

**The Role of Neuropeptide Y in the Hypothalamic Integration of Signals
Mediating Energy Balance and Reproductive Function**

by

Rebecca Elaine Campbell

A DISSERTATION

Presented to the Department of Physiology and Pharmacology
Oregon Health & Science University
School of Medicine
In partial fulfillment of
the requirement for the degree of
Doctor of Philosophy
September 2002

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Rebecca E. Campbell

Has been approved

[Redacted Signature]

Professor in charge of thesis

[Redacted Signature]

Member

[Redacted Signature]

Member

[Redacted Signature]

Member

TABLE OF CONTENTS

	Page
TABLE OF CONTENTS.....	ii-v
LIST OF FIGURES.....	vi-viii
LIST OF TABLES.....	ix
LIST OF ABBREVIATIONS.....	x-xi
ACKNOWLEDGEMENTS.....	xii
PREFACE.....	xiii
ABSTRACT.....	xiv-xvi

I. Introduction.....	1
A. The link between energy homeostasis and reproductive function.....	2
B. The hypothalamus: a site of homeostatic integration and regulation.....	3
C. Overview of the central regulation of food intake.....	5
D. Neuropeptide Y: one potential link.....	12
i. NPY receptors.....	13
ii. The role of NPY in energy balance.....	17
iii. The role of NPY in neuroendocrine reproductive function.....	18
E. Other metabolic systems important in the control of reproductive neuroendocrine function.....	20
i. Orexin.....	20
ii. MCH.....	21
iii. CRH.....	22
iv. Leptin.....	23
F. The model of lactation.....	24
i. Cyclic reproductive function is suppressed during lactation.....	24

ii.	Energy balance is altered during lactation.....	25
iii.	The role of the brainstem: specific activation by the suckling stimulus and input into the arcuate nucleus.....	27
G.	Aims of the thesis and approach.....	29
II.	Distribution of Corticotropin Releasing Hormone Receptor Immunoreactivity in the Rat Brain: Coexpression in Neuropeptide Y and Dopamine Neurons in the Arcuate Nucleus.....	30
A.	Introduction.....	31
B.	Materials and Methods.....	33
C.	Results.....	38
i.	Characterization of CRH R ₁ antibody staining.....	38
ii.	Characterization of CRH R ₁ distribution by single label ICC.....	39
iii.	Colocalization of CRH R ₁ with NPY and TIDA neurons....	41
D.	Discussion.....	42
III.	Neuropeptide Y Y5 Receptor Protein in the Cortical/Limbic System and Brainstem of the Rat: Expression on γ- aminobutyric acid and Corticotropin-Releasing Hormone Neurons.....	56
A.	Introduction.....	57
B.	Materials and Methods.....	58
C.	Results.....	62
i.	Single label immunohistochemistry.....	62
1.	Septum and thalamus.....	63
2.	Hippocampus and cortex.....	63
3.	Brainstem.....	64
ii.	Double label immunofluorescence.....	64
1.	GABA.....	64
2.	Corticotropin-releasing hormone.....	65

3. Neuropeptide Y.....	65
D. Discussion.....	66
IV. Hypothalamic Circuitry of Neuropeptide Y Regulation of Neuroendocrine Function and Food Intake via the Y5 Receptor Subtype.....	80
A. Introduction.....	81
B. Materials and Methods.....	83
C. Results.....	93
i. NPY Y5 receptor protein knockdown following Y5 antisense treatment.....	93
ii. Characterization of the distribution of the NPY Y5 antibody.....	94
iii. Single-label immunohistochemistry.....	95
iv. Double- and triple-label immunofluorescence.....	97
1. Gonadotropin-releasing hormone.....	97
2. Corticotropin-releasing hormone.....	97
3. Neuropeptides.....	97
4. GABA.....	98
5. NPY.....	98
D. Discussion.....	99
V. Neuropeptide Y Y4 Receptor Immunoreactivity in the Hypothalamus of the Rat: Colocalization with Orexin in the Lateral Hypothalamic Area.....	115
A. Introduction.....	116
B. Materials and Methods.....	118
C. Results.....	126
i. Neuroanatomical findings.....	126
ii. Food and water intake.....	128
iii. Investigation of neuronal activation using cFos as a	

marker.....	129
D. Discussion.....	130
VI. Gonadotropin-Releasing Hormone Neurons Coexpress Orexin 1 Receptor Immunoreactivity and Receive Direct Contacts by Orexin	
Fibers.....	146
A. Introduction.....	147
B. Materials and Methods.....	149
C. Results.....	153
D. Discussion.....	154
VII. Summary and Conclusions.....	165
VIII. References.....	180
IX. Appendix.....	236
A. Differential Regulation of NPY mRNA in Adrenergic and Noradrenergic Neurons in the Ventral Lateral Medulla during Lactation.....	236
i. Introduction.....	237
ii. Materials and Methods.....	238
iii. Results and Discussion.....	239

LIST OF FIGURES

Figure 2-1	Characterization of the CRH R ₁ antibody.....	51
Figure 2-2	Representative images of single label CRH R ₁ -ir throughout the rat brain.....	52
Figure 2-3	Single label CRH-R ₁ -ir distribution in representative regions of the hypothalamus.....	53
Figure 2-4	Colocalization of CRH R ₁ -ir with NPY-ir and TH-ir.....	54
Figure 2-5	CRH innervation of CRH R ₁ positive cells in the ARH.....	55
Figure 3-1	Diagrammatic representation of the distribution of Y5 immunoreactivity in the rat brain.....	74
Figure 3-2	Single label Y5 immunoreactivity in the hippocampus of the rat.....	75
Figure 3-3	Single label Y5 immunoreactivity in the cingulated cortex of the rat.....	76
Figure 3-4	Single label Y5 immunoreactivity in the brainstem of the rat...	77
Figure 3-5	Characterization of the phenotypes of Y5 immunoreactive neurons in the dentate gyrus and cortex.....	78
Figure 3-6	Neuropeptide Y innervation of a cortical neuron displaying Y5 receptor immunofluorescence.....	79
Figure 4-1	Antisense or sense oligonucleotide (ODN) treatment effects on food intake.....	108
Figure 4-2	Y5 antisense oligonucleotide (ODN) suppression of Y5 receptor protein levels in the ventromedial hypothalamic nucleus (VMH) and paraventricular nucleus of the hypothalamus (PVH).....	109
Figure 4-3	Western blot analysis of the distribution of the Y5 receptor protein in the rat brain.....	110
Figure 4-4	Single label Y5-immunoreactivity (-ir) in the hypothalamus of the rat brain.....	111

Figure 4-5	Expression of NPY Y5 immunofluorescence (-IF) in GnRH neurons.....	112
Figure 4-6	Double label immunofluorescence (-IF) of Y5 and CRH, neurophysins, and GABA, in the rat hypothalamus.....	113
Figure 4-7	Neuropeptide Y (NPY) innervation of neurons displaying NPY Y5 receptor immunofluorescence (-IF).....	114
Figure 5-1	Single label hypothalamic distribution of Y4-ir.....	136
Figure 5-2	Correlation of Y4 mRNA and protein in central and peripheral tissues.....	137
Figure 5-3	NPY Y4-ir colocalizes with orexin in the LHA.....	138
Figure 5-4	Y4 mRNA is coexpressed in orexin neurons.....	139
Figure 5-5	NPY Y1-ir on fibers and cells of an unknown phenotype in the LHA.....	140
Figure 5-6	Intra-LHA administration of NPY and rPP stimulates food and water intake.....	141
Figure 5-7	Single label cFos expression ipsilateral and contralateral to LHA injections.....	142
Figure 5-8	Intra-LHA rPP, but not NPY, induces a robust increase in cFos expression in orexin neurons.....	143
Figure 5-9	Orexin neurons and cFos expression follow intra-LHA injections of vehicle, NPY or rPP.....	144
Figure 5-10	MCH does not express cFos in response to intra-LHA rPP or NPY.....	145
Figure 6-1	Orexin fibers come in close contact with GnRH neurons.....	161
Figure 6-2	GnRH neurons express OX-R1.....	162
Figure 6-3	Triple label IF demonstrates that GnRH neurons expressing OX-R1 receive close contacts by orexin fibers.....	163
Figure 6-4	NPY Y4/orexin-ir fibers are near GnRH neurons but do not make close contacts.....	164
Figure 7-1	Final Summary Diagram.....	179

Figure A-1	Representative photomicrographs in subregions of the VLM demonstrating NPY mRNA expression during the diestrus stage of the estrous cycle or lactation.....	240
Figure A-2	Quantification of NPY MRNA expression in subregions of the VLM during lactation or diestrus.....	241

LIST OF TABLES

Table 2-1	Relative Levels of CRH R ₁ -ir in Rat Hypothalamus and Forebrain Regions	50
-----------	---	----

LIST OF ABBREVIATIONS

5HT	serotonin
ARH	arcuate nucleus of the hypothalamus
CA1	CA1 region of the Hippocampus
CA2/3	CA2/3 region of the Hippocampus
CC	cingulated cortex
CeAM	central amygdala
CRH R ₁ -ir	CRH R ₁ receptor-like immunoreactivity
CRH	corticotropin releasing hormone
DBB	diagonal band of broca
DG	dentate gyrus
DGg	granular cell layer of the dentate gyrus
DGm	molecular cell layer of the dentate gyrus
DGp	polymorphic cell layer of the dentate gyrus
DMH	dorsomedial hypothalamic nucleus
DR	dorsal raphe
EW	Edinger-Westphal nucleus
FC	fasciola cinerea
FITC	fluorescein isothiocyanate
GABA	γ -aminobutyric-acid
Hab	habenual
Hipoc	hippocampus
ICC	immunocytochemistry
-ir	immunoreactivity
KPBS	potassium phosphate buffer
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
LHA	lateral hypothalamic area
MA	medial amygdala
MDT	mediodorsal thalamic nucleus
Me5	mesencephalic trigeminal nucleus
ML	molecular layer
MnPO	median preoptic nucleus
Mo5	motor 5 nucleus
MRNA	messenger ribonucleic acid
NA	noradrenergic
NPY	neuropeptide Y
OT	oxytocin
OVLT	organum vasculosum of the lamina terminalis
PC	piriform cortex
Pe	periventricular nucleus
PePOA	periventricular preoptic area
PMnR	paramedian raphe nucleus
PnC	pontine reticular nucleus, caudal

PrI	Prolactin
PVH	paraventricular nucleus of the hypothalamus
PVT	paraventricular nucleus of the thalamus
RE	reunion nucleus
RN	red nucleus
RtTg	reticulotegmental nucleus of the pons
SCN	suprachiasmatic nucleus
SNC	Substantia Nigra, compact
SON	supraoptic nucleus
SS	somatosensory cortex
SSC	standard saline citrate
TH	tyrosine hydroxylase
TIDA	tuberoinfundibular dopamine
TRITC	tetramethyl rhodamine isothiocyanate
Tz	trapezoid body
Ucn	Urocortin
VMH	ventromedial hypothalamic nucleus
VP	vasopressin
ZI	zona incerta

ACKNOWLEDGEMENTS

I am extremely grateful to my mentor, Dr. M. Susan Smith, for her gracious support and encouragement throughout my time in her laboratory. She has taught me how to conduct and present scientific research, how to think about scientific issues and how to critically evaluate data and research papers. Her professionalism and the ease with which she handles numerous responsibilities have been inspirational to me. She has prepared me to pursue a career as an independent scientist.

I also want to thank Dr. Kevin Grove for his unending patience and countless hours of scientific mentoring. Dr. Grove has given generously of his time and energy in the support of my graduate studies. His mentoring and encouragement have been vital to my success.

The members of the Smith laboratory, both past and present, have taught me a great deal and have been incredibly supportive. I'd like to thank Dr. Becky Brogan, Dr. Chien Li, Peilin Chen, Andy Schiffmacher, Summer Allen, Dan Copp, Bernadette Grayson, Dr. Patti Williamson, and Dr. Xiao Qui Xiao. I have also gained an enormous amount of training and support from the many members of the Cooley Building. Also, a special thank you goes to Marge Shiningier for all of her support and to Dr. Anda Cornea for all of her assistance in confocal imaging.

PREFACE

In accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health & Science University, Portland, Oregon, I have prepared my dissertation, consisting of a general introduction, five chapters of original data and a general conclusion. The references cited are listed separately in alphabetical order and follow the format of Endocrinology.

Chapters II, III and IV contain data, figures and text as they appear in the original papers that have been published previously (Grove et al., 2000, Campbell et al., 2001, Campbell et al., 2002). Chapters V and VI represent manuscripts that have been prepared for publication and will be submitted to Journal of Neuroscience and Endocrinology, respectively. The Appendix includes a brief description of preliminary data not yet submitted for publication.

ABSTRACT

The rationale for these studies stems from the well established link between the status of energy balance and the function of the reproductive system. There are many conditions in which negative energy balance is associated with a suppression of reproductive function. The mechanisms underlying this link most likely involve the complex integration of endocrine, autonomic and somatosensory systems. The hypothalamus represents a known neuroanatomical center for such integration and regulation of coordinated neuroendocrine responses. Although a number of neuronal and hormonal factors have been implicated in regulating both of these systems, the present thesis focuses specifically on the neuropeptide Y (NPY) system within the arcuate nucleus of the hypothalamus (ARH) as a potential integrating signal in the regulation of energy balance and neuroendocrine reproductive function. Neuropeptide Y is both a potent orexigenic factor and a major regulator of neuroendocrine reproductive function, and as such, may be the key integrating signal between these two systems.

To better understand the neuroanatomical circuitry by which energy balance and reproductive function may be integrated, the first part of this thesis characterizes receptor expression patterns that may play a role, including corticotropin-releasing hormone receptor 1 (CRH R1) and the Y4 and Y5 subtypes of NPY receptors. Using immunofluorescence coupled with confocal microscopy, expression patterns throughout the brain along with selected

phenotypes were identified. The key findings can be summarized as follows: 1) CRH R1 is expressed on a subset of NPY and TIDA neurons in the ARH, suggesting a reciprocal feedback loop between CRH and NPY neurons. 2) The NPY Y5 receptor is widely expressed and colocalizes with GABA and CRH in cortical regions. 3) Within the hypothalamus, the Y5 receptor is coexpressed with approximately 55% of GnRH neurons and a small subset of CRH neurons, in addition to arginine vasopressin and oxytocin neurons in the paraventricular nucleus of the hypothalamus. 4) The Y4 receptor colocalizes with orexin neurons in the lateral hypothalamic area (LHA) and with orexin fibers throughout the brain. 5) Orexin fibers come in close contact with GnRH neurons, the majority of which express the orexin 1 receptor. However, Y4 expressing orexin fibers do not make close contacts with GnRH neurons, suggesting that NPY may have indirect effects on GnRH via orexin cell bodies.

Additional studies investigated the functional role of Y4 receptors on orexin neurons by determining whether they could be activated by a high affinity ligand to the Y4 receptor, rat pancreatic polypeptide (rPP). Injection directly into the LHA of rPP or NPY, but not vehicle, resulted in increased food and water intake. The rPP injections caused a significant increase in cFos expression in orexin but not MCH neurons, suggesting that orexin neurons do indeed express the Y4 receptor and may respond to an endogenous rPP-like ligand. Interestingly, however, while NPY administration resulted in increased cFos expression in the LHA, cFos expression was not significantly elevated in either

orexin or MCH neurons, suggesting the activation of additional phenotypes of neurons.

The final portion of this thesis focused on the role of brainstem NPY during lactation. Lactation represents a natural physiological model of negative energy balance and suppressed reproductive function. Previous studies have demonstrated elevated NPY mRNA in the ARH and dorsomedial nucleus of the hypothalamus (DMH) during lactation. Furthermore, brainstem catecholaminergic neurons, known to express NPY, are stimulated by suckling, as determined by cFos expression, suggesting that this population of NPY neurons may also be activated during lactation. Interestingly, however, NPY mRNA levels in the A1 subregion of the VLM were found to be unchanged during lactation, while NPY mRNA in the C1 subregion of the VLM was suppressed.

The results of the studies presented in this thesis established a neuroanatomical framework by which NPY can mediate the integration of signals between food intake and neuroendocrine reproductive function. This neuroanatomical framework can be used to determine the physiological importance of the various components to the regulation of GnRH neurons.

Chapter I
Introduction

A. The link between energy homeostasis and reproductive function

Changes in energy balance have been clearly shown to have profound effects on reproductive function. For example, there are a number of conditions in women associated with negative energy balance (anorexia nervosa, bulimia, exercise-induced amenorrhea, lactation) that result in the suppression of cyclic reproductive function. Energy balance also appears to play a critical role in the onset of puberty, acting as a gate for “switching on” neuroendocrine reproductive function. Reproduction represents a metabolically expensive physiological process, not necessary for immediate survival, and therefore requires a system to suppress this process in an energy deficient condition.

Several hormonal and neuronal systems are considered to be metabolic signals, which provide a communications link between central and peripheral structures on the status of energy balance of an organism. Because of the interconnected nature of reproductive function and energy balance, it makes physiological sense that important regulators of energy homeostasis also serve as integrating signals to reproductive systems. In fact, numerous regulators of energy homeostasis have also been identified as key players in the regulation of reproductive function. Although some of these metabolic signals act directly at peripheral reproductive tissues, the central site of integration for signals mediating energy homeostasis and regulating reproductive function is most likely within the hypothalamus. The integration of these signals within the hypothalamus is the broad focus of this thesis.

B. The hypothalamus: a site of homeostatic integration and regulation

The hypothalamus is a central structure, located at the base of the brain, below the diencephalon and immediately above the pituitary. The hypothalamus is uniquely positioned to receive, integrate, and communicate information through endocrine, autonomic and somatosensory systems and has long been considered the key central structure in maintaining homeostasis within an animal. By modulating both the nervous and endocrine systems, the hypothalamus is able to integrate the control of complex processes. Via the nervous system, the hypothalamus regulates both autonomic and behavioral processes. These include control of the cardiovascular system, thermoregulatory processes, and visceral functions as well as sexual, maternal, aggressive, and ingestive behaviors. The hypothalamus regulates the endocrine system through a close association with the pituitary gland. By way of releasing or release-inhibiting hormones, the hypothalamus exerts control over the synthesis and secretion of anterior pituitary hormones and even directly releases hypothalamic hormones into the peripheral circulation through the posterior pituitary. Additionally, many cells of the hypothalamus have the ability to act both as nerve cells and as endocrine cells, both receiving and transmitting electrical information as well as secreting hormones into the circulation.

The hypothalamus is made up of clusters of neurons, termed nuclei, and is traversed by several major fiber systems. In addition to intrinsic tracts that travel unidirectionally to the pituitary, these nuclei also have complex connections

with each other and with other major sites in the brain, including the cerebral cortex, the thalamus, the limbic system and the brainstem and spinal cord (Simerly, 1995; Freeman, 1995). Thus, the hypothalamus is postulated as an interface between sensory and motor pathways (Simerly, 1995). Sensory information from a variety of regions is received by the hypothalamus and it in turn sends projections to autonomic neurons, the pituitary gland, and regions controlling somatomotor responses. Understanding this complex neurocircuitry of hypothalamic afferent and efferent connections will provide the foundation for understanding the detailed mechanisms by which the hypothalamus integrates physiological information and processes.

To date, a variety of neurochemical systems have been identified within the hypothalamus, including the classical neurotransmitters, glutamate, γ -aminobutyric acid (GABA) and the catecholamines, along with numerous peptide systems. Although the distribution and physiological characteristics of these phenotypes have been studied extensively, the flow of information through functionally distinct circuits is only now beginning to be defined. Determining the distribution of various neurotransmitter receptors and investigating fiber projections and contacts is a key step in beginning to clarify these circuits.

C. Overview of the central regulation of food intake

The hypothalamus has long been considered a key component in the regulation of food intake and adiposity. The early studies of Hetherington and

Ranson (1940), and Anand and Brobeck (1951) examined the effects of bilateral lesions to various regions of the hypothalamus and initially proposed the idea of a dual center model. They observed that lesions to the lateral hypothalamic area (LHA) resulted in severe hypophagia and therefore this area was labeled a “feeding center”. Conversely, lesions to the ventromedial nucleus (including the adjacent arcuate nucleus and paraventricular nucleus) caused robust hyperphagia and obesity, indicating these areas serve as a “satiety center”. Although these early studies were crude and the findings have undergone much speculation and revision, they clearly implicated the hypothalamus as the epicenter for the neuronal regulation of feeding. Current evidence depicts a much more complex model than the simplistic dual center model, however, the concept of regional networks containing neuronal populations responsible for signals denoting either appetite or satiety remains. This overview will examine both the orexigenic and anorexigenic neuronal and hormonal signals that mediate feeding in the context of some of the different key regions within the hypothalamus.

The arcuate nucleus (ARH), a small nucleus adjacent to the base of the third cerebral ventricle and just dorsal to the median eminence, is considered a pivotal site in the regulation of energy balance. The ARH contains two main populations of neurons involved in the regulation of food intake, including orexigenic neuropeptide Y/agouti-related protein (NPY/AGRP) neurons, and anorexigenic pro-opiomelanocortin/cocaine and amphetamine-regulated transcript (POMC/CART) neurons. NPY has been postulated as the most potent orexigenic agent described. Given centrally, NPY stimulates a robust increase in

food intake and reduces core body temperature (Morley, 1987; Billington et al., 1991). Chronic administration of NPY causes prolonged hyperphagia leading to obesity (Stanley et al., 1986; Beck et al., 1992; Zarjevski et al., 1993). Likewise, fasting causes an increase in NPY mRNA expression in the ARH and increased release in other hypothalamic areas (Brady et al., 1990; Kalra et al., 1991). Evidence to support the role of NPY in the normal maintenance of daily food intake is demonstrated by naturally rising and falling NPY levels in the PVH before and after a meal (Yoshihara et al., 1996a; Yoshihara et al., 1996b).

Conversely, POMC neurons and POMC cleavage products, which make up part of the central melanocortin system, are inhibitory to feeding. Both ACTH and α -MSH (melanocyte stimulating hormone), products of POMC cleavage, act as potent satiety factors when injected centrally (Vergoni et al., 1986; Olszewski et al., 2001). Likewise, POMC gene expression is significantly suppressed following a 48 hour fast (Mizuno et al., 1998). The central inhibitory effects of melanocortins on feeding are thought to act primarily via activation of melanocortin 4 (MC4) receptors, which have been clearly shown to play an important role in the hypothalamic regulation of feeding. Stimulation of MC4 receptors by melanocortin peptides or peptide analogues induces long-lasting inhibition of food intake, while targeted disruption of MC4 receptors results in hyperphagia and obesity (Fan et al., 1997; Huzar et al., 1997), suggesting that tonic activation of MC4 receptors is important in limiting food intake and body fat mass.

AGRP, which is coexpressed in NPY neurons, also binds to MC4 receptors and acts as an endogenous competitive antagonist, resulting in the stimulation of food intake and significant increases in body weight (for review, Vergoni et al., 2000; Marks and Cone, 2001). In contrast to POMC, AGRP mRNA levels are elevated during fasting (Ebihara et al., 1999), and over-expression of AGRP leads to hyperphagia and obesity (Ollmann et al., 1997). Therefore, α -MSH and AGRP work in opposition via MC4 receptors to regulate melanocortin neurotransmission. Although AGRP appears to be colocalized in virtually all ARH NPY neurons and both peptides serve as orexigenic agents, it remains to be determined whether these neuropeptides are released together and whether or not target neurons typically respond to one or both peptides. Analogous to NPY/AGRP coexpression, CART is coexpressed in virtually all ARH POMC neurons and regulates appetite in a similar manner, in that central injections of CART suppress food intake (Lambert et al., 1998). Leptin administration elevates both CART and POMC mRNA (Kristensen et al., 1998, Schwartz et al., 1997). However, the interaction between POMC cleavage products and CART at nerve terminals and at target sites is not known. Determining the release patterns of the peptides and investigating receptor expression patterns in NPY/AGRP and POMC/CART target fields will begin to address these questions.

Both NPY/AGRP and POMC/CART neurons of the ARH have been shown to be direct targets of leptin, a peripheral hormone produced by adipocytes and regarded as a critical component in relaying peripheral signals of adiposity and energy homeostasis to the brain. Leptin is an anorexigenic signal, in that it

reduces appetite and increases energy expenditure. The transgenic *ob/ob* mouse lacks leptin and is severely obese. NPY and POMC neurons both express mRNA for the long form of the leptin receptor (Baskin et al., 1999; Cheung et al., 1997) and are differentially regulated by leptin and metabolic state. Leptin has been demonstrated to positively regulate POMC (α -MSH) and CART (Schwartz et al., 1997, Cowley et al., 2001; Kristensen et al., 1998) and negatively regulate NPY and AGRP (Stephens et al., 1995; Ahima et al., 1996) to maintain energy homeostasis. Moreover, the work of Cowley et al. (2001) has illustrated an interconnected circuit between NPY and POMC neurons in which leptin directly depolarizes POMC neurons and simultaneously hyperpolarizes NPY/GABA neurons. It is suggested that the inhibition of NPY/GABAergic neurons, whose terminals were identified in contact with POMC neurons, would result in decreased GABA release and additional disinhibition of POMC neurons.

Neuronal populations of the ARH are situated in a prime location, virtually outside the blood-brain barrier, to be accessed by leptin and other peripheral signals of metabolism, including insulin, glucose, ghrelin, and a number of peptides produced in the gastrointestinal tract. Insulin, like leptin, reflects the energy availability of the body and acts to reduce energy intake at elevated levels (Baskin et al., 1999). Insulin receptors are also highly expressed in the ARH (Baskin et al., 1988). Gut hormones have also been shown to directly influence hypothalamic circuits. Recent evidence suggests that peripheral PYY₃₋₃₆, a Y2 agonist endogenously released after meals, acts within the ARH to reduce NPY mRNA and food intake (Batterham et al., 2002). Alternatively, ghrelin, an

endogenous ligand for the growth hormone secretagogue receptor that is produced in the stomach, stimulates food intake in a NPY-dependent manner (Nakazato et al., 2001).

Tract tracing and immunocytochemical studies have begun to identify the downstream targets of these ARH populations. Extensive innervation by ARH neurons is observed in a number of other hypothalamic areas implicated in the regulation of food intake and energy balance, including the PVH, dorsomedial hypothalamic nucleus (DMH), VMH, and LHA. The role of each of these regions in the regulation of food intake is briefly discussed below.

The PVH is generally regarded as a site of convergence and integration of many of the systems mediating energy homeostasis. In addition to the axons coming from NPY/AGRP and POMC/CART neurons of the ARH, the PVH receives input from numerous populations implicated in feeding regulation, including neurons containing orexin, serotonin, galanin, noradrenaline, urocortin and opioid peptides. The PVH is extremely sensitive to the microinjection of virtually all of the known orexigenic and anorexigenic signals, resulting in feeding or the inhibition of feeding, respectively.

Among other neuropeptides, the PVH contains CRH and thyrotropin releasing hormone (TRH) neurons. CRH, while well known for its role in the hypothalamic-pituitary-adrenal (HPA) stress axis, is also implicated as an endogenous inhibitor of food intake when administered centrally. Administration of CRH into the brain, and specifically into the PVH, decreases spontaneous or fasting-induced feeding (Krahn et al., 1988; Dunn and Berridge, 1990).

Immunoneutralization or pharmacological blockade of CRH results in enhanced basal and NPY-induced feeding (Heinrichs et al., 1992; Manzaghi et al., 1993). CRH neurons have been suggested as one of the downstream systems modulating food intake elicited by NPY (Heinrichs et al., 1993) and have been demonstrated to receive close contacts by NPY fibers coming from the caudal portion of the ARH (Li et al., 1998; Li et al., 2000). Recently, however, the role of the CRH-like ligand, urocortin (UCN), which binds both CRH R₁ and R₂ subtypes with a higher affinity than CRH, has been suggested to play a more important role in the regulation of food intake than CRH. While CRH may be an important mediator of the inhibitory effects of stress on feeding through the R₁ subtype (Smagin et al., 1999), UCN as well as CRH are proposed to have more potent effects on feeding via the R₂ subtype (Coste et al., 2000).

The PVH is the sole hypothalamic site of increased NPY secretion in response to fasting, and before the initiation of feeding (Kalra et al., 1991; Dube et al., 1992), suggesting that the PVH and the ARH NPY pathway are crucial to the regulation of energy homeostasis (Kalra et al., 1999; Williams et al., 2001). Cowley *et al.* (1999) recently demonstrated that NPY's effect on neuronal activity within the PVH was via modulation of presynaptic GABA release. Similar to the effects of CRH, TRH also promotes negative energy balance by reducing food intake (Kow et al., 1991) and stimulating the thyroid axis. In addition to the strong influence of PVH neurons on pituitary function and other hypothalamic nuclei, the PVH also projects caudally and directly innervates autonomic preganglionic

neurons (Carrasco et al., 2001), providing multiple means of motor output to control peripheral regulation of metabolism.

The VMH and DMH are bilateral nuclei that lie dorsal to the ARH along the third ventricle. Both of these areas have extensive connections with the PVH. The VMH retains the title of the “satiety center” because in addition to lesions in this area resulting in over-feeding, stimulation of this area results in the inhibition of food intake. The VMH is also regarded as an important target for leptin due to the abundance of leptin receptors expressed there (Schwartz et al., 1996). The DMH also contains leptin receptors, as well as an abundance of insulin receptors. The DMH contains NPY expressing neurons, whose expression is greatly increased in genetic models of obesity and in response to lactation (Guan et al., 1998; Li et al., 1998; Kesterson et al., 1997).

The LHA also remains a key area in the regulation of appetite and energy homeostasis. It receives robust innervation from the ARH by NPY, AGRP and α -MSH terminals, and all of these peptides come in close contact with both orexin (also known as hypocretin) and melanin concentrating hormone (MCH) neurons (Elias et al., 1998; Broberger et al., 1998). Both of these recently identified neuropeptides are orexigenic when injected centrally and are regulated by starvation and leptin (Qu et al., 1996; Sakurai et al., 1998; Rossi et al., 1997; Tritos et al., 2001). Leptin receptors are expressed on both orexin and MCH neurons (Hakansson et al., 1999), although it is unknown whether circulating leptin has direct access to these neurons. The MCH knockout mouse was discovered to be hypophagic and lean (Shimada et al., 1998), while

overexpression of MCH results in increased food intake and obesity (Ludwig et al., 2001), providing strong evidence for the role of MCH as a critical effector of energy balance. However, the role of orexin in the physiological regulation of food intake is somewhat less clear. Orexin-A is found to increase daytime feeding, but not overall 24h food intake, and repeated injections have no effect on body weight (Yamanaka et al., 1999). Therefore, it has been suggested that orexin may be important in short-term feeding episodes but not necessarily implicated in obesity. Orexin may play a more important role in arousal and regulation of neuroendocrine function. The LHA also contains glucose-receptive neurons that respond to circulating glucose levels (Bernardis and Bellinger, 1996).

D. Neuropeptide Y: one potential link

NPY is a 36 amino acid peptide expressed and released by neurons throughout the central and peripheral nervous system (Dumonet et al., 1993). It was initially isolated and characterized by Tatemoto and colleagues (1982) from the porcine brain, and has since been discovered in a variety of other species, including rat, guinea pig, rabbit, sheep, and human (Corder et al., 1988; O'Hare et al., 1988; Sillard et al., 1989; Emson et al., 1984). NPY belongs to the pancreatic polypeptide (PP) family, which, in addition to NPY consists of peptides found in the periphery, peptide YY (PYY) and pancreatic polypeptide (PP). *In situ* mRNA mapping studies and immunohistochemical studies in colchicine treated

animals identified the ARH as having the largest group of NPY neurons. A few scattered NPY cells have also been observed in other regions of the hypothalamus (Gehlert et al., 1987), and within the brainstem, another dense population of NPY cells exists colocalized with catecholamine that project to the hypothalamus (Sawchenko et al., 1985). NPY has been shown to play a role in the regulation of numerous physiological systems, including energy homeostasis, reproductive function, cardiovascular function, and seizure activity. Due to its modulation of both energy balance and neuroendocrine reproductive function, it has been suggested that the NPY system may be one of the integrating signals between these two systems.

i. NPY receptors

There are currently five NPY receptor subtypes that have been characterized and pharmacologically distinguished as Y1, Y2, Y4, Y5 and y6. These are all seven transmembrane domain G-protein coupled receptors that inhibit adenylate cyclase activity and in some instances activate protein kinase C pathways (Parker et al., 1998; Selbie et al., 1995). The NPY receptors have unique, tissue specific expression patterns and binding affinities that may account for the diverse effects of NPY.

Y1: The Y1 receptor is identified as the most abundant NPY receptor in the CNS. It has been mapped by selective radioligands throughout the cerebral cortex, thalamus, amygdala, and hypothalamus (Dumont et al., 2000). *In situ* hybridization studies show a similar distribution of Y1 mRNA (Larsen et al., 1994; Parker et al., 1999) and also indicate that the highest concentration of Y1 mRNA

in the hypothalamus is within the ARH (Mikkelsen and Larsen, 1992). In addition, previous immunocytochemical studies from our laboratory and others have identified Y1 receptor expression on many fibers and cells throughout the hypothalamus that may be involved in the regulation of energy homeostasis and reproductive function. Y1 receptors have been identified colocalized with POMC neurons in the ARH (Broberger et al., 1997) and TRH neurons in the PVH (Broberger et al., 1999). Also, Y1 positive nerve terminals of an unknown origin are found in close contact with CRH neurons in the PVH (Li et al., 1999). Y1 positive nerve terminals were also identified making contact with GnRH cell bodies, and GnRH nerve terminals in the median eminence express the Y1 receptor (Li et al., 1999). In addition to neuroanatomical evidence, a number of pharmacological studies have strongly implicated the Y1 receptor in NPY's effect on food intake (for review, Gehlert, 1999) and the LH surge (Jain et al., 1999; Leupen et al., 1997).

The Y1 receptor preferentially binds full-length NPY or NPY analogues such as [Pro34]NPY (Schwartz et al., 1990) and can cause the hydrolysis of phosphatidylinositol or the inhibition of adenylate cyclase (Aakerlund et al., 1990; Herzog et al., 1992).

Y2: Y2 receptors are found throughout the central and peripheral nervous systems and the vasculature, although the Y2 receptor is mainly regarded as an inhibitory autoreceptor. The Y2 receptor is primarily located presynaptically on NPY neurons and activation of the Y2 receptor causes a suppression of NPY release (Naveilhan et al., 1999), consistent with its role as an autoreceptor. Y2

receptors, in contrast with Y1 receptors, have a high affinity for C-terminal fragments such as NPY (13-36) (Swartz et al., 1990). The Y2 receptor has been implicated in the regulation of food intake, gastrointestinal motility, cardiovascular regulation, and neuronal excitability (Kaga et al., 2001). Recently, the production of a conditional hypothalamic Y2 knockout mouse resulted in reduced body weight along with an increase in food intake that correlated with increases in both NPY/AGRP and POMC/CART expression respectively (Sainbury et al., 2002). Also, peripherally produced PYY₃₋₃₆ has been identified as an endogenous ligand of the Y2 receptor and its binding results in an inhibition of feeding (Batterham et al., 2002).

Y4: The Y4 receptor has been thought of primarily as a peripheral receptor, as it binds with a much higher affinity to peripherally produced PP than to NPY (Bard et al., 1995). In the periphery, the main function of Y4 may be mediating the effects of PP on pancreatic secretion, gut motility and gall bladder contraction (Swartz, 1983). However, there is Y4 receptor mRNA and binding within the brain (Parker et al., 1999) and recent evidence reporting the effects of Y4 in the brain, suggesting a central distribution as well. Recently, the Y4 subtype has been implicated in NPY's effect on reproductive function. Stimulation of the Y4 receptor causes a dose-dependent stimulation of GnRH/LH release in male rats and mice (Raposinho et al., 2000) and stimulates basal LH release in female rats (Jain et al., 1999). Although the Y4 receptor has not been classically considered in the central regulation of food intake, recent reports have suggested that it may play a role. The Y4 KO mouse was recently described to have a lean

phenotype (Sainsbury et al., 2002), and PP has been shown to both inhibit food intake when given peripherally and stimulate food intake when given centrally (Katsuura et al., 2002). Studies in this thesis further investigate the role of Y4 receptors in food intake and GnRH modulation through neuroanatomical and physiological approaches.

Y5: The Y5 receptor has a high affinity for both full length and C-terminal fragments, with the highest affinity for NPY and PYY and a lower affinity for rPP. [Ala³¹,Aib³²]NPY is considered a selective agonist for this receptor (Cabrele et al., 2000). Multiple studies employing pharmacological tools and antisense oligonucleotides have suggested that the Y5 receptor is the primary receptor mediating the feeding response and energy expenditure regulation by NPY (Gerald et al., 1996; Schaffhauser et al., 1997; Hwa et al., 1999; Cabrele et al., 2000). In addition, the inhibitory effects of NPY on reproductive function have been linked to activation of Y5 receptors (Raposinho et al., 1999). Furthermore, the Y5 receptor is important in NPY suppression of seizure activity, as the anticonvulsant actions of NPY were absent in the Y5 knockout mouse (Marsh et al., 1999). Y5 mRNA and binding have been reported to be widespread throughout the brain (Dumont et al., 1998; Parker et al., 1999). The data reported in this thesis provide information about Y5 protein distribution throughout the brain and selected phenotypes of cells that express the receptor and are involved in the regulation of energy balance and reproductive function.

y6: The y6 receptor is expressed in mice and rabbits but not in rats. Although the cDNA for the y6 receptor has been cloned in primates and humans, it was found to be a non-functional pseudogene (Gregor et al., 1996).

ii. The role of NPY in energy balance

The overall effect of the central action of NPY is to cause a shift toward positive energy balance. In addition to the potent ability of NPY to stimulate food intake, NPY reduces energy expenditure by decreasing thermogenesis in brown adipose tissue and also facilitates triglyceride deposition by increasing insulin levels (Stanley et al. 1986; Billington et al., 1991). NPY levels are elevated in states of negative energy balance, such as fasting, lactation, and insulin-dependent diabetes mellitus, in an attempt to restore the balance of energy. Data in the streptozotocin-diabetic rat demonstrate an increase in NPY mRNA and peptide levels prior to an increase in food intake (Sahu et al., 1997), suggesting that the increase in NPY drives the hyperphagia following a bout of negative energy balance. NPY increases food seeking and consumption behaviors to the exclusion of other behaviors and may also be the central signal responsible for suppressing other energy costing physiological processes such as reproduction.

One of the most significant roles of NPY in the regulation of food intake may be the recruitment and coordination of other systems. In addition to the direct effects of the NPY system on food intake (i.e. the effects of NPY in the PVH), NPY also indirectly effects food intake by enlisting other mediators of

energy homeostasis. For example, the reciprocal relationship between NPY and both orexin and MCH populations in the LHA allows for the convergence and divergence of signals denoting energy status and controlling the behavioral endpoint.

iii. The role of NPY in neuroendocrine reproductive function

In addition to the well established role for NPY in the control of food intake and energy balance, NPY has also emerged as an essential modulator of reproductive function (for reviews, Kalra and Crowley, 1992; Kalra and Kalra, 1996). There is evidence to suggest that NPY may be involved in the stimulation of both basal and pre-ovulatory surge release of LH, as well as the inhibition of LH release in specific conditions. To this end, NPY has been reported to have both stimulatory and inhibitory effects on GnRH/LH release. In the presence of steroid hormones, NPY administration increases LH release. Endogenous NPY gene expression (Sahu et al., 1995), concentration and secretion (Watanobe and Takebe, 1992) have all been found to rise just prior to the LH surge and blockade of NPY by immunoneutralization or anti-sense oligonucleotides blocks the LH surge in steroid-primed rats and suppresses episodic basal release in OVX rats (Kalra et al., 1995; Xu et al., 1996; Xu et al., 1993; Wehrenberg et al., 1989). Conversely, NPY administration in the absence of steroid hormones, or administered continuously, causes a suppression of LH release in both male and female rats (Catzeflis et al., 1993; Pierroz et al., 1996). Likewise, states of chronically high NPY, such as those observed during lactation, food restriction or

in the leptin deficient *ob/ob* mouse, correlate with a suppression of LH release (Smith, 1993; Wade and Schneider, 1992; Mounzih et al., 1997). Together, these data suggest that NPY most likely plays a stimulatory role in normal cyclicity, but also may contribute to the inhibition of reproductive cyclicity under conditions of negative energy balance.

It is postulated that NPY may exert its effects at multiple levels, including GnRH cell bodies in the preoptic area, GnRH nerve terminals in the median eminence, and also at the level of the posterior pituitary. In support of direct effects on GnRH cell bodies, the primary regulators of preovulatory gonadotropin release, NPY injected centrally has been shown to upregulate GnRH mRNA (Li et al., 1994). In addition, in the primate model, antisense oligonucleotides targeting NPY have been shown to suppress the GnRH surge (Mizuno et al., 2000). Supporting the effect of NPY on GnRH release at nerve terminals, NPY can either stimulate or inhibit GnRH release of median eminence fragments depending on the steroid hormone condition (McDonald, 1990). Also, demonstrated in the primate, NPY infusion into the stalk-median eminence stimulates GnRH release *in vivo* (Woller and Terasawa, 1991). Anatomical studies employing electron microscopy have indicated that GnRH neurons receive synaptic contacts by NPY nerve terminals (Tsuruo et al., 1990), and studies in the Smith lab have established that ARH NPY neurons provide a large proportion of the NPY input to GnRH neurons (Li et al., 1999).

Although the functional involvement of NPY in both of these physiological effects has been well characterized, the neuroanatomical basis for the

transduction of signals affecting these outcomes is not well understood. Studying the afferent contacts and efferent targets of ARH NPY neurons will aid in further defining the role that the hypothalamic NPY neuronal system may play in the integration of the neuroendocrine systems controlling feeding and reproduction. The studies reported in this thesis begin to characterize the potential downstream targets and neuronal circuitry of NPY signaling within the hypothalamus.

E. Other metabolic systems important in the control of reproductive neuroendocrine function:

Similar to the way that NPY recruits and coordinates multiple systems in the regulation of food intake, NPY may also recruit some of the same systems in the integration of responses that modulate reproductive function. Researchers are discovering that many of the systems initially characterized as mediators of energy balance also have profound effects on GnRH/LH secretion. Some of these systems and their interactions with NPY are briefly discussed below.

i. Orexin

As the name implies, the orexins, made up of orexin A and B, are associated with an increase in feeding behavior, although to a lesser extent than other peptides (i.e. NPY, AGRP, MCH). However, similar to NPY, orexin also has bimodal effects on LH secretion. Orexin A has been found to stimulate LH secretion in steroid-primed OXV rats, but to inhibit LH secretion in the absence of ovarian steroids (Pu et al., 1998; Tamura et al., 1999). It was also demonstrated

that immunoneutralization of orexin A in the brain completely abrogated both LH and prolactin surges while icv administration of orexin led to a dose-dependent recovery of fasting suppressed hormonal surges (Kohsaka et al., 2001). Moreover, explant studies have shown that orexin A stimulates GnRH release from the hypothalamic explants of intact male rats and of female rats at proestrus (Russell et al., 2001).

As mentioned previously, orexin neurons are directly contacted by ARH NPY neurons. In addition, studies have demonstrated that the stimulatory effect of orexin on food intake is mediated by NPY, since pretreatment with Y1 receptor antagonists blocks the feeding evoked by orexin (Jain et al., 2000; Yamanaka et al., 2000). Studies presented in this thesis also suggest that orexin may be an intermediate signal mediating NPY's effects on reproductive function. These findings suggest that endogenous orexin plays a role in the neuroendocrine control of reproduction and may also be able to integrate signals associated with energy balance regulation.

ii. MCH

MCH has received a great deal of interest as a regulator of food intake and energy expenditure, but more recently MCH has been shown to have effects on reproductive function. It has been demonstrated that central injections of MCH into the medial preoptic area (mPOA) result in increased female sexual receptivity (Gonzalez et al. 1996) and increased LH release in steroid primed rats (Gonzalez et al., 1997). In addition, blocking MCH with antiserum eliminates progesterone-induced LH release in estrogen-primed rats (Gonzalez et al.,

1996). *In vitro* studies demonstrated that MCH stimulates GnRH release from the median eminence and LH and FSH release from the anterior pituitary of female rats in proestrus (Chiocchio et al., 2001). However, similar to other neuropeptides, the effect of MCH on GnRH/LH release appears to have inverse effects under different steroid conditions. In an estrogen, but not progesterone replaced OVX rat, central MCH administration suppresses LH secretion (Tsukamura et al., 2000). Finally, recent data in the Smith laboratory demonstrated that the majority of GnRH neurons in the rat receive direct contacts by MCH fibers, providing morphological evidence for direct effects by MCH on GnRH activity. Taken together, these data suggest that MCH is another metabolic signal that may play an important role in mediating reproductive function.

iii. **CRH**

In addition to the reported anorectic effects of centrally administered CRH, this peptide has also been shown to disrupt GnRH/LH secretion (Petraglia et al., 1987). Central injection of CRH into either the cerebral ventricles (Ono et al., 1984) or the median eminence (Frias et al., 1997) results in a suppression of LH secretion. It has also been shown by numerous groups that LH activity is suppressed as CRH is increased, and that CRH antagonists can reinstate metabolically suppressed LH pulses (for review, Tellam et al., 2000). Although chronic stress or up-regulation of the HPA axis is commonly associated with a disruption of normal GnRH/LH secretion, it is unclear by what mechanism central

CRH plays a role in the physiological regulation of neuroendocrine reproductive function.

Likewise, although NPY projections and contacts to CRH neurons have been clearly established, as noted above, the mechanism by which NPY may signal through the CRH system to regulate either reproductive function or feeding, is less clear. Acute NPY administration has been suggested to exert a stimulatory effect on CRH gene expression, and ACTH and corticosterone secretion (Suda et al., 1993; Liposits et al., 1988), presumably by activating CRH neurons. Conversely, in states of chronically elevated NPY, such as during lactation or fasting, CRH mRNA expression is suppressed (Fischer et al., 1995; Hwang et al., 1997). Together with the suppressive effects of CRH on GnRH/LH secretion, these data do not support a role for a key NPY-CRH pathway in the regulation of nutritional anestrus; rather CRH inhibition of the GnRH system may be more directly related to stress than metabolic state.

iv. Leptin

Leptin is another metabolic signal that is also an important modulator of reproductive function. Leptin has been shown to play a role in the timing of puberty (Ahima et al., 1997; Chehab et al., 1997; Cheung et al., 1997), and infertility is typically associated with reduced leptin signaling. The genetically obese *ob/ob* mouse lacks a functional leptin gene and is infertile, but fertility is resumed upon administration of exogenous leptin (Stephens et al., 1995; Chehab et al., 1996), an effect that cannot be produced simply by weight loss (Mounzih et al., 1997). It is thought that the effects of leptin on the reproductive system may

be mediated via effects on NPY. For example, in the *ob/ob* mouse, the lack of leptin-mediated inhibition of NPY results in chronically elevated levels of NPY activity (Stephens et al., 1995; Swartz et al., 1996). This in turn is thought to contribute to many of the defects of *ob/ob* mouse, including infertility. Consistent with this, the infertility and obesity of the *ob/ob* mouse are attenuated when crossed with an NPY deficient mouse (Erickson et al., 1996).

F. The model of lactation

The model of lactation is characterized by a negative energy balance due to the energy demands of milk production, and a temporal cessation of reproductive cyclicity due to a suppression of GnRH neuronal activity (for review, Smith and Grove, 2002). Therefore, the lactating rat represents a unique, natural physiological model in which to study the integration between energy balance and the neuroendocrine control of reproduction. The final studies of my thesis, presented in the appendix, examine the lactating model. Some of the known physiological characteristics of the lactating model are briefly described below.

i. Cyclic reproductive function is suppressed during lactation

Lactation results in a suppression of ovarian cyclicity due to the suppression of pulsatile LH secretion. This condition is not dependent upon steroid hormones, as LH is inhibited in both intact and OVX lactators (Lee et al., 1989; Fox and Smith, 1984). The suppression of LH is, however, directly dependent upon the suckling stimulus. Removal of suckling pups at any time

during lactation restores pulsatile LH secretion, while the reinitiation of suckling reinstates LH suppression (Brogan et al., 1999; Lee et al., 1989). Likewise, the intensity of the suckling stimulus correlates with the level of LH inhibition (Fox and Smith, 1984; Lee et al., 1989).

Evidence suggests that decreased LH secretion during lactation is most likely due to a decrease in GnRH activity and release. Support for this contention comes from studies in both primate and rodent models. Lactation in the primate results in a decrease in multi-unit activity in the hypothalamus, indicative of a decrease in GnRH pulse generator activity (Ordog et al., 1998). Studies in the rat have found that lactation results in decreased GnRH receptors in the pituitary (Smith and Lee, 1989), suggesting down regulation as a result of decreased GnRH signaling. Upon administration of pulsatile GnRH to lactating rats, GnRH receptors are upregulated, pituitary sensitivity to GnRH is enhanced, and pulsatile LH secretion is restored (Lee et al., 1989). Similarly, administration of GnRH pulses to lactating rhesus macaques restores LH secretion and ovulation (personal communication, Dr. Harold Spies).

ii. Energy balance is altered during lactation

Due to the energy demands of milk production, the lactating rat increases food intake approximately 4-fold. However, because energy expenditure continues to exceed energy intake, the lactating rat remains in a state of negative energy balance. Therefore, the lactating rat exhibits changes in a number of substances known to regulate or be altered by energy balance. Overall, lactating

animals have an increase in orexigenic signals, including NPY and AGRP and a decrease in anorexigenic signals, including leptin, POMC, and CRH.

More specifically, lactation is associated with a decrease in POMC mRNA expression throughout the ARH (Smith et al., 1993). This likely results in decreased α -MSH levels, reduced signaling to MC4 receptors, and therefore, an increase in food intake. CRH mRNA and content are also suppressed during lactation, although ACTH and corticosterone are apparently elevated (Fischer et al., 1995). Peripherally, serum leptin and thyroid hormone levels are also suppressed (Brogan et al., 1999). The decrease in serum leptin has been found to be due to the metabolic drain of milk production, and not the suckling stimulus alone (Brogan et al., 1999). However, it remains to be determined what factors are responsible for the direct inhibition of leptin levels.

In contrast, hypothalamic NPY peptide levels are significantly elevated during chronic lactation, specifically in the POA, PVH, DMH, and ARH (Ciofi et al., 1991; Malabu et al., 1994; Pickavance et al., 1996). In addition, *in situ* hybridization studies have indicated that NPY mRNA expression is increased during lactation in the caudal portion of the ARH, and is also induced in the dorsal subregion of the DMH (Smith et al., 1993). While the DMH normally lacks detectable NPY mRNA, expression is increased in lactation and several models of obesity, including the lethal yellow agouti mouse and the MC4 knockout mouse (Kesterson et al., 1997). AGRP mRNA, which is colocalized in ARH NPY neurons, is also elevated during lactation (Chen et al., 1999).

The increase in NPY during lactation, coupled with its known modulation of both reproductive function and food intake, point to NPY as an important integrating factor for both the metabolic and reproductive signaling pathways during lactation. Therefore, the central hypothesis of the final portion of my thesis is that NPY acts as an integrating signal within the hypothalamus, contributing to both the increase in food intake and the suppression of GnRH neuronal activity in the lactating rat.

iii. The role of the brainstem: specific activation by the suckling stimulus and input into the arcuate nucleus

Brainstem areas specifically activated by the suckling stimulus: The acute suckling stimulus has been shown to activate cFos expression in neuronal populations of both the brainstem and hypothalamus. Five brainstem regions were identified as specifically activated by the suckling stimulus, including the lateral parabrachial nucleus, peripeduncular area, periaqueductal gray, paralemniscal nucleus, and ventrolateral medulla. Retrograde tracing studies found that all of the activated brainstem populations send direct projections into the ARH (Li et al., 1999). These activated brainstem populations may represent the first signals leading to changes in energy balance and reproductive function observed during lactation. It is reasonable to hypothesize that some of these activated populations make contacts with and modulate the NPY system during lactation. However, studies to date have not indicated the specific contacts of those activated brainstem neurons within the ARH.

The Ventrolateral Medulla (VLM): One population in particular that may prove interesting in mediating signals to ARH NPY neurons are the suckling-activated neurons of the VLM. The majority of cFos expressing VLM neurons have already been identified as noradrenergic. These A1 neurons of the VLM have been well described as the major catecholamine input to the hypothalamus (Sawchenko et al., 1985). Norepinephrine (NE), the primary neurotransmitter of A1 neurons, has been implicated in the regulation of both energy balance and reproductive function. Interestingly, the majority of VLM neurons also colocalize with NPY. The role of NPY in the regulation of catecholaminergic release and function remains unclear. However, it is not unreasonable to speculate that NPY originating in the brainstem may also play a role in some of the effects of NPY at the level of the hypothalamus. Studies presented in the Appendix further investigate this suckling activated population of neurons and the role of brainstem NPY.

Aims of the Thesis and Approach:

Overall Goal: Examine the neuroanatomical targets of NPY neurons in forebrain regions important to reproductive neuroendocrine function and the control of energy balance.

Approach:

- (A) Determine if NPY neurons of the arcuate nucleus have a reciprocal feedback relationship with corticotrophin releasing hormone (CRH) neurons of the paraventricular nucleus of the hypothalamus (PVH) by investigating the expression of CRH receptors in the ARH, specifically in relation to NPY and DA neurons.
- (B) Determine the potential sites of NPY action by characterizing NPY Y5 receptor expression throughout the brain and to specific neuronal phenotypes.
- (C) Determine the potential sites of NPY action via the NPY Y4 receptor subtype by characterizing protein expression throughout the brain and to specific neuronal phenotypes.
 - Investigate the effect of rPP, a potent Y4 ligand, on food and water intake and cFos activation of Y4 expressing cells
- (D) Investigate brainstem regions previously identified as being specifically activated by the suckling stimulus.
 - Characterize changes in the expression of NPY mRNA in the VLM during lactation.

Chapter II

Distribution of Corticotropin Releasing Hormone Receptor
Immunoreactivity in the Rat Brain: Coexpression in Neuropeptide Y and
Dopamine Neurons
in the Arcuate Nucleus

Neuroscience

submitted

Introduction

Corticotropin-releasing hormone (CRH) is a widely distributed brain peptide recognized primarily for its control of the hypothalamo-pituitary-adrenocortical (HPA) axis (Vale et al., 1981). In addition to the well documented neuroendocrine effects of CRH in response to stress, CRH neurons have also been implicated in the regulation of food intake and energy balance (Rothwell, 1990; Richard 1993), reproduction (Rivier and Vale, 1984), inflammatory responses (Vamvakopoulos and Chrousos, 1994), and cardiovascular function (Fisher et al., 1982; Fisher et al., 1983). These effects of CRH are mediated via binding to and activating specific seven-transmembrane domain G protein-coupled CRH receptors of two subtypes, CRH R₁ and CRH R₂. Additionally, two splice variants of the CRH R₂ subtype have been described, CRH R_{2α} and CRH R_{2β}, along with a truncated form of CRH R_{2α} (Kishimoto et al., 1995; Lovenberg et al., 1995; Perrin et al., 1995; Stenzel et al., 1995; Kostich et al., 1998; Miyata et al., 2001). Based on previous characterizations of CRH R₁ and CRH R₂ mRNA (Chalmers et al., 1995; Van Pett et al., 2000) and binding (DeSouza 1987; Rominger et al., 1998), CRH R₂ appears to have a more restricted distribution, while CRH R₁ is found throughout the cortex, forebrain, cerebellum, mesencephalon and pons. Of direct interest to the present study, CRH R₁ binding and mRNA have been demonstrated in the arcuate nucleus of the hypothalamus (ARH) of both the rat and the non-human primate (Wynn et al., 1984; Millan et al., 1986; Wong et al., 1994; Chen et al., 2000). The neuroanatomical distribution of CRH R₁ protein has also been described by use

of immunocytochemistry in the rat and the mouse (Radulovic et al., 1998; Chen et al., 2000), and demonstrates a similarly widespread distribution pattern throughout the brain. However, the study in the rat was limited to reporting the hypothalamic CRH R_1 distribution to the paraventricular nucleus of the hypothalamus (PVH). In addition, many of the phenotypes of cells expressing CRH receptors remain unknown.

Neuropeptide Y (NPY) is a potent orexigenic peptide implicated in several hypothalamic functions including neuroendocrine regulation (Kalra and Crowley, 1992), energy homeostasis (Kalra et al., 1999) and stress responsiveness (Dallman et al., 1995). There is an abundance of physiological data indicating that at least part of NPY's function is mediated through regulation of CRH neurons of the PVH (Rothwell, 1990; Suda et al., 1993; Hwang and Guntz, 1997). Recent studies from this laboratory demonstrated that NPY neurons originating from the ARH project to, and come in close contact with, CRH neurons in the PVH (Li et al., 2000). In addition, PVH CRH neurons are contacted by Y1 expressing fibers and a subset of CRH neurons in the PVH express the NPY Y5 receptor subtype (Campbell et al., 2001).

Conversely, it has been demonstrated that NPY signaling is modulated by CRH (Morley, 1987; Heinrichs et al., 1998; Heinrichs and Richard, 1999). The presence of CRH R_1 mRNA and binding within the ventromedial portion of the ARH suggests that NPY neurons may express the CRH R_1 and may be direct targets of CRH innervation. Taken together, these findings suggest a possible reciprocal feedback regulation between NPY and CRH neurons.

The purpose of the present study was to: 1) characterize the distribution and cellular localization of CRH R₁-like immunoreactivity (CRH R₁-ir) in the rat brain, with an emphasis on the hypothalamic and forebrain distribution, 2) determine if ARH NPY neurons contain CRH R-ir and 3) characterize the relationship between CRH fibers and CRH R₁-ir positive cells in the ARH.

Materials and Methods

Animals:

Virgin cycling female rats (B & K Universal Kent, WA) were housed individually and maintained under a 12:12 light-dark cycle (lights on at 0700h) and constant temperature ($23 \pm 2^{\circ}\text{C}$). Food and water were provided *ad libitum*. All animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee, and every effort was made to minimize the suffering and number of animals used. Tissue was harvested from animals that were either untreated (n=3) or injected with colchicines (n=3), a treatment that is necessary to detect NPY peptide within cell bodies of the ARH. Colchicine-treated rats were injected with 80 μg of colchicine into the lateral ventricle and sacrificed two days following injections. Both groups were sacrificed under pentobarbital anesthesia by intracardiac infusion with ice-cold saline followed by ice-cold 4% paraformaldehyde (pH 7.4). After removal, brains were left in 4% paraformaldehyde for 24 hours then saturated in 25% sucrose. After sinking the brains in sucrose, they were frozen on crushed dry ice and stored at -80°C until sectioned on a sliding microtome at a thickness of 25 μm . Brain tissue

was sectioned into a one in six series and stored in cryoprotectant until processing for immunocytochemistry (ICC).

Single Label ICC:

Single label ICC for CRH R₁-ir was performed on floating sections. Briefly, tissue sections were removed from cryoprotectant and washed in 0.05M potassium phosphate-buffered saline (KPBS) followed by incubation with the CRH R₁ antibody made in the goat (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-1757) for 48 hours at 4 °C. The CRH R₁ antibody, raised against the carboxy-terminus of CRH R₁ protein, is reported to have some cross reactivity with the R₂ subtype. Following washes in KPBS, tissue was incubated for 1h in biotinylated donkey anti-goat antibody (1:600, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), then subsequently washed and incubated in avidin-biotin solution (Vectastain, Vector Laboratories, Inc., Burlingame, CA) for 1h. CRH R₁-ir was visualized with 3,3'-diaminobenzidine enhanced with nickel chloride. Sections were then mounted onto gelatin-coated slides, dried and coverslipped. Distribution of CRH R-ir was observed with a Nikon light microscope (Plan Apo 20X objective, NA 0.75; Plan Apo 4X, NA 0.20) and images were collected using a Micromax Digital Camera (Princeton Instruments Inc.) connected to the MetaMorph Imaging System software package (Universal Imaging Corp., West Chester, PA). The brightness

and contrast of the digital images were adjusted in Photoshop to match visualization by microscope (Adobe Systems Inc., San Jose, CA).

Characterization of the CRH R₁ Antibody:

The CRH R₁ antibody used in the present study has been previously characterized (Chen et al., 2000). To confirm antibody specificity under our laboratory conditions, serial sections were stained with the primary antibody and with the primary antibody preadsorbed with blocking peptide (sc-1757 P). Preadsorption with control peptide eliminated specific antibody staining throughout the brain. Further characterization of the antibody involved performing *in situ* hybridization to detect CRH R₁ mRNA. Patterns of CRH R₁ mRNA expression were then compared to the results obtained using the CRH R₁ antibody.

In Situ Hybridization:

A qualitative assessment of CRH R₁ mRNA distribution was investigated. For this study, adult cycling female rats (n = 2) were sacrificed by decapitation, the brains were removed, frozen and stored at -80 °C until cryostat sectioning at a 20 µm thickness in a one in three series. CRH R₁ mRNA levels were determined in one series of tissue sections by *in situ* hybridization.

In situ hybridization was performed as previously described (Chen et al., 1999; Grove et al., 2001). Briefly, to detect CRH R₁ mRNA, a cRNA probe (a kind gift of W. Vale, Salk Institute, CA) was transcribed from a 550bp cDNA with 50% of the UTP labeled with ³³P. The specific activity of the CRH R₁ cRNA probe was 1.0 x 10⁹ dpm/µg, and the saturating concentration of the probe used in the

assay was 0.3 $\mu\text{g/ml/kb}$. The ^{33}P -UTP labeled CRH R_1 cRNA sense probe resulted in a complete lack specific signal (data not shown). Sections throughout the hypothalamus were fixed in 4% paraformaldehyde and passed through rinses in 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0), 2X standard saline citrate (SSC), a graded series of alcohols, chloroform, and rehydrated in a second series of alcohols. After drying, slides were hybridized with the cRNA CRH R_1 probe overnight in a moist chamber at 50°C. After incubation, slides were washed in 4X SSC, ribonuclease A at 37°C, and in 0.25X SSC at 55°C. Slides were then dehydrated in ethanol and exposed to sheet film (Biomax MR, Eastman Kodak Co., Rochester, NY) for 7 days. Following film development, slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak Co.) diluted 1:1 in 600 mM ammonium acetate and then placed in light-tight boxes containing desiccant and stored at 4 °C for 3 weeks. The slides were then developed and counterstained with cresyl violet and the distribution of silver grains analyzed by darkfield microscopy.

Double Label ICC:

To characterize the phenotypes of cells displaying CRH R_1 -ir, double label immunofluorescence coupled with confocal microscope analysis was performed. Antibody concentrations used for these studies were as follows: goat-anti-CRH R_1 (1:1000); rabbit- anti-NPY (1:5000, a kind gift of M. Brown); mouse-anti-TH (1:250,000, Chemicon; Temecula, CA), rabbit-anti-CRH (1:10,000, Peninsula Laboratories, Inc., San Carlos, CA). Briefly, tissue sections were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline

(KPBS) followed by incubation in a cocktail of primary antibodies containing CRH R₁ and antibodies to either NPY, TH, or CRH for 48 hours at 4 °C. Fluorescence was visualized with either a donkey-anti-goat secondary antibody (CRH R₁) or a donkey anti-mouse secondary antibody (TH) conjugated to an Alexa fluorophore (Alexa 488 or 568, Molecular Probes, Eugene, OR), or with a FITC-conjugated donkey anti-rabbit (NPY, CRH) antibody (Jackson ImmunoResearch Laboratories, Inc). After incubation in the fluorescent secondary antibodies for 1 hour, tissue was washed again in KPBS, then mounted and coverslipped with buffered glycerol.

Confocal Laser Microscopy:

Confocal laser microscopy was performed as previously described (Campbell et al., 2001). Briefly, double-label immunofluorescence was analyzed with the aid of confocal laser scanning microscopy. The Leica Corp. (Germany) TSC SP confocal system consisted of a Leica Corp. RBE inverted microscope equipped with a Ar laser producing light at 488nm (for visualization of FITC and Alexa 488), a Kr laser producing light at 568nm (for visualization of Alexa 568). Various objectives (25X, NA 0.75 and 40X, NA 1.25) were used to scan the images. For each experiment, fluorophore signals were checked individually for bleed through to the apposing detector. All bleed through was eliminated by adjusting laser intensity and detector window width. To assess colocalization of two signals, a series of continuous optical sections, 0.5µm intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. For the study analyzing the close apposition of CRH fibers on neurons expressing CRH

R₁-ir, the optical sections were taken at 0.25 μ m intervals to provide a high resolution for confirmation of close contacts between the fibers and cell bodies. The signals were obtained for each fluorophore on one series of optical sections and were stored separately as a series of 512 x 512 pixel images. The stacks of individual optical slices (0.25 or 0.5 μ m resolution) were analyzed using the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA) to determine close contacts or colocalization. The confocal images are presented as projections of a stack of optical images or as individual slices, as indicated. The brightness and contrast of the images were adjusted in Photoshop to match microscope visualization (Adobe Systems Inc., San Jose, CA).

Results

Characterization of CRH R₁ Antibody Staining:

The specificity of CRH R₁ immunoreactivity was first assessed by preadsorption of the antibody with control peptide. Figure 1A, panels a and b, show representative images of ARH sections that were treated with antibody that was preincubated with either buffer (-CP) or control peptide (+CP), respectively. The extensive CRH R₁-ir staining observed in the -CP tissue was almost completely eliminated in the +CP tissue. Likewise, in regions throughout the brain, preadsorption of the antibody to control peptide completely eliminated specific staining. Panel c shows CRH R₁-ir (red) coupled with a nuclear stain (dapi, blue) and illustrates that although CRH R₁-ir is found throughout the ARH, specific labeling does not include all cells.

Additional confirmation for the specificity of immunoreactive staining observed using this antibody was provided by *in situ* hybridization detection of CRH R₁ mRNA. The CRH R₁ mRNA expression pattern was found to closely match the pattern of CRH R₁ protein expression. Examples are shown for the ARH (Fig. 1B, a and b) and for the central amygdala (ceAM) (Fig. 1B, c and d). Silver grain patterns observed in the ARH (panel b) matched the diffuse but intense staining pattern seen with ICC (panel a). Likewise, the scattered silver grain clusters observed in the ceAM (panel d), reflect the darkly labeled, evenly spaced cell bodies stained by ICC (panel c).

Characterization of CRH R₁ Distribution by Single Label ICC:

Intensely labeled CRH R₁-ir cell bodies and fibers were found throughout the central nervous system. In agreement with previously described studies using another polyclonal antibody in the rat (Radulovic et al., 1998), CRH R₁-ir was observed throughout the cortex, brainstem, and cerebellum. In addition, however, we observed densely labeled cells throughout specific nuclei of the hypothalamus that were not previously reported.

Representative photomicrographs throughout cortical, hippocampal and brainstem regions are shown in Figure 2. The majority of CRH R₁-ir in the cortex was found localized to the soma; however, staining was also present on fibers, probably representing dendritic processes. Although staining was observed at various levels throughout layers 2-6 in the cortex, layer 5 appeared to have the most intense staining observed in the cortex (Fig. 2A,B,C). The staining appeared to be located along membrane surfaces, however granular cytoplasmic

staining indicates some receptors may be docked below membrane surfaces as well. In the hippocampus, granular and punctate staining was observed in pyramidal neurons of the CA1, CA2 and CA3 regions (Fig. 2D), while the most intense staining was present in the GABAergic-like interneurons in the polymorphic cell layer (p) of the dentate gyrus (DG, Fig. 2E,F). The granular cell layer (g) of the DG was also positive for CRH R₁-ir, while the molecular cell layer (m) of the DG lacked specific staining (Fig. 2D,E). The midbrain and brainstem regions displaying the most intense CRH R₁-ir staining are also represented in Figure 2. Dense, homogenous cell body staining was observed in the dorsal raphe nucleus (Fig. 2G), and the locus coeruleus (Fig. 2H). Immunoreactive cells were also observed scattered throughout the reticular region of the substantia nigra (not shown). Furthermore, what appeared to be punctate, membrane bound labeling was found throughout thalamic structures, with the most intense staining in the anterodorsal thalamic nucleus (Fig. 2I).

The hypothalamic and forebrain distribution and relative abundance of CRH R₁-ir is reported in Table 1 and illustrated by representative photomicrographs in Figure 3. Magnocellular neurons in the supraoptic nucleus (SON) and the PVH displayed the most intense level of CRH R₁-ir in the hypothalamus (Fig. 3A,C, higher power of PVH cells in D). Moderate levels of staining were also present in the suprachiasmatic nucleus (SCN) (Fig. 3B), parvocellular region of the PVH (Fig. 3C), and lateral hypothalamus/ perifornical area (Table 1). Of interest, the ARH displayed intense, punctate, cell body staining (Fig. 3E,F). Staining was displayed throughout the rostral to caudal

extent on the ARH, with the most abundant labeling observed in the ventromedial portion and more diffuse patterns of staining observed in the dorsolateral regions. Medium and low levels of CRH R₁-ir immunostaining were also observed in the ventromedial hypothalamic nucleus and the dorsomedial hypothalamic nucleus, respectively (Table 1).

Colocalization of CRH R₁ with NPY and TIDA Neurons:

Double label immunofluorescent studies determined that the CRH R₁-ir in the ARH of the rat was colocalized with NPY neuronal cell bodies. Confocal analysis of these sections demonstrated that the majority of NPY-ir neurons throughout the ARH expressed CRH R₁-ir (Fig 4A-C). However, CRH R₁-ir did not appear to be colocalized with NPY fibers. In addition, there were many single labeled CRH R₁-ir neurons in the dorsomedial portion of the ARH, a region rich in tuberoinfundibular dopamine (TIDA) neurons. Double label immunofluorescence and confocal analysis indicated that nearly every TH-positive cell was colocalized with CRH R₁ in the dorsomedial portion of the ARH (Fig 4D-F). Again, the colocalization appeared to be restricted to TIDA cell bodies and was not seen in fibers. Analysis of single optical slices (0.5µm resolution) was performed to confirm colocalization of CRH R₁-ir to both NPY and TIDA neurons. A high power image of a single co-labeled CRH R₁-ir/TIDA neuron along with a series of optical sections through the z-plane are shown in panel G and G0-4. There was also some staining present in the lateral portion of the ARH; however, the phenotypes of these neurons remain unknown.

To further investigate the hypothesis that CRH is modulating NPY activity directly via CRH R₁, double label immunofluorescence was used to characterize the relationship between CRH fibers and CRH R₁-ir positive cells in the ARH. Figure 5A illustrates that although numerous CRH fibers (green) were found in the ventromedial portion of the ARH, adjacent to the median eminence, CRH fibers were extremely sparse throughout the remainder of the ARH. Within the region of the ARH that was heavily innervated by CRH-ir fibers, many close contacts (yellow) were evident on CRH R₁-ir (red) positive neurons (Fig. 5B and 5B'). For higher magnification analysis of close contacts between CRH-ir fibers and CRH R₁-ir neurons, a nuclear dapi stain (blue) was used to help identify cells (Fig. 5C-E). Panel C shows another example of close contacts in the ventromedial ARH and panel D is a magnification of the box found in C. Additionally, in more sparsely innervated regions of the ARH, scattered fiber processes were also seen coming in close contact with CRH R₁-ir cells (Fig. 5E).

Discussion

This study characterizes the distribution of CRH R₁ protein within the rat brain by immunocytochemistry. The distribution of CRH R₁-ir containing neurons observed throughout the brain closely correlates with previous studies characterizing the distribution of mRNA (Wong et al., 1994) and receptor binding (Wynn et al., 1984) in the rat, suggesting that these areas indeed express CRH receptors. However, while previous immunocytochemical data (Radulovic et al., 1998) presented a similar overall CNS distribution, this study lacked a detailed

description of the hypothalamic regions expressing CRH R₁. The present study focuses on the CRH R₁-ir distribution throughout hypothalamic regions and demonstrates for the first time that CRH R₁ is expressed in both NPY and TIDA neurons of the ARH.

Single Label CRH R₁-ir Distribution:

The representative photomicrographs and distribution described for those areas outside of the hypothalamus (Fig. 2) served to confirm the results of others and to support the use of this particular antibody. Our findings were almost in complete agreement with the distribution described by Radulovic et al. (1998), with the exception of the hippocampus. The current study found a more intense signal throughout the CA1, CA2, CA3 and the DG than did the previous study. However, the present data are in agreement with present and previous *in situ* hybridization findings for this region and the idea of limbic system involvement in CRH signaling pathways (Chalmers, 1995; Van Pett, 2000). It should also be noted that while this antibody has been characterized as specific to the CRH R₁ subtype (Chen et al., 2000), it is produced against the C-terminus, providing a chance for cross-reactivity with the CRH R₂ subtype.

CRH R₁ labeled cells and short fiber processes were found throughout the hypothalamus (Fig. 3, Table 1). Staining was generally observed localized to the membrane, although in some cells, including those in the ARH, CRH R₁-ir was found to outline only part of the cell body, displaying a punctate pattern, and suggesting somatic innervation. The greatest intensity of staining was observed in the magnocellular cells of the PVH and the SON. Parvocellular PVH neurons

in the anterior, medial and lateral regions were also positively labeled in the rat, however not to the same degree as those in the magnocellular division. This is in contrast to findings in the mouse, in which magnocellular neurons were observed to be only lightly labeled while parvocellular cells were found to be intensely labeled (Chen et al., 2000). These data are also in contrast to previous *in situ* hybridization studies, which found surprisingly low levels of CRH R₁ mRNA in the PVH (Potter et al., 1994, Chalmers et al., 1995). While stress has been shown to dramatically increase CRH R₁ mRNA (Rivest et al 1995), we believe the tissue utilized in this study was obtained under relatively stress-free conditions and therefore support the suggestion of others (Chen et al., 200) that high levels of CRH R₁ protein levels may exist concomitantly with low steady state levels of CRH R₁ mRNA. Supporting these data, Radulovic et al. (1998) also reported CRH R₁-ir in the PVH. The presence of CRH R₁-ir on parvocellular neurons is in agreement with the idea of an ultrashort feedback loop, as suggested by Ono et al. (1985), to CRH neurons that may be involved in both neuroendocrine and autonomic functions of CRH. The positively labeled magnocellular cells of both the PVH and SON most likely represent oxytocin (OT) and vasopressin (VP) cells, suggesting that CRH is able to directly affect OT and VP production. The CRH acting on these PVH CRH R₁ may originate from either locally produced CRH (Swanson et al., 1987), or from a variety of other sites known to contain projecting CRH neurons (Champagne et al., 1998).

CRH R₁-ir positive neurons were also abundant in basal forebrain regions, including the medial septum, nucleus of the diagonal band and throughout the

preoptic area. These areas have all been demonstrated to receive fairly robust innervation by CRH fibers (Swanson et al., 1983). Recently it was shown in the mouse that nearly all the cholinergic neurons in this region coexpress CRH R_1 (Sauvage and Steckler, 2000). The expression of CRH R_1 was also observed within the SCN suggesting that CRH may feedback on those neurons thought to be involved in the circadian pacemaker driving the diurnal rhythm of the basal secretory activity of the HPA axis. However, while a clear projection from SCN neurons to CRH producing neurons of the PVH has been established (Vrang et al., 1995), the SCN does not appear to be densely innervated by CRH fibers (Swanson et al., 1983). Finally, the presence of CRH R_1 -ir positive cell bodies in the ARH suggests that NPY neurons could be direct targets of CRH action. This finding led to double label studies to investigate the phenotypes of CRH R_1 - expressing ARH neurons.

Colocalization of CRH R_1 with ARH NPY Neurons:

The presence of CRH R_1 colocalized to NPY neurons in the ARH was confirmed with confocal analysis as depicted in Figure 4. There appears to be an integral relationship between the NPY neurons of the ARH and the CRH neurons of the PVH, and these data provide a potential neuroanatomical substrate for a reciprocal feedback loop between CRH and NPY containing neurons. Typically, CRH activation of the HPA axis is thought to play an important role in enhancing energy storage, with a chronic elevation of circulating glucocorticoids causing a Cushing's syndrome form of obesity, as has been shown in various rat models with perturbed HPA control of glucocorticoid production (Pepin et al., 1992;

Stenzel-Poore et al., 1992; Tronch et al., 1999). In contrast, CRH has also been found to elicit opposing effects to those of NPY on food intake and energy balance. While central administration of NPY elicits a robust increase in food intake, it has been repeatedly shown that central infusion of CRH inhibits food intake and stimulates metabolic activity (Arase et al., 1998). The administration of a CRH antagonist has also been shown to prevent stress-induced decreases in food intake and weight loss (Smagin et al., 1999). Central to the hypothesis of a CRH-NPY feedback loop, injections of CRH were found to impair NPY induced food intake while CRH antagonists enhanced NPY elicited feeding (Morley, 1987; Heinrichs et al., 1998; Heinrichs, 1999). These data suggest that NPY activates CRH that, in turn, is able to inhibit feeding. The present finding of CRH R_1 colocalized to NPY neurons provides a potential neuroanatomical circuit by which CRH may inhibit NPY signaling. However, signaling through CRH receptors activates adenylate cyclase and causes the accumulation of cAMP, therefore, the mechanism by which CRH may inhibit NPY signaling remains to be determined.

Furthermore, the neuronal pathway linking these two systems and the relative importance of CRH verses other CRH like ligands in the modulation of feeding needs further clarification. While it has been shown previously that NPY neurons make synaptic contacts with PVH CRH neurons (Liposits, et al., 1988; Li, et al., 2000) and that CRH neurons express the Y5 receptor subtype (Campbell, et al., 2001), the current study failed to observe a robust innervation of the ARH by CRH fibers (Fig. 5). This lack of dense innervation is in agreement with the findings of others (Swanson et al, 1983). This raises the possibility that

an alternative ligand, such as urocortin (UCN), may be involved in regulating ARH neurons through CRH R₁.

Like CRH, UCN has also been demonstrated as a potent inhibitor of appetite when injected centrally (Bradbury et al., 2000). UCN exhibits a higher affinity than CRH for both the CRH R₁ and CRH R₂ subtypes, but appears to have the highest affinity for the CRH R₂ (Vaughn et al., 1995; Yamamoto et al., 1998). CRH R₂ has not been found expressed in the ARH, but is found in relatively high concentrations in the PVH. Injections of UCN directly into the PVH have been found to decrease NPY induced feeding and suppress respiratory quotient values (Wang et al., 2001, Currie et al., 2001), suggesting that UCN may alter food intake and energy substrate utilization through an interaction with hypothalamic NPY at the level of NPY release. Supporting a role for the R₂ subtype in the regulation of feeding, CRH R₂ but not CRH R₁ antisense treatment attenuates the effects of CRH and blunts the response to UCN on food intake (Smagin et al., 1998) and CRH agonists inhibit food intake in CRH R₁ knockout mice (Cantarino et al., 1999). Taken together with the present findings of the sparse CRH innervation to CRH R₁-ir neurons in the ARH, these data suggest that UCN may be a more potent modulator of NPY neurons in the regulation of feeding, and that this modulation may involve CRH R₂ expressed at NPY nerve terminals in the PVH. However, the presence of CRH R₁ on ARH NPY neurons provides an additional mechanism whereby CRH or UCN may also modulate NPY neuronal function directly. UCN fibers are observed innervating the ARH (Bittencourt et al., 1999), although the origin of these Ucn fibers is unknown.

Colocalization of CRH R₁ with ARH DA Neurons:

In addition to NPY neurons, the presence of CRH R₁ positive neurons in the dorsomedial portion of the ARH suggested that TIDA neurons could also be expressing the receptor. Virtually all the dopaminergic cells in this region were confirmed to colocalize with CRH R₁. The functional implications of this finding may involve the effects of stress on prolactin (Prl) release. Stress is known to induce the release of prolactin (Prl). Under basal conditions, TIDA neurons suppress Prl release by a direct action of DA on pituitary lactotropes (Freeman et al., 2000, review). The presence of CRH receptors on TIDA neurons indicates that CRH induced Prl release may be due to direct effects on TIDA neurons to suppress dopamine release and remove the inhibition Prl secretion. Further studies need to be carried out to confirm this potential mechanism. Finally, this study was in no way a complete evaluation of CRH R₁-ir expressing neurons in the ARH. NPY and TIDA neurons appear to only be a fraction of the CRH R₁-ir neurons of the ARH, which may also include GHRH and pro-opiomelanocortin.

In summary, CRH R₁ protein expression is found expressed on cell bodies and short fiber processes throughout the brain. A detailed description of hypothalamic sites positive for the CRH R₁ provides neuroanatomical evidence for direct effects by the CRH system in a variety of neuroendocrine and autonomic functions. Additionally, the colocalization of CRH R₁ with both NPY and TIDA ARH neurons provides evidence for direct CRH effects on these

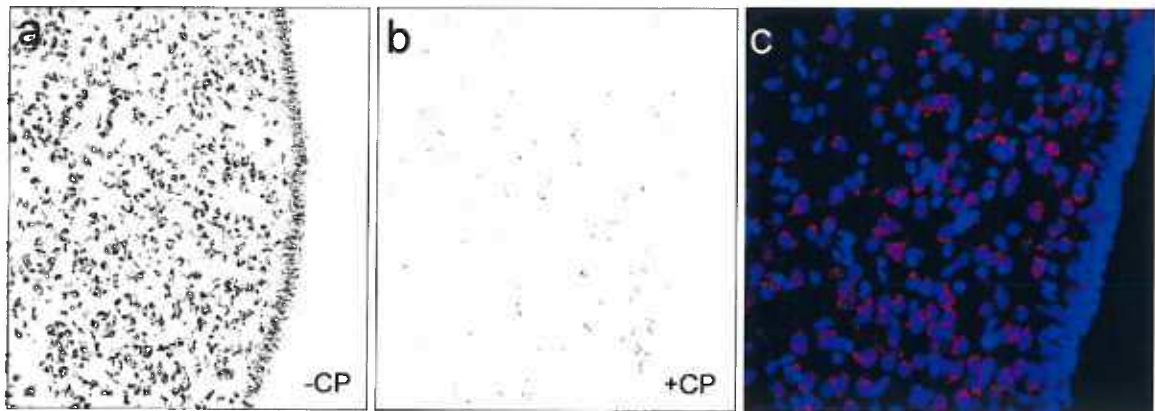
systems, although the specific endogenous ligands and neuronal circuitry involved remain to be determined.

TABLE 1: Relative Levels of CRH R₁-ir in Rat Hypothalamus and Forebrain Regions

Hypothalamic/Forebrain Region	Relative Level of Immunoreactivity ¹
Medial preoptic area	++
Lateral preoptic area	++
Anteroventral periventricular nucleus	+
Suprachiasmatic nucleus	+++
Retrochiasmatic nucleus	+
Supraoptic nucleus	++++
Paraventricular nucleus	
parvicellular division	++
magnocellular division	++++
Ventromedial nucleus	++
Arcuate nucleus	+++
Anterior hypothalamic area	+
Lateral hypothalamic area	++
Dorsal hypothalamic area	+
Dorsomedial nucleus	+
Median eminence	-

¹The relative level of immunoreactivity was assessed by the overall intensity of chromagen staining in each region among 3 different animals. Relative levels: +, light staining; ++, moderate staining; +++, intense staining; +++++, very intense staining.

A



B

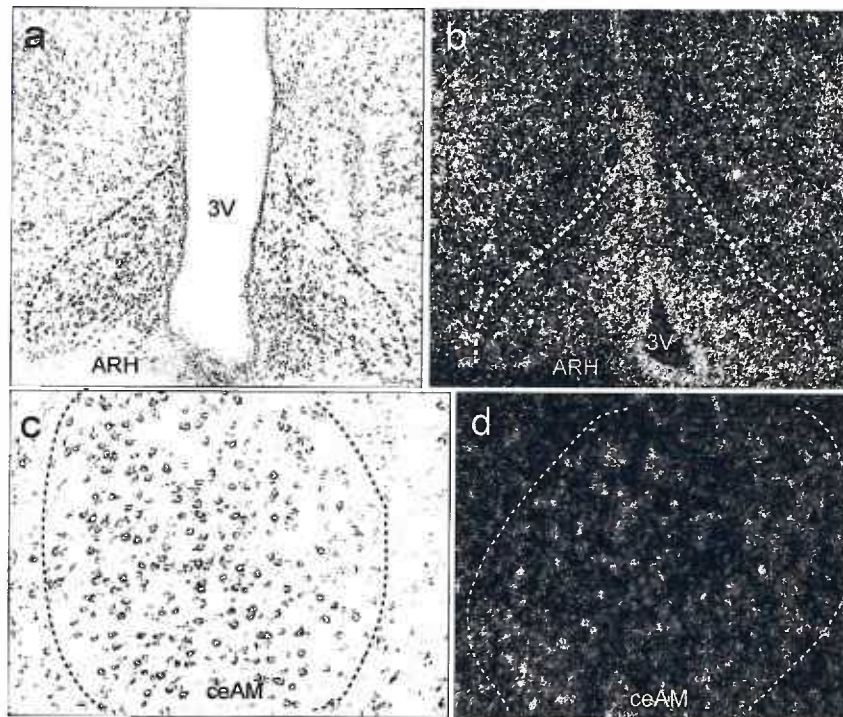


Figure 2-1. (A) Characterization of the CRH R1 antibody in the ARH. (a) Single label CRH R1-ir in the ARH. (b) Pre-incubation of the antibody with CRH R1 control peptide nearly eliminates specific staining. (c) Fluorescent CRH R1-ir (red) co-labeled with a nuclear dapi stain (blue) indicates that while many cells of the ARH are positive for CRH R1-ir, there are many cells not expressing CRH R1-ir. (B) Additional characterization of the CRH R1 antibody: a comparison of patterns of CRH R1-ir and CRH R mRNA expression. Similar patterns of cells displaying chromagen immunoreactivity (a,c) and silver grain clusters (b,d) are shown in side by side panels for the arcuate nucleus (ARH, panels a,b) and central amygdala (ceAM, panels c,d).

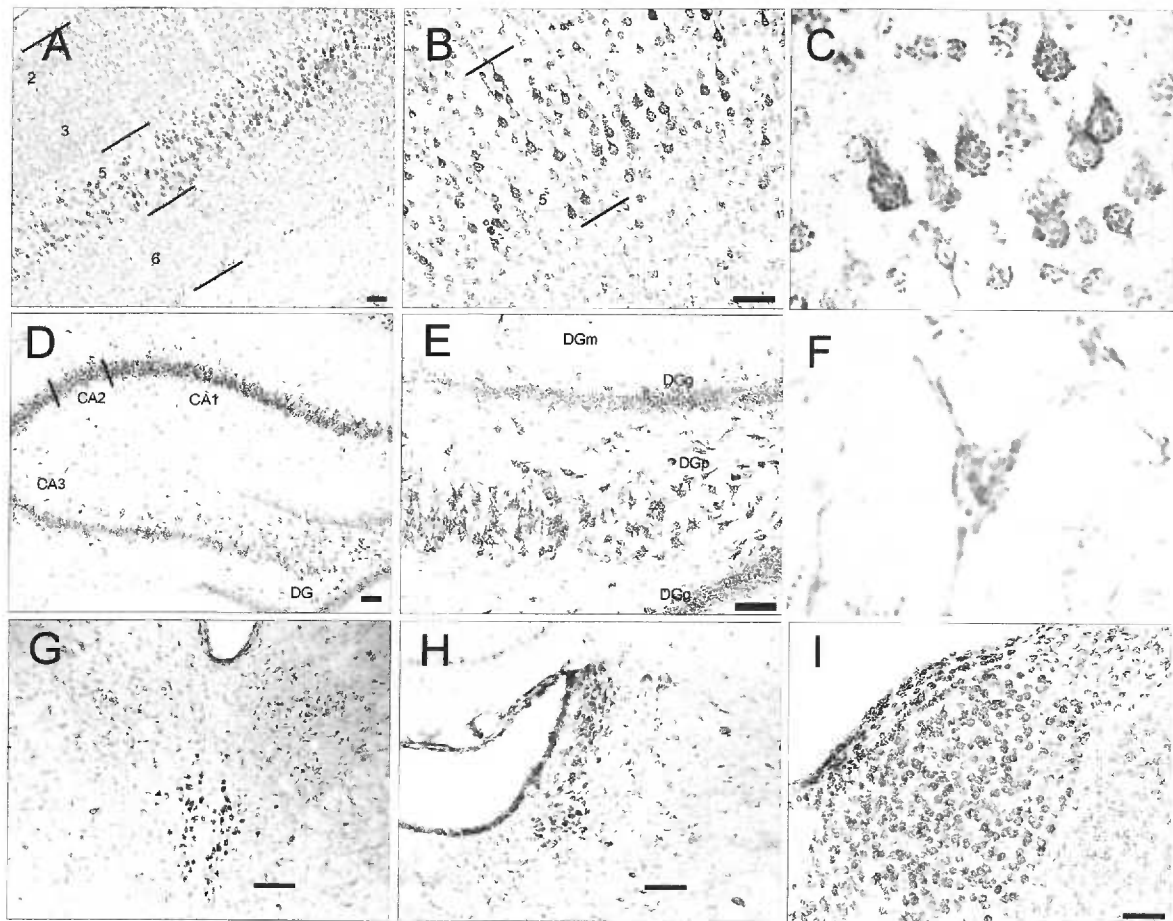


Figure 2-2. Representative images of single label CRH R1-ir throughout the rat brain. Neurons displayed CRH R1-ir throughout layers 2-6 of primary cortex (A), and most intensely on soma membranes and along short fiber processes of layer 5 (B). Panel C shows a high power image of granular staining in cortical neurons. Staining was observed throughout CA1, CA2, CA3 and DG regions (D), with intensely labeled cells in the polymorphic (DGp) and granular (DGg) cell layers of the dentate gyrus (DG, panel E). Panel F shows a high power image of a pyramidal neuron. Brainstem regions with the highest level of CRH R1-ir neuronal staining included the dorsal raphe (G) and locus coeruleus (H). Many thalamic regions, including the anterodorsal thalamic nucleus (I), contained positively labeled cells bodies. Black bars represent 100µm.

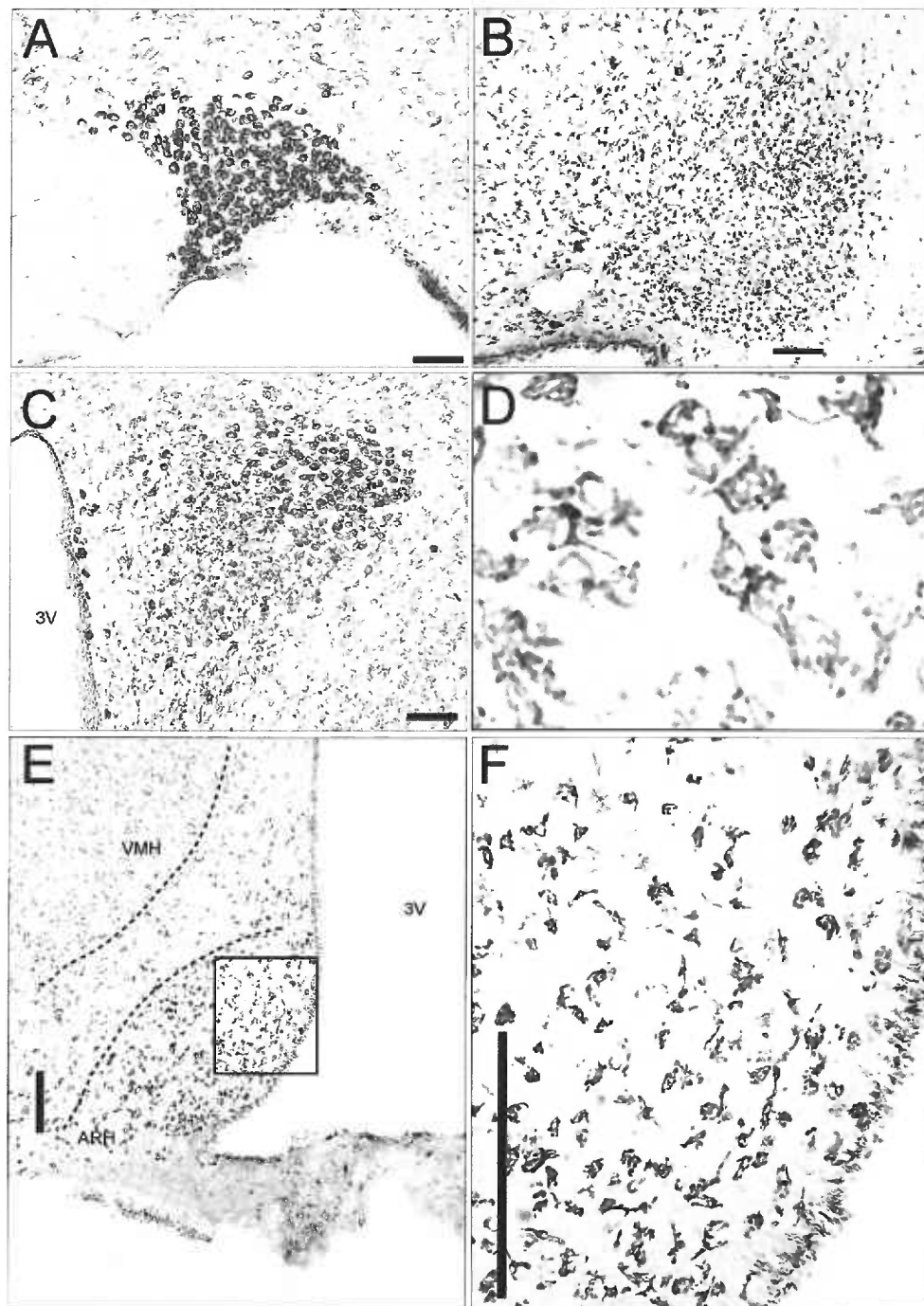


Figure 2-3. Single label CRH-R1-ir distribution in representative regions of the hypothalamus. Intense staining was observed in the supraoptic nucleus (A), suprachiasmatic nucleus (B), paraventricular nucleus (C, higher power image in D), and arcuate nucleus (E). Panel F depicts a higher magnification of neurons in the ARH as outlined by the box in panel E. Black bars represent 100µm.

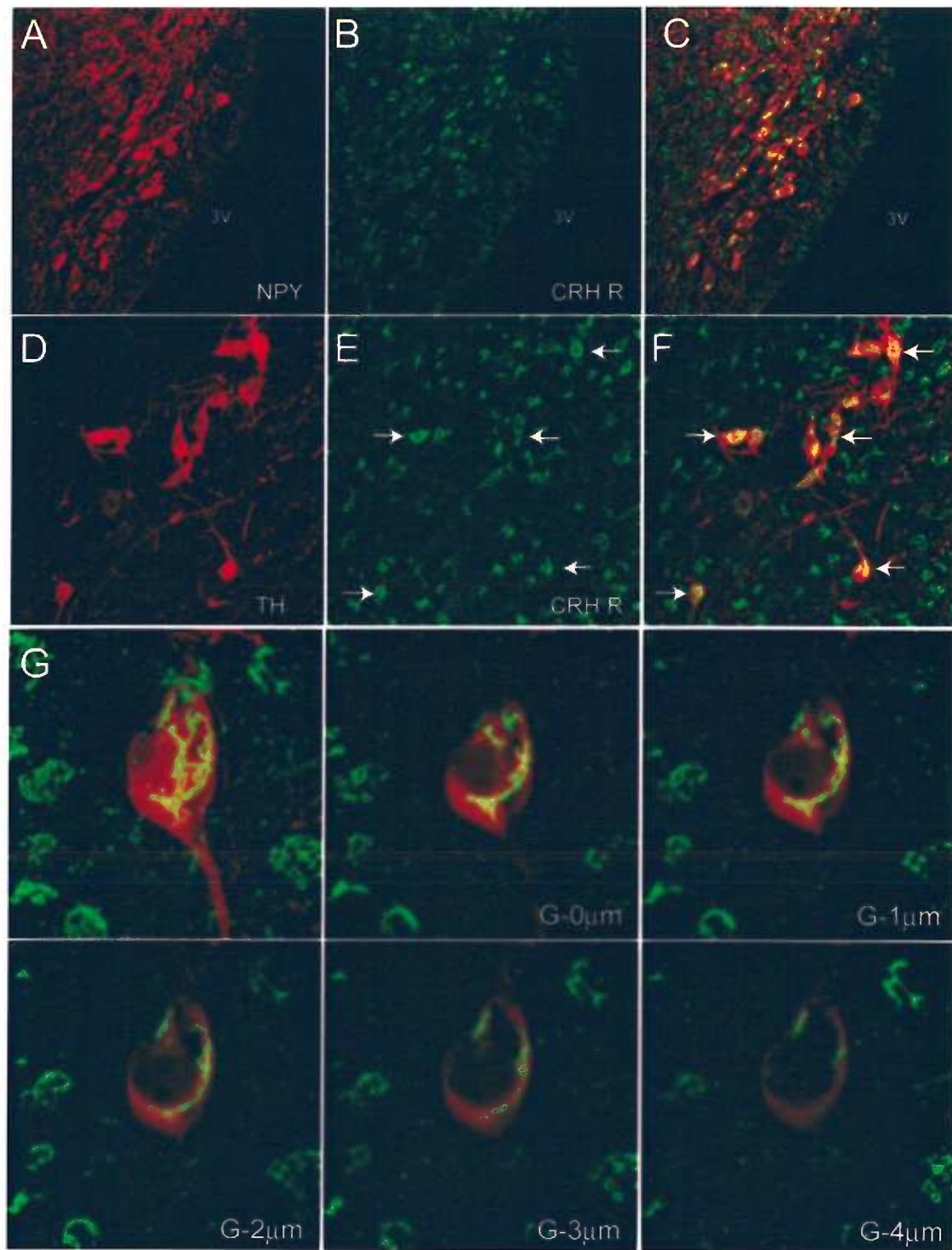


Figure 2-4. Colocalization of CRH R1-ir with NPY-ir and TH-ir. Single label NPY-ir (A) and TH-ir (D) neurons (red) in the ARH; B,E represent single label CRH R1 - ir (green) in the same sections. Colocalization of CRH R1-ir with NPY-ir and TH-ir is shown in yellow (C,F). Panel G shows an example of a co-labeled CRH R1/TIDA neuron, followed by serial optical sections through the z-plane (0.5μm resolution, 1μm separation).

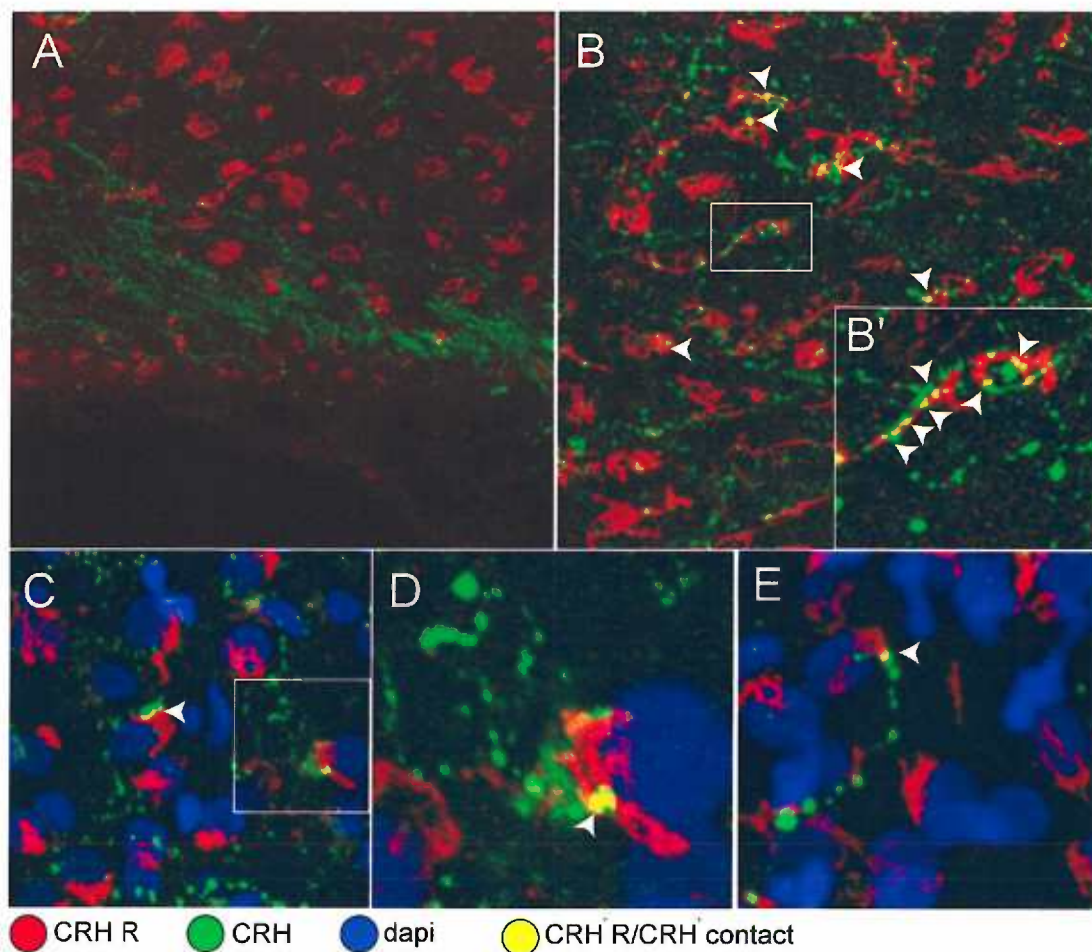


Figure 2-5. CRH innervation of CRH R1 positive cells in the ARH. (A) CRH fibers (green) densely innervate the ventromedial region of the ARH, but are found sparsely throughout the majority of the ARH. (B) In the heavily innervated region, several CRH fibers are seen coming in close contact (yellow) with CRH R1-ir neurons (red). Inset B' shows a higher magnification of the highlighted cell in B. (C-D) A nuclear dapi stain (blue) was used to help identify cells in higher magnification images. Panel C depicts more examples of CRH/CRH R close contacts in the ventromedial ARH. Panel D is a higher magnification of the contacted neuron highlighted in C. Panel E shows a close contact in the sparsely innervated dorsal portion of the ARH. White arrows indicate regions identified as Close contacts, indicated by white arrows, were confirmed by confocal

Chapter III

Neuropeptide Y Y5 Receptor Protein in the Cortical / Limbic System and Brainstem of the Rat: Expression on γ -Aminobutyric acid and Corticotropin Releasing Hormone Neurons.

Kevin L. Grove, Rebecca E. Campbell, Jarleth M.H. French-Mullen, Michael A.
Cowley, M. Susan Smith, *Neuroscience* 100: 731-740, 2000.

Introduction

Neuropeptide Y (NPY) has been shown to have anticonvulsant and anxiolytic actions, likely through activation of NPY receptors in the cortical/limbic system (Colmers et al., 1991; Colmers et al., 1991; Klapstein and Colmers, 1993; Vezzani et al., 1999; Britton et al., 1997; Nakajima et al., 1998). Locally within the cortex and the hippocampus (Hipc), NPY is synthesized in GABAergic interneurons (Hendry et al., 1984 ;Hendry, 1993; Aoki and Pickel, 1990). In addition, the cortex and Hipc also receive afferent NPY projections from the brainstem, where NPY is colocalized in catecholaminergic neurons (Everitt et al., 1984; Hokfelt et al., 1983).

NPY Y receptors belong to the G-protein-coupled receptor superfamily and cause the inhibition of adenylate cyclase, through a coupling to the G protein, G_i . However, recent studies have demonstrated that NPY can stimulate phospholipase C activity, suggesting that some NPY receptors may also be linked to the G_q protein (Parker et al., 1998). Five receptor subtypes for the NPY family of peptides have been cloned from many species and pharmacologically distinguished as Y1, Y2, Y4, Y5 and y6 (Blomqvist and Herzog, 1997). The different receptor subtypes show a large degree of sequence variation (see review (Balasubramaniam, 1997)), yet there are similarities in their binding affinities for the endogenous ligands NPY and the closely related PYY. Pharmacological profiles for chimeric peptides and peptide fragments of the NPY family have been used to characterize differences in the distribution and physiology of the various receptors. However, the lack of subtype specific

ligands has limited the success of these studies to clarify the relative importance of the different subtypes in various functions. The Y5 receptor mRNA and binding is distributed throughout the hypothalamus, cortical limbic system and brainstem (Dumont et al., 1998a; Dumont et al., 1998b; Nichol et al., 1999; Parker and Herzog, 1999). In addition, our group has recently characterized the distribution of the Y5 receptor protein to specific neuronal populations within the hypothalamus involved in both the regulation of food intake and neuroendocrine function [5865]. This study also reported the presence of Y5 receptor protein on hypothalamic GABAergic and CRH neurons. NPY Y5 receptor immunoreactivity (-ir) was also noted in specific cell populations within the cortical limbic system, with a pattern that was suggestive of a colocalization in GABAergic neurons.

The purpose of the present study was to use immunohistochemical techniques to investigate the cellular distribution of the NPY Y5 receptor in the cortical/limbic system and brainstem, as well as to determine some of the phenotypes of cells expressing Y5 receptors. These studies will provide important information about the possible role of the Y5 receptor in the neuronal circuitry involved in cortical/limbic function.

Materials and Methods

Production of the Y5 Receptor Antibody

The C-terminus of the NPY Y5 receptor was synthesized (EVKPEESSDAHEMR) representing amino acids 348-361. This sequence has no similarities to any other cloned NPY receptor or other proteins. The Y5

receptor antibody was produced by Genosys Biotechnologies, Inc, (The Woodlands, TX). Briefly, the peptide was cross-linked with KLH and injected into rabbits. Sera were collected from rabbits after 5 and 6 boosters. A detailed description of the specificity is presented in another manuscript (Campbell et al., 2000).

Characterization of the Distribution of the NPY Y5 Antibody

Animals

Virgin cycling female rats (B & K Universal Kent, WA) were housed individually and maintained under a 12:12 light-dark cycle (lights on at 0700 h) and constant temperature (23 ± 2 °C). Food and water were provided *ad libitum*. All animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care and Use Committee.

Immunocytochemistry (ICC)

For immunohistochemistry studies rats were sacrificed under pentobarbital anesthesia by intracardiac infusion with ice-cold PBS followed by ice-cold 4% paraformaldehyde in NaPO₄ buffer (pH 7.4). The brains were removed and saturated in 25% sucrose, frozen on crushed dry ice and stored at -80 °C. The brains were sectioned at a thickness of 25 µm with a microtome and stored in cryoprotectant until used for immunocytochemistry.

Single label ICC

Single label ICC for NPY Y5-ir was performed on floating sections from three different animals. Tissue sections were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by

preincubation in blocking buffer (KPBS + 0.4% Triton X-100 + 2% normal donkey serum) for 30 min at room temperature. The tissue was then incubated with the Y5 specific antibody (1:5,000) in blocking buffer for 48 hours at 4 °C. Following washes in KPBS, tissue was incubated for 1 hr in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc.), then subsequently washed and incubated in avidin-biotin solution (Vectastain) for 1 hr. NPY Y5-like immunoreactivity (NPY Y5-ir) was visualized with 3,3'-diaminobenzidine enhanced with nickel chloride. Distribution of the single label Y5-ir was characterized using a Nikon microscope (Labphoto-2). Pictures were taken with a Micromax Digital Camera (Princeton Instruments) connected to the Nikon microscope using a Plan Apo 20X (NA 0.75) objective. The MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA) was used to process the images, and the brightness and contrast of the digital images were adjusted in Photoshop (Adobe Systems Inc., San Jose, CA).

Double-label ICC

To characterize the phenotype of cells expressing the NPY Y5 receptor, double label immunofluorescence coupled with confocal microscopic analysis was performed. Each experiment was performed two to three times on tissue from different animals. The polyclonal NPY antibody, made in the goat, (a kind gift of P.Larsen) was used at a concentration of 1:5000. The monoclonal GABA antibody (clone #GB-69; G-141, RBI, Natick, MA) was used at a concentration of 1:1000. The polyclonal CRH antibody, made in the goat, (#SC-1759; Santa Cruz Biotechnology, Inc. CA) was used at a concentration of 1:1000. The double label

immunofluorescence for NPY Y5 and the various other targets was done in series. Briefly, tissue sections were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS), preincubated in blocking buffer for 30 min at room temperature, followed by incubation with the Y5 specific antibody (1:15000) in blocking buffer for 48 hours at 4 °C. Following washes in KPBS, tissue was incubated for 1 hr in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc.), then subsequently washed and incubated in avidin-biotin solution (Vectastain) for 30 min. Y5-ir signal was further amplified using a commercial kit (tyramide signal amplification-indirect kit; NEN Life Sciences Products, Boston, MA) and was visualized using either FITC-conjugated to Neutravidin (1:1000, Pierce, Rockford, IL) or TRITC-conjugated to streptavidin (1:1000, Jackson ImmunoResearch Laboratories, Inc.). The sections were then rinsed and subsequently incubated with the second primary antibody (GABA, CRH or NPY) in blocking buffer for 48 hours at 4 °C. Following washes in KPBS, tissue was incubated for 1 hr in either a donkey anti-goat (CRH and NPY) or a goat anti-mouse (GABA) antibody conjugated to an Alexa fluorophore (Alexa 488 or Alexa 546; Molecular Probes, Eugene, OR).

Confocal Laser Microscopy

Confocal laser microscopy, as previously described (Li et al., 1999), was used to analyze the double label immunofluorescent images. The Leica Corp. (Germany) TSC SP confocal system consisted of a Leica Corp. RBE inverted microscope equipped with a Ar laser producing light at 488 nm (for visualization of FITC and Alexa 488) and a Kr laser producing light at 568 nm (for visualization

of TRITC and Alexa 546). Various objectives (25X/NA 0.75 and 40X/NA 1.25) were used to scan the images. For each experiment, fluorophore signals were checked individually for bleed through to the apposing detector. All bleed through was eliminated by adjusting laser intensity and detector window width. A series of continuous optical sections, at .5 or 1.0 μm intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. For the study analyzing the close apposition of NPY fibers on neurons expressing Y5-ir, the optical sections were taken at 0.5 μm intervals to provide a high resolution for confirmation of close contacts between the fibers and cell bodies. For the studies analyzing Y5-ir and the various phenotype markers (CRH, GABA), a lower resolution is more than adequate to confirm colocalization; therefore, optical slices were taken at 1.0 μm intervals. The signals were obtained for each fluorophore on one series of optical sections and stored separately as a series of 512 x 512 pixel images. The images were then processed with the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA). The confocal images are presented as projections of a stack of optical images. The brightness and contrast of the images were adjusted in Photoshop (Adobe Systems Inc., San Jose, CA).

Results

Single Label Immunohistochemistry

The distribution of NPY Y5-ir is summarized in the diagrams in Figure 1, illustrating localization of the Y5-ir to discrete cell groups throughout the rat brain.

Confocal microscopic analysis of the localization of the Y5-ir within the cell body demonstrated that the staining was mainly associated with the somal membrane and proximal dendritic processes.

Septum and Thalamus (Fig. 1)

In the septum, only a few scattered neurons displayed Y5-ir. In contrast, Y5-ir neurons were present throughout the thalamus, including the paraventricular nucleus of the thalamus, habenula, mediodorsal thalamus, and zona incerta.

Hippocampus and Cortex

In the Hipc Y5-ir staining was present in specific regions, including scattered neurons in the hilar region (Fig. 2B-1), the CA2/3 region (Fig. 2B-2) and the fasciola cinerea (FC; Fig 2B-3). The most intense Y5-ir staining within the Hipc was present within the small CA2 zone (Fig. 2B-2 and 2B-2') and in the FC (Fig. 2B-3) on structures that appeared to be pyramidal cell bodies and/or proximal dendritic processes. A moderate level of Y5-ir was also present on neurons within the hilar region of the dentate gyrus (Fig. 2B-1; black arrows) and the pyramidal cell layer of the CA3 region (Fig. 2B-2). Only a low level of staining was detectable in the granular layer of the dentate gyrus (Fig. 2B-1) and pyramidal cell layer of the CA1 region (Fig. 2B-2).

The most intense Y5-ir staining in the rat brain was present on small (nonpyramidal) cell bodies (10-15 μm in diameter) and proximal dendritic processes scattered throughout the cortex (Fig. 3). The majority of these stained neurons were concentrated in the cingulate cortex (Fig. 3) although cells were

present throughout the whole depth of the cortex, including frontal, parietal, temporal and piriform cortex (Fig. 1).

Brainstem

The majority of Y5-ir staining in the brainstem was limited to the Edinger-Westphal nucleus (EW; Fig. 4A), mesencephalic nucleus (Me5; Fig. 4B) and locus coeruleus (LC; Fig. 4C), with lower levels of staining in the dorsal raphe. A low level of staining was also present in scattered cells throughout the brainstem (Fig. 1).

Double Label Immunofluorescence

GABA

The presence of Y5-ir on scattered neurons in the hilar region of the dentate gyrus (Fig. 2) and throughout the cortex (Fig. 3) strongly suggested that the NPY Y5 receptor was expressed on GABAergic neurons in these two regions. Indeed, double label immunofluorescence for the Y5 receptor and GABA demonstrated that the majority of Y5-ir staining in the hilar region of the Hpc was limited to GABA-ir neurons (Fig. 5). In addition, a few of the GABAergic neurons in the molecular layer (ML) of the dentate gyrus also displayed Y5-ir (Fig. 5). Scattered GABA-ir neurons in the CA1-3 region of the Hpc also displayed Y5-ir (not shown), although the population of neurons coexpressing Y5/GABA represented only a small subpopulation of the total number of GABAergic neurons in these regions. The majority of Y5-ir neurons in the cortex displayed low levels of GABA-ir (Fig. 5; black/white arrows), with only a few of the Y5-ir neurons displaying intense GABA-ir (Fig. 5; white arrow).

However, the majority of neurons in the cortex displaying intense GABA-ir did not contain Y5-ir staining (Fig. 5; white arrow heads).

Corticotropin Releasing Hormone (CRH)

CRH-ir was detected in small cell bodies scattered throughout the cortex (Fig. 5). Although CRH neurons are known to be present in the Hpc (Yan et al., 1998), we were unable to detect them in the present study. This is not surprising since it is difficult to detect CRH-ir in the hippocampus in the adult rat (Olschowka et al., 1982; Sakanaka et al., 1987). All neurons displaying CRH-ir staining in the cortex contained intense Y5-ir (Fig. 5); however, no Y5-ir was evident on the long CRH-ir fibers within the cortex (not shown).

NPY

To characterize functional synaptic interactions between NPY fibers and cells expressing the Y5 receptor, double label immunofluorescence coupled with confocal laser microscopy was used to demonstrate that NPY-ir fibers come in close contact with cells displaying Y5-ir staining. Figure 6 shows an NPY-ir fiber (red color) in the cortex coming in close contact (yellow color) to a neuron displaying Y5-ir (green color). Analysis of single optical slices (0.5 μ m resolution) through this image (Fig. 6 a-f) demonstrated that the NPY-ir fiber (white arrow) comes in close apposition to the Y5-ir neuron and actually wraps around the cell. Note that the Y5-ir cell, which we have shown above to be expressed in a subpopulation of CRH/GABAergic neurons in the cortex (Fig. 5), did not itself display NPY-ir (Fig. 6).

Discussion

The present study demonstrates the presence of Y5-ir neurons throughout the cortical/limbic system and the brainstem, which agrees with our previous findings using Western blot analysis (Campbell et al., 2001). This widespread distribution of the receptor protein also correlates closely with the reported distribution of the Y5 mRNA (Parker and Herzog, 1999; Nichol et al., 1999) and receptor binding (Dumont et al., 1998a; Dumont et al., 1998b). The major limitations to these earlier studies are the inability to determine the cellular localization of the receptor protein and the lack of characterization of the phenotype of the cells expressing the Y5 receptor. The present study demonstrates that the majority of the Y5-ir throughout the rat brain was localized to the neuronal cell body and proximal dendritic processes (Fig 2-6).

Hippocampus

While three subtypes of NPY receptors are abundant in the rat cortex and Hipc, Y1, Y2 and Y5 (Parker and Herzog, 1999; Larsen et al., 1993; Dumont et al., 1998a; Gustafson et al., 1997), there are conflicting reports about the role of each receptor subtype in the regulation of cortical and hippocampal functions. However, all three receptor subtypes likely play an important role in normal physiological conditions as well as in response to pathological hyperactivity in both regions. The uncertainty of the role of the Y5 receptor subtype stems from contradictory reports on the suppression of epileptiform activity in the Hipc by this receptor subtype. Woldbye *et al.* (Woldbye et al., 1997) have demonstrated that the receptor subtype involved in the suppression of seizure activity, as

determined by EEG and markers of cellular activity, had the pharmacological profile of the Y5 receptor. In addition, this group reported that Y2 selective analogues were without effect. Bijak (1999) more recently demonstrated that both Y2 and Y5 selective agonists could play a role in the suppression of epileptiform activity in the Hpc, while Y1 selective analogues were more effective in the cortex. In contrast, Ho *et al.* (Ho et al., 2000) recently reported that an NPY Y2 selective agonist, but not Y5 receptor agonists, was effective at suppressing epileptiform activity in rat hippocampus, using an *in vitro* slice assay. The reason for these discrepancies is unknown; however, the role of the Y5 receptor in the control of pathological hyperactivity has been strengthened by evidence that animals with a knockout of the Y5 receptor gene have a greater sensitivity to epileptic seizures and a higher mortality rate in response to induced seizures (Marsh et al., 1999). Furthermore, the anticonvulsant actions of NPY were absent in Y5 knockout mice (Marsh et al., 1999).

The present study demonstrates that Y5-ir is localized in the pyramidal cell layer of the CA2/3 region, as well as intense staining on short fiber processes (possibly proximal dendritic processes) within this layer (Fig. 2B-2 and 2B-3), suggesting that the Y5 receptor may have post-synaptic actions directly on pyramidal neurons. Ho *et al.* (Ho et al., 2000) used electrophysiological recordings in the CA3 pyramidal cells to demonstrate that a Y5 agonist was ineffective at blocking epileptiform activity in this area, suggesting that the Y5 receptor was not modulating postsynaptic cell membrane excitability. NPY has been shown to inhibit depolarization-induced increases in intracellular Ca^{++} in

dentate granular cells, without directly altering the excitation of the cell (McQuiston et al., 1996). This action was attributed to the Y1 receptor; however, the study used a drug that was later shown to be an agonist for both the Y1 and Y5 receptors. It is possible that the Y5 receptor may have similar actions in the other regions of the Hpc. More study is needed to determine the function of the Y5 receptor in the pyramidal cell layer.

In the hilar region of the dentate gyrus, Y5-ir staining was localized to GABAergic neurons (Fig. 5). The physiological significance of these receptors on the GABAergic neurons in the dentate gyrus is unknown, although these neurons are known to be involved in regulation of dentate granular neuronal function (Brown and Zador, 1990). Since there are several types of GABAergic neurons within this region that coexpress several different neuropeptides (see review (Schwarzer et al., 1996)), more study is needed to further characterize the phenotype of these neurons.

Cortex

In the cortex a subpopulation of small neurons, all containing intense CRH-ir, displayed Y5-ir (Figs. 3 and 5). Interestingly, while GABA-ir could be detected in nearly all of these Y5-ir neurons, the level of staining was low in the majority of them. It is unknown if this is an indication that these neurons preferentially release CRH instead of GABA. The present study also demonstrated that NPY-ir fibers come in close contact with the Y5 receptor expressing neurons (Fig. 6), providing support for a direct functional interaction. The source of these NPY-ir fibers is unknown; however, they likely arise from

NPY/GABAergic interneurons within the cortex (Hendry et al., 1984; Hendry, 1993; Aoki and Pickel, 1990). Although NPY is also colocalized in noradrenergic (NA) neurons in the LC, an area known to extensively innervate the cortex and Hipc, very few of the NA neurons containing NPY project to these regions (Xu et al., 1998; Holets et al., 1988). The normal physiological role of the NPY system in the cortex is unclear; however, NPY has been shown to block epileptiform activity in the frontal cortex of the rat (Bijak, 1999). Bijak (1999) concluded that this suppression of epileptic activity in the cortex likely involved mainly the Y1 receptor subtype. However, this study could not exclude a possible role of the Y5 receptor subtype, since a Y1/5 agonist was used, and the actions of this agonist were not completely blocked by the Y1 selective antagonist BIBP 3226 (Bijak, 1999).

The size (small 10-15 μm) and shape (small volume of cytoplasm and thin primary dendrites) of the cortical GABA-ir neurons containing Y5-ir suggest that they belong to a specific class of GABAergic neurons. These GABAergic neurons are classified as "dendritic targeting" due to their preferential innervation of the distal dendritic shafts of cortical pyramidal neurons (Jakab and Goldman-Rakic, 2000; Kawaguchi, 1995; Kawaguchi and Kubota, 1996). The cortical neurons displaying Y5-ir also coexpressed CRH (Fig. 5), with no evidence of Y5-ir on CRH fibers.

The presence of Y5-ir specifically on CRH neurons in the cortex suggests that the NPY/Y5 receptor system has a very specialized function within the cortex. NPY has been shown to have biphasic effects on CRH release in the

cortex, with low doses stimulating CRH release and high doses inhibiting CRH release (Klapstein and Colmers, 1997). Furthermore, the source of this CRH, whether from CRH neurons projecting from neurons outside the cortex (i.e. amygdala) or from local interneurons, is unknown. Therefore, whether the expression of Y5 receptors on CRH/GABAergic neurons in the cortex represents excitatory or inhibitory actions needs further investigation.

In contrast to the NPY system, the CRH system is considered an excitatory neurotransmitter system in the Hpc and cortex (Siggins et al., 1985; Aldenhoff et al., 1983; Smith et al., 1997), which can induce epileptic seizure activity (Ehlers et al., 1983) and cell death (Causing et al., 1996). The biphasic effects of NPY on CRH release may represent the ability of NPY to differentially regulate CRH release under normal conditions versus pathophysiological hyperactivity. Under normal conditions, the inhibitory effects of NPY would decrease activity of cortical neurons, which if left unchecked, may result in impaired learning or other cognitive abnormalities. Therefore, activation of the CRH system by NPY would have a balancing excitatory effect on cortical/limbic function. In contrast, under conditions where cortical/limbic function is reaching pathological levels, it makes physiological sense for NPY, which is considered to be one of the major neuropeptide anticonvulsant systems, to inhibit CRH release, which itself would only contribute to the pathological condition.

Brainstem

The three regions in the brainstem with the most prominent Y5-ir staining were in the Me5, LC and the EW (Fig. 4), and to a lesser extent in the DR, which

agrees with the presence of high levels of Y5 mRNA in these regions (Nichol et al., 1999; Parker and Herzog, 1999). Although the present study did not investigate the presence of NPY-ir fibers coming in contact with the neurons displaying Y5-ir, as was done in the cortex, NPY-ir fibers are present throughout the brainstem, including in the EW, LC, DR and Me5 (de Quidt and Emson, 1986; Chronwall et al., 1985). The present study also did not characterize the phenotype of the neurons in the brainstem expressing Y5-ir, although the phenotype of neurons in the LC and EW are well defined as being NA and urocortin, respectively (Mongeau et al., 1997; Bittencourt et al., 1999).

Noradrenergic neurons in the LC also coexpress several different neuropeptides, including NPY (Xu et al., 1998; de Quidt and Emson, 1986; Chronwall et al., 1985), galanin (Holets et al., 1988; Xu et al., 1998), and cocaine and amphetamine regulated transcript (Koylu et al., 1999). The NA neurons in the LC project predominantly to the cortical/limbic system (Sawchenko and Swanson, 1981; Zhang et al., 1998). NPY has been shown to act on the LC to induce an anxiolytic response (Kask et al., 1998), likely through suppression of neuronal activity (Illes et al., 1993; Finta et al., 1992); however, both of these actions were mediated through the Y2 receptor. The LC has also been shown to be involved in the suppression of seizure activity (Neuman, 1986; Libet et al., 1977); therefore, some of the anticonvulsant actions of the Y5 receptor may be mediated through modulation of NA neurons in the LC.

The Edinger-Westphal nucleus has recently been characterized as the major source of brain urocortin, a member of the CRH ligand family known to

bind with a high affinity for both the CRH-R1 and R2 receptor subtypes (Vaughan et al., 1995; Bittencourt et al., 1999). The functional role of the urocortin system is similar to that of CRH, although it appears to be more potent at specific CRH related functions, such as suppression of appetite and stimulation of adrenocorticotrophic hormone release (Vaughan et al., 1995; Spina et al., 1996). Although, NPY is known to modulate both of these functions (Kalra and Crowley, 1992; Kalra et al., 1999), there has been no evidence to date that NPY may stimulate or inhibit urocortin release.

The serotonergic neurons in the DR send projections throughout the brain and play a variety of roles. In the cortex and Hipc, NPY and 5-HT have parallel actions on neuronal activity, with both systems eliciting anxiolytic and anticonvulsant responses (Schmitz et al., 1997; Mongeau et al., 1997). In contrast, the two systems have opposing effects on food intake, where 5-HT is a potent suppressor of food intake, an action at least partially involving suppression of NPY neurons in the ARH (Dryden et al., 1996; Dryden et al., 1995; Dryden et al., 1993). If 5-HT neurons in the DR prove to express the Y5 receptor, a case for the NPY/Y5 system could be made for both excitatory and/or inhibitory effects on serotonergic neurons.

The physiological role of the Y5 receptor in the modulation of Me5 neurons is unknown. However, these neurons are the primary afferent fibers to the facial muscles involved in mastication (Waite and Tracey, 1995). Although the phenotype of the neurons expressing Y5-ir in the Me5 was not investigated in

this study, Me5 neurons have been shown to express NPY and galanin (Arvidsson et al., 1994).

In summary, in the present study we used a newly developed NPY Y5 receptor antibody to demonstrate that there is a wide distribution of the Y5 receptor protein, matching the distribution previously reported for the Y5 receptor mRNA in the rat brain. In addition, we demonstrated that the Y5 receptor is expressed on both GABAergic and CRH neurons in the hippocampus and cortex. This wide distribution and expression on these cell types is in agreement with known physiological roles of NPY in the brain. These studies provide the neuroanatomical framework for an important role of the Y5 receptor in mediating the effects of NPY on cortical/limbic function.

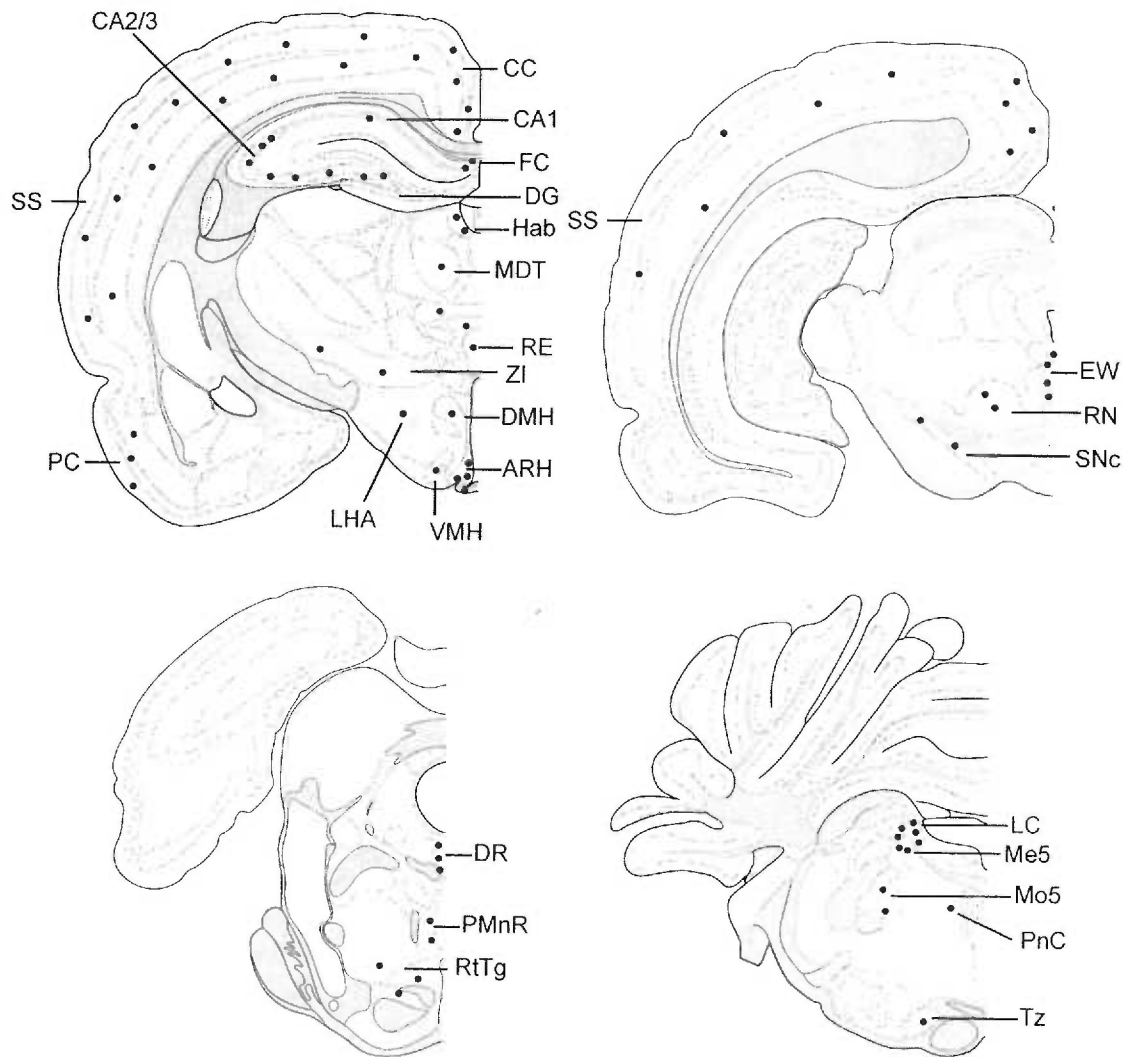


Figure 3-1. Diagrammatic representation of the distribution of Y5 immunoreactivity in the rat brain. Dots represent general localization of Y5 immunoreactive cell bodies. Figures adapted from L.W. Swanson. (1998/1999) Brain Maps: Structure of the Rat Brain, 2nd Edition, Elsevier, Amsterdam.

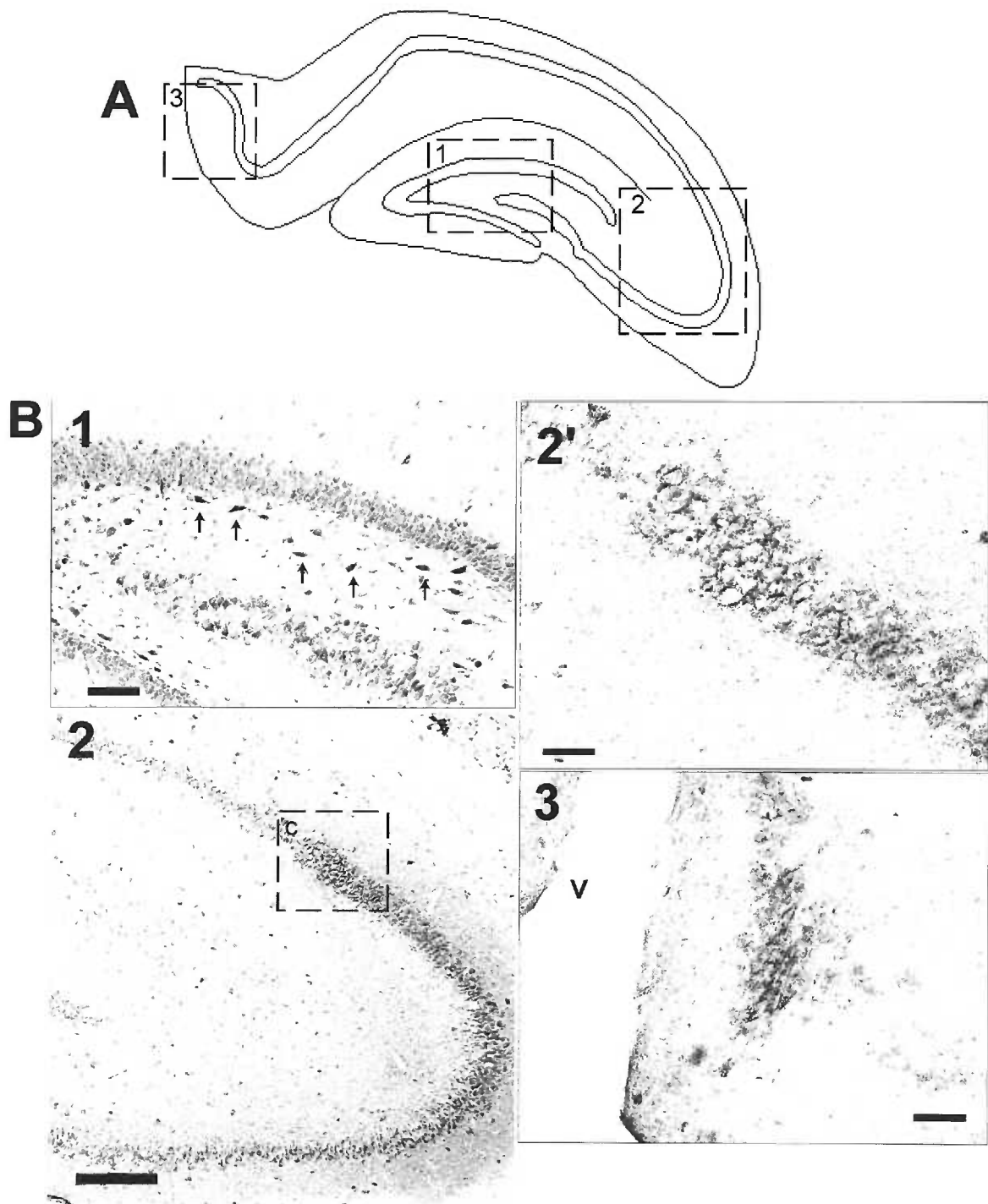


Figure 3-2. Single label Y5 immunoreactivity in the hippocampus of the rat. A. Diagram of the hippocampus indicating the areas (black boxes) shown in B1, 2, and 3. B1. Dentate gyrus. Black arrows indicate intense staining in neurons within the hilar region. Bar represents 50 μm . B2. CA1-3 region of the hippocampus. Black box indicates area shown in c. Bar represents 100 μm . B2'. Y5 immunoreactivity staining in the pyramidal cells and short fiber processes in the CA2 region. Bar represents 25 μm . B3. Y5 immunoreactivity in the cells and fibers in the fasciola cinerea. Bar represents 25 μm .

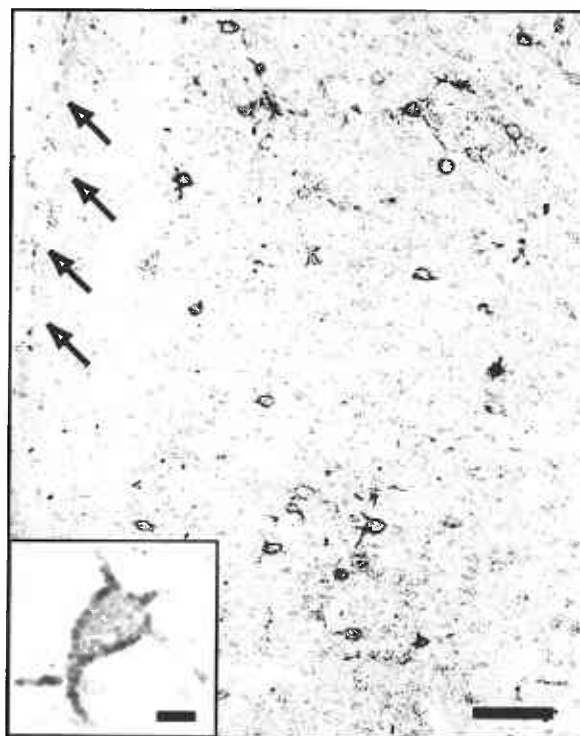


Figure 3-3. Single label Y5 immunoreactivity in the cingulate cortex of the rat. Arrows indicate the midsagittal sulcus. Bar represents 50 μ m. The insert in the lower left displays a high magnification of a typical Y5 immunoreactivity neuron in the cortex (Bar represents 10 μ m).

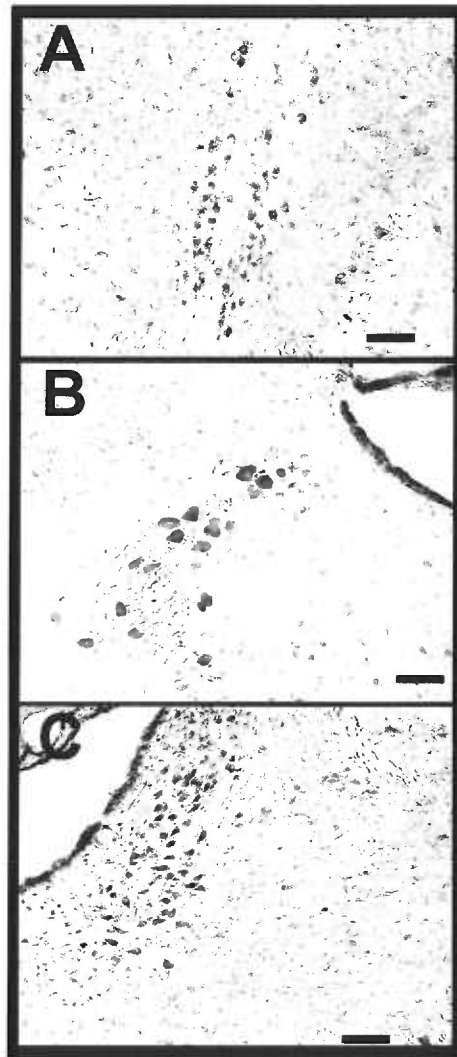


Figure 3-4. Single label Y5 immunoreactivity in the brainstem of the rat. A. Edinger-Westphal nucleus. B. Mesencephalic 5 nucleus. C. Locus coeruleus. Bars indicate 50 μ m.

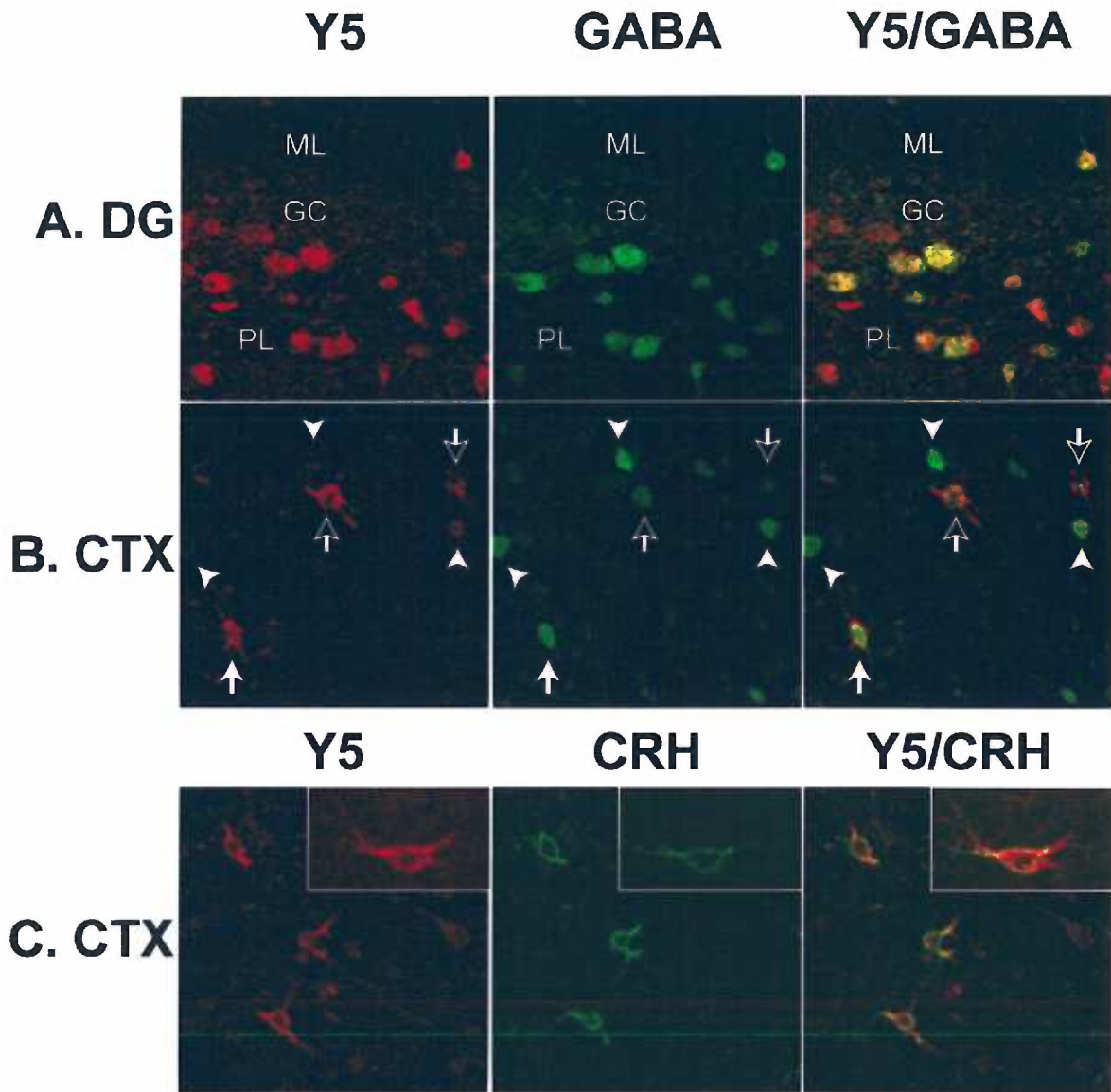


Figure 3-5. Characterization of the phenotypes of Y5 immunoreactive neurons in the dentate gyrus and cortex. Figures represent color confocal digital images of double label immunofluorescence for the Y5 receptor (red) and γ -aminobutyric acid (GABA) or corticotropin releasing hormone (CRH) (green). Neurons displaying colocalization of Y5 and GABA or CRH (yellow) are shown in the right panels. A. Y5 and GABA colocalization in the dentate gyrus (DG). ML - molecular layer; GC - granule cell layer; PL - polymorphic layer. B. Y5 and GABA colocalization in the cortex (CTX). White arrows indicate Y5-IF in a neuron also displaying intense GABA-IF. Black and white arrows indicate Y5-IF in neurons displaying a low level of GABA-IF. White arrowheads indicate GABA-IF in neurons displaying little or no Y5-IF. C. Y5 and CRH colocalization in the cortex (CTX). Images in the upper right corners are higher magnifications of a single cortical neuron displaying Y5 and CRH colocalization.

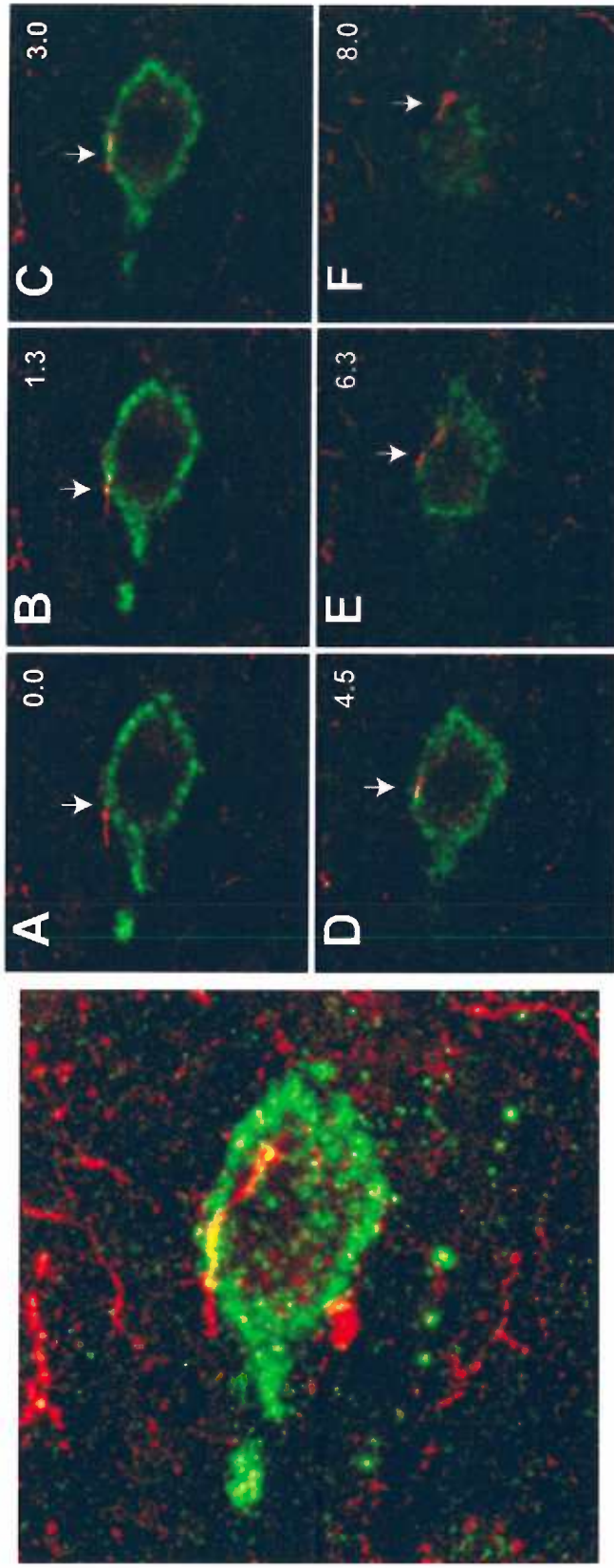


Figure 3-6. Neuropeptide Y innervation of a cortical neuron displaying Y5 receptor immunofluorescence. Figures represent color confocal digital images of double label immunofluorescence for the Y5 receptor (green) and neuropeptide Y (red). A-F. Single optical slices (0.5 μm resolution) through the image shown in the left panel. The number in the upper right corner indicates the distance (μm) from the first image (A). The white arrow indicates the neuropeptide Y immunofluorescence fiber (red) coming in close contact (yellow) with the cortical neuron displaying Y5 immunofluorescence (green). Note how the neuropeptide Y fiber wraps around the neuronal cell body indicating a possible functional synaptic unit between the neuropeptide Y fiber and a neuron expressing the Y5 receptor. Also, note the association of the Y5 receptor with the somal membrane.

Chapter IV

Hypothalamic Circuitry of Neuropeptide Y Regulation of Neuroendocrine Function and Food Intake Via the Y5 Receptor Subtype

Rebecca E. Campbell, Jarlath M.H. French-Mullen, Michael A. Cowley, M. Susan
Smith, Kevin L. Grove, *Neuroendocrinology* 74: 106-119, 2001.

Introduction

Intracerebroventricular administration of neuropeptide Y (NPY) has been shown to modulate a number of physiological functions through actions in the hypothalamus, most notably, the regulation of food intake (Kalra et al., 1999) and neuroendocrine function (Kalra and Crowley, 1992). In the hypothalamus, NPY neurons are found primarily in the arcuate nucleus of the hypothalamus (ARH) with projections throughout the hypothalamus (de Quidt et al., 1986). In the midbrain and brainstem, NPY is colocalized in catecholaminergic neurons projecting to hypothalamic regions (Everitt et al., 1984, Hokfelt et al., 1983). Five receptor subtypes for the NPY family of peptides have been cloned for many species and pharmacologically distinguished as Y1, Y2, Y4, Y5 and y6 (Blomqvist and Herzog, 1997). NPY receptors, in general, belong to the G-protein-coupled receptor superfamily and cause the inhibition of adenylate cyclase and activation of the phospholipase C/ protein kinase C pathway (Leupen et al., 1999, Parker et al., 1998, Selbie et al., 1995). Pharmacological profiles for chimeric peptides and peptide fragments of the NPY family have been used to characterize differences in the distribution and physiology of the various receptors. However, the lack of subtype specific ligands has limited the success of these studies to clarify the relative importance of the different subtypes in various functions.

The NPY Y5 receptor has received increasing interest in recent years due to its importance in the hypothalamic regulation of food intake and energy expenditure (Crisione et al., 1998, Flynn et al., 1999, Hwa et al., 1999, Marsh et

al., 1998, Scheffhauser et al., 1998, Tang-Christensen et al., 1998, Wyss et al., 1998]. In addition, the Y5 receptor has recently been shown to be involved in the regulation of luteinizing hormone (LH) release, specifically in the inhibition of LH in the absence of gonadal steroids (Raposo et al., 1999). This dual action, regulation of food intake and modulation of reproductive neuroendocrine function, indicates that the NPY/Y5 system could function as an intermediate between signals of peripheral energy availability and their modulation of reproductive function.

Previous studies have demonstrated that the Y5 receptor mRNA and binding has a wide distribution throughout the hypothalamus (Dumont et al., 1998, Lupien et al., 1998, Nichol et al., 1999, Parker et al., 1999). However, there is little information available on the cellular localization (i.e. soma and/or fiber terminals) or the phenotypes of cells expressing the Y5 receptor. The purpose of this study was to use immunohistochemical techniques employing a NPY Y5 receptor antibody to investigate the cellular distribution of this receptor in the hypothalamus. In addition this study sought to determine some of the phenotypes of cells expressing Y5 receptors, with a focus on systems involved in the regulation of neuroendocrine function and regulation of food intake. These studies provide important information about the possible role of the Y5 receptor in the NPY neuronal circuits involved in neuroendocrine function, feeding, and energy expenditure.

administered following a recovery period of at least 10 days after surgery. All animal procedures were approved by the AstraZeneca Institutional Animal Care Committee.

Intracerebroventricular Administration of Antisense ODN

Under intraperitoneal ketamine (100 mg/kg) plus xylazine (5 mg/kg) anesthesia, a 23-gauge stainless steel guide cannula was implanted into the third cerebral ventricle at stereotaxic coordinates: -2.1 anteroposterior and 0.0 lateral with respect to the bregma, and 7.5-8.0 dorsoventral from the brain surface as previously described (Plata-Salaman et al., 1998). The location of the cannula tip in the third ventricle was verified by the free outflow of CSF through the guide cannula. A sterile 29-gauge stainless steel obturator was used to ensure the cannula remained patent.

Chronic icv microinfusions were made into the third cerebral ventricle because of the proximity of the hypothalamic feeding-associated sites. Microinfusions were accomplished by using Alzet osmotic minipumps (pumping rate = 1 μ l/h, Model 2001; Alzet, Palo Alto, CA). Following recovery from brain cannula implantation rats were anesthetized with ketamine (100 mg/kg) plus xylazine (5 mg/kg). A filled minipump was connected by a short polyethylene tube (1.1 mm i.d.) filled with test solution to an L-shaped cannula of 29-gauge stainless steel tubing. The minipump was inserted into the subcutaneous interscapular space, and the L-shaped cannula was cemented in place with dental acrylic. All test compounds were dissolved in 0.15 M NaCl. Randomly assigned groups of rats received either vehicle (sterile 0.15 M NaCl), antisense or sense ODNs and

were infused at 5 μ g (1.8 nmol)/rat/24 h. To avoid non-specific adsorption of the compounds to the tubing, all such materials were siliconized. Animals (n = 5/group) were treated for five days, and food, water and body weight were measured daily.

Antisense or sense ODNs (18-mers) were phosphorothioated at all positions to increase resistance to exo- and endo-nucleases (Genosys Biotechnologies, Inc., The Woodlands, TX): antisense to Y5 receptor, 5'-AAGAGGACGTCCATTAGC-3' (MW = 5519), 5' relative to the initiating ATG; sense to Y5 receptor, 5'-GCTAATGGACGTCCTCTT-3' (MW = 5455). Both ODNs were purified by desalting, gel chromatography and HPLC. Antisense and sense constructs were based upon the rat hypothalamic Y5 receptor sequence (Genbank Accession # U56078).

After the chronic infusion period was completed, rats were decapitated and their brains quickly removed (< 30 s from time of decapitation). The brain was immediately placed in oxygenated phosphate-buffered saline solution at 2-4 °C and rinsed several times. The complete hypothalamus was mounted on a block, and serial coronal hypothalamic slices (400 μ m thick) were cut with a vibratome (Oxford, model G). Throughout this procedure, the complete hypothalamus was submerged in oxygenated phosphate-buffered saline solution at 2-3 °C. The right and left ventromedial hypothalamic nucleus and paraventricular nucleus of the hypothalamus were removed from hypothalamic slices by punching them with a micro-puncher (1.0 mm) under a microscope and according to anatomical landmarks. The tissue was then processed for Western blot analysis (described below).

Western blot

Western blot analyses for detection of Y5 protein in animals (n = 3/group) treated with Y5 antisense ODNs were performed as previously described (Vezzani et al., 2000). Briefly, the tissue was homogenized (150 µg/ml) in 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 5 mM EGTA, 1 mM Na-vanadate and a mixture of protease inhibitors. Samples of total proteins (10 µg) were analyzed using SDS-PAGE, 8% acrylamide. Each sample was run in duplicate or triplicate. Proteins were transferred to Hybond nitro-cellulose membrane by electroblotting. For immunoblotting of the Y5 receptor protein, the rabbit anti-Y5 antibody was used at a dilution of 1:2,500. This antibody recognizes a major protein band of an apparent molecular mass of about 57 kD and a minor band at 51 kD (Vezzani et al., 2000). As a control to determine if the Y5 AS ODN treatment was nonspecifically reducing protein expression, western blot analysis was also performed using an antibody directed against the NPY Y2 receptor subtype (developed by J.M.H. French-Mullen), used at dilution of 1:2,500. Membranes were processed for immunoreactivity using enhanced chemiluminescence. Quantitative densitometry, using NIH Image software, was used to determine Y5 and Y2 receptor protein levels in the immunoblots. Exposures with maximal signals below the photographic saturation point were used for densitometric analysis. For the quantification, integrated optic density [IOD; pixel area above threshold * (mean density – background)] was determined using an identical sample box size (encompassing the entire protein band) for all samples. Immunoreactivity of neurofilament 68 (NF; 1:1,000; Chemicon Int., Inc.,

Temecula, CA, USA) was determined in each lane and the value used as an internal standard to correct for possible differences in the protein content of the samples. For statistical analysis, the ratio of the IOD of the Y5 or Y2 receptor band to the NF band of the sense and antisense treated animals were compared.

Statistical Analysis

Results are expressed as means $\pm$ SEM. For comparisons between groups, the two-sample Student's *t* test was applied when the data passed the normality (Kolmogorov-Smirnov) and equal variance (Levene Median) tests; otherwise, the Mann-Whitney test was applied. Differences were considered to be significant only for $p < 0.05$.

Characterization of the Distribution of the NPY Y5 Receptor Protein

Animals

These studies were performed at the Oregon Regional Primate Research Center (Beaverton, OR, USA). Virgin cycling female rats (B & K Universal Kent, WA) were housed individually and maintained under a 12:12 light: dark cycle (lights on at 0700 h) and constant temperature (23 ± 2 °C). Food and water were provided *ad libitum*. All animal procedures were approved by the Oregon Regional Primate Research Center Institutional Animal Care Committee.

For Western blot analysis of the distribution of Y5 protein in the rat brain, animals ($n = 3$) were sacrificed on the day of diestrus by decapitation and the brains quickly removed and dissected on ice. This tissue was processed for Western blot analysis as described below. For immunohistochemistry studies rats ($n = 2-5$, depending on the experiment) were sacrificed under pentobarbital

anesthesia by intracardiac infusion with ice-cold PBS followed by ice-cold 4% paraformaldehyde in NaPO₄ buffer (pH 7.4). The brains were removed post-fixed in 4% paraformaldehyde overnight and then saturated in 25% sucrose, frozen on crushed dry ice and stored at -80 °C. The brains were sectioned using a microtome at a thickness of 25 µm, in a 1 in 4 series, and stored in cryoprotectant until used for immunocytochemistry. For immunofluorescence studies investigating possible expression of NPY Y5-immunoreactivity (-ir) on CRH neurons, animals were treated with colchicine (80 µg; icv) three days prior to sacrifice. The treatment of these animals (n = 4) with colchicine was necessary to detect CRH peptide within the cell bodies of the parvocellular neurons in the PVH. The animals were then anesthetized and perfused and tissue processed as described above.

Western Blot Analysis

For Western blot analysis of the distribution of Y5 protein in the rat brain, the tissue was homogenized in ice-cold hypotonic buffer and then centrifuged at 1,500 rpm (200 X g) for 5 min at 4 °C. The supernatant was additionally centrifuged at 15,000 rpm (20,000 X g) for 20 min at 4 °C. The resulting pellet containing the membrane bound protein was resuspended in sample buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, pH 7.4), frozen and stored at -80 °C. For Western blot analysis, samples were thawed on ice and 50 µg protein samples were further solubilized and separated by SDS-PAGE, using precast gels (Owl Separation Systems, Portsmouth, NH), and subsequently electroblotted onto PVDF membranes (Boehringer Mannheim). NPY Y5 protein

was detected with the NPY Y5 antibody (1:5,000) and visualized using enhanced chemiluminescence by exposure to sheet film (XOMAT, Kodak).

Immunocytochemistry (ICC)

For visualization by chromagen or immunofluorescence using ICC, a titration of the antibody was carried out using dilutions ranging from 1:100 to 1:50,000. The optimum antibody concentration for chromagen visualization was 1:5,000 and for immunofluorescence (using tyramide signal amplification) was 1:15,000. Staining was completely absent for both techniques at a dilution of 1:50,000.

Single label ICC

Single label ICC for NPY Y5 immunoreactivity (-ir) was performed, as previously described (Grove et al., 2000), on floating sections from three animals. Briefly, one series of tissue sections from each animal was removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by preincubation in blocking buffer (KPBS + 0.4% Triton X-100 + 2% normal donkey serum) for 30 min at room temperature. The tissue was then incubated with the Y5 specific antibody (1:5,000) in blocking buffer for 48 h at 4 °C. Following washes in KPBS, tissue was incubated for 1 h in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc.), then subsequently washed and incubated in avidin-biotin solution (Vectastain) for 1 h. NPY Y5-like immunoreactivity (NPY Y5-ir) was visualized with 3,3'-diaminobenzidine enhanced with nickel chloride. Distribution of the single label Y5-ir was characterized using a Nikon microscope (Labphoto-2).

Pictures were taken with a Micromax Digital Camera (Princeton Instruments) connected to the Nikon microscope (Plan Apo 20X objective; NA 0.75) using the MetaMorph Imaging System software package (Universal Imaging Corp., West Chester, PA). The brightness and contrast of the digital images were adjusted in Photoshop (Adobe Systems Inc., San Jose, CA).

Double-label ICC

To characterize the phenotype of cells expressing the NPY Y5 receptor, double label immunofluorescence coupled with confocal microscopic analysis was performed as previously described (Grove et al., 2000). Each experiment was performed two to three times on tissue from different animals. Monoclonal neurophysin I and neurophysin II antibodies (a kind gift of Dr. H. Gainer), corresponding to oxytocin (OT) and arginine vasopressin (AVP) cells, respectively (Whitnall et al., 1985, Ben Barak et al., 1985), were used at a concentration of 1:100. The monoclonal gonadotropin-releasing hormone (GnRH) antibody (a kind gift of H.F. Urbanski) was used at a concentration of 1:1,000. The polyclonal NPY antibody, made in the goat, (a kind gift of P. Larsen) was used at a concentration of 1:5,000. The monoclonal GABA antibody (clone #GB-69; G-141, RBI, Natick, MA) was used at a concentration of 1:1,000. The polyclonal CRH antibody, made in the goat (#SC-1759; Santa Cruz Biotechnology, Inc. CA), was used at a concentration of 1:1,000. The double label immunofluorescence for NPY Y5 and the various other targets was done in series. Briefly, tissue sections were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS), preincubated in blocking

buffer for 30 min at room temperature, followed by incubation with the Y5 specific antibody (1:15,000) in blocking buffer for 48 h at 4 °C. Following washes in KPBS, tissue was incubated for 1 h in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc.), then subsequently washed and incubated in avidin-biotin solution (Vectastain) for 30 min. Y5-ir signal was further amplified using a commercial kit (tyramide signal amplification-indirect kit; NEN Life Sciences Products, Boston, MA) and was visualized using either FITC-conjugated to Neutravidin (1:1,000, Pierce, Rockford, IL) or TRITC-conjugated to strepavidin (1:1,000, Jackson ImmunoResearch Laboratories, Inc.). The sections were then rinsed and subsequently incubated with the second primary antibody in blocking buffer for 48 h at 4 °C. Following washes in KPBS, tissue was incubated for 1 h in either a donkey anti-goat (CRH and NPY) or a goat anti-mouse (neurophysins, GnRH and GABA) antibody conjugated to an Alexa fluorophore (Alexa 488 or Alexa 546; Molecular Probes, Eugene, OR).

Triple-label ICC

To characterize the close contact of NPY-ir fibers on GnRH cell bodies displaying NPY Y5 receptor-ir, triple-label immunofluorescence was used. This technique is similar to that described for double-label immunofluorescence. Briefly, the Y5-ir was detected using the tyramide signal amplification-indirect kit and visualized with TRITC-conjugated to strepavidin (as described above). The tissue was then washed and subsequently incubated with a cocktail of monoclonal GnRH (1:1,000) and goat anti-NPY antibodies (1:5,000) in blocking buffer for 48 h at 4 °C. The monoclonal anti-GnRH antibody was visualized with

FITC conjugated to a donkey anti-mouse antibody (1:200, Jackson ImmunoResearch Laboratories, Inc). The goat anti-NPY antibody was visualized with CY5 conjugated to a donkey anti-goat antibody (1:200, Jackson ImmunoResearch Laboratories, Inc).

Confocal Laser Microscopy

Confocal laser microscopy, as previously described (Grove et al., 2000, Li et al., 1999, Li et al., 2000), was used to analyze the double and triple label immunofluorescent images. The Leica Corp. (Germany) TSC SP confocal system consisted of a Leica Corp. RBE inverted microscope equipped with a Ar laser producing light at 488 nm (for visualization of FITC and Alexa 488), a Kr laser producing light at 568 nm (for visualization of TRITC and Alexa 546) and a HeNe laser producing light at 647 nm (for visualization of CY5). Various objectives (25X/NA 0.75 and 40X/NA 1.25) were used to scan the images. For each experiment, fluorophore signals were checked individually for bleed through to the apposing detector. All bleed through was eliminated by adjusting laser intensity and detector window width. A series of continuous optical sections, at 0.5 or 1.0 μm intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. For the study analyzing the close apposition of NPY fibers on neurons expressing Y5-ir, the optical sections were taken at 0.5 μm intervals to provide a high resolution for confirmation of close contacts between the fibers and cell bodies. For the studies analyzing Y5-ir and the various phenotype markers (GnRH, CRH, Neurophysin, GABA), a lower resolution was adequate to confirm colocalization; therefore, optical slices were taken at 1.0 μm

intervals. The signals were obtained for each fluorophore on one series of optical sections and stored separately as a series of 512 x 512 pixel images. The images were then processed with the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA). The confocal images are presented as projections of a stack of optical slices or as single optical slices. The brightness and contrast of the images were adjusted in Photoshop (Adobe Systems Inc., San Jose, CA).

Results

NPY Y5 Receptor Protein Knockdown Following Y5 Antisense Treatment

Treatment of male Wistar rats with antisense ODNs against the Y5 receptor gene for 5 days significantly decreased ($p < 0.05$) the cumulative (over the entire 5 days of treatment) spontaneous food intake (32%; Fig. 1A) and body weight (11.6%; Fig. 1B), but not the cumulative water intake (Fig. 1C), compared to animals treated with sense ODNs (Fig. 1). There was no difference in food intake, body weight or water intake between animals treated with sense ODNs or saline (not shown); therefore only data from animals treated with antisense and sense ODNs were statistically compared. Animals in all treatment groups displayed a typical decrease in food intake, body weight and water intake in response to the surgery on day 1 (Fig. 1). However, in animals infused with sense ODN, all parameters were approaching pretreatment levels by day 5 post-treatment. In contrast, animals treated with antisense ODNs failed to return to pretreatment levels for food intake and body weight (Fig. 1), with levels being

significantly different from animals infused with sense ODNs starting on day 3. In animals treated with antisense ODNs, the lower level of food intake persisted throughout the treatment period, and body weights continued to decline (Fig. 1).

Western blot analysis of micropunch samples from the ventromedial hypothalamic nucleus (VMH) and paraventricular nucleus of the hypothalamus (PVH) demonstrated that Y5 antisense ODNs significantly reduced the 57 kD Y5 receptor protein band by 51% and 50%, respectively (Fig. 2A, B; $p < 0.05$). Detection of the Y2 protein band was unaffected by the Y5 antisense ODN treatment, suggesting that the effect of the treatment was specific for the Y5 receptor (Fig. 2B).

The above studies provide additional evidence for the specificity of the Y5 receptor antibody by demonstrating: 1) the antibody recognizes a single protein band that corresponds to the estimated molecular weight of the Y5 receptor; and 2) The antibody detects a decrease in the 57 kD Y5 receptor protein following treatment of animals with antisense ODNs to the Y5 receptor.

Characterization of the Distribution of the NPY Y5 Antibody

Western blot analysis revealed a wide distribution of the NPY Y5 receptor protein throughout the rat brain (Fig. 3). For this analysis 50 μ g of protein from different regions of the brain and pituitary was used and the films were exposed to near saturation in order to ensure that all bands could be visualized. The results demonstrate that the antibody directed towards the peptide sequence unique to the Y5 receptor recognized a single major protein band with the approximate molecular weight of 57 kD (Fig. 3), which agrees with the previous

study using this same antibody (Vezzani et al., 2000). In addition, the 57kD molecular weight of the Y5 receptor is unique amongst the known molecular weights of other NPY receptors (Y1 – 73 kD (Wieland et al., 1998); Y2 – 60 kD (Wimalawansa, 1995); Y4 – 51 kA (Eckard et al., 1999) and Campbell, French-Mullen, Smith and Grove, unpublished observation). In these studies a less intense band of a slightly higher molecular weight, likely representing differential glycosylation, was also detected in the hippocampus and brainstem (Fig. 3). The pituitary also displayed a doublet of bands of near equal intensities (Fig. 3) that were of a slightly higher molecular weight than the protein detected in the brain. Vezzani *et al.* (2000) also reported a less intense protein band with a lower molecular weight (51 kD) in specific regions of the hippocampus with this antibody, which was not seen in the present study. This detection of the smaller protein band by Vezzani et al. (2000) is likely due the higher concentration of primary antibody used in this study (1:2500), while the present study used 1:5000.

Single Label Immunohistochemistry

While the vast majority of the Y5-ir was present in the somal membranes, staining was also observed in the cellular compartment and in short fiber processes (not shown) throughout the hypothalamus, including the preoptic area, supraoptic nucleus (SON), PVH and ARH. However, it has yet to be determined if the fiber staining represents dendritic processes or axonal fiber terminals.

NPY Y5-ir was observed throughout the hypothalamus (Fig. 4). In the rostral extent of the hypothalamus Y5-ir was present in neuronal cell bodies in

the organum vasculosa of the lamina terminalis and the diagonal band of Broca (Fig. 4A). The highest intensity of Y5-ir staining within the rostral hypothalamus was present in fusiform shaped bipolar neurons resembling GnRH neurons (Fig. 4B). More caudally, Y5-ir was present in neuronal cell bodies within the preoptic area (Fig. 4C), with the majority of Y5-ir neurons being concentrated medially. A moderate level of Y5-ir was also present in the suprachiasmatic region (not shown). The highest level of NPY Y5-ir in the hypothalamus was present in the magnocellular neurons of the PVH (Fig. 4D) and the SON (Fig. 4E). In addition, neuronal Y5-ir staining was also present in the parvocellular region of the PVH (Fig. 4D) as well as in the periventricular nucleus. In the caudal extent of the hypothalamus, the highest level of Y5-ir was present in the ARH (Fig. 4F). The majority of the Y5-ir in the ARH was located in the medial region with only a few scattered cells in the dorsal and lateral regions. Y5-ir was also present in the VMH as well as a number of scattered neurons spread throughout the dorsomedial hypothalamic nucleus (DMH) and lateral hypothalamic area (LHA) (not shown).

NPY Y5 receptor-ir was also observed in scattered neurons throughout the thalamus, with the highest levels of staining being in the paraventricular nucleus of the thalamus, habenula, mediodorsal thalamic nucleus and zona incerta. As previously reported (Grove et al., 2000), very intense staining was observed in the hippocampus and on the soma and proximal dendritic processes of small (10-15 μm diameter) neurons in the cortex, with the highest concentration of cells being in the cingulate cortex.

Double and Triple Label Immunofluorescence

Gonadotropin Releasing Hormone (GnRH)

As mentioned above, the presence of Y5-ir staining on fusiform bipolar neurons in the preoptic area (Fig. 4A) indicated that the NPY Y5 receptor might be expressed on GnRH neurons. Double label immunofluorescence demonstrated that Y5-ir was indeed present on some, but not all, GnRH neurons (Fig. 5). Quantitative analysis of 287 GnRH neurons from sections through the preoptic area (n = 3 animals) demonstrated that approximately 55% displayed Y5-ir.

Corticotropin Releasing Hormone (CRH)

The presence of Y5-ir on neurons in the parvocellular region of the PVH (Fig. 4D) suggested that NPY Y5 receptors might be present on CRH neurons within this region. Using double label immunofluorescence on tissue from colchicine treated animals (to accumulate CRH within cell bodies; n = 4), Y5-ir was detected on only a small subpopulation (<25%) of CRH neurons scattered throughout the PVH (white arrows; Fig. 6A), while the majority of CRH-ir neurons in the PVH did not display Y5-ir (black/white arrowhead; Fig. 6A).

Neurophysins

A high level of Y5-ir was present in magnocellular neurons in both the SON and PVH (Fig. 4D, E), suggesting that NPY Y5 receptors may be present on oxytocin (OT) and arginine vasopressin (AVP) neurons. Using monoclonal antibodies specific for neurophysin I (representing OT) and neurophysin II (representing AVP), double-label immunofluorescence was used to demonstrate

that nearly all of the OT and AVP neurons displayed Y5-ir (not shown; $n = 2$). However, in each case there were large numbers of Y5-ir neurons that did not contain the neurophysin staining. To determine if Y5-ir was present on neurons within the PVH and SON that did not express neurophysin I or II, a cocktail of the two monoclonal neurophysin antibodies was used. Figure 6B displays Y5-ir and neurophysin-ir staining in the SON and demonstrates that the vast majority of neurons containing neurophysin ($> 95\%$) also contain Y5-ir ($n = 3$). However, a significant portion of the neurons displayed Y5-ir alone. Similar results were found for magnocellular neurons in the PVH (not shown). The phenotype of these non-neurophysin containing neurons, which display Y5-ir, is unknown.

GABA

The only area that we could consistently detect GABA-ir in cell bodies within the hypothalamus was in the DMH. The difficulty in detecting GABA-ir in hypothalamic neurons is a common problem with available GABA/GAD antibodies (unpublished observation). Therefore we were unable to determine if other hypothalamic areas known to contain GABAergic neurons, as assessed by the presence of GAD_{65/67} mRNA (Esclapez et al., 1993, Esclapez et al., 1994) displayed Y5-ir. While most of the GABAergic neurons ($> 75\%$) in the DMH displayed Y5-ir (Fig. 6C; white arrows), there were many Y5-ir neurons in this region that did not display GABA-ir.

NPY

To characterize functional interactions between NPY fibers and cells expressing the Y5 receptor, double label immunofluorescence, coupled with

confocal laser microscopy, was used to demonstrate that NPY-ir fibers make close appositions with cells displaying Y5-ir staining. Figure 7 shows that NPY-ir fibers (shown in red) in the PVH (Fig. 7A) and SON (Fig. 7B) are in abundance in the vicinity of neurons displaying Y5-ir (shown in green). Confocal analysis of single optical slices through these images demonstrated that NPY-ir fibers come in close apposition (shown in yellow) to the Y5-ir neurons. Finally, triple-label immunofluorescence for Y5-ir (red), GnRH-ir (blue) and NPY-ir (green), was used to demonstrate that NPY-ir fibers are abundant in areas with GnRH cells bodies expressing Y5-ir (Fig. 7C, D; Y5/GnRH double-label is represented by magenta). High-resolution confocal microscopy was used to demonstrate that GnRH neurons displaying Y5-ir receive rich innervation by NPY-ir fibers (Fig. 7D, i-iii). Figure 7D (panels i-iii) also illustrates the presence of Y5-ir in the preoptic area that is not localized to GnRH neurons (shown in red). *It should be noted that the patches of single label Y5-ir (red) in Figure 7D (panels i-iii) represent only portions of cells that were captured in the optical slices selected to demonstrate the Y5 colocalization in the GnRH neuron*

Discussion

The present study demonstrates that the rat brain contains a high level of NPY Y5 protein, as determined by Western blot analysis (Fig. 3) and immunocytochemistry (Figs. 4-7). The pattern and high level of Y5 receptor protein expression are consistent with the reported distribution and level of expression of Y5 mRNA (Nichol et al., 1999, Parker and Herzog, 1999).

Although there is an abundance of Y5 mRNA in the hypothalamus, reports on the distribution of Y5-like receptor binding, by *in vitro* receptor autoradiography, have demonstrated only low levels of Y5 receptors in the hypothalamus (Dumonet et al., 1998, Widdowson et al., 1997). Likewise, Statnick *et al.* (1998) reported high levels of Y5 mRNA in the hypothalamus of humans; however, receptor-binding studies failed to detect Y5-like binding in homogenates from the human hypothalamus. The reason for the discrepancy between the high levels of Y5 mRNA and Y5-ir in the hypothalamus and the relatively low levels of Y5-like receptor binding is unknown. One possibility is that the immunocytochemistry likely labels total cellular Y5 protein, while receptor autoradiography likely only labels outer membrane accessible Y5 receptors. The majority of the Y5 receptors may be stored in a manner that makes them inaccessible to released ligands, but under certain physiological conditions the receptors are mobilized to the outer member where they can be activated. However, this possibility remains to be investigated. The major limitations to the earlier *in situ* hybridization studies of the Y5 receptor mRNA distribution, as well as the distribution of the Y5 receptor binding by *in vitro* receptor autoradiography, are the inability to determine the cellular localization of the receptor protein and the lack of characterization of the phenotype of the cells expressing the Y5 receptor.

Neuroendocrine Neuronal Circuitry

Gonadotropin Releasing Hormone

NPY Y5 receptor-ir is widespread throughout the anterior hypothalamus and preoptic area (Fig. 4), although the majority of the labeled cells appeared to

be concentrated medially. The phenotype of the majority of neurons in the preoptic area remains unknown; however, double label immunofluorescence was used to demonstrate that a subpopulation of GnRH neurons, approximately 55%, in the preoptic area displayed Y5-ir (Figs. 5,7). While many groups have demonstrated direct contacts by NPY fibers on GnRH neurons (Li et al., 1999, Tsuruo et al., 1990, Tillet et al., 1989), the present study demonstrated that there is an abundance of NPY-ir fibers making close appositions with GnRH neurons displaying Y5-ir (Fig. 7 C and D). The present data, coupled with our previous findings that the NPY Y1 receptor is present on GnRH nerve terminals in median eminence, but not cell bodies (Li et al., 1999), suggests that NPY can have multiple actions on GnRH neuronal function and release, depending on the site of action and the receptor subtype involved. Indeed, NPY has both excitatory and inhibitory effects on LH release, likely through modulation of GnRH neuronal activity in the preoptic area and release from nerve terminals in the median eminence (Kalra and Crowley, 1992). Pharmacological studies indicated that the NPY receptor mediating the inhibition of LH release has the pharmacological profile of the Y5 receptor (Raposinho et al., 1999), presumably through modulation of GnRH neuronal function. In contrast, the stimulation of GnRH release in the presence of gonadal steroids has been shown to be mediated via the Y1 receptor subtype (Karla et al., 1992; Besecke et al., 1994) and involves the activation of protein kinase C (Leupen et al., 1999).

These results suggest a possible neuronal circuitry by which arcuate NPY neurons, projecting to and coming in close contact with GnRH cell bodies and

nerve terminals (Li et al., 1999), can modulate GnRH neuronal activity. NPY could have direct actions on GnRH cell bodies via the Y5 receptor and on GnRH terminals via the Y1 receptor. In addition, NPY may modulate GnRH neuronal activity by activating Y1 receptors on nerve terminals that come in close contact with GnRH cell bodies in the preoptic area (Suda et al., 1993). The phenotypes of these neuronal populations have efferent projections to GnRH neurons (Gu et al., 1997), are unknown.

Oxytocin-Arginine Vasopressin

The present study further demonstrated that nearly all neurophysin neurons (both OT and AVP) in the SON and magnocellular division of the PVH display Y5-ir (Fig. 6), while others have shown that the Y1 receptor subtype is absent on neurophysin neurons (Broberger et al., 1999). Our results also show that NPY-ir fibers make close apposition with magnocellular neurons displaying Y5-ir (Fig. 7A, B). These findings are in agreement with other groups that have demonstrated NPY-ir fibers making synaptic contacts with magnocellular neurons in the SON and PVH (Iwai et al., 1989, Willoughby et al., 1987). Taken together, the high concentration of NPY-ir fiber contacts on magnocellular neurons in the SON and PVH suggests that NPY may have direct actions on neurophysin neurons to stimulate OT and AVP release through activation of the Y5 receptor (Willoughby et al., 1987, Parker et al., 1993, Sato et al., 1995) (Figs. 4, 6B).

Food Intake

Paraventricular Nucleus of the Hypothalamus

NPY is classically known for its role in the regulation of food intake and energy expenditure, likely through modulation of neurons in the PVH (Kalra et al., 1999, Kotz et al., 1998, Yoshihara et al., 1996). Both the NPY Y1 and Y5 receptor subtypes have been implicated in the regulation of food intake (Kalra et al., 1998, Gerald et al., 1996, Hu et al., 1996, Kanatani et al., 1999, Crisioine et al., 1998). Interestingly, knockout of NPY Y1, Y2, or Y5 receptor genes does not produce leaner animals as would be expected, but rather results in animals that display late onset obesity (Naveilhan et al., 1999, Kushi et al., 1998, Kanatani et al., 2000, Pedrazzini et al., 1998, Marsh et al., 1998). However, it is difficult to anticipate what compensatory mechanisms have taken over in the gene knockout models. It is clear that acute treatment of adult rats with antisense ODNs directed to the Y5 receptor reduces spontaneous food intake, as well as NPY induced food intake (Tang-Christensen et al., 1998, Flynn et al., 1999) indicating that in the normal animal, the NPY Y5 receptor plays a role in regulation of food intake. However, these earlier studies were unable to confirm a decrease in NPY Y5 protein in response to the Y5 antisense treatment. In the present study, we demonstrate that chronic infusion of Y5 antisense ODNs can significantly reduce spontaneous food intake and body weight throughout the treatment period (Fig. 1), as well as a significant decrease in Y5 protein levels in the PVH and VMH (Fig. 2), regions known to be involved in the regulation of food intake.

The phenotype of the downstream neurons modulating the effects of the NPY/Y5 receptor system on food intake are unknown; however, CRH neurons

have been suggested as one of these downstream targets (Heinrichs et al., 1998). There is an integral relationship between the NPY system in the ARH and the CRH system in the PVH. NPY fibers are known to make synaptic contacts with CRH neurons in the PVH (Liposits et al., 1988, Li et al., 2000), although the exact physiological interaction between these two systems is not fully understood. NPY is likely to have both excitatory and inhibitory actions on the CRH system (Suda et al., 1993, Liposits et al., 1988, Fischer et al., 1995, Hwang et al., 1997, Kiss et al., 1994), depending on the mode of action (chronic vs. acute) and the receptor subtype activated. Recent studies from our laboratory and others have demonstrated that CRH neurons in the PVH do not express the Y1 receptor subtype (Li et al., 2000, Broberger et al., 1999). However, Li *et al.* (2000) did demonstrate Y1-ir fibers in close contact with CRH neurons, suggesting that NPY can modulate presynaptic inputs on CRH neurons through activation of the Y1 receptor. The present study demonstrates that while many neurons in the pPVH (Fig. 4D) display Y5-ir, only a small fraction of CRH neurons expressed this receptor (Fig. 6A), suggesting that NPY may modulate the function of a subpopulation of CRH neurons through a direct mechanism involving the Y5 receptor. This hypothesis is supported by the presence of NPY synaptic contacts, as shown by electron microscopy, on CRH neurons (Liposits et al., 1988).

While the majority of Y5-ir neurons in the pPVH were of an uncharacterized phenotype (Fig. 6), cocaine- and amphetamine-regulated transcript (CART) and/or thyrotropin-releasing hormone (TRH) neurons represent

other possible downstream targets in the PVH for the NPY/Y5 system (Kalra et al., 1999). Both CART and TRH neurons in the PVH have been shown to receive afferent inputs from NPY fibers (Lambert et al., 1998, Broberger et al., 1999, Toni et al., 1990) and pretreatment with a CART peptide reduces the stimulation of food intake induced by NPY (Lambert et al., 1998). Broberger *et al.* (1999) recently demonstrated that only the occasional CART neuron displayed NPY Y1 receptor-ir, while the majority of TRH neurons contained Y1-ir, suggesting that some other NPY receptor may be mediating the effects of NPY on CART neurons.

Arcuate Nucleus

NPY neurons in the ARH form a major component of the ARH-PVH pathway involved in the regulation of food intake (see review Sajdyk et al., 1999). The presence of Y5-ir on neurons in the medial portion of the ARH (Fig. 4F) correlates with the distribution of the Y5 mRNA and suggests that the Y5 receptor may be expressed on NPY neurons contained within this region. NPY neurons in the ARH also express the Y2 receptor, with the receptor being expressed on NPY nerve terminals where it likely functions as an autoreceptor (Naveilhan et al., 1999). Together, the presence of Y5 receptors on NPY cell bodies and Y2 receptors on NPY nerve terminals may comprise two mechanisms by which NPY regulates NPY neurons in the ARH. The evidence that Y2 and Y5 receptor knockout mice both exhibit late onset obesity phenotypes would be consistent with the idea that these receptor subtypes may be important for regulating ARH/NPY neuronal function.

Dorsomedial Hypothalamic Nucleus/Lateral Hypothalamic Area

The DMH is considered an integral component of the feeding circuitry of the hypothalamus, mainly due to its integration of projections from the LHA, VMH and ARH, and its subsequent GABAergic projections to the PVH (Boudaba et al., 1996, Bernardis et al., 1998). The DMH and LHA also contain a dense field of NPY fibers (Chronwell et al., 1985). In addition, in specific models of obesity and hyperphagia and during development, NPY mRNA is expressed in the neurons of the DMH and LHA (Kesterton et al., 1997, Li et al., 1998, Smith et al., 1993, Singer et al., 2000), suggesting that the NPY system in the DMH and LHA may play a role in regulation of food intake and adiposity. This hypothesis is supported by evidence that the LHA, specifically the perifornical region, is the most sensitive to the orexigenic effects of exogenous NPY administration (Gillard et al., 1993, Stanley et al., 1998). In the present study, we demonstrated that the DMH (Fig. 6C) and LHA contain a number of scattered neurons displaying Y5-ir. In addition, many of the neurons displaying Y5-ir were GABAergic (Fig. 6C). Cowley *et al.* (1999) recently demonstrated that NPY's effect on neuronal activity within the PVH was via modulation of presynaptic GABA release. The present findings support this hypothesis and suggest that the NPY/Y5 system may participate through direct actions on GABAergic neurons in the DMH.

In summary, there is a wide distribution of the Y5 receptor protein in the hypothalamus, matching the distribution previously reported for the Y5 receptor mRNA in the rat brain. In addition, we demonstrated that the Y5 receptor is expressed on a variety of neuronal types throughout the hypothalamus, including

GnRH, CRH, OT, AVP and GABA neurons. This wide distribution and expression on these cell types is in agreement with known physiological roles of NPY in the brain. Furthermore, the localization of the Y5 receptor to GnRH neurons and to neurons in hypothalamic regions important for the regulation of food intake, such as the PVH, DMH, VMH, LHA and ARH, suggests that the NPY/Y5 system may be a central mediator between signals of energy availability and reproductive neuroendocrine function.

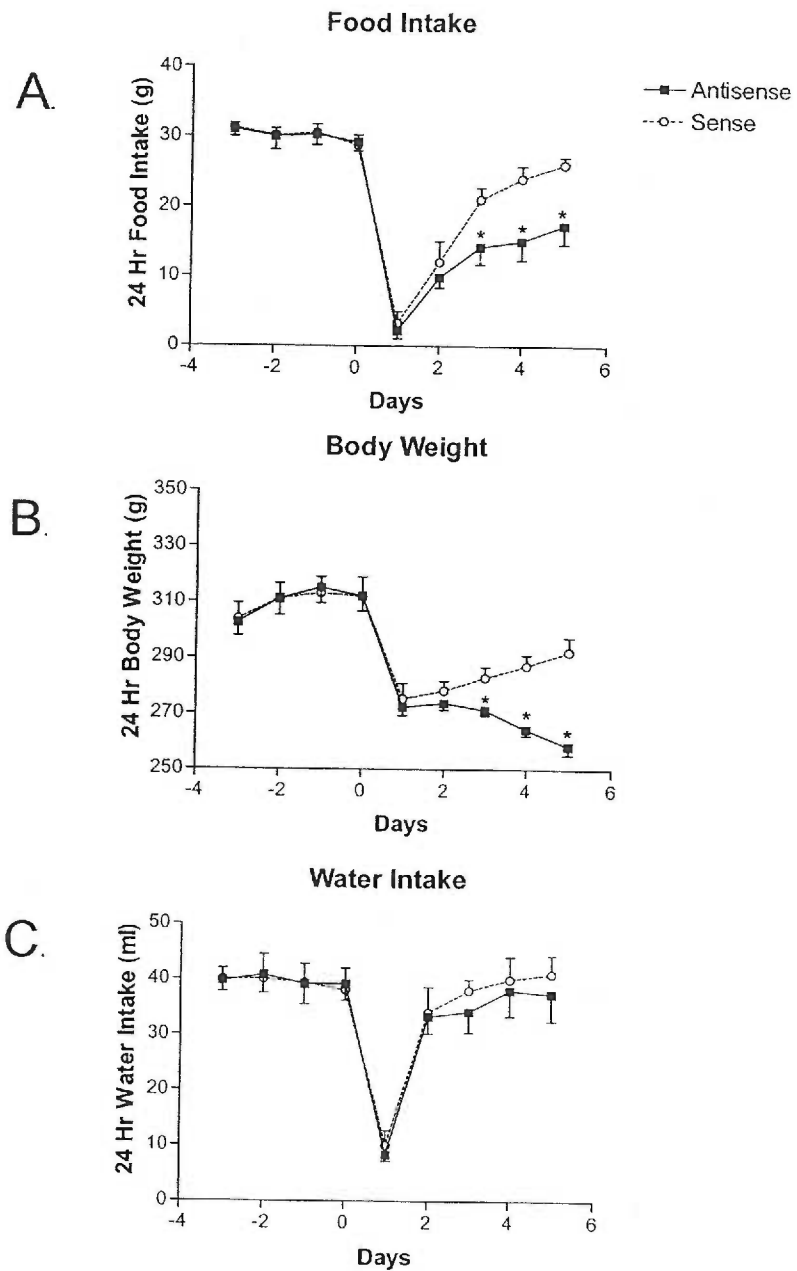


Figure 4-1. Antisense or sense oligonucleotide (ODN) treatment effects on food intake (A), body weight (B) and water intake (C). Values represent daily (24 h) food and water intake or body weight $\pm$ SEM ($n = 5$). Antisense or sense ODN treatment was initiated on day 0 and terminated on day 5. Initial decreases in food and water intake and body weight at day 1 represent a nonspecific surgical effect. Asterisks indicate a significant difference between animals treated with sense and antisense ODNs ($p < 0.05$).

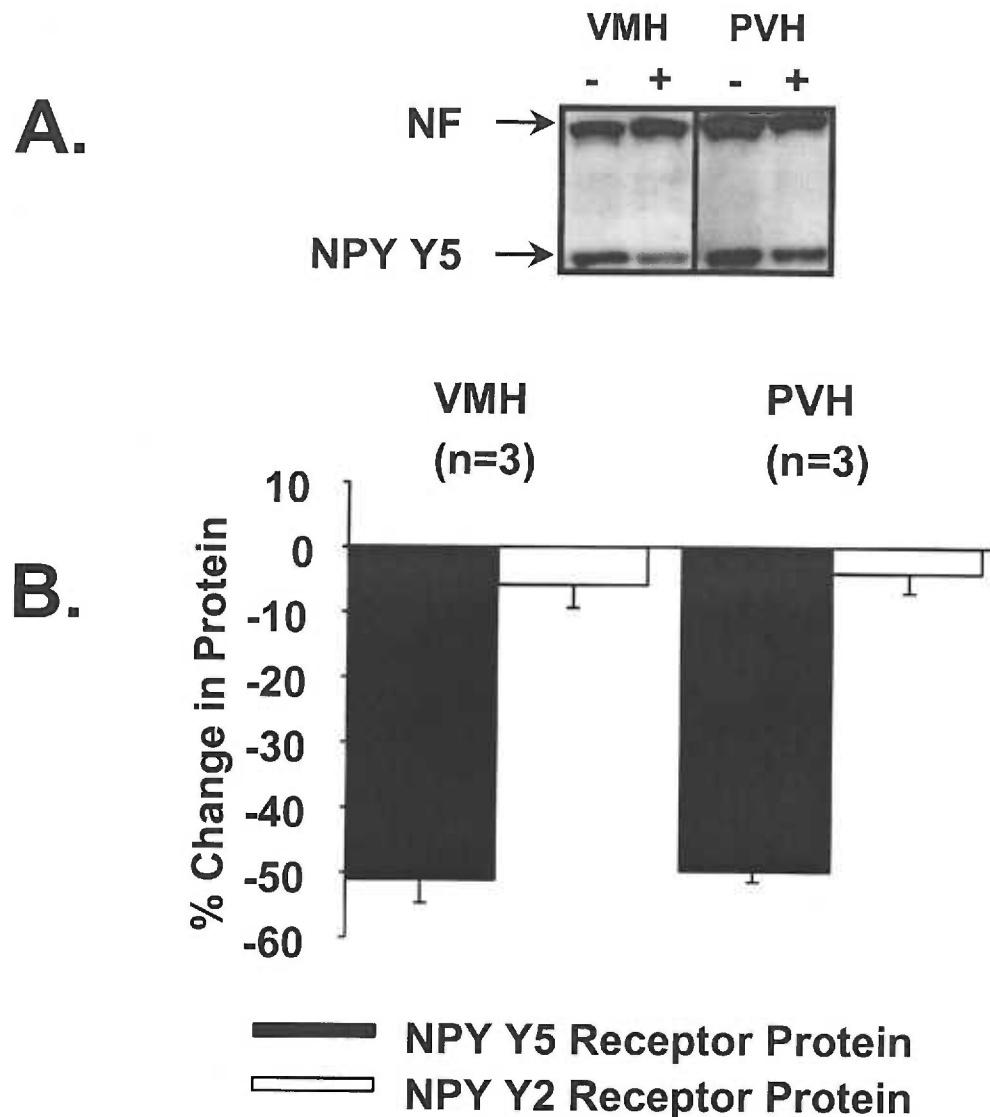


Figure 4-2. Y5 antisense oligonucleotide (ODN) suppression of Y5 receptor protein levels in the ventromedial hypothalamic nucleus (VMH) and paraventricular nucleus of the hypothalamus (PVH). A. Western blots of the 57 kD Y5 protein band in the VMH and the PVH following sense (-) or antisense ODN treatment (+). The Y5 receptor antibody was used at a dilution of 1:2,500. Also shown is the protein band labeled by the neurofilament 68 (NF) antibody, which was used as an internal standard. Ten μ g of protein was used for these assays. B. Quantitative densitometry of the relative optic density of the Y5 and Y2 receptor protein bands. The values represent the mean $\pm$ SEM ($n = 3$) of the percent change of antisense ODN treatment compared to the sense ODN treatment (Y5 and Y2 receptor protein bands were normalized to the NF protein band).

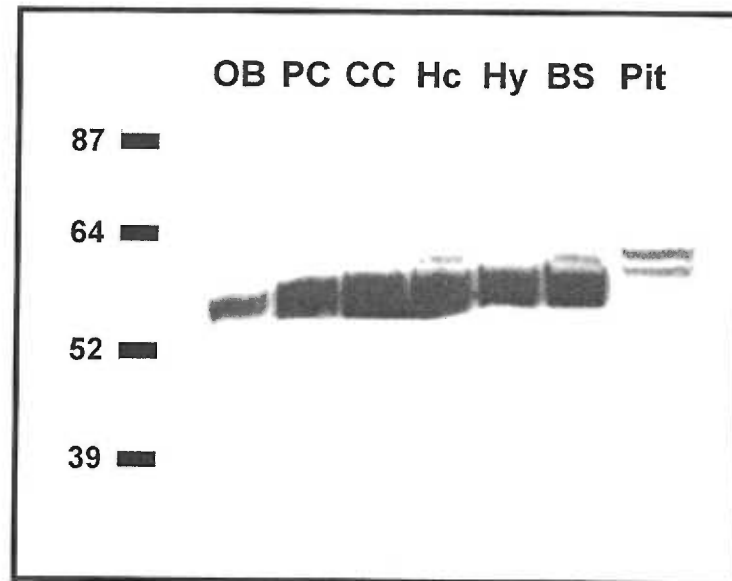


Figure 4-3. Western blot analysis of the distribution of the Y5 receptor protein in the rat brain. The antibody (used at a dilution of 1:5,000) recognized a single major band, with the approximate molecular weight of 57 kD, in the olfactory bulb (OB), piriform cortex (PC), cingulate cortex (CC), hippocampus (Hc), hypothalamus (Hy) and brainstem (BS). A doublet band, with a slightly higher molecular weight, was also detected in the Hc and BS. In the pituitary (Pit), doublet protein bands, with higher molecular weights than seen in the brain, were recognized by the Y5 receptor antibody. For these studies 50 μ g of protein was used and the films were exposed to near saturation to optimize detection of all protein bands recognized by the antibody.

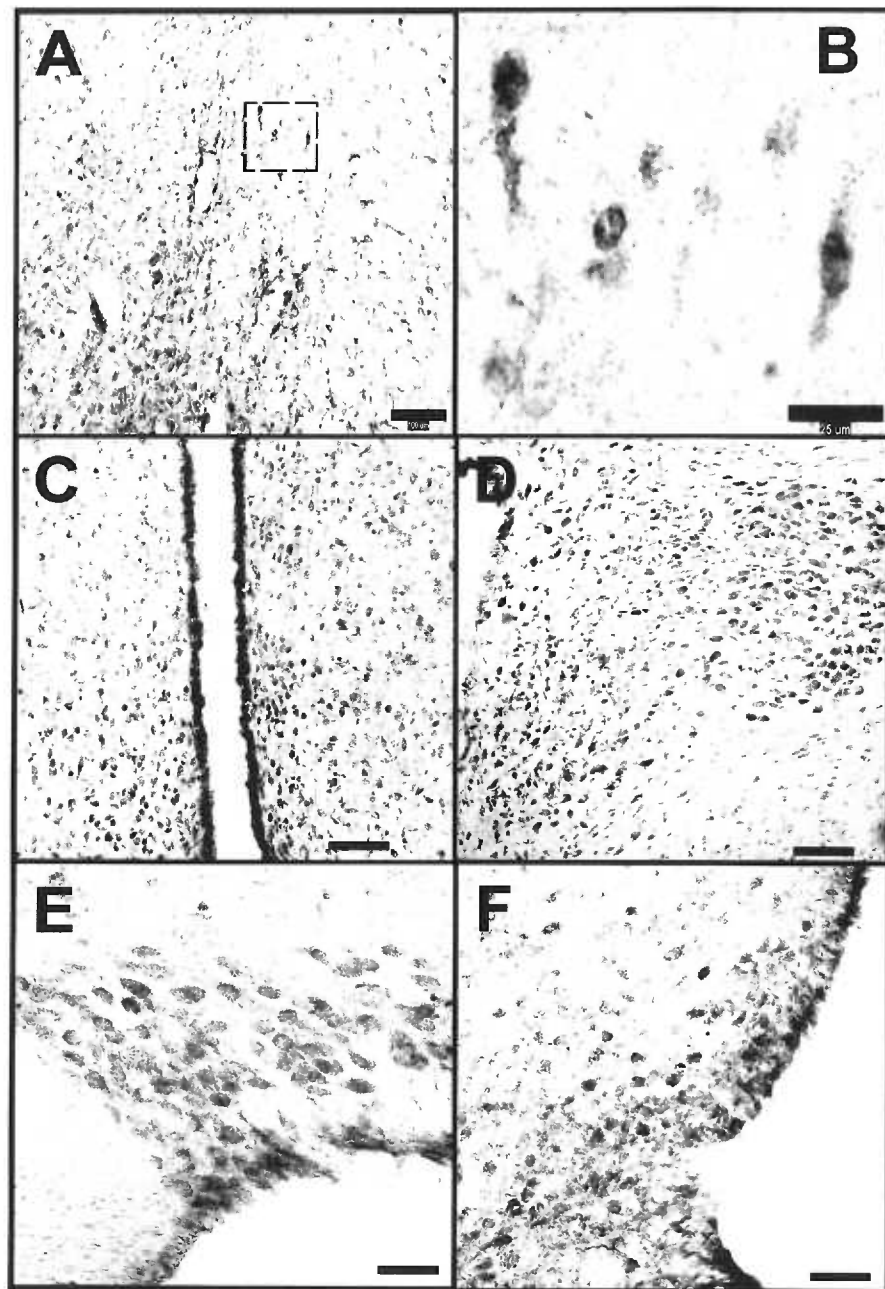


Figure 4-4. Single label Y5-immunoreactivity (-ir) in the hypothalamus of the rat brain. A. Organum vasculosum of the lamina terminalis and diagonal band of Broca. Black box indicates area shown in B. Bar represents 100 μ m. B. Diagonal band of Broca. Bar represents 25 μ m. C. Preoptic area. Bar represents 100 μ m. D. Paraventricular nucleus of the hypothalamus. Bar represents 100 μ m. E. Supraoptic nucleus. Bar represents 50 μ m. F. Arcuate nucleus. Bar represents 50 μ m.

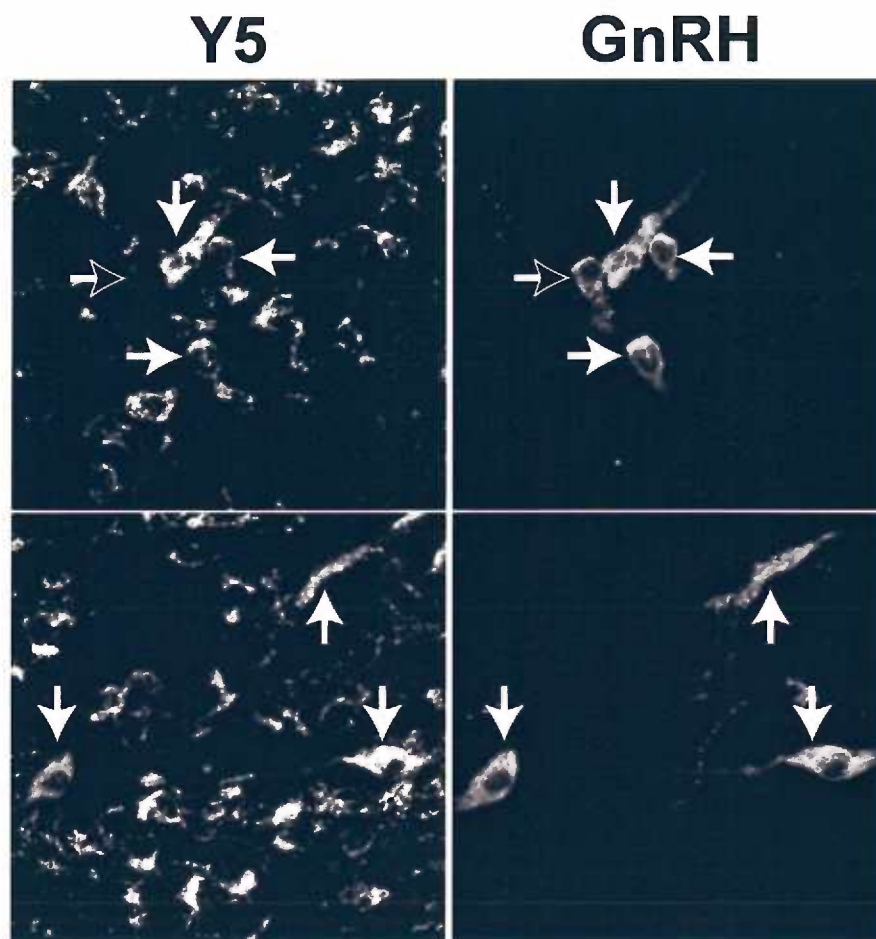


Figure 4-5. Expression of NPY Y5 immunofluorescence (-IF) in GnRH neurons. Figures represent black and white confocal digital images of double label -IF for the NPY Y5 receptor (left panels) and GnRH (right panels) in the lateral region of the diagonal band of Broca. White arrows indicate neurons displaying colocalization of Y5-IF and GnRH-IF. Black and white arrows indicate a GnRH neuron not displaying Y5-IF.

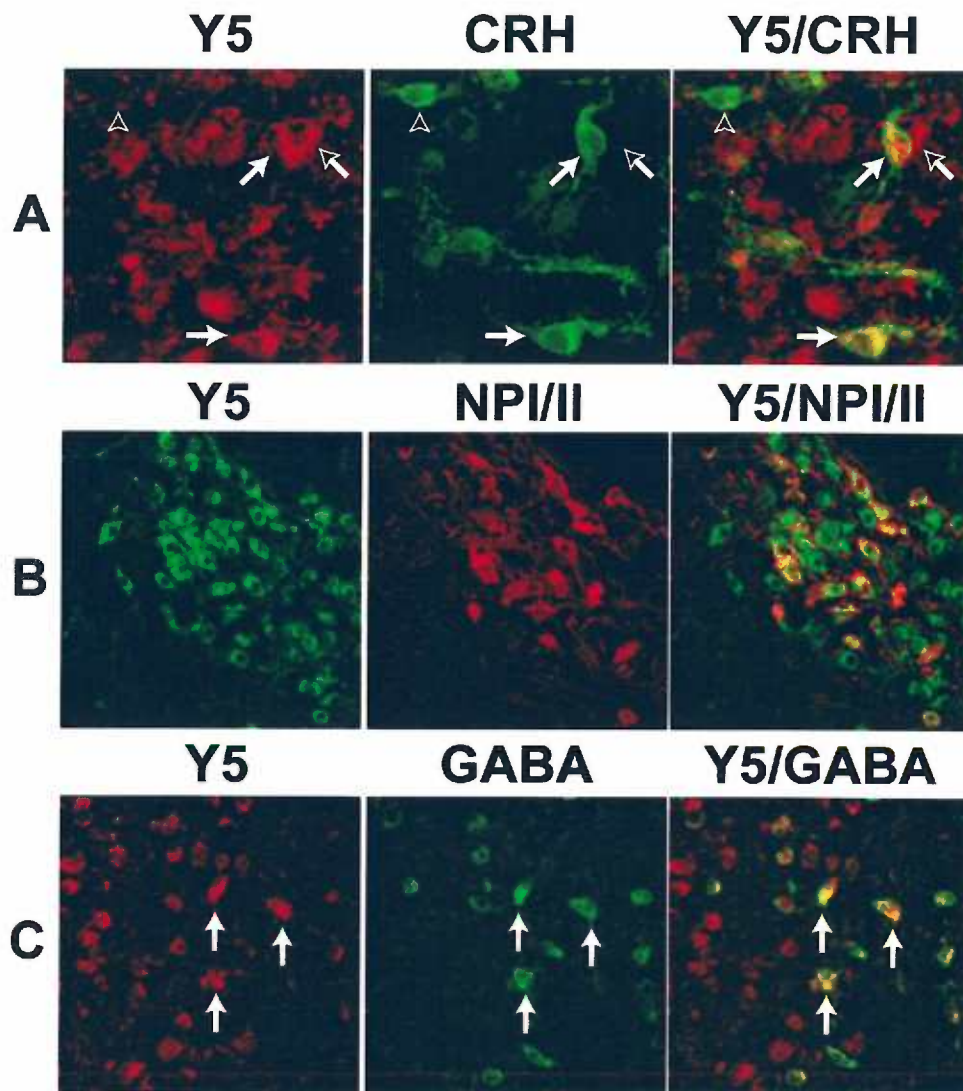


Figure 4-6. Double label immunofluorescence (-IF) of Y5 and CRH (A), neurophysins (B) and GABA (C) in the rat hypothalamus. A) Y5 and CRH double label-IF in the paraventricular nucleus of the hypothalamus. Y5-IF was visualized with rhodamine (red color); CRH was visualized with Alexa 488 (green color). White arrows indicated neurons displaying double label -IF for CRH and Y5 (yellow color). The black and white arrow indicates a single label Y5 neuron lying in close apposition to a CRH neuron displaying low levels of Y5-IF (white arrow). The black and white arrowhead indicates a single label CRH neuron. B) Y5 and Neurophysin I/II (NPI/II) double label-IF in the supraoptic nucleus. Y5-IF was visualized with FITC (green color); NPI/II-IF (representing both oxytocin and arginine vasopressin neurons) was visualized with Alexa 546 (red color). Most of the NPI/II neurons displayed Y5-IF (yellow color), while many neurons in the supraoptic nucleus displayed only Y5-IR. C) Y5 and GABA double label-IF in the dorsomedial hypothalamic nucleus: Y5-IF was visualized with rhodamine (red color); GABA was visualized with Alexa 488 (green color). Nearly all of the GABA-IF neurons displayed Y5-IF (yellow color; indicated by white arrows); while numerous neurons displayed only Y5-IF. 113

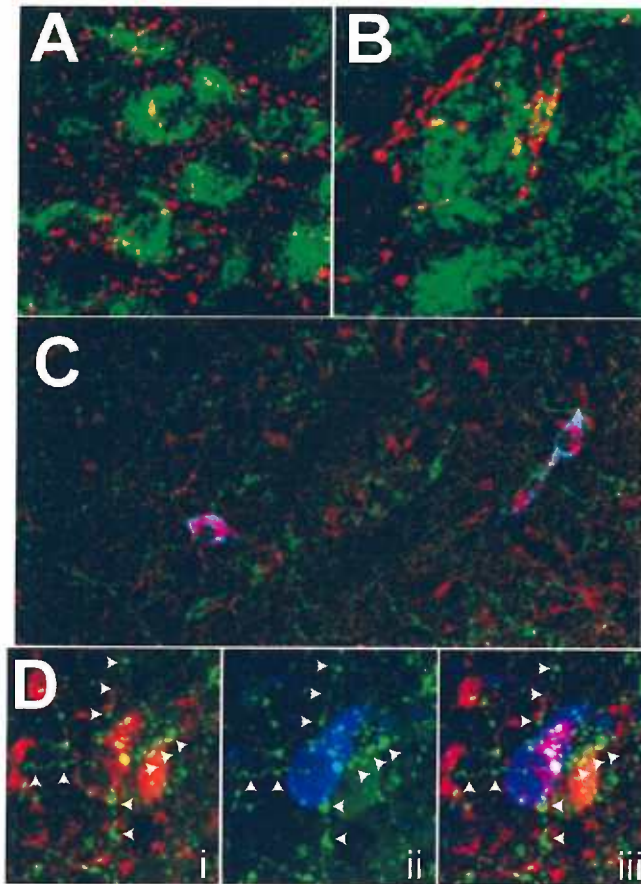


Figure 4-7. Neuropeptide Y (NPY) innervation of neurons displaying NPY Y5 receptor immunofluorescence (-IF). Figures represent color confocal digital images of double label-IF or triple label-IF. A. Paraventricular nucleus. B. Supraoptic nucleus. Y5-IF was visualized with FITC (green color); NPY was visualized with rhodamine (red color). Close appositions between NPY-ir fibers and Y5-ir neurons are shown as the yellow color. C. Triple label confocal image of NPY-IF fibers (green color; Alexa 488) making close appositions with GnRH-IF neurons (blue color; CY5) that display Y5-IF (magenta color; rhodamine). The single label Y5-IF is shown as a red color. Note the presence of Y5-IF in proximal processes of the GnRH neuron. No colocalization of Y5-IF was detected in GnRH fibers D. Higher magnification of triple label -IF, focusing on a single GnRH neuron. i) Digital overlay of Y5-IF (red color) and NPY-IF (green color). The yellow spots represent close contacts between the NPY fiber and the neuronal cell body. ii) Digital overlay of GnRH-IF (blue color) and NPY-IF (green color). The pale blue spots represent close contacts between the NPY fiber and the GnRH neuronal cell body. iii) Digital overlay of Y5-IF (red color), GnRH-IF (blue color) and NPY-IF (green color). Magenta indicates colocalization of Y5-IF and GnRH-IF in the cell body. The white spots indicate close contact of NPY-IF fibers with the GnRH neuron. White arrowheads indicate the path of NPY fibers.

Chapter V

Rat Pancreatic Polypeptide (rPP) Increases Food Intake Through Activation of Neuropeptide Y Y4 Receptors on Orexin Neurons in the Lateral Hypothalamic Area

Journal of Neuroscience

submitted

Introduction:

Neuropeptide Y (NPY) is a member of the pancreatic polypeptide hormone family that also includes pancreatic polypeptide (PP) and peptide YY (PYY). NPY is most notably characterized as a potent orexigenic agent, with exogenous NPY administration causing a robust increase in food intake and body weight (Williams et al., 2001; Morton and Schwartz, 2001). In addition, NPY is also an important modulator of the neuroendocrine reproductive axis (Kalra and Horvath, 1999). It has been suggested that NPY may act as an integrating factor between signals for energy balance and neuroendocrine reproductive function (Smith and Grove, 2002); however, the neuronal circuitry responsible for these effects remains to be elucidated.

Five receptor subtypes have been characterized that bind NPY, PYY and PP with different affinities and have been pharmacologically distinguished as Y1, Y2, Y4, Y5 and Y6. They all belong to the superfamily of G-protein coupled receptors and cause the inhibition of adenylate cyclase and, in some instances, the activation of the phospholipase C/ protein kinase C pathway (Parker et al., 1998; Selbie et al., 1995). Currently, the Y1 and Y5 receptor subtypes are implicated in mediating the effects of NPY on food intake based on a variety of studies using partially selective pharmacological tools and antisense approaches. Both Y1 and Y5 antagonists can inhibit nocturnal feeding as well as fasting and NPY-induced food intake (Crisioine et al., 1998; Kanatani et al., 1999), and administration of a selective Y5 agonist significantly stimulates food intake (Cabrele et al., 2000). Likewise, antisense oligonucleotides directed against both

the Y1 and Y5 receptors have been reported to reduce NPY-induced food intake (Lopez-Valpuesta et al., 1996; Schaffhauser et al., 1997; Campbell et al., 2001). However, knockout studies have provided some unexpected results. Mice lacking the Y1 or Y5 receptor gene exhibit late onset obesity and even hyperphagia rather than the expected lean/hypophagic phenotype (Kushi et al., 1998; Pedreazzini et al., 1998; Marsh et al., 1998). In contrast, Y4 receptor knockout mice do provide expected results, as they are leaner and hypophagic (Sainsbury et al., 2002).

The Y4 receptor has been thought of primarily as a peripheral receptor, since it binds with a much higher affinity to peripherally produced PP than to NPY (Bard et al., 1995). However, both Y4 receptor mRNA and binding within the brain have been reported, including hypothalamic areas involved in the regulation of food intake (Parker and Herzog, 1999; Dumont and Quirion, 2000). Also, administration of 1229U91, a Y4 agonist, or rPP into the third ventricle stimulates (luteinizing hormone) LH secretion, providing additional evidence for centrally active Y4 receptors (Jain et al., 1999; Raposinho et al., 2000). Therefore, although it is unclear whether the lean phenotype of the Y4 knockout mouse is due to peripheral or central mechanisms, it is reasonable to hypothesize that hypothalamic Y4 receptors may play a role in the central regulation of food intake. The present study was designed, therefore, using both anatomical and physiological approaches, to investigate Y4 receptor expression in the hypothalamus and their potential role in the central regulation of food intake.

MATERIALS AND METHODS:

Animals and tissue:

Adult male and female rats (Simonsen, CA) were maintained under a 12:12 light dark cycle (lights on at 0700h) and constant temperature ($23 \pm 2^{\circ}\text{C}$). Food and water were available *ad libitum*. All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

For immunocytochemical studies, animals were sacrificed under pentobarbital anesthesia by cardiac infusion with ice-cold PBS followed by ice-cold paraformaldehyde in NaPO_4 buffer (pH 7.4). The brains and peripheral tissues were then removed, saturated in 25% sucrose, frozen in cooled isopentane, and stored at -80°C until sectioned on a microtome ($25\text{ }\mu\text{m}$). For Western blot analysis, *in situ* hybridization and rtPCR studies, animals were sacrificed by rapid decapitation. Brains and various peripheral tissues were removed, immediately frozen on crushed dry ice, and stored at -80°C until use. Tissue collected for rtPCR was stored in RNeasy (Ambion, Inc., Austin, TX).

Characterization of Y4 receptor antibody:

Production of the Y4 receptor antibody:

Rabbit polyclonal antisera were raised against a peptide derived from a sequence unique to the rat NPY Y4 protein (FVTTRQKEKSNVTN). The Y4 receptor antibody was produced by J.M.H. French-Mullen. The Y4 synthetic peptide was cross-linked with the keyhole limpet carrier, injected into rabbits and sera were collected from rabbits after 5 and 6 boosters.

Single label immunocytochemistry:

These methods have been previously described (Campbell et al., 2001). Briefly, tissue sections (n=3-4, both male and female rats were used in each experiment; 1 series of sections from a 1:3 series) were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS) followed by preincubation in blocking buffer (KPBS + 0.4% Triton X-100 + 2% normal donkey serum) for 30 min at room temperature. Sections were then incubated in the Y4 primary antibody (1:10,000) in blocking buffer for 48 h at 4° C. Following washes in KPBS, tissue was incubated for 1 h in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), then subsequently washed and incubated in avidin-biotin solution (Vectastain, Vector Laboratories, Burlingame, CA) for 1 h. Y4-ir signal was further amplified using a commercial kit (tyramide signal amplification-indirect kit; NEN Life Sciences Products, Boston, MA) and was visualized with either a chromagen label, 3,3'-diaminobenzidine enhanced with nickel chloride, or a fluorescent label, FITC conjugated to strepavidin (1:1,000, Jackson ImmunoResearch Laboratories, Inc.).

To confirm antibody specificity, serial sections were stained with either the primary antibody alone, as described above, or with the primary antibody preadsorbed with control peptide (Phoenix Pharmaceuticals, Inc., Belmont, CA) for 24 h at 4° C. Preadsorption with control peptide eliminated specific antibody staining (not shown).

Western blot analysis:

Western blot analysis was used to investigate the central and peripheral distribution of the Y4 protein. Tissue was homogenized in ice-cold hypotonic buffer and then centrifuged at 1,500 rpm (200 X g) for 5 min at 4° C. The resulting pellet containing the membrane bound protein was resuspended in sample buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, pH 7.4), frozen, and stored at -80 °C. For Western blot analysis, samples were thawed on ice and 25 µg protein samples were further solubilized and separated by SDS-PAGE, using PAGEr Gold pre-cast gels (BioWhittaker Molecular Applications, Rockland, ME), and subsequently electroblotted onto PVDF membranes (Boehringer Mannheim, Indianapolis, IN). NPY Y4 protein was detected with the NPY Y4 antibody (1:5,000) and visualized using enhanced chemiluminescence (SuperSignal, Peirce, Rockford, IL) by exposure to sheet film (XOMAT, Kodak).

Production of NPY Y4 cDNA clone and rtPCR:

For generation of the rat NPY Y4 cDNA clone, total RNA was collected and PCR was performed as previously described (Brogan et al., 2000). The following primers were used: NPY Y4 forward: 5'-GACTTGCTACCCATCCTCATA-3'; and NPY Y4 reverse: 5'-ATCACCACCGCCTCATCTACA-3'. The PCR product was cloned into pGEMT and sequenced to confirm identity. For the qualitative assessment of Y4 mRNA in central and peripheral tissues, rtPCR was performed on total mRNA using the primers given above.

Double label IF:

To characterize the phenotype of cells expressing the NPY Y4 receptor double label IF coupled with confocal microscopic analysis was performed. The polyclonal orexin antibody made in the goat (Santa Cruz, CA) was used at a concentration of 1:10,000 in cocktail with the Y4 primary antibody used at 1:10,000, as described above. In the double label experiment, Y4-ir signal was visualized using FITC conjugated to streptavidin (1:1,000, Jackson ImmunoResearch Laboratories, Inc.) after biotinylated tyramide enhancement, and orexin was visualized with a donkey anti-goat antibody conjugated to an Alexa fluorophore (Alexa 546; Molecular Probes, Eugene, OR).

Double label IF was also used to investigate the phenotype of neurons expressing the NPY Y1 receptor subtype, using a previously characterized Y1 antibody (Li et al., 1999, Li et al 2000). In these experiments, the polyclonal orexin antibody was used in cocktail with the polyclonal Y1 antibody made in the rabbit (CURE/Gastroenteric Biology Center, Antibody/RIA Core, UCLA, Los Angeles, CA, used at a concentration of 1:7,000). The Y1-ir signal was visualized with a donkey anti-rabbit antibody conjugated to FITC following biotinylated tyramide enhancement, and orexin was visualized with a donkey anti-goat antibody conjugated to an Alexa fluorophore (Alexa 546; Molecular Probes, Eugene, OR).

Confocal laser microscopy:

Confocal laser microscopy, as previously described (Campbell et al., 2001), was used to analyze the double label IF images. The Leica Corp.

(Germany) TSC SP confocal system, consisting of a Leica Corp. RBE inverted microscope, an Ar laser producing light at 488nm (for visualization of FITC) and a Kr laser producing light at 568nm (for visualizing Rhodamine or Alexa 546), was used to scan the images. Various objectives (25X, NA 0.75 and 40X, NA 1.25) were used to scan and capture images. For each experiment, fluorophore signals were checked individually for bleed through to the apposing detector. All bleed through was eliminated by adjusting laser intensity and detector window width. To assess colocalization of two signals, a series of continuous optical sections, 0.5 μ m intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. The signals were obtained for each fluorophore on one series of optical sections and were stored separately as a series of 512 x 512 pixel images. The stacks of individual optical slices (0.5 μ m resolution) were analyzed using the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA) to determine colocalization. The confocal images are presented as projections of a stack of optical images. The brightness and contrast of the images were adjusted in Photoshop to match microscope visualization (Adobe Systems Inc., San Jose, CA).

Double label immunocytochemistry/ in situ hybridization for orexin and Y4:

For additional confirmation of Y4-like-ir in orexin neurons, double labeling of Y4 mRNA and orexin-ir was performed. Initially, staining for orexin-ir was performed on floating sections throughout the hypothalamus. Sections were incubated in the orexin primary antibody made in the goat (Santa Cruz, CA), used at a 1:5,000 concentration, and 2 U/ml RNase inhibitor in KPBS + 0.4%

Triton X-100 for 48 h at 4° C. Following washes in KPBS, tissue was incubated for 1 h in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc.) with 10 U/ml RNase inhibitor, then subsequently washed and incubated in avidin-biotin solution (Vectastain, Vector Laboratories, Burlingame, CA) for 1 h. Orexin-ir was visualized with the chromagen 3,3'-diaminobenzidine enhanced with nickel chloride. Sections were then rinsed and fixed in 4% paraformaldehyde, and treated with a fresh solution containing 0.25% acetic anhydride in 1 M triethanolamine (pH 8.0), followed by rinses in 2X SSC.

The NPY Y4 cRNA probe was transcribed from a 400-bp cDNA in which 100% of the UTP was ³³P labeled. Following the rinses above, tissue was incubated in sense or antisense probe (20 million counts/ml of labeled Y4 probe) overnight at 37° C. Following incubation, tissue was washed in SSC that increased in stringency and RNase A at 37° C, and then 0.1X SSC at 55° C. After washing in 0.9% saline, tissue sections were mounted onto slides and air-dried overnight. Slides were then quickly dehydrated through a series of alcohols and exposed to autoradiographic films for 1 day. Slides were then dipped in Kodak NBT2 emulsion (Kodak) diluted 1:1 in 600mM ammonium acetate and stored in light tight boxes for 5 days. Slides were developed and counterstained with cresyl violet and the distribution of silver grains was analyzed by darkfield microscopy.

Cannulation of the LHA and peptide injections:

Male rats (weighing between 250-275 g), under isofluorane gas anesthesia, were implanted with permanent, stainless steel guide cannulas (Plastics One, Inc., Wallingford, CT, 28 gauge, 8.9 mm in length), stereotaxically placed into the lateral hypothalamic area (LHA) according to the atlas of Paxinos and Watson (Paxinos and Watson, 1982, stereotax coordinates: AP -3.3, DV -8.9, L 1.2 in relation to bregma). The guide cannula was secured to the skull by acrylic dental cement and anchored with small stainless steel screws. The guide cannula was plugged with an internal dummy cannula (28 gauge, Plastics One, Inc.) until the time of injections. Only those rats displaying normal food intake and weight gain following surgery were used.

Five to six days after cannulation of the LHA, freely moving rats were remotely injected at 0900 h with rPP (2 μ g/rat in 0.5 μ l), NPY (1 μ g/rat in 0.5 μ l), or vehicle (0.5 μ l sterile artificial CSF) using a Hamilton syringe and PE tubing filled with saline (Grove and Smith, 1998). Immediately following injections, pre-weighed rat chow and water was placed in cages. The amount of food and water consumed was measured at 1 and 3 h post injection and behavior was monitored. At 1200 h, 3 h post injection, animals were sacrificed under pentobarbital anesthesia by cardiac infusion with ice-cold PBS followed by ice-cold paraformaldehyde (4%) in NaPO₄ buffer (pH 7.4). The brains were then removed, post-fixed overnight in 4% paraformaldehyde at 4° C, and saturated in 25% sucrose. Subsequently, brains were frozen on crushed dry ice and stored at -80° C until sectioned on a microtome (25 μ m).

Immunohistochemistry for cFos and LHA neuronal phenotypes:

Triple label immunohistochemistry was used to label cFos, orexin and melanin concentrating hormone (MCH) in order to quantify cFos-positive staining within these two populations of neurons in the LHA. All staining procedures were carried out in one experiment and on every third section throughout the hypothalamus. Briefly, floating tissue sections were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS). Tissue was incubated in 1% H₂O₂ in methanol for 10 min to eliminate endogenous peroxidase activity, followed by washes in KPBS and incubation in blocking buffer for 20 min (KPBS + 0.4% TritonX-100 + 2% normal donkey serum) to reduce background. Sections were then incubated in a cocktail of primary antibodies, including the cFos antibody made in the rabbit (Santa Cruz, SC-52) and used at concentration of 1:25,000, the orexin antibody made in the goat (Santa Cruz, CA) and used at a concentration of 1:10,000, and the MCH antibody made in the chicken (Bachem, CA) and used at a concentration of 1:3,000, for 48 h at 4°C. Following washes in KPBS, tissue was incubated for 1 h in biotinylated donkey anti-rabbit antibody (1:600, Jackson ImmunoResearch Laboratories, Inc.), then subsequently washed and incubated in avidin-biotin solution (Vectastain, Vector Laboratories, Burlingame, CA) for 1 h. c-Fos-ir was visualized with the chromagen 3,3'-diaminobenzidine enhanced with nickel chloride. The sections were then rinsed and incubated with fluorescent secondary antibodies. Orexin was visualized with FITC conjugated to a donkey anti-goat antibody (1:200 Jackson ImmunoResearch Laboratories, Inc.) and

MCH was visualized with rhodamine conjugated to a donkey anti-chicken antibody (1:200 Jackson ImmunoResearch Laboratories, Inc.). Sections were mounted onto subbed slides, cover slipped with glycerol, and sealed.

Quantification of cFos staining in orexin and MCH neurons:

Only those animals with proper cannula placement in the LHA were used in this study. Blinded to the treatment group, orexin and MCH neurons were counted as positive or negative for cFos on both ipsilateral and contralateral sides, relative to the injection site, using a Zeiss Axioskop 2 fluorescence microscope with an adjustable xenon light intensity (Zeiss AttoArc 2). Orexin and MCH positive neurons were counted in series from the rostral to caudal extent of the LHA. On average, 680 orexin and 1226 MCH neurons were counted per rat brain. Images from selected sections were captured using a Leica Corp. (Germany) TSC SP confocal system.

Statistical analysis:

The food and water intake, cell count, and cFos data were analyzed by one-way ANOVA using GraphPad Software, Inc. Prism software (GraphPad Software, Inc., San Diego, CA) followed post-hoc by Newman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

RESULTS:

Neuroanatomical findings:

Analysis of the single label distribution of chromagen Y4-ir demonstrated low levels of staining throughout the hypothalamus (Fig. 1A). The majority of

staining appeared to be in small round cells. Unexpectedly, the most intensely labeled Y4-ir cell bodies were found in the larger cells of the lateral hypothalamic area (LHA, Fig. A,B). Densely labeled Y4-ir cells were apparent throughout the rostral to caudal extent of the LHA.

To provide additional characterization of the Y4 antibody and Y4 distribution in both central and peripheral structures, we correlated Y4 mRNA and protein expression using PCR, Western blot analysis and immunocytochemistry. Centrally, Y4 mRNA was present in both the hypothalamus and cortex (Fig. 2A). In peripheral structures, all the areas investigated, including kidney, liver, duodenum, large intestine, colon, stomach and lung, expressed Y4 mRNA (Fig. 2B). By Western blot analysis, the Y4 antibody identified Y4 protein within membrane extracts of both the hypothalamus and the cortex (Fig. 2C) and all the peripheral areas tested (Fig. 2D). Finally, by immunocytochemistry, Y4-ir was observed in both the hypothalamus and the cortex. The Y4 antibody also identified Y4-ir in peripheral tissues. Figure 2G shows an example of IF labeling in the duodenum in which Y4-ir was observed within goblet cells and along the basal lamina of intestinal villi. Collectively, these data suggest that the Y4 antibody recognizes the membrane bound Y4 receptor in both peripheral and central tissues.

Double label IF experiments indicated that the majority of neurons displaying Y4-ir observed in the LHA (green, Fig. 3A) is colocalized with orexin, whose cell bodies are also entirely restricted to this region (red, Fig. 3B). While there was dense overlap of these proteins in cell bodies, as shown by the yellow

color, there were only a few orexin fibers that colocalized the Y4 receptor in this region (Fig. 3C). Y4-ir was also observed in sparse fibers throughout the hypothalamus (green, Fig. 3D) and all of these fibers were identified as colocalized with orexin (yellow, Fig. 3F). Double labeling of Y4 mRNA by *in situ* hybridization and orexin by chromagen immunocytochemistry provided additional confirmation for the expression of Y4 receptors on orexin neurons. Silver grain clusters are clearly seen colocalized with orexin neurons in the LHA (Fig. 4 A-D). However, there also appear to be a number of non-orexin neurons in the LHA that express Y4 mRNA.

An additional double label IF study investigated the expression pattern of the NPY Y1 receptor subtype within the LHA and in relation to orexin neurons. Y1-ir (green, Fig. 5A-D) was observed on numerous fibers and some cell bodies (yellow arrows) within the LHA. Many Y1-ir fibers surrounded orexin neurons (red), and appeared to make close contact (yellow) as indicated by white arrows (Fig. 5 A-D); however, Y1-ir was not observed colocalized with orexin neurons or fibers.

Food and water intake:

In order to provide additional confirmation for Y4 expression on orexin neurons in the LHA and to investigate the effects of Y4 stimulation on food and water intake regulation, rPP and NPY were administered directly into the LHA. Both NPY and rPP resulted in a significant increase in food and water intake (Fig. 6). NPY elicited a robust feeding response, with 3.06 ± 0.23 g and 4.69 ± 0.71 g of cumulative food intake at 1 and 3 h time points. rPP caused a more moderate

food intake response, with 1.05 ± 0.23 g and 2.60 ± 0.33 g of cumulative food intake at 1 and 3 h time points. Food intake stimulated by rPP was significantly greater than vehicle ($p < 0.01$ at 1h, $p < 0.001$ at 3h) and significantly less than that stimulated by NPY ($p < 0.001$ at 1 h, $p < 0.01$ at 3 h, Fig. 6 A,B). Vehicle injected controls did not consume any food during this period, as would be expected during day light hours. Although, controls consumed a small amount of water; NPY and rPP were equally effective at significantly stimulating water intake at both 1 and 3 h time points ($p < 0.05$, Fig. 6 C,D).

Investigation of neuronal activation using cFos as a marker:

Immunohistochemical detection of cFos induction was used to investigate the LHA cell populations sensitive to either rPP or NPY injections. Both rPP and NPY induced cFos expression ipsilateral to the injection site, while vehicle injections resulted in a low level of cFos induction directly adjacent to the injection site (Figure 7 A-C). Contralateral to the injection, extremely low levels of cFos were observed in all groups (Fig. 7 D-F). rPP administered to the LHA resulted in a robust induction of cFos expression in orexin neurons on the ipsilateral side (Figs. 8, 9). $59.3 \pm 8.5\%$ of orexin neurons were cFos positive 3 h following rPP injection, a significantly greater percentage than that produced by NPY or vehicle injections ($18.5 \pm 4.7\%$ and $21.7 \pm 5.4\%$ respectively, $p > 0.001$, Figs. 8,9). Very few orexin neurons on the contralateral side of the injection displayed cFos expression in any treatment group (Fig. 8B).

The number of cFos positive MCH neurons was extremely low and not significantly different among any treatment group on either the ipsilateral or

contralateral side (Fig. 10 A,B). The number of ipsilateral MCH cells displaying cFos, 10.80 ± 4.0 , 12.29 ± 4.43 , 9.33 ± 4.68 , from vehicle, rPP, and NPY injected animals respectively, represents only a small percentage (~1%) of the number of MCH neurons analyzed per animal.

DISCUSSION:

The present study demonstrates for the first time that orexin neurons in the LHA coexpress the NPY Y4 receptor subtype and are activated by a Y4 specific ligand. Using a newly characterized NPY Y4 antibody, a low level of Y4-ir was observed in small, round cells throughout the hypothalamus. However, the most intensely labeled Y4-ir cell bodies in the hypothalamus were observed in the LHA (Fig. 1A,B). The overall pattern of Y4-ir was in agreement with the *in situ* hybridization pattern of Y4 mRNA presented previously (Parker and Herzog, 1999) and in the current study, as well as the previously reported pattern of Y4 receptor binding (Dumont and Quirion, 2000). Additional characterization of the Y4 antibody demonstrated a complete correlation between Y4 mRNA, as detected by rtPCR, and protein expression, as detected by Western blot analysis and immunocytochemistry, in both central and peripheral regions (Fig. 2A-G), supporting the specificity and sensitivity of this antibody.

Interestingly, by immunocytochemistry, all of the Y4-ir observed in the LHA was colocalized with orexin neurons (Fig. 3). The colocalization of Y4 mRNA expression in orexin neurons supports this finding (Fig. 4); however, Y4 mRNA was also expressed in a number of non-orexin expressing cells. Orexins

have been found to regulate arousal states, influence drinking and feeding, and to play a role in the modulation of neuroendocrine processes, including luteinizing hormone release and thermoregulation (for reviews see Sakurai, 2002; Sutcliffe and de Lecca, 2002; Beuckmann and Yanigasawa, 2002). Central injection of orexin A stimulates food intake in satiated rats (Dube et al., 1999; Haynes et al., 1999) and orexin mRNA is regulated by fasting (Sakurai et al. 1998). However, while orexin-A increases daytime feeding, overall 24h food intake is not increased and repeated injections have no effect on body weight (Yamanaka et al., 1999). Therefore, orexin may be important in short-term feeding episodes or in the initiation of feeding, but is not necessarily implicated in obesity. Alternatively, numerous studies have shown that orexin is key signal in arousal-associated behavior (Sutcliffe et al., 2002). Thus, the orexin system might be responsible for the complex coordination of behavioral and physiological responses to an animal's nutritional needs. Determining the neuroanatomical pathways that influence orexin activity will aid in the understanding of the relative importance of orexin in these physiological roles.

While the neuroanatomical results presented above strongly support a direct role of the Y4 receptor on orexin neurons, this needed to be directly tested. Injection of rPP, a selective Y4 receptor ligand, directly into the LHA of freely moving rats, elicited a robust dipsogenic response and a moderate food intake response (Fig. 5). These results resemble previously reported responses following orexin administration. Orexin A, given icv, increases water intake at a level comparable to angiotensin II, and deprivation of water up-regulates orexin

mRNA (Kunii et al., 1999). As mentioned above, orexin has been implicated in the regulation of food intake; however orexin is a much less potent orexigenic agent than NPY (Edwards et al., 1999). Similar to other studies (Stanley et al., 1993), the present study demonstrated a robust increase in both food and water intake following NPY injection into the LHA. Therefore, the rPP injection closely resembled orexin actions while the NPY injection had a greater effect on food intake, suggesting that rPP may be activating a different population of neurons than NPY.

Indeed, while both rPP and NPY caused a robust induction of cFos expression in the LHA, rPP, but not NPY, induced cFos in orexin neurons. Although these findings cannot determine whether rPP effects are directly on orexin neurons, a direct effect is likely given the neuroanatomical evidence for Y4-ir colocalization on orexin neurons. However, we cannot eliminate the possibility that the stimulation of food intake by rPP is via actions on other populations of neurons. While rPP did not stimulate cFos expression in MCH neurons, it does activate a population of neurons distinct from orexin. Furthermore, although Y4-ir was not observed in non-orexin neurons, Y4 mRNA was observed in non-orexin cells in the LHA (Fig. 4). These data suggest that either the Y4 antibody may not be sensitive enough to detect low levels of Y4 protein, or that Y4 mRNA in these cells may not be translated under the present conditions. Additional studies are necessary to identify the phenotypes of this population, and it remains to be determined if rPP stimulates food and water intake directly via orexin neurons or this unidentified population of neurons.

Surprisingly, NPY injection into the LHA did not significantly stimulate cFos in either orexin or MCH neurons, although, cFos expression was stimulated in the LHA in a population of neurons with an unknown phenotype. This contrasts with previous studies showing that NPY injection into the third ventricle results in the induction of cFos in a small percentage of orexin neurons (Niimi et al., 2001). This discrepancy is most likely due to the mode of administration. Injections into the third ventricle may activate a number of other systems that project to the LHA. Thus, the induction of cFos observed may be a second or third order event. The lack of significant cFos induction in MCH and orexin neurons was particularly unexpected in light of the abundance of NPY terminals in the LHA and in contact with orexin and MCH neurons (Broberger et al., 1998; Horvath et al., 1999). Reciprocal connections of orexin with NPY and α -melanin-stimulating hormone neurons in the ARH have also been described (Elias et al., 1998), and it has been suggested that these are key circuits in the maintenance of food intake signals. However, NPY projections to the LHA may be working through other NPY receptor subtypes, either presynaptically or postsynaptically on different phenotypes of neurons. The lack of cFos induction in orexin and MCH neurons is supported by the lack of Y1 receptor expression on either of these phenotypes (Fig. 4). Y1-ir was found on fibers and cells of an unknown phenotype and in close contact with orexin and MCH neurons, suggesting presynaptic NPY effects via the Y1 receptor. Future studies will attempt to identify the phenotypes of neurons expressing the Y1 receptor. The Y5 receptor has also been identified in the LHA (Campbell et al., 2001), although the phenotype of cells expressing the

Y5 receptor subtype also remains to be determined. In addition to MCH and orexin, the LHA is known to contain large numbers of glucose-receptive neurons (Bernardis and Bellinger, 1996) of unknown phenotypes, as well as the orexigenic neuropeptide galanin (Melanders et al., 1996). What is clear from these studies is that orexin and MCH neurons do not express the Y1 receptor and are not activated by NPY when NPY is administered directly to the LHA.

Although previous reports clearly illustrate the activation of central Y4-like receptors (Jain et al., 1999; Raposo et al., 2000) and the present study demonstrates physiological and cFos responses to the peripheral Y4 ligand, rPP, an endogenous ligand for the *centrally* expressed Y4 receptor has not yet been characterized in the hypothalamus or other regions of the brain. Early studies identified PP-like immunoreactivity in the brain (Inui et al., 1985; Loren et al., 1979; Lundberg et al., 1980; Olschowka et al., 1981); however, later studies were not able to detect PP-like peptide in the brain (Miyazaki and Funakoshi, 1988) leading to the view that earlier studies were probably detecting NPY or another substance altogether. PP, the only known endogenous high affinity ligand for the Y4 receptor, is produced in the periphery, in the islet cells of the endocrine pancreas. Plasma concentration of PP increases rapidly after ingestion of food, serving as an active regulator of digestive function. Peripherally produced rPP, however, does not readily cross the blood brain barrier. Therefore, while the presence and activation of central Y4 receptors has been established, additional studies are required to identify the endogenous ligand and mechanism of activation.

The Y4 receptor has also been implicated in the regulation of reproductive function. In addition to studies implicating the Y4 receptor in the stimulation of LH secretion (Raposinho et al., 2000; Jain et al., 1999), studies extending from the Y4 knockout model also suggest a role for the Y4 receptor in suppression of reproductive function. Crossing Y4 knockout mice with infertile ob/ob mice lacking leptin results in the partial restoration of reproductive function (Sainsbury et al., 2002). The absence of leptin mediated inhibition of NPY results in chronically elevated NPY peptide and mRNA. Sainsbury and colleagues argue that the high hypothalamic NPY levels present in the ob/ob mouse are sufficient to bind Y4 receptors and suppress reproductive function. Interestingly, in the ob/ob mouse, orexin levels are suppressed (Stricker-Krongrad et al., 2002), suggesting that high levels of NPY may indeed be inhibiting orexin neurons via the Y4 receptor in this model. Thus, the Y4 receptor may bind NPY at chronically high levels in the ob/ob mouse. The current data would argue that rPP and NPY bind to different populations of neurons in a wild type rat; however, if NPY is inhibitory through the Y4 receptor on orexin neurons, this would not be detectable using cFos as a marker of neuronal activation, and therefore the lack of cFos induction in the current study would fit with an inhibition of these neurons. The importance of orexin activation in the stimulatory and inhibitory effects of Y4 activation and neuroendocrine reproductive function remain to be addressed.

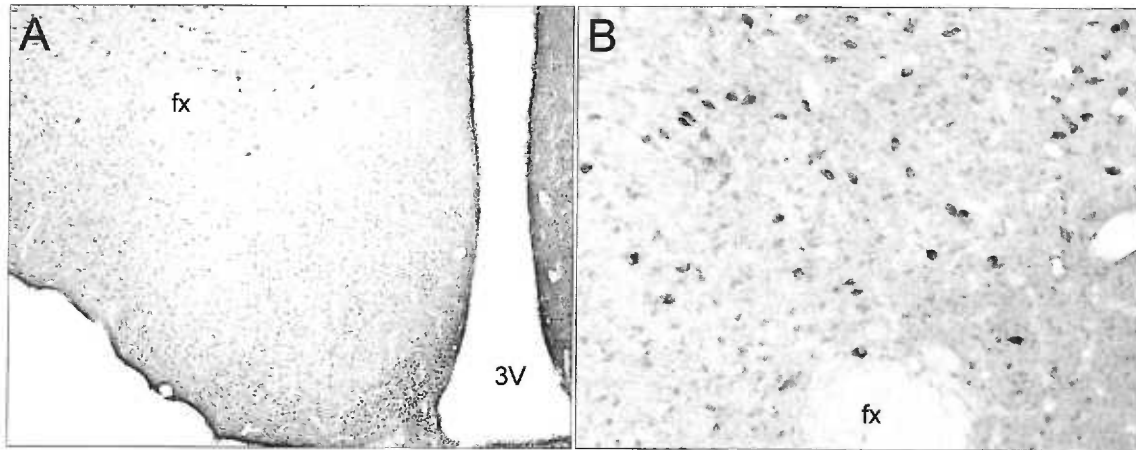


Figure 5-1. Single label hypothalamic distribution of NPY Y4-ir. Panel A shows a low power photomicrograph example of low levels of Y4-ir expressed in small round cells throughout the hypothalamus. A greater density of Y4-ir cells can be seen in the arcuate nucleus, but the most intensely labeled cells were found in the LHA (A). A higher power view of Y4-ir cells in the LHA is shown in panel B.

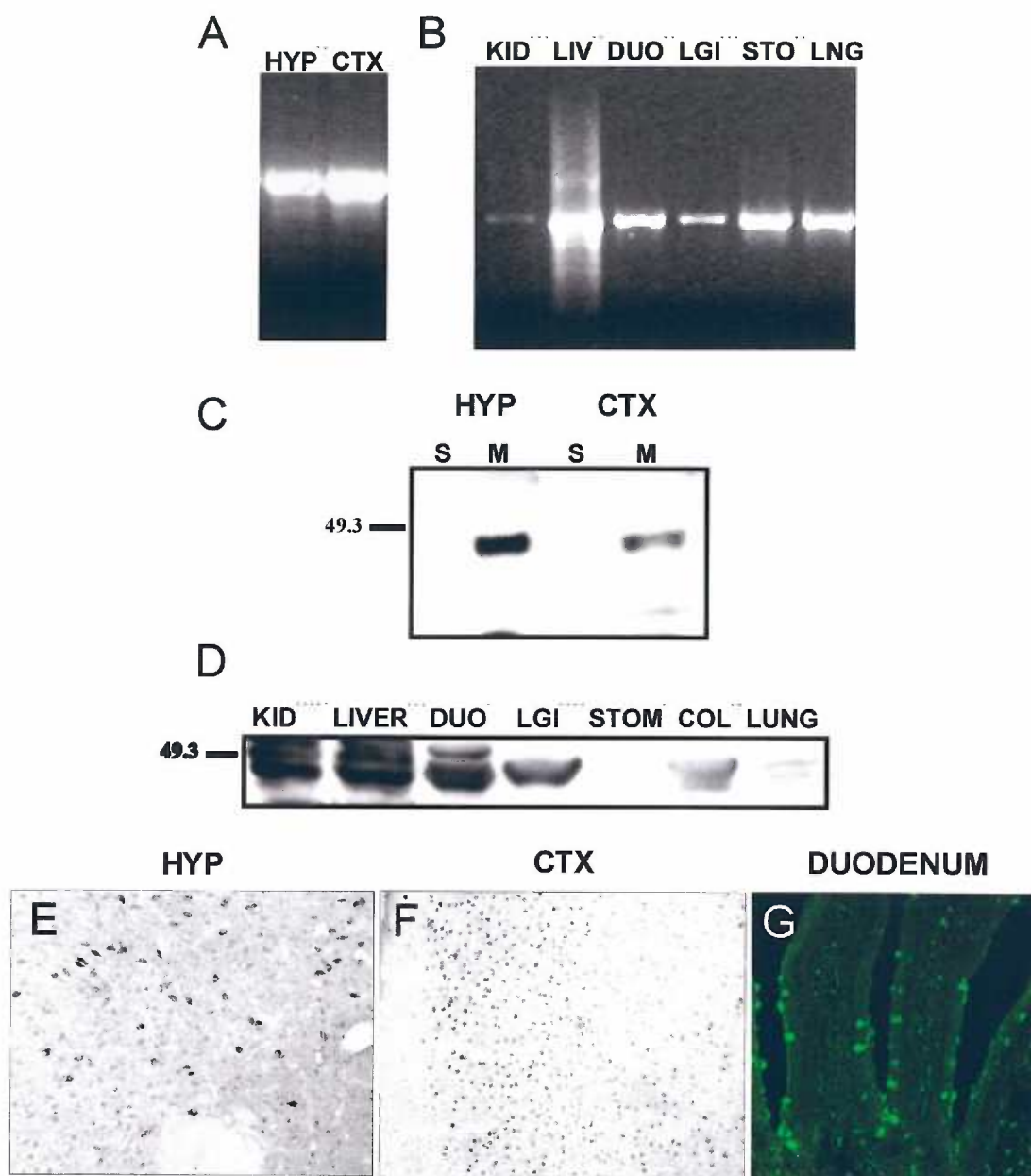


Figure 5-2. Correlation of Y4 mRNA and protein in central and peripheral tissues. Y4 mRNA was found by rtPCR in the hypothalamus (HYP) and cortex (CTX) (A) and throughout peripheral structures (B); including kidney (KID), liver, duodenum (DUO) large intestine (LGI), stomach (STOM) and lung. Western blot analysis shows Y4 protein in membrane (m) fractions of the HYP and CTX (C), and in all peripheral structures (D). Y4-ir was identified in cells of the HYP (E), the CTX (F) and throughout peripheral tissues; the duodenum is shown as an example (G).

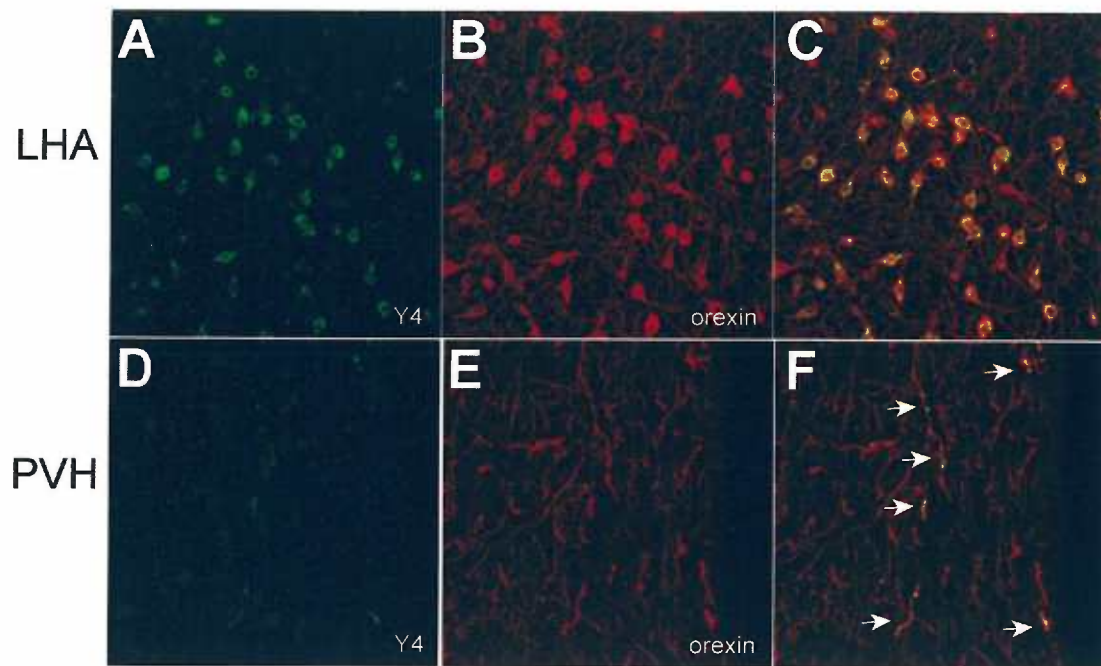


Figure 5-3. NPY Y4-ir colocalizes with orexin in the LHA. Double label IF for Y4 (A,D, green), and orexin (B,E, red) shown in LHA cell bodies (A-C) and a representative fiber in the paraventricular nucleus of the hypothalamus (PVH, D-F). Their colocalization is indicated by the yellow color (C,F).

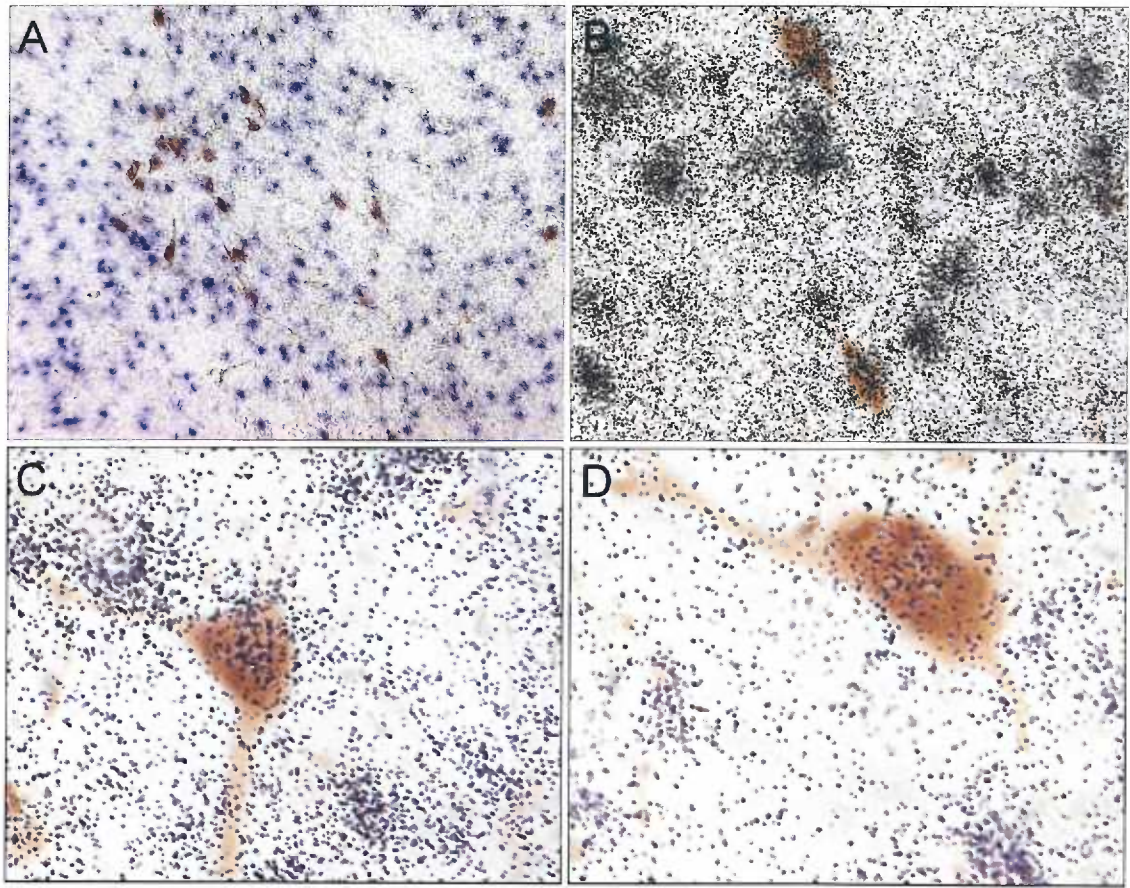


Figure 5-4. Y4 mRNA is coexpressed in orexin neurons. Representative photomicrographs show at low (A) and high (B-D) power, Y4 mRNA, identified by silver grain clusters (purple/black color), throughout the LHA and double labeled with orexin neurons (brown).

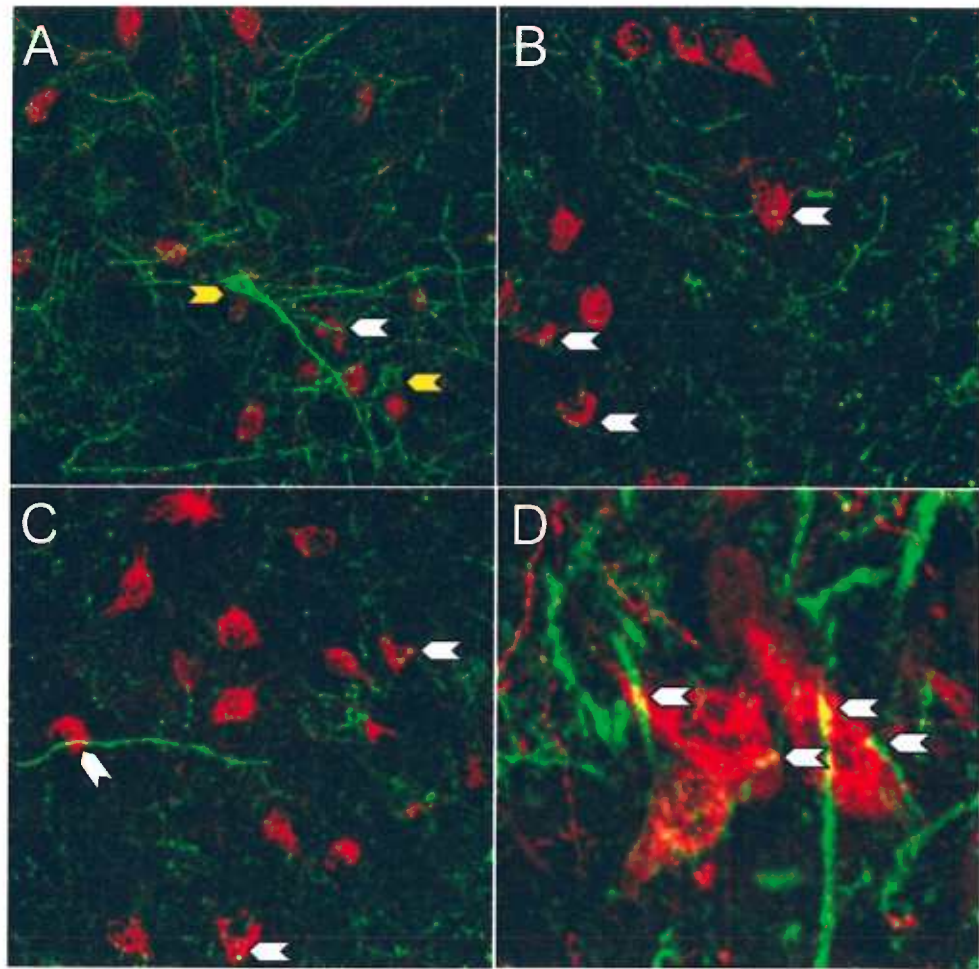


Figure 5-5. NPY Y1-ir on fibers and cells of an unknown phenotype in the LHA. Double label IF for Y1 (green) and orexin (red) in the LHA (A-D) shows Y1-ir on non-orexin cells (yellow arrow) and on fibers surrounding orexin neurons. Y1-ir is not colocalized with orexin neurons, but Y1-ir fibers appear to make close contacts with orexin neurons (white arrows).

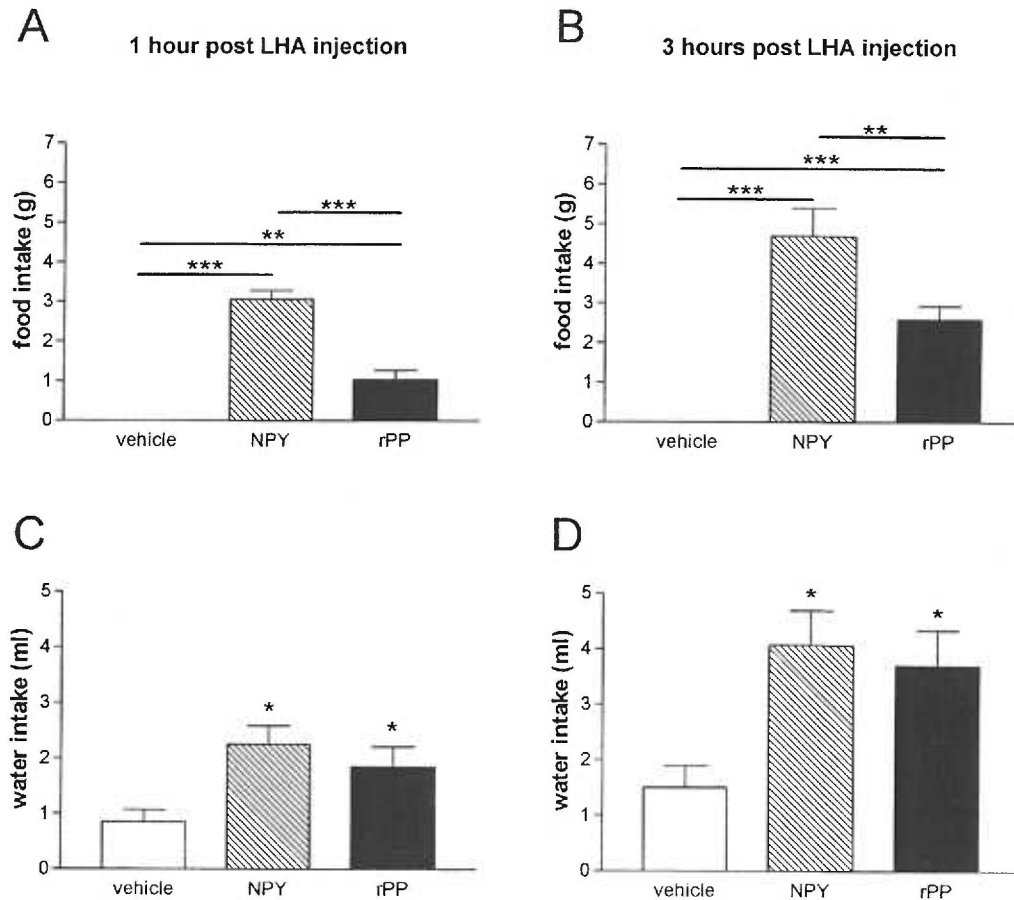


Figure 5-6. Intra-LHA administration of NPY and rPP stimulates food and water intake. Mean $\pm$ SEM cumulative food intake (A,B) and water intake (C,D) 1 and 3 h after intra-LHA injection of vehicle (open bars), NPY (hatched bars), or rPP (solid bars), $n=10-11/\text{group}$. NPY caused a significant increase in food intake compared with both vehicle and rPP injected animals ($**p<0.01$, $***p<0.001$). rPP significantly increased food intake compared to vehicle ($***p<0.001$). Both NPY and rPP significantly increased water intake compared with vehicle ($*p<0.05$).

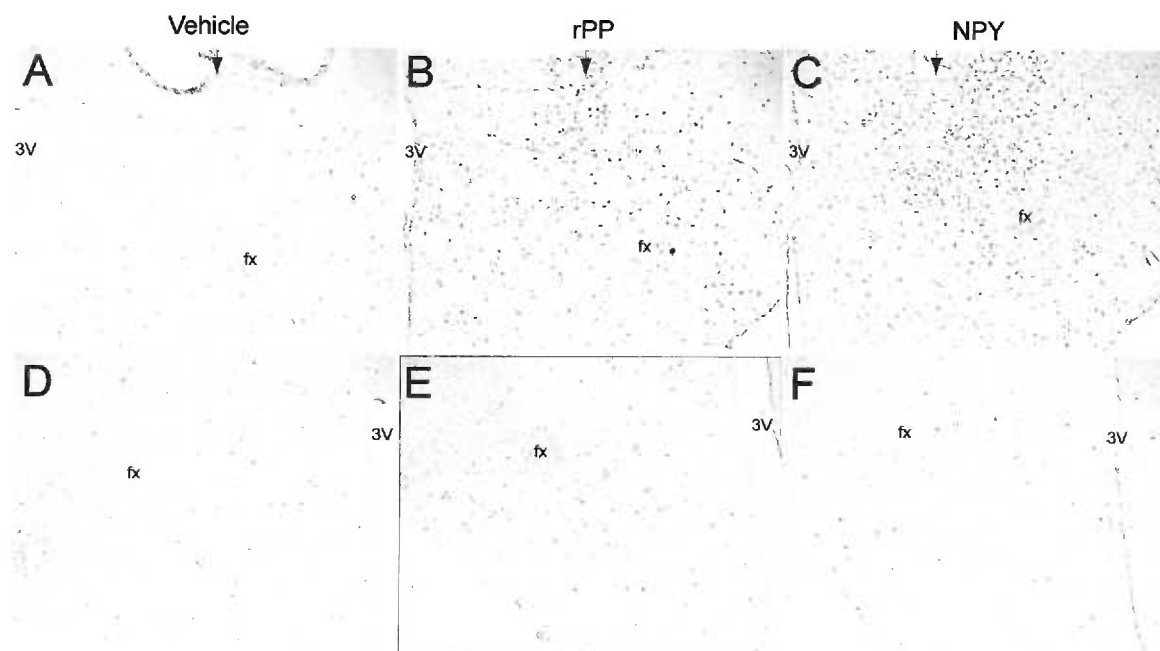


Figure 5-7. Single label cFos expression on ipsilateral and contralateral sides of intra-LHA injections. Representative photomicrographs show cFos expression in the LHA on the ipsilateral (A-C) and contralateral (D-F) side of the injection site (arrow) from animals receiving either vehicle (A,D), rPP (B,E), or NPY (C,F).

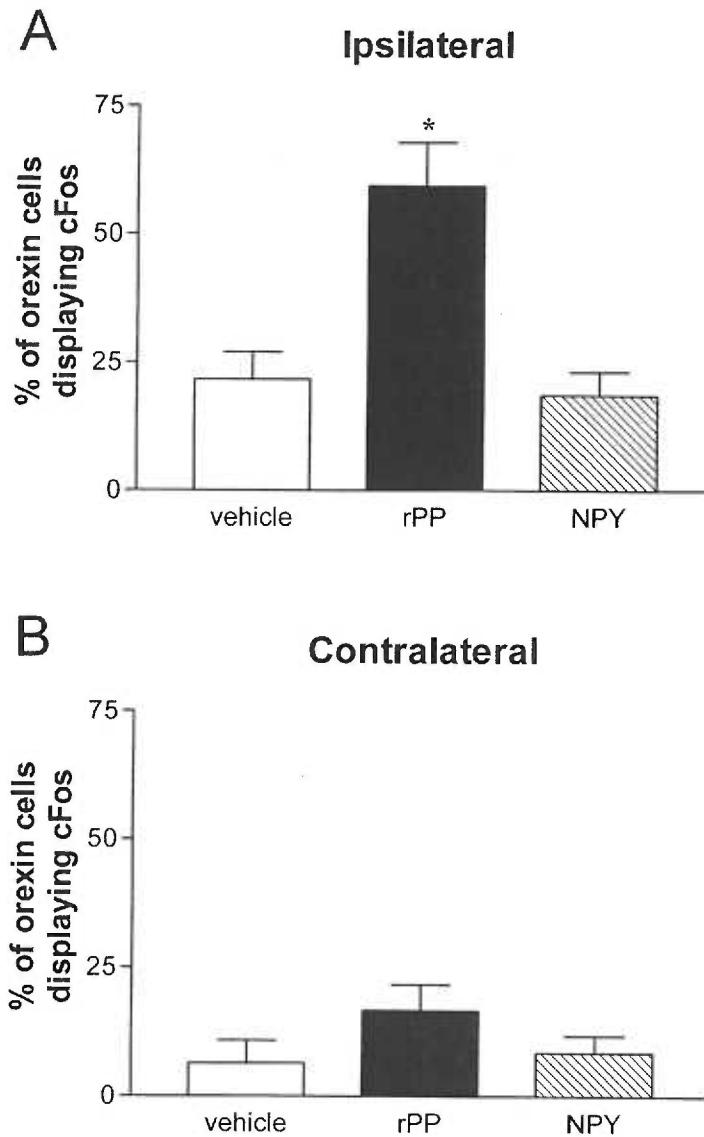


Figure 5-8. Intra-LHA rPP, but not NPY induces a robust increase in cFos expression in orexin neurons. Mean $\pm$ SEM percentage of orexin cells displaying cFos 3 h after intra-LHA injection of vehicle (open bars), NPY (hatched bars), or rPP (solid bars), $n=8-10/\text{group}$. rPP injection resulted in a robust induction of cFos expression ipsilateral to the injections site compared to vehicle and NPY (A, $*p<0.05$). Very low levels of cFos expression was seen in orexin neurons contralateral to the injection site (B) and there were no differences between groups.

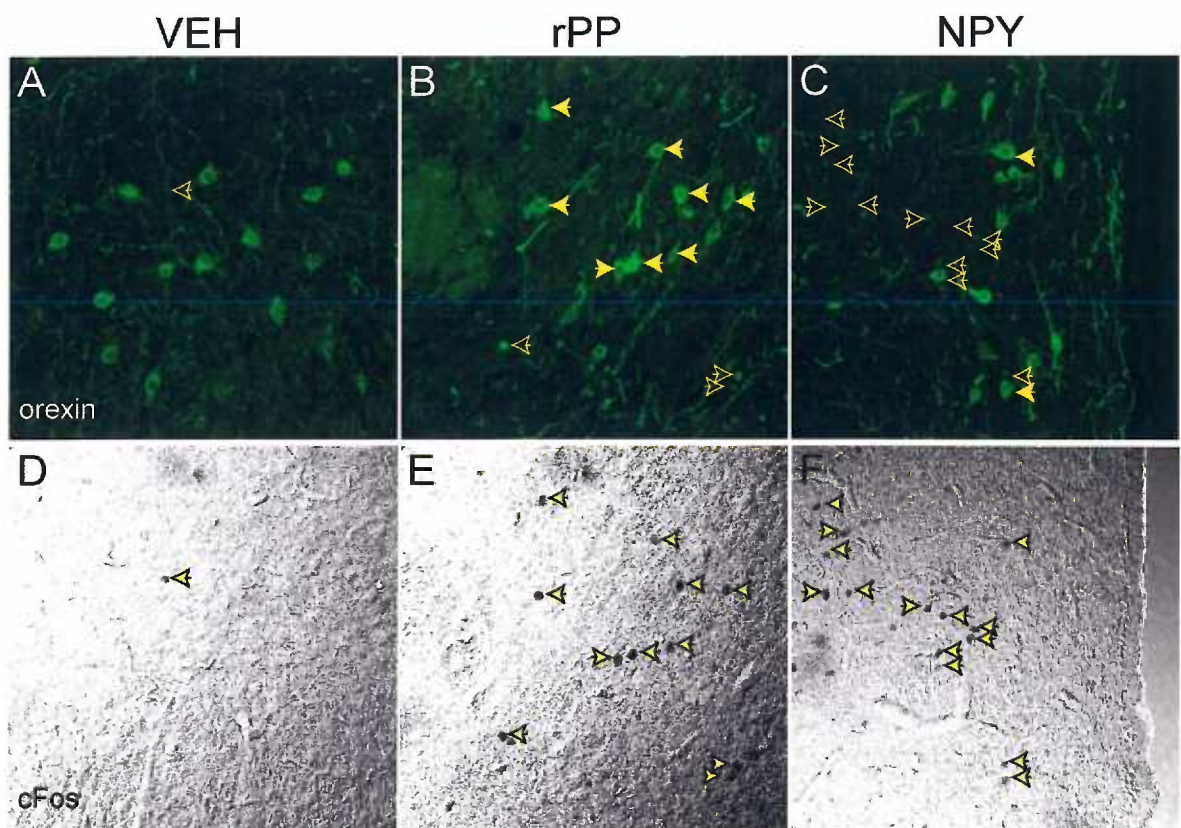


Figure 5-9. Orexin neurons and cFos expression following intra-LHA injections of vehicle, rPP or NPY. Representative images showing orexin-ir (green, A-C) and cFos-ir (black, D-F) in identical fields from animals injected with either vehicle (A,D), rPP (B,E), or NPY (C,F). Arrows in the bottom panels (D-F) identify cFos-ir neurons. In the top panels (A-C), aligned arrows mark the area of cFos expression. Open arrows represent lack of cFos colocalization with an orexin-ir neuron, and filled yellow arrows represent cFos colocalization with an orexin-ir neuron.

Chapter VI

Gonadotropin-Releasing Hormone Neurons Coexpress Orexin 1 Receptor Immunoreactivity and Receive Direct Contacts by Orexin Fibers

Endocrinology

submitted

Introduction

The neuropeptides orexin A and orexin B, also known as hypocretin-1 and hypocretin-2, have recently been characterized (de Lecca et al., 1998; Sakurai et al., 1998) and implicated in the regulation of arousal states, energy metabolism and neuroendocrine reproductive function (for reviews see Sakurai, 2002; Sutcliffe and de Lecca, 2002; Beuckmann and Yanigasawa, 2002). The peptides are derived from a common precursor and their expression has been identified in neuronal cell bodies of the lateral hypothalamic area (LHA). Although orexin expressing cell bodies are restricted to this area, they send widespread projections throughout the brain and spinal cord. Orexin actions are mediated via G-protein coupled receptors of two different subtypes (OX-R1 and OX-R2). OX-R2 is a non-selective receptor that binds both orexin-A and -B, while OX-R1 is highly selective for orexin-A (Sakurai et al., 1998). Complementary to the widespread projections of orexin fibers (Peyron et al., 1998), OX-R1 and OX-R2 mRNA and protein have been found widely expressed in the brain (Trivedi et al., 1998; Marcus et al., 2001; Hervieu et al., 2001; Cluderay et al., 2002).

Recent physiological and neuroanatomical studies suggest that orexin A may play an important role in control of the hypothalamo-pituitary gonadal (HPG) axis. For example, it has been shown that central administration of orexin A stimulates LH secretion. However, similar to other orexigenic peptides, orexin has been shown to have bimodal effects on LH secretion, with orexin stimulating LH secretion in steroid-primed ovariectomized (OVX) rats, but inhibiting LH secretion in the absence of ovarian steroids (Pu et al., 1998; Tamura et al.,

1999). It has also been demonstrated that immunoneutralization of orexin A in the brain completely abolishes both LH and prolactin surges while icv administration of orexin leads to a dose-dependent recovery of fasting suppressed LH (Kohsaka et al., 2001). In addition, explant studies have shown that orexin A stimulates GnRH release from the hypothalamus of both intact male rats and female rats at proestrus (Russell, 2001). Recently, a study employing low dose estrogen replacement in OVX rats, resulting in serum levels similar to those observed in diestrus, demonstrated that low dose estrogen potentiates the suppressive effects of centrally administered orexin A on LH secretion (Furuta et al., 2002), suggesting that orexin A is inhibitory during diestrus, or low estrogen states, and excitatory during surge conditions. Although orexin is clearly implicated in the regulation of GnRH/LH secretion, the neuroanatomical pathway by which orexin may be mediating its effects in the rat remains to be determined. The first aim of the present study was to investigate whether orexin fibers project to and come in close contact with GnRH neurons and if GnRH neurons express OX-R1 in the rat.

Similar to orexin, neuropeptide Y (NPY) has been found to elicit bimodal effects on LH release. In addition to the reported involvement of Y1 and Y5 receptor subtypes in the modulation of LH secretion (Leupen et al., 1997; Raposinho et al., 1999), several lines of evidence support a role for the Y4 receptor subtype in the regulation of GnRH activation and reproductive function. Activation of Y4 receptors, with a Y4 agonist, 1229U91, or a high affinity ligand for the Y4 receptor, rat pancreatic polypeptide (rPP), stimulates GnRH neurons

and in turn LH release in intact male rats (Raposinho et al., 2000) and is important in the basal release of LH in the female rat (Jain et al., 1999). Conversely, when the infertile, leptin deficient *ob/ob* mouse is crossed with the Y4 knockout mouse, fertility is partially restored, suggesting the Y4 receptor is involved in suppressing reproductive function (Sainsbury et al., 2002). Although the exact nature of Y4 receptor action needs clarification, this evidence indicates that it is clearly playing a role in modulation of the HPG axis. Recently, our laboratory identified Y4-like immunoreactivity (Y4-ir) on orexin cell bodies and fibers and also found that injection of the Y4 endogenous ligand, rPP, induced robust cFos activation in orexin neurons (Campbell et al., 2002). Taken together, these data suggest that some of the effects of Y4 activation on LH release may be mediated via orexin. Therefore, the second aim of the present study was to characterize the neuroanatomical relationship between orexin/Y4-ir fibers and GnRH neurons.

Materials and Methods

Animals and Tissue:

Male and virgin cycling female rats (Simonsen, CA) were housed individually and maintained under a 12:12 light dark cycle (lights on at 0700h) and constant temperature ($23 \pm 2^{\circ}\text{C}$). Food and water were available *ad libitum*. All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

For immunocytochemical studies, animals (n=3-4, both male and female rats were used in each experiment) were sacrificed under pentobarbital anesthesia by cardiac infusion with ice-cold PBS followed by ice-cold paraformaldehyde in NaPO₄ buffer (pH 7.4). The brains were then removed, saturated in 25% sucrose, frozen in cooled isopentane, and stored at -80 °C until sectioning on a microtome (25 µm).

Double and Triple Label Immunofluorescence (IF):

All immunofluorescent labeling, as previously reported (Campbell et al., 2001), was performed on floating sections. Double and triple label IF for orexin, GnRH and either synaptophysin, OX-R1, or Y4 were performed using a cocktail of primary antibodies. The polyclonal orexin antibody made in the goat (Santa Cruz, CA) was used at a concentration of 1:10,000. The monoclonal gonadotropin-releasing hormone (GnRH) antibody (a kind gift of H.F. Urbanski, ONPRC, OR) was used at a concentration of 1:500. The polyclonal synaptophysin antibody (DAKO Corp., CA) was used at a concentration of 1:200. The polyclonal OX-R1 antibody (a kind gift of G. Hervieu, Smith Kline Beecham, UK) was used at a concentration of 1:500, and the polyclonal Y4 antibody (a kind gift of J. ffrench-Mullen) was used at a 1:10,000 concentration along with the BLAST technique of biotinylated tyramide enhancement. Each primary antibody was initially titrated and characterized individually.

First, double labeling for orexin and GnRH was performed. Briefly, floating tissue sections were removed from cryoprotectant and washed in 0.05 M potassium phosphate-buffered saline (KPBS). Tissue was incubated in blocking

buffer (KPBS + 0.4%TritonX-100 + 2% normal donkey serum) for 20 min to reduce background and then incubated in a cocktail of the orexin and GnRH primary antibodies for 48 h at 4°C. Following washes in KPBS, tissue was incubated for 1 h in a cocktail of fluorescent secondary antibodies. Orexin was visualized with TRITC conjugated to a donkey anti-goat antibody (1:200 Jackson ImmunoResearch Laboratories, Inc.) and GnRH was visualized with FITC conjugated to a donkey anti-mouse antibody (1:200 Jackson ImmunoResearch Laboratories, Inc.).

Subsequently, double labeling for GnRH and OX-R1 was performed. As in the previous experiment, GnRH was visualized with FITC conjugated to a donkey anti-mouse antibody (1:200) and OX-R1 was visualized with TRITC conjugated to a donkey anti-rabbit antibody (1:200 Jackson ImmunoResearch Laboratories, Inc.).

Triple label experiments for GnRH/orexin/synaptophysin, GnRH/OX-R1/orexin or GnRH/orexin/Y4 were carried out in a similar manner. In each triple label experiment, GnRH was visualized with FITC conjugated to a donkey anti-mouse antibody (1:200). In the first triple label experiment, orexin was visualized with TRITC conjugated to a donkey anti-goat antibody (1:200) and synaptophysin was visualized with Cy5 conjugated to a donkey anti-rabbit antibody (1:200 Jackson ImmunoResearch Laboratories, Inc.). In the second triple label experiment, OX-R1 was visualized with TRITC conjugated to a donkey anti-rabbit antibody (1:200) and orexin was visualized with Cy5 conjugated to a donkey anti-goat antibody (1:200). The final triple label experiment used Cy5 conjugated to a

donkey anti-rabbit antibody (1:200) to visualize Y4 and TRITC conjugated to a donkey anti-goat antibody (1:200) to visualize orexin. Sections from all experiments were mounted onto subbed slides, cover slipped with glycerol, and sealed.

Confocal Laser Microscopy:

Confocal laser microscopy, as previously described (Campbell et al., 2001), was used to analyze the double and triple label IF images for colocalization and close contacts. The Leica Corp. (Germany) TSC SP confocal system, consisting of a Leica Corp. RBE inverted microscope, an Ar laser producing light at 488nm (for visualization of FITC), a Kr laser producing light at 568nm (for visualizing TRITC), and a HeNe laser producing light at 647 (for visualization of Cy5), was used to scan the images. Various objectives (25X, NA 0.75 and 40X, NA 1.25) were used to scan and capture images. For each experiment, fluorophore signals were checked individually for bleed through to the apposing detector. All bleed through was eliminated by adjusting laser intensity and detector window width. To assess colocalization of two signals, a series of continuous optical sections, 0.25 μ m or 0.5 μ m intervals along the z-axis of the tissue section, were scanned for each fluorescent signal. The signals were obtained for each fluorophore on one series of optical sections and were stored separately as a series of 512 x 512 pixel images. The stacks of individual optical slices (0.25 μ m or 0.5 μ m resolution) were analyzed using the MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA) to determine colocalization and contacts. The confocal images are presented as projections of stacks of

optical images or as individual slices, as indicated. The brightness and contrast of the images were adjusted in Photoshop to match microscope visualization (Adobe Systems Inc., San Jose, CA).

Results

Double label IF studies showed that orexin fibers (red) surround GnRH neurons (green) and make close contact with them (yellow color indicates colocalization, Fig.1 A,B,C). Greater than 80% of GnRH neurons observed were contacted by orexin fibers and no differences were observed between male and female animals. Using synaptophysin (a protein localized in synaptic vesicles) as a marker of synaptic contacts, orexin fiber boutons colocalized with synaptophysin were associated with GnRH neurons (Fig. 1 D,E,F). Shown as a stack of images (panel D) or as individual optical slices (0.25 μ m resolution, panels E and F), the spine of an orexin fiber colocalized with synaptophysin makes close contact with a GnRH neuron (white pixels represent triple labeling). Additional double label studies showed that GnRH neurons colocalize with OX-R1-ir (indicated by the yellow color in the low and high power examples in Fig. 2A,B). Consistent with the robust innervation of GnRH neurons by orexin, OX-R1-ir was colocalized with approximately 90% of the GnRH neurons observed. Triple label IF confirmed that orexin fibers, shown in blue, make contacts onto GnRH neurons expressing OX-R1-ir (Fig. 2 C,D).

OX-R1 expression and orexin innervation was also investigated in the region of GnRH terminals in the median eminence (ME). As shown in Figure 3,

although there is some OX-R1 expression (red) in this region and orexin fibers do appear to innervate the dorsolateral ME (blue), there is no coexpression of OX-R1 and GnRH (green) and very little interaction between orexin fibers and GnRH nerve terminals.

An additional triple label study was carried out to investigate whether Y4-like positive orexin fibers make close contacts with GnRH neurons. Figure 4 depicts three GnRH cell bodies surrounded by orexin fibers. The white arrows indicate two fibers that are Y4/orexin positive. The yellow arrows indicate where orexin positive fibers were identified coming in close contact with GnRH neurons. While there were multiple close appositions between orexin fibers and GnRH, as shown previously in Fig. 1, Y4/orexin fibers never appeared to make close contacts with GnRH neurons.

Discussion

The present study provides neuroanatomical evidence for a direct interaction between the orexin system and GnRH neurons. Orexin fibers were observed in close contact with GnRH neurons, and colocalization of orexin with synaptophysin provided further confirmation of synaptic contacts (Fig. 1A-F). Additional evidence for a functional neuroanatomical pathway is demonstrated by the expression of OX-R1-ir in GnRH neurons and the observation that orexin fibers make contacts onto regions of GnRH neurons that express the OX-R1 (Figs. 2A-D). The immunoreactivity of orexin fibers and OX-R1 presented in the present study confirms previous findings demonstrating that both orexin A

immunoreactive fibers (Peyron et al., 1998) and OX-R1 mRNA (Trivedi et al., 1998) and immunoreactivity (Hervieu et al., 2001) are located in the septal and median preoptic regions. Our data is also in agreement with previously reported findings in the ovine hypothalamus, in which orexin fibers, originating from the LHA, were found in close contact with GnRH neurons (Iqbal et al., 2001). However, while the present study found greater than 80% of GnRH neurons contacted by orexin fibers and approximately 90% of GnRH neurons expressing OX-R1, the previous study found contacts on only 30% of GnRH neurons. This discrepancy may be due in part to orexin antibody sensitivity. While the present study shows dense networks of fine immunoreactive fibers (Fig. 1A, Fig. 4), the OX-R1-ir fibers demonstrated in the ovine hypothalamus were less dense, suggesting that the antibody used in the study by Iqbal *et al.* (2001). may not recognize OX-R1-ir in fine fiber processes. Alternatively, the discrepancy between these findings may also be due to species differences between the rat and the sheep.

Several lines of evidence support the contention that orexin A modulates neuroendocrine function. In addition to a number of studies indicating hormonally dependent effects of central orexin administration on LH release (Pu et al., 1998; Tamura et al., 1999; Fututa et al., 2002), studies using *in vitro* explants indicate that the stimulatory effects of orexin are at the hypothalamic level (Russell et al., 2001). It was also reported that orexin A stimulates GnRH release from hypothalamic explants collected from both male rats and female rats in proestrus. These data suggest that the stimulatory effects of orexin on LH release are via

direct stimulation of GnRH secretion. Indeed, the present findings provide the functional neuroanatomical pathway by which orexin may stimulate GnRH neurons and subsequent LH release via direct contact with OX-R1 on GnRH cell bodies. OX-R1 activation leads to both Ca^{2+} influx and direct stimulation of phospholipase C that mediates Ca^{2+} release from intracellular stores (Lund et al., 2000; Smart et al., 1999). OX-R1 is exclusively coupled to the $\text{G}_{q/11}$ subtype of G proteins (van den Pol et al., 1998, Beukmann et al., 2002), suggesting that orexin action via the OX-R1 on GnRH neurons is excitatory. However, orexin has also been shown to inhibit LH secretion in the absence of gonadal steroids. The neuroanatomical pathway by which orexin inhibits LH secretion and the mechanism by which gonadal steroids modulate orexin effects on LH secretion remains to be determined.

As the name implies, the orexins are also associated with an increase in feeding behavior. It has been demonstrated that central injection of orexin A stimulates food intake in satiated rats (Dube et al., 1992; Haynes et al., 2000) and that orexin mRNA is increased with fasting (Sakurai et al., 1998). Furthermore, neutralization of orexin with an anti-orexin antibody dose dependently suppresses spontaneous feeding in fasted rats (Yamada et al., 2000). These data suggest that orexin activity is necessary for normal feeding behavior and that orexin neurons are able to sense the nutritional state of an animal. It has been shown repeatedly that changes in the metabolic status of an animal are tightly linked to changes in reproductive function. Therefore, the ability of orexin to modulate both feeding behavior and reproductive function, along with

the present neuroanatomical findings, suggests that the orexin system is in a key position to serve as an as an integrating signal between energy balance and reproductive function.

In addition, orexin neurons are known to coexpress the long form of the leptin receptor (Ob-Rb) in rat and sheep (Iqbal et al., 2001; Hakansson et al., 1999) Leptin, a peripheral hormone released from adipocytes, is important in denoting adiposity and energy balance to the brain. Also, leptin has been shown to play a role in regulating reproductive function. Administration of leptin restores fasting induced suppression of LH secretion in both sheep and primates (Finn et al., 1998, Nagatani et al., 2000, Nagatani et al., 1998), However, GnRH neurons do not express Ob-Rb. As previously suggested by similar findings in the ovine hypothalamus (Iqbal et al., 2001), direct projections by Ob-Rb expressing orexin neurons to GnRH neurons may provide one potential neuroanatomical circuit by which leptin regulates GnRH activity.

NPY is another neuropeptide system that is suggested as an important integrating signal for both energy balance/food intake and reproductive function (Smith and Grove, 2002). Orexin neurons in the LHA receive direct synaptic contacts by NPY neurons projecting from the arcuate nucleus (ARH) and in turn send reciprocal projections to ARH NPY neurons (Horvath et al., 1999). Similar to the effects of orexin, NPY also elicits steroid dependent effects on LH secretion (Kalra et al., 1999). Currently, there is evidence implicating the Y1, Y4 and Y5 receptor subtypes in NPY's modulation of neuroendocrine reproductive function. Previous studies in our laboratory have investigated the

neuroanatomical distribution of these receptor subtypes. It was determined that approximately 55% of GnRH cell bodies coexpress the Y5 receptor subtype (Campbell et al., 2001), thought to play a role in the inhibitory effects of NPY on LH release in the absence of steroid hormones (Raposo et al., 1999). In addition, Y1 receptors, implicated in NPY stimulation of the LH surge (Jain et al., 1999; Leupen et al., 1997), were identified on fibers of an unknown phenotype making close contacts with GnRH cell bodies and on GnRH nerve terminals in the median eminence (Li et al., 1999). Therefore, NPY may have both pre- and postsynaptic effects directly at GnRH cell bodies and at nerve terminals.

Most recently, Y4-ir and mRNA expression were colocalized with orexin neurons in the LHA (Campbell et al., 2002). In light of the present findings that orexin fibers directly contact GnRH neurons, and that the OX-R1 receptor is abundantly expressed on GnRH neurons, it was hypothesized that the effect of NPY on GnRH/LH release via Y4 receptors may be indirect through orexin neurons. The present study showed that although single label orexin contacts to GnRH neurons were abundant, double labeled Y4/orexin fibers were never observed in close contact with GnRH neurons. Taken together, these data suggest that the major action of NPY on GnRH release via the Y4 receptor is through postsynaptic effects on orexin cell bodies. Again, these data present another means by which orexin may serve as an intermediate signal between the regulation of energy balance and reproductive function.

The presence of Y4-ir on orexin neurons within the hypothalamus, along with orexin contacts directly to GnRH neurons, demonstrates a unique potential

neuronal circuit whereby NPY can exert its effects on LH release via the modulation of orexin. Our recent study characterizing the expression of Y4-ir on orexin neurons also found that administration of rPP, an endogenous ligand of the Y4 receptor, induced robust cFos activation in orexin neurons (Campbell et al., 2002). These data suggest that stimulation of the Y4 receptor by rPP is excitatory to orexin neurons in intact male rats. Activation of orexin may result in an acute increase in orexin, which, acting through the excitatory OX-R1 receptor, would be excitatory to GnRH neurons. We would argue that this is the likely mechanism by which Y4 stimulation results in increased LH secretion as reported by Raposinho and colleagues (2000) and Jain and colleagues (1999). The endogenous ligand of this effect remains to be determined.

Alternatively, recent evidence demonstrated that when the infertile ob/ob mouse was crossed with the Y4 knockout mouse, fertility was partially restored (Sainsbury et al., 2002). These data would suggest that the Y4 receptor is involved in the inhibition of reproductive function. However, the ob/ob mouse represents a unique model in which the absence of leptin results in extremely elevated NPY. These grossly elevated levels of NPY are thought to be responsible for both the obesity and the infertility of the ob/ob mouse (Steohens et al., 1995; Chehab et al., 1996, Mounzih et al., 1997). Therefore, it has been suggested that under conditions of such significantly elevated NPY levels, as in the ob/ob mouse, NPY might act through Y4 receptors to suppress reproductive function (Sainsbury et al., 2002); however additional studies are necessary to

determine the effect of NPY, and potentially other PP family ligands, on orexin neurons via the Y4 receptor.

In summary, these data provide neuroanatomical evidence for a direct link between the orexin system and GnRH neurons. These findings suggest that orexin plays a direct role in the neuroendocrine control of reproduction and may also be uniquely positioned to integrate signals associated with energy balance regulation. Additional studies will be necessary to establish the relative importance of this novel neuronal circuitry in the regulation of reproductive function during normal cyclicity as well as during metabolic changes.

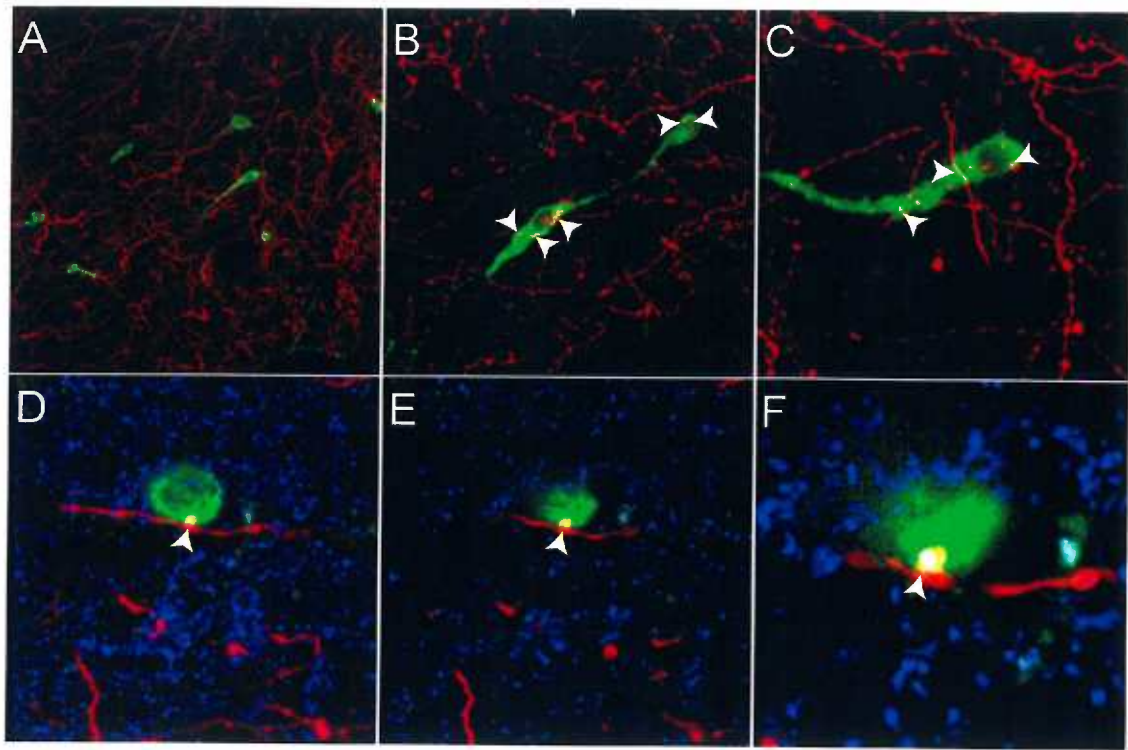


Figure 6-1: Orexin fibers come in close contact with GnRH neurons. Panel A is a low power fluorescent photomicrograph demonstrating GnRH neurons (green) surrounded by orexin fibers (red). Higher power examples of individual GnRH neurons in (B) and (C) show orexin fibers making close contact. Arrowheads indicate apparent close contacts (B, C) and the colocalization of pixels results in a yellow color. Panel D shows an example of an orexin fiber (red), colocalized with synaptophysin (shown in blue) that makes close contact with a GnRH cell body (green). The white pixels, illustrated in a higher power image (F), indicate triple label overlap of all the signals. Panels E and F are serial 0.25mm resolution optical slices demonstrating triple labeling (arrowhead).

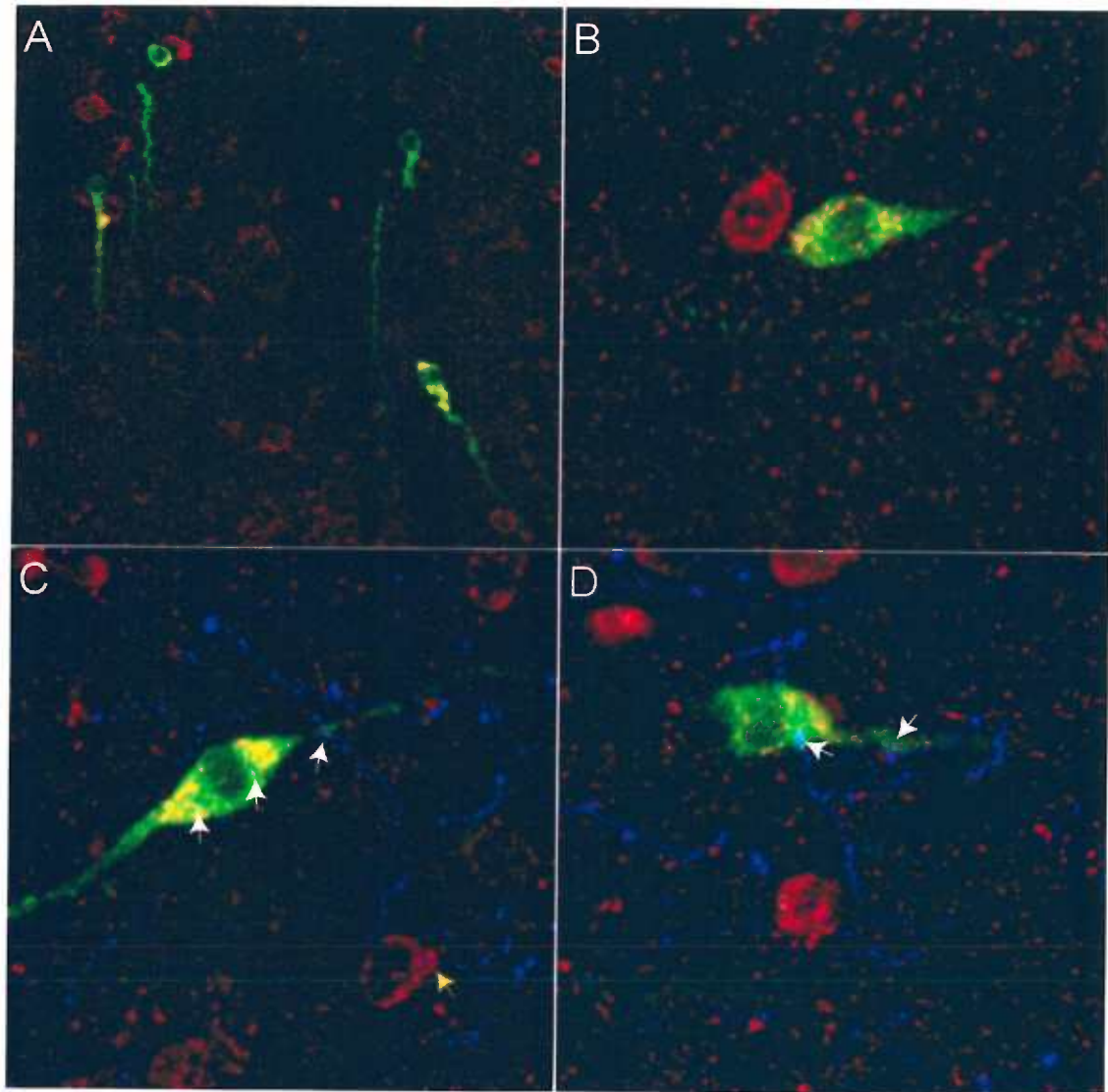


Figure 6-2: Double and triple label IF demonstrates that GnRH neurons express OX-R1-ir and receive close contacts by orexin fibers. Panel A shows a low power example of GnRH neurons (green) and OX-R1 expressing neurons (red) in the mPOA. The yellow color indicates colocalization. A higher power example of an OX-R1 expressing GnRH (green/yellow) neuron and a single labeled OX-R1 neuron (red) is shown in B. Panels C and D show two representative examples of GnRH neurons (green), expressing OX-R1-ir (single label=red, colocalization with GnRH=yellow). Orexin fibers (blue) make apparent close contacts to OX-R1 expressing regions of GnRH neurons as indicated by white arrows. The yellow arrow indicates an orexin fiber contact onto a single label OX-R1 neuron (red).

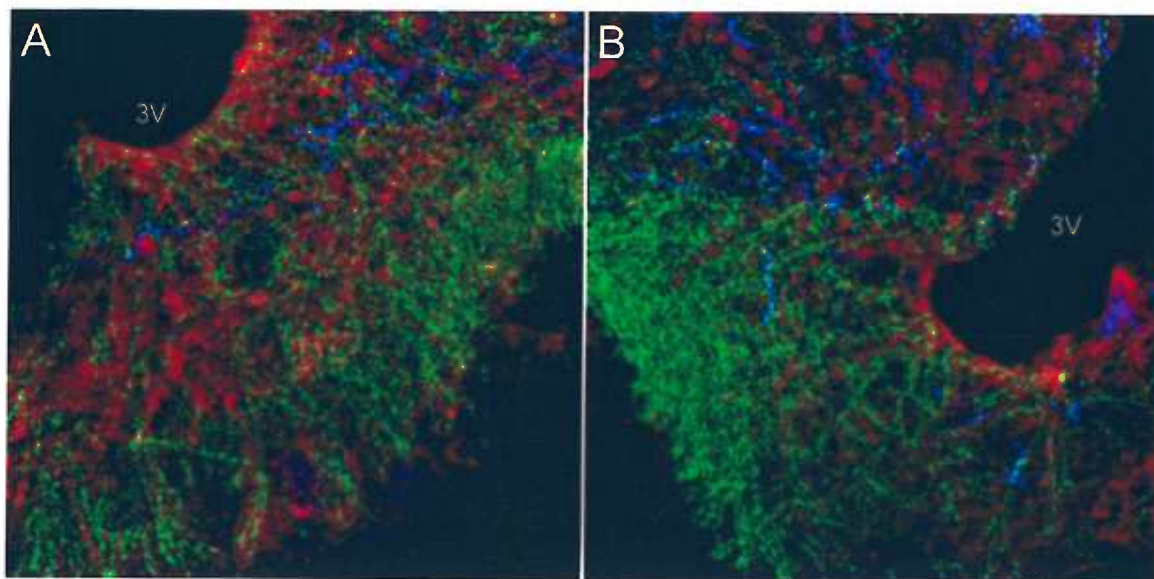


Figure 6-3. GnRH nerve terminals in the median eminence do not express OX-R1. Panels A and B show two representative images of triple label IF for GnRH fibers (green), OX-R1 (red) and orexin fibers (blue) in the ME. No colocalization of OX-R1 and GnRH fibers is apparent and orexin fibers have minimal interaction with GnRH nerve terminals.

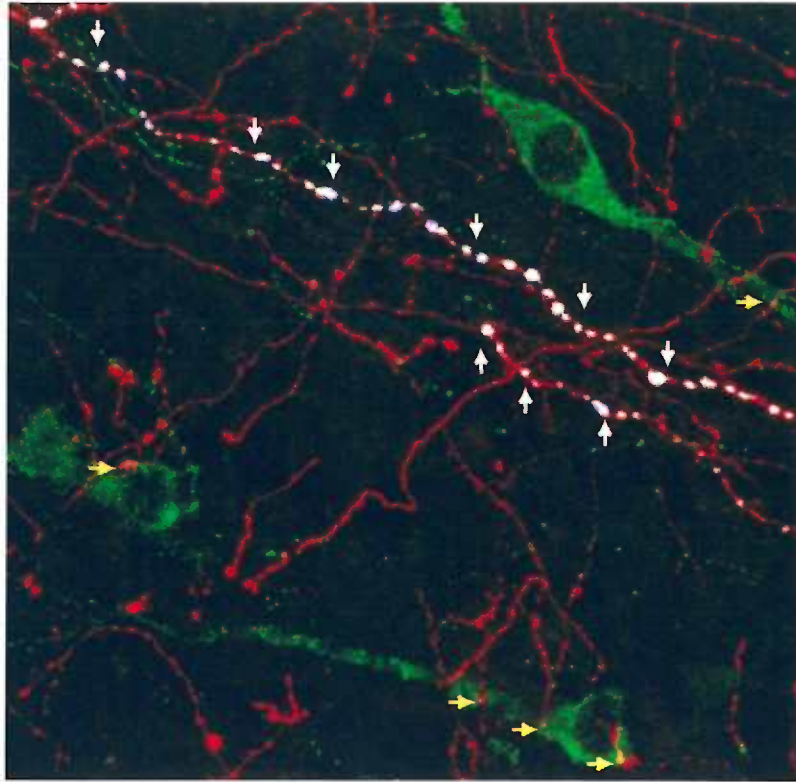


Figure 6-4. NPY Y4/orexin-ir fibers and GnRH neurons. Triple label IF demonstrates GnRH neurons (green) surrounded by orexin fibers (red) and a few Y4/orexin double label fibers (white, indicated by white arrows). Orexin single label fibers coming in close contact with GnRH neurons are indicated by the yellow color and marked by yellow arrows.

The studies described in this thesis provide neuroanatomical evidence for a number of potential circuits mediating NPY's integrative role in the regulation of energy balance and neuroendocrine reproductive function. Using immunohistochemical techniques and confocal microscopy, a number of receptor expression patterns were mapped throughout the rat brain and colocalized with specific phenotypes of neurons. Collectively, these studies provide a better understanding of the neuronal architecture of the NPY system and provide a framework in which to begin to understand the complex mechanism underlying the link between energy homeostasis and reproduction. In order to provide a context in which to summarize the data presented in this thesis, a brief overview of the regulation of feeding and the hypothalamic flow of signals responding to states of negative energy balance are described below. This is followed by a summary of the conclusions drawn from the present data and a brief discussion of future directions. A final summary diagram is provided and referred to in order to facilitate the discussion below.

Brief overview of the hypothalamic response to negative energy balance:

Although the drive to eat and the regulation of energy expenditure are highly complex processes, involving numerous brain regions, most interest has focused on the hypothalamus, as it is uniquely positioned to receive, integrate, and communicate information regarding the maintenance of energy homeostasis. Elaborate arrays of hypothalamic circuits relaying orexigenic or anorexigenic

signals are positioned to respond to shifts in energy balance and to reinstate energy homeostasis.

The metabolic state of an animal is primarily communicated to the CNS via blood borne signals from the periphery (Summary Diagram, A). These include leptin and insulin classically, as well as a number of gut peptides, including PYY and ghrelin. In a state of negative energy balance, resulting from fasting, long-term caloric restriction, or lactation, many of the peripheral signals denoting satiety, including leptin and insulin, are reduced, while those denoting appetite are increased. The hypothalamus contains a number of neurons capable of receiving and mediating the effects of these peripheral metabolic signals; in this regard, NPY/AGRP and POMC/CART neurons in the ARH are considered to be first-order neurons involved in transducing peripheral metabolic signals into neuronal signals (B). These systems are differentially regulated by leptin and have opposing downstream effects on energy balance. States of negative energy balance, in which leptin levels are low, will result in the activation of orexigenic NPY/AGRP systems and the suppression of anorexigenic POMC/CART systems, in an attempt to stimulate food intake.

In addition to interactions with each other, these ARH populations have extensive reciprocal interactions with other hypothalamic regions implicated in the regulation of energy balance, including the PVH, LHA and DMH. The specific downstream targets and the relative importance of each pathway downstream of NPY/AGRP and POMC/CART neurons are just beginning to be recognized. It is becoming apparent that the regulation of energy balance does not involve a

discrete set of pathways in series, but rather involves a number of redundant, parallel circuits that integrate and govern feeding behavior; an unsurprising finding in light of the critical importance of food intake. However, there also exists a wide range of functional diversity among these pathways, allowing for different neural circuits to serve specific functions under specific physiological conditions of altered energy balance.

The PVH is densely innervated by and particularly sensitive to NPY and a number of other neurotransmitters important in energy balance regulation, including α -MSH, serotonin, galanin, noradrenaline, urocortin and opioids. The PVH contains several neuropeptide systems that reduce food intake, including CRH, TRH and OT (C), suggesting that during negative energy balance these neurons would be inhibited by NPY/AGRP and stimulated by POMC/CART neurons. GABAergic terminals within the PVH are an important integrating site for both NPY/AGRP and melanocortin signaling to PVH neural circuits.

The LHA, which also receives dense innervation from the ARH, contains the orexigenic peptides MCH and orexin (D). MCH is consistently elevated in negative energy balance models. Orexin, however, is not elevated during lactation, and is suggested to be involved in the maintenance of daily food intake and general arousal. While NPY would be expected to stimulate these neurons, data presented in this thesis and discussed in greater detail below, suggests that NPY activates an unknown population of neurons in the LHA. MCH and orexin neurons may instead be regulated to a greater extent by AGRP and the

melanocortin system, which would be increased and suppressed, respectively, during negative energy states.

The DMH is also a target of ARH neurons and, in turn, has extensive connections with other hypothalamic nuclei (E). NPY is expressed in a subpopulation of neurons within the DMH during lactation and also in some genetic models of obesity. The role of this population of NPY neurons in mediating downstream systems involved in energy regulation remains to be determined. The VMH, also connected with the DMH and thought to work in concert with the PVH, has a high abundance of leptin receptors. Stimulation of this region inhibits feeding, suggesting that negative energy balance would inhibit this region.

Virtually all of the above mentioned systems also feed back onto NPY/AGRP and POMC/CART neurons in the ARH, suggesting that in addition to a divergence of signals out of the ARH, there is also a convergence of information from a variety of sources, including the brainstem and cortical regions, back into the ARH. While the above mentioned circuits are all implicated in various aspects of the regulation of feeding, the ultimate downstream pathway by which feeding behavior is motivated remains unclear. Most likely, it involves the integration of hypothalamic systems with cortical and limbic systems important in the motivation of behavior.

Summary and conclusions of the present findings:

The initial studies presented here investigated CRH R_1 expression patterns in the hypothalamus and found that NPY neurons in the ARH coexpressed CRH R_1 [see summary diagram, (1)]. An integral relationship has been established between NPY and CRH, with each eliciting opposing effects on food intake and energy balance. Previous studies have clearly demonstrated an anatomical link from ARH NPY neurons to PVH CRH neurons (2) and an abundance of physiological data indicates that at least part of NPY's function is mediated through regulation of CRH. It has been suggested that NPY activates CRH that, in turn, inhibits feeding. Therefore, the demonstration of CRH R_1 expression on NPY neurons provides compelling neuroanatomical evidence for such a reciprocal feedback loop between CRH and NPY systems, whereby CRH may inhibit NPY signaling. However, although CRH fibers were observed in close contact with CRH R_1 expressing NPY neurons using triple label immunofluorescence, the number of fibers present was very sparse (3). Thus, it can be concluded that CRH may only regulate a subset of NPY neurons, and that other CRH R_1 ligands, such as UCN, may be involved in the regulation of ARH NPY neurons. It is becoming apparent from more recent studies that UCN is a more potent inhibitor of food intake than CRH and also interferes with NPY induced feeding. In addition, UCN has a much higher affinity for both receptor subtypes than CRH, while having the highest affinity for CRH R_2 . Likewise, knockout models have suggested that CRH R_1 is not essential in the appetite suppressing effects of UCN. Therefore, while CRH R_1 expression on ARH NPY

neurons may still represent an important circuit for some aspects of regulation by either CRH or UCN, future experiments in this area should probably be focused on the underlying circuits mediating urocortin effects on feeding through CRH R₂.

To begin to understand the roles of the different NPY receptor subtypes in the mediation of NPY's effects, and to identify the efferent targets of NPY neurons, the expression patterns of the Y5 and Y4 receptor subtypes were investigated.

NPY Y5 receptors are strongly implicated in mediating NPY's stimulation of food intake, inhibition of GnRH/LH secretion, and suppression of seizure activity. After the initial characterization of a Y5receptor antibody, Y5-ir was mapped throughout the brain. Y5-ir was found throughout the cortical/limbic system, including expression on pyramidal cells of the CA2/3 region of the hippocampus and on GABAergic and CRH expressing neurons in the cortex. Support for a direct functional interaction was demonstrated with confocal analysis showing NPY fibers in close contact with Y5 expressing neurons in the cortex. Therefore, these data provide a functional neuroanatomical circuit by which Y5 receptors may mediate some of the anticonvulsant effects of NPY.

An investigation of the hypothalamic distribution of Y5-ir found the receptor colocalized with AVP and OT neurons in the PVH (4) and SON, GnRH cell bodies in the mPOA (5), a subset of CRH neurons in the PVH (6), and on GABAergic neurons in the DMH (7), suggesting that NPY can have direct effects on these cell types via the Y5 receptor subtype. The demonstration of Y5 receptors on GnRH cell bodies, along with previous evidence for Y1 receptor

expression on GnRH nerve terminals (8), suggests that NPY can have multiple effects on GnRH neuronal function and release, depending on the site of action and the receptor subtype involved. Taken together with the available pharmacological data for these receptor subtypes, it can be hypothesized that NPY has inhibitory effects in low steroid hormone conditions or, when NPY is chronically elevated, by acting directly on GnRH cell bodies through the Y5 receptor. Conversely, under normal steroid conditions, the ability of NPY to stimulate GnRH release may be at nerve terminals in the median eminence via the Y1 receptor subtype. In addition, Y1 positive fibers of an unknown origin have also been identified coming in close contact with GnRH neurons (9), providing another means of regulation by NPY. Future studies examining the electrophysiological responses of GnRH neurons to specific receptor agonists and antagonists will be necessary to confirm that this hypothesized neuronal circuitry underlies the differential effects of NPY on GnRH neurons. The current development of green fluorescent protein (GFP-) and LacZ-GnRH mice, along with more specific agonists and antagonists, make this a reasonable future aim.

The chronic infusion of Y5 antisense ODNs significantly reduced food intake and body weight, providing further evidence for the role of the Y5 receptor in the regulation of food intake. CRH neurons have been suggested as one of the downstream targets of this regulation and a subset were identified expressing the Y5 receptor (6). Similar to the effects of NPY on GnRH, NPY also has both excitatory and inhibitory actions on CRH neurons. The identification of Y5 receptors present on CRH neurons, along with the previous identification of Y1

positive fibers in contact with CRH neurons (10), again, provides multiple means of regulation through different receptor subtypes.

Y5-ir was also found in a number of other areas associated with NPY's regulation of food intake and neuroendocrine function, consistent with the idea that NPY acts to recruit numerous systems in the integration of these processes. Y5-ir was found within the ARH (11), suggesting that the Y5 receptor may be expressed on NPY neurons and may act as an inhibitory autoreceptor along with the Y2 receptor. Evidence that Y2 and Y5 receptor knockout mice exhibit late onset obesity supports the idea that these receptor subtypes are important in the regulation of ARH NPY neuronal function. Additional studies will need to be conducted in order to confirm Y5-ir in NPY neurons and the regulation of NPY neuronal function by Y5-like agonists.

GABAergic neurons of the DMH also express the Y5 receptor (7). The DMH is considered a major relay center, as it receives fiber projections from the LHA, VMH, and ARH, and then sends GABAergic projections to the PVH (12). Additionally, NPY has been shown to effect presynaptic GABA release in the PVH. It can be hypothesized that NPY may modulate GABA neurons projecting to the PVH at the level of their cell bodies in the DMH, while more direct effects at nerve terminals may be through Y1 receptors. Again, these studies demonstrated that NPY has multiple means of conveying signals through multiple receptor subtypes at multiple levels of neuronal function and release. These neuroanatomical data suggest that the Y5 receptor generally has postsynaptic

effects on neuronal cell bodies, while the Y1 receptor generally regulates presynaptic release.

Other studies in the Smith lab have identified a population of NPY neurons in the non-compact zone of the DMH whose expression is dramatically increased during lactation (13). Increased expression of DMH NPY neurons is also seen in genetic models of obesity that involve the absence of MC4 signaling, however lactation is the only natural physiological state in which the expression of this population of NPY neurons is increased. These neurons also have dense projections to the PVH and most likely contribute to the chronic hyperphagia of lactation. It remains to be determined how these neurons are regulated and what specific role this population of NPY neurons plays in the development of hyperphagia and obesity.

The LHA, an area that has been demonstrated as extremely sensitive to the orexigenic effects of NPY, and contains orexin and MCH neurons, was also found to express Y5 receptors (14). Although the phenotypes of these neurons are unknown, cFos expression patterns following intra-LHA NPY injection imply that the Y5 receptor is not expressed on either of these cell types. Rather, the Y5 receptor may be expressed on GABAergic neurons in this region in addition to those in the DMH. Although GABAergic neurons in this region can be difficult to identify with immunohistochemical methods, future studies using *in situ* hybridization for GAD mRNA coupled with Y5-ir will be able to determine this.

The Y4 receptor subtype was also identified in the LHA and colocalized with orexin neurons (15). rPP, the peripherally produced endogenous ligand for

the Y4 receptor, and NPY, a low affinity ligand for the Y4 receptor, were administered directly into the LHA to examine the effects of a Y4-like activation on orexin neurons. Food and water intake were stimulated by rPP and closely resembled food and water intake stimulated by orexin, while NPY caused a more robust increase in food intake. The immediate early gene product, cFos, was used as a marker of neuronal activation to identify populations of neurons in the LHA specifically activated by rPP and NPY. Both rPP and NPY resulted in a robust induction of cFos activation in the LHA; however, only rPP induced a robust induction of cFos in orexin neurons. Along with the neuroanatomical identification of Y4 receptors on orexin neurons, these data strongly support a direct physiological activation of orexin neurons via Y4 receptors. The lack of cFos induction in orexin neurons by NPY suggests that NPY may not be the endogenous ligand of the Y4 receptor or that NPY may have different downstream effects via the Y4 receptor, perhaps decreased excitability of the neurons that may not result in cFos activation. However, the low affinity of NPY for Y4 receptors and the clear induction of Y4 expressing neurons by rPP indicate that an undiscovered central endogenous ligand for the Y4 receptor may exist. Additional studies, similar to those used to identify ligands for orphan receptors, may be useful in identifying such a peptide.

The lack of cFos induction in either orexin or MCH neurons also raises some interesting questions regarding the populations of neurons that are responding to NPY. In support of the lack of activation, Y1 receptors were not identified on orexin or MCH cell bodies, but on fibers making close contacts with

both of these populations (16). This pattern for Y1 receptor expression again suggests the regulation of presynaptic release. The stimulation of GABA release via Y1 receptors would fit with the lack of cFos activation in orexin and MCH neurons following NPY administration; however this remains to be confirmed in this region. Electrophysiological determination of populations in the LHA that are stimulated or inhibited by NPY may begin to address which phenotypes of neurons are being directly stimulated by NPY.

Orexin, similar to NPY and a number of other neuropeptides that regulate energy balance, also modulates GnRH/LH secretion. The identification of Y4 receptors on orexin neurons made this population of neurons particularly well suited as a potential intermediate of NPY-like regulation of reproductive function. Studies presented in this thesis found the majority of GnRH neurons expressing OX-R1 and orexin fibers making close contacts with these GnRH neurons (17), supporting direct effects via orexin on GnRH neuronal cell bodies. These data demonstrate a unique potential neuronal circuit whereby NPY, or other NPY-like ligands can exert effects on LH release via the modulation of orexin. Further studies are needed to elucidate the relative importance of orexin in Y4 or Y1 regulation of GnRH/LH secretion and subsequent reproductive function.

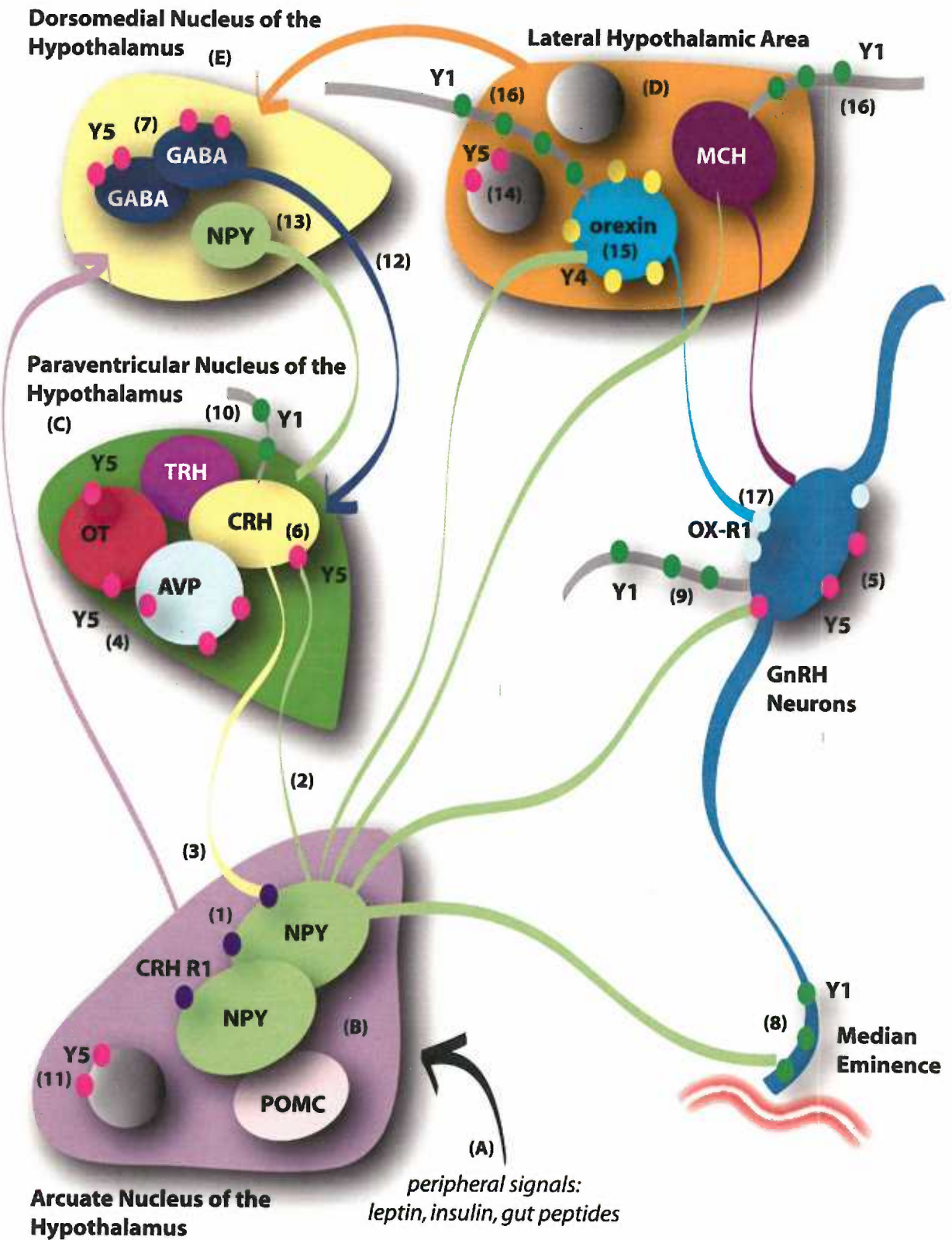
Finally, studies presented in the appendix began to address the role of brainstem NPY by investigating changes in NPY mRNA in subregions of the VLM, an area known to be activated following the suckling stimulus. Lactation did not result in an increase in NPY mRNA expression in the A1 region of the VLM as expected, despite a robust increase in cFos expression in this region following

the suckling stimulus. However, lactation did result in decreased NPY mRNA expression in the C1 region, an area previously demonstrated to not express cFos following a suckling stimulus. Collectively, these data suggest that adrenergic NPYergic C1 neurons are suppressed and noradrenergic A1 neurons are activated during lactation, resulting in a differential regulation of VLM catecholaminergic neurons by the suckling stimulus. This differential regulation could possibly underlie the activation of A1 circuits involved in food intake and the suppression of C1 circuits involved in the activation of stress pathways, reflecting the concomitant hyperphagia and hyporesponsiveness to stress observed during lactation. It remains to be determined if catecholamine levels are changed in these neurons and what synaptic interactions take place between catecholamines and NPY at nerve terminals.

In conclusion, these data identify numerous targets of the NPY system that may represent multiple neuronal circuits by which NPY may act to regulate and integrate the signals mediating energy balance and reproductive function. Mapping of receptor subtypes, investigations of close contacts, and identification of neuronal activation using cFos have described a number of functional neuroanatomical pathways. However, these studies also raise a number of wide-ranging questions that remain to be addressed. First, what is the relative importance of each of the orexigenic systems (NPY, MCH, orexin) to the regulation of GnRH? Also, do they play a role in estrous cyclicity or solely in states of negative energy balance? How can these signals be both stimulatory and inhibitory to GnRH, and how do steroid hormones effect NPY signaling?

What is the effect of activation of Y5 or OX-R1 on GnRH neurons; inhibitory, stimulatory or neuromodulatory? Similarly, what is the effect of Y4 receptor activation on orexin neurons and what is the endogenous brain ligand for this receptor? How are NPY's actions on GnRH neurons integrated with respect to presynaptic input via Y1 receptors versus postsynaptic input via Y5 receptors?

Future studies will provide an even greater understanding of the mechanism by which NPY recruits and regulates multiple systems through multiple receptor subtypes. It is my opinion that investigating the electrophysiological consequences of signaling through these pathways is the next necessary step in pursuing these questions. The development of transgenic animals expressing GFP in a variety of phenotypes, including GnRH and orexin, provides an efficient way to record from known phenotypes of neurons. By collecting tissue under a variety of physiological states, including altered steroid states and altered metabolic states, and using a number of specific agonists and antagonists that are becoming available, some of the above mentioned questions would begin to be addressed.



VIII. References

Aakerlund L, Gether U, Fuhlendorff J, Schwartz TW, Thastrup O 1990 Y1 receptors for neuropeptide Y are coupled to mobilization of intracellular calcium and inhibition of adenylate cyclase. FEBS Lett 260:73-78

Ahima RS, Dushay J, Flier SN, Prabakaran D, Flier JS 1997 Leptin accelerates the onset of puberty in normal female mice. J Clin Invest 99:391-395

Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS 1996 Role of leptin in the neuroendocrine response to fasting. Nature 382:250-252

Aldenhoff JB, Gruol DL, Rivier J, Vale W, and Siggins GR 1983 Corticotropin releasing factor decreases postburst hyperpolarizations and excites hippocampal neurons. Science 221:875-877

Anand BK, Brobeck JR 1951 Localization of a "feeding center" in the hypothalamus of the rat. Proc Soc Exp Biol Med 77:323-324

Aoki C and Pickel VM 1990 Neuropeptide Y in cortex and striatum. Ultrastructural distribution and coexistence with classical neurotransmitters and neuropeptides. Ann N Y Acad Sci 611:186-205

Arase K, York DA, Shimizu H, Shargill N, Bray GA 1998 Effects of corticotropin-releasing factor on food intake and brown adipose tissue thermogenesis in rats. Am J Physiol 255:E255-E259

Arvidsson J, Raappana P, Diez M, and Hokfelt T 1994 Expression of neuropeptides in the rat mesencephalic trigeminal nucleus after peripheral axotomy. *Neuroreport* 5:1269-1272

Backberg M, Hervieu G, Wilson S, Meister B 2002 Orexin receptor-1 (OX-R1) immunoreactivity in chemically identified neurons of the hypothalamus: focus on orexin targets involved in control of food and water intake. *Eur J Neurosci* 15:315-328

Balasubramaniam A 1997 Neuropeptide Y family of hormones: Receptor subtypes and antagonists. *Peptides* 18:445-457

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob G F, Vale WW, Lee KF 2000 Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat Genet* 4:410-414

Bard JA, Walker MW, Branchek TA, Weinshank RL 1995 Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. *J Biol Chem* 270:26762-26765

Baskin DG, Figlewicz Lattemann D, Seeley RJ, Woods SC, Porte D Jr, Schwartz MW 1999 Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res* 848:114-123

Baskin DG, Hahn TM, Schwartz MW 1999 Leptin sensitive neurons in the hypothalamus. *Horm Metab Res* 31:345-350

Baskin DG, Wilcox BJ, Figlewicz DP, Dorsa DM 1988 Insulin and insulin-like growth factors in the CNS. *Trends Neurosci* 11:107-111

Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghattei MA, Cone RD, Bloom SR 2002 Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* 418:650-654

Beck B, Stricker-Krongrad A, Nicolas JP, Burlet C 1992 Chronic and continuous intracerebroventricular infusion of neuropeptide Y in Long-Evans rats mimics the feeding behaviour of obese Zucker rats. *Int J Obes Relat Metab Disord* 16:295-302

Ben Barak Y, Russell JT, Whitnall MH, Ozato K, Gainer H 1985 Neurophysin in the hypothalamo-neurohypophysial system. I. Production and characterization of monoclonal antibodies. *J Neurosci* 5:81-97

Bernardis LL, Bellinger LL 1989 The dorsomedial hypothalamic nucleus revisited: 1998 update. *PSEBM* 218:284-306

Bernardis LL, Bellinger LL 1996 The lateral hypothalamic area revisited: ingestive behavior. *Neurosci Biobehav Rev* 20:189-287

Besecke LM, Wolfe AM, Pierce ME, Takahashi JS, Levine JE 1994 Neuropeptide Y stimulates luteinizing hormone-releasing hormone release from superfused hypothalamic GT1-7 cells. *Endocrinology* 135:1621-1627

Beuckmann CT, Yanagisawa M 2002 Orexins: from neuropeptides to energy homeostasis and sleep/wake regulation. *J Mol Med* 80:329-342

Bijak M 1999 Neuropeptide Y suppresses epileptiform activity in rat frontal cortex and hippocampus in vitro via different NPY receptor subtypes. *Neurosci Lett* 268:115-118

Billington CJ, Briggs JE, Grace M, Levine AS 1991 Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism. *Am J Physiol* 260:R321-R327

Bittencourt JC, Vaughan J, Arias C, Rissman RA, Vale WW, and Sawchenko PE 1999 Urocortin expression in rat brain: evidence against a pervasive relationship of urocortin-containing projections with targets bearing type 2 CRF receptors. *J Comp Neurol* 415:285-312

Blomqvist AG, Herzog H 1997 Y-receptor subtypes--how many more? *Trends Neurosci* 20:294-298

Boudaba C, Szabo K, Tasker JG 1996 Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. *J Neurosci* 16:7151-7160

Bradbury MJ, McBurnie MI, Denton DA, Lee K F, Vale WW 2000 Modulation of urocortin-induced hypophagia and weight loss by corticotropin-releasing factor receptor 1 deficiency in mice. *Endocrinology* 141:2715-2724

Brady LS, Smith MA, Gold PW, Herkenham M 1990 Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology* 52:441-447

Britton K T, Southerland S, Van Uden E, Kirby D, Rivier J, and Koob G 1997 Anxiolytic activity of NPY receptor agonists in the conflict test. *Psychopharm* 132: 6-13

Broberger C, De Lecea L, Sutcliffe JG, Hokfelt T 1998 Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. *J Comp Neurol* 402:460-474

Broberger C, Landry M, Wong H, Walsh JN, Hokfelt T 1997 Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus. *Neuroendocrinology* 66:393-408

Broberger C, Visser TJ, Hokfelt T 1999 Neuropeptide Y innervation and neuropeptide-Y-Y1-receptor-expressing neurons in the paraventricular hypothalamic nucleus of the mouse. *Neuroendocrinology* 70:295-305

Brogan RS, Grove KL, Smith MS 2000 Differential regulation of leptin receptor but not orexin in the hypothalamus of the lactating rat. *J Neuroendocrinol* 12:1077-1086

Brogan RS, Mitchell SE, Trayhurn P, Smith MS 1999 Suppression of leptin during lactation: contribution of the suckling stimulus versus milk production. *Endocrinology* 140:2621-2627

Brown TH, Zador AM 1990 Hippocampus. In: Sheperd GM (ed) *The Synaptic Organization of the Brain*. Oxford University Press, New York, pp 346-388

Cabrele C, Langer M, Bader R, Wieland HA, Doods HN, Zerbe O, Beck-Sickinger AG 2000 The first selective agonist for the neuropeptide YY5 receptor increases food intake in rats. *J Biol Chem* 275:36043-36048

Campbell RE, French-Mullen JM, Cowley MA, Smith MS, Grove KL 2001 Hypothalamic circuitry of neuropeptide Y regulation of neuroendocrine function and food intake via the Y5 receptor subtype. *Neuroendocrinology* 74:106-119

Campbell RE, Smith MS, Allen SE, Grayson BE, French-Mullen J, Grove KL 2002 Orexin Neurons in the Lateral Hypothalamic Area Express the Neuropeptide Y Y4 Receptor and are Activated by Rat Pancreatic Polypeptide (rPP), *J Neuroscience* (submitted)

Carrasco M, Portillo F, Larsen PJ, Vallo JJ 2001 Insulin and glucose administration stimulates Fos expression in neurones of the paraventricular

nucleus that project to autonomic preganglionic structures. *J Neuroendocrinol* 13:339-346

Catzefflis C, Pierroz DD, Rohner-Jeanrenaud F, Rivier JE, Sizonenko PC, Aubert ML 1993 Neuropeptide Y administered chronically into the lateral ventricle profoundly inhibits both the gonadotropic and the somatotrophic axis in intact adult female rats. *Endocrinology* 132:224-234

Causing CG, Makus KD, Ma Y, Miller FD, and Colmers WF 1996 Selective upregulation of T alpha 1 alpha-tubulin and neuropeptide Y mRNAs after intermittent excitatory stimulation in adult rat hippocampus in vivo. *J Comp Neurol* 367:132-146

Chalmers DT, Lovenberg TW, De Souza, EB 1995 Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: Comparison with CRF1 receptor mRNA expression. *J Neuroscience* 15:6340-6350

Champagne D, Beaulieu J, Drolet G 1998 CRFergic innervation of the paraventricular nucleus of the rat hypothalamus: a tract-tracing study. *J Neuroendocrinol* 10:119-131

Chehab FF, Lim ME, Lu R 1996 Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* 12:318-320

Chehab FF, Mounzih K, Lu R, Lim ME 1997 Early onset of reproductive function in normal female mice treated with leptin. *Science* 275:88-90

Chen P, Li C, Haskell-Luevano C, Cone RD, Smith MS 1999 Altered expression of agouti-related protein and its colocalization with neuropeptide Y in the arcuate nucleus of the hypothalamus during lactation. *Endocrinology* 140: 2645-2650

Chen Y, Brunson KL, Muller MB, Cariaga W, Baram TZ 2000 Immunocytochemical distribution of corticotropin-releasing hormone receptor type-1 (CRF(1))-like immunoreactivity in the mouse brain: light microscopy analysis using an antibody directed against the C-terminus. *J Comp Neurol* 420:305-323

Cheung CC, Clifton DK, Steiner RA 1997 Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* 138:4489-4492

Cheung CC, Thornton JE, Kuijper JL, Weigle DS, Clifton DK, Steiner RA 1997 Leptin is a metabolic gate for the onset of puberty in the female rat. *Endocrinology* 138:855-858

Chicchio SR, Gallardo MG, Louzan P, Gutnisky V, Tramezzani JH 2001 Melanin-concentrating hormone stimulates the release of luteinizing hormone-releasing hormone and gonadotropins in the female rat acting at both median eminence and pituitary levels. *Biol Reprod* 64:1466-1472

Chronwall BM, DiMaggio DA, Massari VJ, Pickel VM, Ruggiero DA, and O'Donohue TL 1985 The anatomy of neuropeptide-Y-containing neurons in rat brain. *Neuroscience* 15:1159-1181

Ciofi P, Fallon JH, Croix D, Polak JM, Tramu G 1991 Expression of neuropeptide Y precursor-immunoreactivity in the hypothalamic dopaminergic tubero-infundibular system during lactation in rodents. *Endocrinology* 128:823-384

Cluderay JE, Harrison DC, Hervieu GJ 2002 Protein distribution of the orexin-2 receptor in the rat central nervous system. *Regul Pept* 104:131-144

Colmers WF, Klapstein GJ, Fournier A, St-Pierre S, and Treherne KA 1991 Presynaptic inhibition by neuropeptide Y in rat hippocampal slice in vitro is mediated by a Y₂ receptor. *Br J Pharmacol* 102:41-44

Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ 2001 The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int J Obes Relat Metab Disord* 25:S63-S67

Corder R, Gaillard RC, Bohlen P 1988 Isolation and sequence of rat peptide YY and neuropeptide Y. *Regul Pept* 21:253-261

Cowley MA, Pronchuk N, Fan W, Dinulescu DM, Colmers WF, Cone RD 1999 Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: Evidence of a cellular basis for the adipostat. *Neuron* 24:155-163

Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ 2001 Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411:480-484

Crisioine L, Gigollier p, Batzl-Hartman C, Rueger H, Stricker-Krongrad A, Wyss P, Brunner L, Whitebread S, Yamaguchi Y, Gerald C, Heurich RO, Walker MW, Chiesi M, Schilling W, Hofbauer KG, Levens N 1998 Food intake in free-feeding and energy deprived lean rats is mediated by the neuropeptide Y Y5 receptor. *J Clin Invest* 102:2136-2145

Dallman MF, Akana SF, Strack AM, Hanson ES, Sebastian RJ 1995 The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons. *Ann NY Acad Sci* 771:730-742

Davies L, Marks JL 1994 Role of hypothalamic neuropeptide Y gene expression in body weight regulation. *Am J Physiol* 266:R1687-R1691

de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS 2nd, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG 1998 The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 95:322-327

de Quidt ME, Emson PC 1986 Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system--II. Immunohistochemical analysis. *Neuroscience* 18:545-618

De Souza EB 1987 Corticotropin-releasing factor receptors in the rat central nervous system: characterization and regional distribution. *J Neuroscience* 7:88-100

Dryden S, Frankish HM, Wang Q, Pickavance L, and Williams G 1996 The serotonergic agent fluoxetine reduces neuropeptide Y levels and neuropeptide Y secretion in the hypothalamus of lean and obese rats. *Neuroscience* 72:557-566

Dryden S, McCarthy HD, Malabu UH, Ware M, and Williams G 1993 Increased neuropeptide Y concentrations in specific hypothalamic nuclei of the rat following treatment with methysergide: evidence that NPY may mediate serotonin's effects on food intake. *Peptides* 14:791-796

Dryden S, Wang Q, Frankish HM, Pickavance L, and Williams G 1995 The serotonin (5-HT) antagonist methysergide increases neuropeptide Y (NPY) synthesis and secretion in the hypothalamus of the rat. *Brain Res* 699:12-18

Dube MG, Sahu A, Kalra PS, Kalra SP 1992 Neuropeptide Y release is elevated from the microdissected paraventricular nucleus of food-deprived rats: an in vitro study. *Endocrinology* 131:684-688

- Dumont Y, Fournier A, Quirion R 1998 Expression and characterization of the neuropeptide Y Y₅ receptor subtype in the rat brain. *J Neurosci* 18:5565-5574
- Dumont Y, Jacques D, Bouchard P, and Quirion R 1998 Species differences in the expression and distribution of the neuropeptide Y Y₁, Y₂, Y₄ and Y₅ receptors in rodents, guinea pig, and primate brains. *J Comp Neurol* 402:372-384
- Dumont Y, Quirion R 2000 [(125)I]-GR231118: a high affinity radioligand to investigate neuropeptide Y Y(1) and Y(4) receptors. *Br J Pharmacol* 129:37-46
- Dunn AJ, Berridge CW 1990 Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Res Brain Res Rev* 15:71-100
- Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, Tamaki M, Yoshioka T, Hayase M, Matsuoka N, Aizawa-Abe M, Yoshimasa Y, Nakao K Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* 48:2028-2033
- Eckard CP, Beck-Sickinger AG, Wieland HA 1999 Comparison of antibodies directed against receptor segments of NPY- receptors. *J Recept Signal Transduct Res* 19:379-394
- Edwards CM, Abusnana S, Sunter D, Murphy KG, Ghatei MA, Bloom SR 1999 The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin. *J Endocrinol* 160:7-12

Ehlers CL, Henriksen SJ, Wang M, Rivier J, Vale W, Bloom FE 1983 Corticotropin releasing factor produces increases in brain excitability and convulsive seizures in rats. *Brain Res* 278:332-336

Elias CF, Saper CB, Maratos-Flier E, Tritos NA, Lee C, Kelly J, Tatro JB, Hoffman GE, Ollmann MM, Barsh GS, Sakurai T, Yanagisawa M, Elmquist JK 1998 Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *J Comp Neurol* 402:442-459

Emson PC, Corder R, Lowry PJ 1984 Demonstration of a neuropeptide Y-like immunoreactivity in human pheochromocytoma extracts. *Regul Pept* 8:89-94

Erickson JC, Hollopeter G, Palmiter RD 1996 Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science* 274:1704-1707

Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR 1994 Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. *J Neurosci* 14:1834-1855

Esclapez M, Tillakaratne NJ, Tobin AJ, Houser CR 1993 Comparative localization of mRNAs encoding two forms of glutamic acid decarboxylase with nonradioactive in situ hybridization methods. *J Comp Neurol* 331:339-362

Everitt BJ, Hokfelt T, Terenius L, Tatemoto K, Mutt V, and Goldstein M 1984 Differential co-existence of neuropeptide Y (NPY)-like immunoreactivity with

catecholamines in the central nervous system of the rat. *Neuroscience* 11:443-462

Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD Role of melanocortinerbic neurons in feeding and the agouti obesity syndrome. *Nature* 385:165-168

Finn PD, Cunningham MJ, Pau KY, Spies HG, Clifton DK, Steiner RA 1998 The stimulatory effect of leptin on the neuroendocrine reproductive axis of the monkey. *Endocrinology* 139:4652-4662

Finta EP, Regenold JT, Illes P 1992 Depression by neuropeptide Y of noradrenergic inhibitory postsynaptic potentials of locus coeruleus neurones. *Naunyn Schmiedebergs Arch Pharmacol* 346:472-474

Fischer D, Patchev VK, Hellbach S, Hassan AH, Almeida OF 1995 Lactation as a model for naturally reversible hypercorticalism plasticity in the mechanisms governing hypothalamo-pituitary- adrenocortical activity in rats. *J Clin Invest* 96:1208-1215

Fisher LA, Jessen G, Brown MR, 1983 Corticotropin-releasing factor (CRF): Mechanism to elevate mean arterial pressure and heart rate. *Regul Pept* 5:153-161

Fisher LA, Rivier J, Rivier C, Spiess J, Vale WW, Broan MR 1982 Corticotropin-releasing factor (CRF) Central effects on mean arterial pressure and heart rate. *Endocrinology* 110:2222-2224

Flynn MC, Turrin NP, Plata-Salaman CR, French-Mullen JM 1999 Feeding response to neuropeptide Y-related compounds in rats treated with Y5 receptor antisense or sense phosphothio-oligodeoxynucleotide. *Physiol Behav* 66:881-884

Fox SR, Smith MS 1984 The suppression of pulsatile luteinizing hormone secretion during lactation in the rat. *Endocrinology* 115:2045-2051

Freeman ME 1995 The hypothalamus. In: Conn PM (ed) *Neuroscience in medicine*. JB Lippincott Company, Philadelphia, pp 261-300

Freeman ME, Kanyicska B, Lerant A, Nagy G 2000 Prolactin: structure, function, and regulation of secretion. *Physiol Rev* 80:1523-1631

Furuta M, Funabashi T, Kimura F 2002 Suppressive action of orexin A on pulsatile luteinizing hormone secretion is potentiated by a low dose of estrogen in ovariectomized rats. *Neuroendocrinology* 75:151-157

Gehlert DR 1999 Role of hypothalamic neuropeptide Y in feeding and obesity. *Neuropeptides* 33:329-338

Gehlert DR, Chronwall BM, Schafer MP, O'Donohue TL 1987 Localization of neuropeptide Y messenger ribonucleic acid in rat and mouse brain by *in situ* hybridization. *Synapse* 1:25-31

Gerald C, Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, Smith KE, Vaysse P, Durkin MM, Laz TM, Linemeyer DL, Schaffhauser AO, Whitebread S, Hofbauer KG, Taber RI, Branchek TA, Weinshank RL 1996 A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* 382:168-171

Gillard ER, Dang DQ, Stanley BG 1993 Evidence that neuropeptide Y and dopamine in the perifornical hypothalamus interact antagonistically in the control of food intake. *Brain Res* 628:128-136

Gonzalez MI, Baker BI, Wilson CA 1997 Stimulatory effect of melanin-concentrating hormone on luteinising hormone release. *Neuroendocrinology* 66:254-262

Gonzalez MI, Vaziri S, Wilson CA 1996 Behavioral effects of alpha-MSH and MCH after central administration in the female rat. *Peptides* 17:171-177

Gregor P, Feng Y, DeCarr LB, Cornfield LJ, McCaleb ML 1996 Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. *J Biol Chem* 271:27776-27781

Grove KL, Brogan RS, Smith MS 2001 Novel expression of neuropeptide Y (NPY) mRNA in hypothalamic regions during development: Region-specific effects of maternal deprivation on NPY and agouti-related protein mRNA. *Endocrinology* 142:4771-4776

Hakansson M, de Lecea L, Sutcliffe JG, Yanagisawa M, Meister B 1999 Leptin receptor- and STAT3-immunoreactivities in hypocretin/orexin neurones of the lateral hypothalamus. *J Neuroendocrinol* 11:653-663

Haynes AC, Jackson B, Overend P, Buckingham RE, Wilson S, Tadayyon M, Arch JR 1999 Effects of single and chronic intracerebroventricular administration of the orexins on feeding in the rat. *20:1099-1105*

Heinrichs SC, Cole BJ, Pich EM, Menzaghi F, Koob GF, Hauger RL 1992 Endogenous corticotropin-releasing factor modulates feeding induced by neuropeptide Y or a tail-pinch stressor. *Peptides* 13:879-884

Heinrichs SC, Menzaghi F, Koob GF 1998 Neuropeptide Y-induced feeding and its control. *Vitam Horm* 54:51-66

Heinrichs SC, Menzaghi F, Pich EM, Hauger RL, Koob GF 1993 Corticotropin-releasing factor in the paraventricular nucleus modulates feeding induced by neuropeptide Y. *Brain Res* 611:18-24

Heinrichs SC, Richard D 1999 The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. *Neuropept* 33:350-359

Hendry SHC 1993 Organization of neuropeptide Y neurons in the mammalian central nervous system. In: Colmers WF, Wahlestedt C (ed) *The biology of neuropeptide Y and related peptides*. Humana Press, Totawa, pp 65-156

Hendry SHC, Jones EG, DeFelipe J, Schmechel D, Brandon C, Emson PC 1984 Neuropeptide-containing neurons in the cerebral cortex are also GABAergic. *Proc Natl Acad Sci USA* 81:6526-6530

Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA 2001 Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. *Neuroscience* 103:777-797

Herzog H, Hort YJ, Ball HJ, Hayes G, Shine J, Selbie LA 1992 Cloned human neuropeptide Y receptor couples to two different second messenger systems. *Proc Natl Acad Sci U S A* 89:5794-5798

Hetherington AW, Ranson SW 1940 Hypothalamic lesions and adiposity in the rat. *Anat Rec* 78:149-172

Ho MW, Beck-Sickinger AG, Colmers WF 2000 Neuropeptide Y(5) Receptors Reduce Synaptic Excitation in Proximal Subiculum, But Not Epileptiform Activity in Rat Hippocampal Slices. *J Neurophysiol* 83:723-734

Hokfelt T, Lundberg JM, Tatemoto K, Mutt V, Terenius L, Polak J, Bloom S, Sasek C, Elde R, Goldstein M 1983 Neuropeptide Y (NPY)- and FMRFamide neuropeptide-like immunoreactivities in catecholamine neurons of the rat medulla oblongata. *Acta Physiol Scand* 117:315-318

Holets VR, Hokfelt T, Rokaeus A, Terenius L, and Goldstein M 1988 Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or

galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. *Neuroscience* 24:893-906

Horvath TL, Diano S, van den Pol AN 1999 Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. *J Neurosci* 19:1072-1087

Hu Y, Bloomquist BT, Cornfield LJ, DeCarr LB, Flores-Riveros JR, Friedman L, Jiang P, Lewis-Higgins L, Sadlowski Y, Schaefer J, Velazquez N, McCaleb ML 1996 Identification of a novel hypothalamic neuropeptide Y receptor associated with feeding behavior. *J Biol Chem* 271:26315-26319

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F 1997 Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131-141

Hwa JJ, Witten MB, Williams P, Ghibaudi L, Gao J, Salisbury BG, Mullins D, Hamud F, Strader CD, Parker EM 1999 Activation of the NPY Y5 receptor regulates both feeding and energy expenditure. *Am J Physiol* 277:R1428-R1434

Hwang BH, Guntz JM 1997 Downregulation of corticotropin-releasing factor mRNA, but not vasopressin mRNA, in the paraventricular hypothalamic nucleus of rats following nutritional stress. *Brain Res Bull* 43:509-514

Illes P, Finta EP, Nieber K 1993 Neuropeptide Y potentiates via Y2-receptors the inhibitory effect of noradrenaline in rat locus coeruleus neurones. *Naunyn Schmiedebergs Arch Pharmacol* 348:546-548

Inui A, Mizuno N, Ooya M, Suenaga K, Morioka H, Ogawa T, Ishida M, Baba S 1985 Cross-reactivities of neuropeptide Y and peptide YY with pancreatic polypeptide antisera: evidence for the existence of pancreatic polypeptide in the brain. *Brain Res* 330:386-389

Inui A, Mizuno N, Ooya M, Suenaga K, Morioka H, Ogawa T, Ishida M, Baba S 1985 Cross-reactivities of neuropeptide Y and peptide YY with pancreatic polypeptide antisera: evidence for the existence of pancreatic polypeptide in the brain. *Brain Res* 330:386-389

Iqbal J, Pompolo S, Sakurai T, Clarke IJ 2001 Evidence that orexin-containing neurones provide direct input to gonadotropin-releasing hormone neurones in the ovine hypothalamus. *J Neuroendocrinol* 13:1033-1041

Iwai C, Ochiai H, Nakai Y 1989 Electron-microscopic immunocytochemistry of neuropeptide Y immunoreactive innervation of vasopressin neurons in the paraventricular nucleus of the rat hypothalamus. *Acta Anat (Basel)* 136:279-284

Jain MR, Horvath TL, Kalra PS, Kalra SP 2000 Evidence that NPY Y1 receptors are involved in stimulation of feeding by orexins (hypocretins) in sated rats. *Regul Pept* 87:19-24

Jain MR, Pu S, Kalra PS, Kalra SP 1999 Evidence that stimulation of two modalities of pituitary luteinizing hormone release in ovarian steroid-primed ovariectomized rats may involve neuropeptide Y Y1 and Y4 receptors. *Endocrinology* 140:5171-5177

Jakab RL and Goldman-Rakic PS 2000 Segregation of serotonin 5-HT_{2A} and 5-HT₃ receptors in inhibitory circuits of the primate cerebral cortex. *J Comp Neurol* 417:337-348

Kaga T, Fujimiya M, Inui A 2002 Emerging functions of neuropeptide Y Y(2) receptors in the brain. *Peptides* 22:501-506

Kalra PS, Bonavera JJ, Kalra SP 1995 Central administration of antisense oligodeoxynucleotides to neuropeptide Y (NPY) mRNA reveals the critical role of newly synthesized NPY in regulation of LHRH release. *Regul Pept* 59:215-220

Kalra PS, Dube MG, Xu B, Farmerie WG, Kalra SP 1998 Neuropeptide Y (NPY) Y1 receptor mRNA is upregulated in association with transient hyperphagia and body weight gain: Evidence for a hypothalamic site for concurrent development of leptin resistance. *J Neuroendocrinol* 10:43-49

Kalra SP and Crowley WR 1992 Neuropeptide Y: A novel neuroendocrine peptide in the control of pituitary hormone secretion, and its relation to luteinizing hormone. *Front. Neuroendocrinol* 13:1-46

Kalra SP, Dube MG, Pu S, Horvath TL, Kalra PS 1999 Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine Reviews* 20:68-100

Kalra SP, Dube MG, Pu S, Horvath TL, Kalra PS 1999 Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine Rev* 20:68-100

Kalra SP, Dube MG, Sahu A, Phelps CP, Kalra PS 1991 Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. *Proc Natl Acad Sci U S A* 88:10931-10935

Kalra SP, Fuentes M, Fournier A, Parker SL, Crowley WR 1992 Involvement of the Y-1 receptor subtype in the regulation of luteinizing hormone secretion by neuropeptide Y in rats. *Endocrinology* 130:3323-3330

Kalra SP, Horvath TL 1998 Neuroendocrine interactions between galanin, opioids, and neuropeptide Y in the control of reproduction and appetite. *Ann N Y Acad Sci* 863:236-240

Kalra SP, Kalra PS 1996 Nutritional infertility: the role of the interconnected hypothalamic neuropeptide Y-galanin-opioid network. *Front Neuroendocrinol* 17:371-401

Kanatani A, Kanno T, Ishihara A, Hata M, Sakuraba A, Tanaka T, Tsuchiya Y, Mase T, Fukuroda T, Fukami T, Ihara M 1999 The novel neuropeptide Y Y(1)

receptor antagonist J-104870: a potent feeding suppressant with oral bioavailability. *Biochem Biophys Res Commun* 266:88-91

Kanatani A, Mashiko S, Murai N, Sugimoto N, Ito J, Fukuroda T, Fukami T, Morin N, MacNeil DJ, Van der Ploeg LH, Saga Y, Nishimura S, Ihara M 2000 Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. *Endocrinology* 141:1011-1016

Kask A, Rago L, and Harro J 1998 Anxiolytic-like effects of neuropeptide Y (NPY) and NPY₁₃₋₁₆ microinjected into vicinity of locus coeruleus in rats. *Brain Res* 788:345-348

Katsuura G, Asakawa A, Inui A 2002 Roles of pancreatic polypeptide in regulation of food intake. *Peptides* 23:323-329

Kawaguchi Y 1995 Physiological subgroups of nonpyramidal cells with specific morphological characteristics in layer II/III of rat frontal cortex. *J Neurosci* 15:2638-2655

Kawaguchi Y, Kubota Y 1996 Physiological and morphological identification of somatostatin- or vasoactive polypeptide-containing cells among GABAergic cell subtypes in rat frontal cortex. *J Neurosci* 16:2701-2715

Kesterson RA, Huszar D, Lynch CA, Simerly RB, Cone RD 1997 Induction of neuropeptide Y gene expression in the dorsal medial hypothalamic nucleus in two models of the agouti obesity syndrome. *Mol Endocrinol* 11:630-637

Kishimoto T, Pearce RV 2nd, Lin CR, Rosenfeld MG 1995 A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle. *Proc Natl Acad Sci USA* 92:1108-1112

Kiss A, Jezova D, Aguilera G 1994 Activity of the hypothalamic pituitary adrenal axis and sympathoadrenal system during food and water deprivation in the rat. *Brain Res* 663:84-92

Klapstein GJ, Colmers WF 1993 On the site of presynaptic inhibition by neuropeptide Y in rat hippocampus in vitro. *Hippocampus* 3:103-112

Klapstein GJ, Colmers WF 1997 Neuropeptide Y suppresses epileptiform activity in rat hippocampus in vitro. *J Neurophysiol* 78:1651-1661

Kohsaka A, Watanobe H, Kakizaki Y, Suda T, Schioth HB 2001 A significant participation of orexin-A, a potent orexigenic peptide, in the preovulatory luteinizing hormone and prolactin surges in the rat. *Brain Res* 898:166-170

Kostich WA, Chen A, Sperle K, Largent BL 1998 Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF2gamma receptor. *Mol Endocrinol* 12:1077-1085

Kotz CM, Briggs JE, Grace MK, Levine AS, Billington CJ 1998 Divergence of the feeding and thermogenic pathways influenced by NPY in the hypothalamic PVN of the rat. *Am J Physiol* 275:R471-R477

Kow LM, Pfaff DW 1991 The effects of the TRH metabolite cyclo(His-Pro) and its analogs on feeding. *Pharmacol Biochem Behav* 38:359-364

Koylu EO, Smith Y, Couceyro PR, Kuhar MJ 1999 CART peptides colocalize with tyrosine hydroxylase neurons in rat locus coeruleus. *Synapse* 31:309-311

Krahn DD, Gosnell BA, Levine AS, Morley JE 1988 Behavioral effects of corticotropin-releasing factor: localization and characterization of central effects. *Brain Res* 443:63-69

Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S 1998 Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 393:72-76

Kunii K, Yamanaka A, Nambu T, Matsuzaki I, Goto K, Sakurai T 1999 Orexins/hypocretins regulate drinking behaviour. *Brain Res* 842:256-261

Kushi A, Sasai H, Koizumi H, Takeda N, Yokoyama M, Nakamura M 1998 Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice. *Proc Natl Acad Sci USA* 95:15659-15664

Lambert PD, Couceyro PR, McGirr KM, Dall Bechia SE, Smith Y, Kuhar MJ 1998
CART peptides in the central control of feeding and interactions with
neuropeptide Y. *Synapse* 29:293-298

Larsen PJ, Sheikh SP, Jakobsen CR, Schwartz TW, Mikkelsen JD 1993
Regional distribution of putative NPY Y₁ receptors and neurons expressing Y₁
mRNA in forebrain areas of the rat central nervous system. *Eur J Neurosci*
5:1622-1637

Lee LR, Haisenleder DJ, Marshall JC, Smith MS 1989 Expression of alpha-
subunit and luteinizing hormone (LH) beta messenger ribonucleic acid in the rat
during lactation and after pup removal: relationship to pituitary gonadotropin-
releasing hormone receptors and pulsatile LH secretion. *Endocrinology* 124:776-
782

Lee LR, Paul SJ, Smith MS 1989 Dose response effects of pulsatile GnRH
administration on restoration of pituitary GnRH receptors and pulsatile LH
secretion during lactation. *Neuroendocrinology* 49:664-668

Leupen SM, Besecke LM, Levine JE 1997 Neuropeptide Y Y₁-receptor
stimulation is required for physiological amplification of preovulatory luteinizing
hormone surges. *Endocrinology* 138:2735-2739

Leupen SM, Levine JE 1999 Role of protein kinase C in facilitation of luteinizing hormone (LH)- releasing hormone-induced LH surges by neuropeptide Y. *Endocrinology* 140:3682-3687

Levin BE 1999 Arcuate NPY neurons and energy homeostasis in diet-induced obese and resistant rats. *Am J Physiol* 276:R382-R387

Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, Bilezikjian L, Rivier J, Sawchenko PE, Vale WW 2001 Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA* 98:7570-7575

Li C, Chen P, Smith MS 1998 The acute suckling stimulus induces expression of neuropeptide Y (NPY) in cells in the dorsomedial hypothalamus and increases NPY expression in the arcuate nucleus. *Endocrinology* 139:1645-1652

Li C, Chen P, Smith MS 1999 Morphological evidence for direct interaction between arcuate nucleus neuropeptide Y (NPY) neurons and gonadotropin-releasing hormone neurons and the possible involvement of NPY Y1 receptors. *Endocrinology* 140:5382-5390

Li C, Chen P, Smith MS 2000 Corticotropin Releasing Hormone (CRH) Neurons in the Paraventricular Nucleus (PVH) are Direct Targets for Neuropeptide Y

(NPY) Neurons in the Arcuate Nucleus (ARH): An Anterograde Tracing Study. Brain Res 854:122-129

Libet B, Gleason CA, Wright EW Jr, Feinstein B 1977 Suppression of an epileptiform type of electrocortical activity in the rat by stimulation in the vicinity of locus coeruleus. Epilepsia 18:451-462

Liposits ZS, Sievers L, Paull WK 1988 Neuropeptide-Y and ACTH-immunoreactive innervation of corticotropin releasing factor (CRF)-synthesizing neurons in the hypothalamus of the rat: An immunocytochemical analysis at the light and electron microscopic levels. Histochemistry 88:227-234

Lopez-Valpuesta FJ, Nyce JW, Myers RD 1996 NPY-Y1 receptor antisense injected centrally in rats causes hyperthermia and feeding. Neuroreport 7:2781-2784

Loren I, Alumets J, Hakanson R, Sundler F 1979 Immunoreactive pancreatic polypeptide (PP) occurs in the central and peripheral nervous system: preliminary immunocytochemical observations. Cell Tissue Res 200:179-186

Lovenberg TW, Liam CW, Grigoriadis DE, Clevenger W, Chalmers DT, DeSouza EB, Oltersdorf T 1995 Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. Proc Natl Acad Sci USA 92:836-840

Ludwig DS, Tritos NA, Mastaitis JW, Kulkarni R, Kokkotou E, Elmquist J, Lowell B, Flier JS, Maratos-Flier E 2001 Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance. *J Clin Invest* 107:379-386

Lund PE, Shariatmadari R, Uustare A, Detheux M, Parmentier M, Kukkonen JP, Akerman KE 2000 The orexin OX1 receptor activates a novel Ca^{2+} influx pathway necessary for coupling to phospholipase C. *J Biol Chem* 275:30806-30812

Lundberg JM, Hokfelt T, Anggard A, Kimmel J, Goldstein M, Markey K 1980 Coexistence of an avian pancreatic polypeptide (APP) immunoreactive substance and catecholamine in some peripheral and central neurons. *Acta Physiol Scand* 110:107-109

Lupien SJ, de Leon M, de Santi S, Convit A, Tarshish C, Nair NPV, Thakur M, McEwen BS, Hauger RL, Meaney MJ 1998 Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nature Neurosci* 1:69-73

Malabu UH, Kilpatrick A, Ware M, Vernon RG, Williams G 1994 Increased neuropeptide Y concentrations in specific hypothalamic regions of lactating rats: possible relationship to hyperphagia and adaptive changes in energy balance. *Peptides* 15:83-87

Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK 2001 Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 435:6-25

Marks DL, Cone RD 2001 Central melanocortins and the regulation of weight during acute and chronic disease. *Recent Prog Horm Res* 56:359-375

Marsh DJ, Baraban SC, Hollopeter G, Palmiter RD 1999 Role of the Y5 neuropeptide Y receptor in limbic seizures. *Proc Natl Acad Sci USA* 96: 13518-13523

Marsh DJ, Hollopeter G, Kafer KE, Palmiter RD 1998 Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nat Med* 4:718-721

McDonald JK 1990 Role of neuropeptide Y in reproductive function. *Ann N Y Acad Sci* 611:258-272

McKibbin PE, Rogers P, Williams G Increased neuropeptide Y concentrations in the lateral hypothalamic area of the rat after the onset of darkness: possible relevance to the circadian periodicity of feeding behavior. *Life Sci* 48:2527-2533

McQuiston AR, Petrozzino JJ, Connor JA, Colmers WF 1996 Neuropeptide Y1 receptors inhibit N-type calcium currents and reduce transient calcium increases in rat dentate granule cells. *J Neurosci* 16:1422-1429

Melander T, Hokfelt T, Rokaeus A 1986 Distribution of galanin like immunoreactivity in the rat central nervous system. *J Comp Neurol* 248:475-517

Menzaghi F, Heinrichs SC, Pich EM, Tilders FJ, Koob GF 1993 Functional impairment of hypothalamic corticotropin-releasing factor neurons with immunotargeted toxins enhances food intake induced by neuropeptide Y. *Brain Res* 618:76-82

Mikkelsen JD, Larsen PJ 1992 A high concentration of NPY (Y1)-receptor mRNA-expressing cells in the rat arcuate nucleus. *Neurosci Lett* 148:195-198

Millan MA, Jacobowitz DM, Hauger RL, Catt KJ, Aguilera G 1986 Distribution of corticotropin-releasing factor receptors in primate brain. *Proc Natl Acad Sci USA* 83:1921-1925

Miyata I, Shiota C, Chaki S, Okuyama S, Inagami T 2001 Localization and characterization of a short isoform of the corticotropin-releasing factor receptor type 2alpha (CRF(2)alpha-tr) in the rat brain. *Biochem Biophys Res Commun* 280:553-557

Miyazaki K, Funakoshi 1988 A Distribution of pancreatic polypeptide-like immunoreactivity in rat tissues. *Regul Pept* 21:37-43

Mizuno M, Gearing M, Terasawa E 2000 The role of neuropeptide Y in the progesterone-induced luteinizing hormone-releasing hormone surge in vivo in ovariectomized female rhesus monkeys. *Endocrinology* 14:1772-1179

Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA, Mobbs CV 1998 Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes* 47:294-297

Mongeau R, Blier P, De Montigny C 1997 The serotonergic and noradrenergic systems of the hippocampus: their interactions and the effects of antidepressant treatments. *Brain Res Rev* 23:145-195

Morley JE 1987 Neuropeptide regulation of appetite and weight. *Endocr Rev* 8:256-87

Morton GJ, Schwartz MW 2001 The NPY/AgRP neuron and energy homeostasis. *Int J Obes Relat Metab Disord* 5:56-62

Mounzih K, Lu R, Chehab FF 1997 Leptin treatment rescues the sterility of genetically obese ob/ob males. *Endocrinology* 138:1190-1193

Nagatani S, Guthikonda P, Thompson RC, Tsukamura H, Maeda KI, Foster DL 1998 Evidence for GnRH regulation by leptin: leptin administration prevents reduced pulsatile LH secretion during fasting. *Neuroendocrinology* 67:370-376

Nagatani S, Zeng Y, Keisler DH, Foster DL, Jaffe CA 2000 Leptin regulates pulsatile luteinizing hormone and growth hormone secretion in the sheep. *Endocrinology* 141:3965-3975

Nakajima M, Inui A, Asakawa A, Momose K, Ueno N, Teranishi A, Baba S, Kasuga N 1998 Neuropeptide Y produces anxiety via Y2-type receptors. *Peptides* 19:359-363

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S 2001 A role for ghrelin in the central regulation of feeding. *Nature* 409:194-198

Naveilhan P, Hassani H, Canals JM, Ekstrand AJ, Larefalk A, Chhajlani V, Arenas E, Gedda K, Svensson L, Thoren P, Ernfors P 1999 Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. *Nature Med* 5:1188-1193

Neuman RS 1986 Suppression of penicillin-induced focal epileptiform activity by locus coeruleus stimulation: mediation by alpha 1-adrenoreceptor. *Epilepsia* 27:359-366

Nichol KA, Morey A, Couzens MH, Shine J, Herzog H, Cunningham AM 1999 Conservation of expression of neuropeptide Y5 receptor between human and rat hypothalamus and limbic regions suggests an integral role in central neuroendocrine control. *J Neurosci* 19:10295-10304

Niimi M, Sato M, Taminato T 2001 Neuropeptide Y in central control of feeding and interactions with orexin and leptin. *Endocrine* 14:269-273

O'Hare MM, Tenmoku S, Aakerlund L, Hilsted L, Johnsen A, Schwartz TW 1988 Neuropeptide Y in guinea pig, rabbit, rat and man. Identical amino acid sequence and oxidation of methionine-17. *Regul Pept* 20:293-304

Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS 1997 Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 278:135-138

Olschowka JA, O'Donohue TL, Jacobowitz DM 1981 The distribution of bovine pancreatic polypeptide-like immunoreactive neurons in rat brain. *Peptides* 2:309-331

Olschowka JA, O'Donohue TL, Mueller GP, Jacobowitz DM 1982 Hypothalamic and extrahypothalamic distribution of CRF-like immunoreactive neurons in the rat brain. *Neuroendocrinology* 35:305-308

Olszewski PK, Wirth MM, Shaw TJ, Grace MK, Billington CJ, Giraudo SQ, Levine AS 2001 Role of alpha-MSH in the regulation of consummatory behavior: immunohistochemical evidence. *Am J Physiol Regul Integr Comp Physiol* 281:R673-R680

Ono N, Bedran-DeCastro J, McCann S 1985 Ultrashort-loop feedback of corticotropin (ACTH)- realizing factor to enhance ACTH release during stress. *Proc Natl Acad Sci USA* 82:3528-3531

Ordog T, Chen MD, O'Byrne KT, Goldsmith JR, Connaughton MA, Hotchkiss J, Knobil E 1998 On the mechanism of lactational anovulation in the rhesus monkey. *Am J Physiol* 274:E665-E676

Parker RMC, Herzog H 1999 Regional distribution of Y-receptor subtype mRNAs in rat brain. *Eur J Neurosci* 11:1431-1448

Parker SL, Crowley WR 1993 Central stimulation of oxytocin release in the lactating rat: interaction of neuropeptide Y with alpha-1-adrenergic mechanisms. *Endocrinology* 132:658-666

Parker SL, Parker MS, Crowley WR 1998 Characterization of Y1, Y2 and Y5 subtypes of the neuropeptide Y (NPY) receptor in rabbit kidney. Sensitivity of ligand binding to guanine nucleotides and phospholipase C inhibitors. *Regul Pept* 75-76:127-143

Parker SL, Parker MS, Sweatman T, Crowley WR 1998 Characterization of G protein and phospholipase C-coupled agonist binding to the Y₁ neuropeptide Y receptor in rat brain: Sensitivity to G protein activators and inhibitors and to inhibitors of phospholipase C. *J Pharmacol Exper Therap* 286:382-391

Pedrazzini T, Seydoux J, Kunstner P, Aubert J-F, Grouzmann E, Beermann F, Brunner HR 1998 Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. *Nature Med* 4:722-726

Pepin MC, Pothier F, Barden N 1992 Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. *Nature* 355:725-728

Perrin M, Donaldson C, Chen R, Blount A, Berggren T, Bilezikjian L, Sawchenko P, Vale W 1995 Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. *Proc Natl Acad Sci USA* 92:2969-2973

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS 1998 Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18:9996-10015

Pickavance L, Dryden S, Hopkins D, Bing C, Frankish H, Wang Q, Vernon RG, Williams G 1996 Relationships between hypothalamic neuropeptide Y and food intake in the lactating rat. *Peptides* 17:577-582

Pierroz DD, Catzeflis C, Aebi AC, Rivier JE, Aubert ML 1996 Chronic administration of neuropeptide Y into the lateral ventricle inhibits both the pituitary-testicular axis and growth hormone and insulin-like growth factor I secretion in intact adult male rats. *Endocrinology* 137:3-12

Plata-Salaman CR, Wilson CD, French-Mullen JM 1998 In vivo IL-1 β -induced modulation of G-protein α O subunit subclass in the hypothalamic ventromedial nucleus: implications to IL-1 β -associated anorexia. *Mol Brain Res* 58:188-194

Pu S, Jain MR, Kalra PS, Kalra SP 1998 Orexins, a novel family of hypothalamic neuropeptides, modulate pituitary luteinizing hormone secretion in an ovarian steroid-dependent manner. *Regul Pept* 78:133-136

Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, Maratos-Flier E 1996 A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature* 380:243-247

Radulovic J, Sydow S, Speiss J 1998 Characterization of native corticotropin-releasing factor receptor Type 1 (CRFR1) in the rat and mouse central nervous system. *J Neurosci Res* 54:507-521

Raposo PD, Broqua P, Hayward A, Akinsanya K, Galyean R, Schteingart C, Junien J, Aubert ML 2000 Stimulation of the gonadotropic axis by the neuropeptide Y receptor Y1 antagonist/Y4 agonist 1229U91 in the male rat. *Neuroendocrinology* 71:2-7

Raposo PD, Broqua P, Pierroz DD, Hayward A, Dumont Y, Quirion R, Junien JL, Aubert ML 1999 Evidence that the inhibition of luteinizing hormone secretion by central administration of neuropeptide Y (NPY) in the rat is predominantly mediated by the NPY-Y5 receptor subtype. *Endocrinology* 140:4046-4055

Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE 2001 Urocortin II: a member of

the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci USA* 98:2843-2848

Richard D 1993 Involvement of corticotropin releasing factor in the control of food intake and energy expenditure. *Ann NY Acad Sci* 697:155-172

Rivest S, Laflamme N, Nappi RE 1995 Immune challenge and immobilization stress induce transcription of the gene encoding the CRF receptor in selective nuclei of the rat hypothalamus. *J Neurosci* 15:2680-2695

Rivier C, Vale W 1984 Influence of corticotropin-releasing factor on reproductive functions in the rat. *Endocrinology* 114:914-921

Rominger DH, Rominger CM, Fitzgerald LW, Grzanna R, Largent BL, Zaczek RJ, 1998 Characterization of [¹²⁵I]sauvagine binding to CRH2 receptors: membrane homogenate and autoradiographic studies. *Pharmacol Exp Ther* 286:459-468

Rossi M, Choi SJ, O'Shea D, Miyoshi T, Ghatei MA, Bloom SR 1997 Melanin-concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight. *Endocrinology* 138:351-355

Rothwell NJ 1990 Central effects of CRF on metabolism and energy balance. *Neurosci Biobehav Rev* 14:263-271

Russell SH, Small CJ, Kennedy AR, Stanley SA, Seth A, Murphy KG, Taheri S, Ghatei MA, Bloom SR 2001 Orexin A interactions in the hypothalamo-pituitary gonadal axis. *Endocrinology* 142:5294-5302

Sahu A, Sninsky CA, Kalra SP 1997 Evidence that hypothalamic neuropeptide Y gene expression and NPY levels in the paraventricular nucleus increase before the onset of hyperphagia in experimental diabetes. *Brain Res* 755:339-342

Sahu A, Crowley WR, Kalra SP 1995 Evidence that hypothalamic neuropeptide Y gene expression increases before the onset of the preovulatory LH surge. *J Neuroendocrinol* 7:291-296

Sainsbury A, Schwarzer C, Couzens M, Fetissov S, Furtinger S, Jenkins A, Cox HM, Sperk G, Hokfelt T, Herzog H 2002 Important role of hypothalamic Y2 receptors in body weight regulation revealed in conditional knockout mice. *Proc Natl Acad Sci U S A* 99:8938-8943

Sainsbury A, Schwarzer C, Couzens M, Jenkins A, Oakes SR, Ormandy CJ, Herzog H 2002 Y4 receptor knockout rescues fertility in ob/ob mice. *Genes Dev* 16:1077-1088

Sajdyk TJ, Vandergriff MG, Gehlert DR 1999 Amygdalar neuropeptide Y Y₁ receptors mediate the anxiolytic-like actions of neuropeptide Y in the social interaction test. *Eur J Pharmacol* 368:143-147

Sakanaka M, Shibasaki T, Lederis K 1987 Corticotropin releasing factor-like immunoreactivity in the rat brain as revealed by a modified cobalt-glucose oxidase-diaminobenzidine method. *J Comp Neurol* 260:256-298

Sakurai T 2002 Roles of orexins in regulation of feeding and wakefulness. *Neuroreport* 13:987-995

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M 1998 Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:573-585

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M 1998 Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:573-585

Sato K, Crofton JT, Wang YX, Share L 1995 Effects of gender on the central actions of neuropeptide Y and norepinephrine on vasopressin and blood pressure in the rat. *Brain Res* 689:71-78

Sauvage M, Steckler T 2001 Detection of corticotropin- releasing hormone receptor 1 immunoreactivity in cholinergic, dopaminergic and noradrenergic neurons of the murine basal forebrain and brainstem nuclei-potential implication for arousal and attention. *Neuroscience* 104:643-652

Sawchenko PE, Swanson LW 1981 Central noradrenergic pathways for the integration of hypothalamic neuroendocrine and autonomic responses. *Science* 214:685-687

Sawchenko PE, Swanson LW, Grzanna R, Howe PR, Bloom SR, Polak JM 1985 Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. *J Comp Neurol* 241:138-153

Schaffhauser AO, Stricker-Krongrad A, Brunner L, Cumin F, Gerald C, Whitebread S, Criscione L, Hofbauer KG 1997 Inhibition of food intake by neuropeptide Y Y5 receptor antisense oligonucleotides. *Diabetes* 46:1792-1798

Schaffhauser AO, Whitebread S, Haener R, Hofbauer KG, Stricker-Krongrad A 1998 Neuropeptide Y Y1 receptor antisense oligodeoxynucleotides enhance food intake in energy-deprived rats. *Regul Pept* 75-76:417-423

Schmitz D, Empson RM, Gloveli T, Heinemann U 1997 Serotonin blocks different patterns of low Mg^{2+} -induced epileptiform activity in rat entorhinal cortex, but not hippocampus. *Neuroscience* 76:449-458

Schwartz MW, Baskin DG, Bukowski TR, Kuijper JL, Foster D, Lasser G, Prunkard DE, Porte D Jr, Woods SC, Seeley RJ, Weigle DS. 1996 Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. *Diabetes* 45:531-535

Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG 1996 Identification of targets of leptin action in rat hypothalamus. *J Clin Invest* 98:1101-1106

Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG 1997 Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* 46:2119-2123

Schwartz TW 1983 Pancreatic polypeptide: a unique model for vagal control of endocrine systems. *J Auton Nerv Syst* 9:99-111

Schwartz TW, Fuhlendorff J, Kjems LL, Kristensen MS, Vervelde M, O'Hare M, Krstenansky JL, Bjornholm B 1990 Signal epitopes in the three-dimensional structure of neuropeptide Y. Interaction with Y1, Y2, and pancreatic polypeptide receptors. *Ann N Y Acad Sci* 611:35-47

Schwarzer C, Sperk G, Samanin R, Rizzi M, Gariboldi M, Vezzani A 1996 Neuropeptides-immunoreactivity and their mRNA expression in kindling: functional implications for limbic epileptogenesis. *Brain Res Rev* 22:27-50

Selbie LA, Darby K, Schmitz-Peiffer C, Browne CL, Herzog H, Shine J, Biden TJ 1995 Synergistic interaction of Y1-neuropeptide Y and alpha 1b-adrenergic

receptors in the regulation of phospholipase C, protein kinase C, and arachidonic acid production. *J Biol Chem* 270:11789-11796

Shimada M, Tritos NA, Lowell BB, Flier JS, Maratos-Flier E 1998 Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature* 396:670-674

Siggins GR, Gruol D, Aldenhoff J, Pittman Q 1985 Electrophysiological actions of corticotropin-releasing factor in the central nervous system. *Fed Proc* 44:237-242

Sillard R, Agerberth B, Mutt V, Jornvall H 1989 Sheep neuropeptide Y. A third structural type of a highly conserved peptide. *FEBS Lett* 258(2):263-265

Simerly RB 1995 Anatomical substrates of hypothalamic integration. In: Paxinos G (ed) *The rat nervous system*, second edition. Academic Press, New York, pp 353-376

Singer LK, Kuper J, Brogan RS, Smith MS, Grove KL 2000 Novel expression of hypothalamic neuropeptide Y during postnatal development in the rat. *Neuroreport* 11:1075-1080

Smagin GN, Howell LA, Redmann SJr, Ryan DH, Harris RB 1999 Prevention of stress-induced weight loss by third ventricle CRF receptor antagonist. *Am J Physiol* 276:R1461-1468

Smart D, Jerman JC, Brough SJ, Rushton SL, Murdock PR, Jewitt F, Elshourbagy NA, Ellis CE, Middlemiss DN, Brown F 1999 Characterization of

recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR. *Br J Pharmacol* 128:1-3

Smith MA, Weiss SRB, Berry RL, Zhang L-X, Clark M, Massenburg G, and Post RM 1997 Amygdala-kindled seizures increase the expression of corticotropin-releasing factor (CRF) and CRF-binding protein in GABAergic interneurons of the dentate hilus. *Brain Res* 745:248-256

Smith MS 1993 Lactation alters neuropeptide-Y and proopiomelanocortin gene expression in the arcuate nucleus of the rat. *Endocrinology* 133:1258-1265

Smith MS, Grove KL 2002 Integration of the regulation of reproductive function and energy balance: lactation as a model. *Front Neuroendocrinol* 23:225-256

Spina M, Merlo-Pich E, Chan RK, Basso AM, Rivier J, Vale W, Koob GF 1996 Appetite-suppressing effects of urocortin, a CRF-related neuropeptide. *Science* 273:1561-1564

Stanley BG, Chin AS, Leibowitz SF 1985 Feeding and drinking elicited by central injection of neuropeptide Y: evidence for a hypothalamic site(s) of action. *Brain Res Bull* 14:521-524

Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF 1986 Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides* 7:1189-1192

Stanley BG, Magdalin W, Seirafi A, Thomas WJ, Leibowitz SF 1993 The perifornical area: the major focus of (a) patchily distributed hypothalamic neuropeptide Y-sensitive feeding system(s). *Brain Res* 604:304-317

Statnick MA, Schober DA, Gackenhaimer S, Johnson D, Beavers L, Mayne NG, Burnett JP, Gadski R, Gehlert DR 1998 Characterization of the neuropeptide Y5 receptor in the human hypothalamus: a lack of correlation between Y5 mRNA levels and binding sites. *Brain Res* 810:16-26

Stenzel P, Kesterson R, Yeung W, Cone RD, Rittenberg MB, Stenzel-Poore MP 1995 Identification of a novel murine receptor for corticotropin-releasing hormone expressed in the heart. *Mol Endocrinol* 5:637-645

Stenzel-Poore MP, Cameron VA, Vaughan J, Sawchenko PE, Vale W 1992 Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. *Endocrinology* 130:3378-3386

Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, et al 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530-532

Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, et al 1995 The role of

neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530-532

Stricker-Krongrad A, Richy S, Beck B 2002 Orexins/hypocretins in the ob/ob mouse: hypothalamic gene expression, peptide content and metabolic effects. *Regul Pept* 104:11-20

Suda T, Tozawa F, Iwai I, Sato Y, Sumitomo T, Nakano Y, Yamada M, Demura H 1993 Neuropeptide Y increases the corticotropin-releasing factor messenger ribonucleic acid level in the rat hypothalamus. *Mol Brain Res* 18:311-315

Sutcliffe JG, de Lecea L 2002 The hypocretins: setting the arousal threshold. *Nat Rev Neurosci* 3:339-349

Swanson LW, Sawchenko PE, Lind RW, Rho JH 1987 The CRH motoneuron: differential peptide regulation in neurons with possible synaptic, paracrine, and endocrine outputs. *Ann NY Acad Sci* 512:12-23

Swanson LW, Sawchenko PE, Rivier J, Vale WW 1983 Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. *Neuroendocrinology* 36:165-186

Tamura T, Irahara M, Tezuka M, Kiyokawa M, Aono T 1999 Orexins, orexigenic hypothalamic neuropeptides, suppress the pulsatile secretion of luteinizing hormone in ovariectomized female rats. *Biochem Biophys Res Commun* 264:759-762

Tamura T, Irahara M, Tezuka M, Kiyokawa M, Aono T 1999 Orexins, orexigenic hypothalamic neuropeptides, suppress the pulsatile secretion of luteinizing hormone in ovariectomized female rats. *Biochem Biophys Res Commun* 264:759-762

Tang-Christensen M, Kristensen P, Stidsen CE, Brand CL, Larsen PJ 1998 Central administration of Y5 receptor antisense decreases spontaneous food intake and attenuates feeding in response to exogenous neuropeptide Y. *J Endocrinol* 159:307-312

Tatemoto K, Carlquist M, Mutt V 1982 Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 296:659-60

Tellam DJ, Mohammad YN, Lovejoy DA 2000 Molecular integration of hypothalamo-pituitary-adrenal axis-related neurohormones on the GnRH neuron. *Biochem Cell Biol* 78:205-216

Tillet Y, Caldani M, Batailler M 1989 Anatomical relationships of monoaminergic and neuropeptide Y-containing fibres with luteinizing hormone-releasing hormone systems in the preoptic area of the sheep brain: immunohistochemical studies. *J Chem Neuroanat* 2:319-326

Toni R, Jackson IM, Lechan RM 1990 Neuropeptide-Y-immunoreactive innervation of thyrotropin-releasing hormone-synthesizing neurons in the rat hypothalamic paraventricular nucleus. *Endocrinology* 126:2444-2453

Tritos NA, Mastaitis JW, Kokkotou E, Maratos-Flier E 2001 Characterization of melanin concentrating hormone and preproorexin expression in the murine hypothalamus. *Brain Res* 895:160-166

Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM 1998 Distribution of orexin receptor mRNA in the rat brain. *FEBS Lett* 438:71-75

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G 1999 Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23:99-103

Tsukamura H, Thompson RC, Tsukahara S, Ohkura S, Maekawa F, Moriyama R, Niwa Y, Foster DL, Maeda K 2000 Intracerebroventricular administration of melanin-concentrating hormone suppresses pulsatile luteinizing hormone release in the female rat. *J Neuroendocrinol* 12:529-534

Tsuruo Y, Kawano H, Kagotani Y, Hisano S, Daikoku S, Chihara K, Zhang T, Yanaihara N 1990 Morphological evidence for neuronal regulation of luteinizing hormone-releasing hormone-containing neurons by neuropeptide Y in the rat septo-preoptic area. *Neurosci Lett* 110:261-266

Vale W, Spiess J, Rivier C, Rivier J 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and B-endorphin. *Science* 213:1394-1397

Vamvakopoulos, NC, Chrousos GP 1994 Hormonal regulation of human corticotropin-releasing hormone gene expression: Implications for the stress response and immune/inflammatory reaction. *Endocrine Rev* 15:409-420

van den Pol AN, Gao XB, Obrietan K, Kilduff TS, Belousov AB 1998 Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. *J Neurosci* 18:7962-7971

Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin, M, Vale, W, Sawchenko, PE 2000 Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* 428:191-212

Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, Rivier J, Sawchenko P, Vale W 1995 Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 378:287-292

Vergoni AV, Poggioli R, Bertolini A 1986 Corticotropin inhibits food intake in rats. *Neuropeptides* 7:153-158

Vergoni AV, Schioth HB, Bertolini A 2000 Melanocortins and feeding behavior. *Biomed Pharmacother* 54:129-134

Veazzani A, Moneta D, Mule F, Ravizza T, Gobbi M, French-Mullen J 2000 Plastic changes in neuropeptide Y receptor subtypes in experimental models of limbic seizures. *Epilepsia* 41:S115-S121

Veazzani A, Sperk G, Colmers WF 1999 Neuropeptide Y: emerging evidence for a functional role in seizure modulation. *Trends Neurosci* 22:25-30

Vrang N, Larsen PJ, Mikkelsen JD 1995 Direct projection from the suprachiasmatic nucleus to hypophysiotrophic corticotropin-releasing factor immunoreactive cells in the paraventricular nucleus of the hypothalamus demonstrated by means of Phaseolus vulgaris-leucoagglutinin tract tracing. *Brain Res* 684:61-9

Wade GN, Schneider JE 1992 Metabolic fuels and reproduction in female mammals. *Neurosci Biobehav Rev* 16:235-272

Waite PME, Tracey DF 1995 Trigeminal Sensory System. In: Paxinos G (ed) *The Rat Nervous System* second edition. Academic Press, New York, pp 705-724

Watanobe H, Takebe K 1992 Evidence that neuropeptide Y secretion in the median eminence increases prior to the luteinizing hormone surge in ovariectomized steroid-primed rats: estimation by push-pull perfusion. *Neurosci Lett* 146:57-59

Wehrenberg WB, Corder R, Gaillard RC 1989 A physiological role for neuropeptide Y in regulating the estrogen/progesterone induced luteinizing hormone surge in ovariectomized rats. *Neuroendocrinology* 49:680-682

Whitnall MH, Key S, Ben Barak Y, Ozato K, Gainer H 1985 Neurophysin in the hypothalamo-neurohypophysial system. II. Immunocytochemical studies of the ontogeny of oxytocinergic and vasopressinergic neurons. *J Neurosci* 5:98-109

Widdowson PS 1997 Regionally-selective down-regulation of NPY receptor subtypes in the obese Zucker rat. Relationship to the Y5 'feeding' receptor. *Brain Res* 758:17-25

Wieland HA, Eckard CP, Doods HN, Beck-Sickinger AG 1998 Probing of the neuropeptide Y-Y₁-receptors interaction with anti-receptor antibodies. *Eur J Biochem* 255:595-603

Williams G, Bing C, Cai XJ, Harrold JA, King PJ, Liu XH 2001 The hypothalamus and the control of energy homeostasis: different circuits, different purposes. *Physiol Behav* 74:683-701

Willoughby JO, Blessing WW 1987 Neuropeptide Y injected into the supraoptic nucleus causes secretion of vasopressin in the unanesthetized rat. *Neurosci Lett* 75:17-22

Wimalawansa SJ 1995 Purification and biochemical characterization of neuropeptide Y2 receptor *J Biol Chem* 270:18523-18530

Woldbye DPD, Larsen PJ, Mikkelsen JD, Klemp K, Madsen TM, and Bolwig TG 1997 Powerful inhibition of kainic acid seizures by neuropeptide Y via Y5-like receptors. *Nature Med* 3:761-764

Woller MJ, Terasawa E 1991 Infusion of neuropeptide Y into the stalk-median eminence stimulates in vivo release of luteinizing hormone-release hormone in gonadectomized rhesus monkeys. *Endocrinology* 128:1144-1150

Wong M-L, Licinio J, Pasternak K I, Gold P W 1994 Location of corticotropin-releasing hormone (CRH) receptor mRNA in adult rat brain by in situ hybridization histochemistry. *Endocrinology* 135:2275-2278

Wynn PC, Hauger RL, Holmes MC, Millian MA, Catt KJ, Aguilera, G 1984 Brain and pituitary receptors for corticotropin-releasing factor: Localization and differential regulation after adrenalectomy. *Peptides* 5:1077-1084

Wyss P, Levens N, Stricker-Krongrad A 1998 Stimulation of feeding in lean but not in obese Zucker rats by selective neuropeptide Y Y5 receptor agonist. *Neuroreport* 9:2675-2677

Xu B, Sahu A, Crowley WR, Leranth C, Horvath T, Kalra SP 1993 Role of neuropeptide-Y in episodic luteinizing hormone release in ovariectomized rats: an excitatory component and opioid involvement. *Endocrinology* 133:747-754

Xu B, Sahu A, Kalra PS, Crowley WR, Kalra SP 1996 Disinhibition from opioid influence augments hypothalamic neuropeptide Y (NPY) gene expression and

pituitary luteinizing hormone release: effects of NPY messenger ribonucleic acid antisense oligodeoxynucleotides. *Endocrinology* 137:78-84

Xu Z-Q D, Shi T-J. S, and Hokfelt T 1998 Galanin/GMAP- and NPY-like immunoreactivities in locus coeruleus and noradrenergic nerve terminals in the hippocampal formation and cortex with notes on the galanin-R1 and -R2 receptors. *J Comp Neurol* 392:227-251

Yamada H, Okumura T, Motomura W, Kobayashi Y, Kohgo Y 2000 Inhibition of food intake by central injection of anti-orexin antibody in fasted rats. *Biochem Biophys Res Commun* 267:527-531

Yamanaka A, Kunii K, Nambu T, Tsujino N, Sakai A, Matsuzaki I, Miwa Y, Goto K, Sakurai T 2000 Orexin-induced food intake involves neuropeptide Y pathway. *Brain Res* 859:404-409

Yamanaka A, Sakurai T, Katsumoto T, Yanagisawa M, Goto K 1999 Chronic intracerebroventricular administration of orexin-A to rats increases food intake in daytime, but has no effect on body weight. *Brain Res* 849:248-252

Yan X-X, Toth Z, Schultz L, Ribak CE, and Baram TZ 1998 Corticotropin-releasing hormone (CRH)-containing neurons in the immature rat hippocampal formation: Light and electron microscopic features and colocalization with glutamate decarboxylase and parvalbumin. *Hippocampus* 8:231-243

Yoshihara T, Honma S, Honma K 1996 Effects of restricted daily feeding on neuropeptide Y release in the rat paraventricular nucleus. *Am J Physiol* 270:E589-E595

Yoshihara T, Honma S, Honma K-I 1996 Prefeeding release of paraventricular neuropeptide Y is mediated by ascending noradrenergic neurons in rats. *Am J Physiol* 270:E596-E600

Zarjevski N, Cusin I, Vettor R, Rohner-Jeanrenaud F, Jeanrenaud B 1993 Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity. *Endocrinology* 133:1753-1758

VIII. Appendix

Lactation Differentially Regulates Neuropeptide Y mRNA in Regions of the Ventrolateral Medulla

Introduction:

Previous studies in the Smith laboratory have demonstrated that lactation results in increased neuropeptide Y (NPY) mRNA expression in the arcuate nucleus and dorsomedial nucleus of the hypothalamus (ARH, DMH). NPY is well established as a potent orexigenic agent and modulator of reproductive function. In the lactating rat model, elevated hypothalamic NPY most likely plays an important role in the hyperphagia and suppressed reproductive function associated with the lactating state.

NPY is also coexpressed in a subset of catecholaminergic neurons in the ventral lateral medulla (VLM). It was recently demonstrated that the majority of A1 cells and virtually all of the C1 cells that co-express NPY project rostrally to hypothalamic areas. While the role of NPY in these neurons is unclear, previous studies in our laboratory have shown that noradrenergic A1 neurons, but not adrenergic C1 neurons of the VLM, express cFos following a suckling stimulus, indicating the activation of these cells. It is reasonable to hypothesize, then, that brainstem NPY expression in the VLM may also be increased during lactation and may contribute to the modulation of neuroendocrine and feeding systems. Retrograde tract-tracing studies have shown that these activated VLM neurons have direct projections to the ARH and PVH, providing a means by which NPY produced in the brainstem may modulate these systems.

Therefore, the goal of the present study was to determine if suckling activation of A1 VLM neurons reflects an increase in brainstem NPY mRNA expression.

Materials and Methods:

Animals and Tissue: Pregnant and virgin cycling female rats were used (Simonsen, CA). The day of delivery was considered day 0 postpartum and litters were adjusted to 8 pups on day 2. Lactating rats (n=8) were allowed to suckle their pups undisturbed until sacrifice on day 10. Cycling female rats were sacrificed on diestrus (n=8), as determined by daily vaginal smear. Tissue for in situ hybridization was collected by rapid decapitation, and brains were removed, frozen on dry ice, and stored at -80°C until sectioning at $20\mu\text{m}$ on a cryostat.

In situ Hybridization: Coronal brain sections throughout the VLM were hybridized with a NPY cRNA probe that was transcribed from a 511bp cDNA with 25% of the UTP labeled with ^{35}S . After fixing and dehydrating the tissue, sections were exposed to the cRNA probe overnight in a moist chamber at 55°C . After incubation, the slides were washed in 4xSSC and ribonuclease A at 37°C , and in 0.1xSSC at 60°C . Slides were then dehydrated in ethanol and put on film (Biomax, Kodak) for 36h. The level of NPY mRNA was quantified using NIH image software. The silver grain density X area was measured in arbitrary units using a sampling box that encompassed the entire region of interest. The sampling box was held constant for all brains analyzed and a background reading measured with the same sampling box in an area devoid of specific labeling was subtracted from each measurement. To obtain the mean level of labeling for each animal, an even number of sample values were used from anatomically matched sections in each VLM subregion for each animal. Values

are expressed as mean arbitrary units (area X density) $\pm$ SEM. Data were analyzed using student's t-test (Prism, Graph Pad Software).

Results and Discussion:

In situ hybridization was used to quantify NPY mRNA throughout the VLM, according to the noradrenergic A1, mixed A1/C1, and adrenergic C1 subregions of the VLM as indicated in Figure 1 A, B, C. Quantification of NPY mRNA levels indicated that while A1 and mixed A1/C1 regions expressed similar levels of NPY mRNA in lactating (day 10, 8 pups) and diestrous (day 1 of estrous cycle) animals, lactation resulted in a significant suppression of NPY mRNA in the adrenergic C1 subregion (Fig. 2).

Therefore, lactation did not cause an increase in NPY mRNA expression in the A1 region of the VLM, despite a robust increase in cFos expression in this region following a suckling stimulus. However, lactation did result in a decrease in NPY mRNA expression in the C1 region, an area previously found to not express cFos following a suckling stimulus. Taken together, these data suggest that NPYergic C1 neurons may be suppressed during lactation and that lactation may differentially regulate NPY mRNA in adrenergic and noradrenergic neurons of the VLM. It has been suggested that A1 noradrenergic neurons play a role in stimulating food intake. Alternatively, C1 adrenergic neurons are implicated in the activation of stress pathways. Therefore, activation of A1 neurons and suppression of C1 neurons may be responsible for some of the hyperphagia and hyporesponsiveness to stress that is observed during lactation.

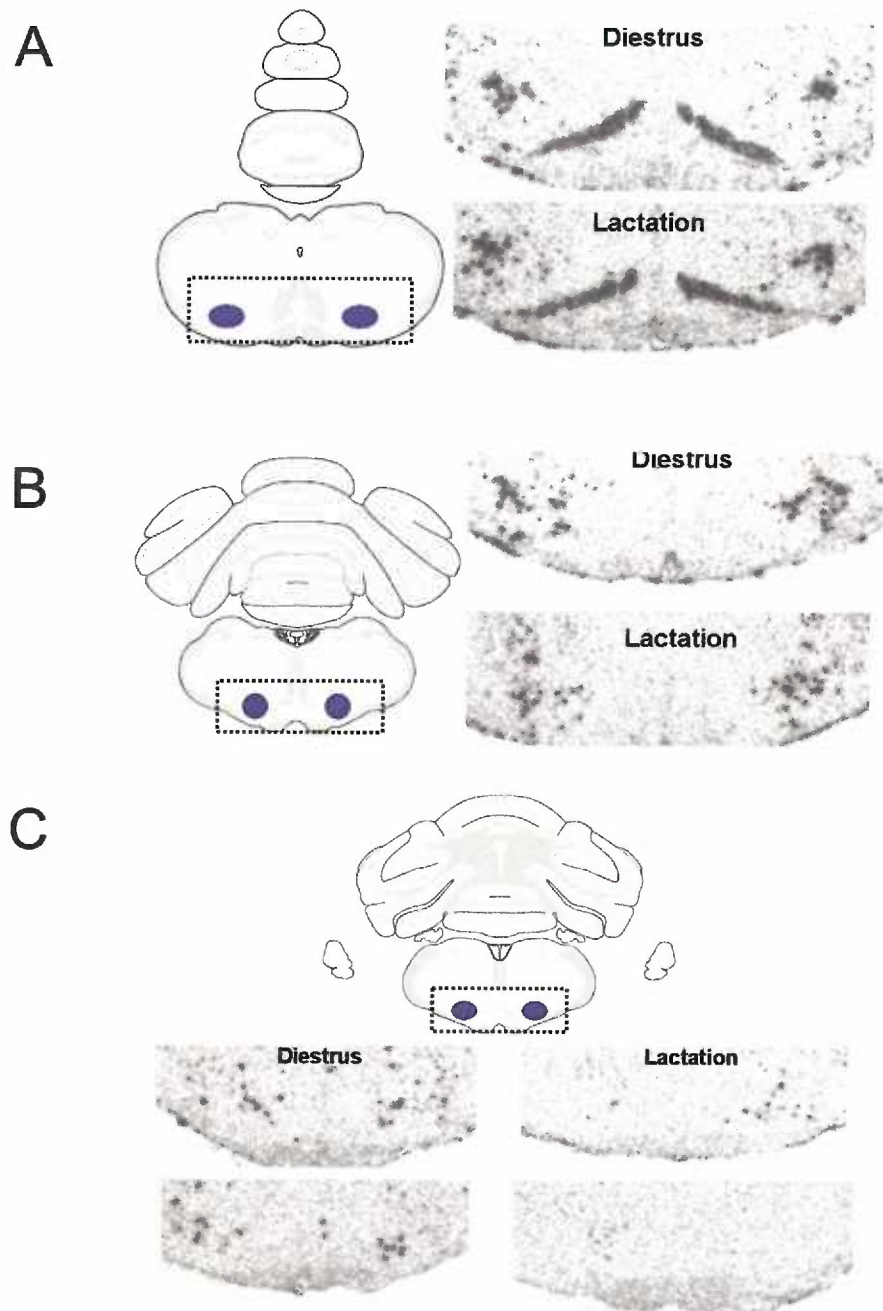


Figure A-1. Representative photomicrographs in subregions of the VLM showing NPY mRNA expression during the diestrus stage of the estrous cycle or lactation. Brain maps indicate the A1 (A), A1/C1 (B), and C1 (C) subregions of the VLM and the region illustrated in the representative images.

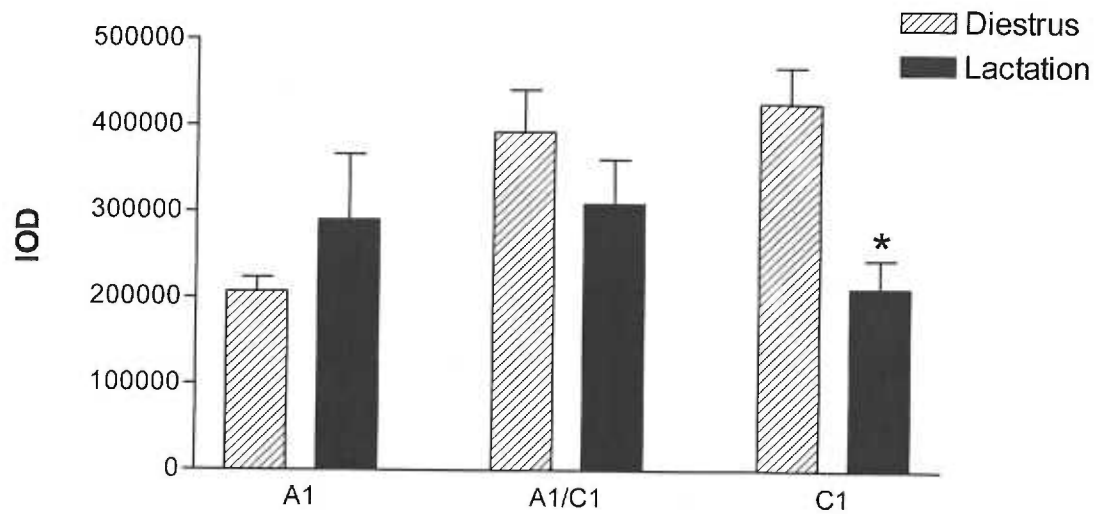


Figure A-2. Quantification of NPY mRNA expression in subregions of the VLM during lactation (black bars) or diestrus (hatched bars). Values represent mean arbitrary units (area X density) $\pm$ SEM. Data were analyzed using a students t-test. * = $p < 0.01$.