat content of the diet rather than the hyperphagia is the main determinant of metabolic adaptations and increased adiposity during the early stages of the DIO (So et al., 2011).

According to the study by Lin et al., mice fed a HFD become rather hypophagic compared to the CD mice after the initial adjustment period, but develop hyperphagia after 16 weeks of HFD treatment (Lin et al., 2000). Our results suggest that NPY induction in the DMH during the later stages of DIO coincides with the late onset of hyperphagia. Interestingly, the reduction of NPY expression in the DMH after fasting suggests that the NPY from these neurons is not a direct driver of the hyperphagia, but rather must be responding to some signals stimulated by the chronic HFD consumption. Similarly, DMH-NPY expression is sensitive to food consumption in developing rats that are normally hyperphagic, and it is greatly reduced by maternal deprivation (Grove et al., 2001). Therefore, DMH-NPY expression is functionally distinct from ARH-NPY

expression which is stimulated by hunger-driven mechanism and is one of the main drivers of refeeding response after fasting (Beck et al., 1990; Brady et al., 1990; White and Kershaw, 1990).

We made a surprising discovery that NPY induced neurons in the DMH co-express CART in HFD fed mice in the later stages of obesity. However, only a small portion of total CART expressing neurons overlaps with NPY induced cells, suggesting that NPY expressing CART neurons are functionally distinct from other CART neuronal populations in the DMH. Furthermore, outside the hypothalamus, we discovered the presence of NPY/CART co-expressing neurons in the brainstem in both DIO and CD mice. Both neuropeptides are abundantly expressed in rodent brain; however, the co-localization of the two has not been reported. While NPY is a potent feeding stimulant in the PVH (Beck, 2006), CART is classically considered a satiety factor and is closely associated with the actions of leptin, and is also thought to counteract the effect of NPY (Kristensen et al., 1998). In rodents, CART is expressed in POMC neurons, but not in NPY/AgRP neurons in the ARH. However, CART co-localization with other orexigenic neuropeptides has been reported in the rodent and human brain. CART is co-expressed in the melanin concentrating hormone (MCH) neurons in the LH (Elias et al., 2001; Menyhert et al., 2007; Vrang et al., 1999). Interestingly, CART is co-expressed in NPY/AgRP neurons and absent from POMC neurons in the infundibular nucleus of human hypothalamus which is equivalent to the ARH in rodents (Menyhert et al., 2007). Lack of POMC/CART co-localization was also observed in the ARH of non-human primate (Grayson et al., 2006).

The purpose of NPY and CART co-localization in the same neuron is puzzling. It is possible that prolonged HFD consumption induces NPY expression in a specific population of CART neurons in the DMH in order to inhibit CART's anorexigenic actions in the downstream targets. Since CART is known to influence energy expenditure by modulating sympathetic nervous outflow and the hypothalamic-pituitary-thyroid axis (Wang et al., 2000), it is also likely that CART may be responsible for increased BAT sympathetic activity mediated by the hyperleptinemia in DIO mice, independently from the anorectic role. However, the anorectic effect of ICV CART injection has recently been questioned since it also causes motor defects, which could explain abnormal feeding behavior (Abbott et al., 2001). In direct contrast with ICV injection, CART injection into specific hypothalamic nuclei including the PVH, ARH and the DMH results in increased feeding responses (Abbott et al., 2001; Hou et al., 2010), suggesting that CART has an orexigenic property. In support of this hypothesis, CART has been demonstrated to stimulate the release of NPY and AgRP, but reduce the release of α-MSH from hypothalamic explants (Dhillo et al., 2002). Therefore, it is likely that CART is not a simple anorectic peptide and has multiple functions in energy homeostasis in diverse regions of the hypothalamus. Future experiments using CART antagonists will be necessary to further investigate the role of CART in food intake and energy expenditure.

In the previous chapter (chapter 3), we demonstrated that DMH-NPY neurons project to the hypothalamic areas including the PVH, implicated in feeding behavior and BAT thermoregulation, in DIO mice. In the present study, CART-ir was observed in DMH-NPY fiber projections in the PVH of DIO mice, confirming the co-expression of NPY and CART in the DMH. The DMH neurons have been also implicated in regulating

hypothalamic outflow to the autonomic nervous system via PVH projections and leptin sensitive cells in the DMH directly innervate the PVH (Elmquist et al., 1998; Gautron et al., 2010). Since DMH-NPY/CART neurons showed direct response to leptin treatment in our study, DMH-NPY/CART neurons may mediate the effect of leptin on sympathtic regulation via PVH projections in DIO condition (Enriori et al., 2011). Leptin receptor expressing neurons located in the dorsal area of the DMH are also synaptically coupled with the rRPa (Zhang et al., 2011b). In the present study, NPY/CART co-expressing neurons responding to leptin are mainly observed in the ventral subdivision of the caudal DMH, and these neurons do not project to the rRPa. Instead, NPY/CART co-localized fibers in the rRPa were co-labeled with the TH (unpublished data), suggesting that brainstem NPY/CART co-expressing neuronal projections to the rRPa are involved in BAT thermoregulation (Zagon, 1993). Therefore, there is a clear anatomical and functional segregation of leptin receptor expressing neurons in the DMH with distinct projections.

Surprisingly, we demonstrated that DMH-NPY/CART neurons are activated by peripheral leptin treatment while ARH-NPY neurons are clearly leptin insensitive in DIO mice. Many studies have shown that neurons in the ARH develop a resistance to high levels of leptin with prolonged HFD feeding, while other brain regions remain leptin sensitive (Enriori et al., 2007; Glavas et al., 2010; Munzberg et al., 2004). A recent study confirmed that DMH neurons respond to central leptin treatment in DIO mice and mediate physiological effects of leptin on sympathetic activity (Enriori et al., 2011). The present finding is the first evidence that leptin directly acts on DMH-NPY/CART neurons to regulate the neuronal functions in DIO mice.

An important question is whether leptin directly regulates DMH-NPY gene expression in DIO condition. Most animal models of obesity (Guan et al., 1998a; Guan et al., 1998b; Kesterson et al., 1997; Tritos et al., 1998) exhibiting DMH-NPY induction have high levels of circulating leptin. Transgenic mice overexpressing leptin exhibit more lean phenotype in normal feeding condition, but they have an increased susceptibility to obesity under a HFD treatment, supporting that a chronic exposure to hyperleptinemia is a predisposing factor to obesity (Ogus et al., 2003). In contrast, leptin–deficient *ob/ob* mice are morbidly obese and hyperphagic, but no DMH-NPY induction has been reported in this model, suggesting the involvement of leptin in DMH-NPY induction. *Ob/ob* mice also have impaired DMH-CART mRNA expression which is normalized by leptin injection (Kristensen et al., 1998). However, *ob/ob* mice have severe developmental defects in the hypothalamus as well as disrupted endocrine and immune functions; therefore, it is difficult to rule out the role of other hormonal and neural factors in DMH-NPY induction.

About 50% of DMH-CART neurons express functional leptin receptors in normal rat brain (Elias et al., 2001), supporting our results that CART gene expression in the DMH is strongly correlated with increased adiposity and leptin levels during DIO development. In contrast, our group has previously demonstrated that DMH-NPY neurons show no expression of the long form of leptin receptor, *ObRb*, during the postnatal leptin surge in mice, when NPY gene expression is unusually high in the DMH (Draper et al., 2010). Our present data suggest that NPY/CART co-expressing neurons in DIO mice may be different from those NPY expressing neurons during postnatal period since NPY/CART neurons showed pSTAT3 response to peripheral leptin treatment.

However, there is no clear correlation between the changes in leptin levels and DMH-NPY mRNA expression in DIO condition, suggesting that leptin may be solely responsible for the direct regulation of CART gene expression, and may stimulate NPY release through the excitation of these cells in DIO mice. DMH-NPY induction in lactating animals (Li et al., 1998a) also indicates that hyperleptinemia is not required for the activation of NPY expression in the DMH. Body weight homeostasis of lactating animals is unique because they exhibit low leptin production despite increased body weight and food intake. Restoring leptin to physiological level does not affect DMH-NPY expression and hyperphagia in lactating rats (Xu et al., 2009). However, lack of CART induction in the DMH (unpublished data) during lactation supports that leptin is critical for the activation of CART. Further electrophysiological experiments will be necessary to confirm the stimulatory effect of leptin on these neurons in DIO condition.

In summary, long term HFD consumption induces NPY and CART induction in the DMH which may be responsible for hyperphagic behavior and increased BAT themogenesis in diet-induced obesity. We discovered two novel findings: 1) NPY and CART are co-expressed in the same neurons in the DMH, projecting to the PVH implicated in food intake and sympathetic regulation and 2) these neurons are activated by peripheral leptin treatment in DIO condition. The present study clearly suggests that DMH-NPY/CART induction is one of the hypothalamic adaptations to increased adiposity and hyperleptinemia. However, the functional roles of NPY and CART expression in the DMH in DIO condition remains to be determined in future studies and will be of great importance to find the potential treatment for obesity

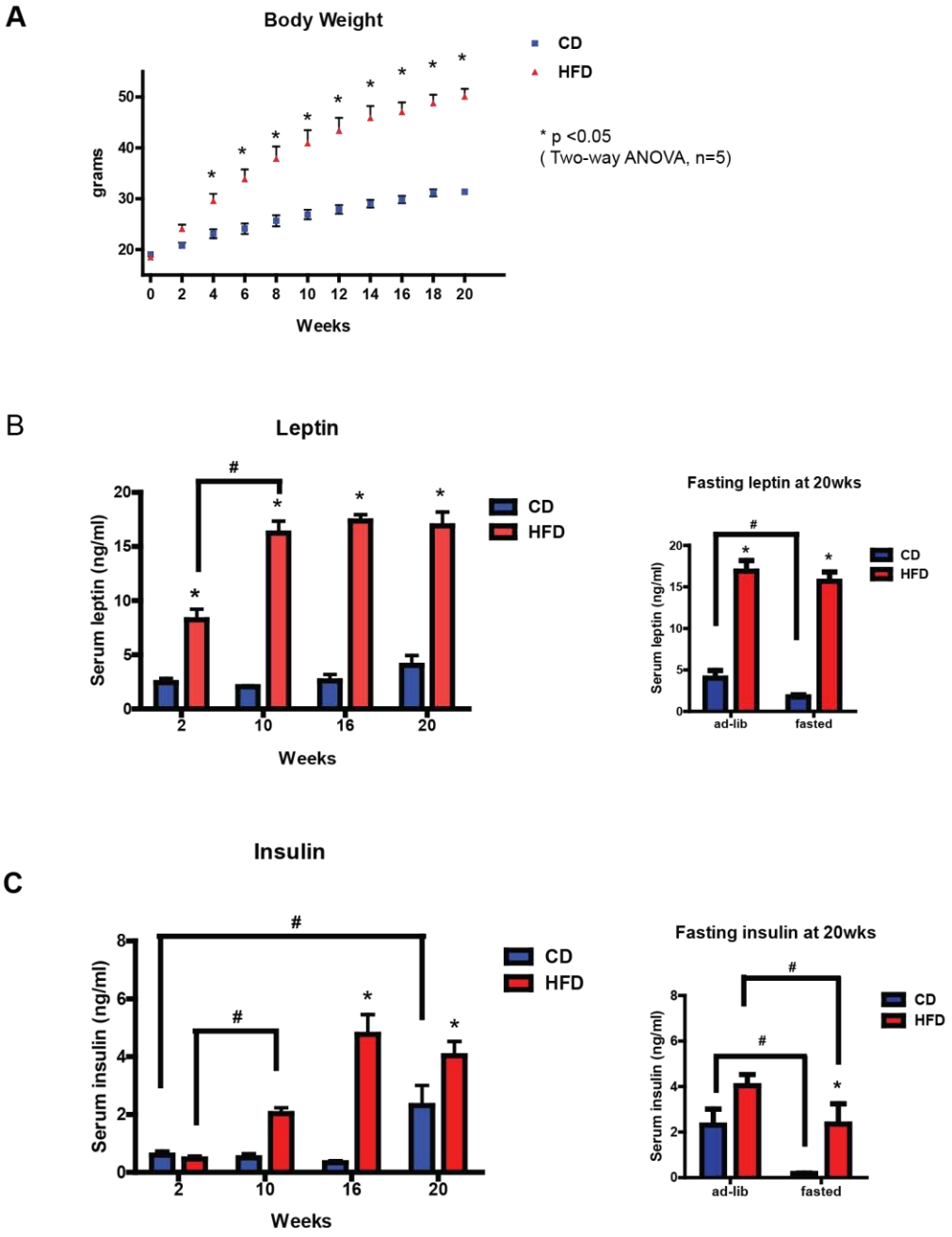

Figure 4-1. Metabolic profile of DIO mice.
5 weeks old C57BL6 Mice are fed with either HFD (n=5) or CD (n=5) for 2, 10, 16 and 20 weeks. For the fasting group, 20 weeks HFD and CD mice are fasted for 24 hrs. The changes in body weight (A), serum leptin concentration (B), serum insulin concentration (C), blood gucose level (D), BAT UCP 1 mRNA expression (E), BAT Adrb3 mRNA expression (F) were measured. Results are shown as mean values ± SEM. * $p < 0.05$ diet effect at each time point analyzed by two-way ANOVA, n=3-5 for each group. # < 0.05 time effect in diet group analyzed by two-way ANOVA, n=3-5.

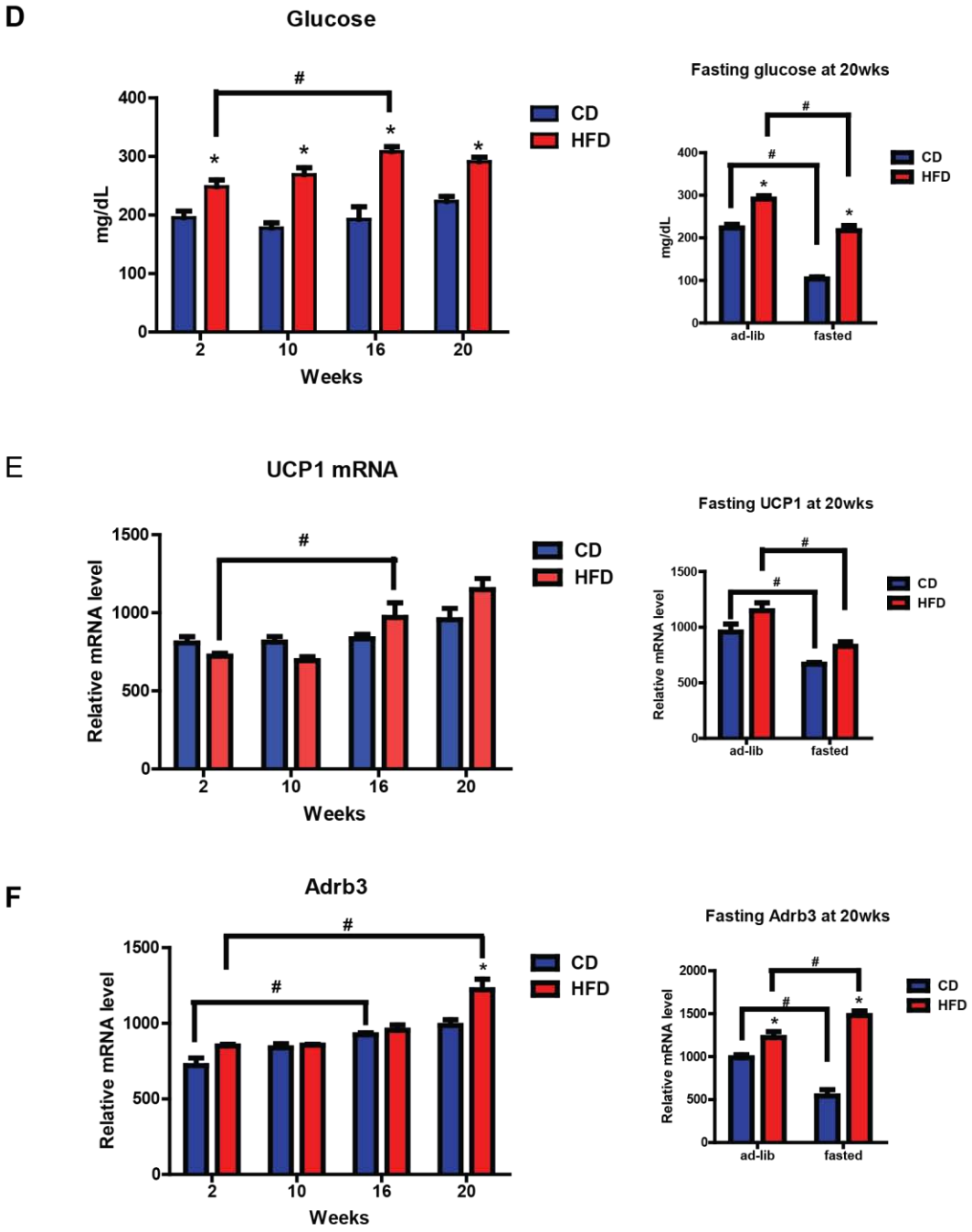

Figure 4-1. Metabolic profile of DIO mice (continued)
5 weeks old C57BL6 Mice are fed with either HFD (n=5) or CD (n=5) for 2, 10, 16 and 20 weeks. For the fasting group, 20 weeks HFD and CD mice are fasted for 24 hrs. The changes in body weight (A), serum leptin concentration (B), serum insulin concentration (C), blood gucose level (D), BAT UCP 1 mRNA expression (E), BAT Adrb3 mRNA expression (F) were measured. Results are shown as mean values ± SEM. * $p < 0.05$ diet effect at each time point analyzed by two way ANOVA, n=3-5 for each group. # < 0.05 time effect in diet group analyzed by two way ANOVA, n=3-5.

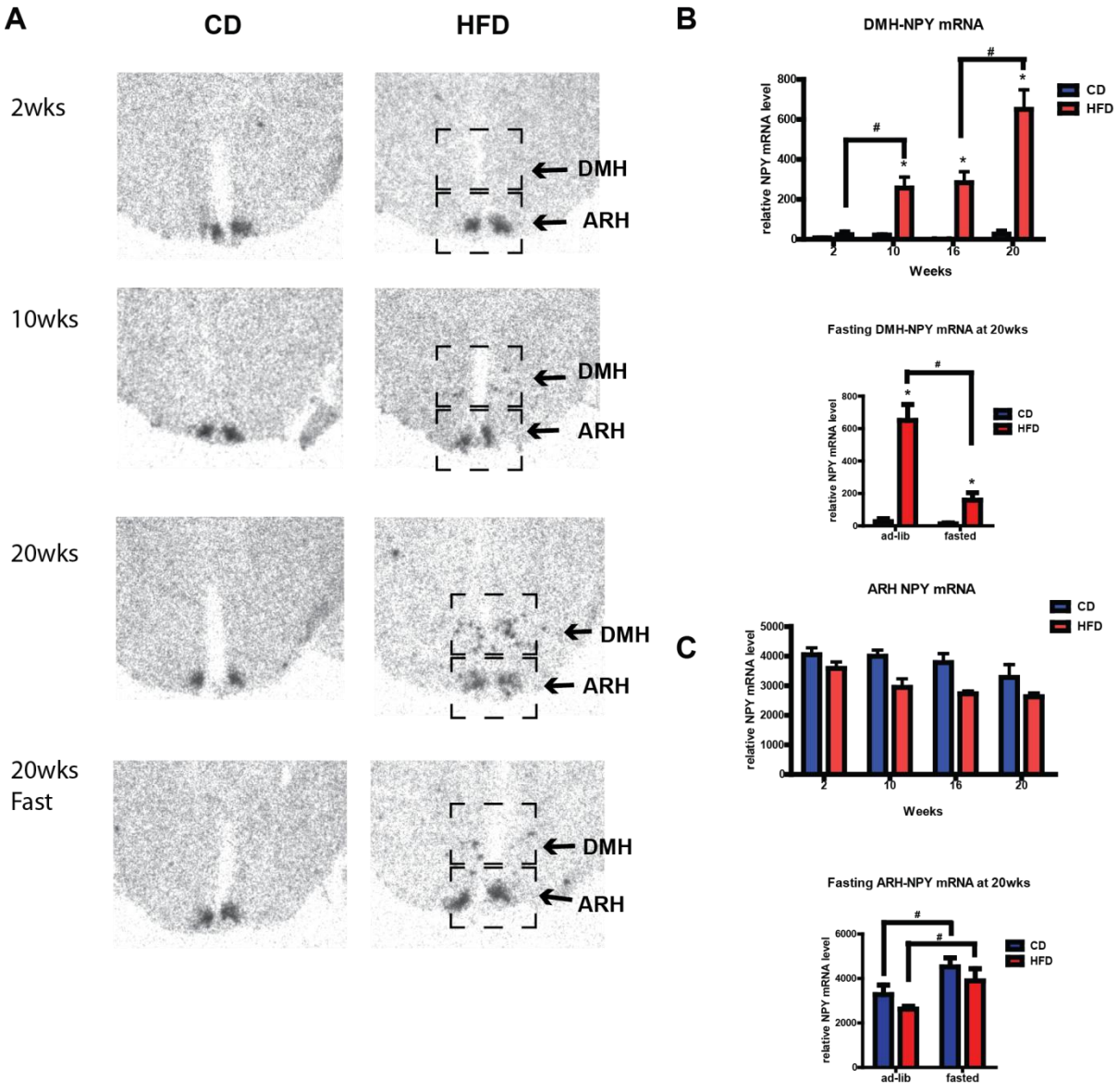

Figure 4-2. Time course of DMH-NPY mRNA expression in DIO mice.
(A) Representative microphotographs for DMH and ARH NPY mRNA expression comparing HFD and CD mice at 2, 10, 16 and 20 weeks fasting from in-situ hybridization films. (B) The comparision of DMH-NPY mRNA expression between HFD and CD mice was quantified using two way ANOVA (*$p < 0.05$, n=4-5). Time effect in each diet group was analyzed by two way ANOVA (# $p < 0.05$) (C)The comparision of ARH-NPY mRNA expression was quantified using two way ANOVA (no statistical significance was found, n=3-4).Time effect in each diet group was analyzed by two way ANOVA (# $p < 0.05$).

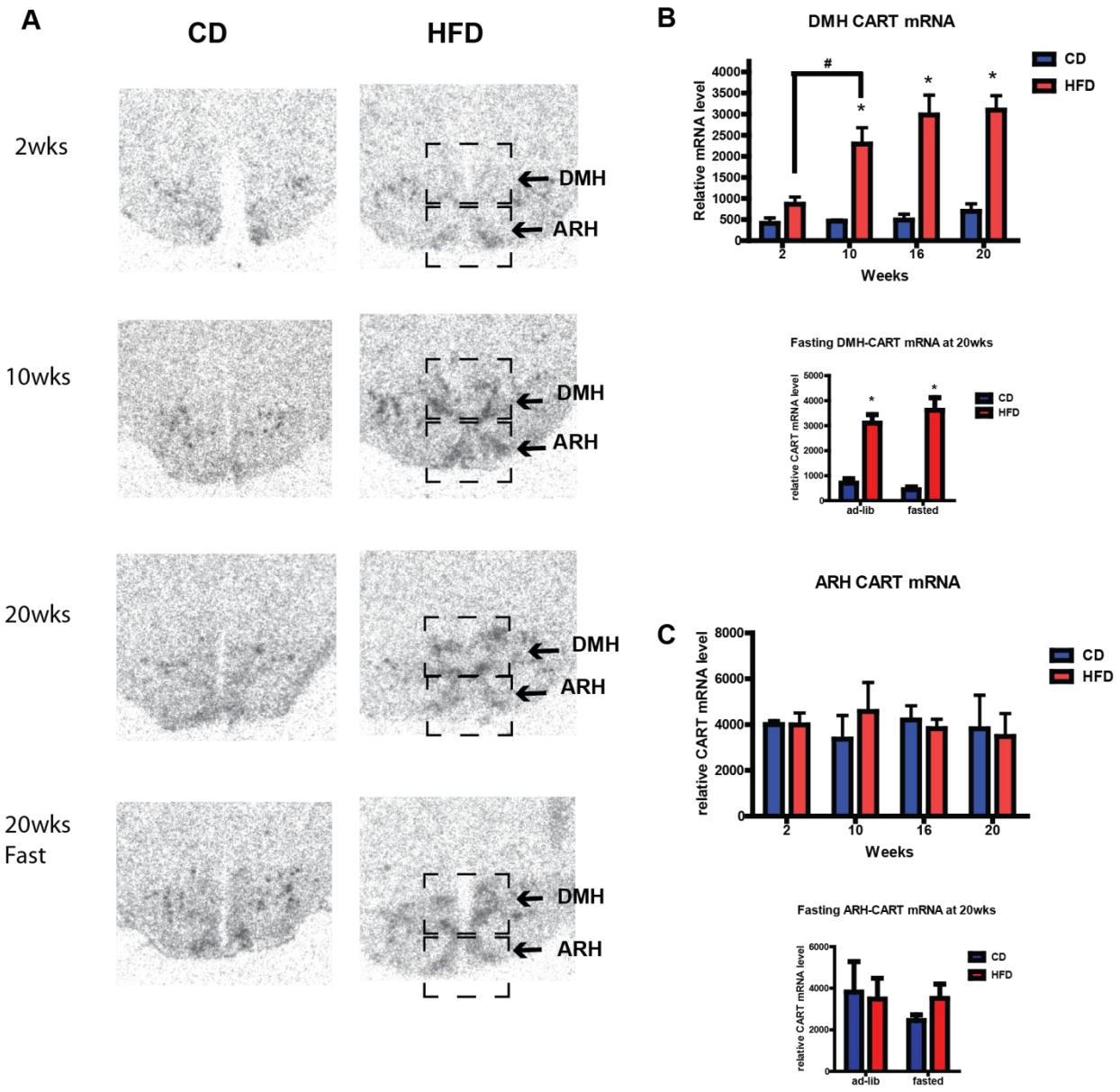

Figure 4-3. Time course of DMH-CART mRNA expression in DIO mice.
(A) Representative microphotographs for DMH and ARH CART mRNA expression comparingHFD and CD mice at 2, 10, 16 and 20 weeks fasting from in-situ hybridization films (B) The comparision of DMH-CART mRNA expression between HFD and CD mice was quantified using two way ANOVA (*$p < 0.05$, n=4-5). Time effect in each diet group was analyzed by two way ANOVA (# $p < 0.05$) (C)The comparision of ARH-CART mRNA expression was quantified using two way ANOVA (no statistical significance was found).

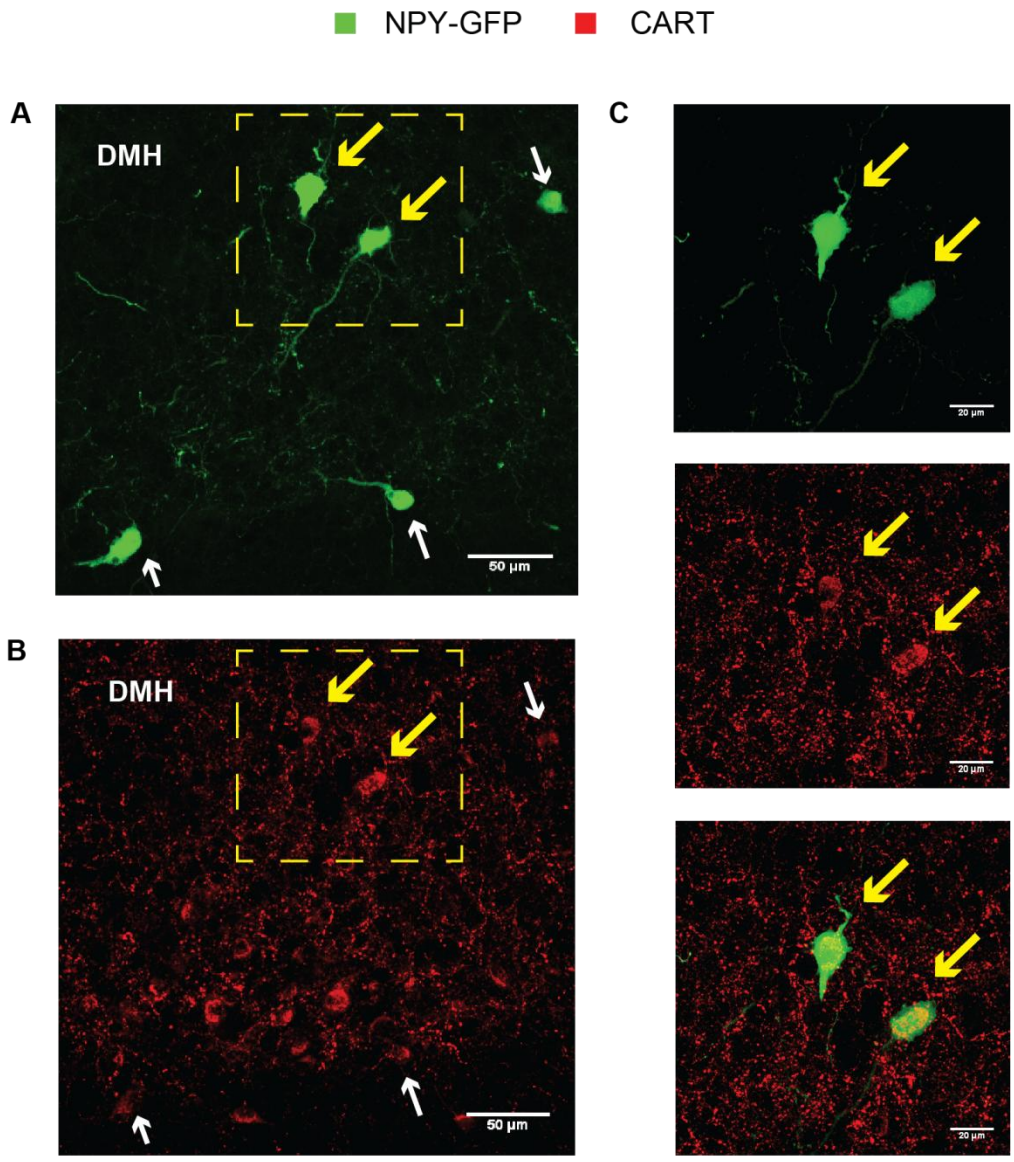

Figure 4-4. NPY-hrGFP and CART peptide co-expression in the DMH of DIO mice. NPY-hrGFP expression (A) and CART peptide (B) in the ventral subdivision of the DMH in NPY-hr GFP mice fed a HFD for 20 weeks. Arrows (white and yellow) indicate the co-expression of CART and GFP in the same cell. 20x confocal images. (C) Upper panel: NPY-hrGFP indicated by yellow arrows. Middle panel: CART peptide indicated by yellow arrows. Bottom panel: Overlay image for NPY-hrGFP and CART expression. 40x confoal images. Co-localization of CART and NPY was not observed in the DMH in CD mice.

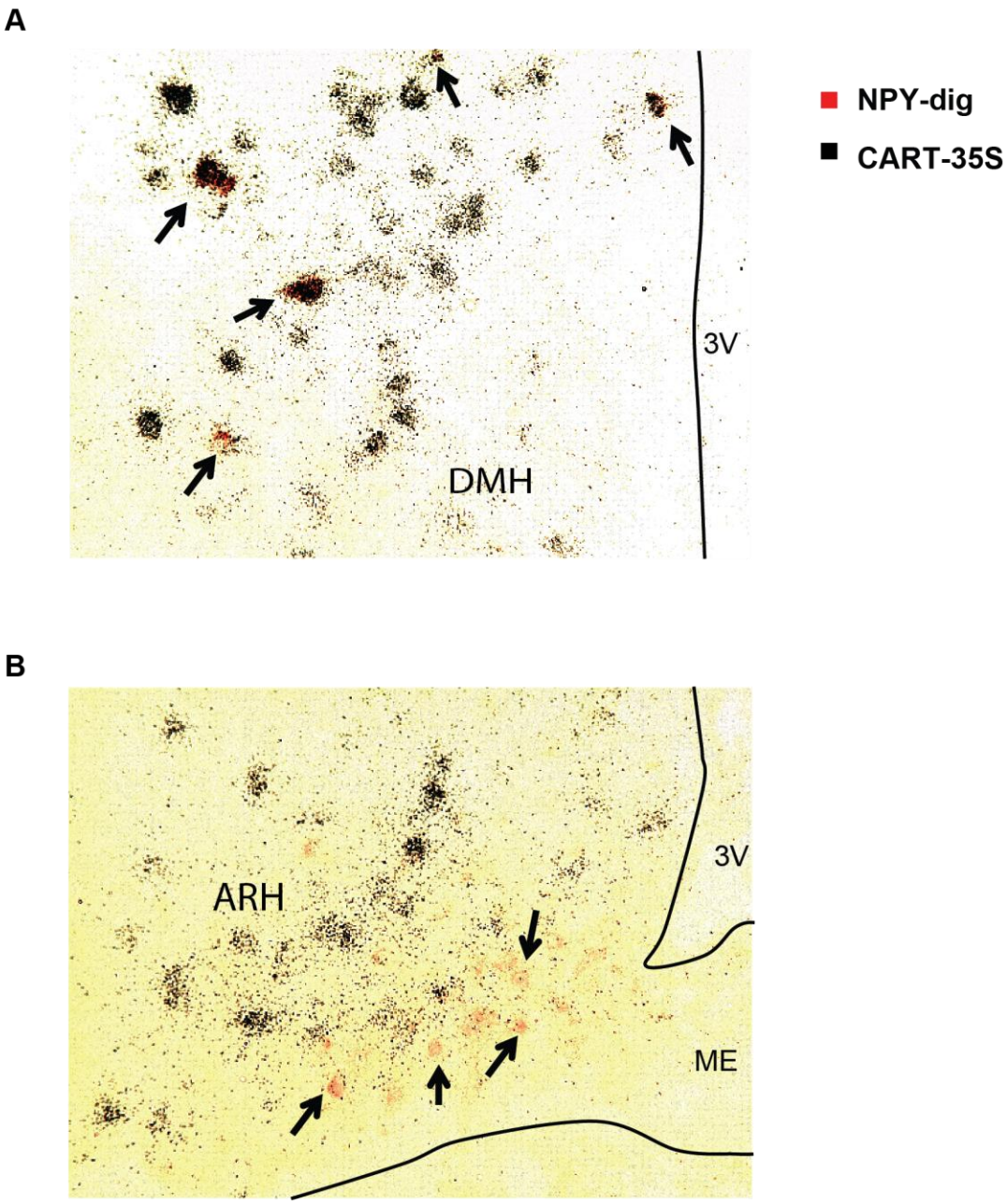

Figure 4-5. NPY and CART mRNA co-expression in DIO mice.
Double in-situ hybridization for NPY-dig (red)and CART-35S (black) in DMH (A) and ARH (B) containing sections of DIO mice. All NPY-dig labled cells were CART positive in the caudal DMH. 12 ± 3 % of total CART mRNA expresing cells (n=3) were co-localized with NPY mRNA expessing cells in the DMH. In the ARH, no co-localization of NPY and CART was observed at any level. Values represent mean ± SEM.

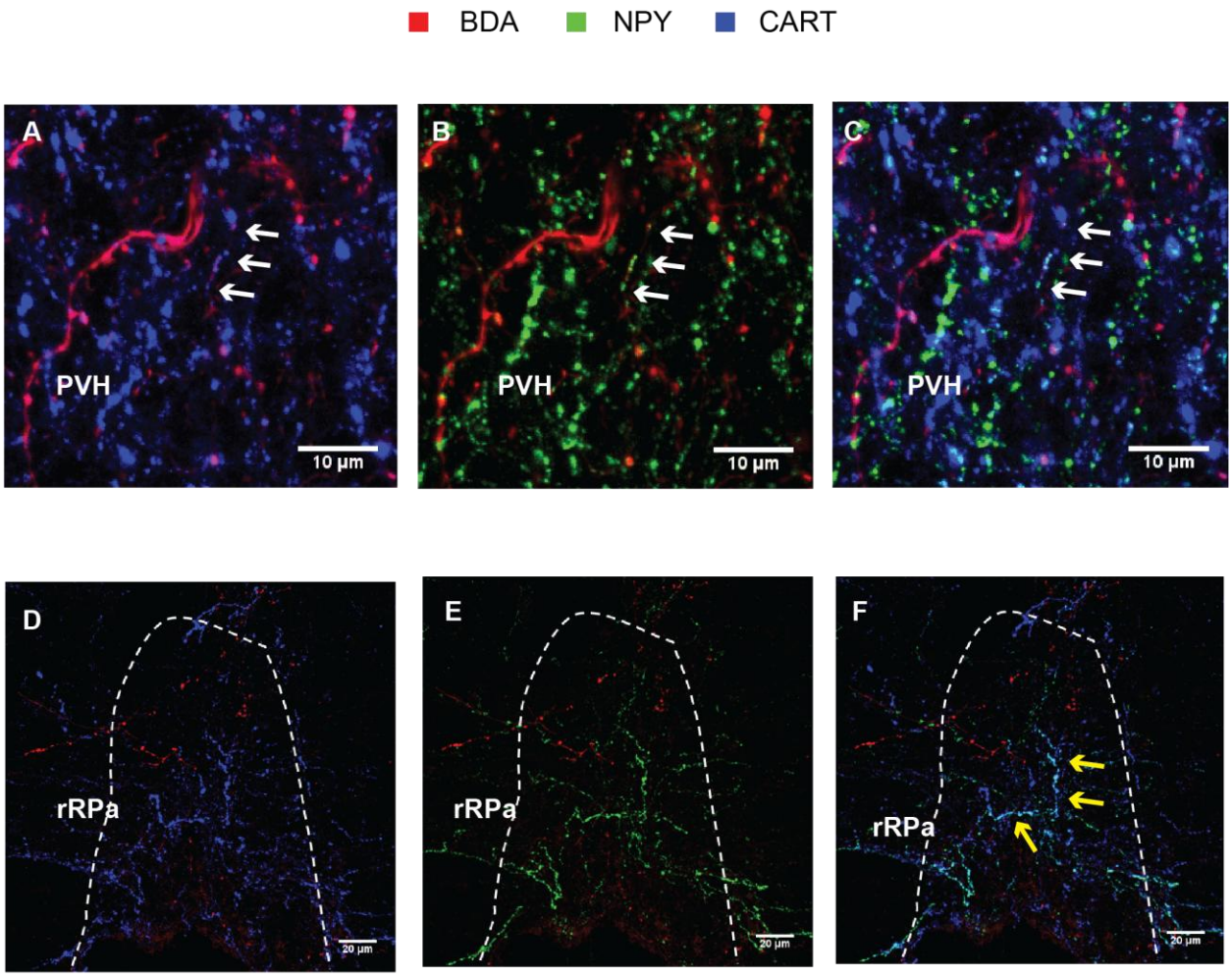

Figure 4-6. DMH-NPY/CART projections to the PVH, but not the rRPa in DIO mice.
(A-C) 40x confocal images (zoomed in) showing BDA/NPY/ CART co-localized fiber in the PVH (A) a BDA (red) and CART (blue) co-localized fiber, indicated by arrows, (B) a BDA and NPY (green) co-localized fiber, (C) overlay image for BDA, NPY, and CART fibers. (D-F) 40x confocal images of BDA, NPY , and CART fibers in the rRPa. (D) BDA and CART fibers in the rRPa. No-colocalization was found. (E) BDA and NPY fibers in the rRPa. No co-localization was found. (F) Overlay of 40x confocal images to show that NPY/CART co-localized fibers (indicated by yellow arrows) in the rRPa are not co-localized with BDA.

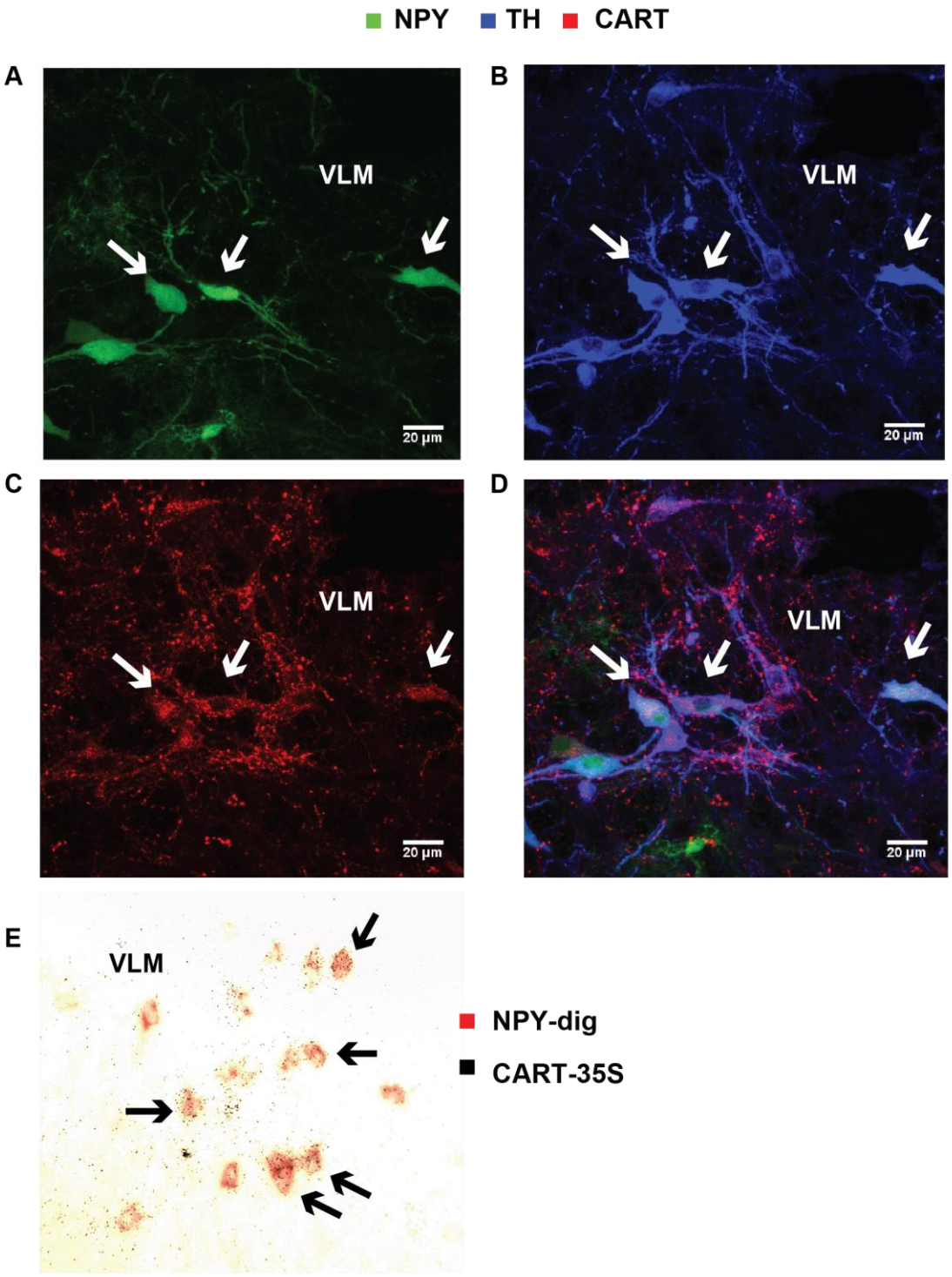

Figure 4-7. NPY and CART co-expressing cells in the brainstem of DIO mice.
(A) NPY-hrGFP neurons (green) in the ventrolateral medulla (VLM) are co-localized with (B) TH (blue) and (C) CART (red), indicated by white arrows. 40x confocal images. (D) Overlay of 40x confocal images showing triple co-localization for NPY, CART, and TH expressing neurons. (E) Double-label in-situ hybridization confirmed NPY-dig (red) and CART-35S (black) mRNA co-expression in the VLM of DIO mice.

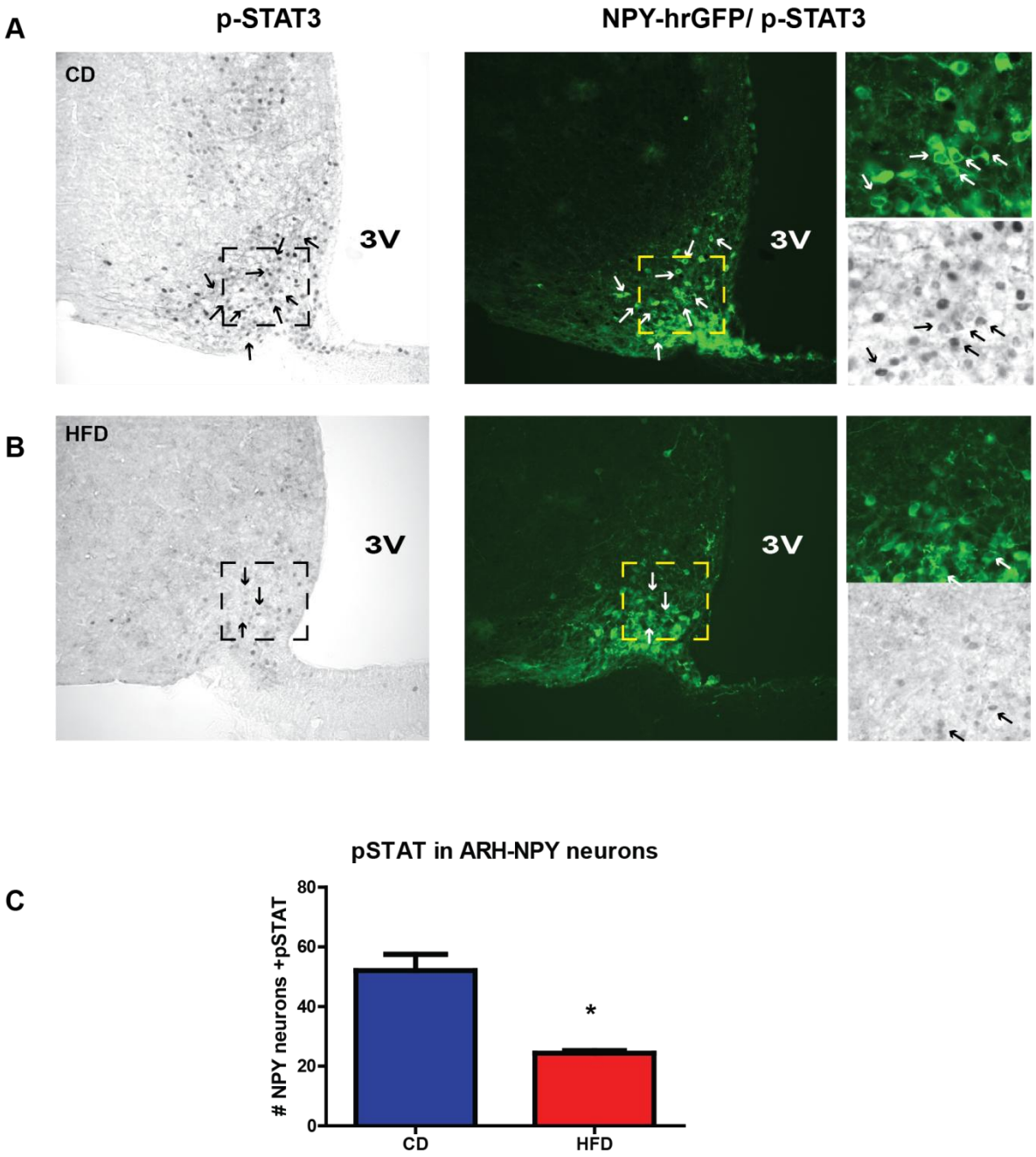

Figure 4-8. p-STAT 3 expression in ARH-NPY neurons in DIO mice.
p-STAT 3 activation in the ARH (left panels) and p-STAT3 in NPY-hrGFP cells (right panels) 45min after i.p. 2 µg/g leptin administration in 20 weeks CD mice (A) and HFD mice (B). NPY neurons are shown as green and p-STAT3-IR is black nuclear staining. The arrows indicate NPY neurons containing p-STAT3 activation.Magnified images showing NPY-hr GFP/p-STAT3 (upper) and p-STAT3 (lower) staining. (C) The bar graph shows the number of NPY neurons containing p-STAT3 staining from two ARH sections per animal (n=3). The results are expressed as mean ± SEM, * $p<0.05$ by unpaired t-test.

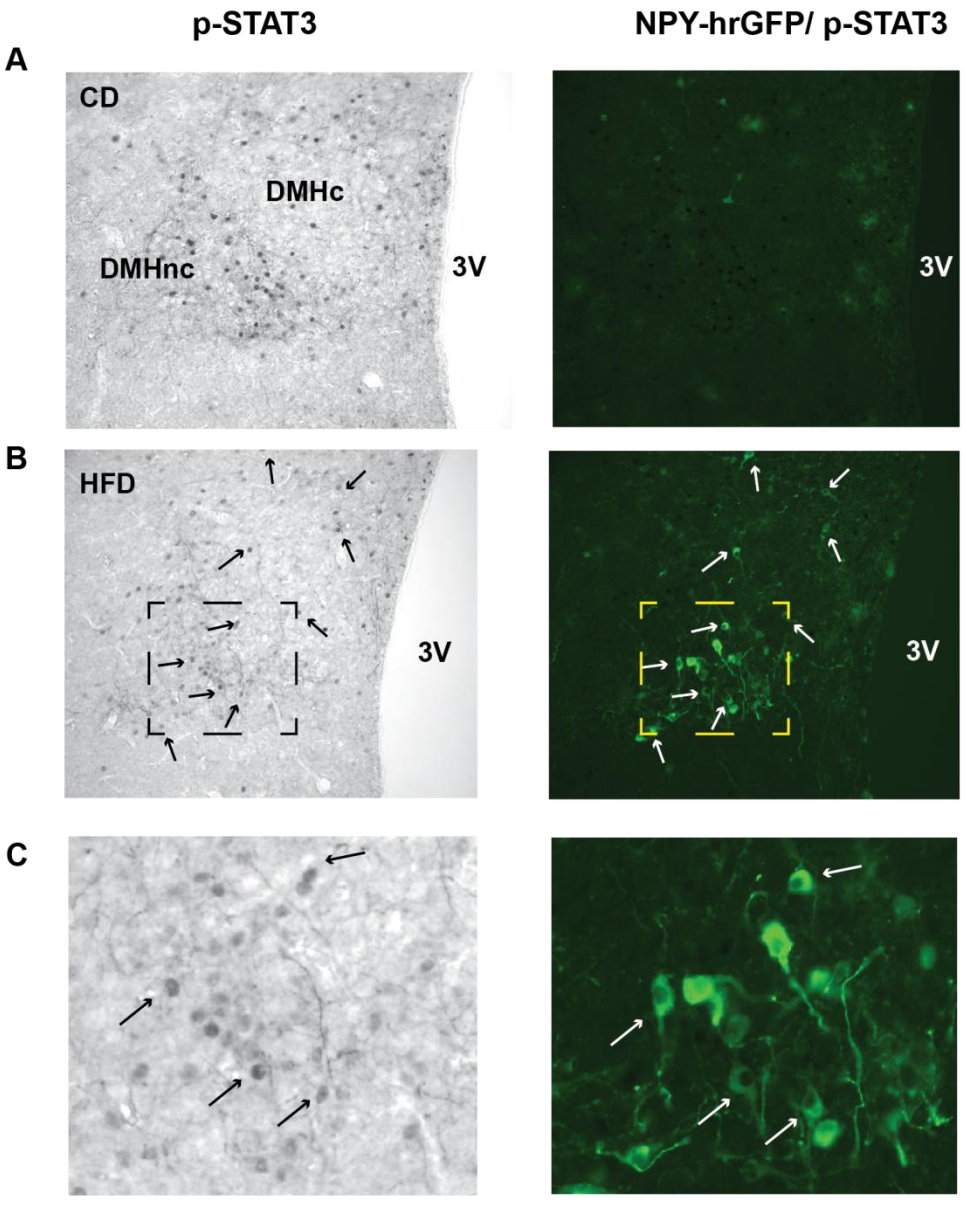

Figure 4-9. p-STAT 3 expression in DMH-NPY neurons in DIO mice.
p-STAT 3 activation in the DMH (left panels) and p-STAT3 in NPY-hrGFP cells (right panels) 45min after i.p. 2 μg/g leptin administration in 20 weeks CD mice (A) and HFD mice (B). NPY neurons are shown as green and p-STAT3-IR is black nuclear staining. The arrows indicate NPY neurons containing p-STAT3 activation. (C) Magnified images showing p-STAT 3 (left) and NPY-hrGFP/p-STAT3 (right). About 65 ± 2 % of of NPY neurons contain p-STAT3 activation (averaged from two DMH sections per animal , n=3). Values represent mean ± SEM.

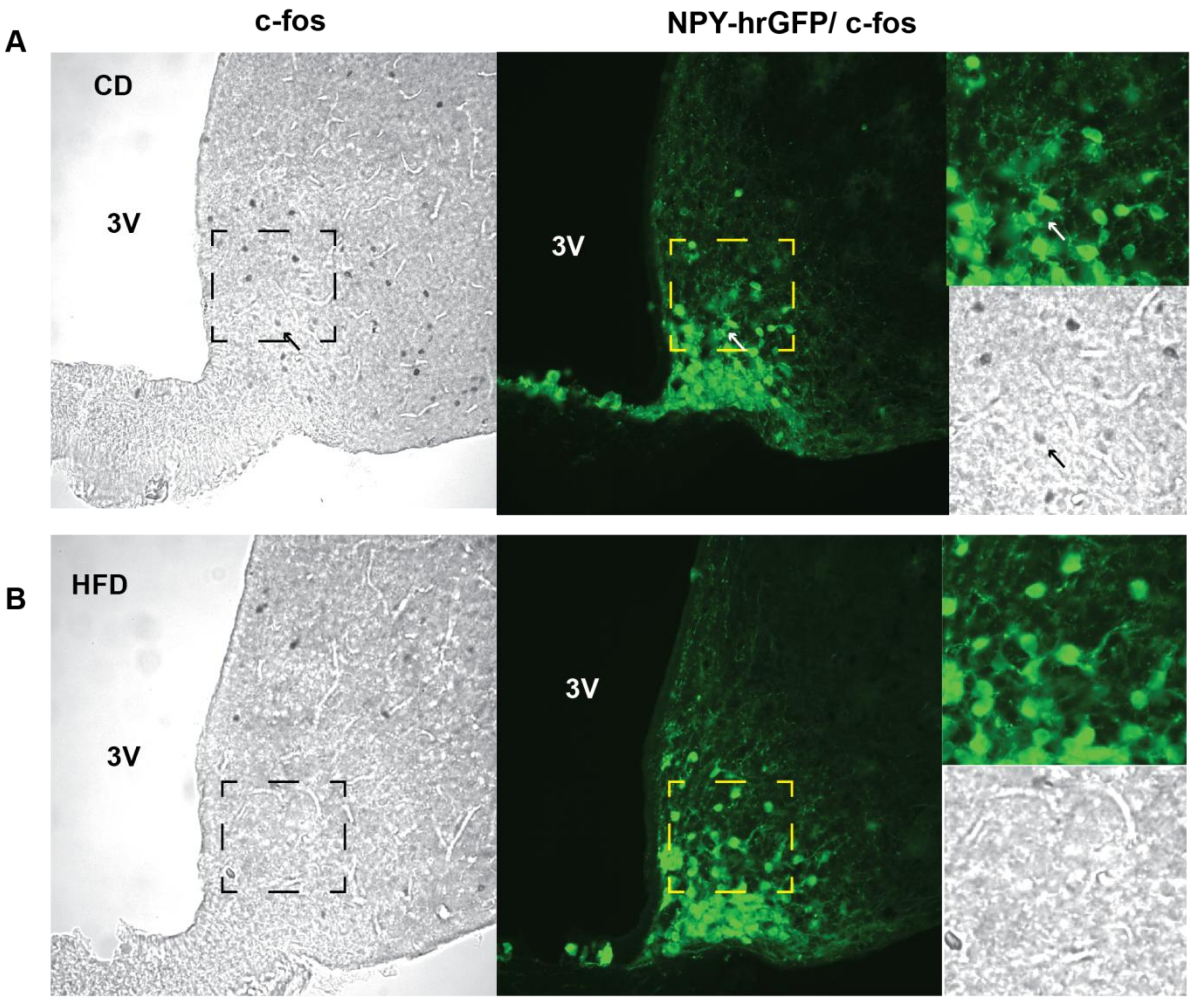

Figure 4-10. c-fos activation in ARH-NPY neurons in DIO mice.
c-fos activation in the ARH (left panels) and c-fos in NPY-hrGFP cells (right panels) 45min after i.p. 2 μg/g leptin administration in 20 weeks CD mice (A) and HFD mice (B). NPY neurons are shown as green and p-STAT3-IR is black nuclear staining. The arrows indicate NPY neurons containing c-fos activation. Magnified images showing NPY-hr GFP/p-c-fos (upper) and c-fos (lower) staining. In both CD and HFD mice, there were few NPY-hrGFP neurons co-expressing c-fos.

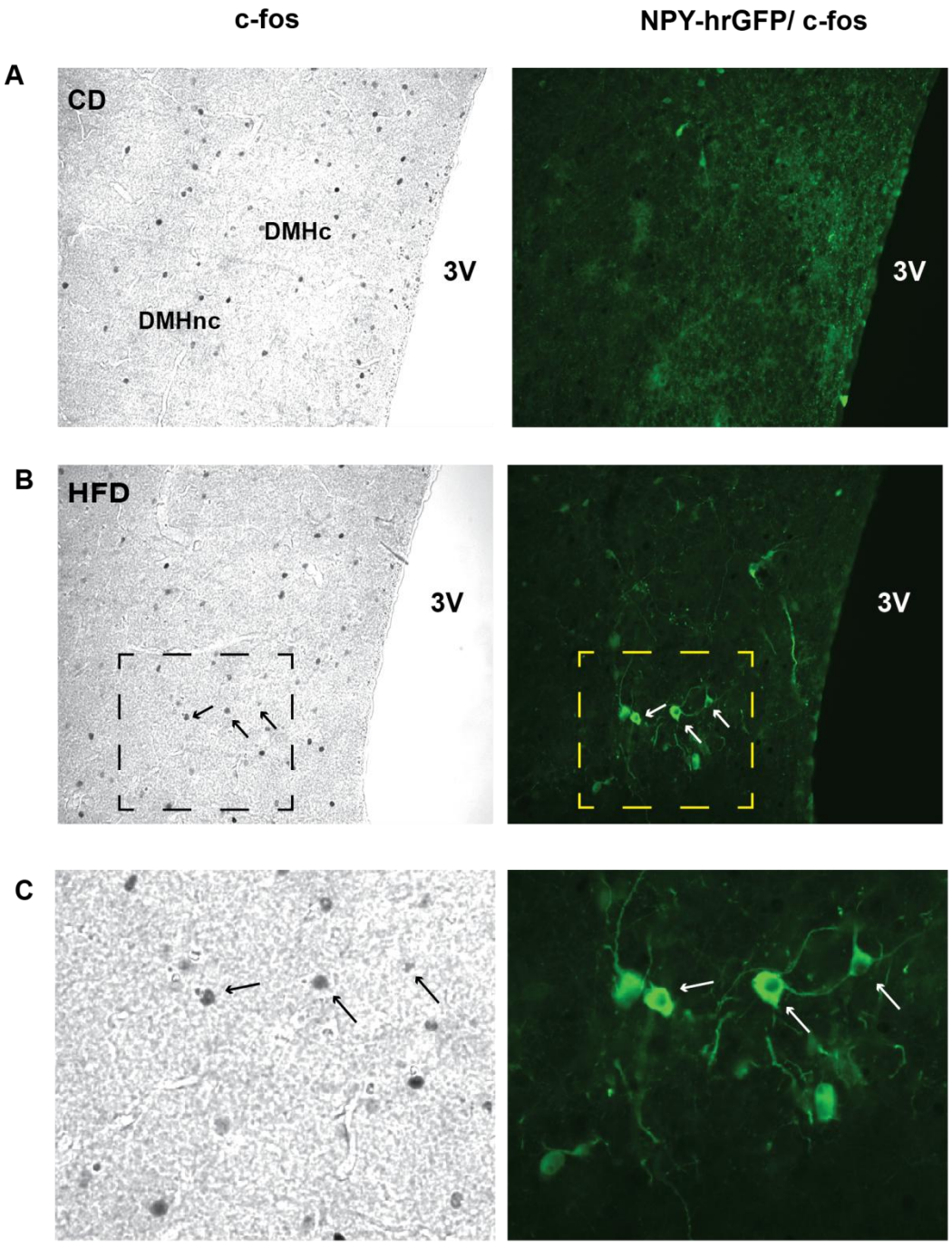

Figure 4-11. c-fos activation in DMH-NPY neurons in DIO mice.
c-fos activation in the DMH (left panels) and c-fos in NPY-hrGFP cells (right panels) 45min after i.p. 2 μg/g leptin administration in 20 weeks CD mice (A) and HFD mice (B). NPY neurons are shown as green and c-fos is black nuclear staining. The arrows indicate NPY neurons containing c-fos activation. (C) Magnified images showing that 47.50 ± 6 % of NPY-hrGFP neurons contain p-STAT3 activation in HFD mice (averaged from two DMH sections per animal , n = 4). Values represent mean ± SEM.

Chapter 5

DISCUSSION

The DMH is an important component of neurocircuitry controlling ingestive behavior and energy expenditure. A primary question in the field now is what integrating role DMH neurons play within the circuitry to modulate these behaviors. Previous studies demonstrated that the DMH is one of the major targets of hormonal/nutrient sensing neurons in the ARH, and viscerosensory and glucosesensing neurons in the brainstem (Bai et al., 1985; Renner et al., 2010; Sahu et al., 1988; Thompson and Swanson, 1998). The neural signals generated in these brain regions are integrated in the PVH where the neurons generate autonomic and neuroendocrine responses to alter feeding behavior and energy expenditure (Engelmann et al., 2004; Hallbeck et al., 2001; Sawchenko and Swanson, 1982a). DMH neurons also send major projections to the PVH (Bai et al., 1985) and serve as a relay station between these key regions in the brain involved in feeding regulation. Altered feeding behavior observed in DMH lesion studies clearly indicates that cell bodies and fibers located in the DMH are critical for maintaining normal energy balance. In addition, emerging evidence suggests that hormonal signals may have direct access to the nucleus and modify the activity of neurons projecting to the PVH (Elias et al., 2000). Despite its important position in feeding neurocircuitry, DMH neurons have not received much attention in the field until recently (Chao et al., 2011; Enriori et al., 2011; Yang et al., 2009; Zhang et al., 2011b), and the mechanisms by which DMH neurons regulate food intake and BAT thermogenesis largely remain unknown. Therefore, it is important to identify and characterize DMH neurons that are sensitive to metabolic signals and are functionally connected to other neurons involved in energy homeostasis. In this thesis, I focused on characterizing NPY neurons in the DMH

that are activated during chornic negative energy balance and obese conditions, and have been linked to hyperphagic behavior which is the common characteristic in these models.

NPY neurons in the DMH

The DMH contains anatomically and functionally distinct populations of NPY neurons (DMHc and DMHnc-NPY neurons), both of which have been implicated in feeding behavior and BAT thermogenesis (Chao et al., 2011; Chen et al., 2004). Animal models exhibiting abnormally high expression of NPY in both subdivisions show hyperphagia and obesity related phenotypes (Chen and Smith, 2004; Grove et al., 2001; Guan et al., 1998a; Guan et al., 1998b; Li et al., 1998a; Yang et al., 2009). Therefore, NPY activation in the DMH generally promotes food intake and energy conservation. However, NPY expression in the DMHc vs. DMHnc appears to be differentially regulated by hormonal and neural signals unique to each animal model. Furthermore, a species difference and temporal nature of DMHnc-NPY expression add more complexity to the characterization of these neuronal functions

Species Consideration

NPY is constitutively expressed in the DMHc in intact rats, while no expression is detected in the DMHc in mice. There is also no evidence of DMHc-NPY expression in other species including the non-human primate either during development or in adults (Grayson et al., 2006), suggesting that DMHc-NPY expression is unique to the rats. NPY neurons in the DMHc have been extensively studied in the OLETF rat model which has congenital CCK 1 receptor deficiency and displays hyperphagic behavior and obesity syndrome (Bi, 2007; Bi et al., 2001). The studies in this model suggest that normal CCK signaling is required for tonic inhibition of NPY gene transcription in the DMHc and a

deficit in CCK signaling is the main cause of NPY overexpression in the DMH, leading to hyperphagia and obesity. Unlike OLETF rats, CCK 1-/- mice do not exhibit NPY expression in the DMHc and do not become hyperphagic and obese (Bi et al., 2004). The absence of NPY expression in the DMHc suggests that DMHc-NPY neurons are not required for normal energy balance in mice. On the other hand, mice exhibit a transient expression of NPY in the DMHnc in specific conditions including development, lactation, and obesity, all of which have unusually high energy demands. While NPY is constitutively expressed in the DMHc, this temporal expression of NPY in the DMHnc is also conserved in similar physiological conditions in rats, suggesting that DMHnc-NPY neurons have similar functions in both species. Transient increase in NPY expression in the DMH during lactation has been also observed in sheep (Sorensen et al., 2002).

DMHc-NPY neurons

Knocking down NPY expression in the DMH in OLETF rats ameliorates the hyperphagia, obesity, and diabetes (Yang et al., 2009). Since viral mediated NPY overexpression in the DMH results in obese phenotypes, the outcomes of this knock down study are not surprising. The main caveat to this study is the relevance of genetically modified NPY expression in the DMHc in normal physiological conditions. When the same experimental approach is subsequently applied in normal rats fed with a high fat diet, NPY knock down in the DMH leads to attenuated weight gain and transforms white adipocytes to brown adipocytes in inguina adipose tissue (Chao et al., 2011), suggesting that DMHc-NPY neurons control BAT thermogenesis and differentiation as well as feeding behavior. Our tracing data together with evidence in the literature suggest that DMHc-NPY neurons may be polysynaptically connected to

adipose tissue via projections to the TRH and CRH neurons in the PVH which are known to regulate WAT and BAT differentiation (Stanley et al., 2010). However, it is unclear whether the physiological effects of NPY knock down were solely due to the loss of DMHc-NPY projections to these neurons. While NPY is induced in the DMHnc in the late stage of diet-induced obesity in mice, DMHnc-NPY expression in DIO rats has not been well characterized. In fact, DMHc-NPY expression does not appear to fluctuate in hyperphagic conditions in the rat such as early postnatal development and lactation, suggesting that blocking NPY induction in the DMHnc may be at least partially responsible for antiobesity effects of DMH-NPY knock down study in DIO rats. Regardless, the potential role of DMHc-NPY neurons in feeding behavior and BAT thermogenesis is also supported by our current findings that these neurons may regulate PVH neurons involved in these homeostatic functions. Increased DMHc-NPY expression has also been associated with weight restoration after long term calorie restriction in rats. Although the increase in NPY expression was mainly described in the DMHc (Bi et al., 2003), it is difficult to conclude that DMHc-NPY neurons are solely responsible for the stimulation of feeding behavior during chronic negative energy balance.

DMHnc-NPY neurons

Due to the transient nature of its expression, DMHnc-NPY expression is often ignored or not distinguished from DMHc-NPY expression. NPY neurons in the DMHnc are distinct from the compact zone populations since they are specifically activated during certain physiological conditions that favor overfeeding and thus have important implications in human obesity. Transient NPY expression in the DMH as well as other

hypothalamic areas supports hyperphagia during development which is necessary for proper growth rather than energy storage and fat accumulation during the critical perinatal period. During maternal deprivation, NPY expression is reduced in the DMHnc, while no change is observed in the DMHc in developing rats (Grove et al., 2001), confirming that DMHnc-NPY expression is a reflection of hyperphagia, but does not appear to drive hunger and food craving. Hyperphagia is also one of the main adaptations during lactation, and NPY induction in the DMHnc is directly stimulated by the suckling stimulus which is a unique phenomenon in the lactation model. DMHnc-NPY expression is still present under food deprivation during suckling stimulus, suggesting that the suckling stimulus is able to sustain activation of these neurons. NPY induction in the DMHnc in DIO mice models is also very unique because of the timing of NPY induction. Despite the rapid weight gain immediately after initiating HFD feeding, a significant NPY induction in the DMH is not detectable until prolonged HFD consumption. Therefore, DMHnc-NPY induction is not the cause of the initial weight gain, but is a consequence of obesity. Like during development, fasting reduces this NPY expression, suggesting a direct link between feeding behavior and DMHnc-NPY expression in DIO model. While DMHc-NPY expression is clearly mediated by CCK signaling in intact rats, the mechanisms of DMHnc-NPY induction in the hyperphagic animal models mentioned above differ and will be discussed in more detail below.

DMHnc-NPY vs. ARH and brainstem NPY neurons

DMHnc-NPY neurons are functionally distinct from ARH-NPY neurons which are the primary driver of immediate feeding response after acute food deprivation. NPY expression in the DMHnc is not induced in response to acute nutritional challenge, but is

increased to prevent negative energy balance when ARH-NPY expression alone is insufficient to maintain normal energy balance. Our tracing data suggest that DMHnc-NPY neuronal projections may target the same neurons in the PVH as innervated by ARH-NPY neurons and provide additional orexigenic inputs to the neurons that modulate feeding behavior during periods of high energy demand. In obese animal models, loss of orexigenic drive from the ARH is compensated by increased NPY expression in the DMHnc. During development and lactation, DMHnc-NPY expression supplements increased orexigenic drive from ARH- NPY neurons. Therefore, DMHnc-NPY neurons mediate hyperphagia and other metabolic adaptations in both negative and positive energy balance through distinct mechanisms.

Differential gene expression of ARH vs. DMHnc-NPY neurons in our microarray study clearly supports that DMHnc-NPY neurons are functionally unique from ARH-NPY populations. ARH-NPY neurons co-express AgRP, which is another potent orexigenic neuropeptide, and also release GABA to neighboring POMC neurons to regulate food intake. GABA is also produced in DMHnc-NPY neurons during development; however, the role of GABA in these neurons in feeding behavior has not been addressed. While the gene expression in ARH-NPY neurons is tightly regulated by fluctuating hormonal levels, DMHnc-NPY neurons do not appear to be regulated by the short term changes in metabolic profile. In fact, NPY and leptin receptor mRNA expression has not been co-localized in DMHc in normal rats (Bi et al., 2003). Furthermore, leptin receptors are not expressed in DMHnc-NPY neurons during the developmental period in mice (Draper et al., 2010), supporting that DMHnc-NPY neurons are not directly responding to leptin. However, our more recent findings

provided the first evidence that DMHnc-NPY neurons exhibit direct response to leptin in mice during DIO condition. Why DMHnc-NPY neurons change their chemical phenotype and become sensitive to leptin in the DIO condition is unclear. In the later stages of obesity, DMHnc-NPY neurons also co-express CART which is clearly stimulated by chronically elevated leptin and may play an essential role in obesity development. Although leptin's effect on NPY gene expression in the DMHnc still remains elusive, increased sensitivity to leptin in these neurons may be some of neural adaptations in response to leptin resistance in the ARH, and increase orexignic drive to support the body weight in severe obese conditions.

The brainstem contains NPY co-expressing catecholaminergic neuronal populations which are functionally and anatomically diverse. The most prominent role of these brainstem NPY neurons in energy homeostasis is glucoprivic feeding regulation (Ritter et al., 2006). Lesioning of specifically the hindbrain catecholamine/NPY neurons that project to the PVH abolish glucoprivic feeding (Li and Ritter, 2004; Ritter et al., 2001), suggesting that brainstem NPY neurons are a key player in glucosensing and are functionally distinct from ARH-NPY neurons that appear to be more critical for sensing adiposity signals such as leptin and insulin. Similar to ARH-NPY neurons, brainstem NPY neurons heavily project to PVH and modulate CRH and TRH neuronal functions implicated in feeding behavior (Fuzesi et al., 2007; Lechan and Fekete, 2006; Sawchenko et al., 1985). NPY/DBH fiber terminals are significantly increased in the PVH in lactating rats (unpublished observation), indicating that brainstem NPY projections may also contribute to the stimulation of food intake during lactation. Furthermore, brainstem neurons activated by the suckling stimulus may induce DMHnc-NPY expression to

stimulate food intake in lactation (Chen and Smith, 2003). Our study demonstrated that NPY/catecholamine neurons in specific regions of the brainstem also express CART. Since NPY and DBH expression in the brainstem are increased by glucoprivation (Li and Ritter, 2004), brainstem CART may also be sensitive to glucoprivic conditions. NPY/CART/DBH triple-labeled fiber terminals were observed in the PVH (unpublished observation), indicating that all three neuropeptides could simultaneously modulate CRH and TRH neuronal functions. The redundancy of NPY inputs from different origins converging into the same target neurons in the PVH likely play different roles depending on the different types of inputs and may explain why our body is well protected from negative energy balance.

Chemical phenotypes of DMHnc-NPY neurons

GABA

Using microarray gene analysis, we attempted to find alternate phenotypes of DMHnc-NPY neurons during the postnatal period in mice so as to be able to study these neurons under normal conditions when they are not expressing NPY. According to our findings, DMHnc-NPY neurons did not express other known neuropeptides that are implicated in feeding behavior. Instead, we identified the expression of GABAergic markers in DMHnc-NPY neurons. GABA is also co-expressed in AgRP/NPY neurons and plays an important role in modulating the activity of POMC/CART neurons to promote feeding (Tong et al., 2008b). It is currently unknown what role GABA plays in both ARH and DMHnc-NPY neurons during development. Although no evidence is available in the literature, DMHnc-NPY neurons may retain their GABAergic phenotype in adult mice.

CART

In adult mice, CART is co-expressed in DMHnc-NPY neurons in the DIO condition. Interestingly, CART expression is not observed in NPY-GFP cells in the DMH during development (unpublished observation), suggesting that these neurons are differentially regulated depending on the metabolic condition. In normal conditions in mice, CART is moderately expressed in the DMHnc where NPY expression is induced during DIO condition. Therefore, it is possible that CART may be the alternate phenotype of DMHnc-NPY neurons when NPY expression is undetectable, possibly due to the sensitivity of the probes for detecting low mRNA level, in normal adult mice. CART expression is also not co-localized with the DMH-NPY neurons in lactating rats (unpublished observation). Consistent with low leptin levels in lactation, CART expression in the DMH appears to be similar to that of normal cycling rats, suggesting that CART expression in the NPY neurons may be mediated by high levels of leptin.

Since CART peptides are anorexigenic in nature, NPY and CART co-production in the same neuron during DIO condition is puzzling. CART is known to interact with NPY as the injection of CART peptide before NPY reduces the increase in feeding caused by NPY injection alone (Lambert et al., 1998). It is possible that early induction of CART expression in the DMH is the defense mechanism against obesity, and prolonged HFD consumption induces NPY expression in a specific population of CART neurons which leads to the inhibition of CART's anorexigenic actions during the late stage of obesity. Another possible explanation is that CART expression in the DMH may have an orexigenic effect which is supported by several findings in the field (Abbott et al., 2001; Hou et al., 2010). In contrast to anorexigenic phenotypes observed with i.c.v

CART injection, a direct injection of CART peptide into specific nuclei in the hypothalamus stimulates feeding behavior, indicating the complexity of CART peptide in feeding regulation. Since CART neurons express leptin receptors and still respond to leptin in DIO mice, the contribution of CART in increased sympathetic activity mediated by hyperleptinemia is also a possible scenario. Future studies will be necessary to further investigate the true nature of NPY/CART co-localization in the DMH during DIO condition

Potential mechanisms of DMHnc-NPY induction

Despite the common hyperphagic behavior, our data suggest that the mechanism of DMHnc-NPY induction may be distinct in each model. An interesting observation is that DMHnc-NPY expression is positively correlated with serum leptin levels in most examples except lactating animals. Although there is no evidence supporting the direct role of leptin in DMHnc-NPY expression during development, it is possible that the hyperleptinemic state is a prerequisite for DMHnc-NPY induction.

Postnatal period

Developing rodents exhibit high levels of circulating leptin levels. The postnatal leptin surge has been implicated in normal development of feeding neurocircuitry rather than being a satiety factor (Bouret et al., 2004b). Although leptin does not affect food intake and body weight during this rapid growth period, NPY/AgRP neurons in the ARH are still regulated by leptin (Caron et al., 2010; Grove et al., 2001; Proulx et al., 2002). Therefore, it is reasonable to speculate that the leptin surge during the perinatal period may also play a role in DMHnc-NPY expression. However, our data showed that DMHnc-NPY neurons do not express leptin receptors or exhibit direct receptor

activation, suggesting that they are not directly regulated by leptin at this early stage. We did not investigate the role of other hormones in DMHnc-NPY expression during the postnatal period. However, our preliminary data showed that DMHnc-NPY neurons from postnatal day 15 mice express insulin receptor mRNA. In addition, the presence of transcription factors involved in insulin signaling pathways in DMHnc-NPY neurons suggests that high levels of insulin during postnatal period may be one of main factors responsible for transient DMHnc-NPY expression.

From our microarray study, we identified several transcription factors that are highly expressed in DMHnc-NPY neurons compared to ARH-NPY neurons during development. FOXA1, FOXO1, and PGC-1α have been implicated in energy homeostasis mainly in peripheral tissues (Liang and Ward, 2006; Sasaki and Kitamura, 2010; Shih et al., 1999), but the role of these transcription factors in hypothalamic gene regulation has not been well characterized. Nonetheless, evidence in the literature suggests that some of these transcription factors may be involved in transcriptional activities in NPY neurons in the hypothalamus (Ma et al., 2010; Sasaki and Kitamura, 2010). In particular, PGC1-α is a member of a family of transcriptional coactivators that play a central role in the regulation of cellular energy metabolism, including adaptive thermogenesis, and glucose metabolism (Liang and Ward, 2006). PGC1-α mRNA is highly expressed in the brain during development, but it declines to an undetectable level in the hypothalamus in adults (Cowell et al., 2007; Tritos et al., 2003), indicating that PGC1-α is a prime candidate for the transcriptional regulation of metabolism during early brain development. Furthermore, we demonstrated that PGC1-1α mRNA/protein expression is localized in the DMH-NPY neurons in P15 mice (Draper et al., 2010),

suggesting that PGC-1α regulates gene exprerssion in DMH-NPY neurons during development.

Emerging evidence suggests that ghrelin also plays an important role during development (Steculorum and Bouret, 2011). Since ghrelin stimulates NPY gene expression in the ARH, the role of ghrelin in DMHnc-NPY expression is also promising and is subject to further investigation. It is also possible that DMHnc-NPY expression is regulated by ARH neuronal projections. ARH projections to the DMH do not fully develop until the second week of the postnatal period (Bouret et al., 2004a; Grove et al., 2003; Grove and Smith, 2003), which coincides with the peak of DMHnc-NPY expression around postnatal day 15 (Grove et al., 2001). The establishment of ARH neuronal projections in the DMH may terminate the transient NPY expression in the DMHnc by providing tonic GABAergic inhibition from NPY/AgRP/GABA neurons or other local GABA neurons under the regulation of the melanocortin system. Although exogenous leptin treatment does not alter feeding behavior in developing animals, leptin is known to promote ARH neuronal projections (Bouret et al., 2004b); therefore, it may indirectly regulate DMHnc-NPY expression via ARH projections during development.

Lactation

DMHnc-NPY expression is clearly activated by the suckling stimulus. While the suckling stimulus is present, DMHnc-NPY neurons are activated even during food deprivation in lactating rats. Since food intake is normally required for DMHnc-NPY expression in other chronic hyperphagic models, the suckling stimulus is unique in the way it activates the entire brain NPY system to drive feeding behavior to produce milk for the pups (Li et al., 1998a, b, 1999a, c, d). In the lactating model, it is important to

consider the contribution of suckling activated hypothalamic and brainstem projections to DMHnc-NPY induction. Although the phenotypes of these neurons activated by suckling have not been characterized, the activation of glucosesensing NPY /catecholamine neurons in the brainstem are particularly interesting since their projections to the hypothalamic nuclei including the PVH, ARH, and DMH play important roles in feeding behavior and reproduction.

Brainstem neurons activated by suckling stimulus project to tuberoinfundibular dopamine (TIDA) neurons in the ARH and modulate the inhibitory effect of dopamine on prolactin secretion (Li et al., 1999d). Prolactin secretion is necessary for milk production during lactation and has been also strongly implicated in lactational hyperphagia and DMHnc-NPY expression (Chen and Smith, 2004; Li et al., 1999d). In the presence of suckling stimulus, bromocriptine treatment attenuates DMHnc-NPY expression in lactating rats, but has no effect in ARH-NPY expression (Chen and Smith, 2004). This specific effect of prolactin in DMHnc-NPY expression is further supported by the presence of prolactin receptors in DMHnc-NPY neurons but not in ARH-NPY neurons. Increased prolactin secretion during lactation therefore may activate NPY expression in the DMHnc via its receptors, and subsequently stimulate food intake and oxytocin release for the "milk let- down" reflex via PVH projections. It would be interesting to determine if prolactin replacement alone can induce DMHnc-NPY expression in normal rats or if other parameters altered during lactation are also required for its induction.

Despite the hyperphagia and increased body weight, lactation is characterized by low leptin levels. Our group previously demonstrated that leptin and insulin replacement to normal physiological level does not affect either food intake or DMHnc-NPY

expression during lactation in rats (Xu et al., 2009), suggesting that low leptin and insulin levels are not the main causes of DMHnc-NPY induction. However, a recent study comparing leptin i.c.v injection vs. peripheral replacement demonstrated that the replacement of leptin in the brain, but not peripheral replacement, leads to food reduction in lactating rats (Suzuki et al., 2010), suggesting that lactation is a state of peripheral leptin resistance. Therefore, low leptin in the brain is the main permissible factor for ARH-NPY neurons to stimulate food intake in lactating rats. The consequence of leptin resistance on DMHnc-NPY expression is less clear.

Since POMC expression is significantly decreased and AgRP is increased in the ARH in lactation, another alternate hypothesis is that reduced melanocortin signaling in the DMH may play a role in NPY induction in the DMH. Our group demonstrated that melanocortin receptor agonist MTII injection into the DMH of lactating rats leads to decreased food intake and DMHnc-NPY expression (Chen et al., 2004). Since melanocortin receptors are not expressed in DMHnc-NPY neurons, these neurons may be normally inhibited by GABAergic neuronal projections that are regulated by melanocortin system. The blockade of GABA release by increasing AgRP/ α-MSH ratio on melanocortin receptors may activate DMHnc-NPY gene transcription during lactation. However, leptin and/or insulin replacement partially recovered ARH-AgRP and POMC expression in the ARH which did not affect DMHnc-NPY expression, providing evidence against this hypothesis.

Diet induced obesity

After prolonged HFD consumption, DIO animals develop leptin resistance which is linked to the development of obesity and metabolic syndrom. In the brain, leptin

resistance is mainly described in the ARH and the severity of the resistance depends on the duration of HFD consumption (Munzberg et al., 2004). According to Lin et al., development of DIO can be divided into three stages (Lin et al., 2000). The early stage of DIO in mice is characterized by relatively normal food intake but elevated body weight and fat gain. During the middle stage (4-15weeks), DIO animals exhibit hypophagia and become resistant to peripheral leptin treatment while the central leptin response is still intact. Late stage DIO animals (after 15weeks) become hyperphagic and develop central leptin resistance. In our study, we confirmed the development of leptin resistance in ARH-NPY neurons in the late stage of DIO mice as the neurons are not sensitive to peripheral leptin treatment. A significant induction of DMHnc-NPY expression was observed during the late stage of DIO, indicating potential implications of systemic leptin resistance in DMHnc-NPY induction. According to Guan et al., ARH-POMC expression is not different between DIO and normal chow fed animals after 24 weeks of diet treatment (Guan et al., 1998a). However, Ziotopoulou et al. showed that POMC gene expression is elevated during the early phase of diet induced obesity (Ziotopoulou et al., 2000). Therefore, it is reasonable to hypothesize that increased leptin levels suppress ARH-NPY and increase POMC expression, resulting in initial hypophagia, and the development of leptin resistance in both ARH neurons subsequently promotes hyperphagia by activating DMHnc-NPY neurons.

GABA release from NPY/AgRP neurons is an important mediator of ghrelin's stimulatory effect on food intake (Tong et al., 2008b). A recent study demonstrated that NPY/AgRP neurons also become resistant to ghrelin in DIO mice (Briggs et al., 2010). Therefore, defective GABA release from NPY/AGRP neurons due to ghrelin resistance

can be one of the potential mechanisms of DMHnc-NPY induction. GABA release from ARH-NPY neurons may also provide tonic inhibition on DMHnc-NPY neurons under the control of melanocortin system. Similar to the lactating model, the removal of this inhibition during systemic leptin resistance may be the potential mechanism of DMHnc-NPY induction. Future studies will be necessary to investigate the potential mechanisms of DMHnc-NPY induction mediated by ghrelin resistance and melanocortin system in DIO model.

Another potential mechanism for DMHnc-NPY induction is a direct action of leptin on the neurons. Leptin receptor expressing neurons are present in the DMH and they are still responsive to leptin after chronic HFD consumption (Matheny et al., 2011). We demonstrated that DMH-NPY/CART co-expressing neurons are also responsive to peripheral leptin treatment in the late stage of DIO, suggesting that both CART and NPY gene expression may be regulated by leptin. Evidence strongly suggests that CART induction in DIO animals is mediated by increased adiposity and high levels of leptin (Elias et al., 2001; Yu et al., 2008). Consistent with this hypothesis, *ob/ob* mice have reduced DMH-CART and NPY expression despite a morbid obesity and hyperphagia. Since leptin is known to inhibit the activity of ARH-NPY neuron, it is difficult to comprehend why leptin would stimulate NPY expression in the DMH during DIO condition. A recent study using hypothalamic cell lines expressing NPY showed that leptin can either inhibit or stimulate NPY secretion depending on the cell type (Dhillon and Belsham, 2011). They also demonstrated that leptin can specifically regulate individual neurons through distinct signaling pathways. Therefore, it is possible that

leptin may exert a stimulatory effect on NPY gene expression via a distinct signaling mechanism from ARH-NPY/AgRP neurons in the late stage of DIO.

However, an inhibitory role of leptin in DMH-NPY gene transcription cannot be ruled out. Although DMH neurons are still responsive to leptin in DIO mice, p-STAT3 signaling is apparently reduced in the DMH neurons in the late stage of DIO compared to normal chow fed animals when injected with leptin, suggesting that DMH-NPY/CART neurons may develop leptin resistance that triggers NPY gene expression. However, using c-fos as a marker, increased neuronal activation is detected in the DMHnc in DIO mice (Xin et al., 2000). Furthermore, our recent data showed that these c-fos positive cells are indeed NPY neurons in DIO mice, suggesting that DMHnc-NPY neurons are chronically activated by hyperleptinemia in DIO mice. Future electrophysiological experiments will be important to reveal the direct effect of leptin in NPY-GFP cells from the DMH of DIO mice. In addition, leptin antagonist injection into the DIO mice will test the role of leptin in NPY and CART gene expression.

In addition to leptin, adipocytes increase the production of other proteins and cytokines in obese conditions (Antuna-Puente et al., 2008). Resistin is another protein secreted by adipocytes, and is implicated in insulin resistance and inflammation. Resistin levels are increased in obesity and resistin deficiency clearly improves glucose homeostasis in several models of severe obesity including DIO and *ob/ob* mice (Qi et al., 2006). Singhal et al. published interesting data showing that central resistin injection in normal mice induces hepatic insulin resistance which seems to be mediated by NPY expression in the ARH and DMH (Singhal et al., 2007). These studies strongly suggest that resistin mediated NPY induction in the DMHnc is the contributing factor for insulin

resistance and diabetes in DIO mice. However, no information regarding the presence of resistin receptors in these NPY neurons is currently available. Therefore, the direct effect of resistin in DMHnc-NPY neuronal activity and gene transcription should be tested in the future studies. Nonetheless, the changes in adipose secreted factors in obesity clearly modulate hypothalamic pathways involved in energy homeostasis and further contribute to the pathogenesis of metabolic disorders associated with obesity.

Efferent projections of DMHnc-NPY neurons

Feeding behavior

Our tracing data from two DMHnc-NPY induction models, the lactating rat and DIO mouse, clearly demonstrated that DMHnc-NPY neurons send major projections to the PVH and LH. NPY injection into the PVH and PFA elicits a powerful stimulatory effect on feeding behavior (Stanley and Leibowitz, 1985; Stanley et al., 1993). The stimulatory effect of NPY on food intake is largely mediated by NPY Y1 and Y5 receptors in the PVH (Beck, 2006; Zhang et al., 2011a). TRH and CRH neurons in the PVH express these receptors and are considered important players in feeding regulation and sympathetic outflow. NPY has a potent inhibitory effect on TRH gene expression in the PVN in the fasting condition (Fekete and Lechan, 2007). Therefore, increased NPY inputs from the DMH should lead to decreased TRH neuronal activity in chronic hyperphagic models. However, TRH gene expression is increased in both lactation and DIO models (Araujo et al., 2010; Sanchez et al., 2007). It is unclear why TRH expression is stimulated in lactation even though NPY inputs from the ARH, DMH and brainstem to the PVH are greatly amplified. Although purely speculative, TRH gene expression may be stimulated by NPY via Y1 or Y5 mediated presynaptic GABAergic inhibition during

lactation. NPY terminals in the PVH also release other neuropeptides such as CART, AgRP, NE (norepinephrine**)**, and E (epinephrine), and the changes in these neuropeptide interactions should be taken into considerations in lactation.

Increased NPY/AgRP inputs from the ARH are known to inhibit CRH mRNA expression in the PVH during fasting condition in rats (Fekete et al., 2001; Fuzesi et al., 2007). In contrary to the activation of endogenous NPY system, central administration of NPY stimulates CRH mRNA expression in rats (Haas and George, 1987; Sarkar and Lechan, 2003). It is possible that increased DMHnc-NPY projections stimulate HPA axis to alter feeding behavior in both lactating and DIO model. There is a clear link between obesity and altered HPA axis activity. Central infusion of glucocorticoids is known to increase food intake and body weight (Cusin et al., 2001), contributing to obesity. Patients with hyperadrenocorticism (Cushing's disease) are obese and have elevated glucocorticoid levels (Anagnostis et al., 2009). Hypercorticosterone is also observed in lactation and is normalized after weaning. Due to technical limitations, we were unable to demonstrate the anatomical relationship between DMHnc-NPY projections and CRH neurons in our models. In future studies, knocking down DMHnc-NPY expression in DIO mice will provide insights into the potential connection between DMHnc-NPY induction and HPA axis in the regulation of feeding behavior.

In attempt to find the downstream target of DMHnc-NPY neurons, we demonstrated that DMHnc-NPY projections are in close apposition to CART expressing cells in the PVH in the lactation model. Although many CART neurons in the PVH are also co-expressed with TRH (Elias et al., 2001), we did not provide direct evidence that CART neurons indeed express TRH. The role of CART neurons in the PVH is best

known to inhibit TRH release, and, therefore, to antagonize prolactin release, which is also linked to milk production and hyperphagia during lactation (Sanchez et al., 2007). It is possible that CART expression is directly inhibited by DMHnc-NPY projections, allowing TRH neurons to stimulate prolactin release during lactation. This hypothesis should be tested in the future with the co-localization study for CART and NPY Y1/Y5-R in the PVH. Manipulating DMHnc-NPY neuronal activation with the suckling stimulus may influence PVH-CART expression and provide functional evidence of DMHnc-NPY regulation of prolactin secretion.

Thermogenesis

DMHnc-NPY induction has been linked to the decrease in BAT thermogenesis in lactating model. On the other hand, DIO mice are characterized with increased BAT thermogenesis late in the course of DIO development. Although central NPY injection decreases BAT themogenesis (Billington et al., 1991; Kotz et al., 1998), we demonstrated that DMHnc-NPY neurons do not send direct projections to the major thermoregulatory site, rRPa. Instead, DMH and PFA orexin neurons have emerged as the critical mediator for BAT thermogenesis via direct projections to rRPa. However, a potential role for DMHnc-NPY neurons in BAT thermoegenesis cannot be ruled out. TRH neurons innervated by DMHnc-NPY projections may indirectly regulate BAT projecting premotor neurons in the rRPa, which contain prominent TRH receptor expressing fibers and varicosities (Barnes et al., 2010). Furthermore, Madden and Morrison demonstrated that disinhibition of neurons in the PVH inhibits BAT sympathetic nerve activity via activation of GABAergic inputs to BAT sympathetic premotor neurons in the rRPa (Madden and Morrison, 2009). Therefore, it is possible that DMHnc-NPY neuronal

projections to the PVH may activate GABAegic neurons that project to the rRPa, resulting in the suppression of BAT activity during lactation.

The difference in circulating leptin levels may explain the discrepancy between lactation and DIO model in BAT thermogenesis. CART expression is significantly increased in the DMH in parallel with leptin levels in DIO mice, while CART expression in the DMH is not as robust in lactating rats. It is likely that DMH-CART is the main player in BAT thermogenensis during DIO development. CART gene polymorphisms have been associated with the modulation of post-absorptive resting energy expenditure and fat-induced thermogenesis in obese individuals (Goossens et al., 2009). Recently, Enriori et al. demonstrated that leptin injection into the DMH stimulates BAT thermogenesis in DIO model, suggesting that CART may be the neurochemical phenotype of leptin receptor expressing cells in the DMH mediating BAT thermogenesis. In support of this hypothesis, DMH-CART expression is increased with leptin treatment in *ob/ob* mice which normalize BAT thermogenesis as well as food intake (Kristensen et al., 1998). Additionally, we demonstrated that DMHnc-NPY/CART expressing neurons are activated by peripheral leptin treatment under hyperleptinemic condition in DIO mice. Similar to lactating rats, the BDA tracing study in DIO mice revealed that DMHnc-NPY/CART expressing neurons do not send direct projections to the rRPa. However, DMHnc-NPY/CART projections were observed in the PVH and LH, suggesting indirect BAT thermoregulatory pathways via neurons in these areas. PVH-TRH and CART expression have been implicated in cold induced thermogenesis and are increased in response to cold exposure (Sanchez et al., 2007). Orexin neurons in the PFA send direct projections to the rRPa and orexin has been shown to modulate brown fat

thermogenesis, and cardiovascular and gastrointestinal functions by acting directly on neurons in the rRPa (Berthoud et al., 2005). Therefore, projections of DMHnc-NPY/CART neurons to these neurons in the PVH and LH should be further investigated in the DIO model.

The role of NPY induction in the CART neurons in regards to energy expenditure is another important area of future study. Despite the increase in sympathetic acivity and BAT thermogenesis, DIO animals are capable of maintaining body weight gain and fat accumulation, indicating that BAT activity is offset by another mechanism such as the reduction of dark cycle activity, potentially mediated by NPY induction (So et al., 2011). It is also possible that NPY modulates CART release at the synaptic terminals or the activity of CART target neurons to promote feeding and reduce energy expenditure. Future studies will be necessary to understand the interactions between these two peptides released from the same neuron in regards to the regulation of food intake and BAT thermogenesis.

Future directions

This thesis work is mainly focused on the neuroanatomical characterization of DMHnc-NPY neuronal circuit. We have discovered several potential targets of DMHnc-NPY neuronal projections and hypothesized the potential mechanisms related to the regulation of feeding, sympathetic activity, and reproduction. However, direct evidence connecting DMHnc-NPY induction and these behavioral changes is still lacking. Melanocortin agonist injection into the DMH in lactating rats provided the first evidence linking DMHnc-NPY expression, and hyperphagia and BAT thermogenesis (Chen et al., 2004). Chao et al. recently used viral mediated RNA interference approach to knock

down DMH-NPY expression in DIO rats (Chao et al., 2011). To study the contribution of DMHnc-NPY induction in obesity, the DIO mouse will be a better model since the mouse does not express NPY in the compact zone. In addition, the time course of DMHnc-NPY induction in DIO mice is well described in this thesis. Since DMHnc-NPY induction was first observed around 10 weeks after HFD treatment, it will be interesting to knock down DMHnc-NPY expression prior to the induction to investigate the effects on food intake, body weight, and other metabolic parameters. Knocking down DMHnc-NPY expression after the maximal induction will be also interesting since these animals have severe metabolic syndrome at this stage and NPY knock down should ameliorate some of these symptoms.

Although the initial discovery of CART revolutionized the field, CART research has been profoundly disabled because the CART receptor has not been identified. CART is expressed in many hypothalamic areas involved in energy homeostasis; however, the role of this CART expression including that in the DMH is vastly unknown. A DMH-CART knock down study in DIO mice will be important to understand the contribution of CART induction in obesity phenotypes. DMH-CART expression may be the defense mechanism against obesity and the main driver of BAT thermogenesis in DIO condition; therefore, loss of CART functions may further increase appetite and body weight. It will also be interesting to see how DMH-CART knock down affects NPY induction in the same neuron in DIO mice.

While efferent projections of DMHnc-NPY neurons have been extensively characterized in this thesis, afferent projections innervating the NPY neurons are unknown. Since NPY-GFP cells can be easily visualized in the DMHnc of DIO mice, it

will be informative to characterize the anatomical relationship between NPY-GFP cells and neuropeptidergic inputs from other hypothalamic regions and brainstem. Specifically, disruption of ARH projections to the DMHnc during DIO may be the key signal permitting NPY induction in the DMHnc. Hormonal signals activating DMHnc-NPY gene expression are poorly understood as well. In addition to the promising role of leptin, a direct role of other hormones such as insulin, ghrelin, and resistin on DMHnc-NPY neurons needs to be tested in DIO mice.

Concluding remarks

Emerging evidence in the field clearly suggests that NPY neurons in the DMH play important roles in energy homeostasis and obesity. Several studies including our own have demonstrated that NPY neurons in the DMHnc have unique functions in feeding behaviors and are differentially regulated from DMHc-NPY and ARH-NPY neurons. It is clear that DMHnc-NPY expression is activated under special physiological conditions that permit hyperphagia to support the energy requirement for growth, milk production and obesity. Characterization of DMHnc-NPY neuronal projections presented in this thesis supports the current hypothesis in the field that DMHnc-NPY neurons have a wide range of roles in energy homeostasis including food intake, BAT thermogenesis, and reproduction. DMHnc-NPY neurons integrate metabolic signals from the ARH, brainstem, and periphery and modulate the PVH neurons to change these behaviors, seen as example in lactation (Fig. 5-1). Specifically in DIO model, DMHnc-NPY neurons co-express CART peptide and may be directly regulated by leptin. Functional implications of NPY/CART co-expression are still unclear, but NPY induction alone or NPY/CART co-expression together may be associated with the palatability-induced hyperphagia.

Evidence suggest that classical feeding neuropeptides such as NPY and CART may interact with the reward associated pathways and participate in food reward and food addiction like behavior. Therefore, NPY/CART expression in the DMHnc may represent functional adaptations within the feeding neurocircuitry in response to long term HFD consumption, allowing the vicious cycle of craving and overeating of the palatable diet (Fig. 5-2). This may explain why calorie restriction regimes often fail in obese humans who are wired to overeat and palatable diet preference. Our findings in this thesis not only contribute to the greater understanding of the mechanisms of hypothalamic feeding regulation, but also provide novel ideas for the development of preventive and treatment strategies for human obesity.

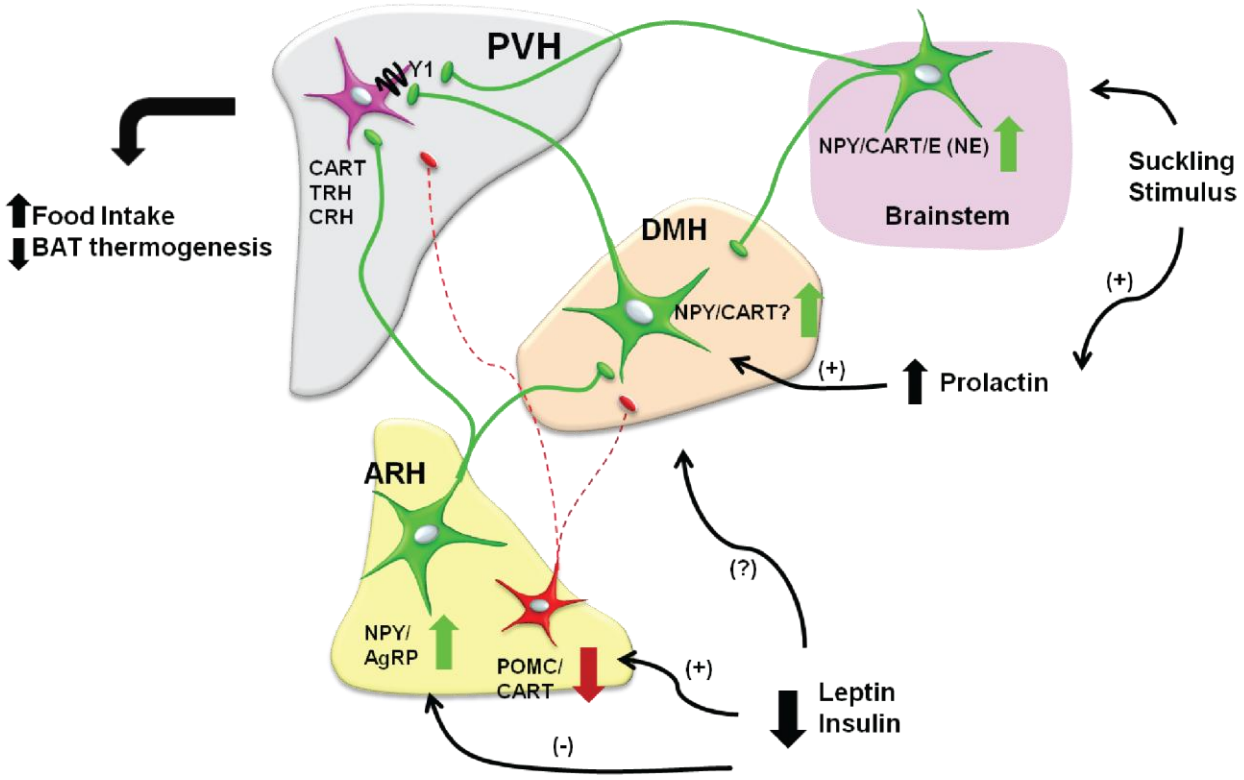

Figure 5-1. Integarting role of DMHnc-NPY neurons in the lactation model.
During lactation, increased NPY signals from the ARH, DMH, and brainstem converge on the PVH to stimulate food intake and decrease BAT thermogenesis. The suckling stimulus is the main driver of NPY expression in all areas and the neural signals generated by the suckling stimulus also increase prolactin secretion which directly regulates DMHnc-NPY expression. The changes in ARH gene expression mediated by circulating leptin and insulin levels may also be an important signal for activating DMHnc-NPY expresion. Therefore, DMH-NPY neurons integrate metabolic signals during lactation and then project to the Y1 expressing cells in the PVH to potentially modulate food intake and BAT thermogenesis.

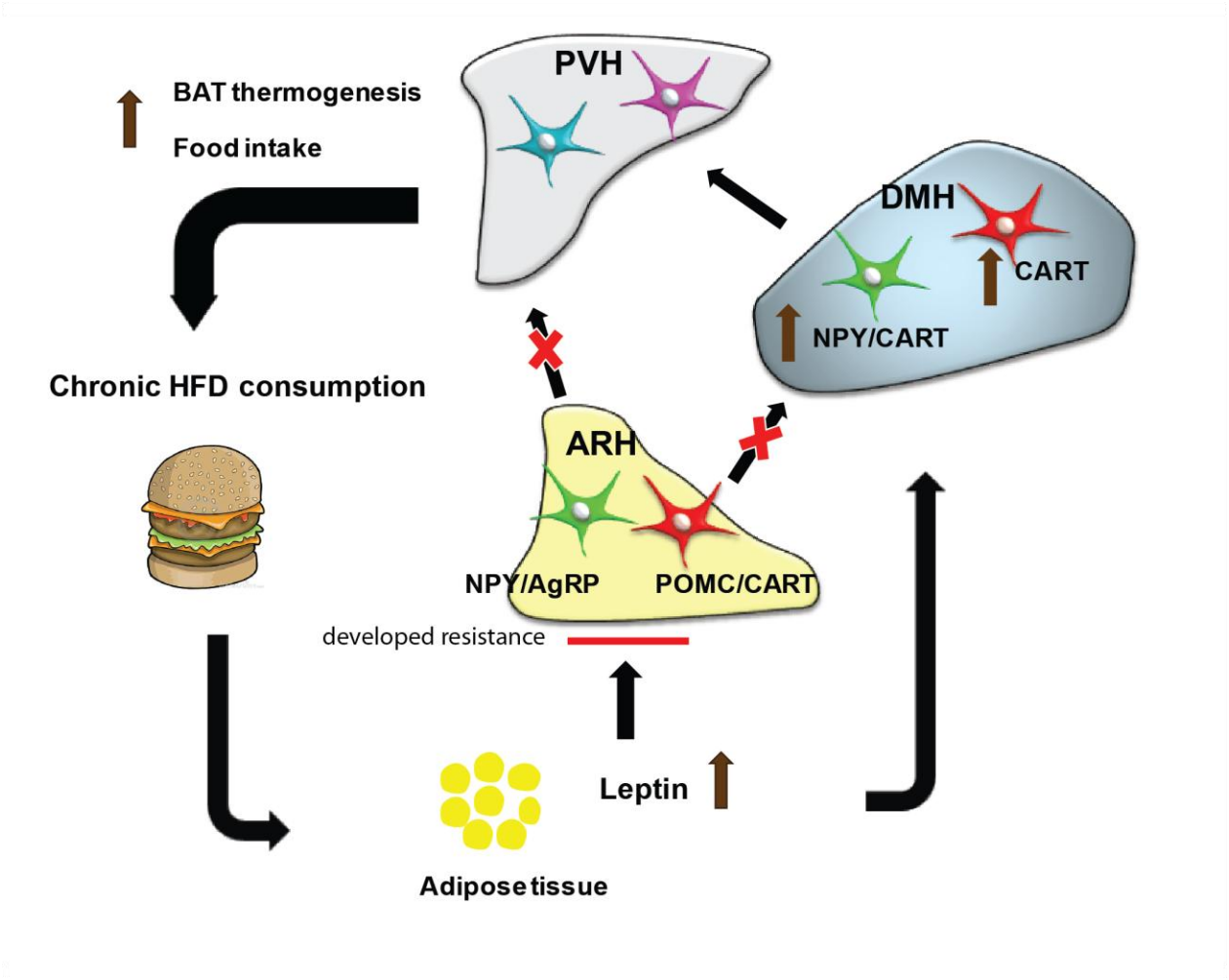

Figure 5-2. Vicious cycle of overeating in chronic HFD consumption.
Chronic HFD consumption leads to high leptin production in adipocytes (shiown in yellow). While ARH NPY/AgRP and POMC/CART neurons become leptin resistant, DMH neurons remain responsive to high levels of leptin, leading to CART and NPY up-regulation. CART and NPY project to the PVH to increase food intake and BAT themogenesis. As a result, a vicious cycle of overeating and palatable diet consumption continues.

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APPENDIX

Supplemental method for RT (reverse transcription)-PCR in Chapter 2

To comfirm the gene expression identified in microarray anaylsys, a new set of isolated DMH-and ARH-NPY neurons were collected from additional P15 NPY-GFP male mice (n=16) as described in the Microdissection and cell dissociation of ARH and DMH for FACS. RNA was extracted using the Pico Pure RNA isolation kit according to the manufacture's protocol. To make RNA solution, 1µl of RNA (concentration 1µg/µl), random primers 0.5 µg/ µg RNA, and 7.5 µl of H_2O were mixed and incubated at 65º C in a dry bath for 5min to reduce secondary structure. For reverse transcription, 4µl of 5x buffer, 4µl of 10mM dNTPs, 2 µl of 0.1M DTT, and 1µl of M-MLV Reverse transcriptase (Fischer Scientific, Pittsburgh, PA) were mixed well and added to the RNA solution. For RT reaction, the sample was incubated at 37º C for 1 hr, then 95º C for 5min, and stored at -20º C until use. The following primer pairs were designed to amplify *Npy*: Forward 5′-GCTAGGTAACAAGCGAATGGGG-3′; Reverse 5′-CACATGGAAGGGTCTTCAAGC-3′, *AgRP*: Forward 5′-GGCCTCAAGAAGACAACTGC-3′; Reverse 5′-TGCGACTACAGAGGTTCGTG-3′, *Pomc*: Forward 5′-GAAGATGCCGAGATTCTGCT-3′; Reverse 5′-GTACTTCCGGGGGTTTTCAG, *Cyclophlin B*: Forward 5′-CAAGACTGAGTGGCTGGATGG; Reverse 5′-ACTTGAAGGGGAATGAGGAAAATA-3′, *Bahl2*: Forward 5′-ACCCATCCACCCACACATAC; Reverse 5′-ATCACCCTCCTCTGCTCTGA, *Foxa1*: Forward 5′-AAACCGGTTATGCACATTGG; Reverse 5′-GCAAGAACTAAAATGGCCACA, *Pgc -1α*: Forward 5′-GGAGCCGTGACCACTGACA; Reverse 5′-TGGTTTGCTGCATGGTTCTG. For PCR

reaction, 45 µl of platinum PCR mix (In vitrogen, Cat# 11306-061), 1.5 µl of 5 µM forward primer, 1.5 µl of 5 µM reverse primer, 2 µl of cDNAs were mixed. PCR amplication was performed as followed: 94° C, 2 min; (94° C, 30 sec; 55° C, 45 sec; 72° C, 1 min) 40 times; 72° C, 7 min. The final PCR product was run on 2% agorase gel.