LACTATION AS A MODEL FOR STUDYING THE NEUROPEPTIDE Y SYSTEM IN THE HYPOTHALAMUS: IMPLICATIONS IN THE REGULATION OF REPRODUCTION AND FEEDING

by

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LIST OF ABBREVIATIONS

3V	Third ventricle
4V	Fourth ventricle
ACTH	Adrenocorticotropin hormone
AGRP	Agouti related protein
AH	Anterior hypothalamus
Aq	Aqueduct
ARH	Arcuate nucleus of hypothalamus
AVPV	Anteroventral periventricular nucleus
BST	Bed nucleus of stria terminalis
Cpu	Caudate putamen
CRF	Corticotropin releasing hormone
csc	Commissure of the superior colliculus
Cy5	Indodicarbocyanine
DC	Dorsal cochlear nucleus
DEPC	Diethyl pyrocarbonate
dlf	Longitudinal fasciculus area
DLL	Dorsal nucleus of lateral lemniscus
DMH	Dorsomedial nucleus of hypothalamus
DMHp	Dorsomedial nucleus, compact zone
FG	Fluorogold
FITC	Fluorescein isothiocyanate

GABA	γ- Aminobutyric acid
GnRH	Gonadotropin releasing hormone
LC	Locus coeruleus
LG	Lateral geniculate area
LH	Lateral hypothalamus
LH	Luteinizing hormone
LPB	Lateral parabrachial nucleus
LSD	Lateral septum, dorsal part
LSV	Lateral septum, ventral part
MC4-R	Melanocortin receptor type 4
ME	Median eminence
MeA	Medial amygdala
MGN	Medial geniculate nucleus
MnR	Median raphe nucleus
Mo5	Motor trigeminal nucleus
MPB	Medial parabrachial nucleus
mPOA	Medial preoptic area
MSH	Melanocyte stimulating hormone
NPY	neuropeptide Y
NTS	Nucleus of solitary tract
opt	Optic tract
OT	Oxytocin

OVLT	Organum vasculosum of the lamina terminals
ox	Optic chiasm
PAG	Periaqueductal gray
PePOA	Periventricular preoptic area
PH	Posterior hypothalamus
PHA-L	Phaseolus vulgaris leucoagglutinin
PIF	Prolactin inhibiting factor
Pir	Piriform cortex
PKA	Protein kinase A
PKC	Protein kinase C
PL	Paraleminiscal nucleus
PMV	Ventral premammillary body
Pn	Pontine nucleus
POMC	Proopiomelanocortin
PP	Peripeduncular area
PRL	Prolactin
PT	Pretectal nuclei
PVH	Paraventricular nucleus of hypothalamus
PVHm	Paraventricular nucleus, magnocellular part
PVHp	Paraventricular nucleus, parvocelluar part
PVT	Anterior paraventricular nucleus of thalamus
rs	Rubrospinal tract

SCN	Suprachiasmatic nucleus
scp	Superior cellebellar peduncle
SNR	Substantia nigra, reticular part
SON	Supraoptic nucleus
Sp5C	Spinal trigeminal nucleus
SUM	Supramammillary nucleus
TH	Tyrosine hydroxylase
TIDA	Tuberoinfundibular dopamine
TRITC	Tetramethyl rhodamine isothiocyanate
VLM	Ventrolateral medulla
VMH	Ventromedial nucleus of hypothalamus

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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, six chapters of original data and a general conclusion. References are listed separately in alphabetical order, and follow the format of Endocrinology.

Chapter II contains data, figures and text as they appear in original paper that have been published previously (Li et al., 1998a). Chapter III and IV contain data, figures and text as they would appear in an original papers that are currently in press (Endocrinology, 1998 and Regulatory Peptides, 1998). Chapter V to VII represents three manuscripts that have been prepared for publication.

ABSTRACT

To better understand the regulation and possible functions of the NPY system in the hypothalamus, this thesis used lactation as a natural physiological state to approach these issues. The first part of the thesis characterized the possible factors that are important in regulating the activity of NPY neurons in the hypothalamus during lactation. It was found that the suckling stimulus is a key factor in activating two distinct populations of NPY neurons in the hypothalamus: the caudal portion of the arcuate nucleus (ARH) and the dorsomedial nucleus (DMH). However, the mechanism by which the suckling stimulus activates the two NPY populations may be different. The activation of ARH NPY neurons is dependent on the neural input activated by suckling, whereas suckling-induced hyperprolactinemia is more important in the activation of the DMH NPY neurons.

The studies described in this thesis used both retrograde and anterograde tracing techniques to identify the downstream target systems for both suckling-activated NPY populations in the hypothalamus. Retrograde tracing combined with *in situ* hybridization demonstrated that the paraventricular nucleus of the hypothalamus (PVH) is a target area for both NPY populations, suggesting that during lactation, NPY input will have a great impact on the activity of PVH, which may be important in mediating the food intake and/or reproduction during lactation. Anterograde tracing combined with multiple immunostaining was used to identify all of the possible target areas in the brain of the ARH NPY neurons. It was found that ARH NPY neurons send projections

into several forebrain regions, including the lateral septum, bed nucleus of stria terminalis and several hypothalamic nuclei. However, ARH NPY neurons do not project heavily into the hindbrain regions. It was also found that the ARH NPY neurons connect directly with both gonadotropin releasing hormone (GnRH) and corticotropin releasing factor (CRF) systems in the hypothalamus, suggesting that ARH NPY neurons may modulate physiological systems such as food intake, stress response and reproduction in part by directly modulating the activity of the two primary regulatory systems in the hypothalamus during lactation.

In order to further understand the neural pathways that may be transmitting the signals of the suckling stimulus to stimulate the ARH NPY neurons, the immediate early gene protein, cFos, was used as a neuronal marker to identify neural populations that were activated by the physical suckling stimulus. Subsequently, suckling-induced cFos expression combined with retrograde tracing was used to determine the neuronal afferent input into the ARH that was specifically activated by the suckling stimulus. Identification of these neural pathways should facilitate an understanding of how somatosensory information may regulate hypothalamic NPY neuronal activity.

The results from this thesis should facilitate our understanding of the regulation of NPY system in the hypothalamus. The anatomical data provide a framework for how the hypothalamic NPY system may be regulated by incoming signals, both neuronal and hormonal. In addition, the identification of

multiple target areas of ARH NPY neurons should allow us to begin to study the function of ARH NPY neurons more precisely and accurately.

CHAPTER I

INTRODUCTION

A. OVERVIEW--THE HYPOTHALAMUS AS A CENTER FOR INTEGRATING NEURAL, HORMONAL, AND METABOLIC SIGNALS THAT ALTER NEUROENDOCRINE FUNCTION

In the central nervous system, the hypothalamus has been considered one of the most important structures in maintaining homeostasis of an amimal's body. The hypothalamus is located in the base of the dienchphalon on both sides of the third ventricle and immediately above the pituitary gland. Histological staining reveals many clusters of neurons throughout the hypothalamic area. Each of the clusters, also known as nuclei, represents a basic functional unit of the hypothalamus. In addition to the well established close relationship between the hypothalamus and the pituitary gland, anatomical studies have shown that many brain areas provide extensive neural input into the hypothalamus, and the hypothalamic efferent nerve fibers also connect to major sites of the brain, including the cerebral cortex, thalamus and limbic system, and the spinal cord (Simerly, 1995). 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). 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 neurotranmission from other, more distant neurons not having direct contact. Thus, the hypothalamus is the logical

place for integrating information derived from both the external and internal environment; it responds to the condition appropriately by modulating various systems of the body. It has been shown that the hypothalamus can regulate the cardiovascular system, thermoregulatory responses, the immune system, the digestive system and even behavior, including aggressive, feeding, maternal and sexual behaviors (Brown, 1994).

Even with the great interest in how the hypothalamus may integrate multiple signals and modulate so many different systems, little is known about the detailed mechanisms by which the hypothalamus may accomplish these complicated tasks. One of the difficulties in studying hypothalamic function is that there are so many substances produced in 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). Each neurochemical system contributes to some of the hypothalamic functions in different degrees. The array of the neurochemical substances in the hypothalamus presents a complicated system to study at several different levels: (1) with few exceptions, most of the neuroactive substances are expressed in more than one particular nucleus in the hypothalamus, and the same substance may participate in different functions in different nuclei; (2) the expression of the substances in different areas of the hypothalamus is sometimes regulated differently; and (3) some neurochemical substances are coexpressed in neurons in one area but not other areas of the hypothalamus.

Thus, in order to understand how the hypothalamus regulates homeostasis of the body, it is necessary to first dissect out the function of each neurochemical system in the hypothalamus in detail, both pharmacologically and anatomically, and to study how the system may function as a sensor to recognize and integrate incoming signals within the hypothalamus. This information may then allow us to study how different systems may interact with each other and how the interaction may contribute to the integration of information in the hypothalamus and the regulation of different systems of the body under different physiological conditions.

B. HYPOTHALAMIC NEUROPEPTIDE Y (NPY): A MODEL SYSTEM TO STUDY HOW MULTIPLE SIGNALS CAN ALTER NEUROENDOCRINE FUNCTION

Neuropeptide Y (NPY) is one of the peptide systems in the hypothalamus which has been extensively studied due to its wide involvement in regulation of many physiological systems. It has been shown that hypothalamic NPY participates in regulating the reproductive axis, energy homeostasis, hormone secretion from the pituitary gland, insulin secretion, and cardiovascular function. Thus, hypothalamic NPY may be a good model system to study how the hypothalamus may convey multiple signals and modulate several physiological systems simultaneously.

1. NPY and its receptors

a. NPY peptide family

Neuropeptide Y (NPY) is a 36 amino acid peptide discovered by Tatemoto and colleagues while searching for C-terminally amidated peptides in extracts of porcine brain (Tatemoto et al., 1982). Subsequently, NPY was also isolated from other species including human (Corder et al., 1984), rat (Corder et al., 1988), guinea-pig, rabbit (O'Hare et al., 1988), and sheep (Sillard et al., 1989), suggesting that the NPY structure has been well conserved during evolution. Amino acid sequence analysis has revealed that NPY belongs to a peptide family including peptide YY (PYY, approximately 70% homology) and pancreatic polypeptide (PP, approximately 50% homology). These peptides exhibit considerable homologies in their primary, secondary and tertiary structures (Glover et al., 1985; Schwartz et al., 1991). The NPY peptide is encoded in a 7.2 kilobase gene with four exons (Larhammer et al., 1987). NPY is first synthesized as a preproNPY peptide with 98 amino acid residues (Minth et al., 1984, 1986). A signal peptide of 29 amino acid precedes the mature NPY peptide, which is followed by a Gly-Lys-Arg processing site. The cleavage at the processing site results in the mature peptide and a carboxyl-terminal peptide of 30 amino acids.

X-ray crystallographic analysis of one of the peptide family, avian PP, revealed that the peptide structure consists of an extended proline helix with three prolines, a turn, and an amphipathic alpha helix (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). In addition to this characteristic

structure, all the peptides in the family have a C-terminal amide that is essential for biological activity.

b. NPY receptors

The receptors for NPY and its family members belong to the seven transmembrane domain-G protein coupled receptor family. All the receptors identified to date are capable of coupling to G_i to mediate an inhibition of adenylate cyclase, though other second messenger systems can be modulated, depending on the cell line in which the receptor is expressed. Currently six receptor subtypes have been identified. Each receptor exhibits slightly different rank order potency in binding to NPY analogues and related peptides. Each of the receptors is discussed briefly below.

Y1 receptor. The Y1 receptor binds with high affinity only full length NPY or full length analogues, such as [Pro34]NPY, and has a much reduced affinity for C-terminal fragments such as NPY (13-36) (Schwartz et al., 1990). Both *in vivo* and *in virto* studies have shown that this receptor can couple either to the hydrolysis of phosphotidylinositol or the inihibition of adenylate cyclase (Aakerlund et al., 1990; Herzog et al., 1992; Hinson et al., 1988). The distribution of the Y1 receptor in the rat brain has been mapped with ^{125}I-PYY as the radioligand, using NPY13-36 to mask Y2-like receptors (Gehlert et al., 1992), or by direct labeling with selective radioligands (Dumont et al., 1996). The Y1 receptor predominates in the cerebral cortex, thalamus, and amygdala and several nuclei in the hypothalamus including the arcuate nucleus. Results from Y1 receptor mRNA mapping studies also show a similar distribution (Larsen et

al., 1994). Y1 receptor is also found in the cerebral vessels surrounding the brain (Bao et al., 1997). In addition, the Y1 receptor has been found in the periphery, including many arteries and veins (Palea et al., 1995; Wharton and Polak, 1990), where it is associated with vasoconstriction or the potentiation of effects of other vasoconstrictors (Malmström and Lundberg, 1997). In the central nervous system, the effects of NPY mediated by the Y1 receptor include anxiolysis-sedation (Heilig et al., 1993; Wahlestedt et al., 1993) and the feeding response (Kalra et al., 1991; Stanley et al., 1992), although its involvement in feeding has been challenged by the study which showed that decreasing the expression of Y1 receptor by antisense oligonucleotide treatment did not alter feeding (Heilig et al., 1993). In addition, the deletion of either one or two of the N-terminal amino acids does not result in a loss of potency to stimulate feeding, despite poor affinities for the Y1 receptor (O'Shea, endo). Recently, it has been shown that the food intake in Y1 receptor deficient mice was modestly reduced, suggesting that the food intake effect of NPY is mediated in part by the Y1 receptor (Pedrazzini et al., 1998). The Y1 receptor is generally considered to be postsynaptic (Wahlestedt et al., 1986), although several studies have also suggested that it can be found in presynaptic sites and functions as a presynaptic receptor (Chen and van den Pol, 1996; Pickel et al., 1998).

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). Y2 receptors are found in a variety of brain regions, including the hippocampus, substantia nigra,

thalamus, hypothalamus, and brainstem (Dumont et al., 1993; Gehlert et al., 1992; Gustafson et al., 1997). In the periphery, Y2 receptors are found in peripheral nervous systems such as sympathetic, parasympathetic and sensory neurons, as well as the vasculature (Dumont et al., 1993; Gehlert et al., 1992; Grundemar et al., 1990; Stjernquist and Owman 1990; Wahletedt and Håkanson 1986; Wahletedt et al., 1986). 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, it has been shown that Y2 receptors can modulate Ca^{2+} currents by selective inhibition of N-type calcium channels (Toth et al., 1993). Many Y2 receptor-mediated effects have been linked to the suppression of transmitter release, and this is consistent with the observation that the Y2-like receptor is predominately located presynpatically (Wahlestedt et al., 1986).

Y3 receptor. A group of receptors with disparate pharmacological profiles have been labeled Y3 receptors. These receptors all share a common low affinity for PYY and display a rank order potency for NPY-related peptides that differs markedly from Y1 or Y2 receptors (Grundemar et al., 1991a,b). However, it has been shown that the Y3 receptor can, like both Y1 and Y2 receptors, inhibit adenylate cyclase activity (Balasubramaniam et al., 1990) and modulate Ca^{2+} influx (Foucart et al., 1993; Wahlestedt et al., 1992). No study has been done to map the distribution of Y3 receptors in the nervous system. Electrophysiological studies suggest that Y3 receptors are expressed in the brainstem, especially in the nucleus of solitary tract (Glaum et al, 1997). Central effects associated with the Y3 receptor include hypotension and bradycardia (Grundemar et al.,

1991a,b). In the periphery, the Y3 receptor has been found in the adrenal medulla (Wehlestedt et al., 1992) and has been suggested to play a role in nicotine-stimulated release of catecholamines (Higuchi et al., 1988a).

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). Like the Y1 receptor, both the C- and N-termini are required for full potency, suggesting that the binding domain is similar in these two subtypes. The Y4 receptor is also coupled to inhibition of cAMP accumulation (Bard et al., 1995; Lundell et al., 1995) and to modulation of Ca^{2+} current influx (Bard et al., 1995). The Y4 receptor is found mostly in the periphery, including the colon, small intestine, pancreas and testis. In the brain the Y4 receptor is found mostly in the circumventricular organs such as the area postrema and the interpedunclular nucleus (Bard et al, 1995; Whitcomb et al., 1990). The main function of the Y4 receptor may be mediating the effects of PP in the inhibition of pancretic exocrine secretion and gall baldder contraction (Schwartz 1983). The central effect of the Y4 receptor is currently unknown.

Y5 receptor. The Y5 receptor has highest affinity for rat NPY and rat PYY with lower affinity for rat PP (Gerald et al., 1996). [Pro34]NPY and C-terminal analogs also exhibit high affinity for this receptor subtype (Gerald et al., 1996). Perhaps the most selective peptide for the Y5 receptor is [dTrp32]NPY, which has low affinity for the other receptor subtypes (Gerald et al., 1996). Interestingly, genomic sequence analysis showed that the reverse complement of part of the human Y5 receptor genomic DNA sequence corresponds to the genomic DNA

sequence for the human Y1 receptor, suggesting that the Y1 and Y5 receptor gene could map, in opposite orientation, to the same locus on chromsome 4q (Gerald et al., 1996). 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 mainly in the nucleus tractus solitarius, area posterma and lateral septum (Dumont et al., 1998). Functionally, the receptor has been suggested as one possible candidate in mediating central NPY induced feeding (Gerald et al., 1996; Schaffhauser et al., 1997).

Y6 receptor. The cDNA for the Y6 receptor was first cloned from mice (Weinberg et al., 1996)). Pharmacological studies showed that it has high affinity for PP and C-terminal fragments (Weinberg et al., 1996). Interestingly, when a similar cDNA was cloned from humans and primates, it was found that the human and primate sequences contain a deletion mutation that results in a premature peptide which does not contain the 7th transmembrane domain (Gregor et al., 1996). These results suggest that the Y6 receptor may not be functional in the human and primate.

2. NPY distribution in the hypothalamus

a. NPY neurons in the hypothalamus

Immunohistochemical studies using antisera against NPY have shown that the mediobasal hypothalamus contains the largest group of NPY-positive neurons (Chronwall et al., 1985; De Quidt et al., 1990). However, NPY staining

of many of these cells could be demonstrated only in colchicine-treated animals. This population of neurons comprised small to medium-sized fusiform cells which occasionally gave rise to thick dendrites at either or both poles and were distributed rostrally from the ventral retrochiasmatic area to the subventricular region of the caudal end of the arcuate nucleus. Some scattered cells were also found in the lateral median eminence. In addition, other groups of NPY-positive neurons were observed in animals treated with colchicine, including those in the supramammillary nucleus, the ventral part of the medial preoptic area, the periventricular nucleus, and within the parvocellular part of the paraventricular nucleus. Also, clusters of large, multipolar cells were observed in the lateral hypothalamic area even without colchicince treatment. NPY mRNA mapping studies by *in situ* hybridization revealed a similar distribution of NPY mRNA positive neurons in the hypothalamus (Gehlert et al., 1987; unpublished observation).

b. NPY fiber distribution in the hypothalamus

A complex network of NPY-positive fibers has been observed throughout the hypothalamus (De Quidt et al., 1990). In the rostral portion of the hypothalamus, a dense plexus of immunostained fibers has been found in the organosum vasculosum lamina terminalis (OVLT) and the continuing anteroventral periventricular nucleus (AVPV). 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 (PePOA) is located.

In the mid-hypothalamic area, a dense plexus of fibers has been observed in the ventral part of the suprachiasmatic nucleus. The paraventricular nucleus (PVH) contains a non-uniform fiber innervation, with the highest density in all of the parvocellular subdivisions and a weak to moderate innervation in the magnocellular portion of the PVH. Scattered fibers and varicosity-like staining, which may represent terminal buttons, have been found throughout the anterior hypothalamic area. The dorsomedial nucleus (DMH) contains dense to moderate levels of 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. NPY-positive fibers have also been detected in the ventromedial nucleus (VMH) but with much less density compared to the neighboring nuclei, with the exception of the dorsomedial portion of the VMH, which has a relatively high density of fibers.

The arcuate nucleus (ARH) contains the densest fiber plexus in the hypothalamus. The fibers are found throughout the ARH, with more fibers in the cadual 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 also found in the external layer of the ME.

The posterior hypothalamus (PH) receives only moderate innervation of NPY fibers, with the exception of the rostral end of the PH 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 which receives moderate levels of NPY innervation.

One of the difficulties in studying the function of NPY in the hypothalamus is that there is more than one population of NPY neurons in the brain that project into the hypothalamus. It has been shown that NPY neurons in the lateral genigulate leaftlet contribute NPY projections to the suprachiasmatic nucleus (Moore et al., 1984; Watts and Swanson, 1987). NPY neurons in the caudal ventrolateral medulla have been shown to innervate multiple areas in the hypothalamus, including the POA and the PVH (Sawchenko et al., 1985). The projections of NPY neurons in the mediobasal hypothalamus (ARH) have not been examined directly, although indirect evidence has suggested that NPY neurons in the ARH also project to the POA, the PVH, and the DMH (Baker and Herkenham, 1995; McShane et al., 1994). The complicated and overlapping projection patterns add yet another level of difficulty in studying the function of NPY in the hypothalamus. Thus, more detailed and sophisticated anatomical studies are needed in order to establish the contribution of NPY projections in the hypothalamus from different populations of NPY neurons.

3. The possible functions of hypothalamic NPY systems

a. NPY and the regulation of energy homeostasis

One of the most widely studied functions of NPY is its potent stimulatory effect in inducing food intake (Lee et al., 1994; Tomaszuk et al., 1996). Central administration, as well as site specific injection of NPY, has shown that NPY can

elicit an increase in food intake even in satiated animals and that the response is greatest when the peptide is infused into the perifornical region as well as the PVH (Stanley and Leibowitz, 1985; Stanley et al., 1985, 1993). Moreover, chronic central injection of NPY can result in obesity (Beck et al., 1992; Zarjevski et al., 1993). In addition, several studies also showed that the levels of endogenous NPY in the hypothalamus fluctuated according to the feeding regimen (Sahu et al., 1988, 1992) and were significantly elevated in fasted or food restricted animals (Brady et al, 1992; White and kershaw, 1990). These results suggest that endogenous NPY in the hypothalamus plays an important role in regulating normal feeding responses. It is unclear which receptor mediates the feeding response of NPY in the hypothalamus. Although Y1 and Y5 receptors have been implicated (Pedrazzini et al., 1998) in mediating the response, it remains to be determined whether the feeding response is mediated by a unique unidentified receptor subtype which may share some similarity to the known receptors or is the result of stimulation of multiple receptor subtypes simultaneously. In addition to the food intake response, hypothalamic NPY can also influence energy homeostasis indirectly by either modulating the activity of the digestive system (Davison and Pearce, 1997), the secretion of insulin and leptin from the pancreas and adipocytes, respectively (Marks et al., 1996; Moltz and McDonald, 1985; Rohner-Jeanrenaud et al., 1996; Van Dijk et al., 1994), or the secretion of growth hormone from the anterior pituitary (McDonald et al., 1985; Okada et al., 1993; Pierroz et al., 1996).

b. NPY regulation of reproduction

In addition to the crucial role in modulating the feeding response, NPY has also been suggested to be one of the essential players in modulating reproductive function. Research on the regulation of reproductive function by NPY has largely focused on its effects in modulating luteinizing hormone (LH) secretion from the anterior pituitary (for reviews see Kalra and Crowley, 1992; Kalra and Kalra, 1996). NPY exhibits both excitatory and inhibitory effects on LH secretion. NPY synthesis and secretion from the ARH correlates positively with serum LH levels during proestrus before the LH surge, which is the key signal in inducing ovulation (Pelletier et al., 1992; Sahu et al., 1989, 1993, 1995). Blockade of hypothalamic NPY function by either central antisense oligonucleotides treatment or central immunization using anti-NPY antibody can abolish the LH surge (Kalra et al., 1995; Sutton et al., 1988). However, NPY can also exert an inhibitory effect on LH secretion. Aubert and colleages (Catzeflis et al, 1993; Pierroz et al., 1996) have shown that continuous intraventricular (icv) infusion of NPY for 7 days inhibits LH secretion in both male and female rats. Furthermore, pulsatile LH secretion is significantly suppressed during physiological conditions when hypothalamic NPY levels are chronically elevated, such as lactation (Smith, 1993) and food restriction (Wade and Schneider 1992). Although the detailed mechanism by which NPY may regulate LH secretion is still under intensive investigation, the hypothalamus and the pituitary gland have been suggested as two of the most likely sites where NPY may exert its effect.

Hypothalamus. Several lines of evidence suggest that NPY may regulate

LH secretion by modulating the activity of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus and the secretion of GnRH from its nerve terminals. A single icv injection of NPY significantly stimulates GnRH gene expression in the preoptic area (Li et al., 1994). NPY can either stimulate or inhibit GnRH release from median eminence fragments, depending on the steroid hormone condition of the animals (McDonald, 1990). Anatomical studies have established synaptic connections between GnRH cell bodies and NPY nerve terminals in the POA (Norgren and Lehman, 1989; Tsuruo et al., 1990), although the origin of the NPY terminals is not completely understood. In the ME, where a high density of GnRH nerve fibers and nerve terminals are located, dense NPY fibers have also been found (Contijoch et al., 1993; McDonald, 1990). However, most of the GnRH terminals are found in the lateral part of the external layer of the ME, whereas the NPY terminals are found mostly in the internal and intermediate layers of the ME, suggesting that NPY may exert its effect on GnRH secretion by paracrine effects instead of by direct contact with the GnRH nerve terminals. In lactating rats, the density of NPY terminals is greatly increased in the external layer of the ME, in contrast to the very low density present in nonlactating animals (Ciofi et al., 1991, 1993). This observation suggests that the regulation on GnRH secretion by NPY in the ME may vary under different physiological conditions.

Pituitary. Accumulating evidence indicates that NPY may also modulate LH secretion at the pituitary level. NPY receptor mRNA (Dyer et al., 1997) and binding sites (Parker et al., 1991) have been identified in the anterior pituitary.

However, NPY alone has no effect on LH secretion from anterior pituitary cells in culture (Crowley et al., 1990). When anterior pituitary cell cultures are treated with the combination of GnRH and NPY, NPY can potentiate the stimulatory effect of GnRH on LH secretion, in part due to a NPY-induced increase in GnRH binding sites on the pituitary cells (Crowley et al., 1990). These results suggest that when NPY is released from its nerve terminals in the ME, it may not only affect the secretion of GnRH from its nerve terminals but also act synergistically to potentiate the effect of GnRH on LH secretion at the pituitary level. In addition to the direct effect of NPY on the GnRH system, it has been suggested that NPY may also affect LH secretion by influencing the activity of the posterior pituitary (O'Conner and Wade, 1996; Vanhatalo and Soinila, 1996). However, it is still unknown which substance from the posterior pituitary may mediate the effect of NPY on LH secretion.

4. Factors involved in regulation of hypothalamic NPY activity

a. Leptin

Leptin is a 16 KD protein hormone secreted into the circulation by adipocytes and, through actions on the central nervous system, regulates food intake, autonomic nervous system activity and hormonal secretion from the pituitary gland (Campfield et al., 1996). Accumulating evidence suggests that leptin may be one of the factors in modulating NPY levels in the hypothalamus. Morphological studies showed that leptin receptors are expressed in NPY-positive neurons in the ARH (Mercer et al., 1996; Hakansson et al., 1998). Exogenous leptin can suppress hypothalamic NPY gene expression and

synthesis (Schwartz et al., 1996b). In addition, in *ob/ob* obese mice, in which endogenous leptin is absent, hypothalamic NPY levels are significantly elevated, and treatment with exogenous leptin decreases NPY expression in the hypothalamus of both normal and *ob/ob* mice (Schwartz et al., 1996a). A feedback loop also appears to exist between hypothalamic NPY and leptin because icv infusion of NPY produces an increase in leptin mRNA in rat white adipose tissue (Rohner-jeanrenaud et al., 1996).

b. Insulin

Before leptin was identified, insulin was considered one of the major factors in regulating hypothalamic NPY. Peripheral or icv injection of insulin suppresses NPY gene expression in the ARH (Malabu et al., 1992; Schwartz et al., 1992). In vitro studies showed that insulin could inhibit NPY release from fetal hypothalamic cultures (Silva et al., 1995). Insulin receptor mRNA has been detected in the ARH area (Marks et al., 1990), although no colocalization study has been done to determine whether NPY neurons in the ARH express insulin receptors.

c. Glucocorticoids

In contrast to insulin and leptin, glucocorticoids have been shown to play a stimulatory role in hypothalamic NPY gene expression (Larsen et al., 1994). Glucocorticoid receptors have been detected in the ARH NPY neurons (Hisano et al., 1988), and more importantly, three copies of the glucocorticoid responsive element (GRE) have been located in the 5'-promoter region of NPY gene, thus suggesting a direct role of glucocorticoids in modulating NPY gene expression

(Misaki et al., 1992).

d. Gonadal steroids

Since it has been shown that NPY plays an important role in modulating the reproductive axis, it is logical to assume that some of the feedback signals regulating the reproductive axis may also be able to modulate NPY in the hypothalamus. Gonadectomy of both sexes produces a decrease in NPY expression in the ARH, and steroid hormone replacement restores the levels of NPY (Camp and White, 1990; Sahu et al., 1993). Interestingly, in the female, progesterone does not have an additional effect on NPY expression when combined with estrogen (Camp and White, 1990). NPY neurons in the ARH concentrate estrogen (Sar et al., 1990), suggesting that estrogen may exert its effect directly on NPY neurons in the ARH, although the presence of estrogen or testosterone receptors on NPY neurons in ARH has not been determined. In addition, the identification of the responsive elements for gonadal steroid hormone receptors in the NPY 5- promoter region has yet to be determined. Thus, more studies need to be done to address the mechanisms by which gonadal steroid hormones may modulate NPY expression in the hypothalamus.

e. Neurochemical substrates

Several neurotransmitters and neuropeptides have been shown to be able to modulate NPY gene expression. These include dopamine (Li and Pelletier, 1986; Pelletier and Simard, 1991) and serotonin (Baker et al., 1996; Rogers et al., 1991). Several peptide systems, including tachykinins and enkephalin, have been shown to make direct synaptic contacts with NPY neurons in the ARH

(Magoul et al., 1993, 1994b), suggesting possible direct effects of these peptides on NPY gene expression, although more studies are needed to elucidate this issue.

f. Second messenger systems

Available evidence suggests that several secondary messenger systems are involved in regulation of NPY gene expression. These include protein kinase A (PKA) (Higuchi et al., 1988b; Pance et al., 1995) and protein kinase C (PKC) (Higuchi et al., 1988b; Lerchen et al., 1995), although the classic cAMP response elements have not been identified in the 5-promoter region of the NPY gene. It has been suggested that the effect of cAMP is mediated by AP-2 site (Balbi and Allen, 1994; Pance et al., 1995), whereas the effect of PKC is mediated by AP-1 sites (Jalava and Mai, 1994). The involvement of PKA and PKC in the regulation of NPY gene expression suggests that any signal which can alter the activity of PKA and/or PKC systems in NPY neurons may potentially modulate NPY gene expression.

C. LACTATION AS A MODEL FOR STUDYING THE HYPOTHALAMIC NPY SYSTEM

Lactation is a natural physiological stage occurring after parturition. During this stage, the newborn offspring are entirely dependent on nutrients provided by the mother. Secretion of milk occurs when the mother receives a suckling

stimulus from the newborns. Most of the interest in lactation has focused on the physiological alterations occurring in the mother that allow her to adapt to the lactating condition. Some of the alterations include the temporal cessation of reproductive cyclicity (McNeilly, 1994), the significant increase in food intake in order to overcome the energy drain due to milk production (Flint and Vernon, 1998; Malabu et al., 1994; Vernon and Flint, 1984), and the significant increase in serum oxytocin and prolactin levels, which are important for maintaining milk synthesis and secretion (Tucker, 1994). It is important that these alterations are reversible after weaning and are related to the intensity of the suckling stimulus (Fox and Smith, 1984; McNeilly, 1994; Tucker, 1994). These characteristics make lactation an interesting model to study the mechanisms causing alterations such as the suppression of reproductive cyclicity and the increase in food intake.

1. NPY activity is elevated during lactation

NPY levels in the hypothalamus have been examined in chronic lactating female rats (Ciofi et al., 1991; Malabu et al., 1994; Pickavance et al., 1996). It was found that hypothalamic NPY peptide levels are significantly elevated during chronic lactation, especially in the POA, PVH, DMH, and ARH. These observations were further extended by measuring NPY mRNA levels by *in situ* hybridization; these studies showed a significant increase in NPY mRNA levels in the ARH (Pape and Tramu, 1996; Smith, 1993). However, the increase in NPY activity in the ARH was observed only in the caudal portion of the ARH, even though NPY-positive neurons are detected throughout the ARH (De Quidt et al., 1990). In addition to the ARH NPY neurons, a population of NPY neurons was

also identified in the DMH area in chronic lactating animals; these neurons were not observed in the nonlactating animals (Smith, 1993). This novel expression of NPY in the DMH of lactating animals underscores the complexity of peptide systems in the hypothalamus, in which peptides may be synthesized in unique places in the hypothalamus under certain conditions such as lactation, to modulate physiological systems that maintain homeostasis of the body.

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

a. Neural signals activated by the suckling stimulus

The mechanical stimulation of the suckling stimulus has been considered one of the key signals in causing many of the alterations associated with lactation, such as prolactin and oxytocin secretion, somatosensory sensations, the sleep-wake cycle, food intake, and the suppression of LH secretion. It has been suggested that the neural impulses activated by suckling are basically transmitted through the spinal cord to the brainstem and then enter the forebrain region including the hypothalamus (Wakerley et al., 1994).

Since there are extensive connections between the hypothalamus and the brainstem, as well as major forebrain regions, it is logical to assume that increased NPY expression in the hypothalamus may be in part due to the neural signals activated by suckling. Currently, NPY neuronal activity has been studied only in chronic lactating animals. It is yet to be determined whether the neural signals induced by suckling play a significant role in activating any of the NPY populations in the hypothalamus. It will be very interesting if NPY neurons indeed

can be activated by neural signals induced by the suckling stimulus, since NPY neurons in the ARH have only been shown to be regulated by peripheral signals such as insulin, leptin, and glucocorticoids. Thus, the ability of neural signals to regulate NPY neuronal activity would suggest that the hypothalamic NPY system plays an important role in integrating multiple incoming signals, including hormonal, visceral and, most importantly, somatosensory input, into the hypothalamus during lactation. The integration may then result in the differential activation of certain populations of NPY neurons, such as observed in lactating animals, which then connect to appropriate downstream neurons to modulate multiple physiological systems, such as suppression of LH secretion and an increase in food intake.

Currently, due to the lack of appropriate techniques, the neural pathways transmitting the suckling stimulus are only vaguely defined, and it has been difficult to determine the phenotypes of the neurons involved in the neural circuitry. In addition, there are also other neural inputs from the visual, auditory, and olfactory stimulation associated with sensory stimuli derived from the offspring. These additional inputs may integrate with the neural signals activated by the physical suckling stimulus at different levels, thus making it very difficult to differentiate the neural circuitry activated only by the suckling stimulus. Recently, the immediate early gene product, cFos, has been used as a marker for identifying activated neurons in the brain when the animal receives certain stimuli such as pain (Hermanson and Bloqvist, 1996) or hypoxia (Teppema et al., 1997). cFos has also been used to identify neural populations in the brain that are

important in mediating behavior (Numan and Numan, 1994, 1995). Thus, cFos expression may be a useful tool for identifying the neural circuitry responsible for transmitting the neural signals activated by suckling.

b. Hormones stimulated by the suckling stimulus

Prolactin. Prolactin is one of the hormones secreted from the anterior pituitary in response to the suckling stimulus. The basic 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 during lactation (Moore et al., 1986; Sauve and Woodside, 1996). It is not clear how elevated prolactin may modulate these events during lactation; although prolactin-specific receptors have been detected in the brain, including the hypothalamus (Bakowska and Morrell, 1997; Chiu et al., 1992; Chiu and Wise, 1994; Crumeyrolle-Arias et al., 1993; Poky et al., 1996). However, the neuronal targets by which prolactin exerts its effects are still unknown. Thus, it is possible that the two NPY systems in the hypothalamus are targets for elevated prolactin in modulating LH secretion and/or the food intake response during lactation.

Oxytocin. Oxytocin is the major hormone released from the posterior pituitary; it stimulates milk ejection during lactation. In addition, oxytocin facilitates maternal behavior and regulates grooming and feeding behavior (Richard et al., 1991). Although it has been shown that oxytocin can modulate

LH secretion in cycling rats, it is not clear whether oxytocin secreted during lactation exerts an inhibitory effect on LH secretion. In addition, the neuronal targets for some of the central effects of oxytocin have not been identified. A study done by Arletti et al. (1993) showed that lesions of the ARH by neonatal administration of monosodium glutamate, which permanently disrupts NPY neurons in ARH, did not alter the effects of oxytocin on feeding and drinking behavior, implying that NPY may not be involved in mediating the effects of oxytocin in the hypothalamus.

c. Metabolic related signals

Glucocorticoids. During lactation, the activity of the hypothalamic-pituitary-adrenal axis (HPA) is elevated, as indicated by the elevated levels of adrenocorticotropic hormone (ACTH) and corticosterone (Walker et al., 1992). Corticotropic releasing factor (CRF) mRNA levels in the hypothalamus are suppressed during lactation (Fischer et al., 1995), suggesting additional factors, such as vasopressin, may be in part responsible for the activation of the HPA axis. As discussed above, glucocorticoids are capable of stimulating NPY synthesis in the ARH as well as in an *in vitro* cell culture system, suggesting that elevated glucocorticoids during lactation may be important in modulating the activation of NPY neurons in the ARH. It is not clear whether glucocorticoids also participate in the activation of NPY neurons in the DMH area during lactation. On the other hand, it has been shown by Bloom and coworkers that NPY can stimulate ACTH release from the anterior pituitary (Small et al., 1997). This study suggests a possible interesting feedback loop between the HPA axis and the

hypothalamic NPY system during lactation.

Leptin. As discussed above, the adipocyte released hormone, leptin, can negatively modulate the synthesis and secretion of NPY from the ARH. Recently, leptin levels were examined during lactation (Brogan et al., 1998) and found to be significantly suppressed in chronic lactating animals. These data suggest that ARH NPY neurons may escape from the inhibitory effect of leptin during lactation.

Insulin. Lactating rats are typically hypoinsulinemic (Flint and Vernon, 1998; Malabu et al., 1994), even though their blood glucose levels remain normal. Since insulin may have an inhibitory effect on NPY activity in the ARH, it is possible that the decrease in insulin levels in lactating animals may contribute to some degree to the increase in NPY levels in the hypothalamus.

3. Possible function of NPY during lactation

a. Suppression of reproductive cyclicity

One of the possible functions of the activated NPY systems in the hypothalamus during lactation is to regulate reproductive function. During lactation, regular ovarian cyclicity is suppressed for at least 20 days in the female rat (Van der Shoot et al., 1978). There is no follicular development in the ovary, due to the absence of pulsatile LH secretion from the anterior pituitary. Currently, the underlying mechanism by which LH secretion is suppressed during lactation is still not completely understood. Earlier studies suggested that the suckling stimulus is the key determinant in inducing anestrus, such that the duration of lactation-induced anestrus depends on the intensity of the sucking stimulus (Fox

and Smith, 1984; Maeda et al., 1987, 1989). In fact, anestrus is prolonged if an intense suckling stimulus is provided beyond day 20 postpartum (Smith, 1978; Smith and Neill, 1977).

Available evidence suggests that the deficits in LH secretion may be due in part to a lack of GnRH stimulation from the hypothalamus. It has been shown that pituitary GnRH receptors and LH subunit mRNAs are inhibited in intact as well as ovariectomized lactating rats (Smith and Lee, 1989). The decrease in GnRH receptors is also directly related to the intensity of the suckling stimulus (Smith, 1984). Since GnRH is known to upregulate its own receptors and stimulate LH synthesis and release, the deficits reflect a deficit in GnRH secretion. In addition, administration of pulsatile GnRH to lactating rats can upregulate GnRH receptors, enhance pituitary sensitivity to GnRH, and stimulate LH secretion (Lee et al., 1989). However, when GnRH content was measured in the ME of lactating rats, it was not different from cycling rats (Jakubowska-Naziemblo et al., 1985). Quantitative *in situ* hybridization also showed that there was no difference in GnRH mRNA between cycling and lactating rats (Marks et al., 1993). Thus, these observations suggest that GnRH release, rather than synthesis, is suppressed during lactation.

One of the possible mechanisms by which NPY may regulate LH secretion is to control the secretion of GnRH from its nerve terminals. This notion is supported by the observation that NPY content in the ME is significantly elevated in lactating females compared to nonlactating animals (Ciofi et al., 1991, 1993). However, it is not clear whether the hypothalamus is the origin of increased NPY

found in the external layer of the ME. NPY neurons in the brainstem and in the sympathetic ganglion both project to the ME (Sahu et al., 1988), but hypothalamic NPY projections, especially from the ARH NPY neurons, into the ME have not been established. In addition, none of the NPY receptor subtypes has been detected in the GnRH cell body or in the nerve terminals. Thus, the functional role of hypothalamic NPY in the regulation of GnRH release in the ME remains to be determined.

b. Lactational hyperphagia

During lactation there is significant energy demand due to milk production, exceeding the whole-body nutrient requirements of the nonlactating animals. In addition, there is hypertrophy and increased synthetic activity of the gastrointestinal tract and liver. These energy demands are met primarily by increased food intake, which rises several fold over nonlactating animals (Flint and Vernon, 1998; Malabu et al., 1994; Vernon and Flint, 1984). Despite this large increase in food intake, lactating animals are hypoinsulinemic (Flint and Vernon, 1998; Malabu et al., 1994) and exhibit various characteristics of chronic undernutrition, including hypothyroidism and decreased thermogenesis in adipose tissue (Flint and Vernon, 1998; Malabu et al., 1994). The undernutrition is probably due to the vast flux of nutrients to the mammary gland for milk production. The mechanism that drives hyperphagia and energy adaptation during lactation is still not completely understood. It has been suggested by many investigators that the mechanism may reside in the hypothalamus because of its important role in the regulation of food intake and energy homeostasis. The

central role of NPY in inducing the feeding response makes it a likely candidate in mediating hyperphagia during lactation. The demonstration of elevated NPY concentrations in PVH, DMH, and ARH, and NPY mRNA levels in the ARH and DMH during lactation, further supports this hypothesis. However, the central mechanism by which NPY mediates the feeding response it still not clear. Corticotropic releasing factor (CRF) in the hypothalamus has been suggested as one of the downstream systems in mediating the food intake response elicited by NPY. It has been shown that alpha-helical CRF, a CRF antagonist, can potentiate the feeding response elicited by central administration of NPY (Heinrichs et al., 1993), and PVH injections of NPY cause an increase in the plasma levels of ACTH and corticosterone (Wahlestedt et al., 1987). Anatomically, NPY innervates CRF positive neurons in the PVHp area (Liposits et al., 1988; Wahlestedt et al., 1987), although the origin of the NPY input on the CRF neurons remains to be determined.

D. AIMS OF THE THESIS AND APPROACH

Aim 1: Identify factors involved in the regulation of the two populations of NPY neurons in the hypothalamus during lactation.

Approach:

An acute resuckling paradigm was used to study the effect of the suckling stimulus as well as suckling-induced hyperprolactinemia in the regulation of NPY neuronal activity in the hypothalamus.

Aim 2: Define the afferent inputs that may be important in modulating NPY neuronal activity in the hypothalamus.

Approach:

1. cFos expression was used to determine the neural circuitry activated by the suckling stimulus.
2. cFos expression combined with anatomical tracing techniques was used to determine the neural inputs that are important in suckling-induced activation of NPY neurons in the ARH.

Aim 3: Define the downstream target areas as well as the phenotype of the neuronal systems in the target areas which may be important in mediating the effect of hypothalamic NPY on reproduction and food intake.

Approach:

1. A PHA-L anterograde tracing technique was used to identify the downstream target areas for the activated NPY neurons in the caudal portion of the ARH.
2. Anterograde tracing combined with multiple histological staining was used to

determine whether the GnRH system is one of the direct target systems of the NPY neurons in the ARH which may mediate reproductive function during lactation.

3. Retrograde tracing was used to determine whether the paraventricular nucleus of the hypothalamus is the target area for the activated NPY neurons in the hypothalamus during lactation.
4. Anterograde tracing combined with multiple histological staining was used to determine whether the CRF system in the PVH is one of the direct target systems of the NPY neurons in the ARH which may mediate food intake during lactation.

CHAPTER II

THE ACUTE SUCKLING STIMULUS INDUCES EXPRESSION OF NEUROPEPTIDE Y (NPY) IN CELLS IN THE DORSOMEDIAL HYPOTHALAMUS AND INCREASES NPY EXPRESSION IN THE ARCUATE NUCLEUS

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Introduction

Lactation in the rat is accompanied by changes in hormone secretion and behavior that participate in adaptation to the lactating condition. The hypothalamus appears to be the site where the majority of these changes are regulated by incoming neural signals induced by the suckling stimulus. For example, several hypothalamic nuclei are involved in regulating the secretion of oxytocin and prolactin (Neill and nagy, 1994; Wakerley et al., 1994), and the medial preoptic area has been shown to be the primary site in the induction of maternal behavior (Numan, 1994). In addition, the suppression of LH secretion and estrous cyclicity during lactation may reflect a change in neuronal activity in the hypothalamus. Thus, identification of neuronal systems in the hypothalamus activated by the suckling stimulus during lactation will contribute to an understanding of how the changes in hormonal secretion and behavior are regulated at the hypothalamic level.

Recently, it has been shown by several laboratories that the levels of neuropeptide Y (NPY) are significantly elevated in a number of hypothalamic areas in chronic lactating animals (Ciofi et al., 1991; Malabu et al., 1994; Pickavance et al., 1996); this is further confirmed by the increase in NPY mRNA levels in the hypothalamus (Pape and Tramu, 1996; Smith, 1993). Most of the NPY-containing neurons in the hypothalamus are found in the hypothalamic arcuate nucleus (ARH) (De Quidt et al., 1990). NPY in the hypothalamus has been shown to participate in regulating several physiological functions, including induction of the feeding response (see review, Lee et al., 1994;

Tomaszuk et al., 1996), hypothalamic neuroendocrine regulation (Blasquez et al., 1995; Hong et al., 1995; Suda et al., 1993) and pituitary hormone secretion (Catzeflis et al., 1993; Kalra and Crowley, 1992; Koenig, 1990; McDonald et al., 1985; Pierroz et al., 1996; Small et la., 1997). The diverse functions of NPY make the peptide a candidate for modulating many of the alterations in hypothalamic function during lactation.

Several key issues have yet to be addressed in considering the involvement of NPY in modulating hypothalamic neuronal activity during lactation. First, it has not been determined if the increase in NPY neuronal activity can be induced by the suckling stimulus. Second, if the activation is indeed associated with the suckling stimulus, then the time course of the activation needs to be determined so as to assess whether it correlates with the time course of suckling-induced changes in other events associated with lactation, such as suppression of LH secretion and increase in food intake.

The aims of the present study were to determine (1) if NPY neurons in the hypothalamus can be activated by the suckling stimulus, and (2) the time course of the activation in response to the suckling stimulus.

Materials and Methods

Animals

Day 18-19 pregnant Sprague-Dawley rats (B & K Universal Inc., Kent, WA) were housed individually and were maintained under a 12:12 light-dark cycle (lights on at 0700 hours) 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 as day 0 postpartum. All the animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee.

Experimental design

An acute resuckling paradigm was designed so that changes in hypothalamic neuronal activity could be correlated with changes in hormone secretion. The paradigm was validated in ovariectomized lactating rats suckling 8 pups so as to more easily assess changes in LH secretion. As shown in Table 2-1, removal of the 8-pup suckling stimulus resulted in a significant reduction in prolactin levels to values approaching the basal levels observed in nonlactating animals (data not shown). The significant rise in LH following removal of the suckling stimulus reflects the ovariectomized state of the animals. Return of the 8-pup litters and resuckling for 24 hours reversed these changes, resulting in increased prolactin secretion and suppression of LH secretion.

Table 2-1. Changes in LH and prolactin secretion in response to acute resuckling.

Hormones (ng/ml)	Day 9, Chronic Suckling	Day 11, -8 pups for 48 hours	Day 12, +8 pups for 24 hours
Prolactin (n=4)	193 ± 29^a	24 ± 3^b	213 ± 32^a
LH (n=4)	$< 5^a$	98 ± 18^b	37 ± 13^c

1. Prolactin values are expressed in terms of rat PRL-RP-3; the RIA was performed as previously described (Abbud et al., 1993).
2. LH values are expressed in terms of rat LH-RP-1; the RIA was performed as previously described (Abbud et al., 1993).

a, b, c: Superscripts reflect differences among the groups within a measure.

In the first experiment, lactating animals had their litters adjusted to eight 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. After pup separation, the females were housed individually in an experimental room under the conditions described above. In a separate room, the pups were kept together in a cage on a heating pad to maintain their body temperature; they had no access to foster mothers. On day 11, groups of animals received either 0 pups (nonresuckled controls, n=5) or 8-pup litters and were allowed to resuckle for 12 (n=5) or 24 (n=6) hours. After the specified time of resuckling,

the animals were sacrificed by decapitation and the brains were quickly removed, frozen on dry ice, and stored at -80° C until use.

From the first experiment, it was found that although 12 hours of resuckling did not result in a significant change in NPY mRNA levels in the ARH, it did induce a strong NPY signal in the dorsomedial nucleus of the hypothalamus area (DMH). To more finely determine the time course of activation of NPY-expressing cells in the DMH area, a second experiment was conducted using the same paradigm described above. Three groups of animals were used: 0 pups (nonresuckled controls, n=3) and 8-pup litters resuckled for 3 (n=3) or 6 (n=3) hours. The brains were processed for *in situ* hybridization to identify NPY-positive neurons in the DMH area.

In situ hybridization

Coronal brain sections (20 μm) were collected through the ARH, and the slides were stored at -80° C until used for *in situ* hybridization. NPY cRNA probe synthesis and the specificity of the cRNA probe and procedure for *in situ* hybridization have been described previously (Smith, 1993). Briefly, the NPY cRNA probe was transcribed from a 511 bp cDNA in which 21% of the UTP was ^{35}S-labeled (Dupont NEN, Boston MA). The saturating concentration of the probe used in the assay was 0.3 μg/ml·Kb. The specific activity of the probe ranged from 1-3 x 10^8 dpm/μg. 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 and rehydrated through a second series of alcohols and then air dried. The slides were exposed to the NPY cRNA probe overnight in moist chambers at 55° C. After incubation, the slides were washed in SSC that increased in stringency, in RNase, and then in 0.1X SSC at 60° C and rehydrated through graded series of alcohols and dried. Slides were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY), exposed for 6-7 days at 4° C and developed. After development, the slides were stained with cresyl violet.

Data Analysis

NPY mRNA in the ARH

The ARH was divided into four subdivisions as described in a previous study (Smith, 1993), using the plates describing coronal sections of the rat brain (Paxinos and Watson, 1982). Briefly,

ARH-A corresponded to the retrochiasmatic area rostrally, to the elongation of the third ventricle caudally (plate 19).

ARH-B continued caudally to the beginning of DMH (plate 20).

ARH-C contained the compact zone of the DMH (plate 21).

ARH-D began with the disappearance of the DMH, to the end of the ARH (plate 22).

The coronal brain sections were anatomically matched across animals from all groups. NPY mRNA was quantitated using the VIDEK HARMONY image analysis system by VIDEK (Rochester, NY). The system identified silver grains

by the brightness of the image. An estimate for silver grains over the entire ARH on each tissue section was given as the area occupied by silver grains within the marked area (because of the close proximity of most NPY cells in the ARH, it was not possible to analyze individual cells). The marked area was constant (1.0 mm x 1.6 mm) for each reading and included the entire ARH. The area occupied by silver grains was typically between 5 - 15% of the marked area.

NPY mRNA in the DMH

NPY mRNA was visualized using dark field optics. The induction of NPY mRNA, represented by clusters of silver grains, was assessed qualitatively.

Statistical analysis

The data for NPY mRNA in the ARH were expressed as the area occupied by grains per section. The mean area occupied by grains per section for each subdivision of the ARH was determined for each animal. Data are presented as mean ± SEM. Differences between groups within a subdivision were evaluated using one-way analysis of variance (ANOVA) and post hoc Scheffe's tests. Differences were considered significant if $p<0.05$.

Results

Effects of acute suckling on NPY mRNA levels in the arcuate nucleus (ARH)

Analysis of NPY mRNA content in each of the four subdivisions of ARH for the different groups revealed that the acute suckling stimulus, during the times examined, did not induce a significant increase in the mRNA content in ARH-A, ARH-B, or ARH-D areas, when compared to the nonresuckled control group (Fig. 2-1). In contrast, the ARH-C area showed a significant increase in NPY mRNA levels when the animals were resuckled for 24 hours compared to the nonresuckled control (Fig. 2-1). As shown in Fig. 2-2, the increase in NPY mRNA in response to suckling was due in part to the recruitment of new cells expressing NPY mRNA; these cells were most evident in the dorsolateral and ventrolateral portions of the ARH-C area. NPY mRNA levels also appeared to be increased in previously expressing cells in the ventromedial portion of the ARH-C. Because of the high density of silver grains over many of the cells in the ventromedial portion, the magnitude of the suckling-induced increase may even be an underestimate. The 12-hour resuckled group showed a moderate increase in mRNA content, which failed to reach significance when compared to the control group (Fig. 2-1).

Effects of acute suckling on NPY mRNA expression in the DMH

In addition to the increase in NPY mRNA in the caudal portion of the ARH, the suckling stimulus also induced expression of NPY mRNA in cells located dorsal and lateral to the compact zone of the DMH (Fig. 2-3). Under the

nonsuckled condition (0 pups, Fig. 2-3), a low level of NPY mRNA signal was observed in the compact zone of the DMH. When viewed under higher magnification, the silver grains appeared to be associated with cell bodies in the compact zone. After 3 hours of resuckling (8-pups, 3 hours, Fig. 2-3), clusters of NPY mRNA were observed in the DMH, although there was variation in the intensity of the signal level found among the animals. After 6 hours of resuckling, the NPY mRNA signals were consistently observed in all the animals examined (8-pups, 6 hours, Fig. 2-3). By 24 hours of resuckling, the level of activation was further increased (8-pups, 24 hours, Fig. 2-3) and was similar to that observed in chronic lactating animals (Smith, 1993). As shown in Fig 2-3, the low levels of hybridization signal detected over the compact zone of the DMH were observed in all the groups; there was no consistent change in silver grains over the compact zone in response to the suckling stimulus. The distribution of NPY expressing cells throughout the DMH in animals resuckled for 24 hours is shown in Fig. 2-4. This group of NPY neurons was scattered around the compact zone of the DMH and extended laterally to the perifornical area of the hypothalamus. Rostrally, few cells were seen when the compact zone emerged from the medial base of the DMH. The density of the cells increased quickly and peaked when the compact zone was fully extended. Caudally, the density decreases rapidly and the cell group disappeared soon after the compact zone disappeared. The rostral-caudal expression of NPY neurons in the DMH occupied the same plane as ARH-C, in which the increase in NPY mRNA was observed.

Discussion

The present study demonstrates that 3 hours of the suckling is sufficient to activate NPY gene expression in the DMH, whereas 24 hours of suckling is required to increase NPY mRNA levels in the caudal portion of the ARH. These data also agree with the results of our earlier study (Smith, 1993), and others (Pape and Tramu, 1996), showing that only a subpopulation of NPY neurons in the ARH expressed elevated mRNA levels in chronic lactating rats. The different time course of activation of NPY neurons in the DMH and ARH could reflect that the two NPY neuronal populations are activated by different signals induced by the suckling stimulus, or that they have different sensitivities to the same signal. On the other hand, the difference could simply result from the difference in the baseline value of NPY mRNA levels in the two areas. It has been shown by both the previous (Smith, 1993) and present study that basal NPY mRNA levels are high in the ARH in diestrous cycling animals, as well as in animals which have been deprived of pups for 48 hours. The high basal NPY mRNA level in the ARH makes it difficult to detect small changes in mRNA. In contrast, there is no detectable NPY message in the area surrounding the compact zone of the DMH in the absence of the suckling stimulus, making it easy to detect increases in signal in response to suckling. Detection of hnRNA instead of mRNA might resolve this issue.

Available anatomical evidence suggests that NPY neurons in the ARH send projections to many hypothalamic areas, including the paraventricular nucleus of the hypothalamus (PVH) and the medial preoptic area (Bai et al.,

1985; Baker and Herkenham, 1995; Magoul et al., 1994a; McShane et al., 1994). The ARH-PVH NPY projection has been shown to play an important role in mediating energy metabolism by regulating food intake. During lactation there is significant energy demand due to milk production; this demand is met primarily by increased food intake (Flint and Vernon, 1998; Malabu et al., 1994; Pickavance et al., 1996; Vernon and Flint, 1984). The central role of NPY in inducing the feeding response makes it a possible candidate in mediating the hyperphagia during lactation. In fact, elevated NPY concentrations in PVH, DMH, and ARH and NPY mRNA levels in the ARH and DMH have been reported in the chronic lactating rat (Malabu et al., 1994; Pape and Tramu, 1996; Pickavance et al., 1996; Smith, 1993). The present study further demonstrates that NPY neurons are activated by the acute suckling stimulus. Taken together, the sustained activation of NPY neurons by the suckling stimulus throughout the course of lactation is likely to play an important role in mediating the increase in food intake to meet the energy demands resulting from milk production.

In contrast to NPY neurons in the ARH, there is little known about the suckling-induced NPY neurons in the DMH. Preliminary data from tract tracing studies in our laboratory have shown that NPY neurons in the DMH project to PVH (unpublished observations), suggesting that DMH NPY neurons may also participate in modulation of PVH neuronal activity. It is yet to be determined if DMH NPY neurons interact with ARH NPY neurons in the PVH. In addition, the

neuronal phenotypes of the PVH neurons controlled by the DMH NPY neurons remain unknown.

The functional significance of suckling-induced activation of NPY neurons in the DMH also remains to be elucidated. In the female rat, this population of NPY neurons does not appear to be activated under basal conditions, nor in response to stimuli that activate NPY neurons in the ARH, such as food deprivation (Marks et al., 1992) and streptozotocin-induced diabetes (Marks et al., 1993). In contrast to the female, the data from male rats are less clear. There are some reports of NPY mRNA expression in the DMH of the male under basal conditions, although it is not possible to determine the location of the cells and the level of expression from the data that has been described (Chen et al., 1996; Kamegai et al., 1996). On the other hand, a study published by White and Kershaw (1990) showed no evidence of NPY mRNA expression in the area of the DMH in the male under basal conditions, except for the low levels of expression found in the compact zone. Preliminary data from our laboratory also indicate that there is no NPY mRNA expression in the DMH in the male (unpublished observation). Furthermore, there are no studies in the male reporting increased expression of NPY in the DMH in response to any stimulus. Thus, in the rat, suckling is the only stimulus that has been shown to activate NPY neurons in the DMH.

Recently, NPY mRNA levels in the hypothalamus were examined in obese, melanocortin receptor type 4 (MC4-R) knock out mice and agouti mice (Kesterson et al., 1997). Both types of animals lack functional MC4 receptors,

either due to the knock out of receptor gene expression or to the functional blockade by endogenous agouti protein (agouti mice). It was surprising that ARH NPY mRNA levels were not altered in these animals compared to normal animals, but there was a significant activation of NPY in the DMH area in both MC4-R knock out and agouti mice. Interestingly, the pattern and distribution of the NPY-expressing neurons in the DMH area in the two mouse models are very similar to those found in the lactating animals. The MC4 receptor, a member of the receptor family for melanocortin peptides including ACTH, MSH, and opioid peptides (Mountjoy et al., 1994), has been shown to play an important role in the induction of obesity in these two animal models (Huszar et al., 1997; kesterson et al., 1997). The data from the MC4-R studies raise the possibility that the function of the MC4-R may be compromised in the lactating rat. The reduction of MC4-R activity may be a factor in the expression of NPY in neurons in the DMH area, which, in turn, may participate in the regulation of increase in food intake during lactation. In fact, the hypothalamic POMC neurons, one of the sources of ligands for activating the MC4-R, showed decreased activity during lactation (Smith, 1993). This data indirectly supports the idea that the function of the MC4-R is compromised during lactation. Taken together, it is possible that the expression of NPY neurons in the DMH area found in these two obese mouse models, as well as in the lactating rat, may play an unique and important role in mediating energy metabolism. Lactation may provide an interesting model to study the role of the DMH NPY neurons, as

well as the possible interaction between the ARH NPY neurons and the DMH NPY neurons in regulating energy metabolism and food intake.

In addition to a possible role in regulating food intake during lactation, increased NPY activity may also modulate reproductive function through changes in GnRH and LH secretion. NPY in the hypothalamus has been shown to play an important role in modulation of GnRH neuronal activity and LH secretion (see reviews, Kalra and Crowley, 1992; Kalra and Kalra, 1996). It is well documented in the rat that NPY can stimulate GnRH and LH secretion during proestrus and in response to steroid priming (Bauer-Dabtoin et al., 1992; kalra and Crowley, 1984; Kalra et al., 1992). However, chronic elevations of NPY in the brain have been shown to suppress LH secretion (Catzeflis et al., 1993; Pierroz et al., 1996). During lactation in the rat, pulsatile LH secretion as well as ovarian cyclicity are suppressed (Fox and Smith, 1984; Lee et al., 1989). The suppression of LH could be accounted for by a diminished release of GnRH from the hypothalamus (Lee et al., 1989), although this relationship has not been established. Interestingly, the time course described in the present study for the increase in NPY activity is inversely correlated with the time course of the suppression of LH secretion in response to acute suckling (Table 2-1). The relationship between the increase in NPY neuronal activity and the suppression of LH secretion by the suckling stimulus raises the possibility that specific populations of NPY neurons in the hypothalamus may be linked to the regulation of LH secretion during lactation. However, further investigation is

needed to understand whether this alteration of NPY neuronal activity by the suckling stimulus is causally involved in the modulation of LH secretion.

Important questions remain as to the nature of the signals induced by suckling that are responsible for activating hypothalamic NPY neurons. The neural impulses from suckling appear to ascend through the brainstem to the thalamus and enter the hypothalamus by ventral and dorsal routes (Tindal and Knaggs, 1971; Tsukamura et al., 1990), resulting in the secretion of oxytocin and prolactin. Thus, it is possible that neural signals or the elevated level of hormones induced by the suckling stimulus, or both, could be responsible for the activation of specific populations of NPY neurons.

In summary, these studies show that the acute suckling stimulus can activate two populations of hypothalamic NPY neurons, one in the caudal portion of the ARH and one in the DMH. The increase in NPY neuronal activity may play an important role in modulating changes in hypothalamic function during lactation.

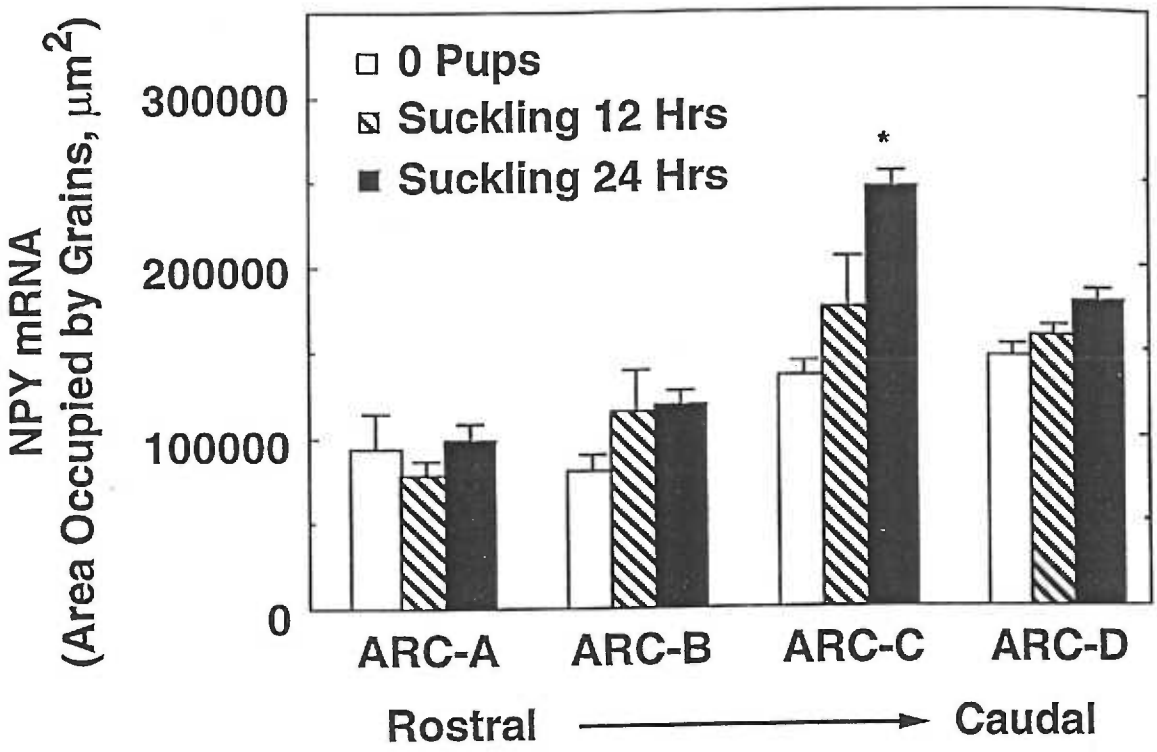


Figure 2-1. NPY mRNA content in the four subdivisions of the ARH in the three experimental groups: 0 pups (n = 5), suckling (+8 pups) for 12 h (n = 5) and suckling for 24 h (n = 6). The data from each subdivision (mean ± SEM) represent the average area occupied by silver grains per section. NPY mRNA content was affected by suckling only in the ARH-C area, where there was a significant increase ($p < 0.0001$, denoted by *) after 24 h of resuckling, when compared with the 0 pups control group. There was no significant difference in the ARH-C area between the control group (0 pups) and 12-h resuckled group ($p = 0.29$).

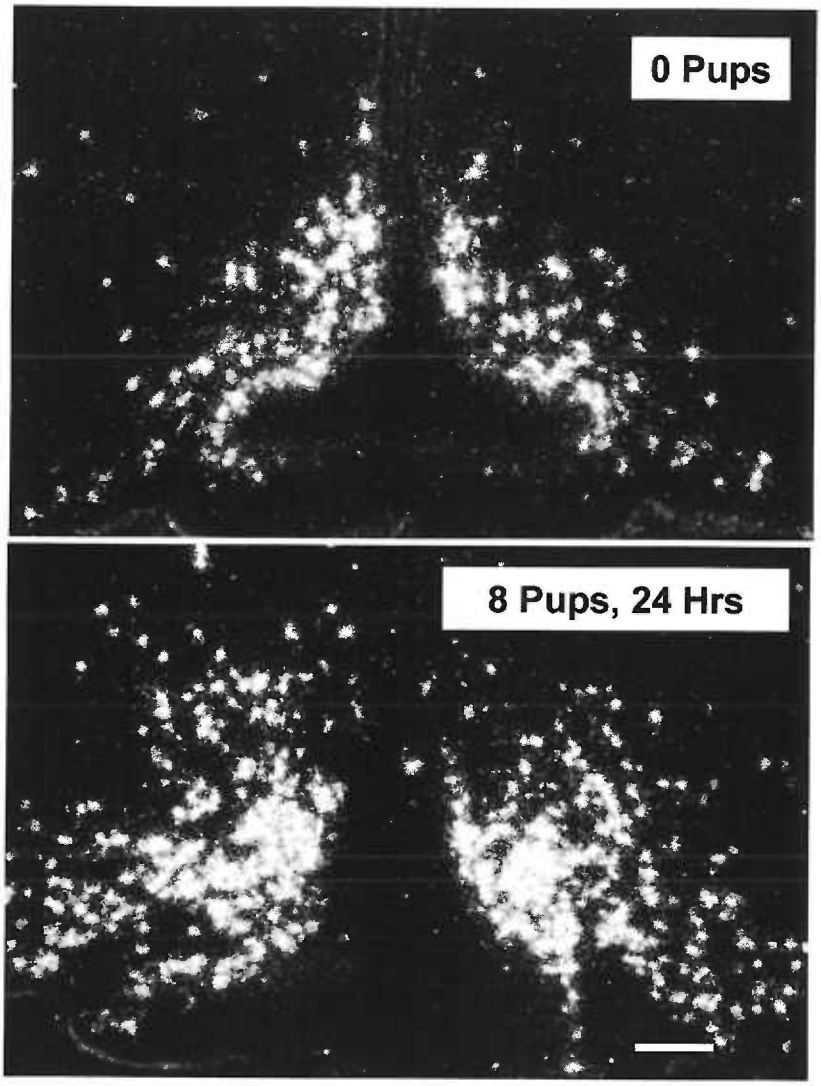
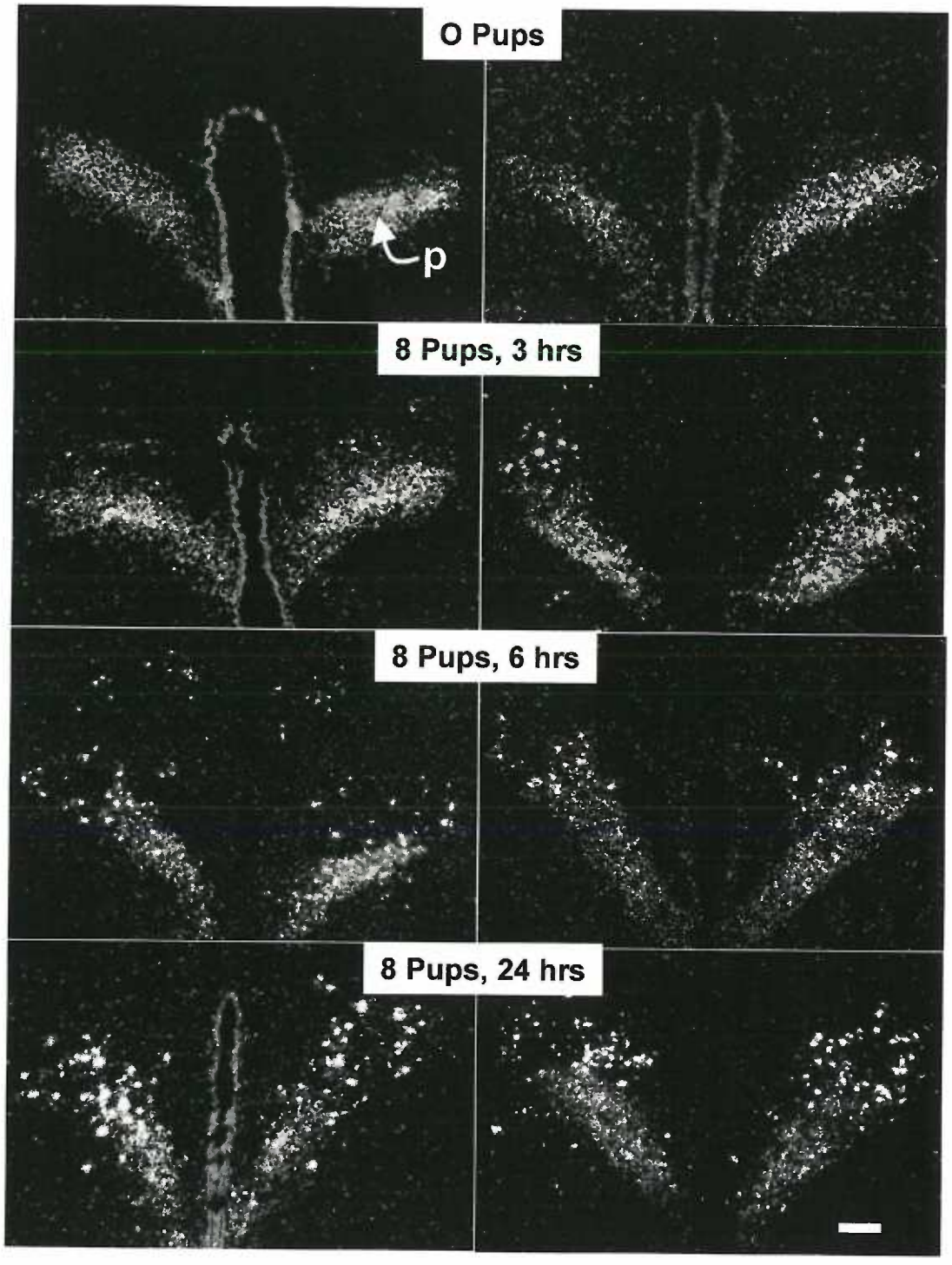
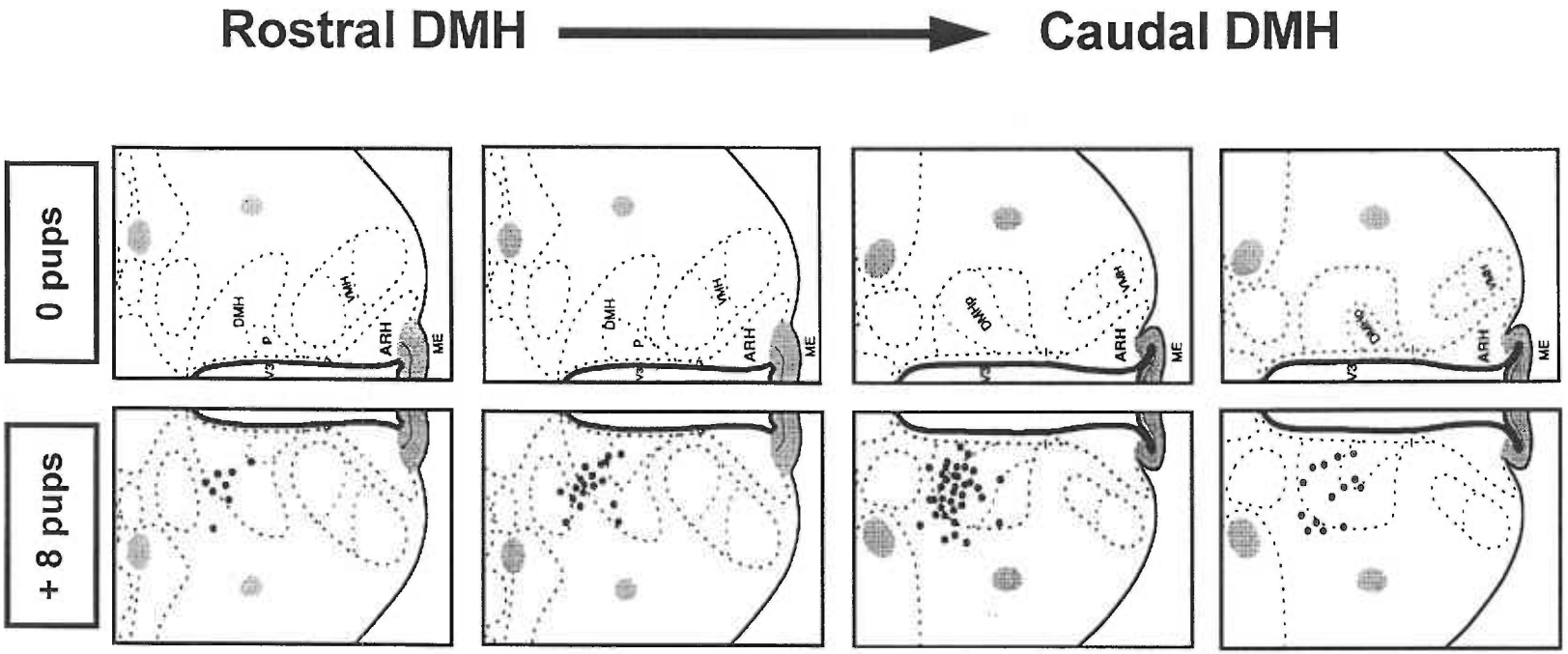


Figure 2-2. Dark-field photomicrographs of the ARH-C area from a control nonsuckled rat (0 pups, top) and a rat resuckled for 24 h (8 pups, 24 Hrs, bottom). The silver grains represent NPY mRNA. Note the marked increase in silver grain expression in the dorsolateral and ventrolateral portions of the ARH-C in the resuckled animal. Scale bar, 200 μm.


O Pups
p
8 Pups, 3 hrs
8 Pups, 6 hrs
8 Pups, 24 hrs


Rostral DMH
Caudal DMH
0 pups
+ 8 pups
DMH
VMH
ARH
ME
3V

CHAPTER III

NEUROPEPTIDE Y (NPY) AND TUBEROINFUNDIBULAR DOPAMINE (TIDA) ACTIVITIES ARE ALTERED DURING LACTATION: ROLE OF PROLACTIN

In press

Endocrinology

Introduction

The activity of several hypothalamic neuronal systems is altered during lactation. These alterations may be important in mediating some of the physiological adaptations occurring during lactation, such as suppression of ovarian cyclicity, increased food intake and suckling-induced milk production.

Neuropeptide Y (NPY) neuronal activity has been shown to be greatly increased in two discrete areas in the hypothalamus during lactation: the caudal portion of the arcuate nucleus (ARH) and the dorsomedial nucleus of the hypothalamus (DMH) (Li et al., 1998a; Smith, 1993). It has been suggested that the increased NPY activity in the caudal portion of the ARH may be important in mediating the increased food intake and the suppression of lutenizing hormone secretion associated with lactation (Lee et al., 1994; Kalra and Crowley, 1992; Kalra and Kalra, 1996; Tomaszuk et al., 1996). Currently, the functional role of the increased NPY in the DMH is still unknown.

In contrast, the activity of the tuberoinfundibular dopaminergic (TIDA) system in the ARH is greatly suppressed during lactation. Dopamine (DA) production as well as the expression of tyrosine hydroxylase (TH), the rate limiting enzyme in the dopamine biosynthetic pathway, is significantly reduced (Demarest et al., 1983; Wang et al., 1993). Under normal conditions, DA produced from the TIDA system is believed to be the main prolactin-inhibiting factor (PIF) that is tonically released to inhibit prolactin (PRL) secretion from lactotrophs in the anterior pituitary (for reviews, see Ben-Jonathan, 1985; Ben-Johnthan et al.,1989). In addition, the activity of the TIDA neurons is regulated

by PRL, such that elevated PRL increases the activity of these neurons and the secretion of dopamine into the median eminence (for reviews, see Moore, 1987). During lactation, however, this feedback regulation of PRL is not operative because elevated PRL levels are not associated with increased TIDA activity. This apparent dissociation between TIDA activity and PRL secretion may be one of the ways by which high levels of PRL are sustained during lactation, although the mechanism for this dissociation is unknown.

Currently, the mechanisms by which the activity of these neuronal systems is altered during lactation are not completely understood. It has been suggested that the suckling stimulus is important in triggering these changes (Li et al., 1998a; Plotsky et al., 1982). Several factors associated with the suckling stimulus, such as the elevated levels of PRL and neural impulses, are possible candidates for mediating the alterations in NPY and the TIDA activities. Thus, in the present study, bromocriptine, a dopamine D_2 receptor agonist, was used to inhibit suckling-induced PRL to characterize its role in modulating NPY and TIDA neuronal activities during lactation.

Materials and methods

Animals

Day 18-19 pregnant Sprague-Dawley rats (B & K Universal Inc., Kent, WA) were housed individually and were maintained under a 12:12 light-dark cycle (lights on at 0700 hours) 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 as day 0 postpartum. All the animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee.

Experimental design

An acute suckling paradigm previously described (Li et al., 1998a) was utilized 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. At that time, the 8-pup litters were removed from the females. On day 11, the animals were randomly divided into the following 3 groups:

(1) 0 pups: nonsuckled controls; animals received 2 subcutaneous vehicle injections (n=7).

(2) 8 pups: 8 pups suckling for 24 hours; animals received 2 vehicle injections (n=8).

(3) 8 pups+B: 8 pups suckling for 24 hours; animals received 2 bromocriptine (B) injections (0.5 mg/rat per injection) (n=8).

Resuckling for 24 hours was chosen since 24 hours of the suckling stimulus, after 48 hours of pup deprivation, was necessary to consistently observe significant changes in NPY gene expression in the ARH (Li et al., 1998a).

Bromocriptine (Sandoz Pharmaceuticals, E. Hanover, NJ) was dissolved in peanut oil containing 25% alcohol (5 mg/ml). Bromocriptine or vehicle was administered subcutaneously 3 hours before returning litters to the dams on day 11 postpartum; a second injection was given at 12 hours after returning the pups. The dose of bromocriptine used in the present study has been shown previously to not cross the blood brain barrier in amounts that have a direct effect in the brain (Demarest et al., 1985; Markey et al., 1978; Seeman and Van Tol, 1994).

After 24 hours of suckling, the animals were sacrificed 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 in a one-in-three series. The slides were stored at -80° C until used for *in situ* hybridization. Trunk blood was also collected and was assayed for rat prolactin by radioimmunoassay. The assay was performed by Dr. Marc Freeman at Florida State University according to methods previously described (Freemand and Sterman, 1978).

In situ hybridization

In the present study, quantitative *in situ* hybridization was utilized to measure the mRNA levels for TH and NPY, respectively, to serve as an indirect measure of neuronal activity. The activity of central neurons has been shown to be related to the cellular levels of mRNA encoding their rate-limiting enzyme, or in peptidergic neurons, to the levels of prepropeptide mRNA (Comb et al., 1987; Young and Zoeller, 1987). Gene expression of TH and NPY in TIDA and NPY neurons, respectively, exhibit a tight parallel relationship with neuronal activity (Hoffman et al., 1994; Li et al., 1998; Malabu et al., 1994; Wang et al., 1993).

NPY and TH cRNA probe synthesis, the specificity of the cRNA probe, and procedures for *in situ* hybridization have been described previously (Li et al., 1998; Smith, 1993; Wang et al., 1993). Briefly, the NPY cRNA probe was transcribed from a 511 bp cDNA in which 21% of the UTP was ^{35}S-labeled (Dupont NEN, Boston MA). The TH cRNA probe was transcribed from a 300 bp cDNA using 50% ^{35}S-labeled UTP. The specific activity for both probes ranged from 1-3 x 10^8 dpm/μg. The saturating concentration for both probes 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 respective cRNA probes overnight in moist chambers at 55° C. After incubation, the slides were washed in SSC that increased in stringency, in RNase, and then in 0.1X SSC at

60° C, and dehydrated through a graded series of alcohols and dried. Slides were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY), exposed for 6-7 days at 4° C and developed. After development, the slides were stained with cresyl violet.

Data analysis

The ARH was divided into four subdivisions (ARH-A, B, C, D), as described in the previous studies (Li et al., 1998a; Smith, 1993), using the rat brain atlas of Paxinos and Watson (1982). We have shown previously (Li et al., 1998a; Smith, 1993) that the suckling stimulus only affects the NPY neurons located in the caudal portion of the ARH (ARH-C). On the other hand, even though the suckling stimulus suppresses TH gene expression throughout the entire ARH (Wang et al., 1993), TIDA neurons are most numerous in the rostral portion of the ARH (ARH-A and ARH-B). Thus, the ARH-C containing sections were used for the NPY study and the brain sections containing ARH-A and ARH-B were used for the TH study. Since the suckling-induced NPY expressing neurons in the DMH occupied the same plane as ARH-C (Li et al., 1998a), the tissue sections covering the ARH-C subdivision were also used to analyze NPY gene expression in the DMH.

The coronal brain sections were anatomically matched across animals from all groups. The hybridization signals were quantitated using the HARMONY image analysis system by VIDEK (Rochester, NY). The system identified silver grains by the brightness of the image. An estimate for silver grains over the entire ARH (for ARH-NPY and TH) or the entire DMH (for DMH-NPY) on each

tissue section was given as the area occupied by silver grains within the marked area.

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. Data are presented as mean ± SEM. Differences between groups were evaluated using one-way analysis of variance (ANOVA) and post hoc Scheffe'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 hours of suckling, while the PRL levels remained low in nonsuckled control animals (Fig. 3-1). Bromocriptine treatment effectively blocked the PRL elevation induced by the suckling stimulus (Fig. 3-1).

NPY gene expression in the ARH

In the ARH-C area, 24 hours of suckling caused a significant increase in NPY gene expression compared to the nonsuckled control animals (Fig. 3-2, 3-3). In addition, there was no difference in NPY gene expression between the 8 pups and 8 pups+B groups (Fig. 3-2, 3-3), indicating that inhibition of PRL secretion did not prevent the suckling-induced activation of NPY gene expression in ARH.

NPY gene expression in the DMH

Suckling 8 pups for 24 hours induced the expression of NPY in a population of neurons located around the compact zone in the DMH (Fig. 3-4). This population of NPY neurons was not observed in the nonsuckled control group (0 pups, Fig. 3-4). In the bromocriptine treated group (8 pups+B, Fig. 3-4), the signal appeared to be less intensive when compared to vehicle treated animals (8 pups, Fig. 3-4). Quantitatively, the levels of NPY mRNA signal in both the 8 pups and 8 pups+B groups were significantly higher than that in the 0 pups control (Fig. 3-5). In addition, the NPY mRNA levels in the 8 pups group were significantly greater than those observed in the 8 pups+B group (Fig. 3-5).

It should be noted that low levels of NPY mRNA signal were observed covering the compact zone of the DMH in all the animals examined (Fig. 3-4), and this expression did not change in response to suckling.

TH gene expression in the ARH

In the ARH-A and B areas, TH gene expression was clearly detected in animals with pups removed for 48 hours (0 pups control, Fig. 3-2). The levels of expression were comparable to those found in normal diestrous females (Wang et al.,1993). TH gene expression in both the vehicle and bromocriptine-treated suckled groups (8 pups and 8 pups+B) were significantly lower than the nonsuckled controls (Fig. 3-2, 3-6). In addition, TH mRNA levels in bromocriptine-treated suckled animals (8 pups+B) were significantly higher than the 8 pups group (Fig. 3-2, 3-6).

Discussion

During lactation, the suckling stimulus plays a key role in mediating the alterations in hypothalamic function. The present studies demonstrated that the suckling stimulus alters both NPY gene expression in the ARH and DMH and TH expression in the ARH. In addition, the results from treatment with bromocriptine indicate that suckling-induced PRL may differentially regulate the activity of different hypothalamic regions during lactation

The results of examining ARH NPY gene expression in the present study are in agreement with the results of our earlier studies (Li et al., 1998a; Smith, 1993), 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. In addition, the ineffectiveness of bromocriptine treatment in altering suckling-induced increases in ARH NPY gene expression indicates that suckling-induced hyperprolactinemia is not important in modulating ARH NPY activity. These results are in agreement with reports showing that PRL treatment does not affect ARH NPY gene expression (Pelletier and Tong, 1992), and immunoneutralization of PRL does not reduce NPY expression during lactation (Pape and Tramu, 1996). In addition, because of the inhibition of PRL secretion, milk production in bromocriptine-treated dams would be minimal; therefore, these animals do not experience a significant change in energy balance. These results thus suggest that the activation of NPY neurons in the ARH is mediated by incoming neural impulses activated by suckling, not by changes in energy balance.

In addition to the ARH NPY neurons, the present study also confirmed our earlier report (Li et al., 1998a) that the suckling stimulus activates a second population of NPY neurons located in the DMH. The blunted NPY expression after the inhibition of elevated PRL by bromocriptine treatment suggests that the activation of the DMH NPY neurons is, to a significant degree, mediated by PRL. The full activation of the NPY neurons is likely achieved by the combination of the suckling-induced neuronal inputs to the DMH and the hyperprolactinemia. The mechanism by which PRL modulates NPY activity in DMH is unknown. Identification of prolactin receptors (PRL-R) in the brain (Bakowska and Morrell, 1997; Chiu et al., 1992; Chiu and Wise, 1994; Crumeyrolle-Arias et al., 1993; Poky et al., 1996) suggests that PRL may act directly in the brain to modulate NPY neruonal activity. It has been shown by immunocytochemistry (Poky et al., 1996) and receptor autoautoradiography (Crumeyrolle-Arias et al., 1993) that PRL-R were found in the DMH, whereas *in situ* hybridization failed to show PRL-R positive neurons in this area (Bakowska and Morrell, 1997; Chiu et al., 1992; Chiu and Wise, 1994). These results suggest that PRL's action in DMH may be presynatpic. On the other hand, PRL-R have also been found in the central nuclei of the amygdala, the medial preoptic area, the lateral septum and the periaqueductal gray (Crumeyrolle-Arias et al., 1993; Bakowska and Morrell, 1997) areas, which have been shown to be activated by the suckling stimulus during lactation (Fleming et al., 1994; Lonestein and Stern, 1997; Numan and Numan, 1994, 1995). The expression of PRL-R in these areas raises the possibility that PRL may modulate DMH NPY

neuronal activity indirectly. Currently, there is very little known about the significance of the suckling-activated DMH NPY neurons during lactation. Recently, a retrograde tracing study conducted in our laboratory demonstrated that the suckling-activated DMH NPY neurons project to the paraventricular nucleus of the hypothalamus (PVH) (Li et al., 1998b), suggesting that these NPY neurons may modulate PVH activity during lactation.

The suppression of TIDA neuronal activity during lactation has been previously reported (Demarest et al., 1983; Plotsky et al., 1982; Wang et al., 1993), although the mechanism by which TIDA activity is suppressed is still not understood. The involvement of PRL in regulating the TIDA neurons during lactation has largely been dismissed because the short-loop feedback regulation, in which elevated PRL levels (Demarest et al., 1984; Lerant et al., 1996; Mohankumar et al., 1997) are normally associated with increased TIDA activity, clearly does not exist during lactation. In addition, exogenous PRL administration failed to activate TIDA neurons during lactation (Demarest et al., 1983). Recently, it was shown that inhibition of suckling-induced PRL in midlactation caused a suppression of DOPA accumulation in the median eminence (Arbogast and Voogt, 1996), suggesting that PRL may play a stimulatory role in regulating TIDA activity during lactation. However, the interpretation of the results is complicated by the following concerns: 1) the suppression of DOPA could not be reversed by coadministation of PRL, and 2) the methods used for measuring TIDA activity. TIDA activity was assessed by treating the animals with a decarboxylase inhibitor NSD 1015. After treatment,

the animals were sacrificed and DOPA accumulation in the median eminence fragments was measured (Moore, 1987). However, the midbrain dopaminergic cell groups (A8, A9, A10), other than TIDA neurons, also terminate in the median eminence (Kizer et al., 1976). Therefore, the changes in DOPA may not solely reflect the activity of TIDA neurons.

In the present study, we utilized an acute suckling model to examine the possible role of PRL in regulating TIDA neurons. In this model, the animals were first deprived of pups for 48 hours before receiving 24 hours of resuckling. The period of pup separation allows the TIDA activity, as well as TH gene expression, to recover to basal levels (Hoffman et al., 1994; Wang et al., 1993). Secondly, *in situ* hybridization was utilized so as to be able to specifically study the changes in TIDA neurons. Consistent with previous reports (Arbogast and Voogt, 1996; Wang et al., 1993), this paradigm confirmed that the acute suckling stimulus greatly inhibited TH gene expression. Surprisingly, TH mRNA levels in these neurons were partially restored when hyperprolactinemia was prevented by bromocriptine treatment. These results suggest that, at least in response to the acute effects of suckling, elevated PRL is involved in the suppression of TH gene expression and, possibly, TIDA neuronal activity. Theoretically, the inhibitory effect of PRL on TIDA neurons observed in the present study should ensure that hyperprolactinemia is maintained during lactation. More importantly, the suckling stimulus appears to be critical in changing the stimulatory effects of PRL on TIDA activity into inhibitory effects. The mechanisms by which elevated PRL negatively modulates TH gene

expression are not clear. Recently, it has been shown that TIDA neurons in the ARH express PRL-R (Lerant and Freeman, 1997). This provides anatomical evidence that PRL can affect TIDA neurons directly by binding to its own receptor. On the other hand, it is also possible that PRL can modulate TH expression indirectly through acting on neurons that connect to the TIDA neurons. Anatomical studies have demonstrated the direct contact between POMC-positive neuronal terminals and TIDA cell bodies (Fitzsimmons et al., 1992; Pelletier and Morel, 1986). It has been shown that μ-opioid receptor antagonists can prevent suckling-induced suppression of TIDA activity (Callahan et al., 1996). Furthermore, immunoneutralization of suckling-induced PRL causes a decrease in the number of activated β-endorphin-positive neurons during lactation (Pape et al., 1996). Therefore, it is possible that PRL may modulate TIDA activity by acting through the POMC system in the ARH.

Anatomical and pharmacological evidence also suggest that ARH NPY can directly modulate TH gene expression and TIDA neuronal activity (Guy and Pelletier, 1988; Hong et al., 1995; Pelletier and Simard, 1991). Thus, upon activation by the suckling stimulus, the ARH NPY neurons may directly modulate TH expression in the TIDA neurons. The possibility that PRL may modulate TH expression through the ARH NPY neurons is ruled out by the present studies which show that ARH NPY expression remained elevated regardless of changes in TIDA neurons that were affected by the suppression of PRL. Taken together, these results suggest that during lactation the activity of TIDA neurons is probably modulated by both PRL-dependent and PRL-

independent mechanisms. Thus, we hypothesize that suckling-induced PRL acts either directly on TIDA neurons or indirectly through other systems, such as the POMC system, but not the NPY system, to modulate TH gene expression during lactation (Fig. 3-7). In addition, PRL-independent mechanisms, such as suckling activated ARH NPY neurons, can also modulate TIDA neurons directly (Fig. 3-7). It is plausible that the negative effect of PRL on TH expression is the result of interactions between these two mechanisms. More studies are needed to resolve this issue.

In conclusion, the present study demonstrated that the suckling stimulus activates two populations of NPY neurons and suppresses TIDA neuronal activity in the hypothalamus. Suckling-induced hyperprolactinemia plays a stimulatory role in suckling-activated NPY neurons in the DMH and an inhibitory role in suckling-induced suppression of TIDA activity.

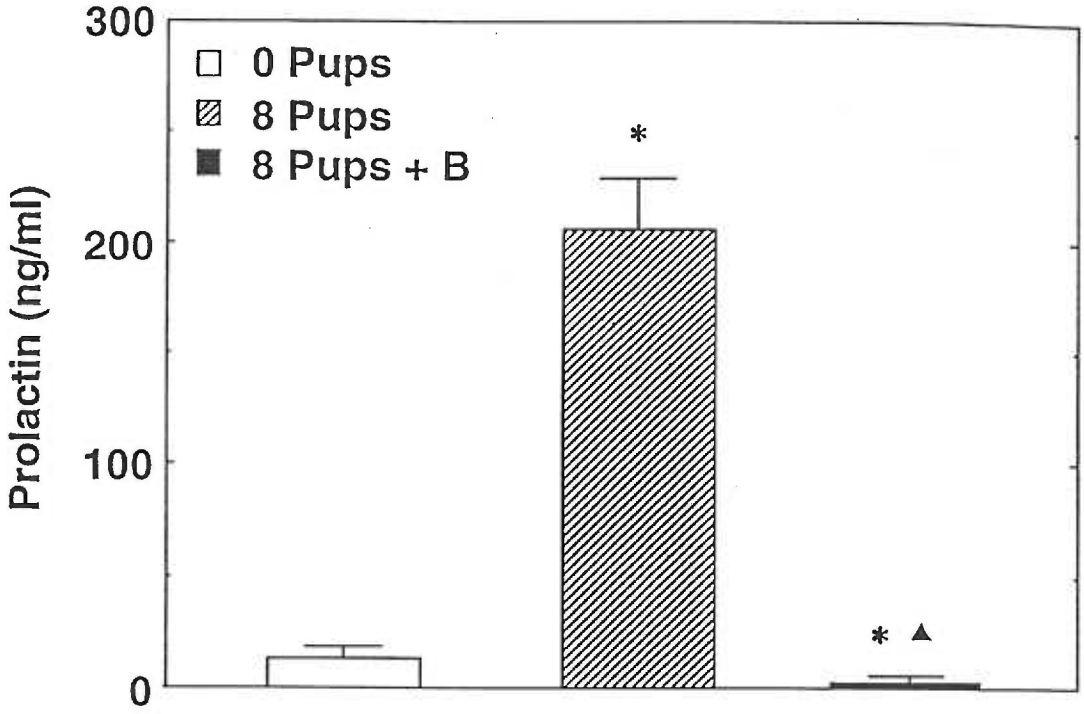
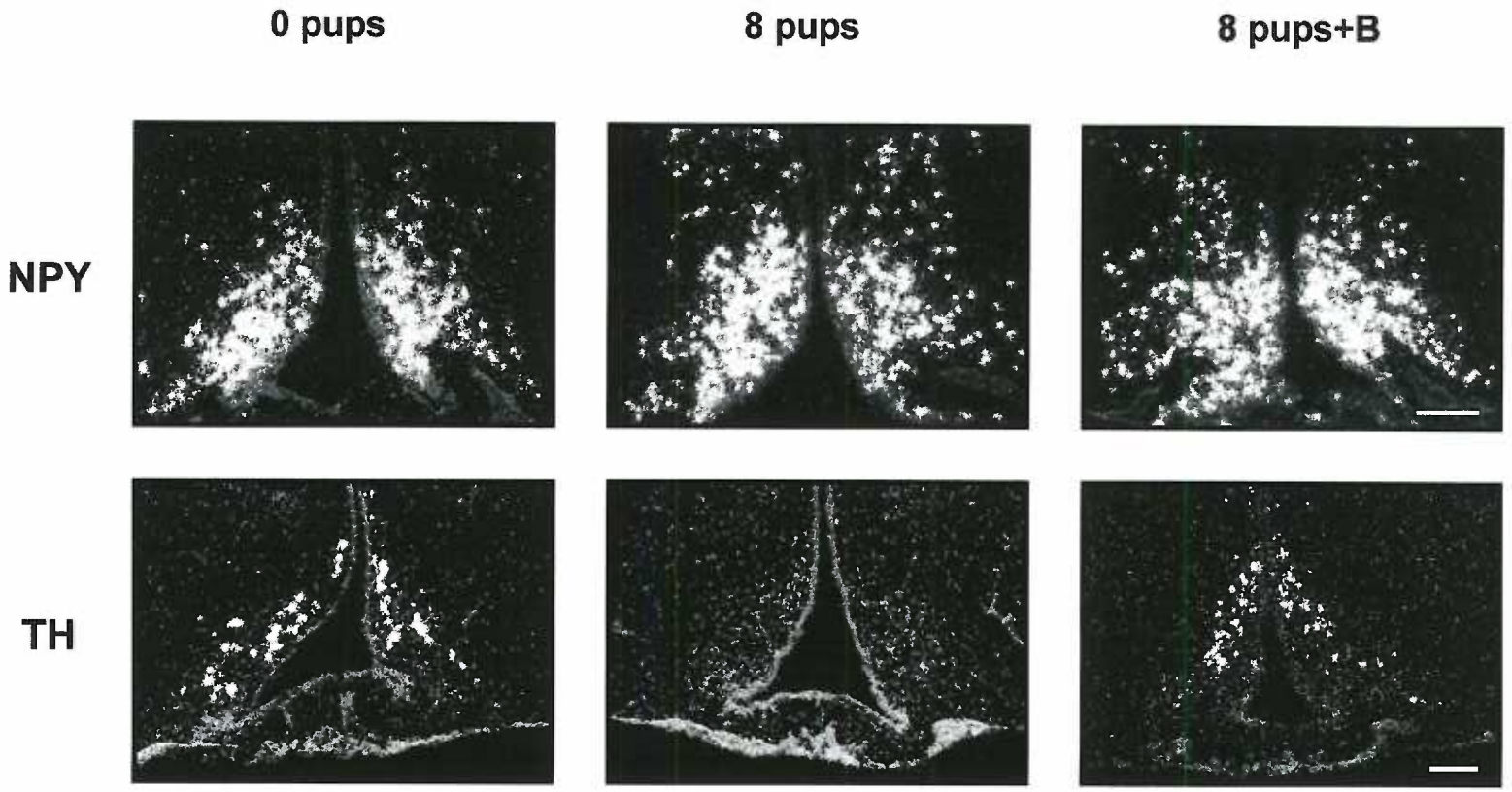


Figure 3-1. Serum PRL levels in the three treatment groups. Animals were deprived of their pups for 48 hours and then were subjected to 24 hours of suckling (8 pups and 8 pups+B) or remained pup-deprived (0 pups control). Bromocriptine treatment (B, 0.5 mg/rat per injection, 2 injections) significantly suppressed suckling-induced PRL secretions. *: Significantly different ($p<0.05$) from 0 pups control. ▲: Significantly different ($p<0.05$) from 8 pups group.


0 pups
8 pups
8 pups+B
NPY
TH

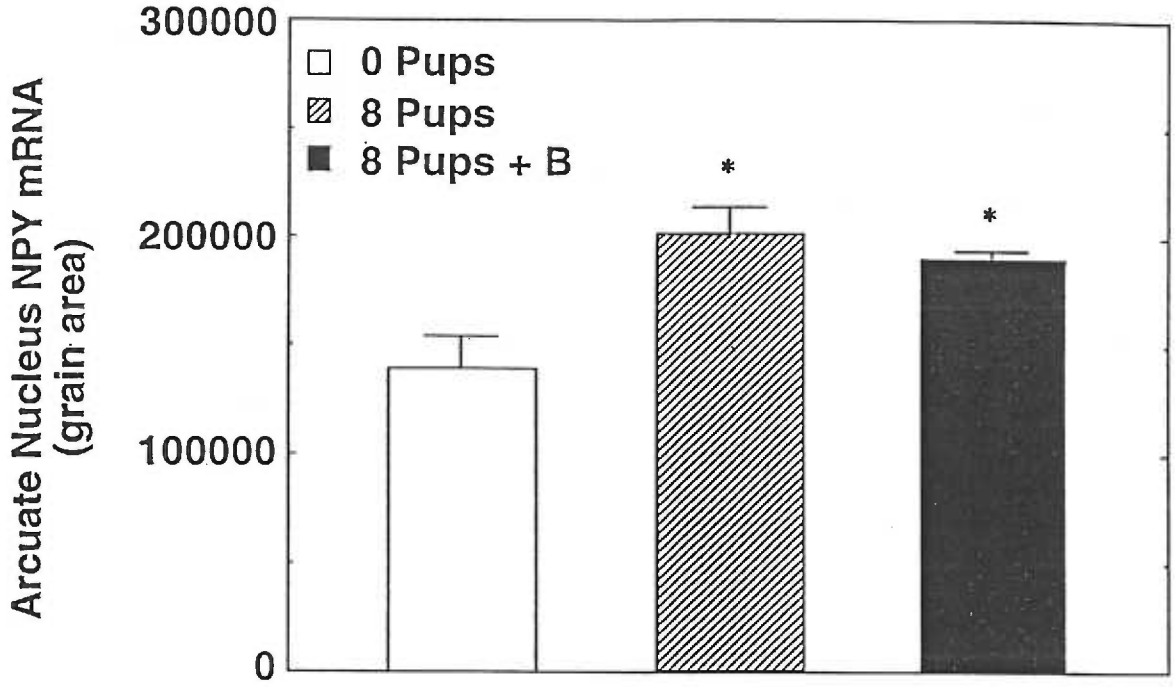
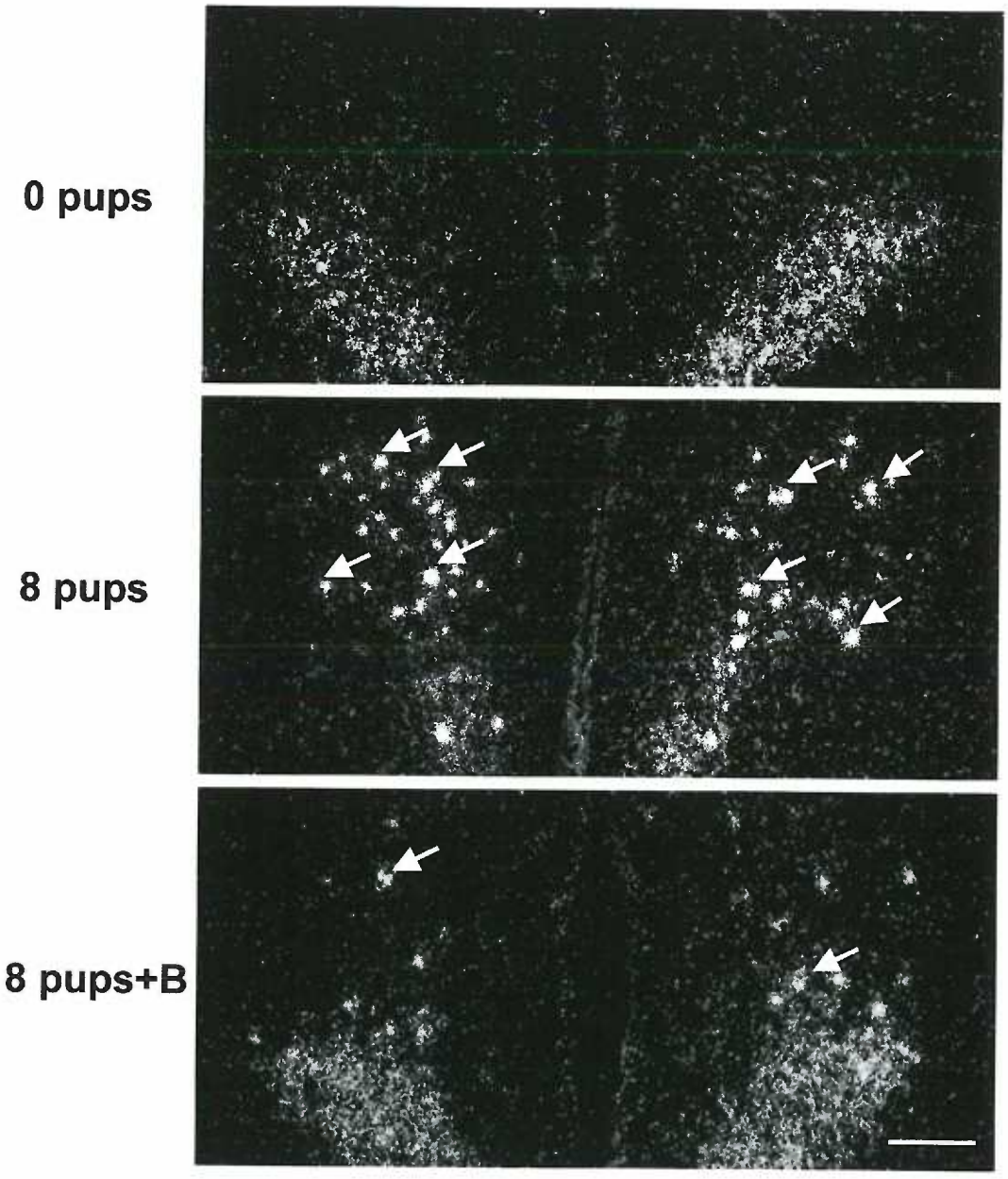


Figure 3-3. NPY mRNA levels in the ARH-C region in the three treatment groups. Acute resuckling for 24 hours, after 48 hours of pup deprivation, induced a significant increase in NPY mRNA levels in this region. Inhibition of PRL secretion by bromocriptine (B) did not prevent the activation of NPY neurons by the suckling stimulus. *: Significantly different ($p<0.05$) from 0 pups control.

NPY



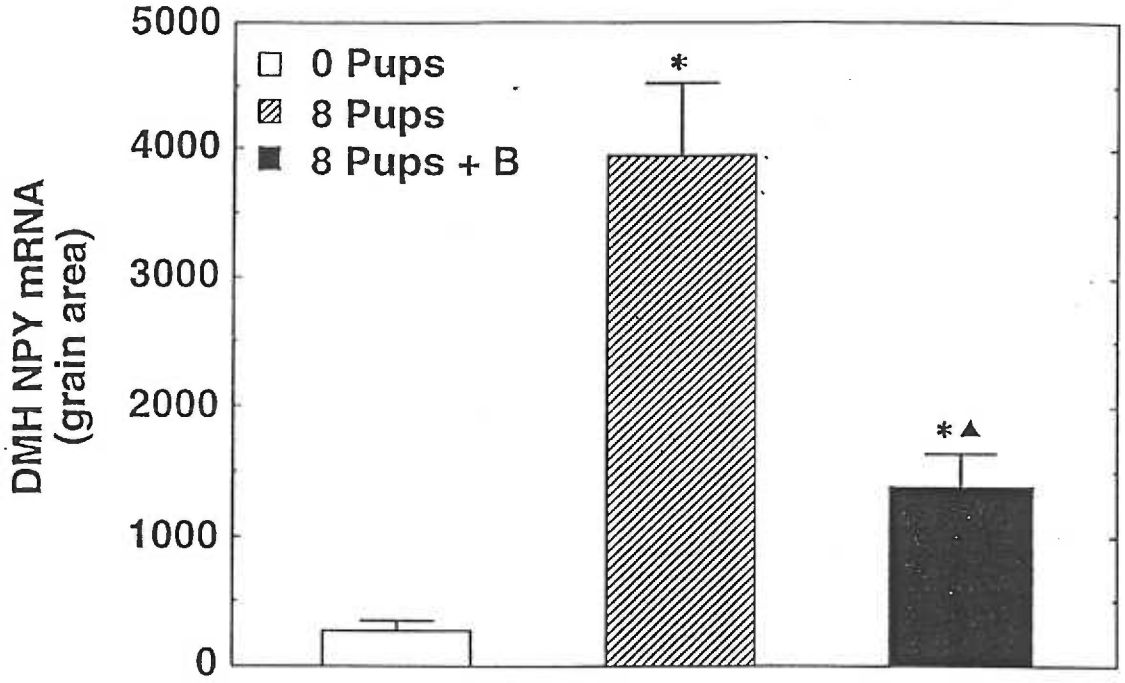


Figure 3-5. NPY mRNA levels in the DMH area in the three treatment groups. Acute resuckling for 24 hours induced a significant increase in NPY mRNA levels in this area, whereas treatment with bromocriptine (B) significantly attenuated NPY gene expression. *: Significantly different ($p<0.05$) from 0 pups control. ▲: Significantly different ($p<0.05$) from 8 pups group.

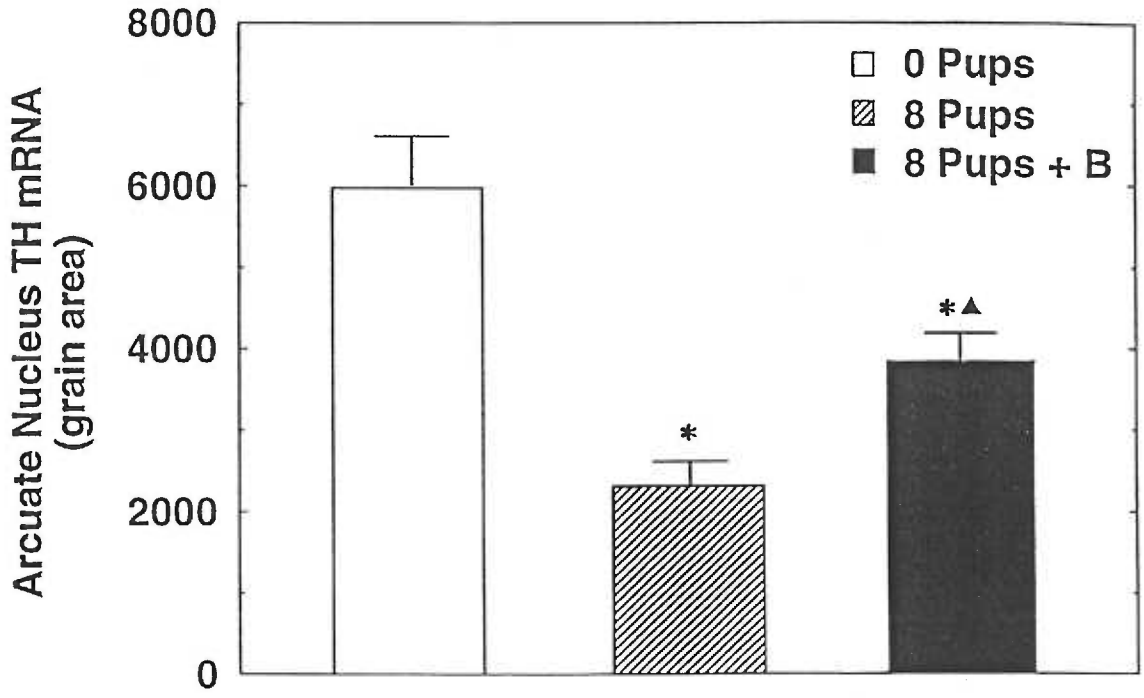


Figure 3-6. TH mRNA levels in the rostral ARH (ARH-A and ARH-B) area in the three treatment groups. TH mRNA levels returned to basal levels in the 0 pups controls, whereas 24 hours of suckling effectively suppressed TH gene expression (8 pups group). Treatment with bromocriptine (B) increased TH mRNA levels compared to the 8 pups group. *: Significantly different ($p<0.05$) from 0 pups control. ▲: Significantly different ($p<0.05$) from 8 pups group.

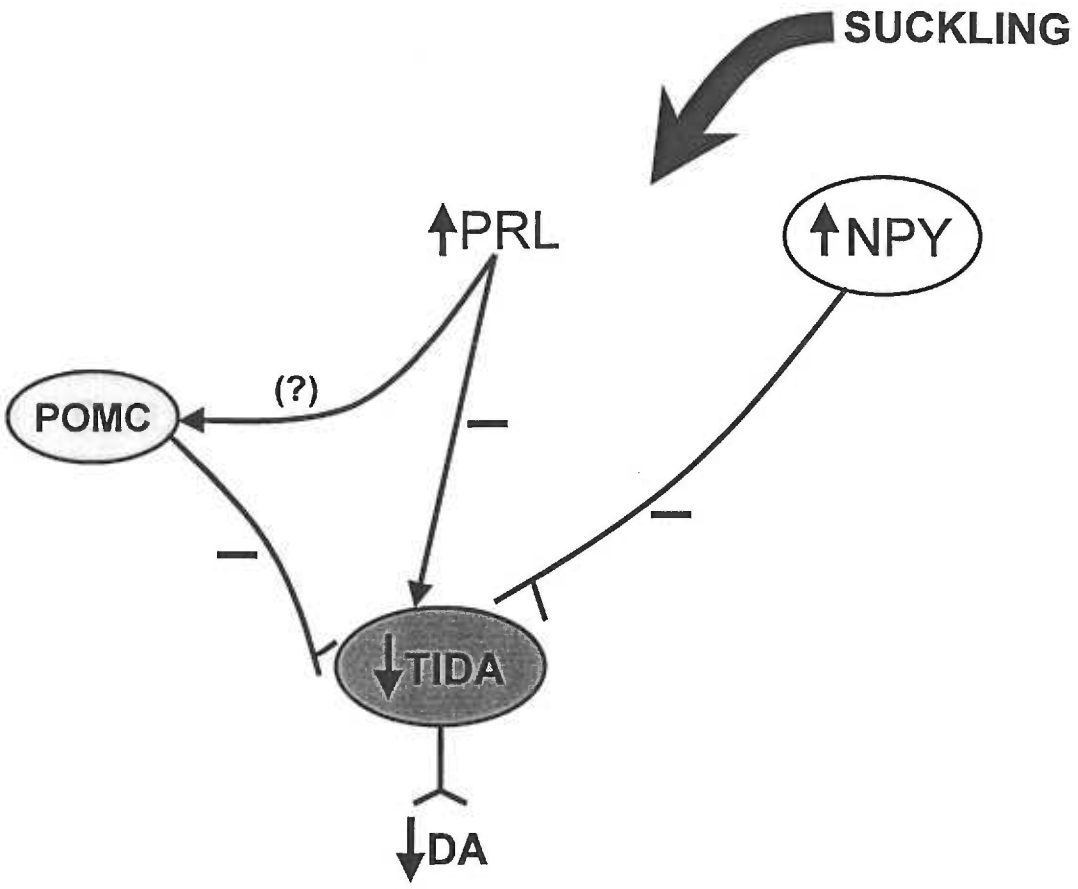


Figure 3-7. A diagrammatic representation of putative pathways in the ARH involved in the modulation of TIDA neurons during lactation. The results of the present study suggest that a PRL-dependent mechanism is involved in the negative modulation of the TIDA neurons. PRL may either act directly on the TIDA neurons or indirectly through other systems in the ARH, such as the POMC system proposed in the diagram, to modulate the TIDA neurons. Also, suckling activated NPY neurons (PRL-independent mechanism) may also directly participate in the modulation of TIDA neurons upon activation by the suckling stimulus.

CHAPTER IV

NEUROPEPTIDE Y (NPY) NEURONS IN THE ARCUATE NUCLEUS (ARH) AND DORSOMEDIAL NUCLEUS (DMH), AREAS ACTIVATED DURING LACTATION, PROJECT TO THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS (PVH)

In press

Regulatory Peptides

Introduction

Neuropeptide Y (NPY) is a 36 amino acid neuropeptide widely expressed in the nervous system including the hypothalamus (De Quidt et al., 1990). Most of the NPY neurons in the hypothalamus are found in the arcuate nucleus of the hypothalamus (ARH) (De Quidt et al., 1990; Gehlet et al., 1987). The hypothalamic NPY system has been shown to be involved in a variety of physiological functions, including food intake (Lee et al., 1994; Tomaszuk et al., 1996) and reproduction (Kalra and Crowley, 1992; Kalra and Kalra, 1996).

It has been shown that during lactation, a condition characterized by hyperphagia and a suppression of reproductive cyclicity (McNeilly, 1994; Pickavance et al., 1996; Vernon and Flint, 1984), NPY neuronal activity was significantly increased in two discrete areas of the hypothalamus: the arcuate nucleus (ARH) and the dorsomedial nucleus (DMH) (Smith, 1993). Interestingly, the increase in NPY activity in the ARH was observed only in the caudal portion of the ARH, even though NPY-positive neurons are detected throughout the ARH (De Quidt et al., 1990). This differential increase in NPY activity in the ARH raised the possibility that NPY neurons in different portions of the ARH may participate in modulating different physiological functions. One possible way to achieve this would be if NPY neurons from different portions of the ARH project to different areas.

The paraventricular nucleus of the hypothalamus (PVH) has been shown to receive extensive NPY innervation from ARH NPY neurons (Bai et al., 1985; Baker and Herkenham, 1995). However, little is known about the NPY projection

patterns from different portions of the ARH into the PVH. Functionally, the ARH-PVH NPY system has been suggested to play an important role in modulating food intake (Akabayashi et al., 1994; Kalra et al., 1991; White and Kershaw, 1990). Thus, it is possible that NPY neurons in the caudal portion but not in the rostral portion of the ARH project to the PVH and may mediate the hyperphagia occurring during lactation.

The function of the NPY neurons in the DMH area that are activated during lactation is unknown. In the female rat, this population of NPY neurons does not appear to be activated under basal condition (Smith, 1993), and suckling is the only stimulus that has been shown to activate them (Li et al., 1998a). Using anterograde tracing techniques, neurons in the DMH were shown to project to the PVH area (Thompson et al., 1996). This evidence suggests that NPY neurons in the DMH area may also project to the PVH as well.

Thus, the objectives of the present study in lactating females were to determine (1) the pattern of NPY projections from different regions of the ARH into the PVH and (2) if PVH is one of the targets of the NPY neurons in the DMH area.

Methods and Materials

Animals

Day 18-19 pregnant Sprague-Dawley rats (B & K Universal Inc., Kent, WA) were housed individually and maintained under a 12:12 light-dark cycle (lights on at 0700-1900) 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 as day 0 postpartum. Lactating animals had their litters adjusted to eight pups on day 2 postpartum. All the animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee.

Retrograde tracer injection

On day 4 postpartum, animals were anesthetized with tribromoethanol (20 mg/100 g body weight) and placed in a stereotaxic apparatus. A glass micropipette with tip diameter of 25-30 μm was filled with the retrograde tracer, fluorogold (FG, 2% w/v, in physiological saline), and inserted into the parvocellular region of the PVH (PVHp). Injection coordinates were 1.8 mm caudal, 0.3 mm lateral to the bregma, and 7.35 mm below the dura, according to the atlas of Paxinos and Watson (1982). FG was injected by iontophoresis with 5 μA current, pulsed at 7 sec intervals for 20 min. The glass pipette was left *in situ* for an additional 10 min to avoid the spread of tracer along the pipette track. The animals were then returned to their 8-pup litters.

Perfusion and tissue sectioning

On day 11 postpartum, animals were anesthetized with an overdose of pentobarbital (125 mg / Kg B.W., i.p.) and perfused transcardially with 150 ml of 2% sodium nitrite in saline followed by 350 ml 3.8% of borax in 4% paraformaldehyde (pH 9.5). The brain was removed and immersed in 25% sucrose at 4 °C for 6 hours. Coronal sections (25 μm) through the whole ARH were cut on a sliding microtome and collected in a one-in-four series. As a control for the retrograde tracing procedure, horizontal sections through the brainstem were also collected for analysis of FG-labeling in the ventrolateral medulla (VLM), an area shown previously to send projections to the PVN (19). The tissue sections were stored at -20 °C until use in multiwell tissue culture plates containing cryoprotectant.

Immunocytochemistry procedures

Tissue sections from all animals were processed in one assay to ensure uniformity of immunostaining. All the solutions were treated with DEPC to prevent RNase contamination. Tissue sections were removed from the cryoprotectant and rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by treatment with 1% $NaBH_4$ -KPBS solution (Sigma, St. Louis MO). Sections were incubated in rabbit anti-FG antibody (1:30,000; Chemicon, Temecula CA) in KPBS with 0.4% Triton X-100 at room temperature for 1 hr, followed by 4°C for 48 hr. After the incubation, the tissues were rinsed in KPBS and incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, 1:600) in KPBS with 0.4%

Triton X-100 for 1 hr at room temperature. This was followed by another 1 hr incubation at room temperature in avidin-biotin complex solution (4.5 μl of A and B each per ml of KPBS-0.4% Triton X-100 (Vectastain ABC Elite Kit, Vector Laboratories)). The FG antibody-peroxidase complex was visualized with a mixture of 3,3 diaminobenzidine (0.2 mg/ml) and 3% H_2O_2 (0.83 μl/ml) in 0.05 M Tris buffer-saline solution. Following the FG staining, tissue sections were mounted on polylysine coated glass slides, air dried and processed for *in situ* hybridization.

In situ hybridization

NPY RNA probe synthesis and the specificity of the cRNA probe and procedure for *in situ* hybridization have been described previously (Smith, 1993). Briefly, the NPY cRNA probe was transcribed from a 511 bp cDNA in which 21% of the UTP was ^{35}S-labeled (Dupont NEN, Boston MA). The saturating concentration of the probe used in the assay was 0.3 μg/ml·Kb. The specific activity of the probe ranged from 1-3 x 10^8 dpm/μg. The brain sections were fixed in 4% paraformaldehyde, digested with proteinase K (10 μg/ml, Sigma), 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 alcohol, delipidated in chloroform, rehydrated through a second series of alcohol, and then air dried. The slides were exposed to the NPY cRNA probe overnight in moist chambers at 55° C. After incubation, the slides were washed in SSC that increased in stringency, in RNase, in 0.1X SSC at 60° C, and rehydrated through

a graded series of alcohol and dried. Slides were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY), exposed for 6-7 days at 4° C and developed.

Data analysis

Semiquantification of FG cells.

The ARH was divided into two subdivisions using the rat brain atlas of Paxinos and Watson (1982). The rostral portion began with the retrochiasmatic area to the beginning of the DMH (plate 19-20). The caudal portion continued caudally through the DMH and to the end of the ARH (plate 21-22).

FG-positive and FG-NPY double-labeled cells in the two subdivisions were identified and counted respectively. A FG-labeled neuron was considered to be double-labeled with NPY if the number of silver grains on top of the cell body was greater than 3 times the background level. Data are presented as mean±SEM. Differences between subdivisions were evaluated using Student's t test. Differences were considered significant if $p<0.05$.

NPY mRNA in the ARH

NPY mRNA in the two ARH subdivisions was quantitated using the VIDEK HARMONY image analysis system by VIDEK (Rochester, NY). The system identified silver grains by the brightness of the image. An estimate for silver grains over the entire ARH on each tissue section was given as the area occupied by silver grains within the marked area. The marked area was constant (1.0 mm x 1.6 mm) for each reading and included the entire ARH. The area occupied by silver grains was typically between 5 - 15% of the marked area. The

data for NPY mRNA in the ARH were expressed as the area occupied by grains per section. The mean area occupied by grains per section for each subdivision of the ARH was determined for each animal. Data are presented as mean±SEM. Differences between subdivisions were evaluated using Student's t test. Differences were considered significant if $p<0.05$.

Results

Retrograde labeling

For semiquantitative retrograde labeling analysis, only the lactating animals in which the injection site was centered in the parvocellular region of the PVH (PVHp) were used (n=5). As shown in Fig. 4-1, the unilateral injection of FG was confined in the region of the PVHp. In addition, the pattern of retrograde labeling was similar in all of the animals with injections centered in the PVHp area.

It has been shown by Sawchenko et al. (1985) that NPY neurons in the ventrolateral medulla (VLM) of the brainstem project to the PVH area. Thus, to serve as a validation of the injection to the PVHp, both FG and NPY mRNA were visualized in the VLM. It was found that in animals in which the injection site was localized in the PVHp area, there were substantial numbers of FG-labeled NPY cells (averaging 70-90% of FG labeled cells) observed in the VLM (Fig. 4-2).

Retrograde labeling and NPY mRNA levels in the ARH

In the ARH, the FG-labeled cells were found throughout the ARH with a higher density of retrogradely labeled cells observed in the caudal portion of the ARH (# FG-labeled cells/section; rostral, 36.4 $\pm$ 4.1 vs. caudal, 84.1 $\pm$ 6.5, $p<0.05$, Fig. 4-3). When the NPY mRNA signal represented by silver grains was viewed simultaneously with the bright field FG image in the same area, it was found that FG-NPY double-labeled neurons were observed both in the rostral and caudal portion of the ARH (Fig. 4-4). However, there were approximately three times more FG-NPY double-labeled neurons in the caudal portion than in

the rostral portion (# FG-NPY double-labeled cells/section: rostral, 12.6 ± 1.7 vs. caudal, 33 ± 3.2, $p<0.05$, Fig. 4-3). When NPY mRNA levels were determined in the ARH, it was found that the mRNA levels (area occupied by silver grains) in the caudal portion of the ARH were about 2.5 fold higher than those in the rostral portion (area occupied by silver grains: rostral, 45883 ± 2712 vs. caudal, 103537 ± 10265; $p<0.05$). This suggests that there were more NPY positive cells in the caudal than the rostral portion of the ARH. Thus, using the respective NPY mRNA levels to normalize the results of FG-NPY double-labeling in both divisions, it was found that the relative proportion of double-labeled cells was similar in the rostral (10 ± 1.5 %) and caudal (12.8 ± 1.7 %) ARH.

Retrograde labeling and NPY double labeling in the DMH

It has been shown that during lactation, the suckling stimulus not only activates NPY neurons in the caudal portion of the ARH, but it also induces expression of NPY in a population of neurons in the DMH area (Li et al., 1998a; Smith, 1993). As shown in Fig. 4-5A, this population of suckling-induced NPY neurons was located dorsolateral to the compact zone of the DMH. Interestingly, many FG positive neurons were also observed in the whole DMH area. Under higher magnification (Fig. 4-5B), it was found that the majority of NPY positive neurons in the DMH area were double-labeled with FG (Fig. 4-5B,C), indicating this population of NPY neurons also sends projections into the PVHp area.

Discussion

In the present study, a combination of retrograde tracing and *in situ* hybridization was used for detecting NPY positive neurons that project to the PVN. The high percentage of neurons double labeled with FG and NPY in the VLM, an area shown previously to send projections to the PVN, provides validation of the technique. It was found that NPY neurons from both rostral and caudal portions of the ARH project to the PVHp area. However, there are significantly more NPY neurons from the caudal portion that send projections into the PVHp area than from the rostral portion. The smaller number of NPY neurons from rostral portion projecting to PVHp is probably due to fewer NPY neurons found in the rostral portion of the ARH. This idea was supported by the observation that there was an approximately 2.5 fold greater expression of NPY mRNA in the caudal portion of the ARH than in the rostral portion. Using NPY mRNA levels as a measure of the number of NPY neurons found in the respective portions of the ARH, it was found that the relative proportion of the double-labeled neurons was similar in the two subdivisions of the ARH. This result suggests that there is no apparent topographical difference in ARH NPY projections into the PVHp area.

Nonetheless, two important observations suggest that NPY neurons in the caudal portion of the ARH are important in modulating activity of PVHp during lactation: (1) there are significantly more NPY neurons from the caudal portion projecting into the PVHp area, suggesting that the caudal NPY population will have a greater influence on PVHp activity than that of the rostral portion and (2)

only the caudal NPY population was activated by the suckling stimulus during lactation. Therefore, it is very likely that the lactation-activated NPY neurons in the caudal portion of the ARH project to the PVHp and will have the greatest effect in modulating the PVHp activity during lactation.

The function of increased NPY input from the caudal ARH into the PVHp during lactation remains to be addressed. One likely function is in regulating the increase in food intake occurring during lactation (Malabu et al., 1994; Pickavance et al., 1996; Wilding et al., 1997). It has been suggested that one possible system in the PVH modulated by NPY and causing hyperphagia is the corticotropin-releasing factor (CRF) system. This is based on the following observations: (1) alpha-helical CRF, a CRF antagonist, can potentiate the feeding response elicited by central administration of NPY (Heinrichs et al., 1993), (2) anatomically, NPY has been shown to innervate CRF positive neurons in the PVHp area (Liposits et al., 1988; Wahlestedt et al., 1987), and (3) PVH injections of NPY cause an increase in the plasma levels of ACTH and corticosterone (Wahlestedt et al., 1987). However, the downstream targets of the feeding responsive CRF neurons have not been studied. The PVH-CRF system has also been suggested to play a role in modulating reproductive function (Rivest and Rivest, 1993). Thus, it is possible that during lactation the NPY system may play a role in the suppression of reproductive cyclicity by modulating PVH-CRF activity. More studies are needed to further elucidate the involvement of the NPY-CRF system in modulating food intake and reproductive function during lactation.

In addition to the NPY neurons in the caudal portion of the ARH, the present study also demonstrated that the population of NPY neurons located in the DMH area and activated during lactation also projects to the PVHp area. Currently little is known about the significance of the DMH NPY neurons during lactation. In the female rat, this population of NPY neurons does not appear to be activated under basal conditions, nor in response to stimuli that activate NPY neurons in the ARH, such as food deprivation (Marks et al., 1992; White and Kershaw, 1990; and unpublished observations) and streptozotocin-induced diabetes (Marks et al., 1993). The present study provides the first anatomical evidence that when they are activated during lactation, these NPY neurons can directly participate in modulating neuronal activity of PVH.

Recently a similar pattern of NPY expression as observed in the DMH area in lactating animals has been reported in two obese mouse models: the agouti mouse and the melanocortin-4 receptor (MC-4 R) knock-out mouse (Huszar et al., 1997; Kesterson et al., 1997). Interestingly, the NPY system in the ARH appeared to be normal in these two mouse models. The results from the obese mouse model studies raise the possibility that the NPY neurons in the DMH area may play an important role in mediating energy metabolism and the feeding response. Thus, it is possible that the NPY neurons in the DMH area may also participate in modulating the increase in food intake during lactation. In addition, the data from the present study provides anatomical evidence that the DMH NPY neurons may be mediating those effects in part through input to the PVH neuronal system.

The results from the present study raise some important questions regarding the functional role of NPY in modulating PVH activity during lactation. First, in addition to the caudal ARH NPY neurons, the DMH NPY neurons provide another source of NPY input into the PVH during lactation. Thus, the level of NPY input to the PVHp during lactation may be even higher than conditions such as food deprivation, in which only the caudal ARH NPY neurons are activated. This suggests that different levels of NPY may modulate PVH activity differently. This idea is supported by the data from *in vitro* electrophysiological studies showing that higher concentrations of NPY decreased PVH neuronal discharge rate, whereas lower concentrations typically increased the discharge rates. (Alberts et al.,1990; Aramakis et al., 1996). Secondly, the phenotypes of the neuronal systems in the PVHp modulated by the DMH NPY neurons remain to be determined as are the possible interactions between the ARH-NPY and DMH-NPY systems in regulating PVHp activity during lactation. Taken together, this information will greatly facilitate an understanding of the mechanisms by which PVH activity is regulated by NPY during lactation.

Important questions remain as to the nature of the signals induced by suckling that are responsible for activating hypothalamic NPY neurons. The neural impulses from suckling appear to ascend through the brainstem to the thalamus and enter the hypothalamus by ventral and dorsal route (Wakerley et al., 1994), resulting in the secretion of oxytocin and prolactin. Thus, it is possible that neural signals or the elevated level of hormones induced by the suckling

stimulus, or both, could be responsible for the activation of specific populations of NPY neurons.

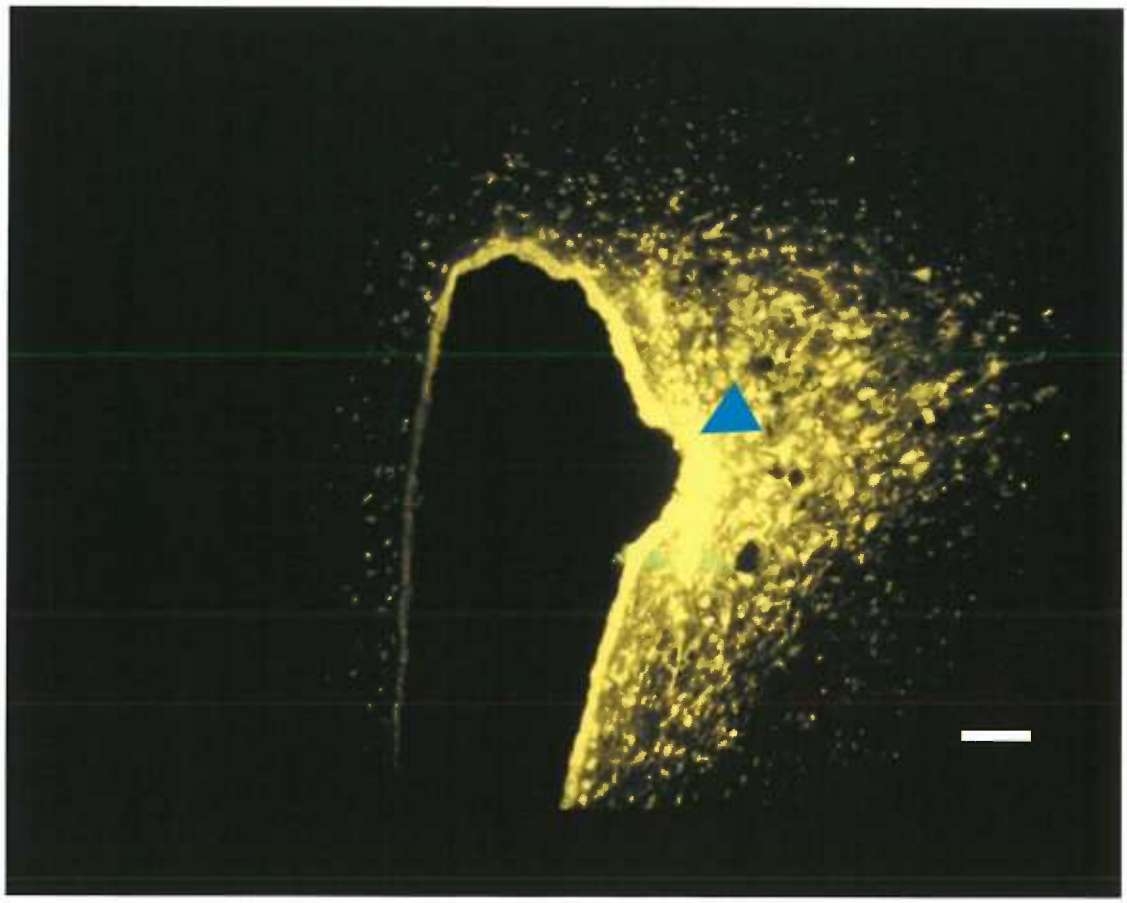


Figure 4-1. Representative fluorescent photomicrograph showing the fluorogold injection site (arrowhead). The unilateral injection was confined to the parvocellular portion of the PVH, as determined by well established landmarks. Scale bar = 20 μm.

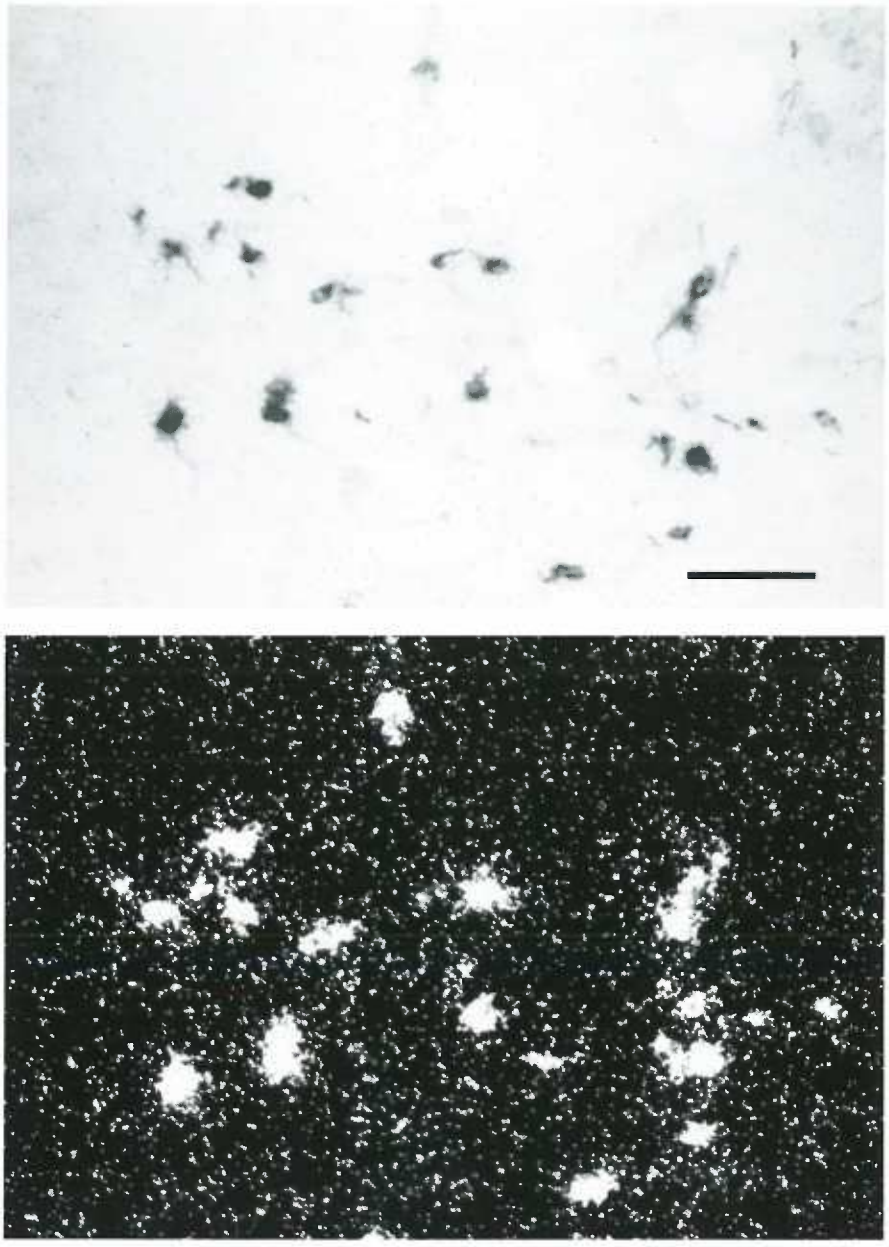


Figure 4-2. Top panel: Bright field image showing FG-positive neurons in the ventrolateral medulla area. Bottom panel: Dark field image showing the same area in which the NPY-positive neurons were identified by clusters of silver grains. It was observed that the majority of the FG-positive neurons were also NPY positive. Scale bar = 10 μm.

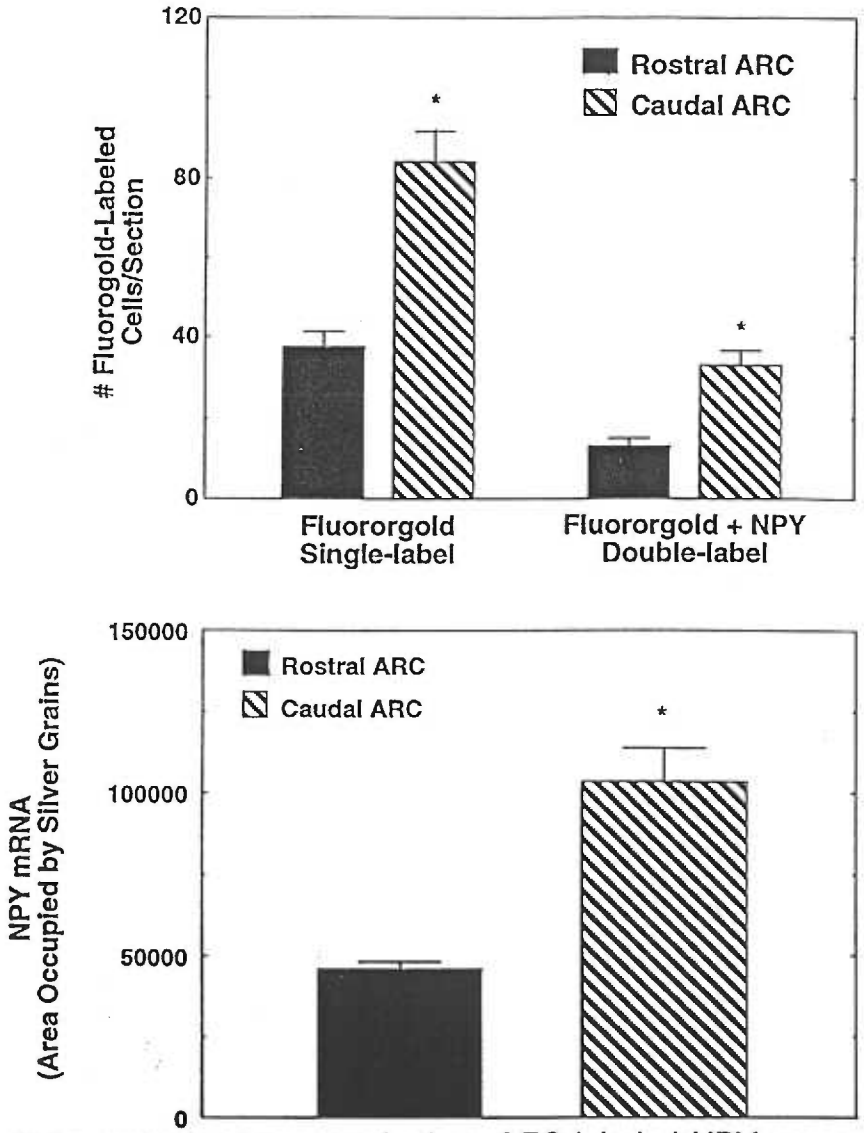
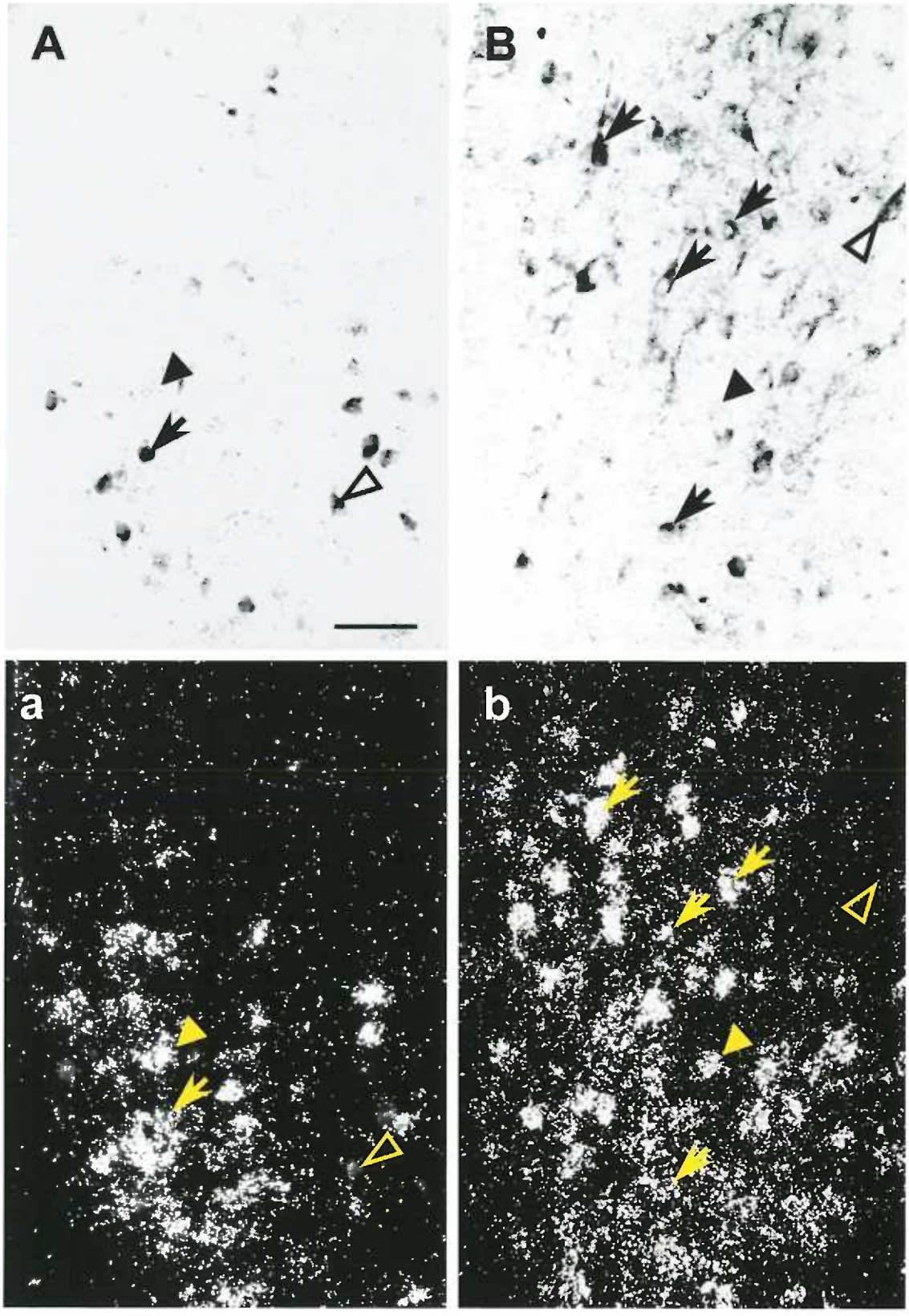
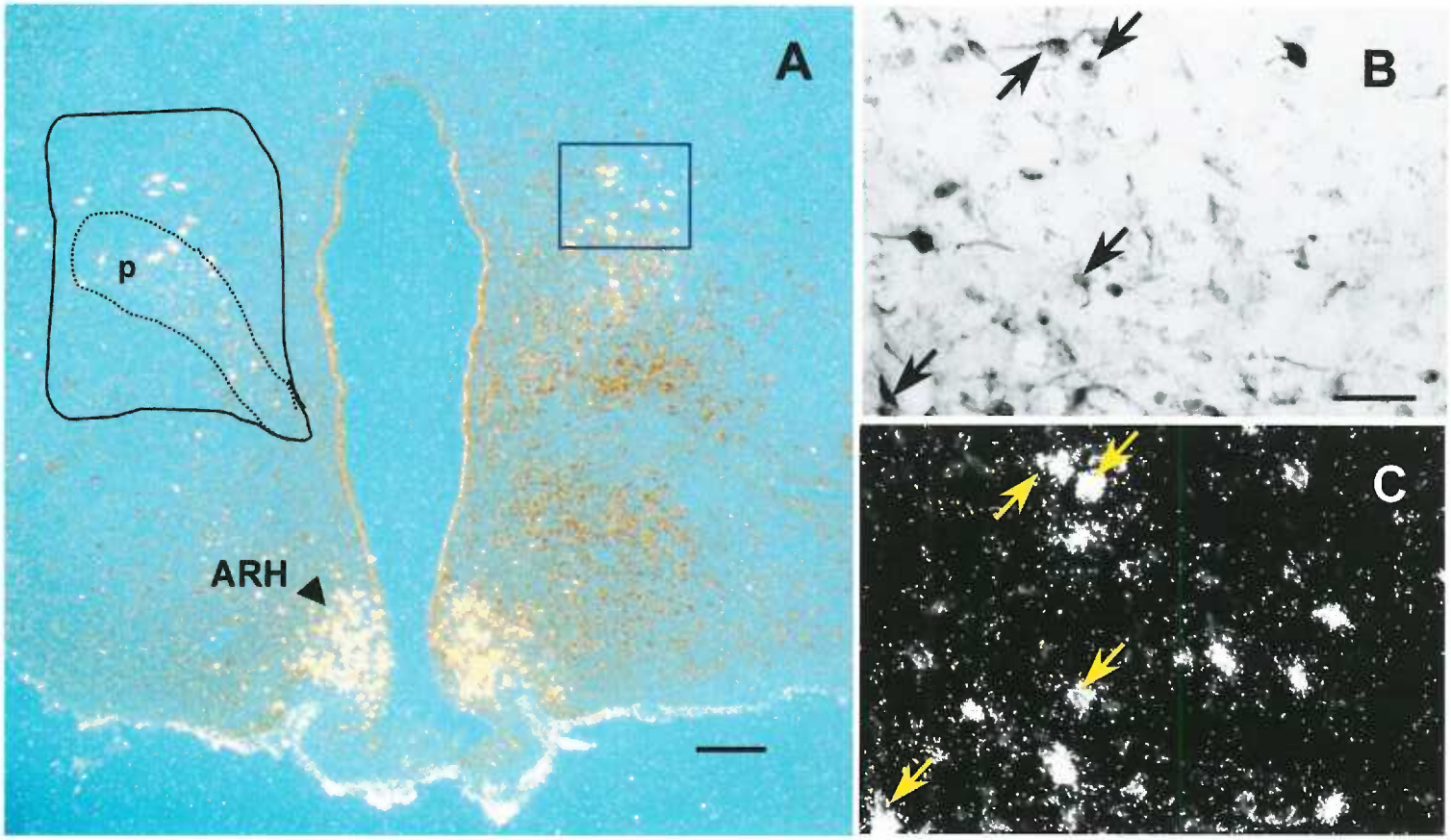


Figure 4-3. Data analysis of the distribution of FG-labeled NPY neurons in the ARH. Top panel: Rostral vs. caudal distribution of the number of FG single-labeled cells per section and FG and NPY double-labeled cells per section. The number of both single- and double-labeled cells were greater (~2.5 fold) in the caudal portion compared to the rostral portion of the ARH. Bottom panel: NPY mRNA content in the two subdivisions of the ARH. The data from each subdivision (mean ± SEM) represent the average area occupied by silver grains per section. *: Indicates $p<0.05$ compared to the rostral ARH.


A
B
a
b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CHAPTER V

IDENTIFICATION OF EFFERENT TARGET AREAS FOR NPY NEURONS IN THE ARCUATE NUCLEUS (ARH): AN ANTEROGRADE TRACING STUDY

Manuscript in preparation

Introduction

Neuropeptide Y (NPY) is a 36 amino acid neuropeptide widely expressed in the rat brain, including the hypothalamus. In the hypothalamus, the largest population of NPY neurons is found in the arcuate nucleus of the hypothalamus (ARH). The function of the ARH NPY neurons has been studied extensively and has been shown to be related to a variety of physiological functions, including food intake (Tomaszuk et al., 1996) and reproduction (Kalra and Crowley, 1992; Kalra and Kalra, 1996).

It has been shown in our laboratory and others that during lactation, a condition characterized by hyperphagia and a suppression of reproductive cyclicity (MeNeilly, 1994; Pickavance et al., 1996; Vernon and Flint, 1984), NPY neuronal activity and NPY levels are significantly increased only in the caudal portion of the ARH, even through NPY-positive neurons are detected throughout the ARH (De Quidt et al., 1990). The results suggested that NPY neurons in the caudal portion rather than the rostral portion of the ARH are important in mediating some of the physiological adaptations during lactation, such as the increase in food intake.

Currently, no detailed study has been done to carefully examine the caudal ARH NPY projections in the brain. Thus, the downstream systems targeted by the caudal ARH NPY neurons that may be important in mediating NPY's effects have not been identified. Retrograde tracing or lesion studies have suggested areas such as the medial preoptic area (mPOA) (McShane et al., 1994) and the paraventricular nucleus (PVH) (Baker and Herkenham, 1995) receive NPY

projections from the ARH, although it is still not clear what the phenotype of the neuronal systems are in those areas that may be directly modulated by ARH NPY input. In order to specifically identify the downstream systems targeted by the caudal ARH NPY neurons, the present study utilized the anterograde tracer, *Paseolus vulgaris* leucoagglutinin (PHA-L), in combination with immunofluorescence staining, to study the projections of the ARH NPY neurons in the brain. In addition, multiple immunostaining was performed to examine whether GnRH and CRF neuronal systems in the hypothalamus are the direct downstream targets for the caudal ARH NPY neurons.

Materials and Methods

Animals

Day 18-19 pregnant Sprague-Dawley rats (B & K Universal Inc., Kent, WA) were housed individually and maintained under a 12:12 light-dark cycle (lights on at 0700-1900) 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 as day 0 postpartum. Lactating animals had their litters adjusted to eight pups on day 2 postpartum. All the animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee.

Anterograde tracer injection

On day 2 postpartum, animals were anesthetized with tribromoethanol (20 mg/100 g body weight) and placed in a stereotaxic apparatus. A glass micropipette with a tip diameter of 5-10 μm was filled with the anterograde tracer, PHA-L (2.5% w/v, in filtered phosphate buffer), and inserted into the region of the caudal ARH. Injection coordinates were 3.0-3.3 mm caudal, 0.2 mm lateral to the bregma, and 9.35 mm below the dura for the caudal portion of the ARH, according to the atlas of Paxinos and Watson (1982). PHA-L was injected by iontophoresis with 5 μA current, pulsed at 7 sec intervals for 20 min. The glass pipette was left *in situ* for an additional 5 min before retrieving. The animals were then returned to their 8-pup litters.

Perfusion and tissue sectioning

On day 13 postpartum, animals were anesthetized with an overdose of pentobarbital (125 mg / Kg B.W., i.p.) and perfused transcardially with 150 ml of 2% sodium nitrite in saline followed by 350 ml 3.8% of borax in 4% paraformaldehyde (pH 9.5). The brain was removed and immersed in 25% sucrose at 4 °C for 6 hours. Coronal sections for the whole brain and horizontal sections for the brainstem (both at 25 μm) were cut on a sliding microtome and collected in a one-in-four series. The tissue sections were stored until use at -20 °C in multiwell tissue culture plates containing cryoprotectant.

Immunocytochemistry procedures

Verification of injection site. Tissue sections containing the ARH were rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) several times and incubated in rabbit anti-PHA-L antibody (1:500; DAKO; Carpinteria, CA) in KPBS with 0.4% Triton X-100 at room temperature for 1 hour. After the incubation, the tissues were rinsed with KPBS and incubated in FITC-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) for 1 hour. After incubation, the sections were rinsed, mounted on glass slides, air dried and coverslipped with buffered glycerol. The staining was examined under epifluorescence microscopy. Only animals with injection sites centered in the ARH were used in the multiple immunofluorescence studies.

Identification of tracer-labeled fibers. Tissue sections were removed from the cryoprotectant and rinsed in KPBS followed by treatment with 1% $NaBH_4$ -

KPBS solution (Sigma, St. Louis MO). Sections were incubated in rabbit anti-PHA-L antibody (1:2500) in KPBS with 0.4% Triton X-100 at room temperature for 1 hr, followed by 4°C for 48 hr. After the incubation, the tissues were rinsed in KPBS and incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, 1:600) in KPBS with 0.4% Triton X-100 for 1 hr at room temperature. This was followed by another 1 hr incubation at room temperature in avidin-biotin complex solution [4.5 μl of A and B each per ml of KPBS-0.4% Triton X-100 (Vectastain ABC Elite Kit, Vector Laboratories)]. The PHA-L antibody-peroxidase complex was visualized with a mixture of 3,3 diaminobenzidine (0.2 mg/ml) and 3% H_2O_2 (0.83 μl/ml) in 0.05 M Tris buffer-saline solution. Following the staining, tissue sections were mounted on glass slides and air dried. The staining on the tissue sections was then OsO_4 intensified before coverslipping with histomount.

Multiple label immunofluorescence. For GnRH triple labeling, the tissue sections were incubated in a mixture of rabbit anti-PHA-L antibody (1:1000), goat anti-NPY antiserum (1:4000, gift from Dr. P.J. Larsen) and the mouse anti-GnRH antibody (1:600, gift from Dr. H.F. Urbanski) in KPBS with 0.4% Triton X-100 and 5% nonfat milk at room temperature for 1 hr, followed by 4°C for 48 hr. After several washes with KPBS, the sections were incubated at room temperature for 1 hour in a mixture of affinity purified secondary antibodies (Jackson ImmunoResearch): donkey anti-rabbit IgG conjugated with biotin (1:600), donkey anti-goat IgG conjugated with tetramethyl rhodamine (TRITC, 1:300), and donkey anti-mouse IgG conjugated with indodicarbocyanine (Cy5,

1:300). This was followed by incubation in avidin-conjugated fluorescein isothiocyanate (FITC, 1:1000) for 1 hour. After the incubation the sections were rinsed, mounted, and coverslipped with buffered glycerol.

For CRF triple labeling, tissue sections were treated in antigen retrieval solution (Biogenex, San Ramon, CA) at 90^0C for 10 min before incubation in the mixture of primary antibodies. The antibody mixture contained rabbit anti-PHA-L antibody (1:2000), goat anti-NPY antiserum (1:4000) and mouse anti-CRF antibody (1:1000, Biogenesis; England, UK). After the incubation, the sections were rinsed and then incubated in a mixture of secondary antibodies (Jackson ImmunoResearch) for 1 hour: donkey anti-rabbit IgG conjugated with FITC (1:350), donkey anti-goat IgG conjugated with TRITC (1:350) and donkey anti-mouse IgG conjugated with Cy5 (1:300). After the incubation, the sections were rinsed, mounted, and coverslipped with buffered glycerol.

Image analysis

The double- and triple-labeling results were analyzed with the aid of confocal faser scanning microscopy. The system consists of a Leica RBE inverted microscope equipped with Ar laser producing monochromatic light at 467 nm and 488 nm, a Kr laser for 568 nm and HeNe laser for 647 nm light. The staining results were examined with both 40x and 100x objectives. A series of adjacent optical sections (approximately 1μm intervals for 40x and 0.5 μm for 100x) along the Z axis were collected for selected fields from 25 μm tissue sections. The signals obtained for each fluorphore on one series of optical

sections were stored separately as a series (stacks) of 512 X 512 pixel images. The stacked images were then used for analyzing the presence of NPY- and PHA-L double-labeled fibers in close apposition to GnRH or CRF positive neurons. The apparent synaptic contacts were confirmed by examining single image planes.

Results

Verification of anterograde tracer injection site

The injection site for each animal was verified by the quick staining process. Only animals with injection sites centered around the caudal portion of the ARH (Fig. 5-1) were used for analyzing the projection pattern and the multiple labeling studies. Some animals showed no projection fibers even though the injection sites were in the ARH. Most of the PHA-L staining in these animals were tanacytes and glial cells (data not shown). These animals were not used for the following multiple-staining studies. Several animals with injection sites outside the caudal ARH (including the ventromedial nucleus and the rostral portion of the ARH) were also processed for fiber staining to compare these projection patterns with those from animals with injection sites in the caudal ARH.

Distribution of PHA-L fibers from the caudal portion of the ARH

Forebrain regions

The majority of the projection fibers was found in the hypothalamus. In general, dense plexuses of PHA-L labeled fibers were found in the organum vasculosum of the lamina terminalis (OVLT, Fig. 5-2a) and continued to the anteroventricular periventricular nucleus (AVPV), the medial portion of the preoptic area (mPOA, Fig. 5-2b, 2e), the periventricular nucleus along the ventricle (PePOA, Fig. 5-2b, 2e), the parvocellular portion of the paraventricular nucleus (PVHp, Fig. 5-2d), the dorsomedial nucleus (DMH, Fig. 5-2g) and the ARH (Fig. 5-2f, 2g). Scattered fibers were observed in the anterior

hypothalamus (AH), the lateral hypothalamus (LH), the rostral end of the posterior hypothalamus (PH) and the tuber cinereum area. Scattered fibers were also found in the median eminence (ME, Fig. 5-2f, 2h). Areas showing no stained fibers include the magnocellular portion of the PVH (PVHm), supraoptic nucleus (SON), suprachiasmatic nucleus (SCN), compact zone of the DMH (DMHp), ventromedial nucleus (VMH), and mammillary body. When the projection patterns were compared among animals with injection sites in the caudal ARH or in the rostral ARH, no apparent topographical difference was found.

In general, when the injection sites were restricted in the ventromedial part of the ARH, the fiber staining was more concentrated along the periventricular zone and the nearby nuclei, including the AVPV, PVHp, DMH, the ARH and the median eminence (ME). When injections were made into the lateral or dorsal parts of the ARH, more fibers were found scattered into more lateral regions in the hypothalamus. When injections were centered in the VMH, the projection patterns were found to be similar to those reported by others (Canteras et al., 1994; Saper et al. 1976), and they presented a different pattern than observed in the ARH injected animals (data not shown).

Extrahypothalamic regions showing strong fiber staining were found in the ventral part of the lateral septal nucleus (LSV) (Fig. 5-2a). The bed nucleus of the stria terminalis (BST) contained moderate levels of PHA-L fibers mostly in the ventromedial and posterior portions (Fig. 5-2a, 2e). Very few fibers were found in the anteromedial and posterolateral portions of the BST. Interestingly,

in animals with injection sites positioned in more lateral portions of the ARH, more fibers were found in the lateral portion of the BST (Fig. 5-2c), although no difference was found in the caudal portion of the BST. Scattered fibers were also observed in the anterior paraventricular thalamus (PVT) and the medial amygdala (MeA). No apparent topographical difference was found in projection patterns between animals with injections sites in the caudal ARH and the rostral ARH.

Midbrain and brainstem

In the midbrain region, fibers were found mostly in the periaqueductal gray (PAG) along the ventricle and the aqueduct (Fig. 5-3a). A very small amount of fibers was found in the peripeduncular area (PP). In the brainstem, all the animals showed moderate levels of staining in the ventral portion of the caudal PAG (Fi.g 5-3b, 3c). When the injection was made into the more lateral or dorsal portions of the ARH, moderate levels of fibers were found in the rostral end of the lateral and medial parabrachial nucleus (LPB, MPB, Fig. 5-3d). Few scattered fibers were found in the areas around the locus coeruleus (LC), the ventrolateral pontine area including the A5 and C2, the ventrolateral medulla (VLM) including the C1 and A1, and the nucleus of solitary tract (NTS). When injections were restricted to the medial portion of the ARH, almost no fibers were found in the above areas except for the PAG and LPB, where a few fibers were found.

Distribution of PHA-L and NPY double-labeled fibers from the caudal portion of the ARH

The sections stained for PHA-L and NPY with FITC and TRITC, respectively, were visualized simultaneously to examine the possible PHA-L/NPY double-labeled fibers by confocal microscopy. In most areas of the forebrain where PHA-L single labeled fibers were found, PAH-L/NPY double-labeled fibers were also detected but with reduced density when compared to the respective single-labeled fibers. In the brainstem, only a few double-labeled fibers were found in the PAG, PP and PBL.

Triple-labeling for GnRH, NPY and PHA-L in the preoptic area and median eminence

Preoptic area (POA). The possible direct apposition between NPY fibers from caudal ARH and GnRH neurons in the POA was determined with triple-label immunofluorescent staining. The staining results were examined using confocal laser scanning microscopy. Many NPY single-labeled, as well as NPY/PHA-L double-labeled fibers, were observed in the POA, with a high density found in the OVLT, AVPV and PePOA areas. These fibers gradually spread out into the diagonal band and the POA. Many NPY single-labeled fibers and nerve terminals were found in close proximity to GnRH cell bodies (Fig. 5-4, 5-5). Occasional direct apposition between the double-labeled fibers and GnRH somas were observed (Fig. 5-4, 5-5).

Median eminence (ME). In the ME, GnRH fibers and terminals were found mainly in the external layer (Fig. 5-6), with low levels of fibers found in the

intermediate layers. NPY, as well as NPY/PHA-L double-labeled fibers, were observed mainly in the intermediate and in externals layer (Fig. 5-6). Occassional close appositions between NPY or NPY/PHA-L double-labeled fibers and GnRH terminals were observed in the intermediate as well as external layers of the ME (Fig. 5-6, 5-7). At higher magnification, some of the close appositions resembled axoaxosynaptic contacts between NPY fibers and GnRH axonal fibers (Fig. 5-8). Occasional double-labeled fibers were also found in the internal layer of ME (Fig. 5-7, 5-8).

Triple-labeling for CRH, NPY and PHA-L in the paraventricular nucleus of hypothalamus (PVH)

Using similar staining procedures as for GnRH, possible close appositions were examined between CRF neurons and ARH NPY nerve fibers in the PVH area. As shown in Fig. 5-9, NPY single labeled fibers and NPY/PHA-L double-labeled fibers were mostly found in the parvocellular portion of the PVH (PVHp). CRF positive neurons were also found in the PVHp area. When multiple staining results were examined simultaneously using confocal microscopy, some NPY/PHA-L double-labeled fibers made close appositions on CRH positive neurons in the PVHp area (Fig. 5-9, 5-10).

Discussion

In the present study, anterograde tracing combined with double-label immunofluorescent staining was used to determine the projections of NPY neurons from the caudal portion of the ARH (Fig. 5-11). The major target areas for the caudal ARH NPY neurons were mostly confined in the hypothalamus, with few extrahypothalamic areas receiving projecting fibers. There was no apparent topographical differences in fiber projecting patterns between NPY neurons located in the rostral and the caudal ARH.

Hypothalamic distributions of ARH NPY fibers

In the hypothalamus, the fibers basically course along the third ventricle and the basal hypothalamic tract. Hypothalamic areas that received heavy ARH NPY projections include OVLT, AVPV, medial POA, PePOA, PVHp, DMH, PH and ARH, areas which have been shown to contain high densities of NPY fibers (De Quidt et al., 1994). These results are in agreement with several retrograde tracing studies showing that the ARH provides NPY input into the mPOA and PVH. In addition, the present study also demonstrated that the AH and LH received innervation from ARH NPY neurons. Thus, the present study indicates that ARH NPY neurons innervate multiple areas in the hypothalamus. In addition, complicated connections exist among hypothalamic nuclei (Ter Horst and Luiten, 1987), which may explain, in part, the involvement of ARH NPY neurons in various physiological regulations.

Projecting NPY fibers were also observed in the ME. Most of the fibers were found in the intermediate layer of the ME with moderate levels of fibers

found in the external layers. This suggests that NPY neurons in the ARH can access the portal blood system and influence the anterior pituitary gland function. It should be pointed out that the NPY staining in the ME observed in the present study may be unique to the lactating rat. Ciofi et. al. showed that NPY staining was dramatically increased in the ME of lactating animals when compared to nonlactating animals (Ciofi et al., 1991, 1993). Thus, the present study further demonstrates that the increased NPY in the ME is, in part, derived from the ARH NPY neurons. Since NPY can act at the pituitary level to modulate the secretion of prolaction (Rettori et al., 1990; Wang et al., 1996), ACTH (Small et al., 1997), and LH (Kalra and Kalra, 1996), it is possible that elevated NPY in the ME from the ARH NPY neurons plays an important role in modulating the secretion of these hormones from the pituitary during lactation. It will be important to determine what cell types express NPY receptors in order to further understand the mechanism by which NPY acts at the pituitary to modulate hormone secretion. In addition, occasional double-labeled fibers were found in the internal layers of the ME, suggesting that the ARH NPY neurons may also send minor input to the posterior pituitary.

Extrahypothalamic distributions of ARH NPY fibers

In the extrahypothalamic areas, the most dense fiber innervation was found in the LSV and several subdivisions of the BST. Few fibers were found in the MeA and PVT. There is no apparent difference in projections to these areas between animals with rostral ARH injections and those with caudal ARH injections. Interestingly, in the BST, ARH NPY fibers were found mostly in the

posterior and rostroventral portions of the BST, and only a few fibers were found in the anteromedial and dorsolateral parts of the BST. However, when the injection sites were positioned in the lateral part of the ARH, more PHA-L single labeled fibers could be seen in the lateral part of the BST. These results suggest that there may be a topographical distribution of projections into different parts of the BST from the neurons in different parts of the ARH. Similar to the LSV, the medial and posterior portions of the BST are extensively connected with the hypothalamus, including the mPOA, LH, and the VMH (Berk and Finkelstein, 1982; Canteras et al., 1994; Magoul et al., 1994; Numan and Numan, 1996; Saper et al. 1976; Simerly and Swanson, 1986); these connections have been suggested to be involved in regulation of neuroendocrine and reproductive behavior. Thus, in addition to direct hypothalamic input, the activated ARH NPY neurons may regulate hypothalamic activity indirectly during lactation by modulating hypothalamic input from areas such as LSV and certain parts of the BST.

In the midbrain regions, most of double-labeled fibers were found in the PAG along the ventricle. Few fibers were found in the PP. In the brainstem region, only a few ARH NPY fibers were observed in the PBL. These results suggest that the ARH NPY system sends very few projections into the brainstem. In contrast, more PHA-L single-labeled fibers were found in the brainstem when the injection site was positioned in the lateral portion of the ARH. Thus, there may be heterogeneity in the brainstem targets of neurons from different portions of the ARH.

Direct interactions between ARH NPY neurons and GnRH and CRF neurons

Synaptic contact between NPY fibers and GnRH perikarya in the POA has been established using electron microscopy (Norgren and Lehman, 1989). The present studies provide further evidence that the ARH NPY neurons provide at least some of the NPY synpatic input. The direct apposition between ARH NPY input and GnRH neurons in the POA also suggests that ARH NPY neurons may directly modulate GnRH neuronal activity. In addition, close contacts between ARH NPY fibers and GnRH nerve terminals were also observed in the ME, suggesting that ARH NPY may modulate the release of GnRH from its nerve terminals in the ME. Interestingly, a recent study conducted in our laboratory has shown that the NPY Y1 receptor is localized in GnRH nerve terminals in the OVLT and ME but not in GnRH cell bodies in the POA (unpublished observation), suggesting that NPY input in the ME may modulate GnRH release by acting through the Y1 receptor.

In addition to the GnRH system, the present study also showed that ARH NPY neurons made direct appositions on CRF neurons in the PVHp. The PVH CRF neurons may be a hypothalamic regulatory factor that inhibits feeding induced by NPY (for review, Heinrichs et al., 1998). The present study provides anatomical evidence that the ARH NPY neurons have direct input to CRF neurons in the PVH. It is possible that during lactation, suckling-activated ARH NPY neurons may induce feeding in part by suppressing CRF neuronal activity. This hypothesis is supported by the observation that CRF gene expression in

the PVH of lactating rats is significantly suppressed compared to nonlactating animals (Fischer et al., 1995). Although several NPY receptor subtypes are found in the PVH area, it is yet to be determined which receptor subtypes mediate the effect of NPY on CRF neurons in the PVH.

In conclusion, the present study identified multiple target areas for the ARH NPY neurons. In future studies, the possible effects of NPY in each of the target areas, both within and outside the hypothalamus, would need to be examined to understand the function of ARH NPY neurons in maintaining body homeostasis during lactation.

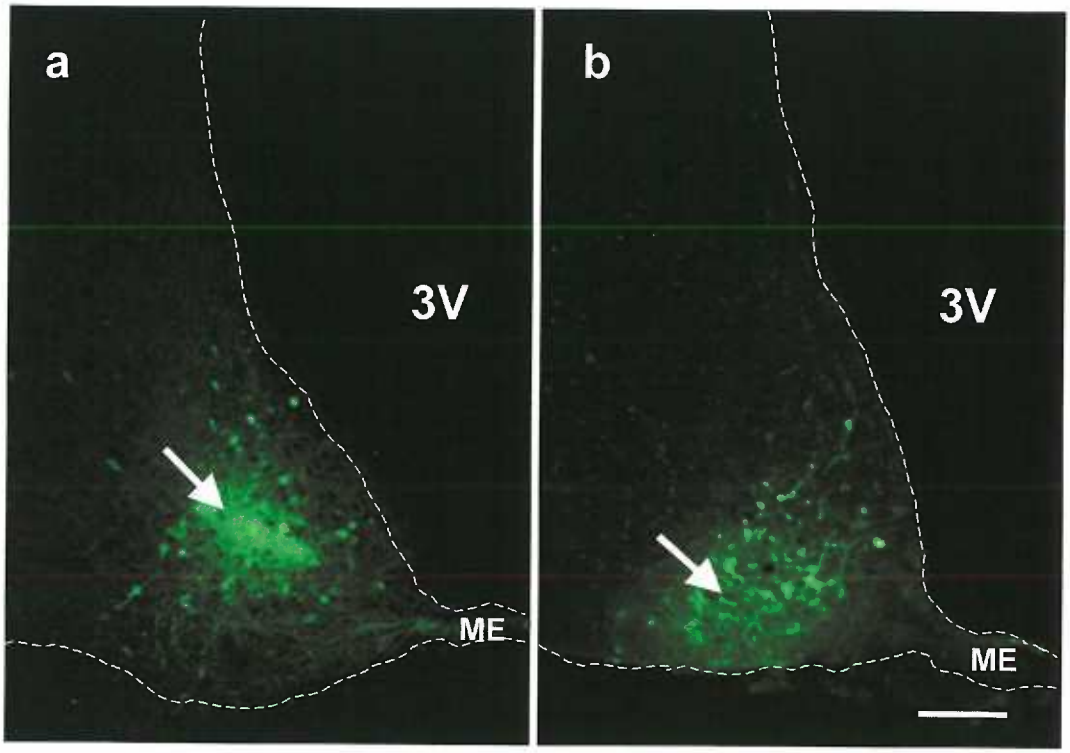
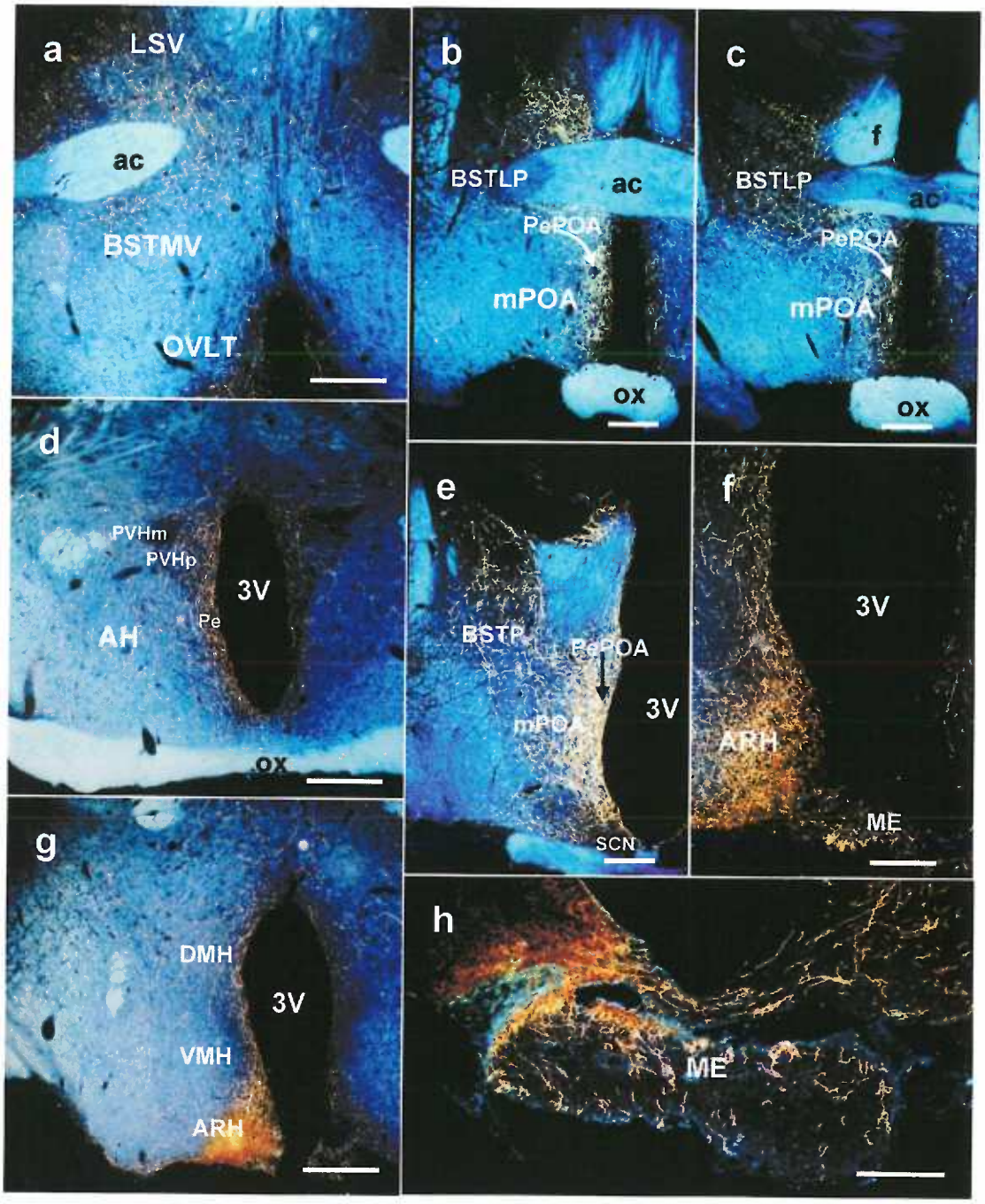


Figure 5-1 Representative fluroescent photomicrographs showing the PHA-L injection site (arrow) that is centered in the ARH (a). In b, the injection site covered the more lateral portion of the ARH. Scale bar = 20 μm.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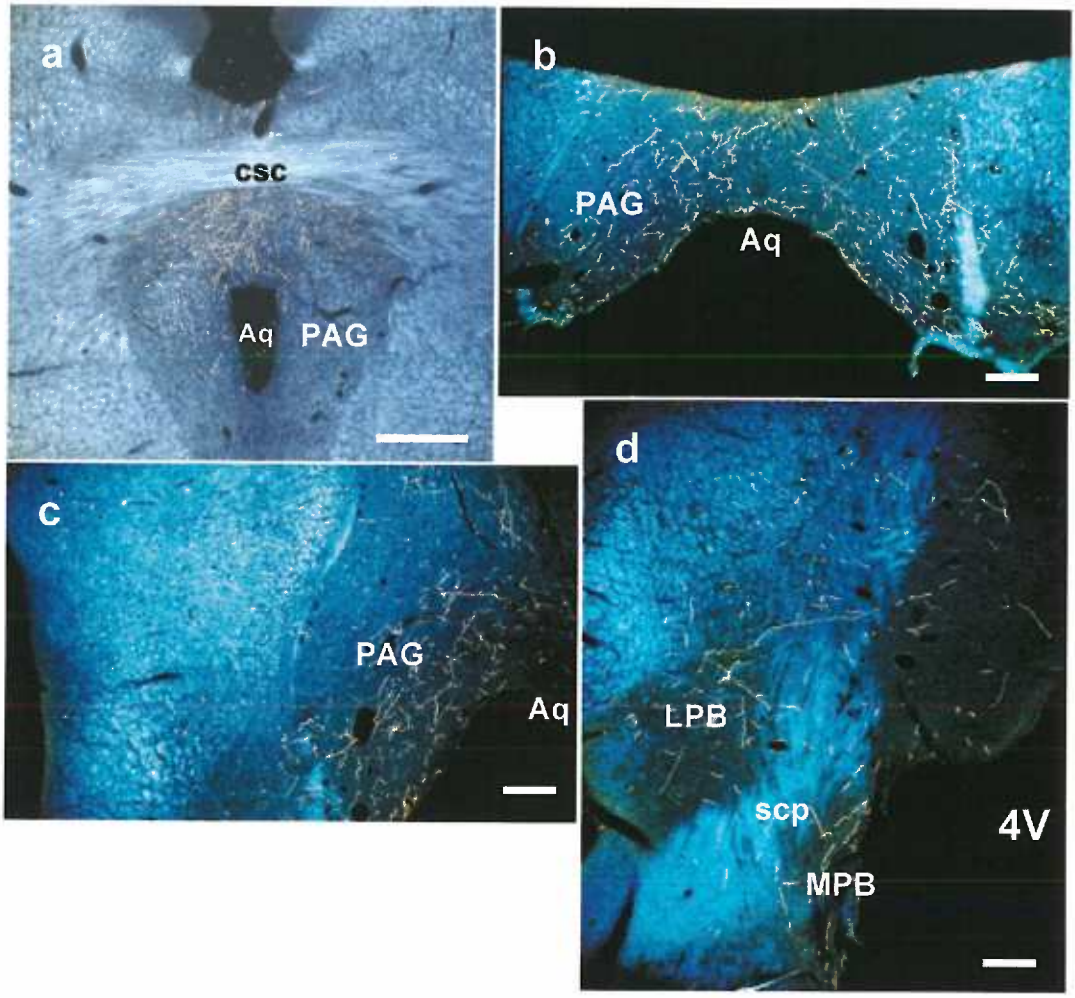
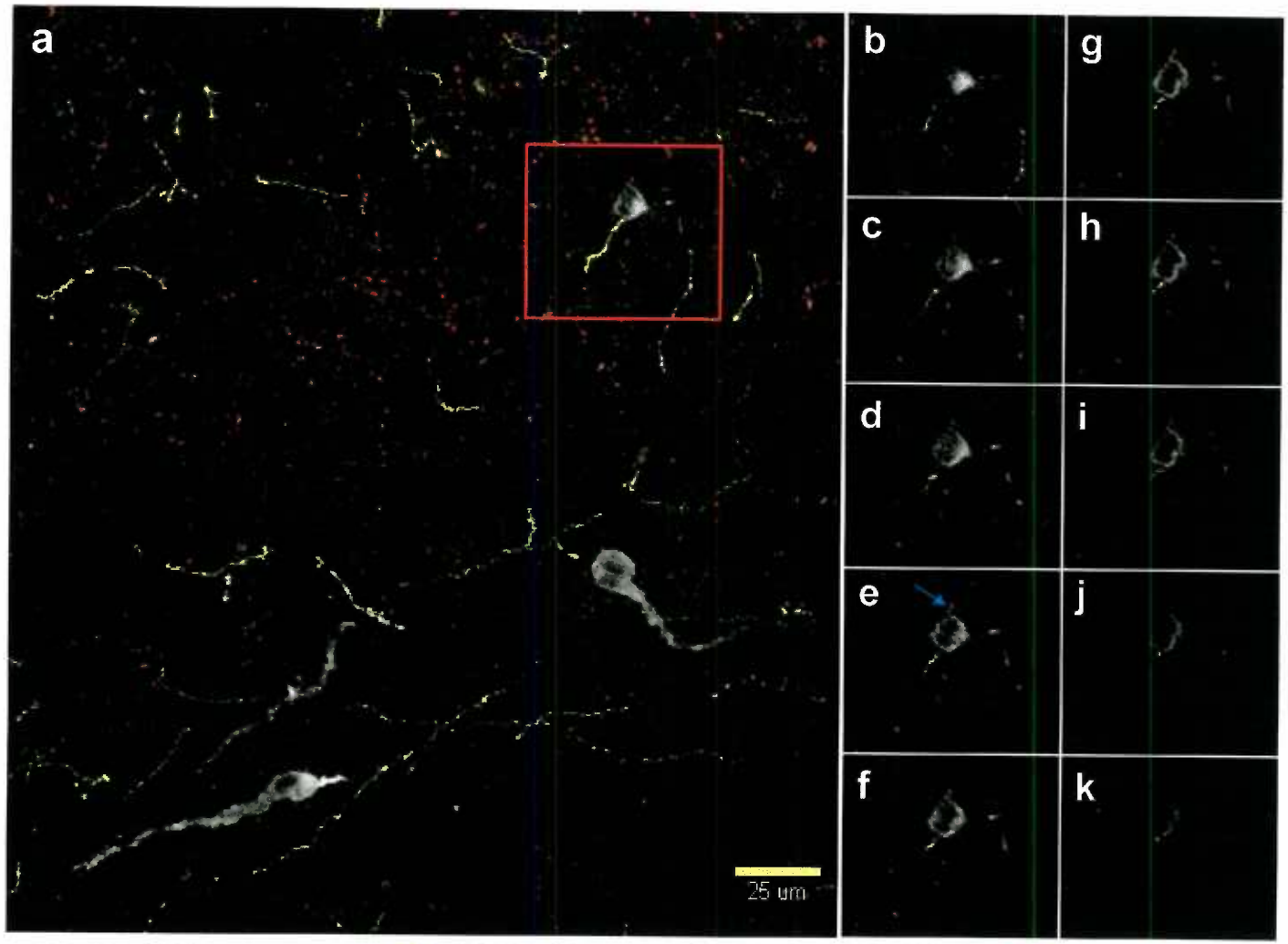


Figure 5-3. Representative dark-field photomicrographs show in the distribution of PHA-L positive fibers within the rostral (a), mid (b) and caudal portion (c) of the PAG. PHA-L fibers were also found in the LPB and MPB area (d). Aq: aqueduct; csc: commissure of the superior colliculus; LPB, MPB: lateral and medial parabrachial nucleus; PAG: periaqueductal gray; scp: superior cerebellar peduncle. Scale bars = 40 μm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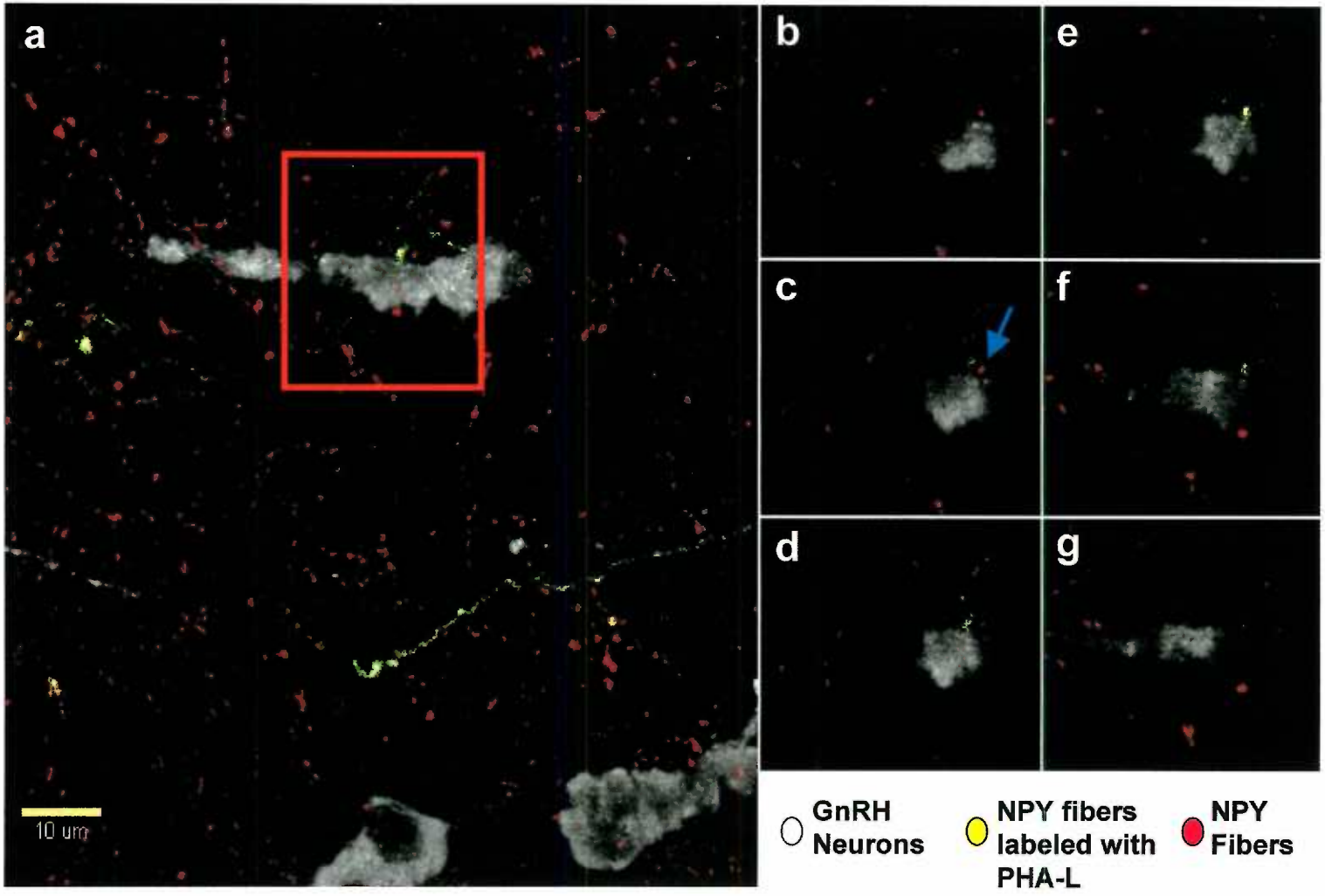
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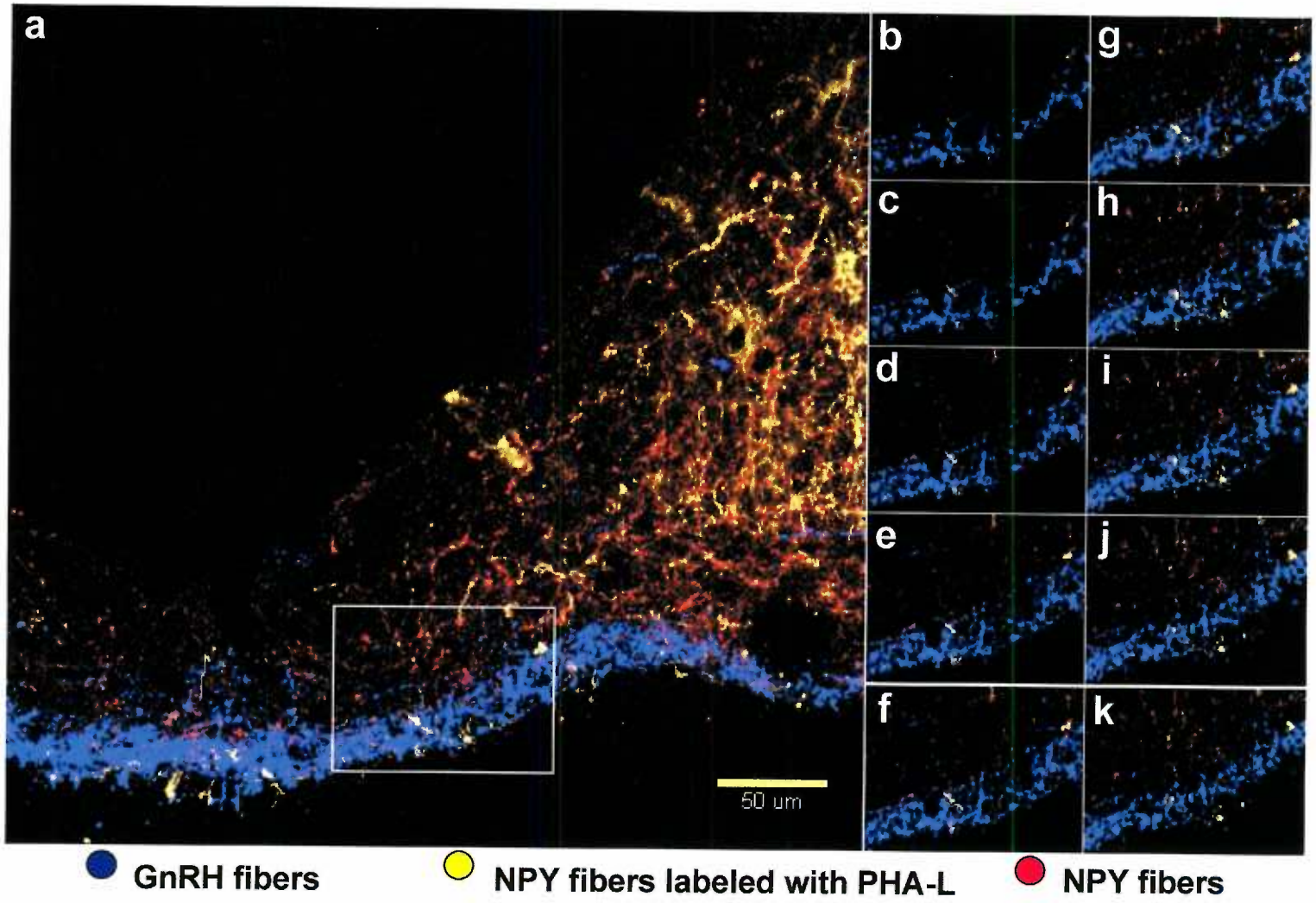
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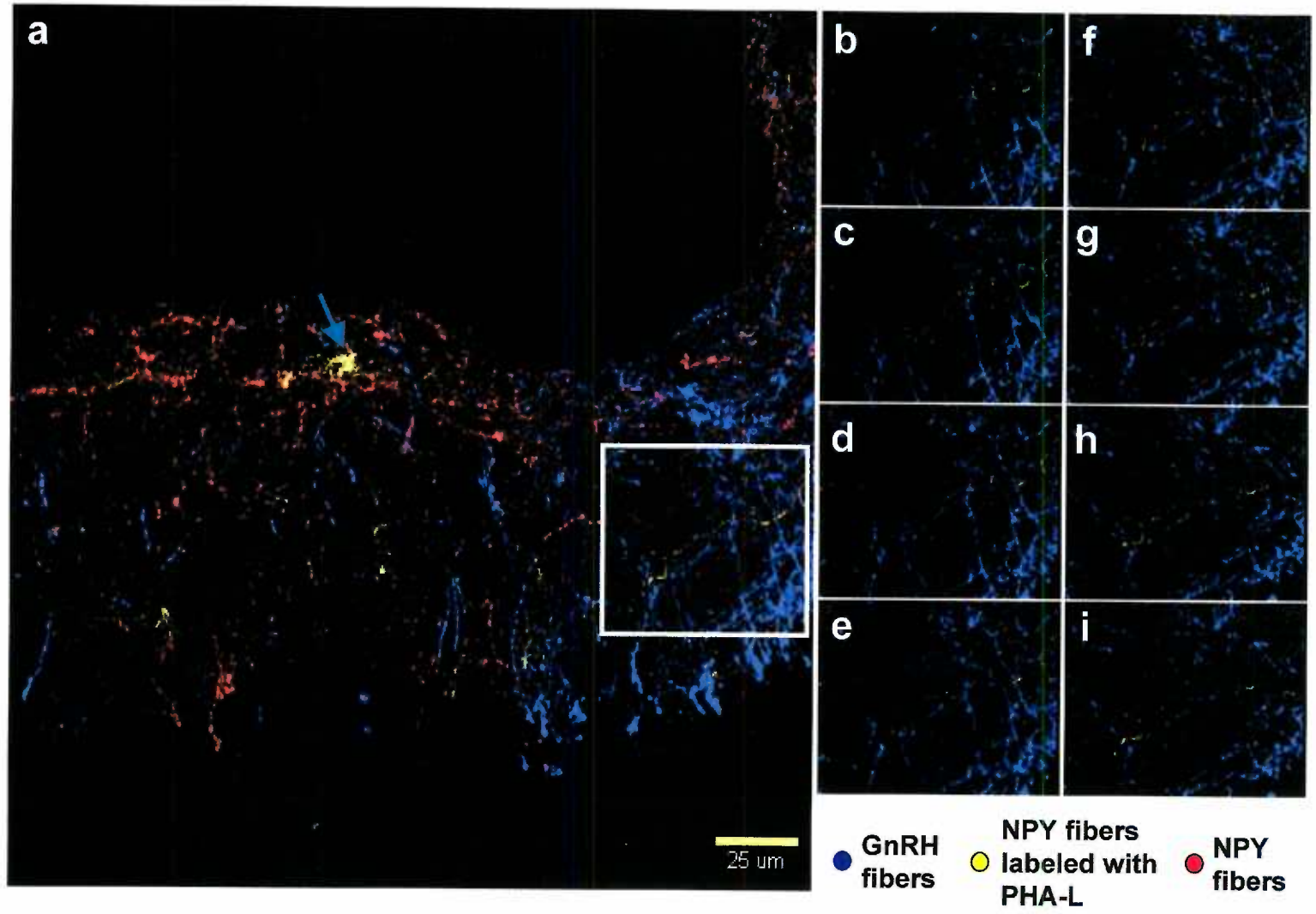
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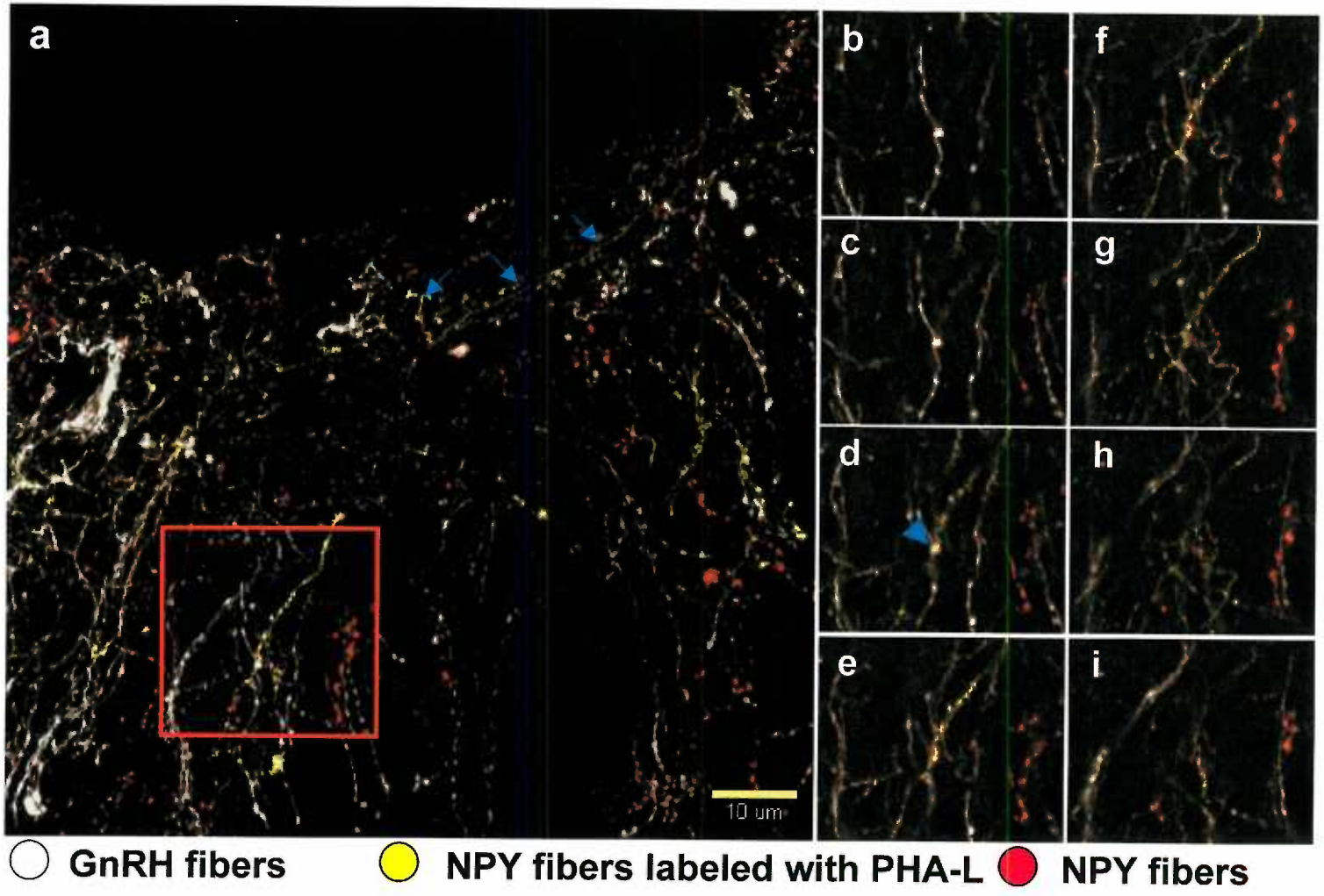
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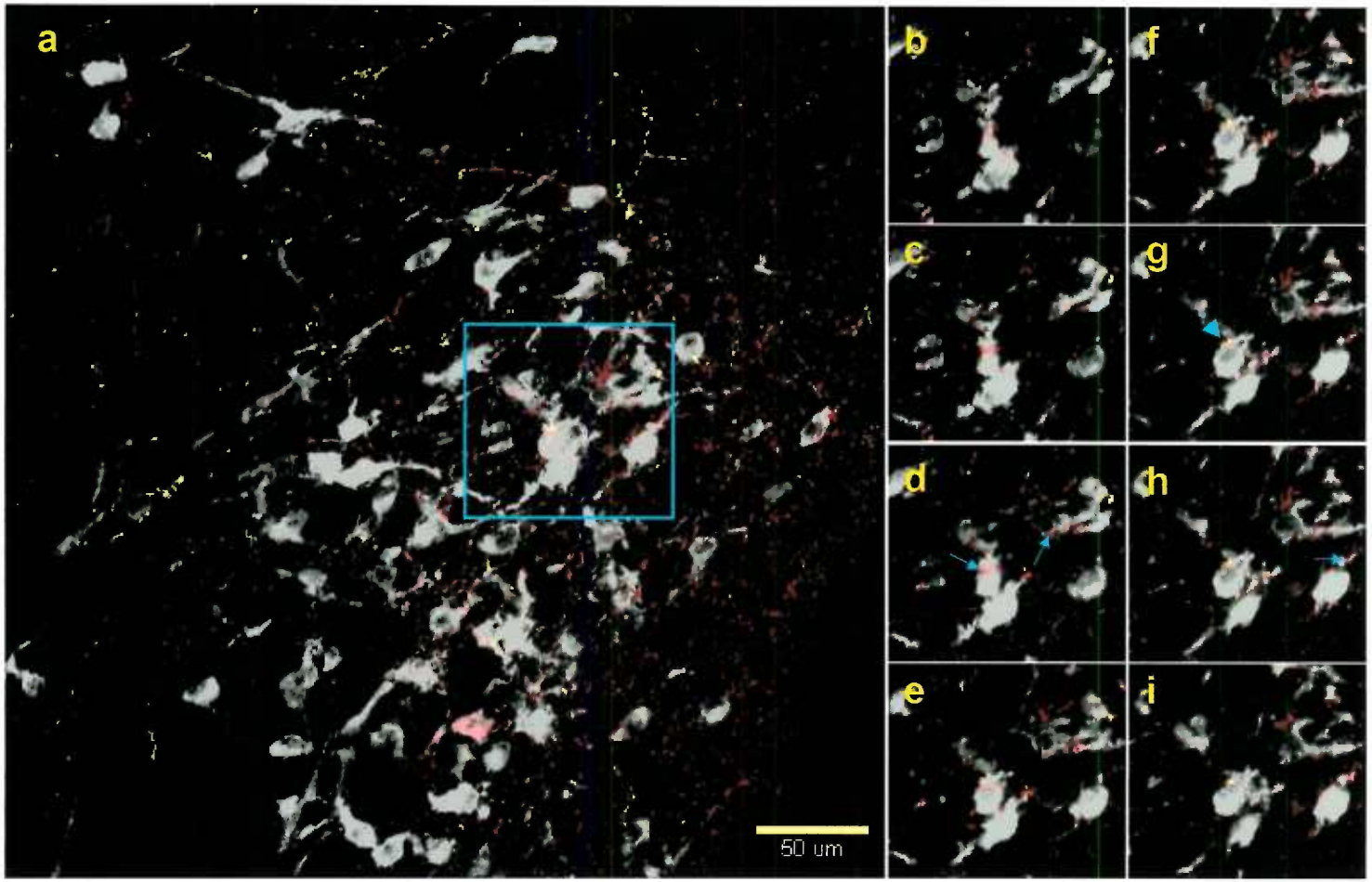
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CRF neurons
NPY fibers labeled with PHA-L
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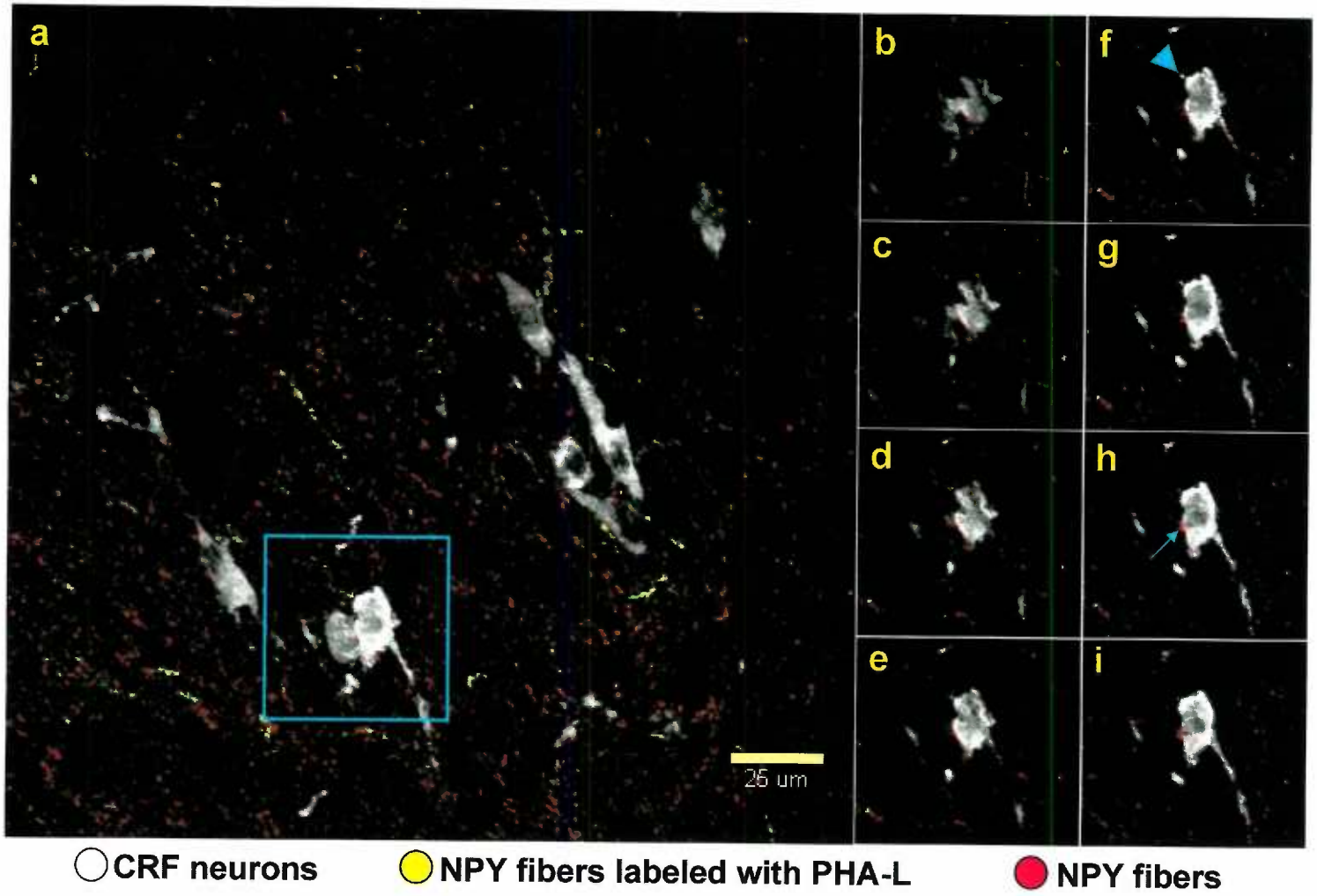
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CRF neurons
NPY fibers labeled with PHA-L
NPY fibers

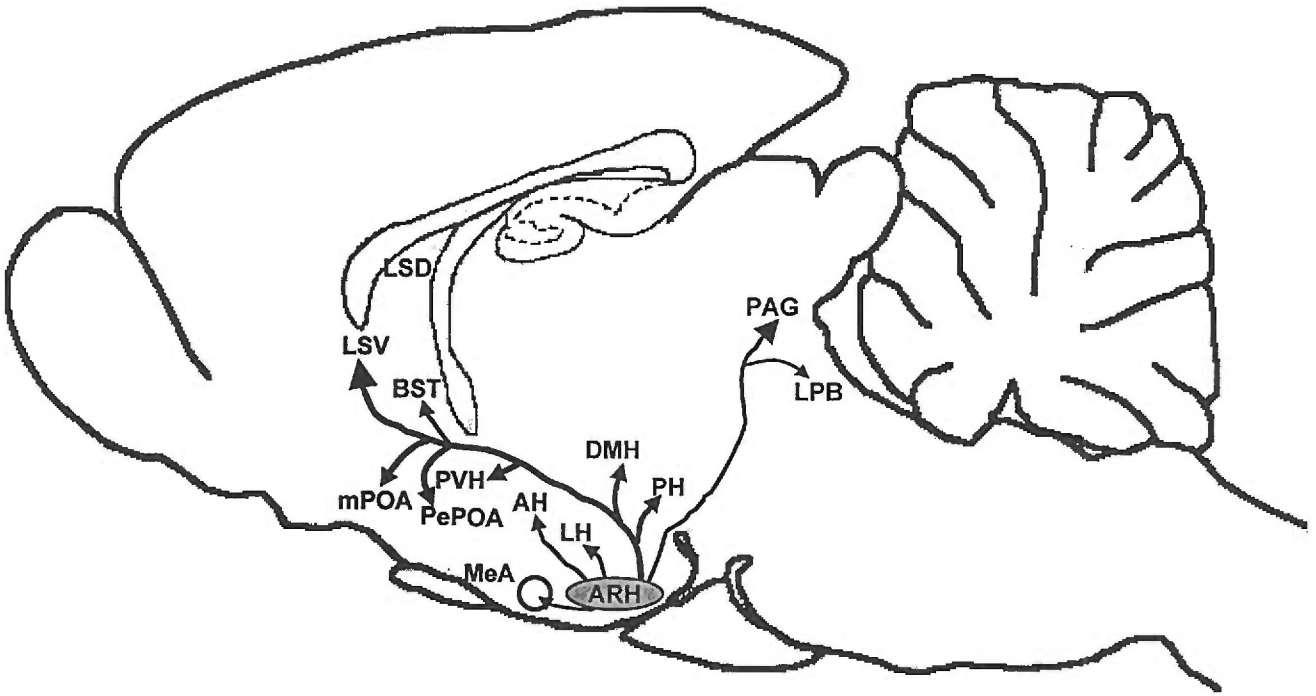


Figure 5-11. A diagrammatic representation showing the major projections from NPY neurons in the caudal portion of the ARH. The relative fiber density of each projection is roughly proportional to the thickness of the lines associated with it.

CHAPTER VI

NEURAL PATHWAYS ACTIVATED BY THE SUCKLING STIMULUS AS DEMONSTRATED BY CFOS EXPRESSION: NEURAL STIMULI DERIVED FROM SUCKLING VERSUS PUP EXPOSURE

Submitted to Neuroscience

Introduction

During lactation, the suckling stimulus induces many changes in hypothalamic function which contribute to adaptation to the lactating condition. For example, it has been shown that suckling stimulates the release of oxytocin (OT) (Knobil and Neill, 1994), prolactin (Knobil and Neill, 1994), ACTH and corticosterone (Walker et al., 1992), as well as causing a suppression of pulsatile LH secretion (Fox and Smith, 1984; Smith, 1984; Maeda et al., 1987, 1989; and Tsukamura et al., 1990). Thus, knowledge of the neural pathways activated by the suckling stimulus will greatly facilitate an understanding of the mechanisms by which suckling induces changes in hypothalamic function.

Earlier studies used either lesions (Tindal and Knaggs, 1971; Juss and Wakerley, 1981; and Dubois-Dauphain et al., 1985a, 1985b, 1985c) or electrical stimulation (Tindal et al., 1967, 1968, 1969; and Tindal and Knaggs, 1971) to identify the central pathways for the milk-ejection reflex. These studies showed that neural signals activated by suckling traveled through the spinal cord and were relayed through the lateral cervical nucleus before entering the brainstem. In the brainstem, however, the pathway was less clear, which may reflect the complex neuronal connections in this area. The techniques employed by the earlier studies limit the interpretation of the data and the identity of the suckling-sensitive pathways. First, brainstem lesions may interrupt a variety of somatosensory inputs, which may profoundly complicate the interpretation of the results. Second, lesion or electrical stimulation damages the area of study

and thus does not permit further study of the neurochemical substrates which may be involved in the pathway.

A newer technique, expression of the immediate early gene product, cFos, has proven to be a useful marker for neuronal activation. cFos serves as a transcription factor that can alter the expression of other genes (Sheng and Greenberg, 1990; Morgan and Curran, 1991). cFos is rapidly and transiently activated in response to a variety of stimuli in the brain (Morgan and Curran, 1991; Hoffman et al., 1993; Ceccatelli et al., 1989). It has been shown that the expression of cFos is the result of Ca^{2+} influx into the activated cells upon membrane depolarization caused by the incoming excitatory input (Morgan and Curran, 1991). Thus, in the present study, I used the induction of cFos expression by the acute suckling stimulus to map the neural populations in the brain which may be involved in transmitting suckling-induced neural signals into the brain, including the hypothalamus. In addition, I compared the patterns of cFos expression in the brain induced by the suckling stimulus with those induced by sensory stimuli of pup exposure in order to differentiate the neural populations that were activated only by the physical suckling stimulus. In addition, since it has been shown that several catecholaminergic populations in the brainstem send projections into the hypothalamus (Bjorklund and Hokfelt, 1984), double-label immunostaining was used to investigate whether catecholaminergic neurons in the brainstem are activated by the suckling stimulus.

Methods and Materials

Animals

Day 18-19 pregnant Sprague-Dawley rats (B & K Universal Inc., Kent, WA) were housed individually and maintained under a 12:12 light-dark cycle (lights on at 0700-1900) 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 as day 0 postpartum. All the animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee.

Experimental design

Lactating animals had their litters adjusted to eight 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. After pup separation, the females were housed individually in an experimental room under the conditions described above. On day 11, animals were placed into one of the following treatment groups: 0 pups suckling (controls, 0 pups, n = 4); 8 pups exposure for 90 min (the dams were separated from the pups with a wire screen that did not interfere with sensory stimuli from the pups, 8PE, n = 5); 8 pups suckling for 90 min (all pups were nursing within 1-2 min of placement in the cage with the dam, 8 pups, n = 6).

Perfusion and tissue sectioning

After 90 min of pup exposure or suckling, animals were anesthetized with an overdose of pentobarbital (125 mg/Kg B.W., i.p.) and perfused transcardially with 150 ml of 2% sodium nitrite in saline followed by 150 ml 2.5% acrolein (EM grade, Polysciences) in phosphate-buffered 4% paraformaldehyde (pH 7.4). The brain was removed from the skull and the brainstem was blocked from the beginning of caudal periaqueductal gray to the end of the medulla, excluding the cerebellum and inferior colliculi. Both brain and brainstem were immersed in 25% sucrose at 4 °C overnight. Coronal sections (25 μm) were cut through the forebrain region and horizontal sections (25 μm) were cut through the whole brainstem on a sliding microtome and collected in a one-in-three series. The tissue sections were stored until use at -20 °C in multiwell tissue culture plates containing cryoprotectant.

Immunocytochemistry procedures

Tissue sections from all animals were processed in one assay to ensure uniformity of immunostaining. Immunocytochemistry of cFos was performed as described elsewhere (Lee et al., 1990). Tissue sections were removed from cryoprotectant and rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by treatment with 1% $NaBH_4$ -KPBS solution (Sigma). Sections were incubated in rabbit anti-cFos antibody (*sc*-52, Santa Cruz, 1:15,000) in KPBS with 0.4% Triton X-100 at room temperature for 1 hr, followed by 4°C for 48 hr. After the incubation, the tissues were rinsed in KPBS and incubated in

biotinylated goat anti-rabbit IgG (Vector Laboratories, 1:600) in KPBS with 0.4% Triton X-100 for 1 hr at room temperature. This was followed by another 1 hr incubation at room temperature in avidin-biotin complex solution [4.5 μl of A and B each per ml of KPBS-0.4% Triton X-100 (Vectastain ABC Elite Kit, Vector Lboratories)]. The cFos antibody-peroxidase complex was visualized with a mixture of $NiSO_4.6H_2O$ (25 mg/ml), 3,3'-diaminobenzidine (0.2 mg/ml) and 3% H_2O_2 (0.83 μl/ml) in 0.175 M sodium acetate solution. When the staining was appropriate, tissue sections were transferred into sodium acetate solution to stop the color reaction, rinsed in KPBS and 3% H_2O_2 (0.83 μl/ml) in 0.05 M Tris buffered saline solution. Following the staining, tissue sections were mounted on gelatin-coated glass slides, countered stained with neutral red, and then dehydrated through graded alcohols, cleared in xylenes, and coverslipped with Histomount (National Diagnostics). For cFos and tyrosine hydroxylase (TH) double-label immunocytochemistry, after the color reaction for the cFos staining, the tissue sections were rinsed and then incubated in the monoclonal mouse anti-TH antibody (MAB318, Chemicon, 1:250,000). The same immunocytochemistry procedures described above were followed except that horse anti-mouse IgG (Vectorlaboratories, 1:600) was used as the secondary antibody. The TH antibody-peroxidase complex was stained with a mixture of 3,3'-diaminobenzidine (0.2 mg/ml) and 3% H_2O_2 (0.83 μl/ml) in 0.05 M Tris buffer-saline solution. Following the TH staining, tissue sections were mounted on slides, dehydrated and coverslipped.

Data analysis

Quantification of cFos-positive cells. cFos-positive cells were identified by the evident blue-black reaction products in cell nuclei. In the forebrain, the number of cFos-positive cells in the dorsal and ventral lateral septum (LSD and LSV), medial preoptic area (mPOA), the periventricular preoptic area (PePOA) and the supraoptic nucleus (SON) were quantified. In the brainstem, the number of cFos-positive cells in the lateral and ventrolateral caudal periaqueductal gray ($cPAG_{l,vl}$) and lateral parabrachial nucleus (LPB) was quantified. A microscope-video camera-computer image analysis system was used for the quantification. The 4X bright field image of the sections was acquired using a video camera (Cohu) connected to a Pentium-based microcomputer. The image was captured and analyzed using the Harmony group image analysis program (Videk Corporation, Rochester, NY). Control animal tissue was used to set the threshold of brightness for the analysis, so that all the cFos-positive cells were visible, while the gray background color was eliminated. The size of the region analyzed remained constant and covered the entire area of interest.

Quantification of cFos-positive TH cells. cFos-positive TH cells were identified as cells with brown cytoplasmic deposits and blue-black nuclei. The number of single- and double-labeled TH cells was counted for each animal in the nucleus of the solitary tract (NTS, noradrenergic A2 neurons) and the ventral lateral medulla (VLM, noradrenergic A1, and C1 neurons). Since the TH-positive cells within the locus coeruleus (LC, noradrenergic A6 neurons) were too densely

packed to discern the boundary between cells, only the number of cFos-positive TH cells were determined.

Statistical analysis. The number of cFos-positive cells of the LSD, LSV, mPOA, PePOA, SON, $cPAG_{l,vl}$ and LPB, and the number of cFos-positive TH cells of the LC were expressed as the number of cFos or cFos-positive TH cells per section, respectively. The mean number of cells per section for each of the areas was determined for each animal. The data from cFos-TH double-labeled cells from VLM and NTS were expressed as the percent of cFos-positive TH cells. Data are presented as mean ± SEM. Differences among groups within an area of interest were evaluated using one-way analysis of variance (ANOVA) and post hoc Scheffe's tests. Differences were considered significant if $p < 0.05$.

Results

Distribution of cFos-positive cells in the forebrain

The induction of cFos in the forebrain in response to pup exposure or suckling is summarized in Table 6-1.

Table 6-1 Forebrain areas showing cFos expression (= no cFos; +, ++, +++ = degree of cFos)

Area	0 Pups Suckling	8 Pups Exposure	8 Pups Suckling
LSD			++
LSV			++
BST		+	+
mPOA		+	+++
PePOA			++
AH		++	++
LH		+	+
PH		+	+
PVHm		+	+
PVHp		+	+
SON			++
DMH		+	+
VMH			
PMV		+	+
SUM		+	+
MeA		++	++
PVT		+	+
Cpu		+	+
Cortex		++	+++

Suckling of 8 pups for 90 min induced cFos expression in discrete nuclei in the hypothalamus including mPOA, PePOA, SON, anterior hypothalamus (AH), the lateral hypothalamus (LH), the dorsomedial nucleus (DMH) except the compact zone, the zone between the ventromedial nucleus (VMH) and arcuate

nucleus (ARH), the posterior hypothalamus (PH), the ventral premammillary body (PMV) and the supramammillary nuleus (SUM). In the extrahypothalamic areas, suckling induced cFos expression in the LSD and LSV, the bed nucleus of stria terminalis (BST), the cortical areas, the paraventricular nucleus of thalamus (PVT), the caudate putamen (CPu), and the amygdala (Fig. 6-2B). Pup exposure alone did not induce cFos expression in the PePOA and SON in the hypothalamus or in the LSD and LSV. In other areas, pup exposure alone induced less cFos positive cells than observed in response to suckling (Table 6-1). The parvocelluar portion of paraventricular nucleus of the hypothalamus (PVHp) and the areas surrounding the PVHp showed very inconsistent cFos expression in response to both suckling and pup exposure. The magnocellular portion of the PVH showed little cFos expression in response to either suckling or pup exposure alone.

Distribution of cFos-positive cells in the brainstem

The induction of cFos in the brainstem in response to pup exposure or suckling is summarized in Table 6-2.

Table 6-2. Brainstem and midbrain areas showing cFos expression (= no cFos; +, ++, +++ = degree of cFos)

Area	O Pups Suckling	8 Pups Exposure	8 Pups Suckling
VLM/A1			+++
NTS/A2	+	+	+
LC/A6			+
PAG	+	++	+++
LPB			++
PL			+++
Pn	+	+++	+++
Sp5C		+++	+++
DC	+	++	++
Reticular Formation		++	+++
PT	-	+	+
PP		++	++
dlf	+	++	++
MGN		++	++
LG		+	+
Colliculli	+	+++	+++

Suckling of 8 pups for 90 min induced cFos expression in VLM, NTS, LC, $cPAG_{l,vl}$, LPB, pontine nucleus (Pn), spinal trigeminal nucleus (Sp5C), reticular formation, dorsal cochlear nucleus (DC), and a less well defined area which is located around the caudal portion of the paralemniscal nucleus (PL) close to the rubrospinal tract (Fig. 6-1), when compared to the nonsuckled controls (0 pups, Table 6-2). Pup exposure (8PE) alone induced levels of cFos expression in $cPAG_{l,vl}$, and reticular formation that were intermediate between the 0 pups and 8 pups groups. Similar levels of cFos-positive cells were found in 8 pups and 8PE groups in Pn, Sp5C, and DC (Table 6-2), which may be related to the

sensory input associated with smell, sound, and visual stimuli from the pups. Several areas, including NTS (Table 6-2), the cuniform nucleus, medial raphe, ventral tegmentum and area postrema (data not shown), were found to express similar levels of cFos in all three groups.

cFos expression was also examined in the midbrain region. cFos positive staining was found in the peripedunclar area (PP), the dorsal longitudinal fasciculus area (dlf), the pretectal nuclei (PT), the medial geniculate nuclueus (MGN), the lateral geniculate area (LG) and the superior and inferior colliculli in both suckled and pup exposure groups with no apparent difference in cFos staining pattern and intensity. In the control nonsuckled animals, few cFos positive cells were detected in the dlf and the colliculli areas.

Quantitative analysis of cFos expression

Forebrain regions

m*POA*. The suckling stimulus induced a significant increase in cFos positive cells in the mPOA when compared to the same area in the nonsuckled control group (number of cFos cells per section: 0 pups, 8±2.3; 8PE, 38.1±12.1; and 8 pups, 150.6±13.4; Fig. 6-2A, 6-3). The cFos positive cells were observed generally in the lateral portion of the mPOA with relatively fewer cells detected in the medial portion of the mPOA (Fig. 6-2A). Pup exposure induced a small but significant number of cFos positive cells in the mPOA compared to the control (Fig. 6-2A, 6-3). The number of cFos cells induced by pup exposure was significantly lower than that in the suckled animals, and there was no apparent topographical distribution as was observed in the suckled animals (Fig. 6-2A).

PePOA. Along the ventricle, the suckling stimulus induced a significant number of cFos positive cells in the PePOA area (number of cFos cells per section: 0 pups, 2.5±0.7; 8PE, 3±1; and 8 pups, 73.8±10.7; Fig. 6-2A, 6-3). However, pup exposure did not induce cFos expression in this area (Fig. 6-2A).

SON. The suckling stimulus induced a significant number of cFos positive cells in the SON compared to both the nonsuckled control and pup exposure groups (number of cFos cells per section: 0 pups, 2.8±1.8; 8PE, 9.3±4.1; and 8 pups, 88.3±12.1; Fig. 6-2B, 6-3). Pup exposure induced a small number of cFos positive cells in 2 out of 5 animals, but the number was still significantly smaller compared to each animal in the suckled group. In the suckled group, most of cFos positive cells were detected in the anterodorsal portion as well as the caudolateral and caudoventral portion of the SON, where the oxytocinergic as well as the vasopressinergic neurons are located. Fewer cFos cells were found in the center of the nucleus.

LSD and LSV. In the lateral septal area, the suckling stimulus induced a significant number of cFos positive cells in the dorsal as well as ventral divisions (LSD: 0 pups, 3.5±0.9; 8PE, 11.5±4.4; and 8 pups, 86.6±7.4; and LSV: 0 pups, 11.4±1.8; 8PE, 22.8±2.4; and 8 pups, 134.1±12.9; Fig. 6-2A, 6-3). A few cFos positive cells were also detected on the border between the dorsal and internal division of the LS. Pup exposure did not induce a significant increase in cFos positive cells when compared to the control group (Fig. 6-2A, 6-3).

Brainstem regions

LPB. Suckling-induced significant numbers of cFos-positive cells in the lateral parabrachial nucleus (LPB; Fig. 6-4), whereas pup exposure had no effect on induction of cFos in this area (number of cFos cells per section: 0 pups, 2.6±1.4; 8PE, 3.8±4.4; 8 pups, 23.4±5.4; Fig. 6-4, 6-6).

$cPAG_{l,vl.}$ In the caudal PAG, most of the cFos staining was found in the lateral and ventrolateral regions (Fig. 6-4). Both suckling and pup exposure induced greater numbers of cFos-positive cells than observed in the control group (8PE, 140.2±56.0, or 8 pups, 210.0±34.3 vs. 0 pups, 39.0±13.7; $p<0.05$ respectively, Fig. 6-4, 6-6). In addition, the 8 pups group had significantly higher levels of cFos expression than the 8PE group (Fig. 6-4, 6-6).

Quantification of cFos and TH double-labeled cells in the brainstem

VLM/A1. The staining patterns and numbers of single-labeled TH neurons were similar in all three groups (Fig. 6-5). However, significant numbers of cFos-positive TH neurons were found in the 8 pups group (percent of cFos-positive TH neurons per section: 0 pups, 0.5±0.4; 8PE, 1.4±0.7; 8 pups, 37.4±4.1; Fig. 6-5, 6-6).

VLM/C1. In contrast to the TH neurons in the A1 area, TH neurons in the C1 area were not activated in response to suckling. Although the 8 pups group had a slightly higher percent of cFos-positive TH neurons, it failed to reach significance (0 pups, 0.2±0.1; 8PE, 0.3±0.3; 8 pups, 5.3±2.2; $p=0.06$; Fig. 6-6).

NTS/A2. Pup exposure or the suckling stimulus caused no further increase in cFos expression in TH neurons, when compared to basal

expression in the 0 pups group (0 pups, 14.8±2.9; 8PE, 16.7±2.7; 8 pups, 22.8±4.0; p=0.26; Fig. 6-5, 6-6). The numbers and staining patterns of single-labeled TH neurons were similar across the three groups (data not shown).

LC/A6. Significantly higher numbers of cFos-positive TH cells were found in the 8 pups group than in the 8PE or 0 pups groups. The numbers of cFos-positive cells per section within the TH-labeled A6 area were: 0 pups, 3.0±0.7; 8PE, 3.3±0.9; and 8 pups, 24.1±3.9; Fig. 6-6).

Discussion

In the present study, the immediate early gene product, cFos, was used as a marker for neuronal activation to identify neural populations in the brain that were activated by the acute suckling stimulus. After 90 min of suckling, many areas in the forebrain, as well as brainstem regions, exhibited substantial cFos nuclear immunoreactivity compared to the nonsuckled control group. The transient nature of cFos expression, as demonstrated previously by several laboratories (Numan and Numan, 1994, 1995; Lonestein and Stern, 1997; Fleming et al., 1994), necessitated the use of an acute resuckling paradigm. cFos expression is remarkably absent in the CNS of the chronically suckled rat (Smith and Lee, 1990). Furthermore, cFos expression induced by the acute suckling stimulus has completely disappeared by 8 hours after the onset of resuckling (Smith et al., 1994). Taken together, these results demonstrate that suckling induced cFos expression provides a valuable model to identify central pathways activated by the suckling stimulus.

Using pup-exposure to dissociate neural populations activated by sensory stimuli from those activated by suckling (Fig. 6-7), it was found that several areas, including mPOA, PePOA, SON, LSD and LSV in the forebrain and A1, LPB, $cPAG_{l,vl}$, A6, and PL in the brainstem, showed significant increases in cFos expression in response to suckling when compared to pup-exposure. These results suggest that these areas may play an important role in mediating suckling-induced alterations during lactation. The importance of each of the areas is discussed below.

Medial preoptic area (mPOA)

It has been shown by Numan and his colleages that the mPOA plays an important role in mediating maternal behavior (Numan, 1994). They observed that female rats exhibiting maternal behavior showed significantly more cFos positive cells in this region than control animals (Numan and Numan, 1994, 1995). The pups of FosB knock-out mice died soon after parturition primarily because the female knock-out mice failed to exhibit maternal behavior and take care of the pups (Brown et al., 1996). Thus, the FosB knock-out mice study further suggests that the expression of cFos in neurons in the mPOA area plays a crucial role in mediating maternal behavior during lactation. The present study demonstrated that sensory stimuli from pup exposure can induce a small but significant amount of cFos expression in the mPOA, suggesting that the sensory cues may play a minor role in facilitating maternal behavior. The efferent connections of mPOA neurons have been extensively studied. The medial portion of the mPOA mainly projects within the periventricular zone of the hypothalamus, including PVH and ARH, whereas the lateral portion of the mPOA sends projections to the DMH, LH, LSV and is the major area of mPOA that projects to the brainstem (Simerly and Swanson, 1988). Functionally, the mPOA can modulate LH and prolactin secretion, food intake responses, and autonomic responses (Simerly, 1995). Taken together, it is possible that in addition to maternal behavior, the mPOA may play an important role in mediating alterations in a number of physiological functions during lactation.

Periventricular preoptic area (PePOA)

PePOA is a longitudinal structure sandwiched between the mPOA and the third ventricle. The function of PePOA has not been examined in detail. Several studies have suggested that neurons in the PePOA may participate in the regulation of the reproductive system by modulating GnRH neuronal activity in the preoptic area: (1) anatomical studies showed that the PePOA neurons send efferent connections directly on to GnRH perikarya (Berghorn et al., 1996), (2) the PePOA is coactivated with GnRH neurons during the afternoon of the LH surge and the activation is progesterone dependent (Le et al., 1997), and (3) the majority of neurons in the PePOA express progesterone receptors (Le et al., 1997). However, the present study showed that sensory stimulation from pup exposure did not activate PePOA. Thus, it is possible that the PePOA is one of the sites involved in suckling-induced suppression of LH secretion and subsequently the suppression of reproductive cyclicity during lactation.

Supraoptic nucleus (SON)

The SON is the major site where oxytocin is released into the posterior pituitary in response to the suckling stimulus. The majority of oxytocin neurons and vasopressin neurons are located in the outside rim of the SON (Armstrong, 1995), which coincides with the distribution of cFos expression induced by suckling in the present study. Thus, it is likely that most of the suckling-induced cFos cells in the SON are probably either oxytocin positive or vasopressin positive or both.

Lateral septum

One of the few extrahypothalamic areas showing differences in cFos staining between the suckled group and the pup exposure group is the lateral septum, including both the dorsal part (LSD) and the ventral part (LSV). Anatomical studies have revealed that the LS sends dense projections into hypothalamic areas, including the mPOA, PVH, DMH, LH, the perifonical region and the mammillary bodies (Jakab and Leranth, 1995). Moderate levels of efferent fibers have also been found in the ARH and median eminence. This heavy septal input into the hypothalamus suggests that LS may play an important role in mediating suckling-induced alterations in the hypothalamus. Neurochemical studies have showed that the major LS input into the hypothalamus is GABAergic (Jakab and Leranth, 1995), suggesting that the main function of LS on hypothalamic function may be inhibitory. Hypothalamic areas especially the mPOA and LH also send input into the LS, suggesting a possible feedback control between the hypothalamus and LS. In addition, LS has been suggested to be involved in behavioral regulation, including consumptive behavior, sleep, and the initiation of specific motivated behaviors (Jakab and Leranth, 1995), suggesting that LS may play a role in mediating some of the behavior patterns observed in lactation.

Ventrolateral medulla (A1)

The acute suckling stimulus induced a significant increase in the number of TH neurons expressing cFos in the A1 noradrenergic cell group but not in the nearby C1 adrenergic group. The A1 noradrenergic neurons have been shown

to send direct projections into many hypothalamic nuclei, including PVH, SON, and ARH of the hypothalamus (Sawchenko and Swanson, 1982, and unpublished observation). The function of the A1 noradrenergic groups has been related to OT and vasopressin secretion from PVH and SON, the secretion of anterior pituitary hormones, and the regulation of autonomic function (Sawchenko and Swanson, 1982). The results from the present study further demonstrate that this group of neurons can be activated by the suckling stimulus and may play an important role in regulating hypothalamic function during lactation.

Locus coeruleus (A6)

Another noradrenergic cell group showing significant cFos expression in response to the suckling stimulus was the A6 group in the LC. A6 provides most of the noradrenergic inputs into the cortex and hippocampus (Aston-Jones et al., 1995). During lactation the cortex and hippocampus of the female rat is refractory to stimulation by excitatory amino acid treatment, which induces severe seizures when given to nonlactating rats (Abbud et al., 1992). The activation of the A6 group by the suckling stimulus may play an important role in mediating this effect.

Lateral parabrachial nucleus (LPB)

LPB showed strong cFos expression only in the resuckled animals. 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, 1995). Anatomically, LPB has been shown to send direct projections into various hypothalamic areas, including the preoptic area, PVH, DMH and VMH (Saper and Loewy, 1980; Fulwiler and Saper, 1984; Bester et al., 1997). These findings suggest that LPB could be important in regulating hypothalamic control of autonomic function during lactation. Future studies employing double label immunocytochemistry will permit further characterization of the neuronal phenotypes in the LPB area activated by suckling.

Lateral and ventrolateral of caudal Periaqueductal gray ($cPAG_{l,vl}$)

Lateral and ventrolateral PAG also showed strong cFos induction by the suckling stimulus, although pup exposure alone also induced significant cFos expression. This result is in agreement with a similar study by Stern and coworkers (1997). However, it is important to point out that earlier studies showed that lesions of PAG did not block OT release and milk ejection (Juss and Wakerley, 1981), suggesting that PAG is probably involved in other alterations associated with suckling stimulus. In fact, Stern and coworkers have suggested that PAG may be involved in mediating maternal kyphosis and aggression during lactation (Lonestein and Stern, 1997). It has been shown that the hypothalamus provides the richest afferent inputs into the PAG area, with dorsomedial and lateral hypothalamus sending most of the input into the $cPAG_{l,vl}$ (Beitz, 1995). In addition, PAG also receives minor afferent input from the brainstem and spinal cord (Beitz, 1995). Thus, it is possible that cFos

expression in PAG may be secondary to activation of the hypothalamus by the suckling stimulus.

Paralemniscal nucleus

In the present study, a group of cFos-positive cells induced by the suckling stimulus was found in the cadual portion of the paralemniscal nucleus medial to the rubrospinal tract. The functional role of this group of neurons during lactation is unknown. It is worth noting that the retrograde tracing study described in chapter VII showed that this neuronal population sends projections into the ARH. Thus, this area may be important in altering hypothalamic function when activated by the suckling stimulus. Further investigation is needed to understand the significance of the activation of this population of neurons during lactation.

Several areas, including DC, Pn, Sp5C and reticular formation, showed strong cFos expression in both resuckled and pup-exposure groups. These data suggest that these areas can be activated by the sensory stimuli associated with the pups, in the absence of suckling. In fact, these areas have been shown to be involved either in relaying or in integrating sensory information into higher brain centers (Jones, 1995; Ruigrok and Cella, 1995; Waite and Tracy, 1995; Webster, 1995). However, it is important to recognize that areas like the reticular formation have been shown to send long projections into the hypothalamus (Jones and Yang, 1985; Vertes et al., 1986) and, therefore, may play some role in regulating hypothalamic function during lactation.

Among the areas identified in the present study showing activation by the suckling stimulus, only VLM/A1 has been shown by earlier studies to be a part of suckling-sensitive pathways (Crowley and Armstrong, 1992). On the other hand, several areas, including NTS/A2 (Crowley and Armstrong, 1992), medial raphe nucleus (Clarke et al, 1985) and ventral tegmentum (Juss and Wakerley, 1981), which have been implicated in transmitting the effects of suckling, did not show significant increases in cFos expression in response to the acute suckling stimulus. The NTS/A2 noradrenergic group has received considerable attention in regulating suckling-induced OT secretion, based on the following observations: (1) tracing studies indicated that A2 sends projections into SON and PVH (Sawchenko and Swanson, 1982; Cunningham and Sawchenko, 1988; Day and Sibbald, 1988), (2) electrical stimulation of A2 activated OT neurons in the PVH and SON (Day et al., 1984) and (3) stimulation of NTS in lactating rats increased OT releases (Plotsky et al., 1988). However, the results from the present study do not support the notion of involvement of A2 in the suckling-sensitive pathways. It is possible that A2 may not be important under normal physiological conditions in transmitting the neuronal signals induced by suckling, even though it is capable of stimulating OT release from the hypothalamus. In contrast to the lack of effect of suckling, several stimuli have been shown to induce a significant increase in cFos expression in NTS; these include acute stress (Larsen and Mikkelsen, 1995), changes in blood pressure (Dun et al., 1993; Chan and Sawchenko, 1994; Krufoff et al., 1995), gastric distention (Willing and Berthoud, 1997), and even administration of CCK (Chen

et al., 1993; Day et al., 1994) Thus, further investigation is needed in order to resolve the issue of whether there is NTS involvement in mediating the effects of suckling.

The advantage of using cFos as a marker for identifying the functional pathways conveying the neuronal signals of the suckling stimulus is two-fold. First, double-label immunocytochemical staining can be employed to identify the possible neurochemical substrates involved in the pathways. Second, cFos staining can be combined with tracing techniques so that the projections from cFos-positive cells can be traced to determine the down stream neuronal systems in the pathway. This approach will permit characterization of the functional pathways transmitting the suckling stimulus and will greatly facilitate an understanding of the mechanisms by which hypothalamic function is altered during lactation.

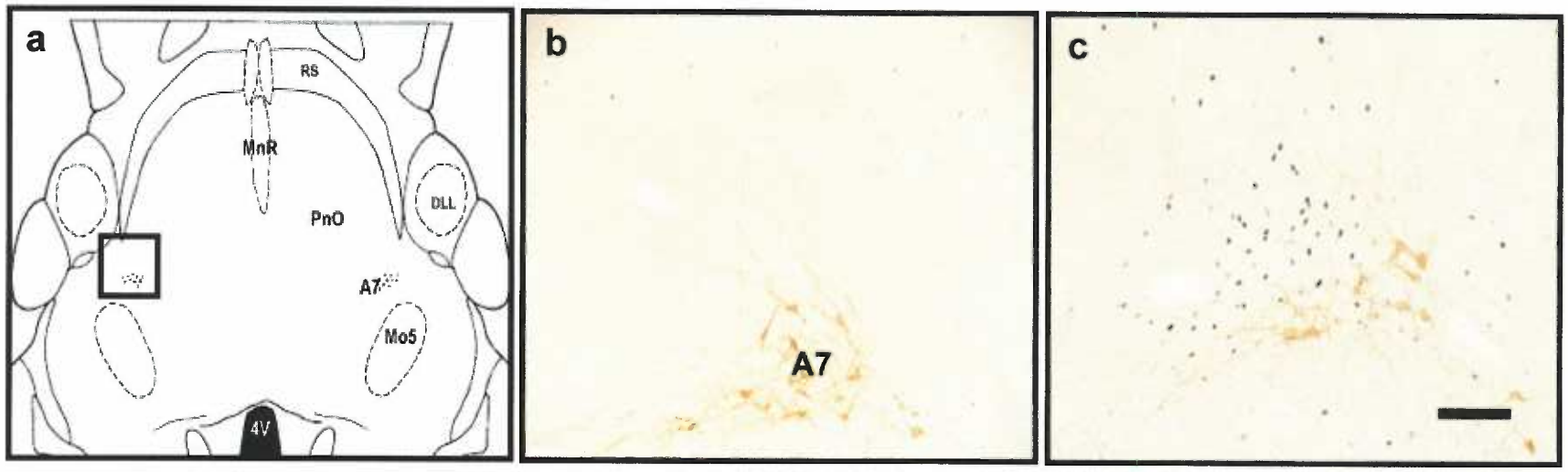
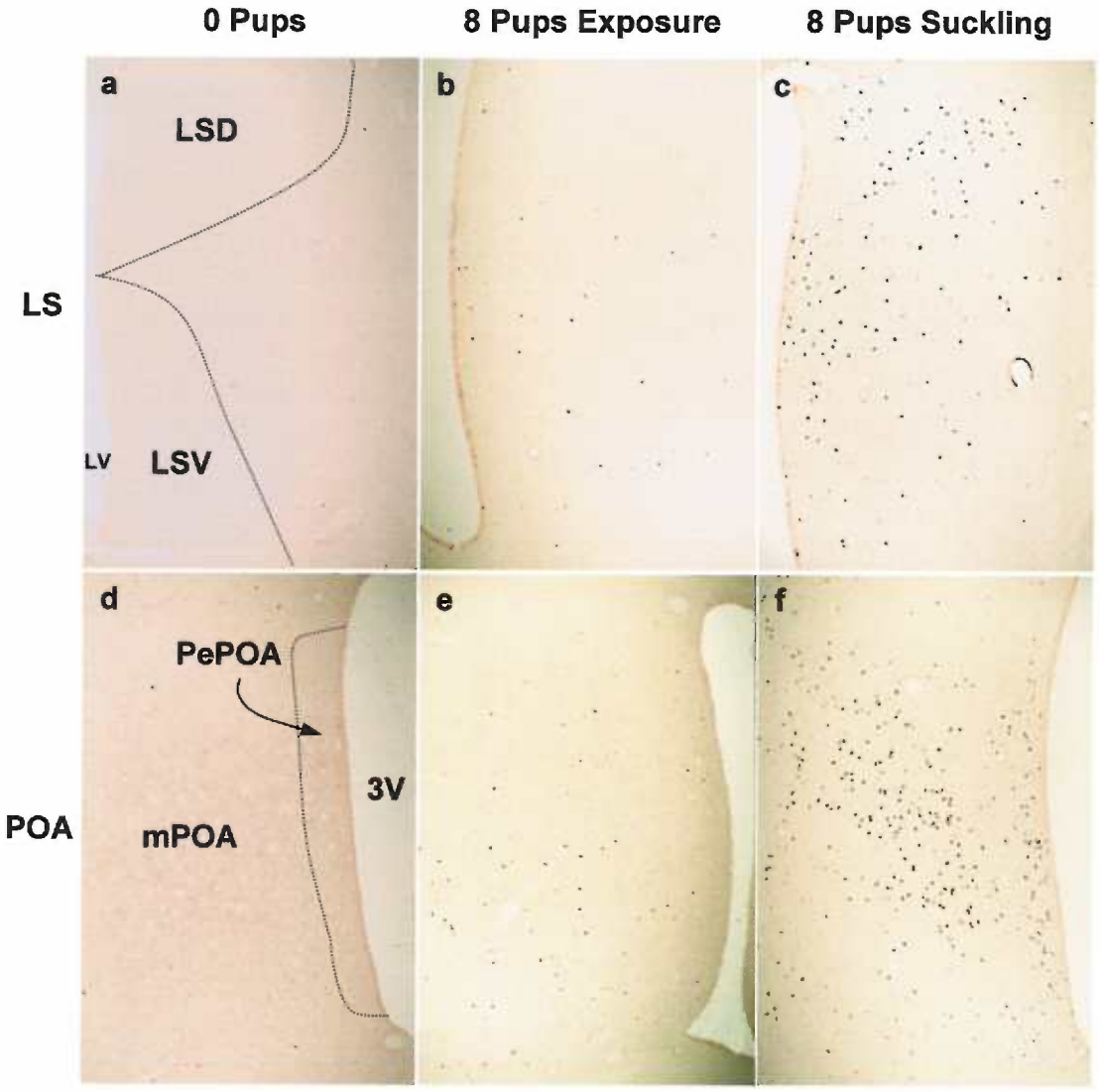
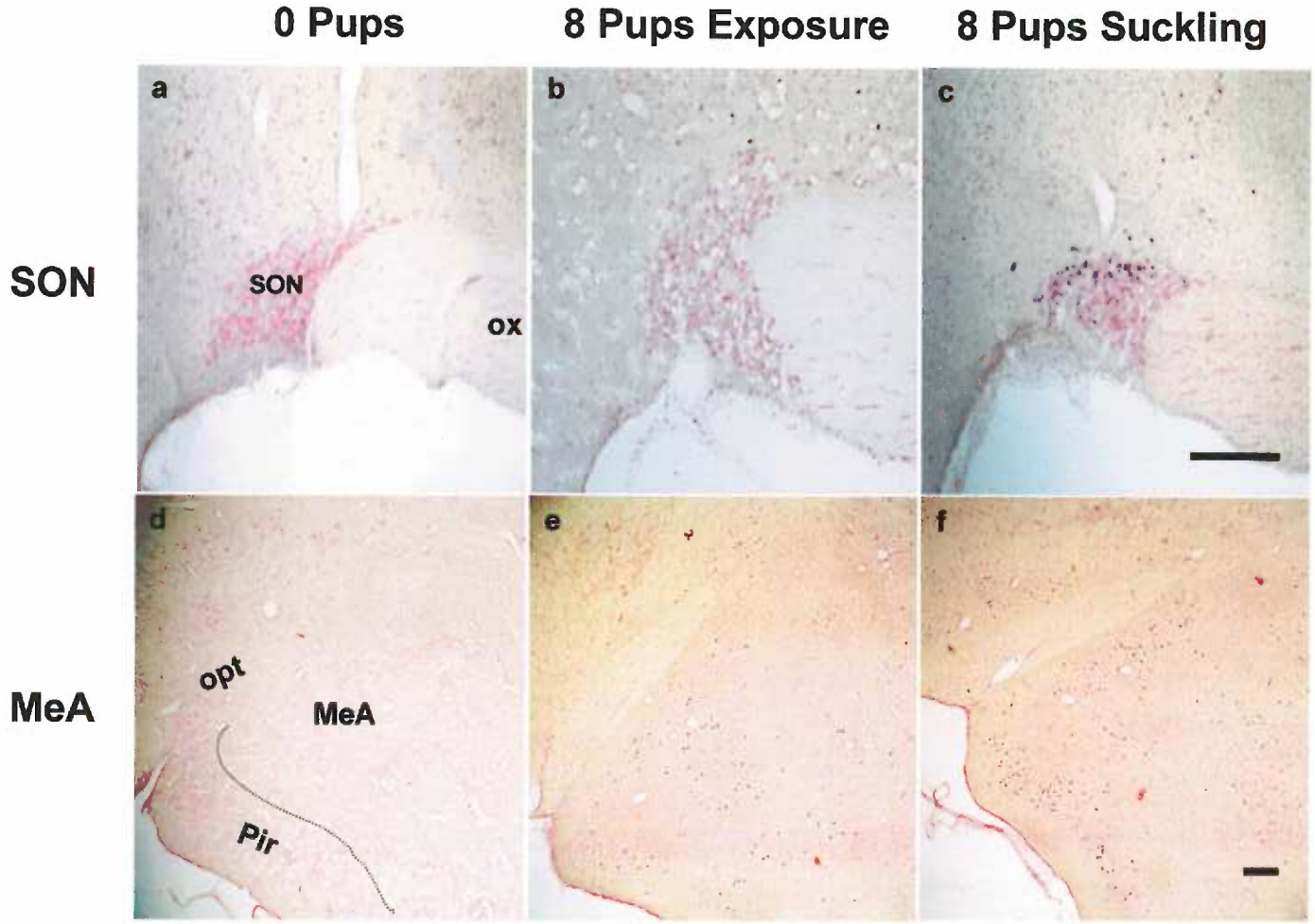


Figure 6-1. Suckling-induced cFos expression in the cadual portion of the paralemniscal nucleus (PL) close to the rubrospinal tract. A simplified horizontal brainstem drawing (left, plate 93, Paxinos and Waston, 1982) indicates the approximate location of this suckling-induced cFos population in the brainstem. This population of cFos-positive cells (dark nuclear staining) is located rostrally to the A7 noradrenergic neurons (brown cytoplasmic staining) and medially to the rubrospinal tract. The box in the drawing indicates the area depicted in the photomicrographs from a 0 pups control animal (middle) and from a 8 pups suckling animal (right). A7: A7 noradrenerigc neurons; MnR: median raphe nucleus; Mo5: motor trigeminal nucleus; rs: rubrospinal tract; DLL: dorsal nucleus of lateral lemniscus; 4V:4th ventricle. Scale bar = 15 μm.


0 Pups
8 Pups Exposure
8 Pups Suckling
a
b
c
LS
LSD
LV
LSV
d
e
f
POA
PePOA
3V
mPOA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SON
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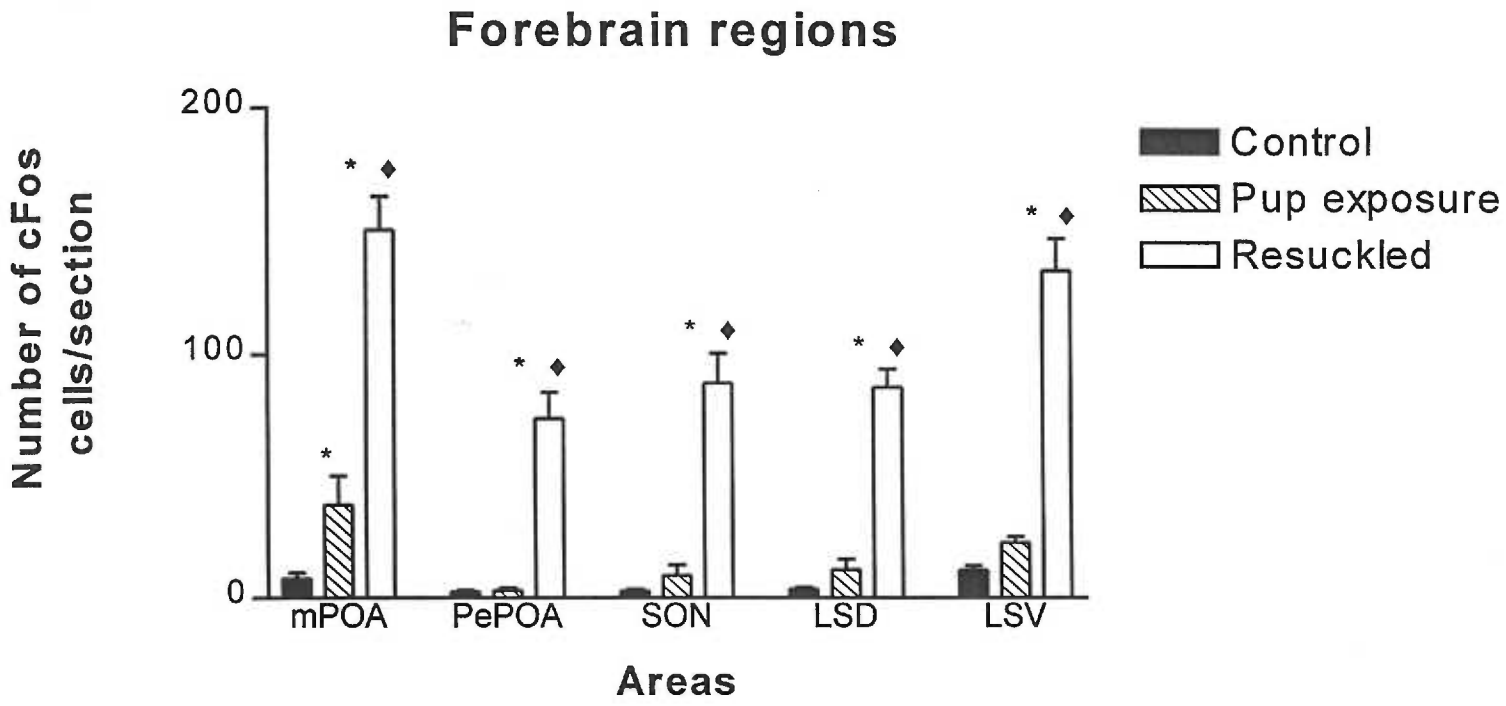


Figure 6-3. Numbers of cFos-positive cells in the mPOA, PePOA, SON, LSD and LSV of the 3 treatment groups. Data are presented as mean ± SEM. *: significantly different ($p<0.005$) from the 0 pup nonsuckled control. ♦: significantly different ($p<0.005$) than the 8 pups exposure group.

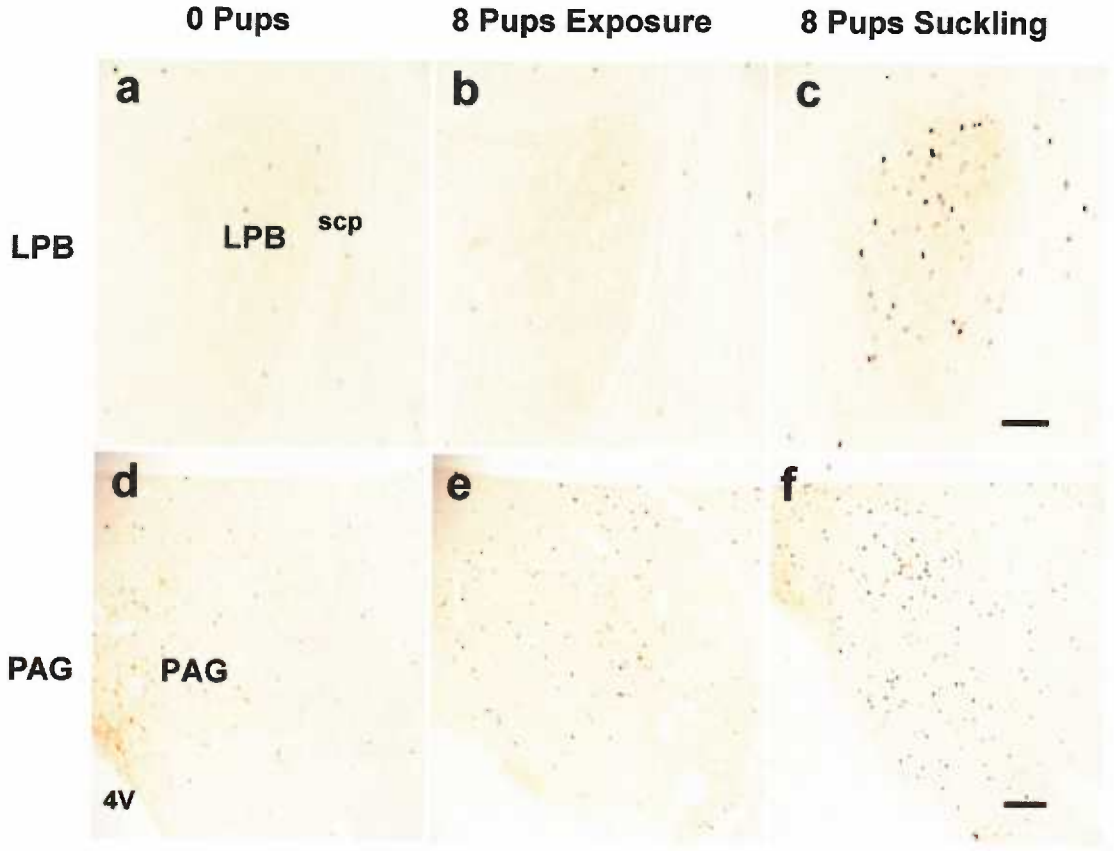


Figure 6-4 cFos expression in the LPB and cPAG areas. Representative photomicrographs of cFos immunoreactivity in the LPB (a, b, c; scale bar =10 μm) and cPAG (d, e, f; scale bar =15 μm) from 0 pup control (a, d), 8 pup exposure (b, e) and the 8 pup suckling group (c, f). In the cPAG area, TH-positive neurons were observed around the dorsal raphe area and medial portion of the cPAG, but cFos-positive TH neurons were rarely found across the three groups. LPB: lateral parabarchial nucleus; PAG: periaqueductal gray; scp: superior cerebellar peduncle; 4V: fourth ventricle.

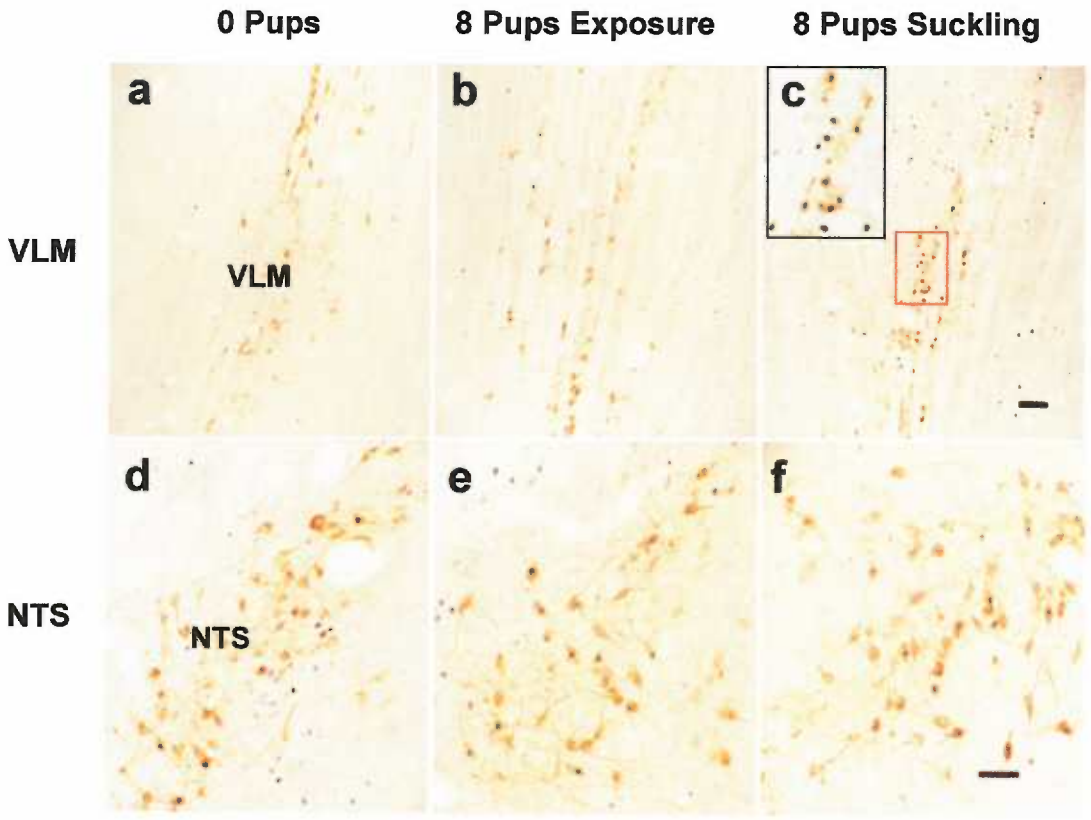


Figure 6-5. Suckling-induced cFos expression in the VLM/A1 and NTS/A2 catecholaminergic cell groups. Representative photomicrographs of cFos (dark black nuclear staining) and TH (brown cytoplasmic staining) immunoreactivity in the VLM/A1 (a, b, c; scale bar=15 μm) and NTS/A2 (d, e, f; scale bar= 15 μm) from 0 pup control (a, d), pup exposure (b, e) and 8 pups suckling group (c, f). The inset shows a magnified view of the area indicated by the box. In the VLM, cFos/TH double-labeled cells were observed in the 8 pups suckling animal (c) but the double-labeled cells were rarely found in the 0 pup control (a) and pup exposure (b) groups. In the NTS, cFos/TH double-labeled cells were found in all three groups of animals (d, e, f). NTS: nucleus of solitary tract; VLM: ventrolateral medulla.

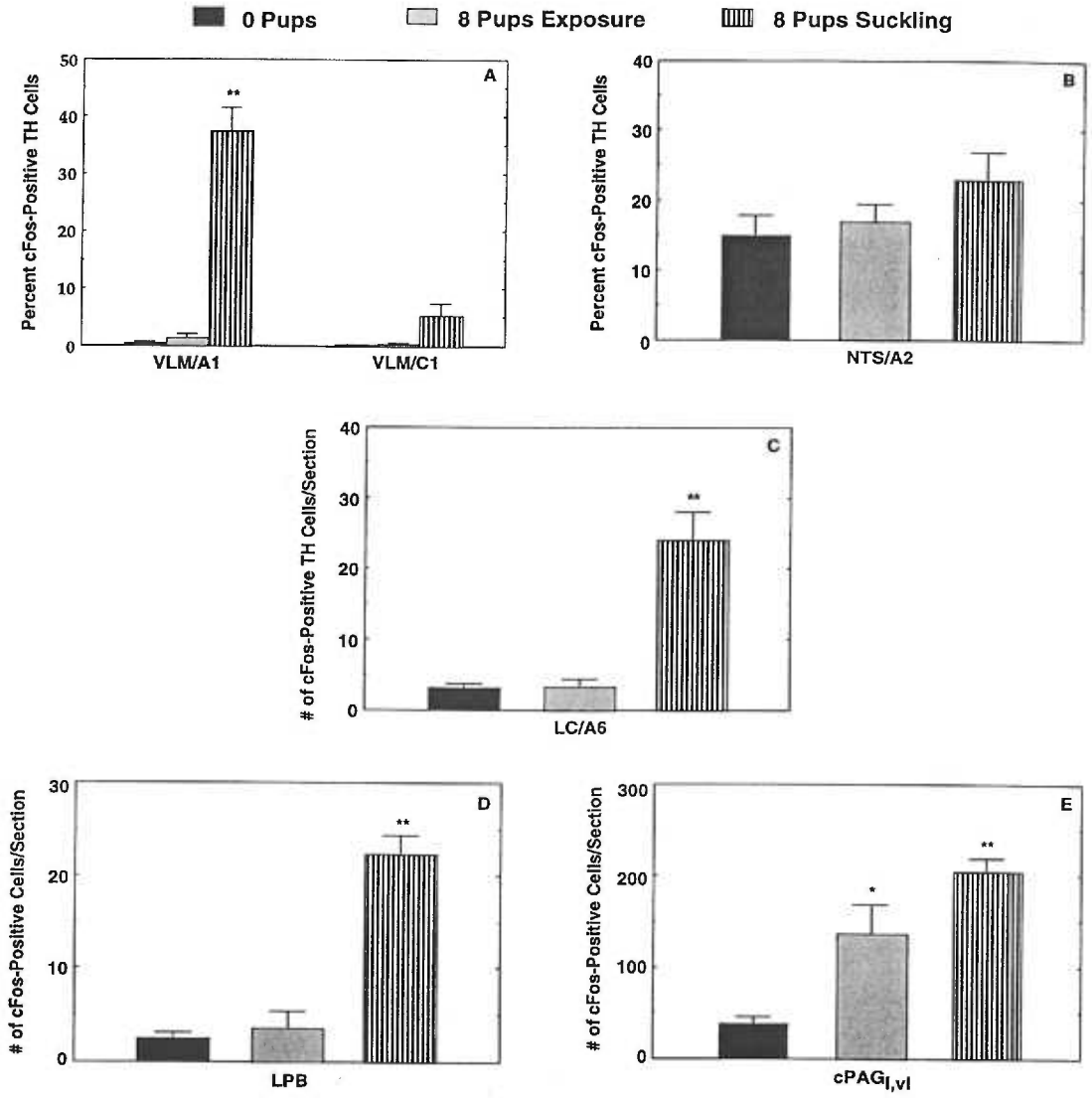


Figure 6-6. Effects of the suckling stimulus or pup exposure on cFos expression in TH-positive neurons in VLM/A1 (A), VLM/C1 (A), NTS/A2 (B), and LC/A6 (C) and on cFos expression in LPB (D) and cPAG (E). In VLM/A1, VLM/C1, and NTS/A2, the data were expressed as the percent of cFos-positive TH cells. In LC/A6, the data were expressed as the number of cFos-positive TH cells per section. In LPB and cPAG, the data were expressed as number of cFos-positive cells per section. **: indicates $p<0.05$ compared to 0 pup control and pup exposure. *: indicates $p<0.05$ compared to 0 pup control.

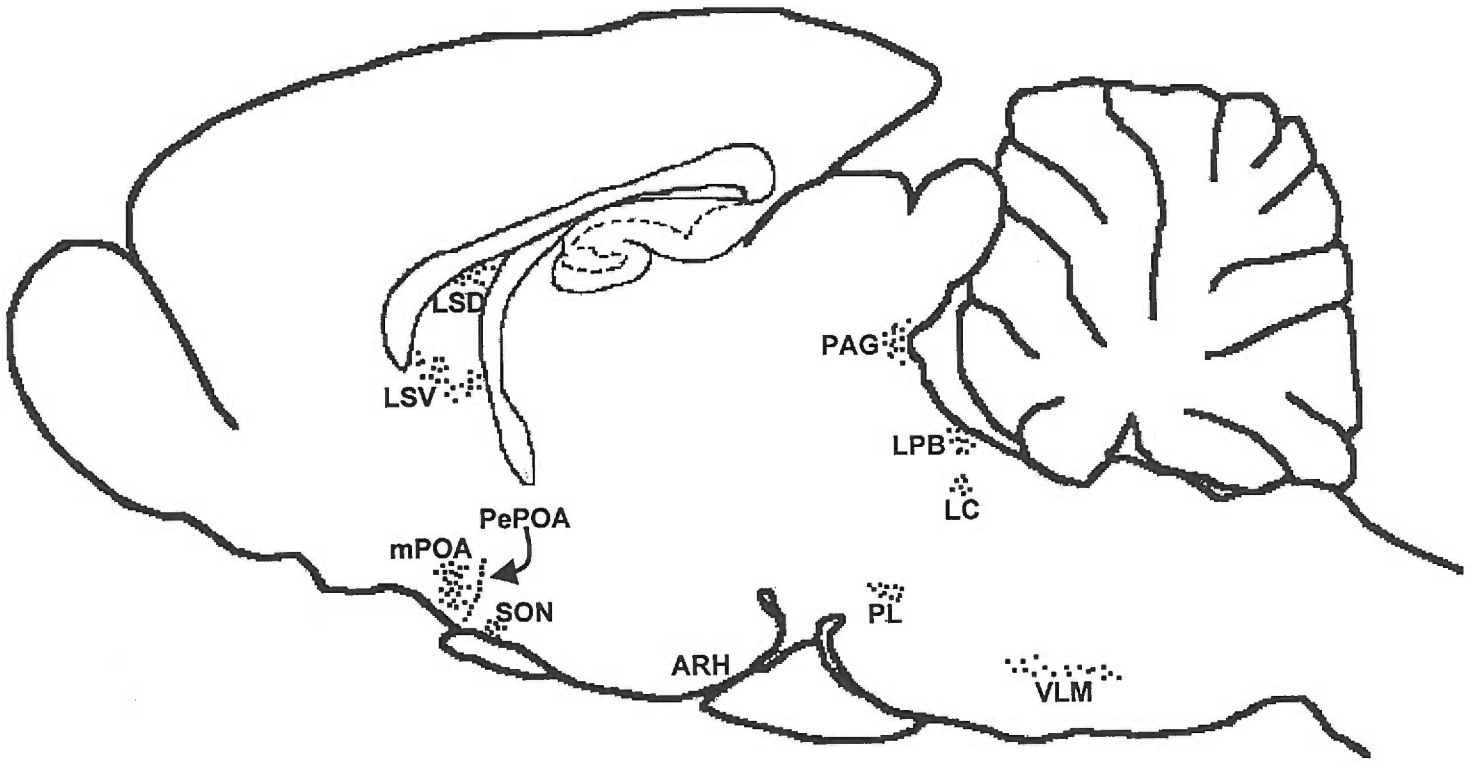


Figure 6-7. A diagrammatic representation showing the major neural populations activated specifically by the suckling stimulus. The black dots represent the expression of cFos-positive neurons in each area. The relative abundance of cFos expression in each area is roughly proportional to the number of the dots associated with it. Areas activated by both pup exposure and suckling are not shown in this drawing.

CHAPTER VII

IDENTIFICATION OF NEURONAL INPUT TO THE ARCUATE NUCLEUS (ARH) ACTIVATED BY THE SUCKLING STIMULUS

Manuscript in preparation

Introduction

Neuropeptide Y (NPY) neuronal activity in the arcuate nucleus of the hypothalamus (ARH) has been shown to be greatly increased during lactation (Li et al., 1998a; Smith, 1993). The increase in NPY activity in the ARH was observed only in the caudal portion of the ARH, even though NPY-positive neurons are detected throughout the ARH (De Quidt et al., 1993). It has been suggested that the increased NPY activity in the caudal portion of the ARH may be important in mediating some of the physiological alterations associated with lactation, such as the increased food intake (Tomaszuk et al., 1996) and the suppression of lutenizing hormone secretion (Kalra and Crowley, 1992; Kalra and Kalra, 1996).

Currently, the mechanisms by which the caudal ARH NPY neurons are activated during lactation are not completely understood. It has been shown that the suckling stimulus is important in triggering this alteration (Li et al., 1998a; Smith, 1993). Removal of suckling-induced hyperprolactinemia does not affect the activation of the ARH NPY neurons (Li et al., 1998b). The lack of involvement of hyperprolactinemia in activation of ARH NPY neurons also implies that the metabolic signals induced by changes in energy balance due to milk production, which is induced by the elevation of prolactin, are not required for the activation of the ARH NPY neurons. Taken together, these results suggest that the activation of NPY neurons in the ARH may be mediated by incoming neural impulses activated by suckling.

It has been shown that the immediate early gene protein product, cFos, can be used as a marker for neuronal activation (Morgan and Curran, 1991; Hoffman et al., 1993; Ceccatelli et al., 1989). Several laboratories, including ours, have shown that a discrete pattern of cFos expression in the brain is observed after the female rat has received an acute suckling stimulus (Li et al., 1997; Lonestein and Stern, 1997; Numan and Numan, 1994, 1995). Thus, in the present study, retrograde tracing from the caudal ARH area combined with acute suckling-induced cFos expression was used to identify neuronal populations in the brain that may be important in activating the caudal ARH NPY neurons in response to suckling.

Materials and Methods

Animals

Day 18-19 pregnant Sprague-Dawley rats (B & K Universal Inc., Kent, WA) were housed individually and maintained under a 12:12 light-dark cycle (lights on at 0700-1900) 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 as day 0 postpartum. Lactating animals had their litters adjusted to eight pups on day 2 postpartum. All the animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee.

Experimental design

An acute suckling paradigm previously described (Li et al., 1988a) was utilized 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. At that time, the 8-pup litters were removed from the females. On day 11, the 8-pup litters were returned to the dams to allow suckling for 90 min (all pups were nursing within 1-2 min of placement in the cage with the dam).

Retrograde tracer injection

On day 4 postpartum, animals were anesthetized with tribromoethanol (20 mg/100 g body weight) 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 caudal portion of the ARH. Injection coordinates were 3.2 mm caudal, 0.25 mm lateral to the bregma, and 9.75 mm below the dura, according to the atlas of Paxinos and Watson [18]. FG was injected by iontophoresis with 5 μA current, pulsed at 7 sec intervals for 20 min. The glass pipette was left *in situ* for an additional 10 min to avoid the spread of tracer along the pipette track. The animals were then returned to their 8-pup litters.

Perfusion and tissue sectioning

After 90 min of pup suckling, animals were anesthetized with an overdose of pentobarbital (125 mg / Kg B.W., i.p.) and perfused transcardially with 150 ml of 2% sodium nitrite in saline followed by 150 ml 2.5% acrolein (EM grade, Polysciences) in phosphate-buffered 4% paraformaldehyde (pH 7.4). The brain was removed, divided into forebrain and brainstem from the collicular area and immersed in 25% sucrose at 4 °C overnight. Coronal sections for the forebrain and horizontal sections for the brainstem (both at 25 μm) were cut on a sliding microtome and collected in a one-in-four series. The tissue sections were stored until use at -20 °C in multiwell tissue culture plates containing cryoprotectant.

Immunocytochemistry procedures

Immunocytochemistry of cFos and FG was performed as described elsewhere (Lee et al., 1990). Tissue sections were removed from cryoprotectant and rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by treatment with 1% $NaBH_4$ -KPBS solution (Sigma). Sections were incubated in rabbit anti-cFos antibody (*sc*-52, Santa Cruz, 1:15,000) in KPBS with 0.4% Triton X-100 at room temperature for 1 hr, followed by 4°C for 48 hr. After the incubation, the tissues were rinsed in KPBS and incubated in biotinylated goat anti-rabbit IgG (Vectorlaboratories, 1:600) in KPBS with 0.4% Triton X-100 for 1 hr at room temperature. This was followed by another 1 hr incubation at room temperature in avidin-biotin complex solution [4.5 μl of A and B each per ml of KPBS-0.4% Triton X-100 (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 (0.2 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 transferred into sodium acetate solution to stop the color reaction, rinsed in KPBS, and then incubated in the rabbit anti-FG antibody (Chemicon, 1:30,000 for the forebrain sections and 1:15,000 for the brainstem sections). The same immunocytochemistry procedures described above were followed except that horse anti-mouse IgG (Vector Laboratories, 1:600) was used as the secondary antibody. The FG antibody-peroxidase complex was stained with a mixture of 3,3'-diaminobenzidine (0.2 mg/ml) and 3% H_2O_2 (0.83 μl/ml) in 0.05 M Tris buffer-saline solution. Following the FG staining, tissue

sections were mounted on gelatin coated glass slides, dehydrated through graded alcohols, cleared in xylenes, and coverslipped with Histomount (National Diagnostics).

Results

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

Only the lactating animals in which the injection site was located in the caudal portion of ARH were used (n=5). As shown in Fig. 7-1, the unilateral injection of FG was confined in the region of the ARH. In addition, the pattern of retrograde labeling was similar in all of the animals with injections centered in the caudal ARH.

The pattern and density of cFos expression in all of the experimental animals were similar to those observed in acute resuckled animals reported in the study described in chapter VI. In addition, in that study, several areas were identified that showed significant increases in cFos expression only in response to the physical suckling stimulus (Table 6-1, 6-2). Therefore, the present study focused on the areas shown in Table 6-1 and 6-2 to examine for the presence of cFos and FG double-labeled neurons.

Forebrain input to the caudal ARH

When the forebrains of FG-injected animals were examined, most of the FG-positive cells were found in the hypothalamus, and the densest concentration of FG-positive cells was found in the ventral and medial portions of the mPOA (Fig. 7-2), PePOA (Fig. 7-2), AH, PVH, VMH, the ventral premamillary nucleus (PMV), ARH, and the zone between the VMH and ARH. Scattered FG-positive cells were found in the LH and DMH. Outside the hypothalamus, dense FG-positive cells were observed in the ventral part of the

lateral septal area, the BST and the central and the medial amygdaloid nuclei (Fig. 7-2). A few scattered FG cells were also found in the paraventricular nucleus of the thalamus.

FG/cFos double-labeled cells were found throughout various hypothalamic areas. Double-labeled cells were found in the mPOA and PePOA, where suckling-induced cFos expression was detected (Fig. 7-3). Occasional, scattered double-labeled cells were found in the AH, DMH, LH, and the zone between VMH and ARH. Outside the hypothalamus, the BST and amygdaloid area (Fig. 7-3) showed some double-labeled cells. Very few double-labeled cells were detected in the lateral septal area, even though large numbers of cFos- and FG- single labeled cells were found in this area.

Brainstem input to the ARH

In the brainstem, FG-positive cells were found in the peripeduncular area (PP) (Fig. 7-4A), cPAG, the external layer of LPB (Fig. 7-4A), the area between pontine pedunculoreticular area and LPB, the area surrounding the LC, the barington nucleus, the caudal part of the paralemniscal nucleus (PL) (Fig. 7-4B), and the VLM (Fig. 7-4B).

Most FG/cFos double-labeled neurons were found in the PP, LPB, PL and the VLM (Fig. 7-4A, B). A few scattered double-labeled cells were found in the cPAG.

Disscussion

In the present study, retrograde tracing combined with cFos expression induced by the suckling stimulus was used to identify neural populations in the brain which may be important in suckling-induced NPY activation in the caudal ARH.

Forebrain input to the ARH

In the hypothalamus, most double-labeled cells were found in the mPOA and PePOA, suggesting that once activated by the suckling stimulus, these two areas may directly influence NPY neuronal activity in the ARH. In agreement with the present study, it has been shown that the mPOA, specifically the medial portion, sends heavy projections throughout the ARH, including areas of high density of NPY neurons (De Quidt et al., 1990). However, the efferent projections of PePOA have not been systemically examined. In future studies, it will be important to characterize the phenotype of the cFos positive neurons in both areas in order to further understand how these areas may mediate the activation of ARH NPY neurons.

Occasional scattered double-labeled cells were also found in several additional hypothalamic areas, including the AH, DMH, LH, and the zone between the VMH and ARH, although the cFos expression in these areas can also be induced by sensory input activated by pup exposure alone. These results suggest the possibility that sensory input induced by pup exposure may also be able to positively modulate ARH NPY activity to some extent, but the full

activation may require the combined input of sensory cues and the somatosensory input activated by the physical suckling stimulus.

In extrahypothalamic areas, double-labeled cells were found in the BST and amygdaloid areas, although cFos expression in these areas can also be induced by pup exposure. This suggests that the BST and amygdala may be another routes by which the sensory stimuli associated with pup exposure may modulate NPY activity in the ARH. However, only a few double-labeled cells were found in the lateral septal area, even though a very high density of both single labeled cells were found in this area. This result suggests that the lateral septum may not directly modulate ARH NPY activity. The present study cannot rule out the possibility that the lateral septum may indirectly modulate NPY activity by sending input to other neural sites such as the mPOA, which then send direct input into the ARH NPY area (Simerly and Swanson, 1988).

Brainstem input to the ARH

In the brainstem, most of the FG/cFos double-labeled cells were found in the areas where cFos expression was induced specifically by the suckling stimulus, including the external layer of the LPB, VLM, PL and some small number of scattered double-labeled cells in the cPAG. Thus, these areas appear to play an important role in relaying the neural input derived from suckling into the ARH area where the NPY neurons are located.

The LPB is considered one of the relay centers that convey visceral information from the body through the NTS into the hypothalamus (Saper, 1995). The LPB is involved in regulating a variety of physiological functions,

including control of body fluid homeostasis, energy metabolism, and blood oxygenation (Saper, 1995). Thus, it is possible that the NPY system in the ARH is one of the down stream systems in the hypothalamus activated by LPB neural input in response to the suckling stimulus. The NPY system in turn would regulate body homeostasis by modulating hypothalamic neuroendocrine function. The efferent projections of the LPB have been examined using several different anterograde tracing techniques (Saper and Loewy, 1980; Fulwiler and Saper, 1984; Bester et al., 1997). The LPB mainly projects into hypothalamic areas, including VMH, DMH, and LH, whereas ARH only receives a moderate input from LPB. Thus, the extensive retrograde labeling in the LPB seen in the present study after FG injections into the ARH of lactating animals could be due to either FG leakage into the nearby VMH or to a potential modification of neuronal input from the LPB into the ARH occurring specifically during lactation. A comparison between lactating and nonlactating animals in the efferent projections into the ARH from LPB should resolve this issue.

VLM has been shown to send extensive projections into the hypothalamus mainly in the PVH (Sawchenko et al., 1985). Studies of the function of VLM have mainly focused on the regulation of oxytocin and vasopressin release from the PVH (Bealer and Crowley, 1998; Crowley and Armstrong, 1992; Day and Sibbald, 1989; Shioda et al., 1998). The present study adds another potentially important function of this area during lactation, that of modulating the activity of the NPY system in the ARH. Because the distribution of the double-labeled cells observed in the present study resembled

the pattern of the suckling activated tyrosine hydroxylase (TH) positive cells found in the same area (Sawchenko et al., 1985), it is plausible to hypothesize that suckling-activated catecholamine neurons in the VLM send projections into the ARH to modulate the NPY system during lactation. In addition, it has also been shown that many of the VLM TH-positive neurons also express NPY (Sawchenko et al., 1985), suggesting that NPY input from the VLM may act together with catecholamines from the VLM to modulate NPY activity in the ARH during lacation. Further studies are needed in order to elucidate this issue.

A less well known area identified in the present study as containing double-labeled cells is the paralemniscal nucleus (PL). Previous studies using acute suckling to induce cFos expression showed that only the physical suckling stimulus was capable of inducing cFos expression in this area, suggesting a unique role for this area during lactation. Currently, little is known about the function of neurons located in this area. The present study thus provides the first anatomical evidence that the neurons in the PL may also participate in the regulation of hypothalamic function such as the NPY activity in the ARH during lactation.

Finally, a substantial number of double-labeled cells were found in the peripeduncular area (PP) located just dorsal to the substantia nigra. The induction of cFos in this area is not specific to suckling, since pup exposure has been shown to induce an equivalent density of cFos-positive cells. The efferent projections out of this area have not been examined in detail, although this area has been shown to provide neural input into the mPOA (Simerly and Swanson,

1986). Results from several lesion studies suggest that this area is important in mediating maternal behavior as well as maternal aggression during lactation (Factor et al., 1993; Hansen and Ferreira, 1986). Thus, the demonstration of double-labeled cells in the peripeduncular area implies that it represents another route by which sensory cues may activate the ARH and possibly the NPY system during lactation.

The present studies provide anatomical evidence about the possible sites that, upon activation by the suckling stimulus or even just the sensory input from pup exposure, may provide neural input into the ARH and possibly the NPY system during lactation (Fig. 7-5). Anterograde tracing from each area should provide the final proof whether these areas make direct contacts with the NPY system in the ARH. Characterization of the phenotype of the neurons in the areas showing direct contact with the ARH NPY system should facilitate our understanding of the mechanisms by which the suckling stimulus activates the ARH NPY system during lactation.

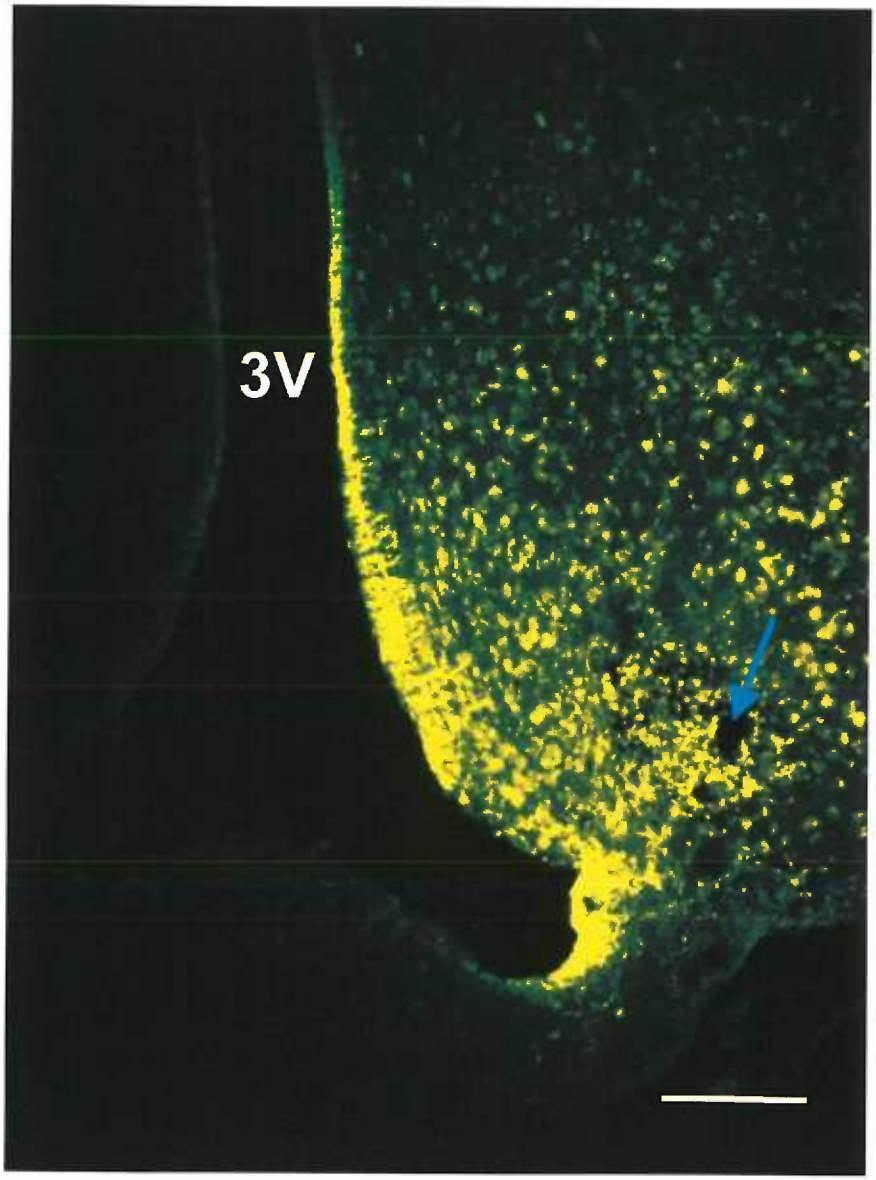


Figure 7-1. Representative fluroescent photomicrograph showing the FG injection site (arrow) in the ARH. 3V: third ventricle. Scale bar = 20 μm.

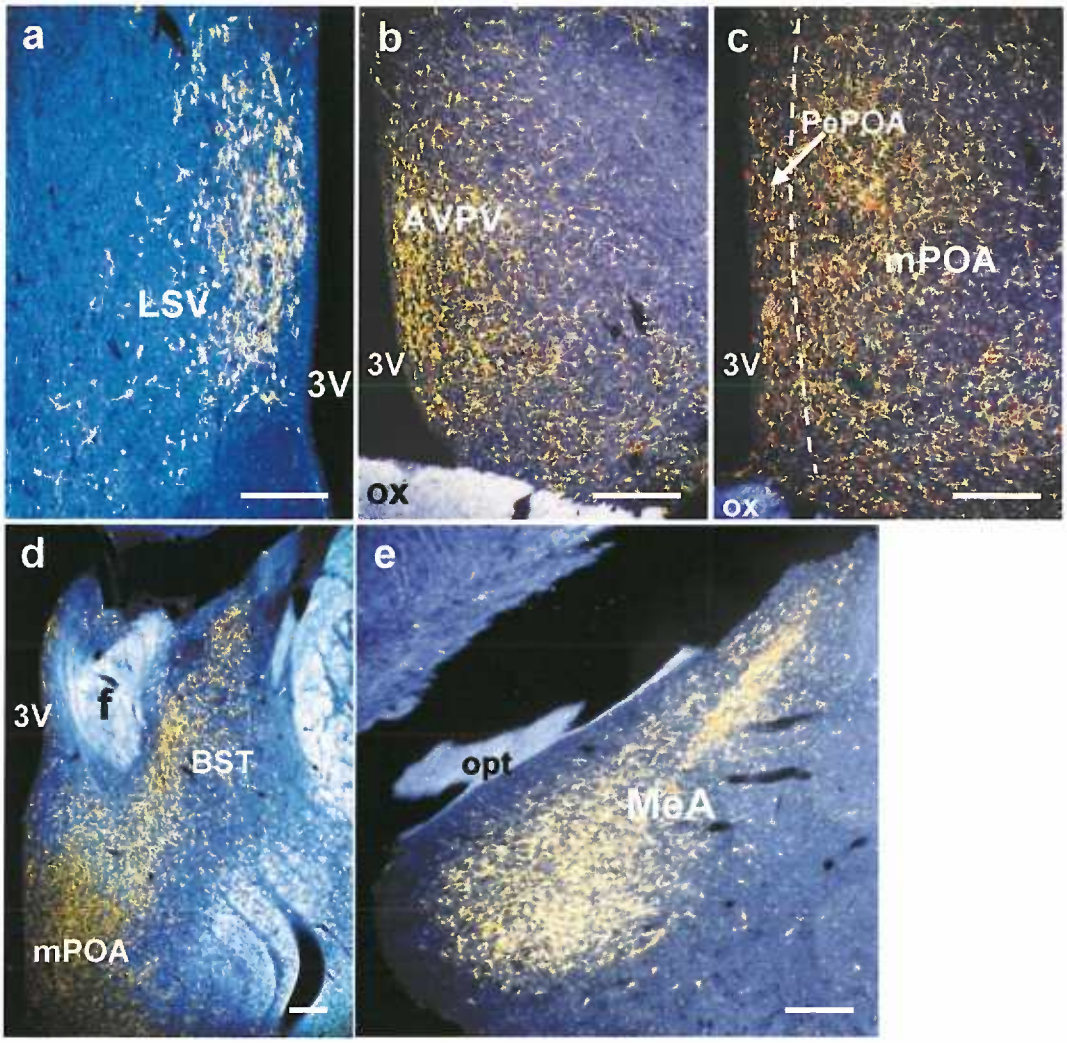
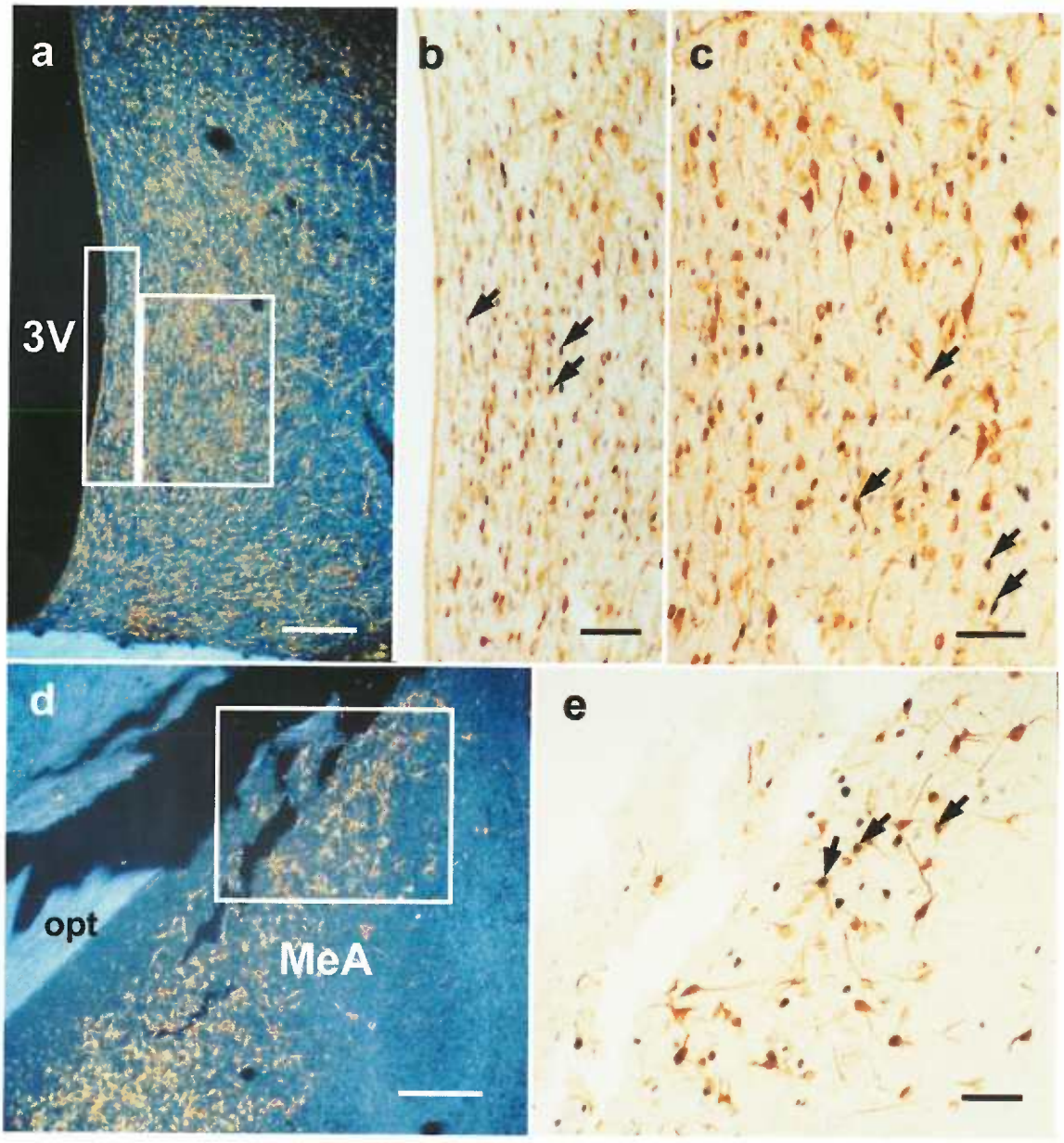
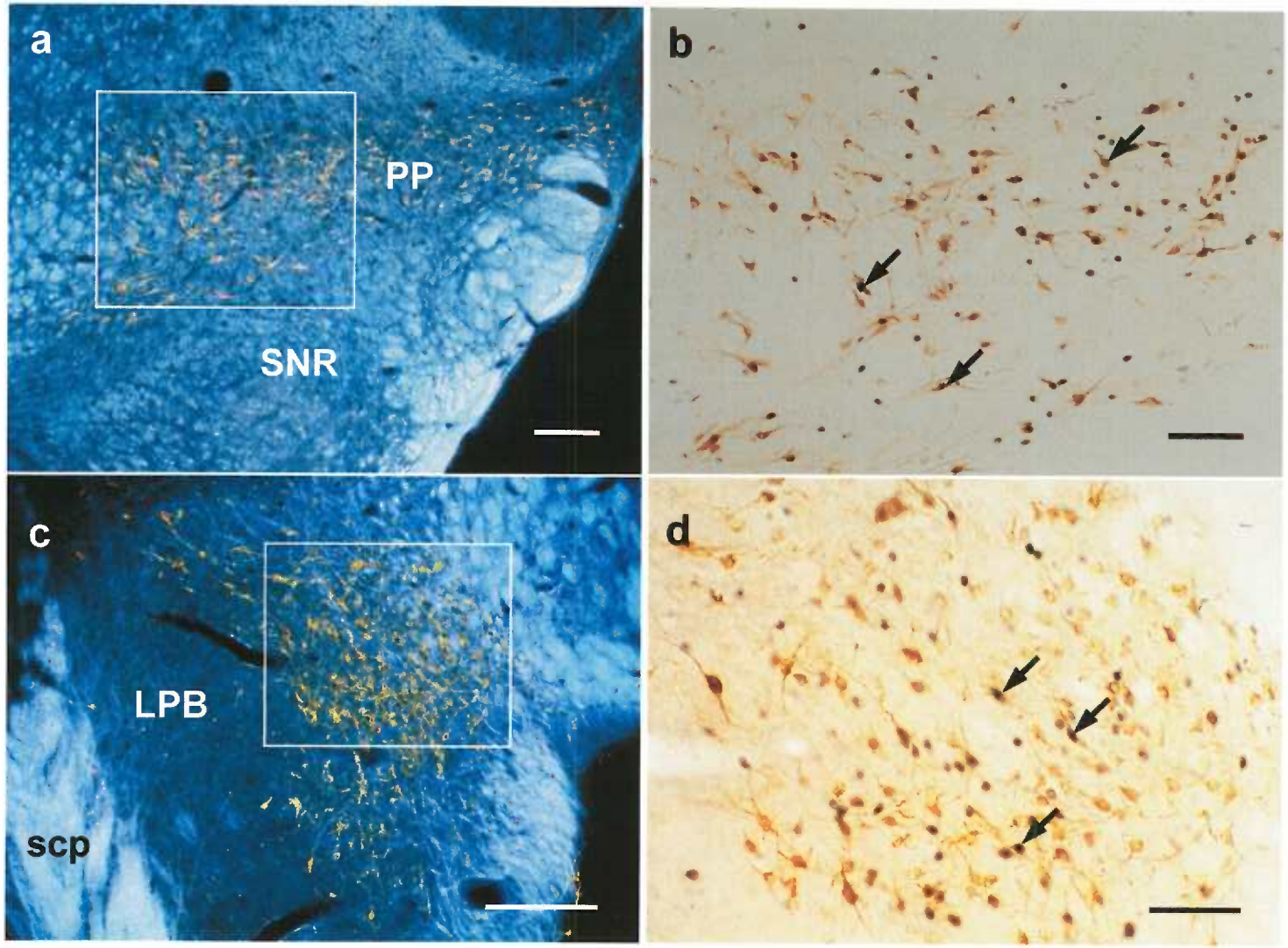
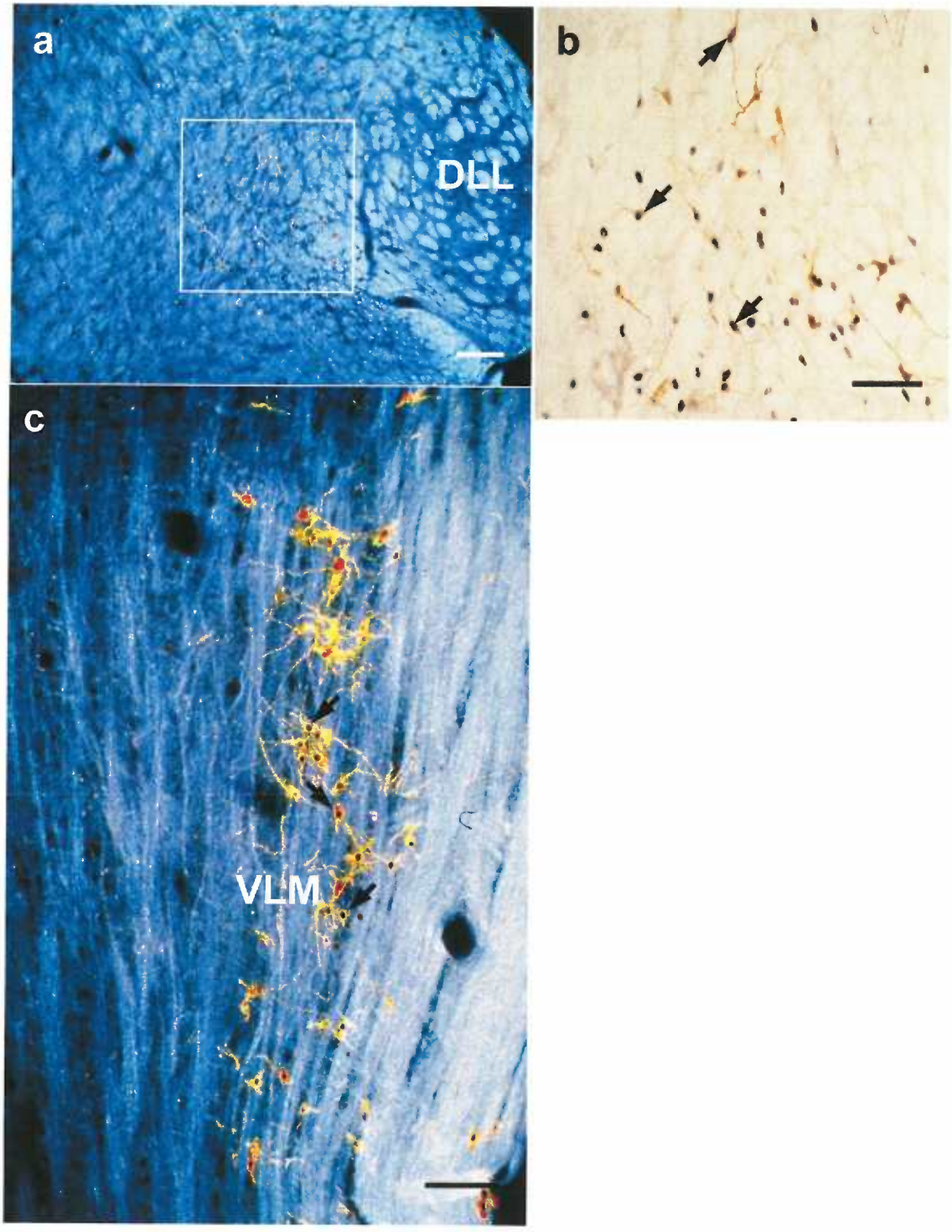


Figure 7-2. Representative low power dark-field photomicrographs show FG positive cells (golden staining) in LSV (a), AVPV (b), mPOA and PePOA (c, d) BST (d) and MeA (e). AVPV: anteroventral periventricular nucleus; BST: bed nucleus of stria terminalis; f: fornix; LSV: lateral septum, ventral part; mPOA: medial preoptic area; MeA: medial amygdala; ox: optic chiasm; opt: optic tract; PePOA: periventricular preoptic area; 3V: third ventricle. Scale bars = 25 μm.


a
3V
b
c
d
opt
MeA
e


a
PP
SNR
b
c
LPB
scp
d


a
DLL
b
c
VLM

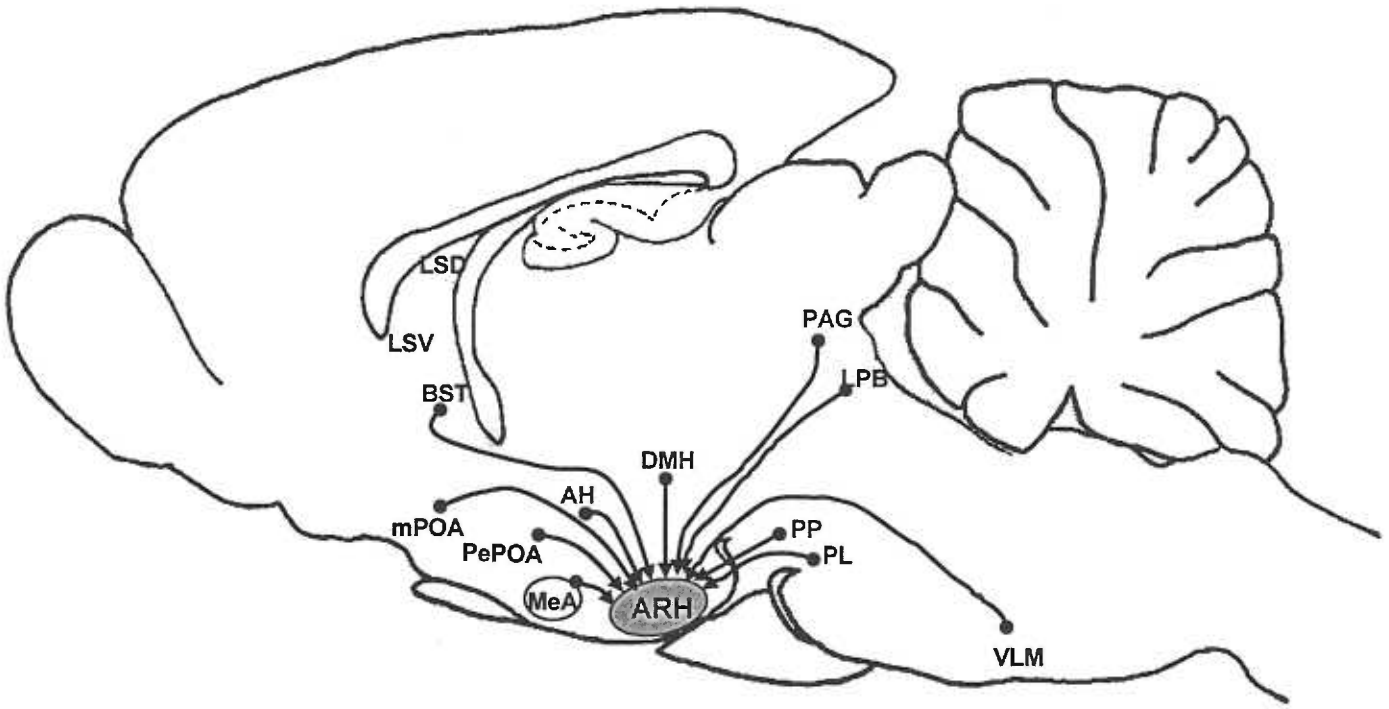


Figure 7-5. A diagrammatic representation showing the afferent inputs to the caudal portion of the ARH that were activated during lactation. According to the cFos study conducted in chapter VI of this thesis, afferent inputs that were activated specifically by the physical suckling stimulus include mPOA, PePOA, PAG, LPB, PL and VLM. Afferent inputs from areas that were activated by both suckling and sensory stimuli associated with pup exposure include BST, AH, DMH, MeA and PP.

CHAPTER VIII

CONCLUSIONS

By using the acute suckling paradigm, I found that the suckling stimulus could activate a significant increase in NPY neuronal activity in two distinct hypothalamic areas: the caudal portion of the ARH and the DMH area. Both NPY populations were significantly activated by 24 hours of the suckling stimulus. These results suggest that the suckling stimulus is the key determinant in activating NPY neurons in the hypothalamus during lactation. However, the two populations of NPY neurons showed different sensitivity to hyperprolactinemia. The activation of NPY neurons in the ARH is mediated by the neural signals induced by suckling and is independent of hyperprolactinemia, whereas the activation of NPY neurons in the DMH is a combination of neural signals and hyperprolactinemia. The mechanism by which PRL may mediate the activation of DMH NPY neurons is unknown. Even though PRL receptors have been identified in the brain, it is not clear whether the DMH NPY neurons express PRL receptors. In addition, PRL may exert its effect by modulating the suckling-induced neural input into the DMH area.

The involvement of hyperprolactinemia in DMH NPY activation also suggests that the DMH NPY neurons may mediate some of the central effects of PRL during lactation. PRL has been suggested to participate in the regulation of maternal behavior, feeding behavior and the suppression of LH secretion during lactation. The study from this thesis showed that the DMH NPY neurons project to the PVH, an important area in regulating food intake and also reproduction. Thus, the DMH NPY neurons may be one of the target systems in the hypothalamus that mediates some of the effects of PRL during lactation.

In contrast to the DMH NPY neurons, the activation of NPY neurons in the ARH depends on the neural signals induced by the suckling stimulus. Currently, all of the signals that have been shown to affect NPY neuronal activity arise from the periphery, including leptin, glucocortcioids and insulin, suggesting that the ARH NPY system may function as a sensor to recognize the alteration of body energy homeostasis. The ARH NPY system in turn maintains homeostasis by stimulating feeding behavior. On the contrary, the results from the suckling study suggest that the ARH NPY neurons can be quickly activated by neural signals through somatosensory pathways, implying that during lactation, the ARH NPY neurons are stimulated even before peripheral signals are altered. This rapid activation should provide a strong drive to increase food intake, allowing the female to prevent a significant energy drain due to milk production. However, the present study does not completely rule out the involvement of peripheral signals in modulating ARH NPY neuronal activity. Peripheral factors may provide a long-term signal in conjunction with the neural signals from suckling to maintain the activity of ARH NPY neurons at maximal levels.

To begin to understand the mechanisms by which suckling-induced neural input may activate the ARH 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 physical suckling stimulus. cFos expression induced by the sensory stimuli associated with pup exposure was also identified in order to determine the specific neural populations activated specifically by the physical suckling stimulus. Several

neural populations, both in the forebrain and the brainstem, were found to be rapidly activated by suckling. Then retrograde tracing from the caudal ARH area combined with suckling-induced cFos expression was used to identify the afferent input to the caudal ARH that was activated by the suckling stimulus. This study demonstrated that several areas, including mPOA, PePOA, PBL, PAG and VLM, may play important roles in modulating the ARH NPY neuronal activity during lactation. In addition, several areas that were activated by pup exposure alone, including BST, PP and MeA, also send projections into the ARH, suggesting that the neural input activated by sensory stimuli associated with pup exposure may also be capable of activating ARH NPY neurons to a certain extent. In future studies, the phenotype of the suckling-activated ARH afferent input needs to be determined to further characterize the neurochemical substrates that may be involved in the afferent pathway. In addition, anterograde tracing from each of the identified areas needs to be conducted to confirm whether neurons in each area establish direct connection with NPY neurons in the ARH.

It is still not clear why the suckling stimulus activates only the caudal portion of the ARH even though NPY neurons are found throughout the ARH. It is possible that there are subtle differences in the afferent input into different parts of the ARH. Further retrograde tracing studies to determine the afferent input to different parts of the ARH are needed to elucidate this issue.

The present thesis also began to address the possible functions of the NPY systems during lactation by first establishing the neural architecture of the NPY

system in the brain. Retrograde tracing combined with *in situ* hybridization demonstrated that the PVH is a target area for both suckling-activated ARH and DMH NPY populations, suggesting that both NPY systems may modulate the activity of neurons in the PVH during lactation.

Furthermore, in order to facilitate the identification of all of the possible target areas whose neuronal activity may be affected by ARH NPY neurons, anterograde tracing combined with immunofluorescent staining was used to determine the efferent targets of the ARH NPY neurons. It was found that NPY neurons in the caudal ARH project mainly to the forebrain regions, including LSV, several divisions of BST, MeA, PAG, and several hypothalamic nuclei, including mPOA, PePOA, PVH, DMH, PH and ARH. ARH NPY neurons do not appear to send heavy projections into the brainstem areas. These results clearly indicate that the function of ARH NPY neurons is not limited to the hypothalamus. Areas such as LSV, BST, and PH send projections into hippocampus and even cortical regions, suggesting that the ARH NPY neurons may not only modulate neuroendocrine function in the hypothalamus but may also modulate behavior or even cognitive function indirectly by modulating the afferent input into the higher brain centers. Although the involvement of NPY in modulating the activity of neurons in the PVH has been established, the function of NPY in the remaining areas is not clear. Thus, the anatomical tracing study first identified the major target areas for the caudal ARH NPY neurons; then in future studies, the effects of NPY in each individual area need to be

examined to understand the complete function of the ARH NPY system during lactation.

The triple labeling study in the present thesis demonstrated that the ARH NPY neuronal fibers make direct apposition on GnRH neurons in the mPOA and its nerve terminals in the ME. The direct connection between the ARH NPY system and the GnRH system suggests that during lactation, the ARH NPY neurons may modulate the animal's reproductive function by directly modulating GnRH neuronal activity, its secretion from the nerve terminals, or both. It is also possible that NPY may reach the pituitary to modulate the effect of GnRH on the pituitary.

The close relationship between NPY and the CRF neuronal system has been well established. The demonstration of a direct anatomical link between the ARH NPY neurons and the PVH CRF neurons further indicates that the ARH NPY neurons provide at least part of the NPY afferent input to CRF neurons. In addition, several other NPY populations, including the suckling-activated DMH NPY neurons and the NPY neurons in the VLM, also send projections into the PVH area, thus suggesting that additional NPY inputs may also make direct connections with CRF neurons. It will be interesting to determine whether there is an interaction between different NPY inputs in modulating CRF neuronal activity and whether different NPY input contacts different subpopulations of CRF neurons to modulate different physiological functions.

The demonstration that ARH NPY neurons provide direct anatomical links to both GnRH and CRF systems suggests that the ARH NPY can modulate several physiological systems simultaneously. These anatomical data may explain why alterations of energy homeostasis and the stress response are sometimes associated with changes in reproductive function. The results from this thesis suggest that the NPY system may play an important role in integrating incoming signals from the outside as well as inside environments and in turn regulate multiple neuroendocrine systems to respond to the conditions of lactation.

In contrast to the ARH NPY neurons, the projections of the DMH NPY neurons are difficult to study. The DMH NPY neurons, which range from 700-900 cells per animals, are not clustered together like the NPY neurons in the ARH. Instead, they are scattered around the compact zone of the DMH area. Thus, anterograde tracing studies are very difficult to conduct. A typical anterograde tracer injection normally labeled 70-90 cells surrounding the center of the injection site. Therefore, one typical tracer injection into the DMH area would probably label less than ten DMH NPY neurons in a lactating animal. Thus, alternative tracing techniques may need to be considered for studying the projections of the DMH NPY neurons during lactation.

Recently the importance of the NPY system in the hypothalamus has been challenged by the NPY knockout mouse. Apparently the mouse without NPY is still fertile, can maintain normal body weight and food intake, and metabolic parameters appear to be normal. The only defect observed was that the mouse

was more susceptible to neuronal damage by seizure. Thus, this knockout study suggests that there may be redundant systems in the hypothalamus that maintain normal energy homeostasis or reproductive function. It is possible that many neuropeptides as well as neurotransmitter systems act in a parallel fashion to control one or several common pathways that maintain energy and reproductive homeostasis. Therefore, removing one of the systems such as NPY may not seriously alter overall regulation. More importantly, the demonstration of colocalization of several neurotransmitters and peptides, such as GABA and agouti-related peptide (AGRP), with NPY in the same neurons in the ARH further argues that removing the expression of one peptide may not necessarily mean that the neurons are no longer functional. In addition, it is not clear whether the knockout of NPY also affected the expression of other PP family members. Thus, it is possible that the remaining members of the family were still functional and could activate NPY receptors to regulate various physiological systems.

In conclusion, the present thesis demonstrated that the sucking stimulus activates two discrete populations of NPY neurons in the hypothalamus. The mechanism by which the suckling stimulus activates the two NPY populations may be different. Suckling-induced hyperprolactinemia may be more important in the activation of the DMH NPY neurons, whereas the activation of ARH NPY neurons is dependent on the neural input activated by suckling. Several afferent inputs to the ARH were identified which may be important in mediating the activation of ARH NPY neurons during lactation. PVH is a target area for both

NPY populations during lactation. Anterograde tracing identified multiple target areas in the brain for the ARH NPY neurons. The ARH NPY neurons may directly modulate the activity of GnRH and CRF systems in the hypothalamus; this modulation may be important in regulating the physiological alterations in reproduction and food intake during lactation.

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