LACTATION AS A PHYSIOLOGICAL MODEL FOR STUDYING INDUCTION OF NEUROPEPTIDE Y IN THE DORSOMEDIAL NUCLEUS OF THE HYPOTHALAMUS: IMPLICATIONS IN THE REGULATION OF ENERGY HOMEOSTASIS

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LIST OF ABBREVIATIONS

3V	Third ventricle
ACTH	Adrenocorticotropin hormone
AGRP	Agouti related protein
AH	Anterior hypothalamus
ARH	Arcuate nucleus of hypothalamus
AVPV	Anteroventral periventricular nucleus
BDNF	Brain-derived neurotrophic factor
BST	Bed nucleus of stria terminalis
CART	Cocaine and amphetamine-regulated transcript
CCK	Cholecystokinin
CNS	Central nervous system
CRF	Corticotropin-releasing factor
DMH	Dorsomedial nucleus of hypothalamus
DMHp	Dorsomedial nucleus, compact zone
F	Fornix
FG	Fluorogold
FITC	Fluorescein isothiocyanate
GABA	γ- Aminobutyric acid
GAD	Glutamate decarboxylase
H / O	Hypocretin / Orexin
IBAT	Interscapular brown adipose tissue

IHC	Immunohistochemistry
ISH	*In situ* hybridization
LH	Lateral hypothalamus
LHA	Lateral hypothalamus
LPB	Lateral parabrachial nucleus
LS	Lateral septum
LV	Lateral ventricle
MCH	Melanin-concentrating hormone
MC1/2/3/4/5R	Melanocortin 1/2/3/4/5 receptor
ME	Median eminence
MeA	Medial amygdala
mPOA	Medial preoptic area
α-MSH	α-Melanocyte stimulating hormone
MTII	Melanotan-II
NPY	Neuropeptide Y
NTS	Nucleus of solitary tract
OB-Rb	Leptin receptor, long form
opt	Optic tract
OVLT	Organum vasculosum of the lamina terminals
OVX	Ovariectomy
PAG	Periaqueductal gray
PePOA	Periventricular preoptic area
PFR	Prefrontal cortex

PH	Posterior hypothalamus
PMv	Ventral premammillary nucleus
POMC	Proopiomelanocortin
PP	Peripeduncular area
PRL	Prolactin
PS	Parastrial nucleus
PVH	Paraventricular nucleus of hypothalamus
PVHm	Paraventricular nucleus, magnocellular part
PVHp	Paraventricular nucleus, parvocelluar part
PVp	Posterior periventricular nucleus of hypothalamus
PVT	Anterior paraventricular nucleus of thalamus
RIA	Radioimmunoassay
SCN	Suprachiasmatic nucleus
scp	Superior cerebellar peduncle
SFO	Subfornical organ
SUBv	Ventral subiculum
SuM	Supramammillary nucleus
TMd	Tuberomammillary nucleus, dorsal part
TRITC	Tetramethyl rhodamine isothiocyanate
VLM	Ventrolateral medulla
VMH	Ventromedial nucleus of hypothalamus
UCP1	Uncoupling protein 1

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PREFACE

In accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health Sciences University, Portland, Oregon, I have prepared my dissertation, consisting of a general introduction, three chapters of original data and a general conclusion. References are listed separately in alphabetical order, and follow the format of *Journal of Neuroscience.*

Chapter II, III and IV contain data, figures and text as they appear in original papers that were published previously (Chen and Smith, 2003, Chen and Smith, 2004, Chen et. al., *Journal of Neuroscience* 2004, *in press*).

ABSTRACT

NPY neurons in the DMH have been implicated in playing an important role in regulating energy homeostasis. This thesis used the lactating rat as a model to better understand the regulation and possible functions of the NPY system in the DMH. The first part of this thesis used the cFos protein as a neuronal marker in combination with retrograde tracing to identify the neuronal afferent input into the DMH that was specifically activated by the suckling stimulus. Several forebrain areas, including the mPOA and the LS, and brainstem areas, including the LPB and the VLM, were identified as important in relaying suckling-induced neural signal into the DMH. Identification of these neural pathways provides insights into how suckling-generated somatosensory information may mediate the activation of DMH NPY during lactation.

The second part of the thesis investigated the role of suckling-induced hyperprolactinemia in NPY expression in the DMH. Lactating rats treated with bromocriptine to block suckling-induced PRL secretion showed significantly reduced NPY expression in the DMH. Exogenous PRL injection to lactating rats treated with bromocriptine reversed the suppressive effect of bromocriptine on DMH NPY expression, indicating that PRL is an important factor in the elevation

of NPY activity in the DMH. Furthermore, double label ISH for NPY and PRL-R showed NPY-positive neurons in the DMH also express PRL-R mRNA. These data suggests that PRL may act directly on DMH NPY neurons to modulate NPY gene expression during lactation.

The aim of last part of the thesis was to investigate, both anatomically and pharmacologically, the relationship between the melanocortin system and NPY neurons in the DMH. The neuroanatomical studies showed that abundant α-MSH and AGRP fibers are in close apposition to NPY -positive cells in the DMH. In addition, MC4R mRNAs were found to express in close proximity to the DMH NPY neurons, suggesting that melanocortin peptides may affect DMH NPY neurons by modulating the activity of neurons upstream of NPY-positive cells in the DMH. To study the function of the proposed ARH melanocortin-to-DMH NPY pathway, a MC3/4Rs selective agonist, MTII, was injected bilaterally into the DMH. MTII injection significantly suppressed feeding induced by 24 h fasting or suckling-induced hyperphagia. Furthermore, MTII treatment greatly attenuated suckling-induced NPY expression in the DMH. MTII treatment also stimulated UCP1 activity in the brown adipose tissue of suckling female rats, indicative of increasing sympathetic outflow. These results demonstrated that melanocortin

peptides in the DMH play an important role in inducing NPY expression in the DMH of lactating rats and in regulating energy homeostasis, at least in part by modulating appetite and energy expenditure.

In conclusion, studies conducted in this thesis identified suckling-induced neural impulses, PRL, and decreased melanocortin signaling as important factors in activating NPY expression in the DMH. DMH NPY neurons contribute to the regulation of energy homeostasis, at least in part, by modulating feeding and peripheral sympathetic activity. In the future, a further phenotypic characterization of DMH NPY neurons will greatly facilitate our understanding in the significance of this population of neurons in energy homeostasis.

CHAPTER I

INTRODUCTION

A. OVERVIEW—HYPOTHALAMUS, A PRIMARY SITE FOR CENTRAL NERVOUS SYSTEM REGULATION OF FOOD INTAKE AND ENERGY HOMEOSTASIS

The ability to maintain energy homeostasis is a vital survival mechanism of animals, including humans. Energy intake and energy expenditure of an individual are constantly monitored and balanced in response to a change in energy state by an array of complex physiological systems. It has been shown that individuals of normal weight match cumulative energy intake to energy expenditure with great precision when measured over the course of months to years (Edholm, 1977; Leibel et al., 1995; Levine et al., 1999). This precise matching of energy intake and energy expenditure is controlled by the central nervous system, primarily in the hypothalamus. The hypothalamus receives and integrates peripheral metabolic, endocrine and neuronal signals, and coordinates a response that either changes food intake or energy expenditure to correct the disrupted energy equilibrium. An imbalance between energy intake and expenditure will result in metabolic-related disorders such as obesity. In fact, obesity has become an increasing threat to the overall health of the human population. Obesity is the result of an increase in excessive energy intake without concurrent increase in energy expenditure. Its etiology involves both genetic and environmental factors. It has been hypothesized that as the result of natural selection, all of us, particularly certain populations, have powerful genetic predispositions to retain excess calories in preparation for

famine (Neel, 1962; Bjorntorp, 2001). This genetic propensity for energy conservation, in combination with the modern obesity-prone life style of excess caloric intake and decreased physical activity, may contribute to the current obesity epidemic.

Obesity should no longer be regarded simply as a cosmetic problem. It has been linked to increased risk of the development of severe health problems, such as type 2 diabetes mellitus, coronary heart disease, stroke, and certain forms of cancer (Visscher and Seidell, 2001). Therefore, there is a pressing need to find treatments to fight obesity. As the hypothalamus is central to controlling the body's overall energy balance by modulating feeding and energy expenditure, the knowledge of how the hypothalamus integrates multiple signals and modulates energy balance will provide insights for developing effective therapeutic treatments to control excessive weight gain. The focus of this thesis is to utilize the lactating rat as a model to understand the integration of energy related signals within the hypothalamus and how several neurosubstrates may interact in the hypothalamus to modulate feeding and energy expenditure in the periphery.

B. GENERAL ANATOMY OF THE HYPOTHALAMUS

The hypothalamus is located in the middle of the base of the diencephalon, and encapsulates the ventral portion of the third ventricle and the area immediately above the pituitary gland. The hypothalamus is composed of

several clusters called nuclei, each of which represents a basic functional unit of the hypothalamus and underlies its various, specific function. These nuclei received extensive neural input from many brain areas and send efferent nerve fibers to major sites of the brain, including the cerebral cortex, thalamus and limbic system, and the spinal cord (Simerly, 1995b). Moreover, several studies have demonstrated that some nuclei in the hypothalamus exhibit very extensive and complicated connections with each other (Ter Horst and Luiten, 1987). These hypothalamic nuclei also communicate with the peripheral systems by directly releasing hypothalamic hormones into the peripheral circulation via the posterior pituitary, or indirectly by discharging releasing hormones or inhibiting hormones into the portal vein blood and, via receptors in the endocrine cells, directly influence the output of anterior pituitary tropic hormones. In addition to the extensive neural connections between the hypothalamus and the rest of the nervous system, the hypothalamus is also well supplied with blood vessels. This suggests that hypothalamic nuclei can be influenced by a wide variety of chemical messengers from both the blood and CSF, as well as neurotransmitters from other more distant neurons not having direct contact. Thus, the unique cytoarchitecture of the hypothalamus has positioned it to receive and integrate information derived from both the external and internal environment. It responds to a condition appropriately by adjusting an array of autonomic, endocrine, and behavioral responses to restore homeostasis. The hypothalamus serves this integrative function by regulation of several physiological needs critical for basic life support, including such activities as

fluid and electrolyte balance, food ingestion and energy metabolism, thermoregulation, immune response, and even behavior, including aggressive, feeding, maternal and sexual behaviors (Brown, 1994).

To date, the broad outlines of the hypothalamic control systems, their anatomical localization, and integrative properties have gradually emerged from cumulative experimental studies of the past few decades. However, the detailed mechanisms by which the hypothalamus may accomplish these complicated tasks are not well understood. This could be attributed to the complex nature of the neural circuitries and large numbers of substances produced within the hypothalamus. In addition to classical neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA) and the catecholamines, several dozen neuropeptides have been identified in the hypothalamus (Brown, 1994). Importantly, additional new neurosubstrates have been discovered in the hypothalamus in the past several years, and the pace of discovering new substances is not slowing down, due to the availability of more sophisticated tools and extensive genome databases. Although a large body of data concerning the distribution and physiological properties of these neurochemical systems has been generated, the information about the interactions between different neurochemical systems is still relatively lacking. Thus, in order to understand how the hypothalamus regulates homeostasis of the body, it is essential to determine anatomically and pharmacologically the function of each neurochemical system in the hypothalamus and how the hypothalamic circuitries may function as a sensor to recognize incoming signals within the

hypothalamus. This information will provide great insight in understanding the interaction among different systems and how the interaction may contribute to the integration of information in the hypothalamus to maintain homeostasis.

C. HYPOTHALAMIC REGULATION OF FOOD INTAKE AND ENERGY HOMEOSTASIS

The hypothalamus has long been considered as the pivotal center in the regulation of the food intake and energy homeostasis. Early studies carried out in the 1940s and 1950s first demonstrated that lesions to the ventromedial hypothalamus (Hetherington and Ranson, 1940) induced hyperphagia and obesity whereas damage to the lateral hypothalamus (Anand and Brobeck, 1951) resulted in decreased food intake, even aphagia, such that starvation to death often ensued. These observations led to the notion that ingestive behavior is controlled by the interaction between LHA feeding and VMH satiety "centers" in the hypothalamus (Steller, 1954). Later on, studies making more discrete lesions to the LHA or VMH did not reproduce the aphagia or overeating behavior and led to the recognition that damage of nerve tracts in these nuclei leading to other hypothalamic nuclei underlies the original observations. Although these early lesion studies were crude and the findings have undergone much challenge and revision, they clearly established that the hypothalamus plays a pivotal role in the regulation of food intake, although the regulatory mechanisms have become much more complex. Current understanding of the intricate interconnections in the hypothalamus and the

physiological actions of a number of feeding-related neuropeptides or hormones indicate that feeding is controlled by highly complex neuronal circuits. This overview will summarize orexigenic and anorexigenic neuronal and hormonal input signals, their interconnections, as well as their efferent projections of the feeding-related neural circuits.

Arcuate nucleus (ARH). The ARH is situated in the mediobasal aspect of the hypothalamus adjacent to the third ventricle and just above the medium eminence. ARH is widely considered as the "primary sensory" center of the hypothalamic neuronal circuits in regulating energy balance because of its location and its connections with other neural systems that are involved with food intake regulation. The strongest inputs to the ARH are from other parts of the periventricular zone of the hypothalamus, including the paraventricular hypothalamic nucleus (PVH) and lateral hypothalamic nucleus (LHA) (Sawchenko and Swanson, 1983; Li et al., 1999a). ARH also receive neuronal inputs from extrahypothalamic nuclei, such as medial amygdala, bed nucleus of stria terminalis, and nucleus of solitary tract (NTS) (Li et al., 1999a). These areas may play an important role in relaying visceral or emotional feedback signals to the hypothalamus to modulate feeding. In addition to receiving neural signals from various afferent inputs, ARH can also assess blood-borne circulating molecules that normally would not exit the vascular system. In addition to being physically located immediately above the ME, ARH also has a very close vascular connection with ME (Ambach et al., 1976), which contains the primary network of the hypophyseal portal system. This unique property

allows ARH to monitor circulating hormone or nutrient levels such as glucose, leptin, ghrelin, or insulin that reflect the energy state of the whole organism. The ARH integrates these signals and relays the information via its efferent projections to other brain areas. Anatomical studies have shown that ARH neurons project to adjacent hypothalamic areas with the strongest projections to the PVH (Li et al., 1999a, 2000). ARH also sends major projections to areas that are important for the regulation of energy balance such as the dorsomedial nucleus (DMH), perifornical area, and LHA.

The ARH contains a number of distinct populations of neurons that produce different neurotransmitters or neuropeptides. Among them, two populations of neurons have been shown to play key roles in controlling energy balance. One population of neurons expresses neuropeptide Y / agouti-related protein (NPY/AGRP), while the other population expresses proopiomelanocortin / cocaine and amphetamine-regulated transcript (POMC/CART). NPY is regarded as the most potent orexigenic agent. A single bolus intracerebroventricular injection of NPY potently stimulates feeding in rodents and other mammalian species (Clark et al., 1984; Miner et al., 1989; Larsen et al., 1999). Repeated injection of NPY directly into the PVH over a period of several days results in sustained hyperphagia, decreased energy expenditure, reduced sympathetic outflow to brown adipose tissue, and increased lipogenesis by stimulating the expression of lipogenic enzymes in white adipose tissue (Stanley et al., 1986; Billington et al., 1991). Thus, exogenous NPY administration leads to a state of positive energy balance and increases fat

storage. In addition, the expression of NPY in the ARH is elevated in response to negative energy states such as fasting. This increase in expression is accompanied by an increase in release of NPY in other hypothalamic areas (Brady et al., 1990; Kalra et al., 1991). Yoshihara et al. showed that NPY levels rise and fall in the PVH before and after a meal (Yoshihara et al., 1996), suggesting that endogenous NPY is closely involved in the normal maintenance of daily food intake.

AGRP is co-localized with NPY neurons in the ARH (Chen et al., 1999). AGRP was isolated and cloned based on its sequence homology to the agouti protein, a natural antagonist to the MCRs (Ollmann et al., 1997; Shutter et al., 1997). Unlike agouti protein, AGRP is expressed predominately by NPY neurons in the ARH (Shutter et al., 1997; Haskell-Luevano et al., 1999). AGRP is an endogenous antagonist to the MC4R (Ollmann et al., 1997; Shutter et al., 1997). It stimulates feeding by preventing the binding and activation of α-MSH to MC4R (Rossi et al., 1998). AGRP mRNA levels are elevated during fasting (Ebihara et al., 1999), and transgenic mice overexpressing AGRP develop an obesity syndrome analogous to the MC4R null mice (Ollmann et al., 1997). These results support a role for AGRP in the normal control of body weight and suggest that antagonist as well as agonist concentrations can influence MC4R signaling.

In contrast to NPY neurons, POMC-derived peptides, such as α–melanocyte stimulating hormone (α-MSH), have been shown to potently suppress food intake and increase energy expenditure through binding to the

melanocortin-4 receptor (MC4R) in the brain. ARH POMC gene expression is significantly suppressed following a 48-hour fast (Mizuno et al., 1998). Central administration of α-MSH or MC4R agonist leads to prolonged inhibition of food intake (Fan et al., 1997). Conversely, antagonism of MC4R in the hypothalamus leads to hyperphagia, reduced energy expenditure and obesity (Raposinho et al., 2000; Adage et al., 2001). Furthermore, MC4R-deficient mice exhibit a hyperphagic obesity syndrome (Huszar et al., 1997). Therefore, melanocortin signaling in the brain appears to be required for normal control of food intake and body weight.

Analogous to NPY/AGRP coexpression, CART is co-localized extensively with ARH POMC neurons (Flier and Maratos-Flier, 1998; Kristensen et al., 1998). CART is also a powerful inhibitor of feeding behavior. Central administration of CART peptide leads to inhibition of NPY-induced food intake in rodents (Kristensen et al., 1998; Lambert et al., 1998). Chronic administration of CART decreases food intake and body weight (Larsen et al., 2000), while passive immunization of CART increases food intake (Kristensen et al., 1998; Lambert et al., 1998). In addition, food deprivation results in a decrease of CART mRNA in the ARH (Kristensen et al., 1998). Currently the underlying mechanisms that mediate the effects of CART are still poorly understood. Studies show that CART peptide injection to the brain causes cFos induction in several hypothalamic areas, such as PVH, DMH, and ARH (Vrang et al., 2000). These areas could be the potential sites of action of CART. However, to date, the receptor has not yet been identified.

Accumulating evidence suggests that both NPY/AGRP and POMC/CART neurons of the ARH are the downstream effectors of leptin action. Leptin is a peripheral hormone secreted by adipose tissue in proportion to the mass of fat content. Circulating leptin is actively transported into the brain, where it binds to the long-form of its receptor (OB-Rb). Leptin is essential in the regulation of energy homeostasis since rodents with a genetic deficiency in producing leptin or its receptor exhibit profound hyperphagia and severe obesity (Schwartz et al., 2000; Woods and Seeley, 2000). Central administration of leptin to rodents with normal body weight acutely decreases food intake and increases sympathetic activity, thus increasing energy expenditure (Haynes et al., 1997; Havel, 2000). ARH is one of the brain areas that contains high levels of OB-Rb. Histological studies show that both NPY/AGRP and POMC/CART neurons coexpress OB-Rb (Cheung et al., 1997; Baskin et al., 1999). Leptin has been shown to negatively regulate NPY/AGRP and positively regulate POMC/CART (Cowley et al., 2001). Central leptin treatment can reduce elevated ARH NPY gene expression induced by fasting, a condition marked by low levels of leptin (Schwartz et al., 1996). Moreover, genetic leptin deficient *ob/ob* mice exhibit elevated NPY gene expression in the ARH, which can be reversed by leptin injection (Stephens et al., 1995). On the other hand, leptin administration to fasted animals increases hypothalamic POMC gene expression, whereas leptin injection to *ob/ob* mice reverses the low POMC mRNA expression in the ARH (Schwartz et al., 1997).

Similar to leptin, insulin has been proposed as another humoral factor that signals peripheral energy status to the brain. This hypothesis is based on evidence that insulin also circulates in proportion to body fat. Central administration of insulin results in a negative energy balance (Woods et al., 1998) and neuron-specific deletion of its receptor causes obesity (Bruning et al., 2000). Moreover, the insulin receptor has been shown to colocalize with ARH NPY/AGRP and POMC/CART neurons, which are negatively and positively regulated by insulin, respectively (Schwartz et al., 1992; Ahima et al., 1996; Mercer et al., 1996). These observations suggest that insulin and leptin exert overlapping effects on ARH neurons involved in energy homeostasis. It remains to be determined whether the effects of both hormones are mediated through similar CNS circuits.

In addition to being receptive to long-term adiposity signals, ARH also expresses receptors for several meal-to-meal regulators such as PYY_{3-36} and ghrelin. PYY_{3-36}, a gut hormone that is secreted in response to the ingestion of food, acts within the ARH via NPY Y2 receptors to reduce NPY mRNA and food intake (Batterham et al., 2002). Ghrelin, a peptide secreted from the stomach and upper intestine, has been shown to stimulate food intake by activating ARH NPY/AGRP neurons (Nakazato et al., 2001).

PVH. The PVH is located in a central position to receive input signals from other feeding-related hypothalamic and brainstem nuclei. The major inputs are the direct pathways between ARH and PVH that carry NPY/AGRP and α-MSH/CART peptide signals. Other PVH projecting areas that have been

implicated in feeding include DMH, VMH, and LHA. In addition, the PVH is innervated by adrenergic as well as noradrenergic fibers from the hindbrain. These pathways may be important in relaying visceral and peripheral metabolic status to the CNS. PVH neurons provide extensive descending projections to the pituitary, the autonomic-related structures in the brainstem and spinal cord, including both parasympathetic and sympathetic preganglionic neurons (Swanson and Sawchenko, 1983). These extensive descending projections suggest that the PVH serves as a motor output from the hypothalamus in regulating energy homeostasis via its modulation of autonomic and neuroendocrine functions.

PVH consists of a magnocellular division, with three distinct parts, and a parvocellular division, with five distinct parts. A great variety of neuropeptides are synthesized in the nucleus. Among them, Corticotropin-releasing factor (CRF) and thyrotropin-releasing hormone (TRH) neurons have been shown to be closely involved in the central regulation of food intake and energy expenditure. CRF is a 41 amino acid peptide best known for its role in regulating the hypothalamic-pituitary-adrenal axis. CRF is also a potent anorexigenic peptide. Central administration of CRF or direct injection of the peptide into the PVH inhibits night time and fasting-induced feeding (Krahn et al., 1988; Dunn and Berridge, 1990), and immunoneutralization or pharmacological blockade of CRF results in enhanced basal and NPY-induced feeding (Heinrichs et al., 1992; Menzaghi et al., 1993). CRF neurons have been suggested to act downstream of leptin (Uehara et al., 1998), NPY (Heinrichs et

al., 1993), and MC4R (Vergoni et al., 1999) signaling, which is in agreement with the neuroanatomical evidence that PVH receives strong afferent NPY/AGRP and POMC/CART input originating from ARH. Besides the effect on food intake, CRF increases sympathetic nervous system activity, thereby increasing thermogenesis, energy expenditure and lipolysis (Masaki et al., 2003).

TRH is an important stimulator of energy expenditure through regulating the hypothalamus-pituitary-thyroid axis. TRH also acts downstream from leptin, NPY and melanocortin pathways (Legradi and Lechan, 1998; Mihaly et al., 2000). TRH has also been suggested to work as a brain neuropeptide to suppress food intake (Kow and Pfaff, 1991).

DMH. The role of the DMH in feeding behavior has been enigmatic. Although lesions of the area resulted in reduction of food and water intake, it was believed that the reduction was an adaptive response to a lower body weight. The lesioned animals were able to defend their new body weight and hydration status, showing normal responses to homeostatic challenges, such as food or water restriction (Bellinger et al., 1976). These observations suggested that DMH is probably in a nodal position to integrate multiple inputs and modulate multiple physiological responses. Therefore, ablation of the nucleus results in a change that is the sum of altered multiple factors rather than changes of a single modality. In addition to ingestive behavior, DMH has been implicated in a variety of behavioral and physiological responses, including reproduction;

endocrine, autonomic, and behavioral aspects of stress; circadian rhythms; and thermogenesis (Bernardis and Bellinger, 1998).

Neuroanatomical and neurochemical studies have provided more definitive evidence on the involvement of DMH in regulating energy balance. First, DMH is in a position to receive information about the metabolic state of an animal via projections from the ventrolateral medulla and parabrachial nucleus, where gastric and gustatory signals originating from NTS are integrated. Several anorexigenic peptide receptors have been found in DMH. For example, glucagon like peptide-2 receptor (GLP-2) has been localized in the compact zone of the DMH (Tang-Christensen et al., 2001) and cholecystokinin-A (CCK-A) receptors are found throughout the DMH (Woodruff et al., 1991). Complementing the receptor expression, GLP-2 fibers from the NTS heavily innervate the DMH, and CCK neuronal fibers originated from the NTS and the parabrachial nucleus have been shown to project to the DMH. Moreover, rats with a spontaneous mutation that results in deletion of the CCK-A receptor gene are obese and diabetic (Takiguchi et al., 1997), further implicating the hindbrain CCK-DMH pathway as a potentially important route of energy homeostatic regulation. Second, DMH receives inputs from ARH, LHA, VMH, and PVH, the hypothalamic nuclei that are important in regulation of energy balance. The DMH contains leptin, insulin, and MC4R receptors (Marks et al., 1990; Mountjoy et al., 1994; Mercer et al., 1996). Moreover, glucose-receptive neurons have been reported in the DMH (Niijima, 1989). Finally, DMH sends efferent projections to various brain areas that are important in energy balance and

feeding; the densest output projection is to the PVH (Thompson et al., 1996). This neuroanatomical evidence suggests DMH is an important second order integration center to receive and integrate multiple feeding related signals, that in turn projects to the PVH. So far, only a few orexigenic or anorexigenic peptides have been shown to be expressed in DMH; these include galanin and TRH. The function of these two peptides in this nucleus is unknown. Recently several groups reported DMH contains NPY expressing neurons. Importantly, the expression is observed only in certain genetic models of obesity (Kesterson et al., 1997), diet induced obesity (Guan et al., 1998), and lactation (Li et al., 1998b). All these models have a common thread, hyperphagic behavior. Using the lactating rat as a model, Li et al. showed that the DMH NPY neurons project to PVH (Li et al., 1998a). Taken together, it is conceivable the DMH NPY might be one of the important peptide systems that integrates the output signal from DMH to PVH to modulate energy balance.

LHA. The LHA covers a relatively large but loosely outlined area in the middle and caudal parts of the hypothalamus, with the medial forebrain bundle running along the rostrocaudal axis. The LHA has long been considered essential in regulating food intake and body weight. Lesions of this area result in severe hypophagia or even aphagia. This view of LHA was later revised, as smaller lesions of the area did not reproduce aphagia. The effect probably is a result of severing fiber tracks of the medial forebrain bundle rather than the LHA per se. The importance of the LHA in the regulation of appetite and energy homeostasis has been revived with the identification of two neuropeptide

systems, hypocretin/orexin (H/O) and melanin-concentrating hormone (MCH) in this area. Anatomical and functional studies of the two peptides have provided insights into the role of LHA in feeding behavior and energy balance. Both of these peptides stimulate food intake when injected centrally and are upregulated by fasting (Qu et al., 1996; Szekely et al., 2002). Both neuronal populations receive ARH NPY/AGRP innervation and are the downstream targets of NPY-induced feeding (Broberger et al., 1998; Elias et al., 1998). In addition, evidence has suggested that both of the neuropeptide systems are influenced by leptin, with leptin receptors coexpressed with both H/O and MCH neurons (Hakansson et al., 1999). Ablation (Shimada et al., 1998) or overexpression (Ludwig et al., 2001) of MCH results in hypophagia and leanness or hyperphagia and obesity, respectively, further supporting a role for the MCH system in energy balance and feeding behavior. The role of H/O in feeding behavior is less clear since the major phenotype of the H/O null mice is narcolepsy, and the reduced food intake is the result of less awake time for food consumption.

VMH. Early Lesion studies suggested that the VMH is a satiety center in the brain. Yet, this view was challenged when small lesions restricted to the nucleus failed to recapitulate the hypothalamic obesity syndrome (Gold, 1973). In addition, the observations that vagotomy could prevent the massive VMH lesion-induced overeating (Powley and Opsahl, 1974) suggested that the lesion-induced hyperphagia is secondary to alterations in peripheral metabolism. Although these data did not support VMH as a satiety center, they

showed that VMH plays an important role in regulating peripheral metabolism. The underlying mechanism by which the VMH modulates peripheral metabolism is not well understood because so far no neurochemical markers for the VMH have been identified. Recently Xu et al. showed that brain derived neurotrophic factor (BDNF) is highly expressed in the VMH, and its level decreases in obese MC4R knockout mice and increases in response to fasting (Xu et al., 2003). In addition, a mouse model with only one copy of the BDNF gene is hyperphagic and obese. These data provide insights to the underlying mechanism of VMH's action in energy homeostasis. However, it remains to be determined if BDNF functions as a local neural circuit modifier or as the efferent neurotransmitter. VMH is receptive to several appetite-regulating signal molecules, including leptin and urocortin-3, through leptin and type 2 CRH receptors, respectively (Elmquist et al., 1998; Li et al., 2002). VMH neurons are also sensitive to glucose (Niimi et al., 1995). VMH might modulate peripheral metabolism via its projection to the periaqueductal gray, which in turn sends projections to the brainstem and spinal cord autonomic centers.

D. HYPOTHALAMIC NEUROPEPTIDE Y (NPY) IS A MAJOR OREXIGENIC SYSTEM IN ENERGY HOMEOSTASIS

1. NPY and its receptors

a. NPY peptide family

Neuropeptide Y (NPY) is a 36 amino acid peptide widely expressed in the central and peripheral nervous system. (Lundberg et al., 1982; Adrian et al., 1983; Allen et al., 1983b) as well as in cells of neural crest origin such as adrenal chromaffin cells (Allen et al., 1983a; Higuchi and Yang, 1986). Tatemoto and colleagues first discovered the peptide from the extracts of porcine brain (Tatemoto K, 1982). Subsequently, NPY was also isolated from a variety of other species, including human (Corder et al., 1984), rat (Corder et al., 1988), guinea-pig, rabbit (O'Hare et al., 1988), sheep (Sillard et al., 1989), alligator (Wang and Conlon, 1993), frog (Griffin et al., 1994), and sea bass (Cerda-Reverter et al., 2000), revealing a remarkable degree of conservation during evolution. NPY belongs to a peptide family that also includes peptide YY (PYY, approximately 70% homology, (Wahlestedt et al., 1986) and pancreatic polypeptide (PP, approximately 50% homology (Gilbert et al., 1988). All these peptides have an amidated carboxyl-terminus and exhibit considerable homologies in their primary, secondary and tertiary structures (Glover et al., 1984; Schwartz et al., 1990). The gene encoding rat NPY spans 7.2 kilobase pairs and contains four exons (Larhammar et al., 1987). NPY is first synthesized as a preproNPY peptide with 98 amino acid residues (Minth et al., 1986). A signal peptide of 29 amino acids precedes the 36-residue NPY sequence, followed by a proteolysis/amidation Gly-Lys-Arg site. The cleavage at the processing site results in the mature peptide and a carboxyl-terminal peptide of 30 amino acids (Higuchi et al., 1988).

The general structure of the family of peptides has been established using x-ray crystallography of avian PP (Glover et al., 1983), and confirmed in several nuclear magnetic resonance studies of PYY and synthetic analog [Leu^{31}, PRO^{34}]NPY (Khiat et al., 1998; Keire et al., 2000). The peptide structure consists of an extended proline helix with three prolines, a turn, and alpha helix, and the four most carboxy-terminal residues are in a flexible loop conformation (Glover et al., 1983). It has been shown that this folded structure is important for binding to some of the receptor subtypes (Beck-Sickinger, 1997).

b. NPY receptors

The NPY family of peptides exhibits a variety of central and peripheral functions mediated by at least six receptor subtypes denoted as Y1, Y2, Y3, Y4, Y5, and y6. These receptors are members of the seven transmembrane domain G-protein coupled receptor family. All except Y3 have been cloned. All cloned receptors have been shown to couple to inhibitory G-protein (G_i) and thus mediate inhibition of cAMP synthesis and, in some instances, activate protein kinase C pathways (Selbie et al., 1995; Parker et al., 1998). The Y3 receptor was postulated based on the pharmacological profile of a low affinity for PYY and on a rank order potency for NPY-related peptides that differs markedly from Y1 or Y2 receptors (Grundemar et al., 1991a, b). Electrophysiological and binding studies suggest that Y3 receptors are expressed in the NTS (Glaum et al., 1997) to mediate inhibitory cardiovascular effects of NPY (Grundemar et al., 1991a, b). However, Y3 has not yet been identified. Following the nomenclature rule of IUPAR, the y6 receptor has been given the lower case

designation because it encodes a truncated receptor in most mammals, including humans and rats (Michel et al., 1998).

Y1 receptor. The Y1 receptor requires both the N- and C-terminal portion of NPY for recognition and activation (Schwartz et al., 1990). Both *in vivo* and *in vitro* studies have shown that this receptor can couple either to phosphotidylinositol hydrolysis or inhibition of adenylate cyclase (Herzog et al., 1992). In the periphery, the Y1 receptor is present at the vascular sympathetic neuroeffector junction and mediates pressor responses of NPY (Grundemar and Hogestatt, 1992; Grundemar and Hakanson, 1993). In the brain, the Y1 receptor has been located in the cerebral cortex, hippocampus, thalamus, amygdala and several nuclei in the hypothalamus, including ARH (Dumont et al., 1996). In the central nervous system, the Y1 receptor has been demonstrated to mediate the anxiolytic effect of NPY in the amygdala (Heilig et al., 1993; Wahlestedt et al., 1993) and is involved in stimulating feeding within the hypothalamus (Kalra et al., 1991; Stanley et al., 1992), although its involvement in feeding has been challenged by the study showing that decreasing expression of Y1 receptor by antisense oligonucleotide treatment did not alter feeding (Heilig et al., 1993). In addition, counter intuitively, knockout of Y1 receptors results in moderate obesity, increased white adipose tissue weight, and elevated basal levels of plasma insulin (Kushi et al., 1998). However, closer analysis showed that the Y1 receptor deficient mice have slightly decreased food intake and NPY-induced feeding, whereas fasting-induced feeding was markedly reduced, suggesting that the food intake effect of

NPY is mediated in part by the Y1 receptor (Pedrazzini et al., 1998; Pedrazzini and Seydoux, 2000).

Y2 receptor. The Y2 receptor, in contrast to Y1, has a much higher affinity for C-terminal fragments than for substituted analogues such as [Pro34]NPY (Schwartz et al., 1990; Grundemar et al., 1993). Like the Y1 receptor, the Y2 receptor can couple to the inhibition of adenylate cyclase (Colmers and Pittman, 1989; Foucart and Majewski, 1989). In addition, Y2 receptor has been shown to modulate Ca^{2+} currents by selective inhibition of N-type calcium channels (Toth et al., 1993). In the periphery, Y2 receptor is located on the autonomic fibers, as well as the vasculature (Wahlestedt and Hakanson, 1986; Wahlestedt et al., 1986; Grundemar and Hakanson, 1990; Stjernquist and Owman, 1990; Gehlert et al., 1992; Dumont et al., 1993). In the CNS, Y2 receptors are found in a variety of brain regions, including the hippocampus, substantia nigra, thalamus, hypothalamus, and brainstem (Dumont et al., 1993; Gehlert and Gackenheimer, 1997; Gustafson et al., 1997). The Y2 receptor is mainly located presynaptically where it acts as an autoreceptor to inhibit further release of transmitters (Wahlestedt et al., 1986). This may explain why many of Y2 receptor-mediated effects oppose Y1-mediated effects. For example, Y1 agonists are anxiolytic whereas Y2 agonists appear to be anxiogenic (Sajdyk et al., 2002). The same holds true for the central effects of NPY on blood pressure as Y2 agonists increase while activation of central Y1 receptors decrease blood pressure (Morton et al., 1999). The Y2 receptor has been implicated in seizure modulations via its

presynaptic inhibition of glutamate release in the hippocampus (Colmers et al., 1991). Y2 receptor deficient mice show increased body weight, food intake and fat deposition, but a normal response to NPY-induced food intake and intact regulation of the re-feeding response after food restriction (Naveilhan et al., 1999). These data support the notion that the Y2 receptor likely plays a modulatory role in NPY-mediated activity via its presynaptic regulation of transmitter release.

Y4 receptor. The Y4 receptor is characterized by its high affinity for rat PP with lower affinity for NPY and PYY (Bard et al., 1995). The Y4 receptor is also coupled to inhibition of cAMP accumulation (Bard et al., 1995; Lundell et al., 1997) and to modulation of Ca^{2+} current influx (Bard et al., 1995). In the periphery, the Y4 receptor is found mostly in the colon, small intestine, pancreas and testis. The main function of Y4 may be mediating the effects of PP on pancreatic hormone secretion, gut motility and gall bladder contraction (Schwartz, 1983). In the brain the Y4 receptor is found mostly in the circumventricular organs such as the area postrema and the interpeduncular nucleus (Bard et al., 1995). By *in situ* hybridization, low levels of mRNA encoding Y4 have been reported in the rat hypothalamus (Parker and Herzog, 1999). Recently, the Y4 receptor has been implicated in the central regulation of food intake, since Y4 deficient mice have a lean phenotype (Sainsbury et al., 2002), and PP has been shown to both inhibit food intake when given peripherally and stimulate food intake when given centrally (Katsuura et al., 2002). Campbell et al. showed the effect of PP in stimulation of food intake may

be mediated by the H/O neurons that coexpress the Y4-receptor (Campbell et al., 2003).

Y5 receptor. The Y5 receptor has the highest affinity for rat NPY and rat PYY, with lower affinity for rat PP (Gerald et al., 1996). [Ala^{31}, Aib^{32}]NPY is considered a selective agonist for this receptor (Cabrele and Beck-Sickinger, 2000). When expressed in mammalian cell lines, this receptor also couples to the inhibition of adenylate cyclase (Gerald et al., 1996). The mRNA for the Y5 receptor has been found in the brain and testis (Gerald et al., 1996). In the brain, this receptor is found in a number of hypothalamic nuclei, lateral septum, hippocampus, cingulated cortex, central and anterior cortical amygdaloid nuclei, nucleus tractus solitarius, and area postrema (Gerald et al., 1996; Dumont et al., 1998). The role of the Y5 receptor in NPY-induced feeding has been demonstrated by antisense knockdown studies (Tang-Christensen et al., 1998; Flynn et al., 1999), and Y5-selective agonists (Wyss et al., 1998; McCrea et al., 2000). Similar to Y1 deficient mice, genetic knockout of Y5 receptor results in the unexpected phenotype of late-onset obesity characterized by increased food intake, body weight, and adiposity. As seen with the Y1 receptor knockout, the Y5 receptor null mice exhibit a reduced feeding response to centrally administered NPY, and this response is completely eliminated by injection of Y1 receptor antagonist (Marsh et al., 1998). These results suggest that both the Y1 and Y5 receptor subtypes mediate NPY-induced feeding. The Y5 receptor also contributes to the anticonvulsant effects of NPY, as the anticonvulsant actions of NPY were absent in the Y5 knockout mouse (Marsh et al., 1999).

2. NPY distribution in the hypothalamus

a. NPY neurons in the hypothalamus

Anatomical studies have identified ARH as the major site of NPY synthesis in the hypothalamus (Gehlert et al., 1987; Morris, 1989). NPY-producing neurons are distributed in the entire rostrocaudal extent of the nucleus, with the highest density at the caudal half of the ARH. The NPY neurons form a cluster in the ventromedial part of the ARH at all levels, with some scattered cells extending to the lateral median eminence. In addition, a few scattered NPY-positive neurons are observed in the LHA, zona incerta, and intermediate periventricular nucleus of the hypothalamus.

Accumulating evidence has shown that NPY is also expressed in the DMH under certain conditions. Smith first demonstrated NPY mRNA expression in a population of neurons in the non-compact zone of the DMH in lactating rats (Smith, 1993). Unlike ARH NPY neurons, the activity of the DMH NPY neurons does not increase in response to negative energy balance. In fact, this elevation of NPY synthesis in the DMH is not seen in animals that have normal body weights and food intake, nor in the fasting animals. A similar expression pattern of NPY neurons in the DMH later was reported in mice with high fat diet-induced obesity and in several lines of mouse genetic obese models involving the disruption of the melanocortin system (Kesterson et al., 1997; Guan et al., 1998). Interestingly, all the animal models that exhibit DMH NPY neuronal induction share a commonality, that is, hyperphagia and/or obesity. Taken together, this information suggests that the novel expression of NPY in the DMH

is involved in causing the hyperphagia observed during lactation and in some obesity models. Currently, the role of DMH NPY neurons in modulating food intake has not been examined in detail. More importantly, no study has been done to examine the signals or neuroactive substrates that are important for modulating the expression of NPY in DMH neurons. This information will not only facilitate our understanding of how NPY expression is regulated under the obese state but may also provide important insights into pharmacological intervention for certain types of obesity. Investigating the involvement of DMH NPY in mediating hyperphagia in lactating animals is the major focus of this thesis.

b. NPY fiber distribution in the hypothalamus

Immunohistochemical studies using antisera against NPY have shown a complex network of NPY-positive fibers distributed throughout the brain including the hypothalamus (de Quidt et al., 1990). NPY neuronal fibers in the hypothalamus originate from multiple sources. It has been shown that NPY neurons in the lateral geniculation leaflet contribute NPY projections to the suprachiasmatic nucleus (Moore et al., 1984; Watts and Swanson, 1987). NPY neurons in the caudal ventrolateral medulla innervate multiple hypothalamic areas, including the POA and the PVH (Sawchenko et al., 1985). The discovery of AGRP, which is coexpressed in NPY neurons in the ARH (Hahn et al., 1998; Chen et al., 1999), provides a more definitive picture of ARH NPY projections because the ARH is the only location where AGRP neurons are produced (Haskell-Luevano et al., 1999). It has been shown that the majority of AGRP-

immunoreactive (ir) fiber terminals also are NPY-ir (Grove et al., 2003). Therefore, the AGRP neuronal fiber distribution mirrors ARH NPY fiber projections. Based on the AGRP-ir and NPY-ir staining (de Quidt et al., 1990; Haskell-Luevano et al., 1999), NPY neurons in the ARH course along the third ventricle and innervate many parts of the periventricular zone. In the rostral portion of the hypothalamus, ARH NPY neurons heavily innervate the organosum vasculosum lamina terminalis (OVLT) and the continuing anteroventral periventricular nucleus. The fiber density gradually decreases into the more lateral areas of OVLT before reaching the lateral septum, where extensive NPY fibers are found. The preoptic area (POA) contains a moderate to dense plexus of fibers in the medial portions as well as the ventricular border where the periventricular preoptic area is located.

In the mid-hypothalamic area, the PVH contains a non-uniform NPY fiber innervation, with the highest density in all of the parvocellular subdivisions and a weak to moderate innervation in the magnocellular portions of the PVH. Scattered fibers and varicosity-like staining, which may represent terminal buttons, are found throughout the anterior hypothalamic area. The DMH also contains dense innervation in the dorsal and ventral portions, whereas the compact zone of the DMH, sandwiched between the dorsal and ventral portion of the DMH, contains only few scattered fibers. Dense fibers have also been found in the medial portion of the lateral hypothalamus and the perifornical hypothalamic area. The VMH receives relatively less NPY innervation

compared to the neighboring nuclei, with the exception of the dorsomedial portion of the VMH, which has a relatively high density of fibers.

The ARH contains the densest fiber plexus in the hypothalamus. The fibers are found throughout the ARH, with more fibers in the caudal portion of the ARH. The median eminence (ME) also contains some NPY-positive fibers, mostly in the internal to intermediate layers of the ME. Few fibers and varicosity-like staining are found in the external layer of the ME.

The posterior hypothalamus receives only moderate innervation of NPY fibers, with the exception of the rostral end of the posterior hypothalamus located dorsal to the caudal end of the DMH, where a very dense plexus of NPY fibers is found. The ventral premammillary nucleus is one of the few subdivisions of the mammillary body that receives moderate levels of NPY innervation.

Little is known about the projections of NPY neurons in the DMH, since this population of NPY neurons is only activated in certain conditions. Li et al. showed that in the lactating rats, the suckling-activated DMH NPY neurons send major projections to the parvocellular part of PVH (Li et al., 1998a).

3. Potential underlying mechanism of NPY in regulation of energy homeostasis

Hypothalamic NPY is an important contributor to energy balance through its pleiotropic effects on food consumption, energy expenditure, and energy storage. Central administration of NPY has been shown to stimulate feeding,

decrease energy expenditure, reduce sympathetic outflow to brown adipose tissue, and increase lipogenesis by stimulating the expression of lipogenic enzymes in white adipose tissue (Clark et al., 1984; Stanley et al., 1986; Miner et al., 1989; Billington et al., 1991; Larsen et al., 1999). Thus, the overall effect of the central action of NPY is to cause a shift toward positive energy balance. Consistent with this notion, it has been shown that expression of NPY in the ARH and the peptide release at the PVH are both elevated during fasting, which is a state of negative energy balance (Brady et al., 1990; Kalra et al., 1991). These results suggest that endogenous NPY in the hypothalamus plays an important role in coordinating a host of physiological responses, including regulation of feeding and energy expenditure and energy stores, to reverse the negative energy balance.

The hypothalamic NPY system may mediate and coordinate the wide variety of effects in energy balance via its extensive projections and connections with other mediators of energy homeostasis. In addition to the well-established direct connection with PVH to control feeding and autonomic output, NPY has been shown to modulate feeding via its reciprocal connections with MCH and H/O neurons in the LHA. Although ARH NPY projects heavily to the DMH, the involvement of this pathway in energy homeostasis regulation has not been examined. Since the DMH NPY has been implicated in mediating hyperphagic behavior, it is conceivable that ARH NPY may coordinate a hyperphagic response in certain physiological conditions via its connection with

the NPY neurons in the DMH. This hypothesis will be tested and discussed in this thesis.

E. HYPOTHALAMIC MELANOCORTIN SYSTEM IS A MAJOR ANOREXIGENIC SYSTEM IN ENERGY HOMEOSTASIS

1. Melanocortin peptides and their receptors in the brain

a. The melanocortin peptides

Melanocortins include α-, β-, and γ-melanocyte-stimulating hormone (α-, β-, and γ-MSH) and adrenocorticotropic hormone (ACTH). These peptides are posttranslational products of the proopiomelanocortin (POMC) prohormone. This prohormone also gives rise to the opiate peptide β-endorphin, hence the name pro-opio-melanocortin. Among all the peptide products of POMC, the melanocortins are classified as a peptide family based on the fact that all melanocortin peptides contain the key pharmacophore amino acid sequence His-Phe-Arg-Trp, which is necessary for the binding to the melanocortin receptors.

POMC is posttranslationally processed in different tissues into various melanocortin peptides. For example, POMC is processed in the adrenocorticotrope of the anterior pituitary to produce ACTH, while POMC in the intermediate pituitary is processed by different peptidases to obtain α-MSH. In the brain, the predominate mature product is α-MSH. A few neurons expressing ACTH in the hypothalamus have also been reported.

b. Endogenous melanocortin antagonists

A uniqueness of the melanocortin system is the presence of endogenous antagonists, agouti and agouti-related protein (AGRP). Agouti is a 134 amino acid protein normally expressed in the skin and is a high-affinity antagonist of the type 1 melanocortin receptor (MC1R). Upon binding to the receptor, agouti prevents the binding of MSH to the receptor and thus results in an inhibition of eumelanin pigment synthesis (Voisey and van Daal, 2002). Pharmacological studies have shown that agouti is also a competitive antagonist of the MC4R (Lu et al., 1998). AGRP is a 132 amino acid protein, structurally related to agouti (Shutter et al., 1997), but found predominately in the CNS. Similar to agouti in the skin, it is believed that AGRP acts as an antagonist of the MC3R and MC4R in the brain. Interestingly, both agouti and AGRP have been shown *in vitro* to be inverse agonists (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001), suggesting that they have the potential to regulate their respective MCRs, even in the absence of agonists.

c. Melanocortin receptors

Five melanocortin receptors (MCRs) have been identified. All the MCRs are seven-transmembrane G-protein coupled receptors, which are linked to cAMP generation via the stimulatory G protein, G_s, and adenylate cyclase (Adan and Gispen, 1997). The MC1R is expressed primarily in the cutaneous melanocytes and plays a key role in determining skin and hair pigmentation (Jordan and Jackson, 1998). MC2R is the ACTH receptor, expressed primarily in the adrenal cortex (Mountjoy et al., 1992) and its primary function is to

mediate the effect of ACTH to stimulate the synthesis and secretion of glucocorticoids in the adrenal cortex. MC3R and MC4R are neural melanocortin receptors. The MC3R is expressed in the brain in regions of the hypothalamus and limbic system. In the periphery MC3R has been found in the placenta and gut (Gantz et al., 1993; Roselli-Rehfuss et al., 1993). MC4R is restricted primarily to the brain, where it is widely expressed (Gantz et al., 1993; Mountjoy et al., 1994). Both MC3R and MC4R have been implicated in regulation of energy homeostasis. The MC5R, on the other hand, is widely expressed at low levels throughout the body (Griffon et al., 1994; Labbe et al., 1994; Fathi et al., 1995). MC5R has been shown to play a role in sebaceous gland secretion (Chen et al., 1997).

2. Melanocortin distribution in the hypothalamus

a. Melanocortin positive neurons

POMC-positive neurons are found primarily in two brain areas: the ARH in the hypothalamus and NTS in the brainstem. In the ARH, POMC neurons are found throughout the ARH along the longitudinal axis. Most of ARH POMC neurons cluster in the ventrolateral part of the nucleus. Almost all of the POMC neurons in the ARH also express another anorexigenic peptide, CART. The ARH POMC can also be cleaved into the endogenous opioid, β-endorphin, and is proposed to be coreleased with α-MSH from POMC neuronal terminals. Central injections of opioids has been shown to stimulate food intake (Kalra and Horvath, 1998; Glass et al., 1999). However, the results from β-endorphin-specific knockout studies indicate otherwise. Mice lacking β-endorphin are

hyperphagic and obese, and the effects of endorphin on food intake are independent of α-MSH (Appleyard et al., 2003). POMC neurons in the ARH also express leptin receptors, indicating that these neurons are downstream targets of circulating leptin. In addition, the expression of POMC and CART in the ARH is closely associated with an animal's metabolic status. For example, POMC expression in the ARH is reduced in food deprived animals, while a high fat diet results in elevated expression (Mizuno et al., 1998; Torri et al., 2002). AGRP is expressed exclusively in the NPY neurons in the ARH with no overlap with POMC-positive cells in the ARH (Hahn et al., 1998; Chen et al., 1999). Similar to POMC neurons, NPY/AGRP neurons in the ARH also express leptin receptors. However, as an endogenous antagonist, AGRP has opposite effects to those of POMC and acts as an orexigenic agent. Its expression is sensitive to metabolic states and increases in response to negative energy balance, suggesting an important role of endogenous AGRP in energy balance regulation.

b. Melanocortin fibers

Immunohistochemical studies using antisera against α-MSH have shown a complex network of α-MSH-positive fibers distributed throughout the brain (Jacobowitz and O'Donohue, 1978; O'Donohue et al., 1979). α-MSH-producing neurons in the ARH course along the third ventricle and innervate many parts of the periventricular zone. In the rostral portion of the hypothalamus, α-MSH neurons heavily innervate the anteroventral periventricular nucleus, the POA, the lateral septum, and the nucleus interstitialis stria terminalis. In the mid-

hypothalamic area, high density of α-MSH-ir staining was found in the parvocellular subdivisions of PVH, which overlaps with a high density of NPY/AGRP-ir fibers, although no colocalization of peptides was found within the same fiber terminals (Cowley et al., 1999). A high density of fibers is found throughout the anterior hypothalamic area. In the caudal portion of the hypothalamus, the DMH contains dense innervation in the dorsal and ventral portions, while the compact zone contains only few scattered fibers. Dense fibers have also been found in the medial portion of the lateral hypothalamus and the perifornical hypothalamic area. The VMH receives only very little α-MSH innervation. The ARH and the ME also contain a dense fiber plexus of α-MSH-ir fibers.

The posterior hypothalamus receives only moderate innervation of α-MSH fibers, with the exception of the rostral end of the posterior hypothalamus located dorsal to the caudal end of the DMH, where a very dense plexus of α-MSH fibers is found.

Since AGRP is produced almost exclusively in the NPY neurons in the ARH (Hahn et al., 1998; Chen et al., 1999; Haskell-Luevano et al., 1999), it is proposed that NPY and AGRP are cotransported to and coreleased at the fiber terminals. This premise is supported by the observations that the majority of AGRP-immunoreactive (ir) fiber terminals also are NPY-ir (Grove et al., 2003), and that the fiber projection pattern is identical for ARH NPY and AGRP (Bagnol et al., 1999; Haskell-Luevano et al., 1999).

3. The role of the melanocortin system in energy balance regulation

A large body of pharmacological and genetic evidence has demonstrated that the central melanocortin system plays a pivotal role in regulating energy homeostasis. Central injection of melanocortin agonists, including α-MSH or NDP-α-MSH, can reduce food intake and body weight and increase energy expenditure. In addition, POMC null mice are hyperphagic and obese (Yaswen et al., 1999).

Of the five melanocortin receptors, MC3R and MC4R are found in the brain and are likely mediating the effects of melanocortins on energy homeostasis. In support of this notion, central injection of MTII, a high affinity agonist for MC3/4R, significantly reduces food intake and body weight, while injection of SHU-9119, a nonselective MC3/4R antagonist, increases food intake and inhibits the effect of leptin on food intake and body weight, along with reducing UCP-1 mRNA expression in brown adipose tissue (Zimanyi and Pelleymounter, 2003). Mice with either MC3R or MC4R deletion have been generated (Huszar et al., 1997; Butler et al., 2000; Chen et al., 2000). It was found that MC4R null mice are hyperphagic and obese while MC3R null mice exhibit elevated energy expenditure, albeit normal food intake. These results suggest that MC4Rs play a primary role in mediating the anorectic effects of melanocortin peptides, while MC3Rs are primarily involved in the metabolic effects.

F. THE INTERACTION OF NPY AND MELANOCORTIN SYSTEMS IN THE REGULATION OF ENERGY BALANCE

Accumulating evidence suggests there is an interaction between hypothalamic NPY and POMC systems in the hypothalamus to regulate energy balance. In the ARH, abundant NPY terminals are found in close contact with POMC neurons and POMC neurons express Y1 receptors (Broberger et al., 1997). Electrophysiological studies have shown that NPY has an inhibitory effect on ARH POMC neuronal activity (Cowley et al., 2001). In addition to the ARH, NPY/AGRP-immunoreactive (ir) and α-MSH-ir fibers are found in close proximity to each other in the PVH. Electrophysiological studies showed that both NPY and α-MSH have an effect on PVH neurons, sometimes on the same neuron, with NPY being inhibitory and α-MSH being stimulatory (Cowley et al., 1999). Recent evidence has suggested that the DMH is another potential site where the two systems may interact in modulating energy balance. Both POMC and AGRP immunoreactive fibers are found in the DMH (Bagnol et al., 1999; Haskell-Luevano et al., 1999), and MC4Rs are expressed in the DMH (Kishi et al., 2003). Finally, NPY mRNA expression is elevated in a population of neurons in the DMH in several hyperphagic rodent models, which are also associated with reduced melanocortin signaling in the brain. One of the major aims of this thesis was to investigate the possible interaction between NPY and POMC in the DMH in energy balance regulation.

G. LACTATION AS A MODEL FOR STUDYING THE INTERACTION BETWEEN NPY AND MELANOCORTIN SYSTEMS IN ENERGY HOMEOSTASIS

Lactation is a natural physiological state occurring after parturition, in which a host of concerted changes in behavior and in the rate of metabolism in various body tissues occurs in the dams to insure that proper adaptations are in place to successfully support the young. It has been shown that the activity of several hypothalamic neuronal systems is altered during lactation; these alterations may mediate some of the physiological adaptations occurring during lactation, such as temporary cessation of reproductive cyclicity (McNeilly, 1994), significant increase in food and water intake in order to overcome the energy demand due to milk production (Malabu et al., 1994; Pickavance et al., 1996; Flint and Vernon, 1998; Smith and Grove, 2002), induction and initiation of maternal behavior (Bridges, 1994), and a significant increase in serum oxytocin and prolactin levels, which are important for maintaining milk synthesis and secretion (Tucker, 1994). It is noteworthy that all these alterations can be "switched" on and off easily by initiating and terminating the suckling stimulus. In addition, these alterations are related to the intensity of the suckling stimulus (Fox and Smith, 1984; McNeilly, 1994; Tucker, 1994). Therefore, each physiological alteration can be assessed in a "dose-dependent" manner. These characteristics make lactation a useful model to study the central mechanisms involved in physiological alterations, such as the dramatic increase in food intake.

1. Changes in food intake and energy balance during lactation

During lactation there is significant energy demand due to milk production, which greatly exceeds the whole-body nutrient requirements of nonlactating animals. This energy demand is met by a 3- to 4-fold increase of food intake (Malabu et al., 1994; Flint and Vernon, 1998; Smith and Grove, 2002). Due to the vast flux of nutrients to the mammary gland for milk production, the energy expenditure continues to exceed the energy intake, despite this large increase in food intake. Therefore, the lactating animals exhibit several characteristics of a negative energy state, such as hypoleptinemia (Woodside et al., 1998; Brogan et al., 1999), hypoinsulinemia (Malabu et al., 1994; Flint and Vernon, 1998), hypothyroidism (Oberkotter and Rasmussen, 1992; van Haasteren et al., 1996), decreased thermogenesis in adipose tissue (Flint and Vernon, 1998), and decreased lipogenesis (Steingrimsdottir et al., 1980). The mechanisms that drive hyperphagia and energy adaptation during lactation are not completely understood. It has been suggested that the mechanisms may reside in the hypothalamus because of its important role in the regulation of food intake and energy homeostasis (Smith and Grove, 2002).

It has been shown that the neuronal activities of several hypothalamic neuropeptide systems are altered during lactation. Overall, and analogous to negative energy balance models, such as fasting; lactating rats have suppressed anorexigenic and heightened orexigenic peptide signaling in the hypothalamus. During lactation, ARH POMC mRNA expression is decreased (Smith, 1993). On the other hand, hypothalamic NPY peptide levels are

significantly elevated during chronic lactation, especially in the POA, PVH, DMH, and ARH (Ciofi et al., 1991; Malabu et al., 1994; Pickavance et al., 1996). In addition, *in situ* hybridization studies indicate that NPY/AGRP neuronal activity is greatly increased in the caudal portion of the ARH (Smith, 1993; Li et al., 1998b; Chen et al., 1999).

In addition to the ARH NPY neurons, there is an induction of high levels of NPY mRNA in neurons scattered dorsolaterally to the compact zone of the DMH during lactation (Smith, 1993; Li et al., 1998b). To date, lactation is the only physiological adult model showing the induction of NPY expression in the DMH, and the suckling stimulus is required for the activation (Li et al., 1998b). This induction of DMH NPY expression is not seen in normal females or males. Furthermore, fasting animals, which share several physiological characteristics of a negative energy state with lactating animals, also show no induction of DMH NPY neurons, albeit the ARH NPY neuronal activity is greatly increased. This suggests that, unlike the ARH NPY, the activation of DMH NPY neuronal population is not a result of the energy homeostatic adaptations in response to a negative energy state. Rather, the activation of DMH NPY may play a role in modulating other physiological responses that are unique to the lactation, such as hyperphagia.

The notion that elevated NPY mRNA expression in the DMH may play an important role in mediating the hyperphagia observed during lactation is supported by reports that a similar expression pattern of NPY neurons in the DMH has been observed in mice with high fat diet-induced obesity (Guan et al.,

1998) and in several lines of obese mouse genetic models that lack functional MC4R signaling (Kesterson et al., 1997). All of these models are hyperphagic and obese. More importantly, NPY levels in the ARH are unchanged in the agouti obese mouse and the MC4R null mice, further emphasizing the potential importance of NPY expression in the DMH as the underlying mechanism of the hyperphagic phenotype observed in these obese mouse models. These data suggest that the activation of DMH NPY may play an important role in the hyperphagia observed during lactation and in some obesity models.

Currently, the mechanisms by which the DMH NPY population is activated and its role in mediating food intake/energy homeostasis is not completely understood. Little is known about the phenotypic characteristics of these neurons when they are not expressing NPY, which is their common state. In addition, it remains to be determined which signals or neuroactive substrates are important for inducing and modulating the expression of NPY in DMH neurons. Using the lactating rat as the model, the major goal of my thesis is to investigate the regulation and functional role of this population of NPY neurons, which might provide insights into potential treatments of obesity or cachexia. The central hypothesis of this thesis is that factors that are cardinal to lactation play important roles in signaling the activation of NPY neurons in the DMH during lactation.

2. Factors that may be involved in modulation of DMH NPY activity during lactation

a. Neural signals activated by the suckling stimulus

It has been shown that the suckling stimulus is one of the key signals in causing many of the alterations associated with lactation, such as prolactin and oxytocin secretion, somatosensory sensations, food intake, and the suppression of LH secretion. Neural impulses activated by suckling travel through the spinal cord and are relayed to the brainstem and then enter the forebrain region including the hypothalamus (Wakerley et al., 1994; Li et al., 1999b).

Our laboratory previously showed that similar to the ARH, NPY expression in the DMH is induced rapidly after the onset of the suckling stimulus. Significant NPY mRNA expression in the DMH is apparent after just 3 hours of the suckling stimulus, and the number of neurons and the intensity of NPY mRNA expression reach maximal levels after 12-24 h of suckling (Li et al., 1998b). These results suggest that the acute onset of the suckling stimulus is involved in stimulating DMH NPY expression. Using immunostaining for cFos, a marker of neuronal activation, in combination with neuronal tract tracing techniques, our previous studies identified suckling activated neural afferent inputs into the caudal ARH, where NPY neurons are located (Li et al., 1999a). Using similar approaches, we were able to demonstrate that suckling-activated DMH NPY neurons project to the PVH (Li et al., 1998a). For my thesis research, similar approaches were used to investigate suckling activated neural afferents into the DMH to identify the pathways that are potential candidates for activating NPY expression in the DMH during lactation.

b. Suckling-induced hyperprolactinemia

Prolactin is one of the hormones secreted from the anterior pituitary in response to the suckling stimulus. The classic function of elevated prolactin during lactation is to stimulate the mammary gland to synthesize several important ingredients in milk (Neill and Nagy, 1994). In addition to this basic function, prolactin stimulates progesterone secretion from the ovary during lactation. It has been suggested that elevated prolactin during lactation can negatively modulate LH secretion (McNeilly, 1994) and stimulate feeding (Moore et al., 1986; Sauve and Woodside, 1996). Our previous studies showed that treatment of lactating rats with bromocriptine, which serves to suppress suckling-induced PRL secretion, caused a significant reduction of DMH NPY mRNA expression. These data suggested that suckling-induced hyperprolactinemia is permissive for elevating NPY activity in the DMH. It remained to be determined whether PRL is an integral part in modulating NPY neuronal activity in the DMH during lactation.

c. Melanocortin input from the ARH

Several lines of evidence have suggested the importance of the melanocortin system in the ARH in the expression of NPY in the DMH: (1) POMC and AGRP neurons in the ARH send extensive projections into the DMH, (2) MC4R is found in the DMH, and (3) during lactation, ARH POMC and AGRP expression is down- and up-regulated, respectively, which results in a reduced melanocortin signaling. This is consistent with the observation that NPY expression in the DMH is only observed when a reduced central MC4R signaling is achieved (Kesterson et al., 1997). It is thus conceivable that

decreased melanocortin input from the ARH may play a direct role in activating NPY expression in the DMH. One of the aims of this thesis was to investigate the involvement of melanocortin and its receptor, likely MC4R, in the activation of NPY in the DMH during lactation.

H. AIMS OF THE THESIS AND APPROACH

Aim 1: Identify afferent neural populations activated by the suckling stimulus that may modulate DMH NPY neuronal activity during lactation.

Approach:

Afferent neuronal populations that send direct projections to the DMH were defined by using retrograde tracing from the DMH. An acute resuckling paradigm was used to activate neurons and induce the expression of the immediate early gene protein, cFos, which identifies suckling activated neuronal populations. Double labeling of neurons with the tracer and cFos identifies neurons that are activated by suckling and send direct projections to the DMH.

Aim 2: Characterize the role of suckling-induced hyperprolactinemia in the activation of DMH NPY neurons during lactation.

Approach:

1. An acute resuckling paradigm was used in combination with pharmacological manipulations of plasma prolactin levels to examine the role of suckling-induced hyperprolactinemia in modulating DMH NPY neuronal activity.

2. The expression of prolactin receptors was characterized in the hypothalamus with a specific emphasis on DMH NPY neurons to provide an anatomical basis for PRL to act in the DMH.

Aim 3: Investigate the potential effect of the melanocortin system in the DMH in modulating energy homeostasis and DMH NPY expression during lactation.

Approach:

1. The neuroanatomical relationship between the melanocortin system and NPY neurons was characterized in the DMH. Double-label fluorescent *in situ* hybridization and immunohistochemistry were used to determine whether αMSH-ir fibers and AGRP/NPY-ir fibers make close contacts onto DMH NPY neurons. Double label *in situ* hybridization was used to visualize MC4R and NPY in the DMH to determine whether DMH NPY neurons express MC4R.
2. A high affinity MC3/4R agonist, MTII, was injected into the DMH of lactating rats to investigate the function of the melanocortin system in the DMH on suckling-induced NPY expression and energy homeostasis including feeding and energy expenditure.

CHAPTER II

SUCKLING-INDUCED ACTIVATION OF NEURONAL INPUT TO THE DORSOMEDIAL NUCLEUS OF THE HYPOTHALAMUS: POSSIBLE CANDIDATES FOR MEDIATING THE ACTIVATION OF DMH NEUROPEPTIDE Y NEURONS DURING LACTATION.

Peilin Chen and M. Susan Smith, *Brain Research* 984: 11-20, 2003.

Introduction

Neuropeptide Y is a 36 amino acid peptide highly expressed in the brain including the hypothalamus (Tatemoto K, 1982). In the hypothalamus, NPY is involved in the regulation of multiple physiological functions including feeding and reproduction (Kalra and Crowley, 1992; Zarjevski et al., 1993; Kalra and Kalra, 1996; Tomaszuk A, 1996). Although the arcuate nucleus (ARH) is the major site of NPY expression in the hypothalamus, accumulating evidence suggests that NPY can also be expressed in other hypothalamic areas under special conditions. For example, a group of NPY neurons in the non-compact zone of the dorsomedial nucleus of the hypothalamus (DMH) is activated during lactation (Smith, 1993; Li et al., 1998b). A similar expression pattern of NPY neurons in the DMH has also been reported in mice with high fat diet-induced obesity (Guan et al., 1998) and in several lines of mouse genetic obese models. Interestingly, all the animal models that exhibit DMH NPY neuronal induction share a commonality, that is, hyperphagia and/or obesity. These data suggest that the expression of NPY in the DMH may play an important role in the hyperphagia observed during lactation and in some obesity models.

Currently, the mechanisms by which the DMH NPY neurons are activated during lactation are not completely understood. It has been shown that the suckling stimulus is essential in triggering this alteration (Li et al., 1998b). Our laboratory previously showed that NPY neurons in the DMH are activated quite rapidly after the onset of the suckling stimulus. Significant NPY mRNA expression in the DMH is apparent after only 3 hours of the suckling stimulus,

and the number of neurons and the intensity of NPY mRNA expression reach maximal levels after 12-24hours of suckling. Previous studies from our laboratory also showed that suckling-induced hyperprolactinemia is involved but is not sufficient to stimulate full NPY gene expression in the DMH (Li et al., 1999c). These results suggest that the activation of NPY neurons in the DMH may be mediated by additional factors. One of the likely signals that may be involved in stimulating DMH NPY expression is suckling-induced neural impulses transmitted through the brainstem into the hypothalamus.

The immediate early gene protein product, cFos, has been extensively used as a marker for neuronal activation (Ceccatelli et al., 1989; Lee et al., 1990; Morgan and Curran, 1991). Several laboratories, including ours, have observed a discrete pattern of cFos expression in the brain of the lactating rat in response to an acute suckling stimulus (Numan and Numan, 1995; Lonstein and Stern, 1997; Li et al., 1999b). We demonstrated that cFos expression specifically activated by the physical suckling stimulus was observed in the lateral septum (LS), medial preoptic area (mPOA), periventricular preoptic area (PePOA), supraoptic nucleus, ventrolateral medulla (VLM), locus coeruleus, lateral parabrachial nucleus (PBL) and paralemniscal nucleus (PL) (Li et al., 1999b). On the other hand, cFos expression related to the sensory input associated with pup exposure was observed in the anterior hypothalamus (AH), medial amygdala (MeA), periaqueductal gray (PAG), and a number of sensory relay structures in the brainstem areas (Li et al., 1999b).

The purpose of the present study was to determine which of the neuronal populations activated by the suckling stimulus project to the DMH. This was accomplished by using retrograde tracing from the DMH area, combined with acute suckling-induced cFos expression. The results from this study will greatly facilitate the identification of neuronal populations that are potential candidates for mediating the activation of the DMH NPY neurons in response to suckling.

Materials and methods

Animals

Day 18-19 pregnant Sprague-Dawley rats (n=30; Simonson, Carpertina, CA) were housed individually and maintained under a 12:12 light-dark cycle and constant temperature. Food and water were provided *ad libitum*. The day of delivery was considered as day 0 postpartum. All the animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

Experimental Design

An acute suckling paradigm previously described was utilized to control the onset and intensity of the suckling stimulus (Li et al., 1998b). Briefly, lactating animals had their litters adjusted to 8 pups on day 2 postpartum and the pups remained with their mothers until day 9. At that time, the 8-pup litters were removed from the females for 48 hours. On day 11, the 8-pup litters were returned to the dams to allow suckling for 90 min.

Retrograde Tracer Injection

On day 4 postpartum, animals were anesthetized with tribromoethanol (20 mg/100 g body weight [B. W.]) and placed in a stereotaxic apparatus. A glass micropipette with a tip diameter of 25-30 μm was filled with the retrograde tracer, fluorogold (FG, 2% w/v, in physiological saline), and inserted into the non-compact zone portion of DMH, an area containing a high density of suckling-activated NPY neurons. Injection coordinates were 3.2 mm caudal, 0.6 mm lateral to the bregma, and 8.6 mm ventral to the dura, according to the atlas of Paxinos and Watson(Paxinos and Watson, 1998). FG was injected by iontophoresis with 5 μA current and pulsed at 7-s intervals for 20 min. The glass pipette was left *in situ* for an additional 5 min to avoid the spread of tracer along the pipette track. After the injection, the animals were returned to their 8-pup litters to resume suckling.

Perfusion and Tissue Sectioning

After 90 min of pup suckling, the animals were anesthetized with an overdose of pentobarbital (125 mg/kg B. W., ip) and perfused transcardially with 150 ml of 2% sodium nitrite in saline followed by 300 ml of 4% borax-paraformaldehyde (pH 9.5). The brain was removed and 25 μm sections were cut and collected for immunohistochemistry procedures.

Immunohistochemistry procedures

Tissue sections from all ten animals were processed in one assay to ensure uniformity of immunostaining. One series of tissue sections (a 1 in 4 series of forebrain sections, a 1 in 3 series of brainstem sections) from each animal were rinsed in 0.05 M potassium phosphate-buffered saline (KPBS)

followed by treatment with 1% $NaBH_4$-KPBS solution. Sections were incubated in rabbit anti-cFos antibody (*sc*-52, Santa Cruz, 1:15,000) in KPBS with 0.4% Triton X-100 (KPBSX) for 48 hours. After incubation, the tissue was rinsed in KPBS and incubated in biotinylated donkey anti-rabbit IgG (Jackson Laboratories, 1:600) in KPBSX for 1 hour at room temperature. This was followed by another 1-hour incubation at room temperature in avidin-biotin complex solution (Vectastain ABC Elite Kit, Vector Laboratories). The cFos antibody-peroxidase complex was visualized with a mixture of $NiSO_4.6H_2O$ (25 mg/ml), 3,3-diaminobenzidine 90.2 mg/ml), and 3% H_2O_2 (0.83 μl/ml) in 0.175 M sodium acetate solution. When the staining was appropriate, the tissue was rinsed in KPBS and then incubated in the rabbit anti-FG antibody (Chemicon, 1:30,000). The same immunohistochemistry procedures described above were followed. The FG antibody-peroxidase complex was visualized with a mixture of 3,3-diaminobenzidine 90.2 mg/ml), and 3% H_2O_2 (0.83 μl/ml) in 0.05M Tris buffer-saline solution. Following the FG staining, tissue sections were mounted on gelatin coated glass slides, dehydrated and coverslipped with DPX mounting medium (BDH Laboratory Supplies, Poole, England).

Data analysis

The slides were analyzed under a light microscope. All the areas analyzed were anatomically matched across the 10 animals. The areas in the brain were defined according to the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1998). cFos-positive cells were identified by the evident blue-black staining in cell nuclei, and FG-positive cells were identified as cells

with golden-brown cytoplasmic deposits. Cells with golden-brown cytoplasmic and blue-black nuclear staining were defined as FG/cFos double-labeled cells.

Results

Verification of FG injection site in the DMH and cFos expression induced by suckling

Only the suckled animals with the injection site within the DMH area were included in the analysis (n=10). As shown in Fig.1, the FG deposit was restricted primarily within the area where most suckling-induced NPY neurons were observed, that is outside the compact zone of the DMH (DMHp) and within the borders of the DMH (Li et al., 1998b).

Seven of the 10 rats had the injection sites centered in the ventral part of the DMH; the others had the placement of the needle in the dorsal part of the DMH (Fig 1). However, the pattern of distribution of FG-labeled cells was similar among the 10 animals. The FG-labeled cells in the forebrain region were mostly ipsilateral of the injections site with a few scattered cells in the contralateral side, whereas in the brainstem, FG-labeled cell were largely bilateral. Our results are in general agreement with previous retrograde tracing studies (Berk and Finkelstein, 1981; Kita and Oomura, 1982; Fahrbach et al., 1984; Thompson and Swanson, 1998)

The patterns of cFos expression in all animals receiving a 90 min suckling stimulus were similar to those reported in previous studies (Li et al., 1999a, b)

Forebrain input to the DMH

The expression patterns of FG and cFos in the brain are summarized in Table 1. Outside the hypothalamus, a large number of FG-positive cells were observed in the bed nucleus of stria terminalis (BST, Fig. 2a), the lateral septum (LS, Fig. 2a), and the ventral subiculum (SUB). Few scattered FG neurons were found in the lateral habenula (LH), paraventricular thalamic nucleus (PVT), and the subfornical organ (SFO). The majority of forebrain projections to the DMH arise in the hypothalamus. In agreement with previous retrograde tracing studies (Thompson and Swanson, 1998), the single greatest density of FG cells was seen in the parastrial nucleus (PS, Fig. 2b). Substantial numbers of FG-positive cells were also found in the medial preoptic area (mPOA, Fig. 2c), anterior hypothalamic nucleus (AH, Fig. 2d), the posterior periventricular nucleus hypothalamus (PVp), and the dorsal part of tuberomammillary nucleus (TMd). Moderate levels of FG-positive cells were observed in the periventricular part of preoptic area (PePOA), the anterior parvicellular part of periventricular nucleus, (AVPV), arcuate nucleus (ARH), anterior parvicellular part of the paraventricular nucleus (PVHap), suprachiasmatic nucleus (SCN), ventromedial nucleus (VMH), and the medial part of the supramammillary nucleus (SuM). Scattered FG-positive cells were found in the vascular organ of the lamina terminalis (OVLT) and lateral hypothalamic area (LHA).

FG/cFos double-labeled neurons were found outside and within various hypothalamic areas. Outside the hypothalamus, the majority of double-labeled cells were found in the LS (average of 24 cells/section; Fig. 3a, 3b). A high

concentration of double labeling was also found in the BST. In addition, a few scattered FG/cFos double-labeled cells were found in the SUB. Within the hypothalamus, double-labeled neurons were found primarily in the mPOA (average of 28 cells/section) (Fig. 3c, 3d) and PePOA (average of 6 cells/section). Few scattered double-labeled cells were found in the AH, ARH, AVPV, PVp, and SuM. Several areas, including the cortex and the medial amygdala, which have no FG-positive cells, showed a high number of cFos single-labeled cells, which is consistent with earlier reports (Li et al., 1999a, b)

Midbrain and brainstem input to the DMH

A summary of FG and cFos labeling in the midbrain and brainstem is listed in Table 1. In the midbrain and brainstem regions, a large number of FG-positive cells were found in the peripeduncular nucleus (PP), the ventrolateral medulla (VLM, Fig. 4a), and the lateral parabrachial nucleus (LPB, Fig. 4c). Scattered FG cells were found in the caudal part of the periaqueductal gray (PAG) and the raphe nuclei (central linear, medial and dorsal nuclei). Occasional FG-positive staining was found in the nucleus of solitary tract.

FG/cFos double-labeled cells were found mainly in the PP (average of 22 cells/section), and in the two areas where suckling-induced cFos expression has been identified [VLM (average of 7 cells/section, Fig. 4b) and LPB (average of 5 cells/section, Fig. 4d)]. A few scattered double-labeled cells were found in the PAG. Several areas including the collicular area, the pontine nuclei and reticular formation, in which no FG positive cells were observed, showed a large

number of cFos single-labeled cells, which is consistent with earlier reports (Li et al., 1999a, b).

Discussion

In the present study, retrograde tracing combined with cFos expression was used to identify neural populations in the brain that are activated by the suckling stimulus and project to the DMH. These neural populations may be potential candidates for suckling-induced activation of NPY neurons in the DMH. We have reported that DMH NPY neurons are activated very rapidly after the onset of the suckling (i.e., 90 min [unpublished observation] to 3 hr (Li et al., 1998b)). In the present study, the dams were sacrificed 90 min after the resumption of suckling; thus, it is likely that activation of NPY neurons in the DMH had already begun, although it was too early to detect cFos expression in this area. After 90 min of acute resuckling, the neural impulses resulting from the suckling stimulus, suckling-induced hyperprolactinemia and induction of maternal behavior are all possible factors that could be responsible for the activation of DMH NPY neurons. The 90 min time period is too short for a change in energy balance associated with milk production to be playing a major role, since milk synthesis and production had to start de novo at the onset of resuckling, following the 48 hr period of pup removal. In addition, cFos expression patterns after 90 min of suckling are not altered when the hyperprolactinemia is blocked by pretreatment with bromocriptine (unpublished observation). Therefore, the FG/cFos double-label populations identified in the

present study can be mostly attributed to the neural impulses of suckling and/or the induction of maternal behavior.

cFos protein has been routinely used as the marker for neuronal activation (Lonstein and Stern, 1997; Li et al., 1999a, b). Nonetheless, it has been shown that not all neuronal populations express cFos protein when activated. In addition, cFos protein cannot be used to identify neuronal populations that are inhibited by the suckling stimulus. However, even though cFos expression may not identify all neuronal changes induced by the suckling stimulus, any activated neuronal populations that are identified provide important new information. In future studies, retrograde tracing combined with other immediate early gene products will yield a more comprehensive list of suckling-induced changes in neuronal inputs to the DMH area

Although the retrograde tracer was injected in the area of the DMH where NPY expression is induced, it remains likely that cell types other than NPY, such as galanin and γ-aminobutyric acid neurons, receive the afferent inputs and then relay their signals to NPY neurons. Studies of anterograde tracing from identified regions will be needed to provide definitive information about whether activated afferent inputs directly contact NPY neurons as well as other cell types in the DMH.

The pattern of cFos expression in the suckled animals observed in the present study was similar to that reported previously by our laboratory and others (Lonstein and Stern, 1997; Li et al., 1999a, b). In addition, the pattern and distribution of FG labeled cells after FG injection into the DMH were also

similar to those reported by others (Berk and Finkelstein, 1981; Kita and Oomura, 1982; Thompson and Swanson, 1998). Fig 5 summarizes the afferent inputs into the non-compact zone of the DMH that were activated during lactation. Based on the results from our previous study (Li et al., 1999b), some of the afferent inputs are activated specifically by the physical suckling stimulus, such as the mPOA PePOA, LS, LPB, PAG and VLM. These neuronal populations are the most likely candidates that might be involved in activating DMH NPY neurons during lactation. Other afferent inputs to the DMH, including the AH, BST, and SuM, are more likely associated with sensory stimuli resulting from pup exposure.

Forebrain input to the DMH

In the hypothalamus, the highest density of double-labeled cells were found in the mPOA and PePOA, suggesting that once activated by the suckling stimulus, these areas may potentially influence NPY neuronal activity in the DMH. Simerly and Swanson (Simerly and Swanson, 1988) injected PHA-L, an anterograde tracer, into the mPOA and reported that the medial part of mPOA sends significant projections to the DMH. The present results generally agree with this finding. Other anterograde tracing studies have shown that all preoptic nuclei, including PePOA, project to the DMH (Simerly and Swanson, 1988; Gu and Simerly, 1997; Thompson and Swanson, 1998). In future studies, it will be important to characterize the phenotype of the FG/cFos-positive neurons in the mPOA and PePOA in order to further understand how these areas may mediate the activation of DMH neurons. The suckling-induced activation of the mPOA

(present results, [21]) may be related to the induction of maternal behavior. This area has also been shown to express high levels of cFos protein in virgin females expressing maternal behavior (Numan and Numan, 1995). Since our current suckling paradigm is unable to dissociate the maternal behavior component from the suckling stimulus, it is possible that some of the cFos - labeled cells in the mPOA were associated with the maternal behavior. Therefore, we cannot rule out the possibility that the induction of maternal behavior might be involved in the activation of the NPY mRNA expression in the DMH. More studies are needed in order to elucidate this issue.

A few double-labeled cells were also found in several additional hypothalamic areas, including the AH, PMv and SuM. Our previous study showed that sensory stimuli associated with pup exposure alone is sufficient to induce cFos expression in these areas (Li et al., 1999b). These results suggest that sensory input induced by pup exposure may play some role in modulating DMH neuronal activity.

In extrahypothalamic areas, a significant number of double-labeled cells were found in the LS. Several tracing studies have confirmed that LS sends dense inputs to the DMH (Risold and Swanson, 1997; Thompson and Swanson, 1998). As reported in our previous study (Li et al., 1999b), LS is one of the brain areas that requires the physical suckling stimulus for cFos induction. The high number of cFos/FG double label neurons in this area suggests that LS may directly modulate DMH activity during lactation. The phenotype of cFos/FG double-labeled neurons is yet to be identified. It has

been shown that a large number of neurons in this area are GABA-ergic (Ferraguti et al., 1990; Okamura et al., 1990), which provide inhibitory inputs to the DMH. Interestingly, substantial numbers of GABA neurons are found in the DMH (Okamura et al., 1990). It is possible that afferent inputs from LS may provide an enhanced inhibitory tone on local inhibitory GABA neurons in the DMH during lactation, resulting in "disinhibition" and activation of NPY neurons. It has been suggested that LS, especially the ventral part of the structure, is involved in modulating ingestive behavior (Risold and Swanson, 1996), further substantiating a potential role of DMH NPY on feeding regulating during lactation. A large number of double-labeled cells were also found in the BST, although cFos expression in this area can be induced by pup exposure alone, suggesting that the BST may serve as a route by which the sensory stimuli associated with pup exposure may modulate neuronal activity in the DMH.

Brainstem input to the DMH

Inputs to the DMH from the brainstem are primarily limited to the LPB, VLM, and caudal part of PAG, which are also the areas where cFos was induced specifically by the suckling stimulus (Li et al., 1999b). FG/cFos double-labeled cells were found in all three areas which suggests that these areas in the brainstem may play an important role in relaying the neural input derived from suckling into the DMH area where the NPY neurons are located.

Projections from the LPB to the DMH have been reported previously using anterograde (Saper and Loewy, 1980; Fulwiler and Saper, 1984; Kesterson et al., 1997) or retrograde (Berk and Finkelstein, 1981) tracing

methods. In general agreement with our results, these projections appear to mainly arise from the lateral parts of the nucleus. The LPB has been shown to play an important role in relaying visceral information into the higher brain centers and to be involved in a variety of physiological regulations, including control of body fluid homeostasis, energy metabolism and blood oxygenation (Saper, 2002). It has been shown that the majority of cells in the LPB express cholecystokinin (CCK) (Fulwiler and Saper, 1984), and the CCK receptor type A (CCK_A) was found in the DMH (Carlberg et al., 1992; Mercer and Beart, 1997). The LPB-CCK input to the DMH might play a role in modulating energy balance and food intake during lactation since rats lacking CCK_A are hyperphagic and obese (Bi and Moran, 2002). Future studies are needed to determine whether DMH NPY neurons express CCK_A and whether the efferent projections from LPB neurons make direct connections on DMH NPY neurons.

VLM has been shown to send direct projections into many hypothalamic nuclei, such as paraventricular (PVH), suparaoptic (SON) (Sawchenko and Swanson, 1981, 1982) and ARH (Li et al., 1999a). The main function of the VLM is to regulate oxytocin and vasopressin secretion from PVH and SON, the secretion of anterior pituitary hormones and regulation of autonomic function (Sawchenko and Swanson, 1981, 1982). The direct VLM projections to the DMH established in the present study and others (Thompson and Swanson, 1998) suggests that DMH may be a modulator, or an intermediate modulator upstream to the PVH (Li et al., 1998a), to regulate the stress response and

autonomic function by the VLM during lactation (Sawchenko and Swanson, 1981; Leibowitz et al., 1989).

In the caudal part of PAG, FG-labeled neurons were sparse compared to other brainstem areas. However, a few FG/cFos double-labeled neurons were found in this area. Our previous studies showed strong cFos induction by the suckling stimulus in this area, although pup exposure alone could also induce cFos expression to some degree (Li et al., 1999b). It has been shown that PAG probably does not play a role in the suckling-induced milk ejection pathway. Rather, it is more likely involved in other alterations associated with the suckling stimulus, such as maternal kyphosis and aggression (Juss and Wakerley, 1981; Lonstein and Stern, 1997).

There have been no previous reports of a projection from the PP, which is located close to the peripeduncular nucleus and dorsal to the substantia nigra, to the DMH. However, a significant labeling of FG was found in this area in the present study. The discrepancy between the present study and other DMH regrograde tracing studies is most likely due to the difference in the placement of the tracer. Several cases from the present study have the FG deposit in the more lateral and ventral parts of the DMH, compared to previous studies (Berk and Finkelstein, 1981; Fahrbach et al., 1984; Thompson and Swanson, 1998). In addition, the possibility that the tracer is taken up and transported by fibers-of-passage cannot be ruled out. Anterograde tracing from the PP will be needed to confirm the projection between the PP and DMH. A substantial number of FG/cFos double-labeled cells were found in the PP. The

induction of cFos in this area is not specific to suckling, since pup exposure alone can induce a similar amount of cFos as does the suckling stimulus (Li et al., 1999b). The PP has been implicated in mediating maternal behavior, as well as maternal aggression during lactation (Hansen and Ferreira, 1986; Factor et al., 1993). The present finding of double-labeled cells in the PP suggests that DMH might play a role in mediating maternal behavior during lactation.

In summary, the present studies provide anatomical evidence about the possible neuronal pathways for transmitting neural impulses from the suckling stimulus to the DMH during lactation. Whether neurons from these areas make direct contacts with the DMH NPY neurons need to be further elucidated by anterograde tracing from each identified area. Characterization of the phenotype of the FG/cFos double-labeled neurons in the areas will be pursued in the future studies to facilitate our understanding of the mechanisms by which the suckling stimulus activates the DMH NPY system during lactation.

Table 1. Summary of FG-, cFos- and FG/cFos-positive cells identified in the retrogradely labeled areas after FG injection into the DMH

Areas	FG-positive cells	CFos-positive cells	FG/cFos double-labeled cells
Forebrain Regions			
AVPV	++	+	+
mPOA	++++	+++	++
PePOA	++	++	+
PS	+++	+/-	+/-
OVLT	+	+	+/-
SFO	++	-	-
PVHap	++	+/-	+/-
AH	++++	++	+
LHA	+	+	-
SCN	++	+	+/-
ARH	++	+	+
VMH	++	+/-	-
PVp	+++	+	+
PMv	++	+	+
TMd	+++	+	+/-
SuM	++	+	+
PVT	+	+	-
LH	+	-	-
MeA	+/-	++	+/-
BST	+++	++	++
LS	+++	++	++
PFR	+	-	-
SUBv	++	+	+
Midbrain and Brainstem			
PAG	+	+++	+
LPB	++	++	+
PP	++	++	++
Raphé Nuclei	+	+	+/-
NTS	+/-	+	-
VLM	++	+++	+

Cell density (cells/section): ++++, >150; +++, 50-150; ++, 10-50; +, <10; +/–, very rare.

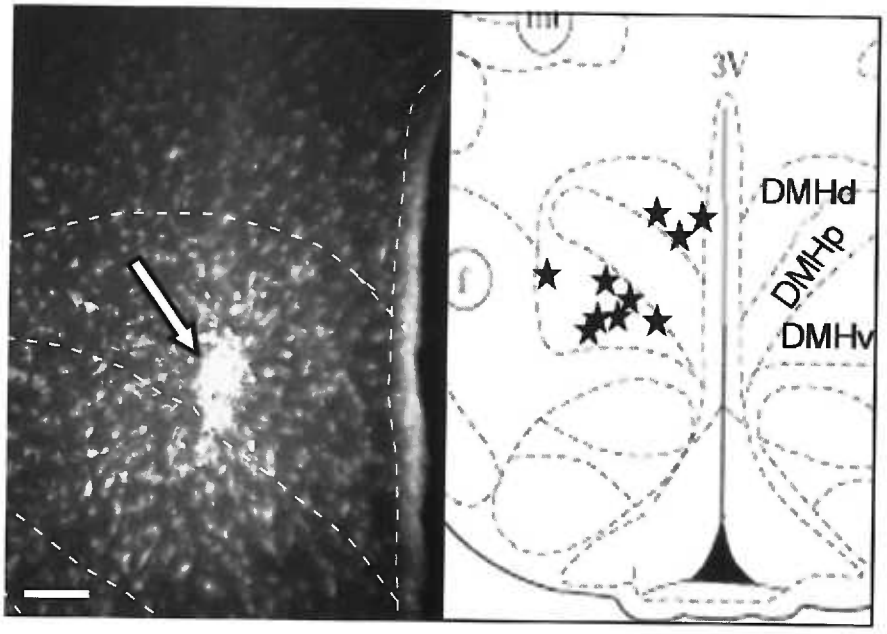


Fig. 2-1. Representative fluorescent photomicrograph of FG injection site (arrow). Right panel: Coronal brain map showing the ten placements of FG (★) that are within the boundary of the noncompact zone of the DMH. *Scale bar* = 50 μm.

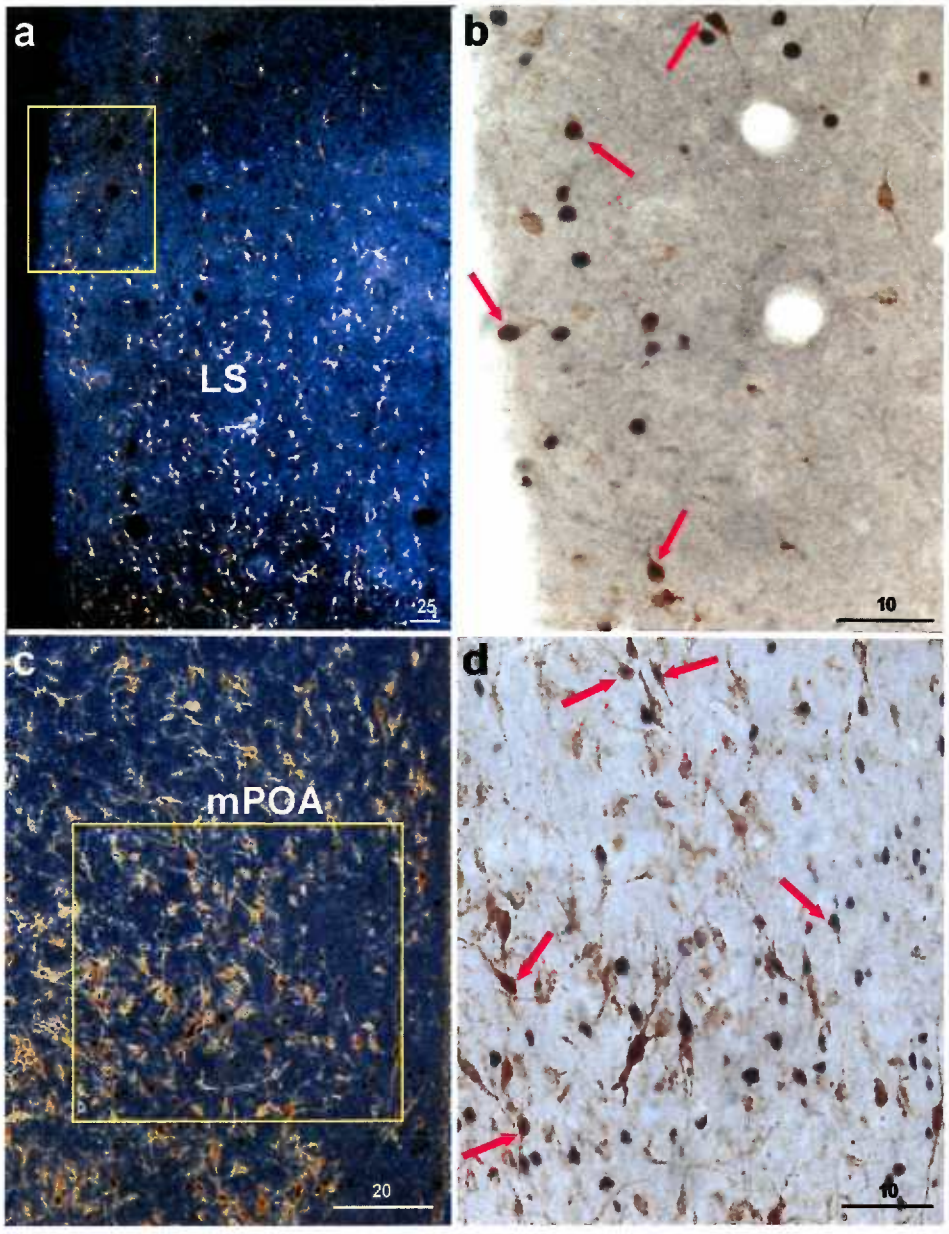


Fig. 2-3. FG/cFos double-labeled cells in the LS and mPOA. Low power dark-field phtomicrograph of LS(a) and mPOA(c) showing the FG labeled cells (golden staining) found within each area. High power bright-field phtomicrographs of the boxed areas in LS (b) and mPOA (d) showing the colocalization (arrows) of FG (brown staining) and cFos (black nuclear staining) in respective areas. *Scale bars* unit = μm.

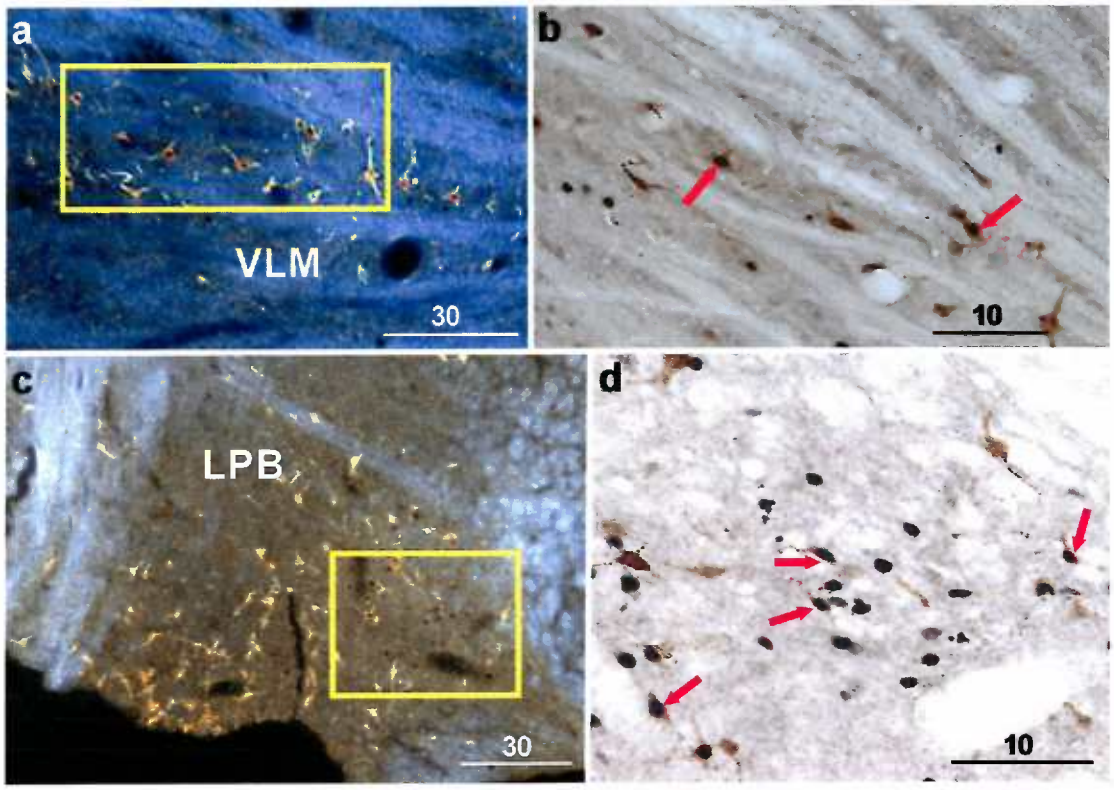


Fig. 2-4. FG/cFos double-labeled cells in the VLM and LPB. Low power darkfield phtomicrograph of VLM(a) and LPB(c) showing the FG labeled cells (golden staining) found within each area. High power bright-field phtomicrographs of the boxed areas in VLM (b) and LPB (d) showing the colocalization (arrows) of FG (brown staining) and cFos (black nuclear staining) in respective areas. *Scale bars* unit = μm.

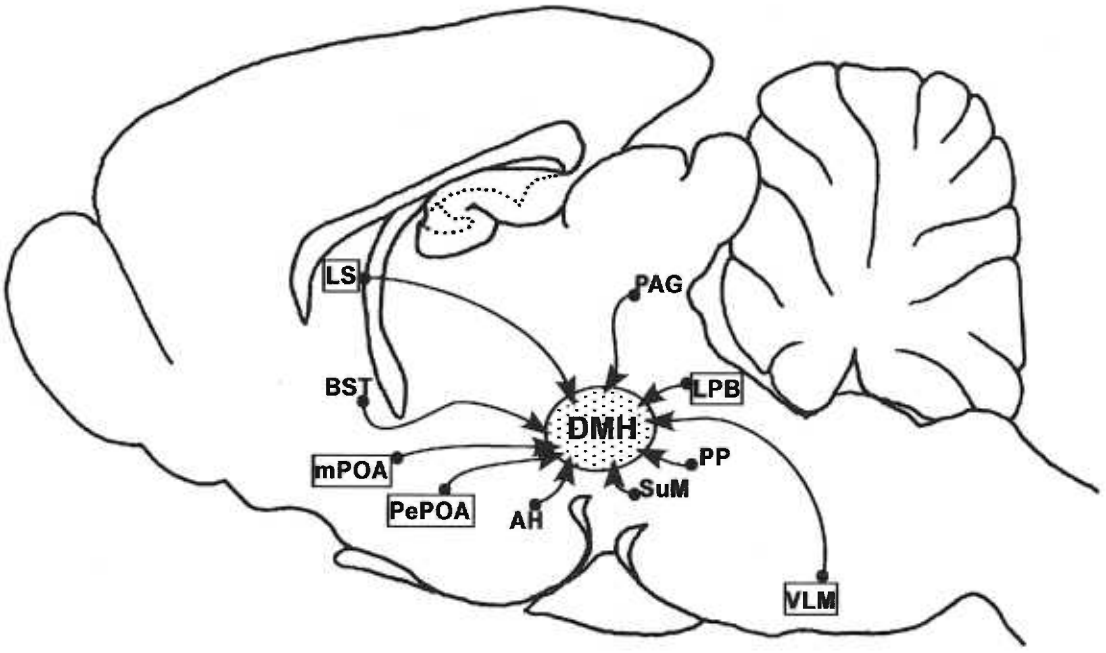


Fig. 2-5. A diagrammatic representation showing major afferent inputs to the non-compapct zone of the DMH. Areas within the rectangles represent the afferent pathways to the DMH that are activated by the physical suckling stimulus and possible candidates for playing a role in activation of DMH NPY neurons during lactation.

Introduction

Lactation is a natural physiological state occurring after parturition, in which a host of concerted changes in behavior and in the rate of metabolism in various body tissues occurs in the dams to insure that proper adaptations are in place to successfully support the young. It has been shown that the activity of several hypothalamic neuronal systems is altered during lactation; these alterations may mediate some of the physiological adaptations occurring during lactation, such as suppression of ovarian cyclicity, increased food intake and suckling-induced milk production (Smith and Grove, 2002).

During lactation, Neuropeptide Y (NPY) neuronal activity has been shown to be greatly increased in the caudal portion of the arcuate nucleus (ARH-C) and dorsomedial nucleus of the hypothalamus (DMH) (Smith, 1993; Li et al., 1998b). The functional role of ARH NPY has been extensively studied in various experimental models. It has been suggested that the increased NPY activity in the ARH-C may be important in mediating the sustained hyperphagia and suppression of luteinizing hormone secretion associated with lactation (Kalra and Crowley, 1992; Lee et al., 1994; Kalra and Kalra, 1996; Tomaszuk A, 1996). On the other hand, the functional role of the increase in NPY in the DMH is still unknown; however, the DMH has been implicated in the control of food intake and energy balance (Bellinger and Bernardis, 2002). A similar expression pattern of NPY neurons in the DMH has been reported in mice with high fat diet-induced obesity (Guan et al., 1998) and in several lines of mouse genetic obese models(Kesterson et al., 1997). Interestingly, all the animal models, including

lactation, that exhibit DMH NPY neuronal induction share a commonality, that is, hyperphagia and/or obesity. These data suggest that the activation of DMH NPY may play an important role in the hyperphagia observed during lactation and in some obesity models.

Currently, the mechanisms by which the DMH NPY neurons are activated during lactation are not well understood. It has been shown that the suckling stimulus is essential in triggering this alteration (Smith, 1993; Li et al., 1998b). Several factors associated with the suckling stimulus, such as the neural impulses arising from suckling (Chen and Smith, 2003) and the elevated levels of PRL (Li et al., 1999c), have been suggested to be involved in mediating the alterations of NPY activity in the DMH. Our previous studies showed that treatment of lactating rats with bromocriptine, which serves to suppress suckling-induced PRL secretion, caused a significant reduction of DMH NPY mRNA expression. These data suggested that suckling-induced hyperprolactinemia may play an integral part in elevating NPY activity in the DMH. In the present study, PRL replacement was given back to bromocriptine-treated dams to directly examine whether PRL is one of the suckling-associated factors that is important in modulating NPY neuronal activity in the DMH during lactation. We also surveyed the anatomical distribution of PRL receptors (PRL-R) in the brain, with a special focus on its relationship to the DMH NPY neurons, in an attempt to provide a neuroanatomical basis for PRL's actions on DMH NPY neurons during lactation.

Materials and Methods

Animals

Day 18-19 pregnant Sprague-Dawley rats (B & K Universal, Inc., Kent, WA) were housed individually and maintained under a 12-h light, 12-h dark cycle (lights on at 0700 h) 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. All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

Experimental design

An acute suckling paradigm, as previously described (Li et al., 1998b), was used in the present study to control the onset of the suckling stimulus more precisely. Briefly, lactating animals had their litters adjusted to eight pups on day 2 postpartum, and the pups remained with their mothers until day 9, when the eight-pup litters were removed from the females. On day 11, or 48 hr after pup removal, the animals were randomly divided into the following four groups: 1) nonsuckled controls, animals received subcutaneous (s.c.) vehicle injections (0 pups + V; n=7); 2) eight pups suckling for 24 h, animals received vehicle injections (8 pups + V; n=7); 3) eight pups suckling for 24 h, animals received bromocriptine injections (0.5 mg/rat/injection; 8 pups + B; n=9); and 4) eight pups suckling for 24 h, animals received injections of bromocriptine (0.5 mg/rat/injection) plus ovine prolactin (oPRL, 1 mg/rat/injection; 8 pups + B + P;

n=10). Resuckling for 24 h was chosen because it consistently induced maximum NPY gene expression in the DMH and ARH after 48 h of pup deprivation (Li et al., 1998b, 1999c). This dose of bromocryptine has been shown previously to completely inhibit suckling-induced prolactin secretion (Li et al., 1999c), as was confirmed in the present studies by the absence of milk in the pups' stomachs at the end of the 24-h resuckling period. The treatment regimen for ovine prolactin, which has been reported to restore prolactin-dependent processes (Smith, 1978; Smith and Lee, 1989), resulted in pup stomachs that were full of milk after 24 h of resuckling.

Bromocriptine (Sandoz Pharmaceuticals Corp., East Hanover, NJ) was dissolved in peanut oil containing 25% alcohol (5 mg/ml). Ovine prolactin (AFP-10677C, NIDDK-NHPP) was dissolved in 50% polyvinylpyrrolidone (5 mg/ml). Each animal received two injections, with the first treatment at 3 h before returning the litters to the dams on day 11 postpartum, and the second treatment at 12 h after returning the pups.

After 24 h of suckling, the animals were killed by decapitation, and the brains were quickly removed, frozen on dry ice, and stored at –80 °C. Coronal brain sections (20 μm) were collected through the ARH (the full extent of the DMH is dorsal to this area) in a one in three series. The slides were stored at –80°C until used for *in situ* hybridization. Trunk blood was collected to assay for plasma levels of rat PRL by radioimmunoassay (RIA), which was performed by Dr. Marc Freeman at Florida State University, according to methods previously described (Freeman and Sterman, 1978).

In situ hybridization

In the present study, quantitative *in situ* hybridization was used to measure NPY mRNA levels to serve as an indirect measure of neuronal activity. NPY complimentary RNA (cRNA) probe synthesis, the specificity of the cRNA probe, and procedures for *in situ* hybridization have been described previously (Smith, 1993; Li et al., 1998b). Briefly, the NPY cRNA probe was transcribed from a 511-bp complementary DNA (cRNA) in which 21% of the UTP was ^{35}S-labeled (PerkinElmer, Boston MA). The specific activity of the probe was 5~6x10^8 dpm/μg. The saturating concentration for the probe used in the assay was 0.3 μg/ml•Kb.

The brain sections were fixed in 4% paraformaldehyde and treated with a fresh solution containing 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), followed by a rinse in 2X SSC, dehydrated through a graded series of alcohols, delipidated in chloroform, rehydrated through a second series of alcohols, and then air-dried. The slides were exposed to the cRNA probes overnight in moist chambers at 55°C. After incubation, the slides were washed in SSC that increased in stringency, followed by incubation in ribonuclease A, and in 0.1X SSC at 60°C to remove non-specific binding. Slides were then dehydrated through a graded series of alcoholsand dipped in NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), exposed for 5-7 days at 4°C, and developed. After development, the slides were stained with cresyl violet.

Double-label ISHH of NPY and PRL-R mRNAs

Fresh frozen tissue sections from lactating animals (day 12 postpartum, n=4) were used in this NPY/PRL-R mRNA dual-labeling study. A 365-bp fragment of cDNA coding for the long and short forms of rat prolactin receptor (PRL-R) was generated by PCR and subcloned into the pGEMT vector (Promega). Antisense and sense rat PRL-R cRNA probes were transcribed from the cDNA in which 25% of the UTP was ^{33}P-labeled (PerkinElmer, Boston MA). The specific activity of the probe was 5~6x10^{9} dpm/□g. The saturating concentration for the probe used in the assay was 6x10^{7} cpm/ml. The NPY cRNA probe was transcribed from a 511-bp cDNA in which the digoxigenin (dig)-UTP was incorporated. Brain sections were fixed and washed as described above and were exposed to the mixture of ^{33}P-PRL-R (6x10^{7} cpm/ml) / dig-NPY (2 μg/ml) cRNA probes in the moist chamber for 15 h at 55°C. After incubation and posthybridization washes, the slides were incubated in alkaline phosphatase (AP) conjugated goat antidigoxigenin antibody (1:2000, Roche Molecular Biochemicals) at 4°C overnight. The AP-complexes were visualized with the substrate of nitroblue tetrazolium and 5-bromo-4-chloro-3-inodyl phosphate toluidinum (Roche Molecular Biochemicals). Slides were checked under light microscopy to insure that the staining intensity of dig-NPY was satisfactory. Slides were then dehydrated, dipped in 3% parlodion followed by NTB-2 emulsion (Eastman Kodak Co.), exposed for 21 days at 4°C and developed.

Data analysis

Brain sections containing the DMH (corresponding to ARH-C sections in Li et. al., 1999) were used for the NPY mRNA analysis (20 μm coronal sections in a 1-in-3 series). The coronal brain sections were anatomically matched across animals from all groups. The hybridization signals were quantified using the OPTIMUS image analysis system, version 6.2 (Media Cybernetics, Silver Spring, MD). Brain section images were captured individually by a CCD camera (Cohu) and displayed on a computer monitor. The system identified silver grains by the brightness of the image. For the ARH, NPY cells were too close together to analyze individually, an estimate for silver grains over the entire ARH on each tissue section was given as the area occupied by silver grains within the region-of-interest (ROI). The ROI was kept constant for all the sections analyzed. Similar principles of quantification were used for analyzing NPY mRNA expression levels in the DMH area. A ROI was drawn to encompass all the NPY-positive neurons in the DMH. However, since the NPY neurons were scattering around the compact zone, it was not possible to exclude part of the compact zone, where low levels of NPY mRNA were present in both lactating and nonlactating animals. Therefore, additional steps were taken to ensure that only silver grain clusters associated with NPY neurons were counted. First, the area occupied by silver grains within the entire ROI was read and recorded (value A). Second, on the same image, all the NPY silver grain clusters associated with NPY neurons were retouched with the background color and a second reading was recorded (value B); this value

reflected total background for the ROI. Finally, value B was subtracted from value A to yield the silver grain area occupied by the NPY neurons only. The sum of the silver grain areas was divided by number of sections quantified for each animal and was expressed as silver grain area per section. To elucidate whether PRL may modulate DMH NPY neuronal activity by increasing the number of NPY neurons or by increasing the intensity of NPY mRNA expression in each neuron, the total number of DMH NPY neurons was counted for each animal. The sum of the silver grain areas was divided by the total number of DMH NPY neurons to yield silver grain area per cell.

Statistical analysis

The data were expressed as grain area per section, NPY positive cells per section and grain area per cell. The means for each of these measurements were determined for each animal. Data are presented as the mean±SEM. Differences between groups were evaluated using one-way ANOVA and *post-hoc* Fisher's tests. Differences were considered significant if $p<0.05$.

Results

Effects of bromocriptine treatment on PRL levels

PRL levels were significantly elevated by 24 h of suckling (8 pups+V), compared to the low levels in nonsuckled control animals (0 pups+V, Fig.1). Bromocriptine treatment effectively suppressed suckling-induced PRL secretion (8 pups+B, Fig.1). Although the rat PRL RIA did not detect the exogenously

administered oPRL (Fig1), similar treatment has been shown to cause a large increase in oPRL levels (Doherty et al., 1985) and to completely restore PRL-dependent physiological functions inhibited by bromocriptine (Smith, 1978; Smith and Bartke, 1987; Smith and Lee, 1989). To support the validity of the PRL treatment, we inspected the stomach of each pup for the existence of milk. All pups from the suckled groups, except those from the 8 pups+B, had full stomachs of milk after 24 h of suckling.

NPY gene expression in the DMH

Suckling eight pups for 24 h induced the expression of NPY in a population of neurons located around the compact zone in the DMH (8 pups+V, Fig. 2a). This population of NPY neurons was not observed in the nonsuckled control group (0 pups+V, Figs. 2a). This data was in agreement with our previous studies (Li et al., 1998b, 1999c). Animals that received bromocriptine treatment had a significantly less NPY mRNA signal (silver grain area / section: 6.07±1.28 vs.27.54±3.68, Figs 2a and 3a) than that of the vehicle-treated animals,; however, this was still significantly higher than the nonsuckled controls (6.07±1.28 vs.0.14±0.06, Figs 3a). PRL replacement significantly reversed the effect of bromocriptine treatment on suckling-induced NPY expression, as the NPY mRNA signal of the 8 pups+B+P group was significant higher than that of the 8 pups+B group (16.46±2.87 vs. 6.07±1.28), albeit it did not reach the same levels as the 8 pups+V group (Figs. 2a and 3a). Bromocriptine treatment also resulted in fewer NPY mRNA positive cells in the

DMH induced by suckling stimulus (cells / section: 4.40±0.54 vs. 9.98±0.27, Fig 3b). PRL treatment nearly reversed the effect of bromocriptine on the number of NPY neurons expressed in the DMH (cells / section: 7.32±0.76 vs. 4.40±0.54, Fig 3b), even though a significant difference was still apparent between the 8 pups+B+P and 8 pups+V groups (Fig 3b). As the result of this closeness in numbers of NPY cells, when the data was expressed as silver grain area / cell, there was no significant difference between the 8 pups+B+P and 8 pups+V groups (Fig 3c). It should be noted that low levels of NPY mRNA signal were observed in the compact zone of the DMH (DMHp) in all animals examined, and this expression did not change in response to either suckling or any of the treatments (Fig 2a).

NPY gene expression in the ARH

In the ARH-C area, 24 h of suckling caused a significant increase in NPY gene expression compared with the nonsuckled group (Fig. 2b and 3d). In addition, there was no difference in NPY gene expression among the groups of 8 pups+V, 8 pups+B, and 8 pups+B+P (Fig. 2b and 3d), indicating that inhibition of PRL secretion did not prevent the suckling-induced activation of NPY gene expression in the ARH.

PRL-R expression in the brain

We first performed ^{33}P-PRL-R single-label *in situ* hybridization on brain sections from lactating and diestrous animals to validate the specificity of the

cRNA probe. In agreement with previous studies (Chiu and Wise, 1994; Bakowska and Morrell, 1997), we detected PRL-R mRNA in discrete regions in the brain from both groups of animals, with the highest hybridizing signal observed in the choroids plexus. Prominent PRL-R mRNA signal was observed mainly in the hypothalamus and limbic structures (data not shown). In the hypothalamus, PRL-R mRNA was observed in the preoptic areas, paraventricular nucleus (PVH), ventromedial nucleus, anterior hypothalamic area, ARH and DMH. In the limbic area, PRL-R mRNA was found in the lateral septum and medial portion of the bed nucleus of stria terminalis. In the midbrain, PRL-R was observed in the periaqueductal gray. The specificity of the PRL-R cRNA probe was confirmed by a lack of hybridization signal on the tissue sections that were hybridized with sense probe.

PRL-R and NPY double labeling in the DMH

To investigate the anatomical relationship between NPY and PRL-R expressing neurons in the DMH and ARH, PRL-R and NPY double label *in situ* hybridization was carried out only on the brain sections containing ARH-C. Dig-NPY mRNA containing neurons were identified under bright field as dark blue deposits in the cytoplasm. ^{33}P-PRL-R mRNA containing neurons were identified under bright field as clusters of black grains clustered over and surrounding the cytoplasm. When both of the signals were examined under bright field illumination, the majority of the DMH NPY-positive neurons were labeled with PRL-R mRNA signal (Fig. 4, a, b). However, in the ARH, most PRL-R

expressing neurons cluster more in the dorsal-medial part of the ARH, while NPY containing neurons congregate in the more ventral-lateral part of the nucleus. Hence, almost no NPY/PRL-R coexpression was observed in the ARH (Fig 4, c, d).

Discussion

During lactation, a significant elevation of NPY mRNA is observed in neurons located in ARH-C and DMH. The results for ARH NPY gene expression in the present study are in agreement with those of our earlier studies (Smith, 1993; Li et al., 1998b, 1999c) and others (Pape and Tramu, 1996), showing a significant increase in NPY gene expression in neurons in the caudal ARH in response to the suckling stimulus. We also showed that bromocriptine does not alter suckling-induced NPY mRNA upregulation in this area, suggesting that suckling-induced hyperprolactinemia is not essential in modulating NPY gene expression in the ARH during lactation (Li et al., 1999c). This notion is substantiated in the present study in that oPRL replacement failed to modify NPY gene expression in the ARH. Finally, while PRL-R is abundant in the ARH and has been shown to be expressed on tyrosine hydroxylase neurons (Arbogast and Voogt, 1997; Lerant and Freeman, 1998), almost no colocalization of PRL-R and NPY neurons was found in this region. These results are further supported by reports showing that PRL treatment or pituitary graft-induced hyperprolactinemia does not affect ARH NPY gene expression (Pelletier G, 1992; Garcia et al., 2003), and immunoneutralization of PRL does

not reduce NPY expression during lactation (Pape and Tramu, 1996). It is noteworthy that the bromocriptine-treated dams experience a relatively low energy demand because of the suppression of milk production resulting from the inhibition of PRL secretion, suggesting these animals do not experience a significant change in energy balance. Thus, the activation of NPY in the ARH in the bromocriptine-treated animals occurs in the absence of a change in energy balance. These results suggest that the activation of NPY neurons in the ARH is mediated by incoming neural impulses activated by suckling, and not by elevated levels of PRL or changes in energy balance.

The present study also confirmed our earlier reports that the suckling stimulus activates a second population of NPY neurons located in the DMH (Smith, 1993; Li et al., 1998b, 1999c). Inhibition of elevated PRL by bromocriptine treatment resulted in significantly lower NPY expression levels, this reduction was reflected by a decrease in the number of NPY neurons activated and in the intensity of mRNA levels of individual neuron. PRL replacement to the bromocriptine-treated animals significantly increased NPY expression levels in the DMH, albeit the replacement did not completely restore the expression to the levels of control suckling animals. Exogenous PRL reversed the effect of bromocriptine on NPY mRNA expression levels of individual neurons, but only partially restored its effect on the number of NPY neurons activated. The inability of oPRL to completely restore bromocriptine suppressed DMH NPY mRNA expression could be due to that fact that the oPRL treatment may not completely mimic the physiological actions of

endogenous rat PRL. Doherty et al., using a similar regimen, showed that even though the serum concentration of oPRL was in the range of several micrograms per milliliter, the treatment was not as effective in suppressing copulatory behavior of male rats as the much lower elevation of serum rat PRL levels produced by ectopic pituitary grafts (Doherty et al., 1985). In the present study, the physiological potency of oPRL treatment was determined by the fact that all pups from oPRL-treated dams had full bellies of milk, similar to those of 8 pups+V group, suggesting that the pharmacological oPRL treatment was effective. However, it is possible that some effects of endogenous rat PRL were not fully restored by the oPRL replacement.

The mechanism by which PRL modulates NPY activity in DMH has not been fully explored. Several studies reported the identification of PRL-R in the brain (Chiu et al., 1992; Crumeyrolle-Arias et al., 1993; Chiu and Wise, 1994; Roky et al., 1996; Bakowska and Morrell, 1997), suggesting a possible pathway for PRL to act directly in the brain to modulate NPY neuronal activity. It has been shown previously by immunohistochemistry (Roky et al., 1996) and receptor autoradiography (Crumeyrolle-Arias et al., 1993) that PRL-R is found in the DMH, whereas other groups failed to show PRL-R in this area, either by immunocytochemistry or *in situ* hybridization (Chiu et al., 1992; Chiu and Wise, 1994; Bakowska and Morrell, 1997; Pi and Grattan, 1998; Bakowska and Morrell, 2003). In the present study, we were able to identify PRL-R mRNA in the DMH area by *in situ* hybridization. Compared to previous studies, we used a different portion of the PRL-R gene sequence to generate the antisense cRNA

probe and a higher energy isotope ^{33}P, instead of ^{35}S, to label the probe. In addition, studies have shown that PRL-R expression levels in several brain areas were altered during lactation (Sakaguchi et al., 1994; Sugiyama et al., 1996; Pi and Grattan, 1999; Pi and Voogt, 2000, 2001). Hence another possible explanation for the discrepancy with earlier studies is that the PRL-R expression in the DMH observed in the present study is a result of lactation-induced upregulation. However, we observed PRL-R positive neurons in the DMH in both lactating and nonlactating animals and the levels between the two groups appeared to be similar.

In the present study, we showed that in the lactating rats, most of the DMH NPY neurons were also PRL-R-positive. These results suggest that PRL may act directly on its receptor to activate NPY gene expression in the DMH. In addition to the DMH, PRL-R mRNA is also observed in the medial preoptic area, the lateral septum, and periaqueductal gray areas. Studies from our laboratory have shown that neurons in these areas send direct neural inputs to the DMH and, more importantly, these neurons are activated in response to the suckling stimulus (11). The expression of PRL-R in these areas raises the possibility that PRL may also modulate DMH NPY neuronal activity via indirect pathways by modulating neural populations upstream of the DMH. More studies are needed in order to resolve this issue.

Another possible signal for activation of DMH NPY neurons is the change in energy balance that is normally associated with milk production. As stated above, the bromocriptine treatment not only blocked PRL secretion but also

suppressed milk production, which presumably would alter the status of energy balance compared to that of the suckled, vehicle-treated animals. Conversely, administration of exogenous PRL to replace the suppressed endogenous PRL caused by bromocriptine not only restored PRL levels, but also milk production, thus, changing energy balance back to the high demand state. However, it has been shown that DMH NPY neurons are activated rapidly after the onset of the suckling (i.e., 90 min [unpublished observation] to 3 h (Li et al., 1998b)), and the expression levels reach maximum levels around 9-12 h (unpublished observation and (Li et al., 1998b)). Therefore, the early induction of DMH NPY occurs before any significant milk production and change in energy balance has taken place. It is possible that the altered energy status in lactating animals may be involved in maintaining the expression of NPY in the DMH, even if not in the induction of expression. Future experiments in which both PRL levels and milk production of the dams are independently controlled are needed to elucidate the involvement of energy balance in modulating DMH NPY activity.

Currently, the physiological significance of the suckling-activated DMH NPY neurons during lactation remains unknown. Our previous retrograde tracing study demonstrated that the suckling-activated DMH NPY neurons project to the PVH (19), an area shown to be the key site in the brain in controlling energy homeostasis. These data suggest that one of the possible functions of DMH NPY neurons during lactation may be to modulate activity of the PVH, resulting in the regulation of energy homeostasis. Interestingly, this information suggests that, during lactation, there is an exaggerated NPY input into the PVH, because

ARH NPY also provide prominent projections into this nucleus (Simerly, 1995a; Li et al., 1999a, d).One can argue that this exaggerated NPY input to the PVH is to ensure that high levels of hyperphagia are achieved during lactation in order to offset the large energy demand due to milk production. On the other hand, previous studies have illustrated that the neural pathways activating the two groups of NPY neurons do not overlap (Thompson and Swanson, 1998; Li et al., 1999a; Chen and Smith, 2003). Furthermore, the present study also demonstrated that PRL differentially modulates the activity of the two NPY populations. Finally, anatomical studies show that the projections of neurons from the ARH and DMH only partially overlap (Simerly, 1995a; Thompson et al., 1996; Li et al., 1999d), suggesting the function of NPY neurons in the DMH and ARH may not be redundant and each population may serve different physiological functions during lactation.

In conclusion, the present study demonstrated that the suckling stimulus activates two populations of hypothalamic NPY neurons, in the DMH and ARH. We also demonstrated that suckling-induced hyperprolactinemia plays a stimulatory role in the activation of NPY neurons in the DMH but not in the ARH. In addition, NPY-positive neurons in the DMH express PRL-R mRNA, whereas most ARH NPY neurons do not express PRL-R mRNA. These data suggest that PRL could act directly on DMH NPY neurons to modulate NPY gene expression during lactation. Therefore, DMH and ARH NPY neuronal populations are differentially regulated by PRL.

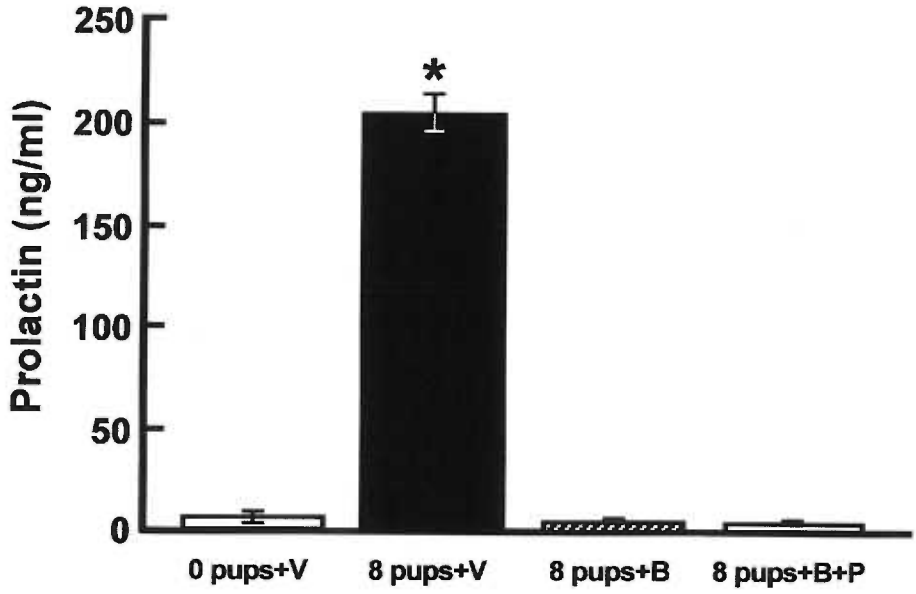


Fig. 3-1. Plasma levels of rat PRL as determined by a rat PRL RIA. Suckling induced a significant elevation of plasma levels of rat PRL [8 pups + vehicle(V); *solid bar*]. Bromocriptine (B) treatment significantly suppressed suckling-induced PRL secretion. Because the PRL antibody used in the assay is specific to rat PRL, the PRL levels remained suppressed int he group treated with bromocriptine and ovine PRL (P) (8 pups + B + P group). *, Significantly different ($p<0.05$) from all other groups.

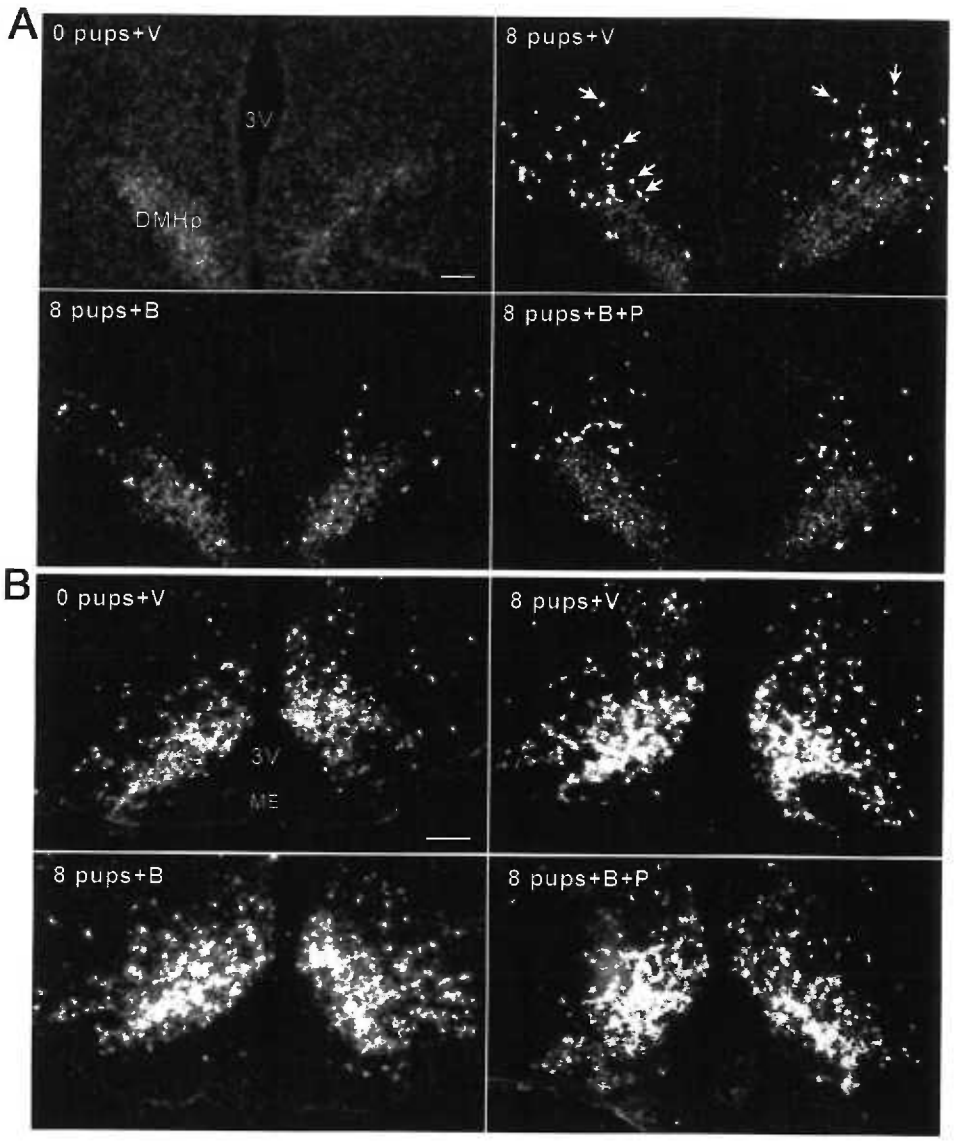


Fig. 3-2. A, Dark-field photomicrographs of the DMH area from the four treatment groups showing the expression of NPY mRNA. Acute resuckling for 24 h [8 pups + vehicle (V)] induced clusters of silver grains (representative clusters indicated by the arrows) scattered in the DMH, compared with the 0 pups + V group. The low levels of signal covering the DMHp was observed in all the animals examined. Bromocriptine (B) treatment significantly blunted NPY mRNA expression (8 pups + B), although PRL replacement partially restored the NPY mRNA expression levels (8 pups + B + P). *Scale bar*, 200 μm. B, Dark-field photomicrographs of the ARH area from the four treatment groups. The silver grains represent NPY mRNA. Note the marked increase in silver grain expression in all groups that received the suckling stimulus. Bromocriptine and/or ovine PRL (P) treatments did not change the NPY mRNA levels in the ARH. *Scale bar*, 200 μm

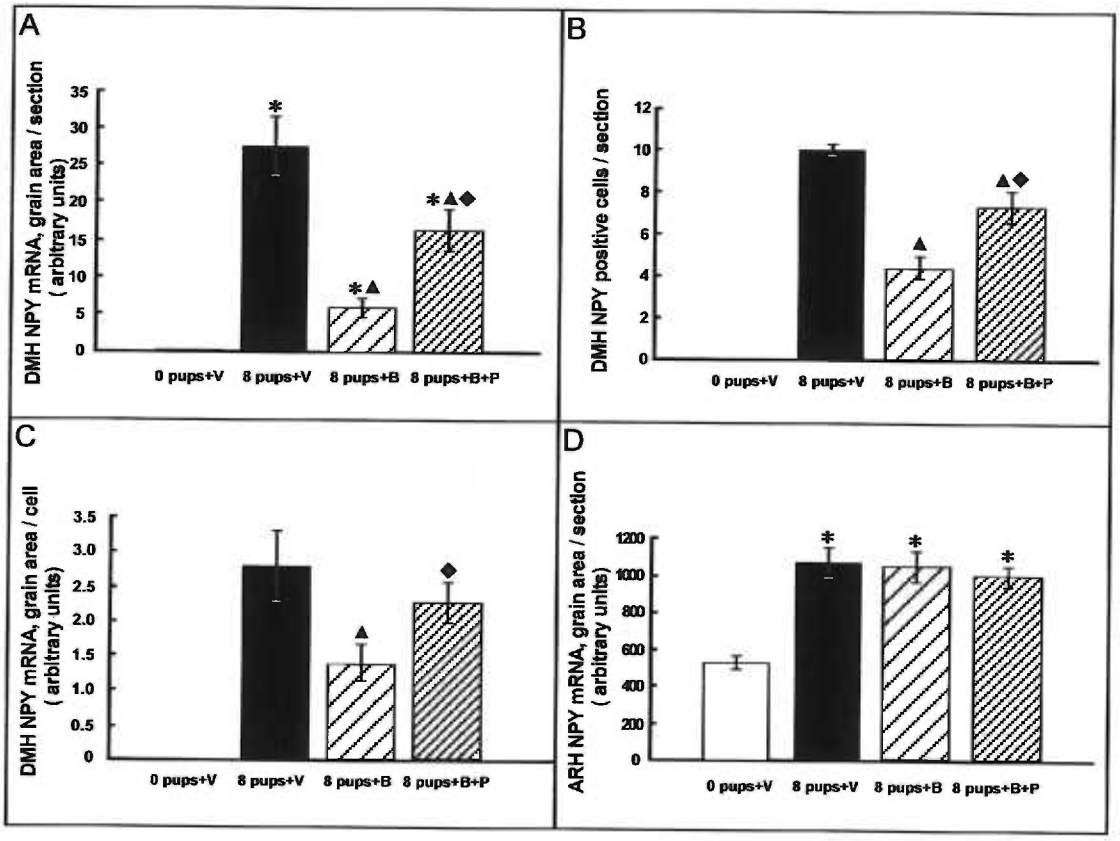


Fig. 3-3. A, NPY mRNA levels in the DMH area. Resuckling for 24 h induced a significant increase in NPY mRNA levels in this area. Bromocriptine (B) tratment greatly attenuated suckling-induced NPY gene expression. Ovine PRL (P) injections to the suckling rats treated with B partially restored NPY mRNA expression. B, Number of NPY mRNA-positive neurons found int he DMH area in the four treatment groups. C, NPY mRNA levels in the DMH expressed as grain area per cell. It should be noted in panels B and C that there were no NPY cells detected in the 0 pups + vehicle (V) group. D, NPY mRNA levels in the ARH-C region in the four treatment groups. Resuckling for 24 h after a 48-h pup separation induced a significant increase in NPY mRNA levels in this region. Inhibition of PRL secretion by bromocriptine failed to suppress the increase in NPY gene expression induced by the suckling stimulus. Furthermore, exogenous PRL treatmetn to bromocriptine-treated animals did not alter NPY mRNA expression levels. *, Significantly different ($p<0.05$) from 0 pups + V group. ▲, Significantly different ($p<0.05$) from 8 pups + V group. ◆, Significantly different ($p<0.05$) from 8 pups + B group.

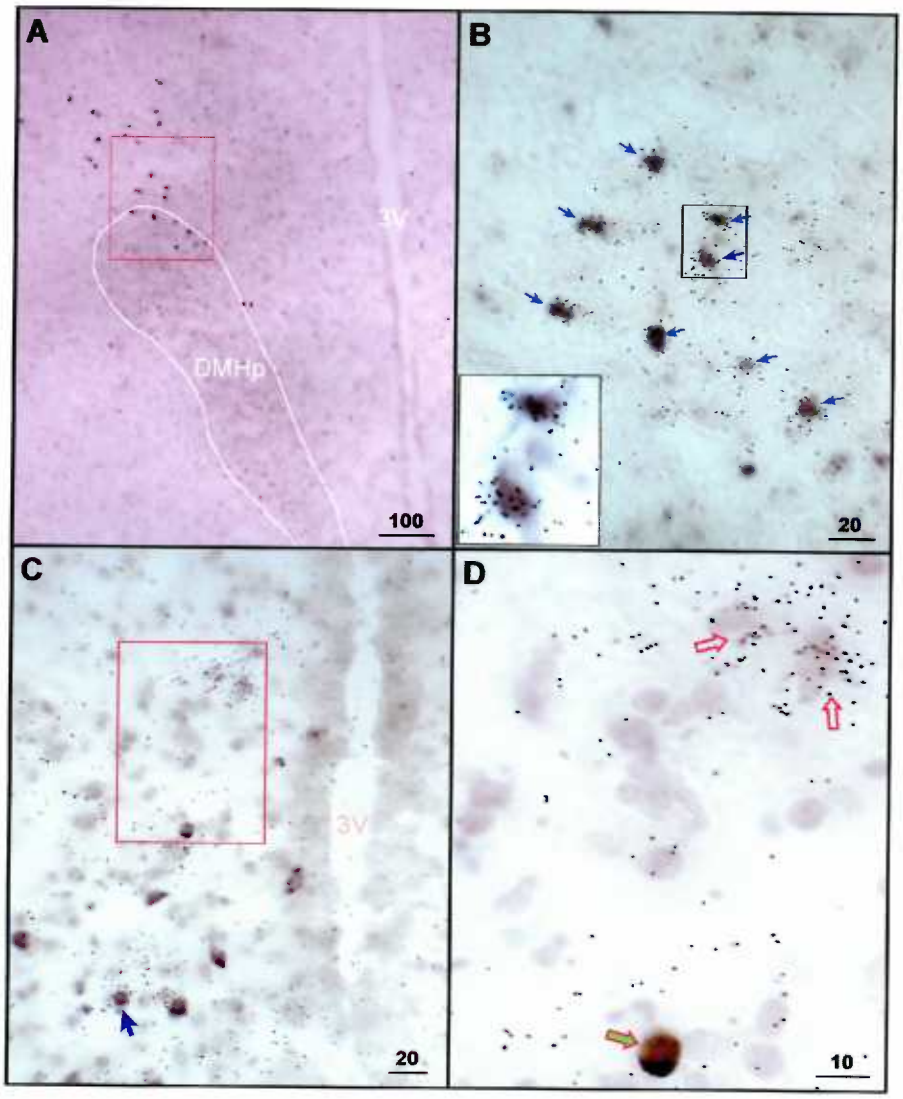


Fig. 3-4. NPY and PRL-R double labeling in the DMH. A: Lower power bright-field photomicrographs of DMH showing digoxigenin (dig) labeled NPY-positive neurons (*blue-black deposit*) scatter around the compact zone of DMH (DMHp, outlined by *solid white line*). B: High magnification of the red boxed area in A showing examples of dig-NPY/ ^{33}P-PRL-R mRNA double labeled neurons (*blue arrows*) in the DMH. The insert further illustrated ^{33}P-PRL-R mRNA (as *clusters of black grains*) scattered over and surrounding the dig-NPY mRNA (as *purple-blue staining*) nucleus. C: Representative photomicrograph showing dig-labeled NPY neurons (*blue-black deposit*) In the ARH. Only occasional NPY/PRL-R double labeled cells (*blue arrow*) were observed. D: Higher magnification of the red box in C showing examples of NPY (*filled green arrow*) and PRL-R (*open arrows*) single labeled cells in the ARH. 3V: third ventricle. *Scale bars* unit =μm.

CHAPTER IV

MELANOCORTIN 4 RECEPTOR-MEDIATED HYPERPHAGIA AND ACTIVATION OF NEUROPEPTIDE Y (NPY) EXPRESSION IN THE DORSOMEDIAL HYPOTHALAMUS DURING LACTATION

Peilin Chen, Sarah M. Williams, Kevin L. Grove, and M. Susan Smith, *Journal of Neuroscience* 24, 2004, *in press*

Introduction

Proopiomelanocortin (POMC) gene-derived peptides in the hypothalamus are a pivotal anorectic system in regulating energy balance. Central administration of α-melanocyte stimulating hormone (α-MSH), one of the POMC gene products, suppresses feeding and elevates energy expenditure (Cone, 1999; Vergoni and Bertolini, 2000), whereas POMC null mice develop overt obesity (Yaswen et al., 1999). In the hypothalamus, most POMC-producing neurons are found in the arcuate nucleus (ARH), and these neurons project into widespread brain regions, including hypothalamus, septum, amygdala and brainstem (Mezey et al., 1985).

Of the 5 melanocortin receptor (MCR) subtypes for POMC peptides (Gantz and Fong, 2003), MC3R and MC4R are the two major subtypes found in the central nervous system, and the MC4R is thought to be responsible for mediating the central effects of POMC peptides in energy homeostasis. For example, central administration of MC4R specific antagonists causes an increase in feeding and obesity (Kask et al., 1998). Mice with MC4R deletion display hyperphagia, reduced energy expenditure and obesity (Huszar et al., 1997). Moreover, over-expression of agouti-related protein (AGRP), an endogenous MC4R antagonist (Shutter et al., 1997), results in hyperphagia and obesity (Ollmann et al., 1997), further reinforcing the importance of MC4Rs in energy balance.

One underlying mechanism of the anorectic effects of MC4R action may involve the inhibition of hypothalamic neuropeptide Y (NPY) expression, a

potent orexigenic system. In MC4R null mice there is an induction of NPY mRNA in neurons in the dorsomedial hypothalamus (DMH, Kesterson et al., 1997). Interestingly, neural activity of NPY neurons in the ARH is not altered in this obese model, in spite of the well documented role of ARH NPY neurons in feeding regulation (Levine and Morley, 1984). These results suggest that the hyperphagia associated with decreased MC4R action may involve induction of NPY expression in the DMH. Similar NPY expression in the DMH has been reported in two other hyperphagic models, lactation (Li et al., 1998b) and the diet-induced obese mouse (Guan et al., 1998). The characteristic of hyperphagia associated with these animal models has led to the hypothesis that NPY in the DMH may be involved in feeding regulation mediated by reduced MC4R signaling.

Lactation is a natural physiological state in which the energy expenditure due to milk production is met by a large increase in food intake [3-4-fold compared to nonlactating rats; (Wade and Schneider, 1992; Wade et al., 1996; Brogan et al., 1999)]. Associated with the hyperphagia of lactation, the expression of the two orexigenic peptides in the ARH, NPY and AGRP, is elevated (Li et al., 1998b; Chen et al., 1999), whereas the anorectic peptide, POMC, is reduced (Smith, 1993). Thus, lactating rats also exhibit reduced MC4R signaling as a consequence of reduced POMC and elevated AGRP expression, consistent with the notion that reduced melanocortin signaling is associated with the induction of NPY expression in the DMH (Li et al., 1998b).

In the present study, the lactating rat was used as a model to first determine the neuroanatomical relationship among POMC and AGRP nerve fibers and terminals, MC4R, and NPY neurons in the DMH. Second, a MC4R / MC3R agonist, MTII, was administered into the DMH to determine whether activation of MC4Rs in the DMH can suppress DMH NPY expression and food intake during lactation.

Materials and Methods

Animals and tissue

Pregnant or cycling female Sprague-Dawley rats (B & K Universal, Inc., Kent, WA) were housed individually and maintained under a 12-h light, 12-h dark cycle (lights on at 0700 h) 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 8 pups on day 2. All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

For immunohistochemistry (IHC) studies, the animals were anesthetized with an overdose of pentobarbital (125 mg/kg b. w., ip) and perfused transcardially with 150 ml of 2% sodium nitrite in saline followed by 300 ml of 4% borax-paraformaldehyde (pH 9.5). The brain was removed and 25 μm sections were cut and collected in cryoprotectant, and stored at –20°C until use. For *in situ* hybridization (ISH) and real-time PCR studies, animals were killed by

rapid decapitation. The brains and interscapular brown adipose tissue (IBAT) were quickly removed, frozen on powdered dry ice, and stored at –80°C. Coronal brain sections (20 μm) were collected through the DMH/ARH areas and stored at –80°C until use. Trunk blood was collected to assay plasma leptin levels by radioimmunoassay (RIA).

The distribution of melanocortin system peptides in the DMH and their anatomical relationship with DMH NPY neurons

IHC for α–MSH immunoreactivity (-ir) or AGRP-ir. To investigate whether ARH POMC or AGRP neurons sends fiber projections to the DMH area, single-label IHC for α–MSH or AGRP was performed. A 1-in-6 series of 25 μm tissue sections from 3 diestrous and 3 lactating animals and covering the entire rostral to caudal extent of the DMH was used for each antibody. Tissue sections were rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by treatment with 1% $NaBH_4$-KPBS solution. Sections were incubated in sheep anti-α-MSH antibody (1:5,000; Chemicon, Temecula, CA), or rabbit anti-AGRP (1:5000, Phoenix Pharmaceuticals, Bont, CA,), in KPBS with 0.4% Triton X-100 (KPBSX) for 48 h. After incubation, the tissue was rinsed in KPBS and incubated in FITC-conjugated donkey anti-sheep or anti-rabbit IgG (1:300; JacksonImmuno Research Laboratories, West Grove, PA) in KPBSX for 1 h at room temperature. The tissue sections were then mounted on gelatin-coated glass slides and coverslipped with buffered glycerol.

Double-label ISH-IHC for NPY mRNA and α-MSH-ir, AGRP-ir, or NPY-ir. To investigate the anatomical relationship between melanocortin peptide fibers and

DMH NPY neurons, double-label ISH/IHC was performed. Free-floating tissue sections (1 in 4 series, covering the entire rostral to caudal extent of the DMH) from lactating D12 animals were used in this study. NPY cRNA probe was made from a 511-bp rat cDNA into which digoxigenin (dig)-UTP (Roche Molecular Biochemicals, Indianapolis, IN) was incorporated (it should be noted that we could not use IHC to label NPY cell bodies since NPY-ir is not visible in hypothalamic cell bodies without colchicine treatment). The tissue sections were rinsed with sodium phosphate buffers, treated with a fresh solution containing 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), followed by a rinse in 2X SSC (standard saline citrate). After the pre-hybridization wash, the free-floating sections were pre-hybridized in hybridization buffer containing 2mg/ml torula yeast RNA for 2 h at 55°C, followed by incubation in the hybridization buffer containing 2 μg/ml dig-NPY cRNA for 15 h at 55°C. After incubation, the tissue sections were washed in SSC that increased in stringency, in ribonuclease, in 0.1X SSC at 60°C, and then were incubated in a primary antibody mixture of mouse anti-digoxigenin monoclonal antibody IgG (1:10,000; JacksonImmuno Research Laboratories, West Grove, PA), and one of the following antibodies: 1) sheep anti-α-MSH antibody IgG (1:5,000; Chemicon, Temucula, CA); 2) rabbit anti-AGRP (1:5,000, Phoenix Pharmaceuticals, Belmont, CA), and 3) rabbit anti-NPY (1:4,000, provided by Dr. Phillip J Larsen at Rheoscience, Rodovre, Denmark). When a double-label IHC and ISH was performed to detect AGRP-ir and NPY mRNA simultaneously, the AGRP antibody failed to detect AGRP-ir staining in the DMH in this

particular procedure. Thus, NPY immunostaining was used to identify NPY positive fibers as an alternative marker for AGRP-ir fibers. This is based on the findings that almost all ARH NPY neurons co-express AGRP (Hahn et al., 1998; Chen et al., 1999). Furthermore, our lab has shown that the majority of NPY fibers in the DMH are also AGRP-positive (Grove et al., 2003). Therefore, NPY-ir fiber staining is representative of AGRP, providing evidence of the neuroanatomical connections between AGRP-ir fibers and NPY neurons in the DMH. After incubation for 48 h at 4°C, the tissue sections were washed and incubated in donkey biotinylated anti-mouse IgG (1:600), and donkey anti-sheep, anti-rabbit, or anti-goat IgG-TRITC (1:300; Jackson ImmunoLaboratories) for 1 h at room temperature (RT). This was followed by 30 min incubation at RT in avidin-biotin complex solution (1:225. Vectastain ABC Elite Kit, Vector Laboratories) followed by application of biotinyl tyramide (1:200, TSA-Indirect Kit, NEN Life Sciences: Boston, MA) for 15 min. Finally, streptavidin-Cy2 (1:1000, 1 hr at RT) was applied to the tissue sections to visualize mRNA encoding NPY. The tissue sections were then mounted on gelatin-coated glass slides, counterstained with Hoechst 33258 (Molecular Probes, Eugene, OR) for visualization of cell nuclei, and coverslipped with buffered glycerol.

Confocal microscopy image analysis. Close appositions between α–MSH- or AGRP-positive fibers and DMH NPY neurons were analyzed with confocal laser scanning microscopy as described previously (Li et al., 1999d). The Leica Corp. (Germany) TSC NT confocal system consisted of a Leica RBE inverted

microscope equipped with an Ar laser producing light at 467 nm and 488 nm, a Kr laser for 568 nm, and a HeNe laser for 647 nm. Various objectives (25X / NA 0.75, 40X / NA 1.25, and 100X / NA 1.4) were used to scan the images. A series of continuous optical sections, at 0.2 μm intervals along the z-axis of a tissue section, were scanned for fluorescent signals. The signals obtained for each fluorophore on one series of optical sections were stored separately as a series of 512 x 512 pixel images. The stacks of individual optical slices (0.2 μm resolution) were analyzed using the MetaMorph Imaging System (Universal Imaging Corporation, West Chester, PA) to determine the close appositions of melanocortin system fibers on the NPY neurons. The confocal images are presented as projections of a stack of optical images.

Double-label ISH of dig-NPY and ^{33}P-MC4R mRNAs. Fresh frozen tissue sections from lactating D12 animals (20 μm, 1-in-3 series) were used. A 600-bp antisense rat MC4R cRNA probe was transcribed from the cDNA in which 25% of the UTP was ^{33}P-labeled (PerkinElmer, Boston MA). The specific activity of the probe was 5~6x10^9 dpm/μg. The same dig-NPY cRNA probe, as described above, was used in this study. Brain sections were fixed and washed as described above and were exposed to the mixture of ^{33}P-MC4R (5x10^7 cpm/ml) + dig-NPY (2 μg/ml) cRNA probes in a moist chamber for 15 h at 55°C. After incubation and posthybridization washes, slides were treated with a similar IHC protocol as described above, except that chromogen (3,3'-diaminobenzidine, DAB) was used to visualize NPY mRNA. The slides were air-dried and dipped in NTB-4 emulsion (Eastman Kodak, Rochester, NY), exposed for 30 days at

4°C, developed, counterstained with cresyl violet, and coverslipped with DPX mounting medium (BDH Laboratory Supplies, Poole, England).

Double-label ISH of dig-NPY and ^{35}S-GAD^{67} mRNAs. The same protocol for double-label ISH, as described above, was used. A 220-bp antisense rat GAD^{67} cRNA probe was transcribed from the cDNA in which 20% of the UTP was ^{35}S-labeled (PerkinElmer, Boston MA). The specific activity of the probe was 4~5x10^{8} dpm/ug. The slides were dipped and exposed to NTB-4 emusion for 7days.

Effects of an MC4R agonist injected into the DMH on food intake, DMH NPY expression, and peripheral markers of energy metabolism

Bilateral cannulation of the DMH area. Female rats were anesthetized with tribromoethanol (20 mg/100 g b. w.) and placed in a stereotaxic apparatus. The double-guide cannula (28 gauge; center-to-center: 1.0 mm; tubing length below pedestal: 8.4 mm; Plastics One Inc., Roanoak, VA) was inserted into the area surrounding the compact zone of DMH [DMHp, coordinate: 3.3 mm caudal, 0.5 mm lateral to the bregma, and 8.4 mm ventral to the dura, according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998)], the area containing a high density of suckling-activated NPY neurons. The guide cannula was secured to the skull by acrylic dental cement and anchored with stainless-steel bone screws. A dummy cannula (32 gauge; tip extends 0.5 mm below the guide cannulae; Plastics One Inc.) was inserted into the guide cannula to maintain patency until the time of injections. Rats were handled daily during the recovery

period and only animals displaying normal food intake and weight gain after surgeries were used.

MTII treatment paradigm.

1) Nonlactation groups. A group of 8 cycling females were ovariectomized and implanted with a bilateral DMH cannula 8 days before the MTII (Phoenix Pharmaceuticals, Mountain View, CA) treatment. The rat chow was removed from the animals 24 h before the treatment. On the day of treatment, freely moving rats were remotely injected at 0900 h with MTII (0.3 nmol in 0.4 μl per side per animal, n=4), or vehicle (0.4 μl double-filtered sterile artificial CSF [aCSF], n=4) through the internal cannula (32 gauge, tip extends 0.5 mm below guide cannula) that was connected via PE50 tubing to a Hamilton syringe. Injected solutions were manually pushed through over a 1-min period, and the internal cannula stayed in situ for 1 min after the injection. Immediately after injections, pre-weighted rat chow was placed in cages. The dose of MTII was extrapolated from the literature as causing a maximal suppression of food intake when given intracerebroventricularly or directly into specific brain nuclei (Fan et al., 1997). Food intake was measured 2, 4, 8, and 24 h after injection. After the completion of the experiment, animals were killed by decapitation. The brains were collected, sectioned, and counterstained with thionin to verify the placements of guide cannulae.

2) Lactation groups. The acute suckling paradigm was used to induce DMH NPY expression. Sixteen lactating animals with 8-pup litters were implanted with bilateral DMH cannulae on day 2 postpartum, and the animals were

returned to their litters to resume suckling and to recuperate from the surgery. At 1830 h on day 9 postpartum, litters were separated from their dams and placed with foster animals until 6 h before the MTII injection. After 48 h of pup separation, the dams received bilateral injections of MTII (n=8, 0.4 nmol in 0.4 μl per side) or vehicle (n=8, 0.4 μl double-filtered sterile aCSF per side). Pups were returned to the animals immediately after the injection to resume suckling for 9 h. A 9-h acute suckling stimulus was chosen based on preliminary studies showing that 9 h of suckling was sufficient to induce near maximal expression of NPY mRNA in the DMH while minimizing alterations in energy expenditure due to milk production. Thus, in this acute suckling paradigm, the expression of NPY in the DMH is primarily the result of neural stimulation induced by suckling. Animals had free access to food and water during the experiment. The interactions between the dams and the pups were observed during the first 15 min and the last 15 min of the 9-h suckling period. Most dams started retrieving and suckling their pups within 3 min of returning the litter. During the last 15 min of the suckling period, most dams were observed to be in the kyphosis suckling position. To ensure that suckling and milk production occurred, each of the 8 pups' bellies was dissected and graded 0 to 3, depending on the amount of the contents (24 would be the maximum score for a 8-pup litter). The food intake of each dam was measured at the end of the 9-h period of resuckling. Brain tissue, BAT, and trunk blood were collected. Brain tissue was sectioned (20 μm coronal sections) and then subjected to ISH for NPY mRNA.

To ensure that alteration of DMH NPY expression was a direct result of the MTII treatment and not secondary to the feeding suppressive effect of MTII, another group of 16 lactating animals (8 received MTII injections, 8 received vehicle injections) were subjected to the same experiment paradigm except that during the 9-h period of resuckling, animals had no access to food but free access to water. Our preliminary studies showed that animals receiving 9 h of acute suckling in the absence of food express a comparable intensity of DMH NPY mRNA as fed animals.

ISH to detect DMH NPY mRNA. The same rat NPY cRNA and ISH procedures as described above were used except that the NPY cDNA probe was labeled with ^{35}S-UTP. The specific activity of the probe was 5~6x10^{8} dpm/μg, and the saturating concentration for the probe used in the assay was 0.3 μg/ml•Kb.

Quantification of DMH NPY mRNA. Only animals with the cannula necrosis tracks within the DMH boundary were used for the analysis. Brain sections containing DMH were used for the NPY mRNA analysis (20 μm, 1-in-3 coronal sections). Tissue sections from all groups were processed in one batch to avoid inter-assay variations. For data analysis, the brain sections were anatomically matched across animals from all groups. The hybridization signals were quantitated using the OPTIMUS image analysis system, version 6.2 (Media Cybernetics, Silver Spring, MD). Brain section images were captured individually by a CCD camera (Cohu) and displayed on a computer monitor. The system identified silver grains by the brightness of the image. An estimate for silver grains over the entire DMH on each tissue section was given as the

area occupied by silver grains within the region-of-interest (ROI). The brightness, threshold, and ROI were kept constant for all the sections analyzed. A ROI was drawn to encompass all the NPY-positive neurons in the DMH. However, since the NPY neurons were scattering around the compact zone, it was not possible to exclude the compact zone, where low levels of NPY mRNA were present in both lactating and nonlactating animals. Therefore, another ROI was drawn to outline the compact zone and the silver grain area within was analyzed and subtracted from the readout obtained from the ROI that covered the whole DMH, so that silver grain clusters associated only with NPY neurons were counted. The sum of the silver grain area was divided by number of sections quantified for each animal and was expressed as silver grain area per section.

Statistical analysis. The data were expressed as the area occupied by grains per section. The mean area occupied by grains per section was determined for each animal. Because all of the slides could not be processed in one assay, the results were normalized to the vehicle-treated controls. Data are presented as the mean±SEM. Differences between groups were evaluated using one-way ANOVA and *post-hoc* Fisher's tests. Differences were considered significant if $p<0.05$.

Real time PCR assay for UCP expression in BAT. BAT was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and total cellular RNA was isolated according to the manufacturer's specifications. The quality and completeness of the RNA were determined by nano-assay bioanalysis using the Agilent 2100

Bioanalyzer (Agilent, Foster City, CA) and measuring the area under the curve of the peaks corresponding to 18S and 28S rRNA. The concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm. Real-time quantitative PCR amplification reactions were carried out in an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) to measure UCP1 mRNA levels. The principle of TaqMan real-time PCR is based on DNA amplification and cleavage of an internal probe that is hybridized to the amplified DNA by the 5'-3' exonuclease activity of the *Taq* DNA polymerase during PCR cycles. 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 final concentration of the RT product was determined by measuring the absorbance at 260 and 280 nm. The reaction was then diluted to 10 ng/μL for PCR analysis. Reactions were conducted in duplicate for increased accuracy. Ten μL of reaction mixture contained 5 μL TaqMan Universal PCR Master Mix, 300 nM specific target gene primers, 80 nM GAPDH gene primers, 250 nM specific probes, and 2 μL (20 nM) 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 Sequence Detector System. After PCR was completed, baseline and threshold values were set to optimize the amplification plot, and the data was exported to an Excel spreadsheet. Standard curves were drawn on the basis of the log of the input RNA versus the critical threshold (CT) 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 CT values to be converted to relative RNA concentration for each sample. The primers and probes were designed using the Primer Express software from Applied Biosystems. The sequence of the primer and probes used were as follows:

UCP1 forward: TCC CTC AGG ATT GGC CTC TAC

reverse: GTC ATC AAG CCA GCC GAG AT

probe: Fam-AAC GCC TGC CTC TTT GGG AAG CAA-Tamra

GAPDH forward: AGA ACA TCA TCC CTG CAT CCA

reverse: GGC CAT GCC AGT GAG CTT

probe: Vic-TGG TGC CAA GGC TGT GGG CAA-Tamra

RIA for serum leptin. Serum leptin levels were determined with a commercial leptin RIA kit (Linco Research Inc., St. Charles, MO). The range of standard concentrations was between 0.5 and 50 ng/ml, which is within the limit of linearity. All samples were measured in the same assay.

Results

Neuroanatomical connections between AGRP, α-MSH fibers and NPY neurons in the DMH

IHC and ISH techniques were used to establish the neuroanatomical relationship between melanocortin-producing neuronal projections (AGRP, α-MSH) and DMH NPY neurons. Analysis of single-label IHC demonstrated that there was abundant α-MSH-ir (Fig. 1a) and AGRP-ir (Fig. 2a) fiber staining throughout the DMH area of the lactating rats. To investigate the anatomical

relationship between the melanocortin peptides and suckling-activated NPY neurons in the DMH, we applied double-label fluorescent histological staining to simultaneously label the fibers and NPY cell bodies. The double-label fluorescent IHC/ISH technique was sensitive enough to stain a comparable amount of α-MSH-ir fibers (red) as was detected with single label IHC, and NPY mRNA-containing neurons (green) were observed scattering around the DMHp (Fig. 1b). With the aid of confocal microscopy, many close appositions were observed between α-MSH-ir fibers and NPY neurons. An examination of 0.2-μm single optical sections through each NPY neuron confirmed the close apposition of α-MSH-ir fibers with the cell bodies (Fig. 1c,d). In the AGRP-ir / NPY mRNA double IHC / ISH studies, AGRP-ir was represented by NPY-ir. As shown in Fig. 2b, we observed robust NPY-ir fibers and NPY mRNA staining in the DMH. When examined under higher magnification, many close appositions between NPY-ir fibers and NPY neurons were found, and the close appositions were confirmed after examining a series of 0.2-μm confocal optical sections.

MC4R and NPY mRNA expression in the DMH

These studies examined the relationship between the melanocortin signaling system, MC4R, and DMH NPY neurons. Double-label ISH of MC4R and NPY was examined with darkfield and brightfield microscopy. In the DMH area, both MC4R (silver grain clusters, Fig. 3a,c) and NPY (brown cytoplasmic staining, Fig. 3b,d) neurons were scattering next to each other, outside of the DMHp; however, no colocalization of the two substances was observed. To ensure that the lack of coexpression was not due to decreased sensitivity of the double-

label ISH technique, other brain regions were examined where both MC4R and NPY were found. Examples of neurons co-expressing MC4R and NPY were observed in the cortical area (Fig. 3e-f).

GAD^{67} and NPY mRNA expression in the DMH MC4R and NPY mRNA expression

These studies examined the anatomical relationship between GABA-ergic and NPY neurons in the DMH. Double-label ISH of GAD^{67} and NPY was examined with darkfield and brightfield microscopy. In the DMH area, both GAD^{67} (silver grain clusters, Fig. 3g) and NPY (brown cytoplasmic staining, Fig. 3h) neurons were scattering next to each other outside of the DMHp. However, no colocalization of the two substances was observed. To ensure that the lack of coexpression was not due to decreased sensitivity of the double-label ISH technique, ARH NPY neurons, the majority of which have been shown to be GABA-ergic (Horvath et al., 1997), were examined. Fig. 3i shows several examples of GAD^{67} / NPY double-labeled neurons in the ARH.

Effect of DMH MTII injection on the refeeding response of 24-h food-deprived nonlactating female rats

The effects of MTII were first examined in nonlactating animals to determine whether direct injections into the DMH and the dose of MTII were effective in suppressing food intake. The refeeding response after a 24-h fast was used so as to maximize food intake in the vehicle-treated animals. Bilateral administration of MTII into the DMH after a 24-hr period of fasting produced a significant inhibition of food intake at all time points measured when compared

to the vehicle-treatment (Fig. 4). The suppressive effect of MTII on the refeeding response appears to be long lasting, since the cumulative food intake consumed by the MTII-treated group was still significantly lower than that of the vehicle-treated animals 24 hr after the drug infusion (Fig. 4).

Effect of DMH MTII injection on food intake and DMH NPY expression in lactating rats

Having established the effectiveness of the MTII treatment, an acute suckling paradigm was used to examine the effects of increased melanocortin signaling in the DMH on suckling-induced hyperphagia and induction of NPY expression. Rats receiving vehicle or MTII injections into DMH exhibited normal maternal behavior. When 8-pup litters were returned to the injected animals, they retrieved the pups and started suckling within 3 min. After 9 h of suckling, the stomach contents of pups from MTII and vehicle groups were examined and found to not be different (vehicle: 19.71 ± 1.40, MTII: 16.25 ± 2.02; p=0.09), indicating a similar level of milk ingestion by the pups. The vehicle-treated lactating animals consumed about 13 g of rat chow in the 9-h time period. The hyperphagic effects of the suckling stimulus were already in evidence since animals that received no suckling stimulus after 48 hr pup separation ate only about 56% of the amount of food consumed by acute resuckled rats during a similar 9-h period. Suckled animals receiving bilateral DMH MTII injections ate significantly less than the vehicle-injected suckled animals in the same period of time (74% decrease, Fig. 5d).

Suckling 8 pups for 9 h induced the expression of NPY in a population of neurons located around the compact zone in the DMH (Fig. 5a). This expression pattern was comparable to that found in animals resuckled for 24 h or during chronic lactation (Li et al., 1998b). In animals receiving MTII treatment, DMH NPY mRNA was 33% of the level observed in the vehicle-treated animals ($p < 0.05$, respectively, Fig. 5b-c). It should be noted that the low levels of NPY mRNA signal observed in the DMHp did not change in response to treatment.

Suckling 8 pups for 9 h in the absence of food induced a comparable intensity of DMH NPY mRNA expression as was observed in the *ad libitum* fed group. Treatment with MTII caused approximately a 70% reduction of NPY mRNA expression compared with the vehicle-treated animals ($p < 0.05$). There was no difference in stomach contents of the pups between the vehicle- and MTII-treated groups (vehicle: 15.67 ± 2.50; MTII; 15.33 ± 2.20; p=0.46), indicating a similar level of milk ingestion by the pups.

Effect of DMH MTII administration on the IBAT UCP1 gene expression in the lactating rats

It has been shown that central injection of an MC4R agonist activates a host of peripheral responses to increase energy expenditure, including increases in oxygen consumption (Hwa et al., 2001), sympathetic outflow to BAT, (Haynes et al., 1999), UCP1 mRNA expression (Williams et al., 2003), and core body temperature (Murphy et al., 2000). In addition, the DMH has been implicated in playing a key role in regulating sympathetic activity and thermogenesis. To

provide additional information of the effects of MTII injections in the DMH, UCP1 mRNA levels in IBAT were used as a marker for changes in peripheral energy metabolism. Figure 6a shows the standard curves of UCP1 and GAPDH RNA, which serves as a reference standard to normalize the DNA quality and quantity among different samples. Along the 5 serial ten-fold dilutions, both tested genes showed linear amplification within the range used for this study, with correlation coefficients greater than 0.99.

Animals suckling 8 pups for 9 h had significantly lower UCP1 mRNA in the BAT than nonlactating females (nonlactating females: 0.63 ± 0.16, acute resuckled: 0.25 ± 0.03 relative units, n = 7 for both groups, $p < 0.05$), similar to that previously described (Xiao et al., 2004). In animals resuckled for 9 h in the presence of food, bilateral MTII injections into the DMH prevented the suckling-induced suppression of UCP1 activity in the BAT (Fig. 6b) and restored levels to those observed in nonlactating females. In contrast, MTII failed to prevent the suckling induced suppression in UCP1 mRNA in BAT in lactating rats suckling for 9 h in the absence of food (Fig. 6c).

Effect of DMH MTII administration on plasma leptin levels in the lactating rats

Serum leptin measurements were made to obtain another measure of the effects of MTII administration on peripheral signals of energy metabolism. Our previous studies showed that lactating rats have markedly decreased serum leptin levels, and removal of the suckling stimulus for 48 h results in leptin levels that are much higher than normal, reflecting the increased body fat of lactating

animals (Brogan et al., 1999; Xiao et al., 2004). Compared to the results obtained in our previous study, the leptin levels (2.8 ± 0.5 ng/ml, Table 1) in animals receiving 9 h of acute suckling after 2 days of pup removal were lower than animals receiving no acute suckling stimulus (approximately 4.5 ng/ml) but not as suppressed as levels observed in chronic lactating rats (approximately 0.4 ng/ml). These data suggest that animals receiving 9 h of suckling were still in the process of adjusting their peripheral physiological responses to adapt to the negative energy balance associated with prolonged milk production. Rats suckling for 9 h in the absence of food exhibited significantly lower plasma leptin levels than the *ad libitum* fed lactating rats (Table 1). DMH MTII injections resulted in a modest reduction of plasma leptin levels in both *ad libitum* and food-deprived groups; however, the differences were not statistically significant (Table 1). It has been suggested that the sympathetic nervous system exerts an inhibitory control over leptin production and release (Donahoo et al., 1997); Pierroz et al. observed a significant downregulation of plasma leptin levels in mice receiving 4 days of icv MTII treatment, but not in the pair-fed controls (Pierroz et al., 2002). These results might explain why we failed to see a significant effect of MTII on circulating leptin levels of resuckled animals after only 9 h of MTII treatment.

Discussion

In the present study we employed an acute resuckling paradigm as a model to determine whether MC4Rs in the DMH play an important role in (1)

the regulation of food intake and energy expenditure, particularly under conditions where there is a strong hyperphagia, such as lactation, and (2) the activation of NPY expression in the DMH during lactation. The short suckling duration (9 h) used in the study reflects, to a greater extent, the neural effects of suckling and, to a lesser extent, the state of negative energy balance that comes from long-term milk production. This conclusion is supported by the results showing that leptin levels in animals receiving 9 h of suckling were not suppressed to the same level as those of chronic lactating rats. The further reduction in serum leptin levels in food-deprived suckled animals provides additional support for the notion that leptin levels are a good indicator of the degree of negative energy balance.

In agreement with other studies (Mezey et al., 1985; Haskell-Luevano et al., 1999), we found intense α-MSH and AGRP fibers and terminals within the DMH area. The close apposition of α-MSH fibers with NPY neurons in the DMH provides a neuroanatomical framework for melanocortin peptides to affect NPY expression. At this time, it is not possible to provide an empirical estimation of the relative contribution of ARH-derived α-MSH fibers identified in the DMH. In addition to the ARH, POMC/α-MSH producing neurons are also found in the nucleus of the solitary tract (Palkovits et al., 1987; Bronstein et al., 1992). However, DMH retrograde studies have shown that the majority of inputs to the DMH arise in the hypothalamus, with only a few projections from brainstem (Thompson and Swanson, 1998; Chen and Smith, 2003). Therefore, it is reasonable to conclude that POMC neurons in the ARH are the major source of

α-MSH fiber projections to the DMH that come in close apposition to NPY neurons.

Similarly, it is not possible to directly determine the relative input of ARH-derived NPY-AGRP fibers to the DMH. Some of the NPY fibers could be originating locally from DMH NPY cells. In addition, NPY-producing neurons in the ventrolateral medulla have been shown to send modest projections to the DMH area (Allen et al., 1983b; Thompson and Swanson, 1998; Chen and Smith, 2003).Therefore, it remains possible that some of the NPY-ir fibers making close appositions on DMH NPY neurons might come from NPY cells other than ARH. Taken together, these results provide a neuroanatomical framework for α–MSH and AGRP effects on NPY neurons in the DMH.

In the present study, we failed to detect NPY/MC4R double-labeled cells in the DMH. This is not surprising, since most studies have demonstrated that MC4Rs couple to G_s to activate adenylyl cyclase (Cone et al., 1996). Thus, one would predict that activation of MC4R results in increased cell activity, which is opposite to our hypothesis that decreased melanocortin system signaling during lactation causes increased DMH NPY neuronal activity. A likely explanation of the anatomical results is that MC4Rs may be expressed on a separate population of neurons within the DMH, which in turn make local synapses onto DMH NPY neurons. These presynaptic terminals that express MC4Rs may be the actual targets of the α-MSH and AGRP projections (Fig. 7). Thus, the melanocortin peptides may synapse on the inhibitory terminals that impinge on the NPY neurons. During lactation, the decreased melanocortin system

signaling could result in reduced inhibitory input to the NPY neurons, leading to activation of NPY expression (Fig. 7). GABA is the prime candidate for the inhibitory input to the DMH NPY neurons, since we and others have reported there are abundant GABA neurons in the DMH (Okamura et al., 1990). In addition, the results from double ISH of GAD^{67} and NPY indicate that, like MC4Rs, GABA is not coexpressed with NPY but GABA neurons are in the vicinity of the NPY neurons in the DMH. In addition, α-MSH has been shown to modulate its downstream effectors via presynaptic GABA-ergic inputs in the paraventricular nucleus [PVH; (Cowley et al., 1999)]. A demonstration of colocalization of GABA and MC4R in the DMH would provide additional support for this hypothesis.

To further substantiate the physiological significance of the anatomical link between the ARH melanocortin system and DMH NPY neurons, MTII, a MC3R/MC4R receptor agonist, was injected directly into the DMH of both nonlactating and lactating rats to examine (1) if DMH is a target of POMC through MC4R in modulating appetite and energy homeostasis and (2) whether reversal of reduced melanocortin tone in the DMH of lactating rats would prevent the activation of NPY neurons in the DMH. In agreement with others (Kim et al., 2000), the present study first showed that bilateral injections of MTII into the DMH of OVX nonlactating rats significantly attenuated the 24-h fasting–induced refeeding. One possible downstream target of the DMH that is mediating this effect is the PVH, as the DMH projects heavily to the parvicellular part of the PVH (Thompson et al., 1996), which is a final common pathway

through which the brain controls feeding (Swanson and Sawchenko, 1983). Although extensive studies have suggested a direct pathway from ARH POMC neurons to MC4R expressing cells in the PVH to suppress feeding (Vergoni and Bertolini, 2000), our findings suggest yet an additional pathway for ARH POMC peptides to regulate feeding, by way of the MC4R in the DMH. This indirect pathway may allow the POMC system to fine-tune its final output to PVH to regulate appetite.

Using the acute resuckling model, the present study showed that 9 hr of resuckling was sufficient to not only induce hyperphagia but also to induce DMH NPY expression. This short period of resuckling is not sufficient to increase NPY expression in the ARH (Li et al., 1998b), suggesting that the induction of NPY expression in the DMH is an important component of the hyperphagia of lactation. Activation of MC4Rs by direct injection of MTII in the DMH of lactating rats greatly decreased (by 77%) the suckling-induced increase in food intake. The MTII-induced decrease in food intake was coupled with a greatly attenuated expression of suckling-induced DMH NPY mRNA (Fig. 5). The similar reduction of DMH NPY expression by the MTII treatment, whether the animals were fed or not fed during the 9 h period of acute suckling, suggests that the reduction was a direct result of the DMH MTII injections and not secondary to the inhibitory effect of the MTII treatment on feeding. It is noteworthy that MTII treatment was equally effective in suppressing food intake in nonlactating females that lack NPY expression in neurons in the DMH. This result suggests that the appetite circuitry involving DMH exerts a tonic input into

the PVH to modulate daily energy intake. When a large energy demand is present, such as milk production, NPY expression in the DMH will provide an additional signal to the PVH to further increase food intake. This view is supported by the findings from Crowley et al. showing that heightened NPY expression and lowered MC4R signaling is the main driving force for the hyperphagia in lactation (Crowley et al., 2003). This study showed that central injections of a NPY antagonist and α-MSH (at a dose much higher than normally used to suppress feeding in normal female rats) reduced food intake of lactating rats to approximately 40% of normal. Their results are in agreement with our finding that when the extra population of NPY neurons in the DMH was suppressed by the activation of MC4Rs in the DMH, food intake of the lactating rats was significantly reduced by 77%, indicating that DMH NPY neuronal population plays a key role in inducing hyperphagia during lactation. Recently, Hill and Levine showed that lactating mice lacking functional NPY were still able to mount a comparable hyperphagia as the wildtype controls (Hill and Levine, 2003), which seems to undermine the importance of NPY in feeding during lactation. However, the apparent normal feeding regulation in the NPY knockout mice could be a result of the developmental compensation by other redundant systems or that the non-selective loss of NPY throughout the brain may not be an accurate reflection of the role of the DMH NPY system in feeding under certain circumstances such as lactation. The results obtained in the present studies clearly showed that DMH NPY plays a pivotal role in modulating lactation associated hyperphagia.

In the present study, we showed that direct DMH MTII injections prevented the suckling-induced reduction of IBAT UCP1 mRNA in the *ad libitum* fed resuckled rats. This result demonstrates for the first time that MC4R signaling in the DMH is involved in regulating sympathetic outflow to the BAT. There are several possible pathways by which MC4R in the DMH may modulate UCP1 expression in the BAT. First, the information may be relayed via projections to the PVH, which in turn influence sympathetic neuronal activity that innervates BAT. Second, DMH sends modest projections to the medullary nucleus raphé pallidus (Thompson et al., 1996), an area shown to be the essential integration area mediating the thermogenic response in rodent models (Madden and Morrison, 2003). Finally, it has been shown that BAT UCP1 gene expression can be regulated by thyroid hormones (Masaki et al., 2000); therefore, it is possible the DMH MC4R-responsive pathway may regulate TRH neuronal activity in the PVH, which in turn modulates UCP1 gene expression in the BAT via releasing thyroid hormones. Furthermore, Fekete et al. showed NPY in the PVH can suppress the hypothalamic-pituitary-thyroid axis (Fekete et al., 2002), and i.c.v. infusion of NPY greatly decreased the expression of UCP1 in the BAT (Zakrzewska et al., 1999). Taken together, it is conceivable that MTII in the DMH might stimulate UCP1 gene production in the BAT via its suppression of DMH NPY output to the PVH. However, the same MTII treatment to the food-deprived lactating group did not prevent the reduction in UCP1 mRNA expression in the BAT, even though NPY mRNA in the DMH was decreased. This data suggests that the combination of energy expenditure (milk

production) and no energy intake (fasting) may activate protective energy adaptations to prevent further energy loss, and thus override the effect of DMH MTII treatment in activating UCP1 activity in the BAT.

In conclusion, the present study provides a neuroanatomical framework for the action of the melanocortin signaling system in the DMH. The melanocortin system in the DMH not only plays an important role in inducing NPY expression in the DMH of lactating rats but also in regulating energy homeostasis, at least in part by modulating appetite and energy expenditure.

Table 1. Plasma leptin levels in lactating rats received bilateral injections of MTII or vehicle to the DMH.

	Leptin (ng/ml)	
	Vehicle (n = 7)	MTII (n = 8)
Ad libitum -fed	$2.81 \pm 0.50^{a\ c}$	$2.07 \pm 0.43^{a\ d}$
Food-deprived	$1.27 \pm 0.32^{b\ c}$	$0.74 \pm 0.25^{b\ d}$

a: $p = 0.11$, b: $p = 0.11$, c: $p = 0.01$, d: $p = 0.01$

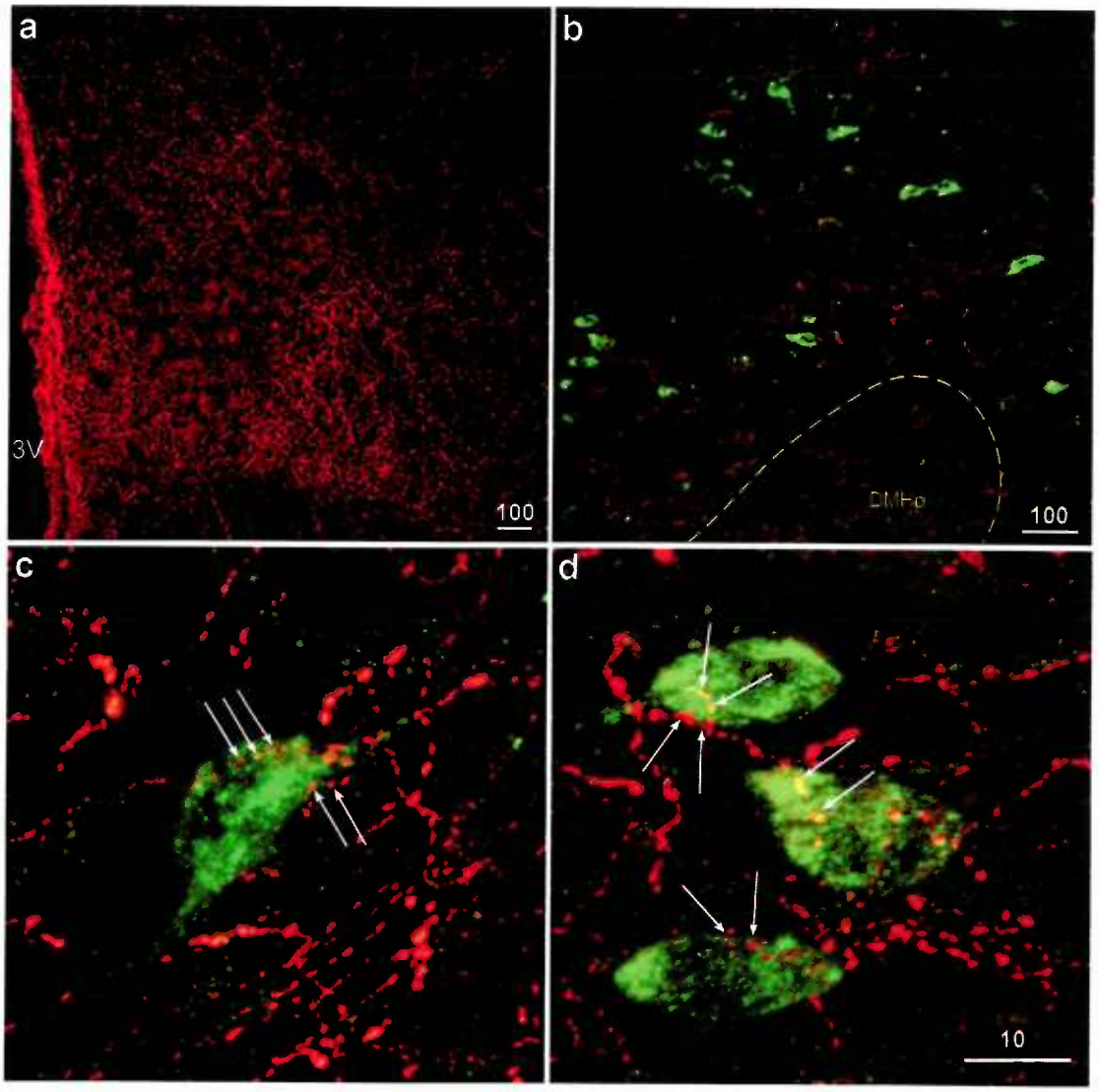


Fig. 4-1. Localization of NPY mRNA (green) and α-MSH-ir fibers (red) in the DMH. **a**: Low power stacked confocal images showing abundant single labeling of α-MSH-ir fibers in the DMH. **b**: Many NPY mRNA containing neurons (green) were found scattering outside the compact zone of DMH (DMHp). **c-d**: Representative high magnification confocal images showing NPY mRNA (green) and α-MSH-ir fibers (red) in the DMH. Close appositions (arrows) of α-MSH-ir fibers on NPY neurons were observed in this area. 3V: third ventricle. *Scale bar* unit = μm.

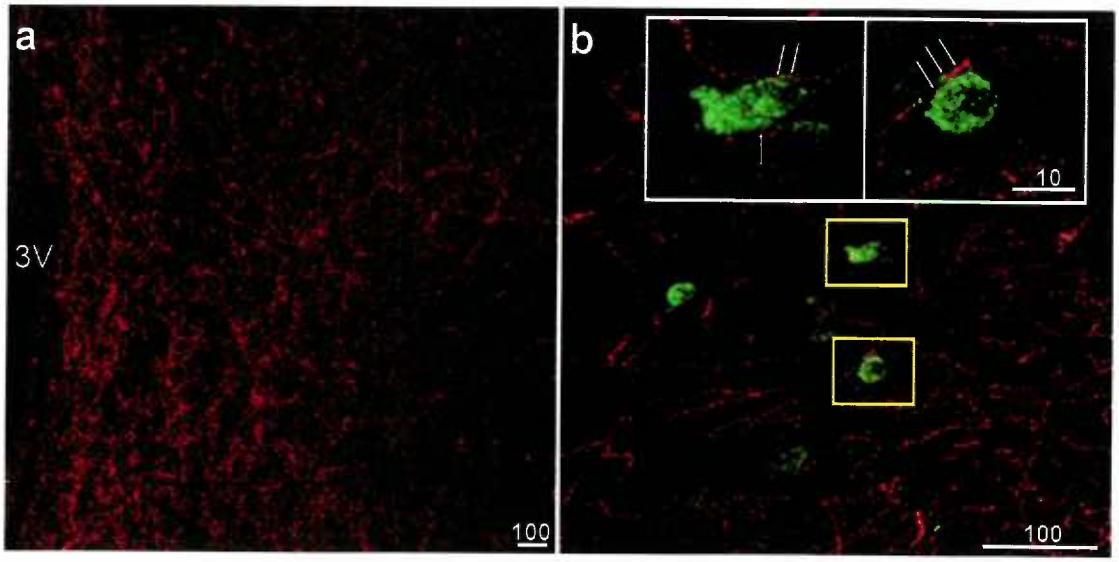


Fig. 4-2. **a**: Low power stacked confocal images showing abundant single labeling of AGRP-ir fibers in the DMH. **b**: Many NPY mRNA containing neurons (green) were surrounded by NPY-ir fibers (red) in the DMH. Insets are high magnification confocal images of the yellow-boxed areas showing NPY-ir fibers (red) in close apposition (arrows) to NPY-producing neurons in the DMH. 3V= third ventricle, *Scale bar* unit = μm.

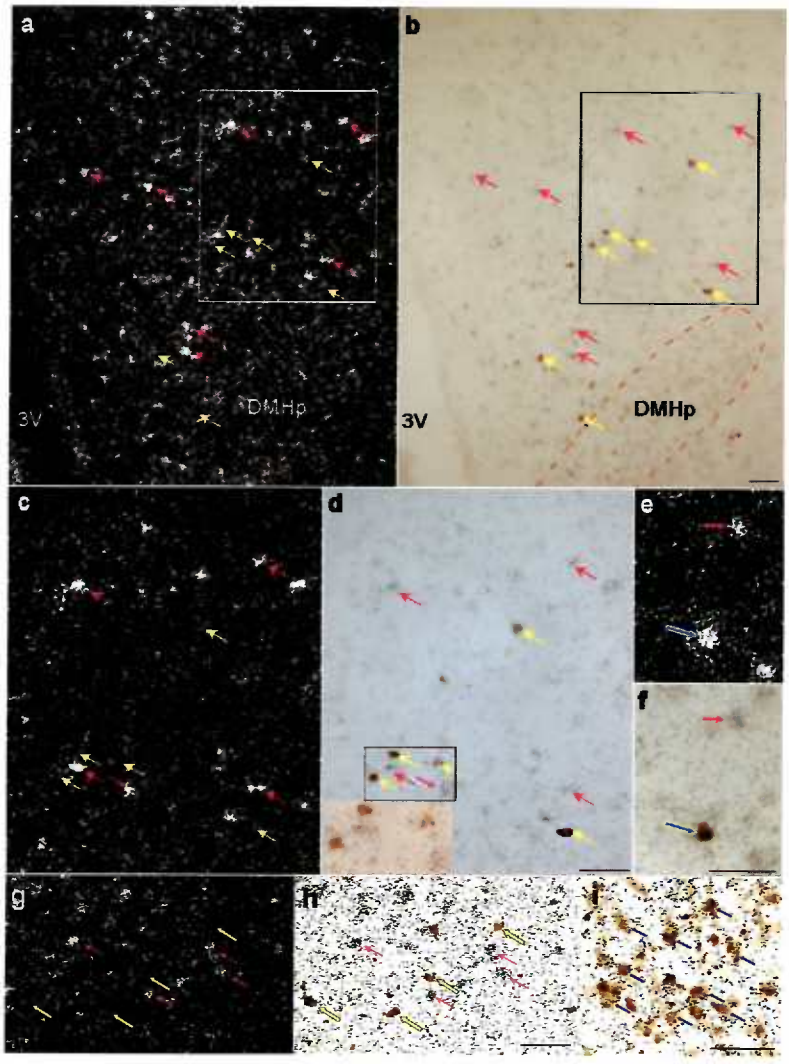


Fig. 4-3. Expression of MC4Rs, GAD^{67}, and NPY mRNAs in the rat brain. **a** and **b**: Low power darkfield (a) and brightfield (b) photomicrographs of DMH showing the distribution of MC4Rs (silver grains clusters, red arrows) and NPY neurons (brown cytoplasmic deposit, yellow arrows) around the compact zone of DMH (DMHp). No apparent colocalization between the two signals was observed in this area. **c** and **d**: High power photomicrographs of the boxed areas in a and b to further illustrate that no colocalization of MC4R and NPY was found in the DMH. The inset in d depicts the high magnification bright-field photomicrograph of the area delineated by the black rectangle in d. **e** and **f** show an example of colocalization of MC4R and NPY mRNAs (indicated by the blue arrows) in the cerebral cortex. **g** and **h**: Low power darkfield (g) and brightfield (h) photomicrographs of DMH showing GAD^{67} mRNA positive (silver grains clusters, red arrows) and NPY mRNA positive neurons (brown cytoplasmic deposit, yellow arrows). Note that little colocalization was observed between the two signals in the DMH. **i**: Low power brightfield photomicrograph of the ARH showing that the majority of NPY neurons (brown cytoplasmic deposit, indicated by the blue arrows) coexpressed GAD^{67} (clusters of black speckles, indicated by the blue arrows). 3V: third ventricle. *Scale bar* = 100 μm.

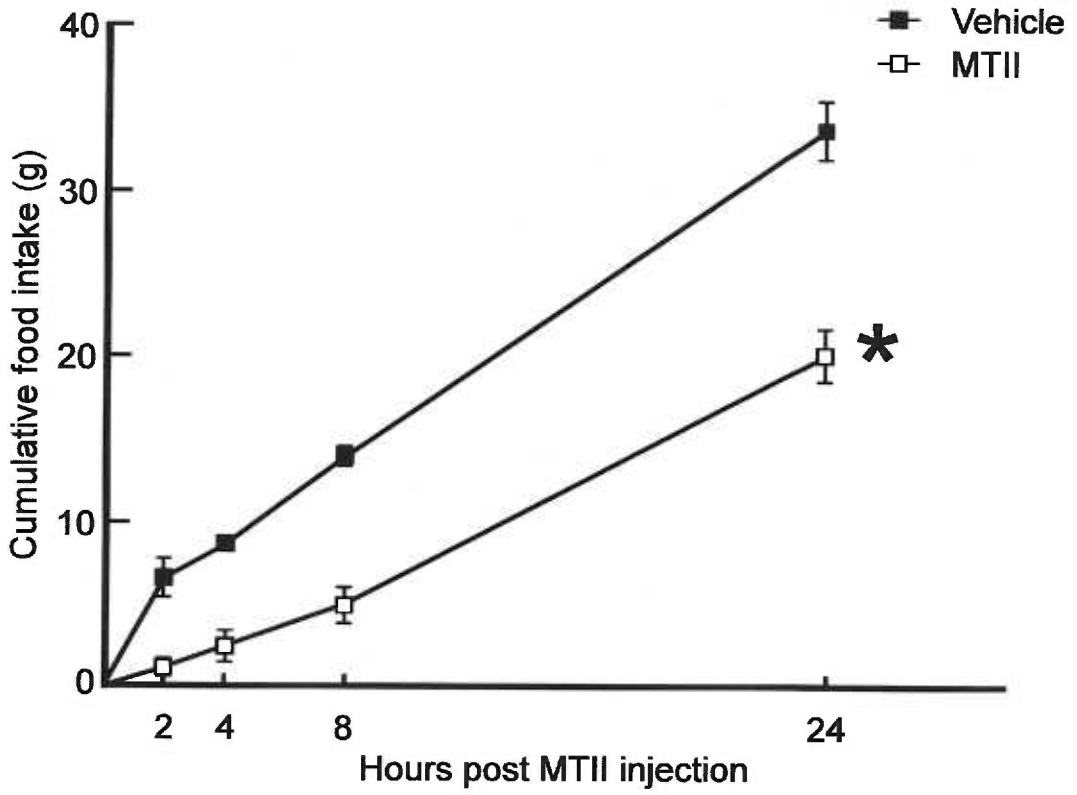


Fig. 4-2. Bilateral DMH administration of MTII suppresses feeding. 0.4 nmol MTII injected directly to the DMH of 24-h food-deprived ovariectomized rats produced a potent inhibition of cumulative food intake (g, mean±SEM, n=4 for each group) compared to vehicle control. * Significantly different from vehicle-treated rats at all time points, $p < 0.05$.

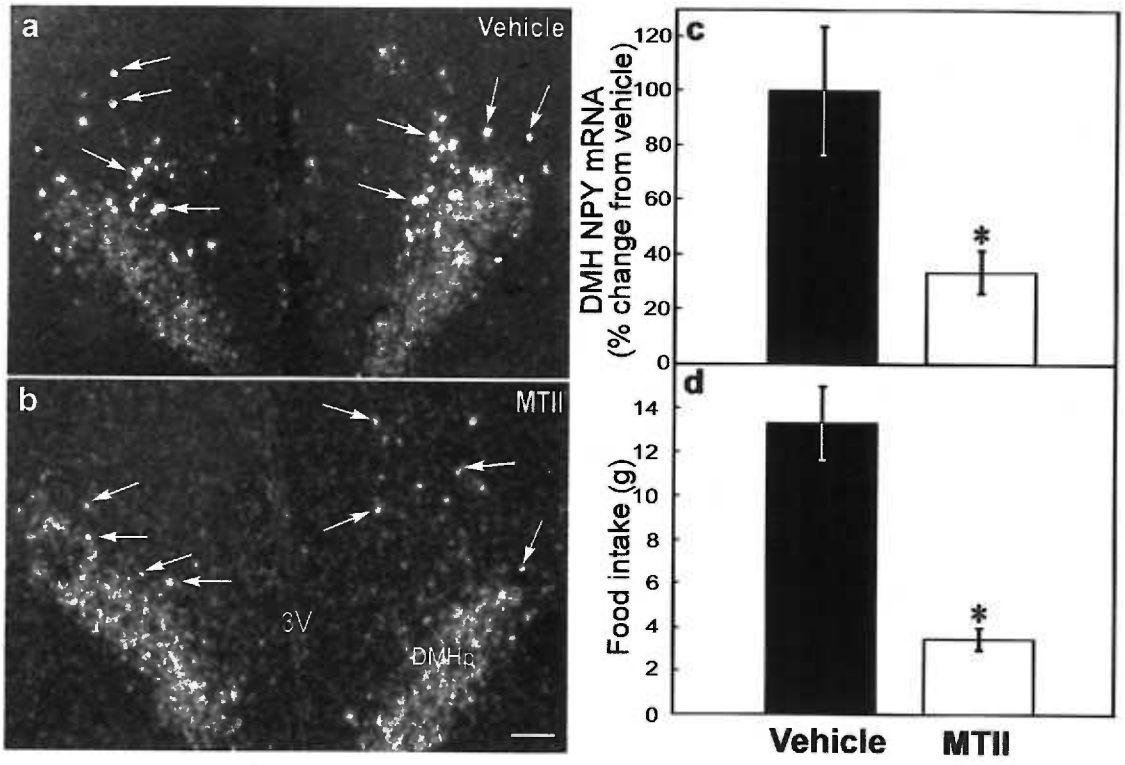


Fig. 4-5. Bilateral MTII administration into the DMH suppresses suckling-induced DMH NPY expression and hyperphagia in the lactating rats. **a**: Representative dark-field photomicrograph showing the expression of NPY mRNA in the DMH area from a lactating rat treated with vehicle. Suckling for 9 hr induced prominent NPY silver grain clusters (representative clusters indicated by the arrows) scattered in the DMH. **b**: DMH MTII injection resulted in a reduction in both number and size of the silver grains clusters (several examples indicated by the arrows). The low level of signal covering the DMHp was observed in all the animals examined. **c**: Quantitative analysis of DMH NPY expression levels in the vehicle and MTII treated groups. **d**: Summary of accumulative food intake of animals treated with vehicle or MTII. MTII-treated lactating rats ate significantly less than the vehicle-treated group. Data were expressed as mean±SEM. * Significantly different from vehicle-treated group. $p < 0.05$. 3V: third ventricle. *Scale bar* = 200 μm.

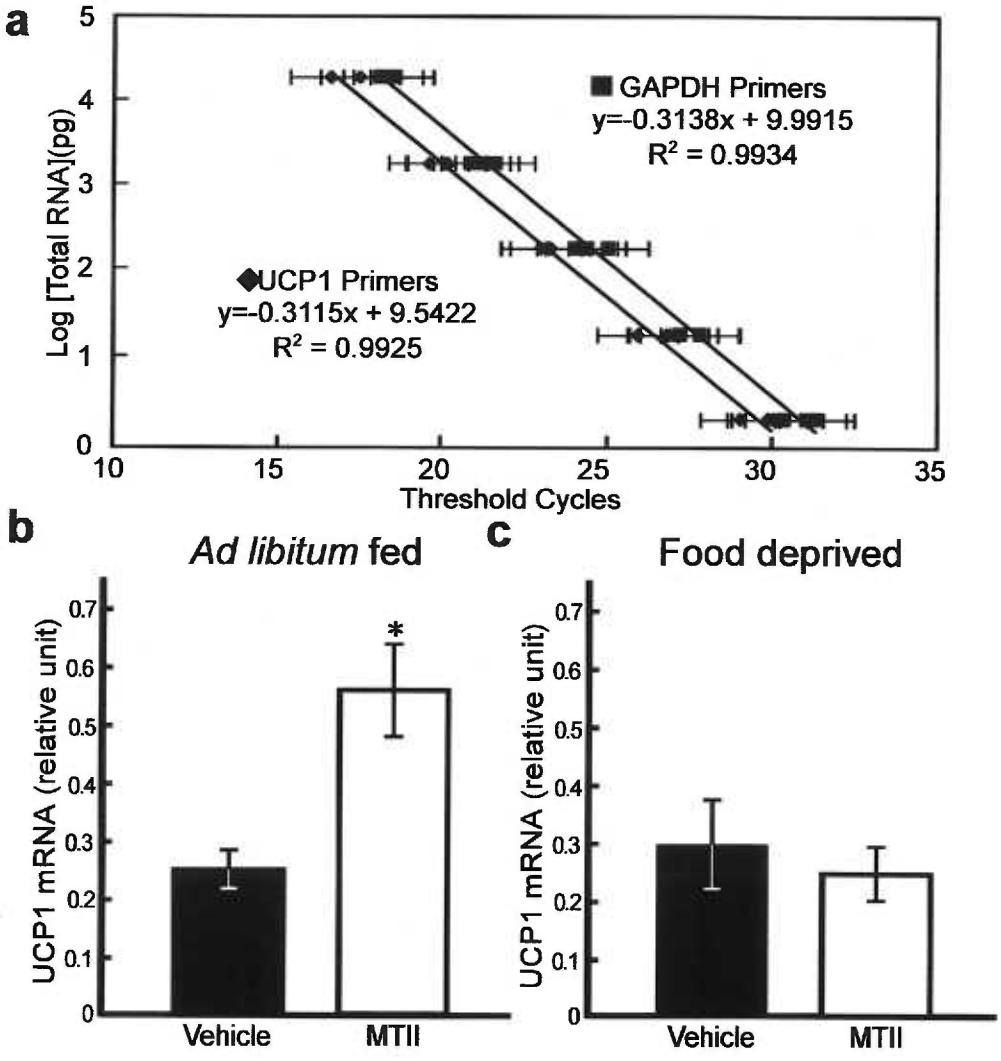


Fig. 4-6. UCP1 mRNA expression in the IBAT. **a**: The standard curves of UCP1 (◆) and GAPDH (■) for real time PCR. GAPDH was selected as a reference standard to normalize the RNA quality and quantity from IBAT across different groups. **b**: Bilateral DMH MTII administration significantly stimulated IBAT UCP1 mRNA expression in the *ad libitum* fed lactating rats. MTII injection caused approximately a 128% increase of UCP1 mRNA expression compared with the vehicle group. **c**: Direct DMH MTII injections to the food-deprived lactating rats did not alter UCP1 mRNA expression in the IBAT when compared to the vehicle-treated rats. *: Significantly different from vehicle group, $p < 0.05$.

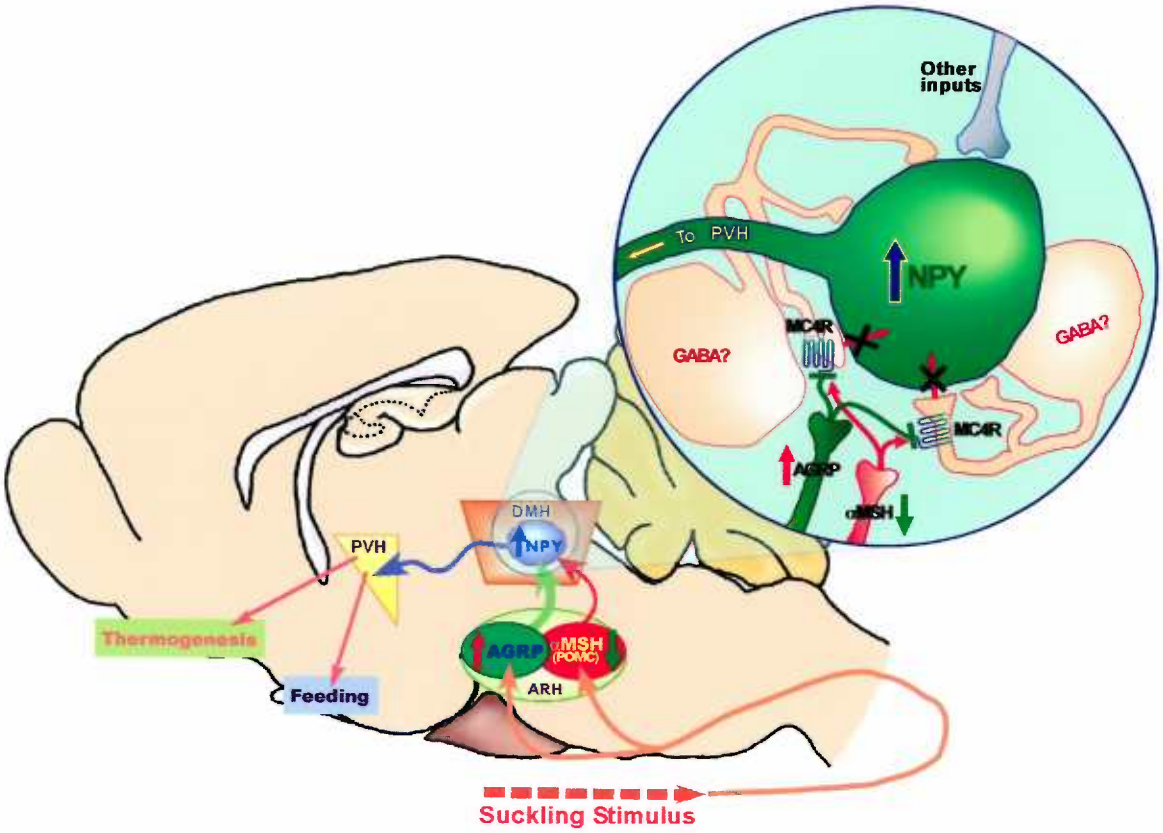


Fig. 4-7. Schematic diagram summarizes the proposed hypothesis for MC4R-mediated activation of DMH NPY neurons and the hyperphagic response during lactation. During lactation, ARH AGRP/NPY input into the DMH is elevated whereas ARH POMC tone into the DMH is reduced. Most of these inputs likely terminate on inhibitory interneurons expressing MC4R (i.e., GABA-ergic) in the DMH. The insert depicts MC4R signaling. The increased AGRP input in combination with reduced POMC input from the ARH causes a reduction in MC4R signaling leading to a decrease in GABA-ergic inhibition on the DMH NPY neurons, resulting in the activation of NPY mRNA expression during lactation. The activated NPY neurons in the DMH may be involved in a number of modulations during lactation, including hyperphagia and energy expenditure, probably via projection to the PVH.

CHAPTER V

SUMMARY AND CONCLUSIONS

Extensive studies have shown that hypothalamic neuropeptide systems are important central nervous system modulators of energy homeostasis. As obesity and obesity-associated pathological complications have becoming prevalent, it is essential to understand what the role of each system is and their interaction in the regulation of energy homeostasis. The expression of NPY, a powerful orexigenic peptide, in the DMH has been reported in several rodent models that are hyperphagic or obese, including lactation, diet-induced obesity and in mice that are deficient in central melanocortin signaling. These observations strongly suggest a potentially important function for NPY produced in the DMH in energy homeostasis. This thesis focused on understanding the regulation of expression of NPY in the DMH and the potential role of the DMH NPY in energy balance. The lactating rat was employed in these studies to examine possible signals involved in regulating NPY expression in the DMH. The possible mechanisms for the activation of DMH NPY neurons during lactation were: 1) Suckling-induced neural input that directly projects to the DMH to activate NPY neurons, 2) Suckling-induced hyperprolactinemia, and 3) Melanocortin signaling from the ARH. Finally, these studies also examined the potential role of NPY neurons in the DMH in energy homeostasis during lactation.

Our previous studies showed that DMH NPY neurons were rapidly activated by suckling-induced neural signals through somatosensory pathways (Li et al., 1998b), implying that during lactation, the DMH NPY neurons are stimulated even before peripheral signals are altered. Presumably, this rapid

activation should trigger a whole host of neural signals to induce a strong drive to increase food intake, allowing the female to adjust preemptively to prevent the anticipated significant energy drain due to milk production. This hypothalamic anticipatory phenomenon during lactation is similar to the metabolic "fight or flight" syndrome (Smith and Grove, 2002).

To begin to understand the mechanisms by which suckling-induced neural input may activate the DMH NPY neurons, the immediate early gene product, cFos, was used as a marker for neuronal activation to identify the neural populations in the brain that were activated specifically by the suckling stimulus. Then, retrograde tracing from the DMH area was combined with suckling-induced cFos expression to identify the afferent input to the DMH that was activated by the suckling stimulus. The pattern of cFos expression in all animals receiving a 90 min acute suckling stimulus was similar to that reported in our previous studies (Li et al., 1999b). In addition, afferent inputs to the DMH observed in the present study are in general agreement with previous retrograde tracer studies (Thompson and Swanson, 1998).

This study found that several areas, including mPOA, PePOA, LS, LPB, and VLM, were activated by physical suckling stimulus and sent direct projections to the DMH. Hence, these areas may play important roles in modulating the DMH NPY neuronal activity during lactation. In addition, several areas that were activated by pup exposure alone, including AH, BST, PP, PMv, and SuM, also send projections into the DMH, suggesting that the neural input activated by sensory stimuli associated with pup exposure may also be capable

of activating DMH NPY neurons to a certain extent.

Several suckling activated areas are particularly important concerning the modulation of energy status during lactation. The LPB has been shown to play an important role in relaying visceral information into higher brain centers and to be involved in a variety of physiological regulations, including control of body fluid homeostasis, energy metabolism and blood oxygenation. Several receptors, including μ-opioid, MC4, and Neuropeptide FF, are expressed in the LPB and are involved in modulating feeding (Kishi et al., 2003; Nicklous and Simansky, 2003; Wilson et al., 2003). However, not many neurosubstrates have been reported as being expressed in this area. The predominate one is cholecystokinin (CCK) (Fulwiler and Saper, 1984), and the CCK receptor type A (CCK_A) is found in the DMH (Carlberg et al., 1992; Mercer and Beart, 1997). The LPB-CCK input to the DMH might play a role in modulating energy balance and food intake during lactation, since other studies have shown that rats lacking CCK_A are hyperphagic and obese (Bi and Moran, 2002).

The main function of the VLM is to regulate oxytocin and vasopressin secretion from PVH and SON, the secretion of anterior pituitary hormones and regulation of autonomic function (Sawchenko and Swanson, 1981, 1982). Because DMH projects heavily into the PVH, the projection of VLM to the DMH established in the present study (Li et al., 1998a) suggests that DMH may be an intermediate upstream modulator to regulate the stress response and autonomic function by the VLM during lactation (Sawchenko and Swanson, 1981; Leibowitz et al., 1989). It has been shown that the majority of VLM

neurons are catecholaminergic and/or NPY-positive (Sawchenko and Swanson, 1981), and suckling-activated VLM afferents might modulate DMH neuronal activity via these neurochemicals. Future studies are needed to confirm this speculation.

A significant number of cFos/FG double-labeled cells were found in the LS. It has been shown that a large number of neurons in this area are GABA-ergic (Ferraguti et al., 1990; Okamura et al., 1990), which provide inhibitory inputs to the DMH. Interestingly, substantial numbers of GABA neurons are found in the DMH (Okamura et al., 1990). It is conceivable that the LS may provide a strong inhibitory tone on local inhibitory GABA neurons in the DMH during lactation, resulting in "disinhibition" and activation of NPY neurons. It has been suggested that LS, especially the ventral part of the structure, is involved in modulating ingestive behavior (Risold and Swanson, 1996), thus supporting a potential role of DMH NPY in feeding regulating during lactation.

In summary, this study provides anatomical evidence about the possible neuronal pathways for transmitting neural impulses from the suckling stimulus to the DMH during lactation. Whether neurons from these areas make direct contacts with the DMH NPY neurons needs to be further elucidated by anterograde tracing from each identified area. Characterization of the phenotype of the FG/cFos double-labeled neurons in the areas will further facilitate our understanding of the mechanisms by which the suckling stimulus activates the DMH NPY system during lactation.

In addition to neural input induced by suckling, this study also examined

the involvement of prolactin (PRL) in activating DMH NPY expression during lactation. We previously showed that blocking the suckling-induced high PRL levels with dopamine D2 receptor agonist, bromocriptine, significantly attenuated DMH NPY mRNA expression, suggesting that circulating high PRL levels might be involved in modulating DMH activity during lactation (Li et al., 1999c). In this study, ovine PRL (oPRL) was given back to the bromocriptine-treated lactating rats to examine whether PRL is required for the activating of NPY expression in the DMH.

In agreement with our previous studies, the suckling-induced NPY mRNA upregulation in the ARH was not altered by the bromocriptine treatment. In addition, the inability of oPRL replacement in modifying ARH NPY gene expression further substantiated the notion that ARH NPY neurons are not regulated by the PRL during lactation. Finally, while PRL-R is abundant in the ARH and has been shown to be expressed on tyrosine hydroxylase neurons (Arbogast and Voogt, 1997; Lerant and Freeman, 1998), almost no colocalization of PRL-R and NPY neurons was found in this region. These results are further corroborated by reports showing that PRL treatment or pituitary graft-induced hyperprolactinemia does not affect ARH NPY gene expression (Pelletier G, 1992; Garcia et al., 2003), and immunoneutralization of PRL does not reduce NPY expression during lactation (Pape and Tramu, 1996).

On the other hand, inhibition of elevated PRL by bromocriptine treatment resulted in significantly lower NPY expression levels in the DMH, and this reduction was apparent in the number of NPY neurons activated and the

intensity of mRNA levels of individual neuron. PRL replacement to the bromocriptine-treated animals significantly increased NPY expression levels in the DMH, albeit the replacement did not completely restore the expression to the levels of suckled animals. In addition, we showed that NPY mRNA silver grains signal per cell is not different between 8 pups + B + P and 8 pups + V groups. Taken together, these data suggested that exogenous PRL reversed the effect of bromocriptine on the NPY mRNA expression levels of individual neurons, but only partially restored its effects on the number of NPY neurons activated. The inability of oPRL to completely restore bromocriptine suppressed DMH NPY mRNA expression could be due to the fact that the ovine prolactin treatment may not completely mimic the physiological actions of endogenous rat PRL.

To provide possible mechanisms for which PRL modulates NPY activity in DMH, we used *in situ* hybridization to investigate whether PRL-R expresses in the DMH. We showed that in the lactating rats the majority of the DMH NPY neurons are also PRL-R-positive. These results suggest that PRL may act directly on its receptor to activate NPY gene expression in the DMH. In addition to the DMH, PRL-R mRNA is also observed in the medial preoptic area, the lateral septum, and periaqueductal gray areas. Earlier studies from our laboratory have shown that neurons in these areas send direct projections to the DMH and, more importantly, these neurons are activated in response to the suckling stimulus (Li et al., 1999b). The expression of PRL-R in these areas raises the possibility that PRL may also modulate DMH NPY neuronal activity

via indirect pathways by modulating neural populations upstream of the DMH. More studies are needed in order to resolve this issue.

Another possible signal for activation of DMH NPY neurons is the change in energy balance that is normally associated with milk production. In these studies in which the role of prolactin in the induction of NPY expression in the DMH was examined, bromocriptine treatment not only blocked PRL secretion but also suppressed milk production. Previous studies from our laboratory showed that similar treatment with bromocriptine prevented the suppression of leptin associated with the suckling stimulus (Brogan et al., 1999). Therefore, the status of energy balance in the bromocriptine-treated rat would be different compared to that of the suckled, vehicle-treated animals. Conversely, administration of exogenous PRL to replace the suppressed endogenous PRL caused by bromocriptine not only restored PRL levels, but also milk production, thus changing energy balance back to the high demanding state. However, it has been shown that DMH NPY neurons are activated rapidly after the onset of the suckling [i.e., 90 min (unpublished observation) to 3 h (Li et al., 1998b)]; therefore, the early induction of DMH NPY occurs before any significant milk production and change in energy balance has taken place. It is possible that the altered energy status in lactating animals may be involved in maintaining the expression of NPY in the DMH, even if not in the induction of expression. Future experiments in which both PRL levels and milk production of the dams are independently controlled will further elucidate the direct involvement of PRL versus signals associated with energy balance in modulating DMH NPY activity.

Lastly, the importance of the melanocortin system on DMH NPY expression and on energy homeostasis during lactation was investigated. The anatomical studies established a close relationship between the melanocortin system, including α-MSH, AGRP and MC4R, and NPY neurons in the DMH. The close apposition of α-MSH or NPY/AGRP fibers with NPY neurons in the DMH provides a neuroanatomical framework for melanocortin peptides to affect NPY expression. We also showed there are abundant MC4Rs expressed in close proximity with NPY neurons in the DMH. However, no NPY/MC4R double-labeled cells were found in the area. This result, in combination with the anatomical data, suggests that MC4R may be expressed on a separate population of neurons within the DMH, which in turn makes local synapses onto DMH NPY neurons. These presynaptic terminals expressing MC4R may be the actual targets of the α-MSH and AGRP projections. During lactation, the decreased melanocortin system signaling could result in reduced inhibitory input to the NPY neurons, leading to activation of NPY expression. GABA is the prime candidate for the inhibitory input to the DMH NPY neurons, since we also showed that there are abundant GABA neurons in the DMH. Like MC4R neurons, the GABA neurons are not coexpressed with NPY but are in the vicinity of the NPY neurons in the DMH. A demonstration of colocalization of GABA and MC4R in the DMH would provide additional support for this hypothesis.

A pharmacological approach was then taken to further substantiate the physiological significance of the anatomical link between the ARH melanocortin

system and DMH NPY neurons. MTII, a MC3R/MC4R receptor agonist, was injected directly into the DMH of both nonlactating and lactating rats. This study first showed that bilateral injections of MTII into the DMH of ovariectomized female rats significantly attenuated the 24 h fasting–induced hyperphagia. This result argues that the DMH may be a part of the neural circuitry of melanocortin signaling in regulating feeding. One possible downstream target of the DMH is the PVH, as the DMH projects heavily to the parvicellular part of the PVH, a final common pathway through which the brain controls feeding (Swanson and Sawchenko, 1983). Thus, in addition to an extensive melanocortin input into the PVH from the ARH, the current findings suggest yet an additional pathway for ARH POMC peptides to regulate feeding, by way of the MC4R in the DMH. This indirect pathway may allow the POMC system to fine-tune its final output to PVH to regulate appetite.

This study also showed that activation of MC4R in the DMH of lactating rats greatly diminished (77%) the induced increase in food intake in response to 9 h suckling. The drug-induced decrease in food intake was coupled with a greatly attenuated expression of suckling-induced DMH NPY mRNA. The similar reduction of DMH NPY expression by the MTII treatment whether the animals were fed or not fed during the 9 h period of acute suckling suggests that the reduction was a direct result of the DMH MTII injections and not secondary to the inhibitory effect of the MTII treatment on feeding. In addition to the suppression of feeding, DMH MTII injections also prevented the suckling-induced reduction of IBAT UCP1 mRNA in the *ad libitum* fed resuckled rats.

This result demonstrates for the first time that MC4R signaling in the DMH is involved in regulating sympathetic outflow to the BAT. However, the same MTII treatment to the food deprived lactating group did not prevent the reduction in UCP1 mRNA expression in the BAT, even though NPY mRNA in the DMH was decreased. This data suggests that the combination of energy expenditure (milk production) and no energy intake (fasting) may activate protective energy adaptations to prevent further energy loss, and thus override the effect of DMH MTII treatment in activating UCP1 activity in the BAT.

Although the results from this thesis study implicate these suckling-activated DMH NPY neurons as an important player in driving hyperphagia and decreasing peripheral energy expenditure, an important issue that remains to be resolved is the phenotypic characteristics of these neurons. What neurosubstrates characterize these neurons during the vast majority of time when they are not expressing NPY? Identification of additional neurosubstrates expressed by these neurons would greatly extend our understanding of the role this group of neurons plays in physiological states other than lactation, the downstream targets and the functional consequence after activating this system. This information will be potentially important for developing drug treatments for feeding-related disorders, such as obesity, anorexia, and cachexia. Conventional techniques available for resolving this issue include double-label ISH to screen all the known neurosubstrates that have been reported to be expressed in the DMH, one by one to see if any of them coexpress with NPY. Using this approach, we have eliminated a few, GABA and

galanin, from a large pool of candidates. However, this approach has proven inefficient and time consuming. More efficient approaches are pressingly in need for this endeavor.

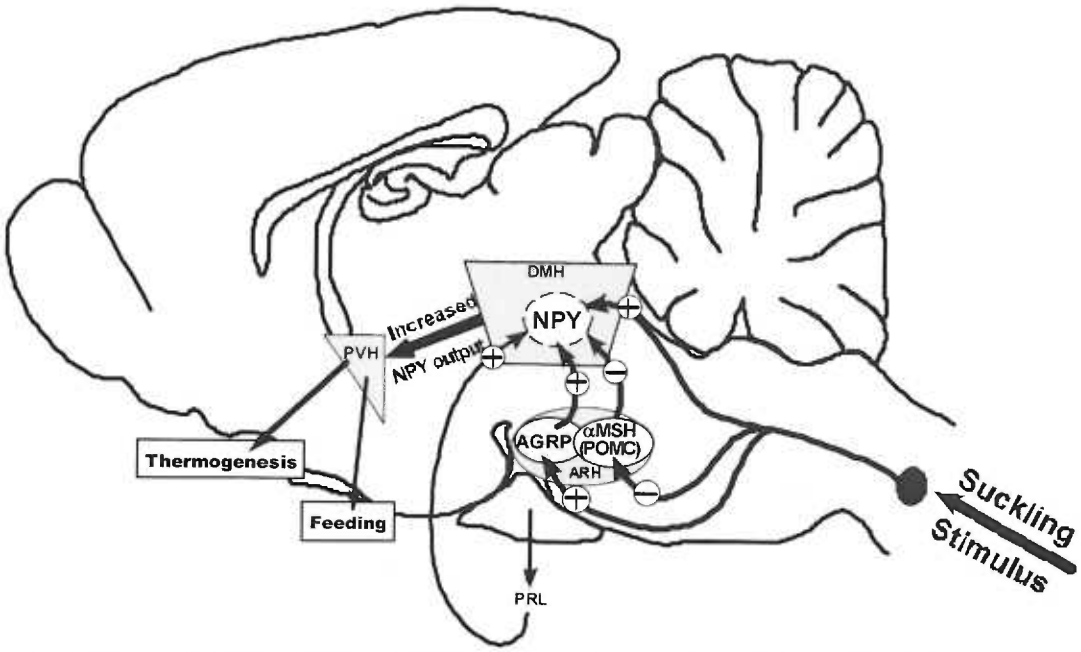
Recently, a high throughput gene profiling tool, DNA microarray technology, has become increasingly popular in various fields of biomedical research. The expression profile of thousands of genes can be examined simultaneously in a given sample using this technology, which makes DNA microarray an excellent tool for achieving our goal of finding other neurochemicals produced in the DMH NPY neurons. Therefore, the key issue is to get anatomically correct and high quality RNA from tissue samples. In our case, the ultimate goal would be to dissect out each individual NPY neuron and extract RNA from these cells. This goal could potentially be achieved by using laser capture microdissection (LCM).

LCM is a microscope equipped with laser beams to dissect out tissue at a single cell resolution. Several cancer pathology studies have demonstrated that LCM is capable of dissecting out tissue- or cell-specific high quality RNAs when the cellular clusters are easily discernable by their morphology under microscope. In our case, the suckling-activated NPY neurons need first to be visualized by IHC. Normally, colchicine is employed to aid in the visualization of NPY-ir cell bodies. However, it is impossible to treat lactating rats with colchicine because the severe side effects from the treatment cause the rats to stop nursing their pups, which would prevent suckling-induced NPY in the DMH. Furthermore, less success has been reported in using LCM to dissect cells that

have to be labeled with IHC, since the quantity and quality of RNAs decrease with each procedure of IHC. I have tried several strategies to circumvent these issues in attempt to label DMH NPY neurons in lactating rats. First, a cocktail of various polyclonal and monoclonal NPY antibodies was combined with signal amplification techniques to facilitate the detection of NPY in cell bodies in the lactating rats without colchicine treatment. This method, however, failed to detect any NPY cytoplasmic staining. Secondly, I used a line of NPY knockout mice (Erickson et al., 1996), in which the gene encoding NPY was replaced with lacZ gene, such that the NPY neurons express lacZ instead of NPY. The lacZ can be visualized by a quick reaction with its substrate, which might significantly reduce the risk of losing quality and quantity of the RNAs. However, due to the low lacZ promoter activity, only a few lacZ–positive cells were visible in the ARH, while no lacZ-positive cells were found in the DMH of the lactating NPY KO mice.

Another line of transgenic mice expressing sapphire green fluorescent protein (SFP) under the control of NPY regulatory elements (NPY-SFP mice) has been generated by Dr. Friedman's laboratory at the Rockefeller University (Cowley et al., 2003). The lactating NPY-SFP mice would be a great model for LCM, since SFP is visible under fluorescent microscopy. In addition, MC4R null mice can be used as an alternative model to detect and collect NPY neurons in the DMH. Injection of colchicine to MC4R null mice to prevent transportation of NPY out of the cell body would aid in the visualization of NPY neurons in the DMH.

In conclusion, this thesis identified suckling activated neural afferent into the DMH. This information will facilitate the understanding of DMH NPY expression induced by suckling. I also demonstrated that hyperprolactinemia during lactation is an important factor involved in modulating NPY expression in the DMH. The expression of PRL-R on DMH NPY neurons suggests that prolactin may exert its effect directly on the NPY neurons in the DMH. Finally I provided a neuroanatomical framework for the melanocortin signaling system in the ARH to affect NPY neuronal activity in the DMH. The melanocortin system in the DMH not only plays an important role in modulating NPY expression in the DMH of lactating rats but also in regulating energy homeostasis, at least in part by modulating appetite and energy expenditure. The ultimate goal for our laboratory in this project is to determine the phenotypic characteristics of the DMH NPY neurons. To solve this problem, using LCM in combination with global gene profiling by DNA microarray technology will potentially be the most efficient approach. Although there are several technical obstacles that need to



be resolved, the NPY-SFP mouse model could be a key for the understanding of the function and regulation of the suckling-activated NPY neurons in the DMH (Chen and Smith, 2004).

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