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Leptin and glucocorticoids exert opposing actions on neuropeptide Y and corticotropin releasing hormone secretion within the hypothalamus.

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Nutritional Science

Major professor

Date 12-13-99

LEPTIN AND GLUCOCORTICOIDS EXERT OPPOSING ACTIONS ON NEUROPEPTIDE Y AND CORTICOTROPIN-RELEASING HORMONE SECRETION WITHIN THE HYPOTHALAMUS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1999

ABSTRACT

LEPTIN AND GLUCOCORTICOIDS EXERT OPPOSING ACTIONS ON NEUROPEPTIDE Y AND CORTICOTROPIN-RELEASING HORMONE SECRETION WITHIN THE HYPOTHALAMUS

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Leptin is proposed to rapidly control food intake by influencing secretion of hypothalamic neuropeptide Y (NPY), a stimulator of food intake, and/or corticotropin-releasing hormone (CRH), an inhibitor of food intake. Glucocorticoids appear to oppose leptin actions on these hypothalamic neuropeptides to control food intake and body fat balance. The present studies were thus conducted to determine if leptin rapidly influences secretion of hypothalamic NPY and/or CRH, and also to determine if leptin effects on secretion of these neuropeptides are altered by glucocorticoids. Lean and ob/ob mice, 7 - 8 wk old intact or adrenalectomized (ADX) mice, were used.

I measured changes in concentrations (as an index of secretion) of NPY and CRH in specific hypothalamic regions (i.e. arcuate nucleus, paraventricular nucleus, ventromedial nucleus, and dorsomedial nucleus) 1 h or 3 h after intracerebroventricular leptin administration and also directly assessed leptin effects on *in vitro* NPY and CRH secretion. No rapid-onset (i.e. within 1-3 h) effects of leptin on hypothalamic NPY and CRH concentrations were observed in intact mice, consistent with the ineffectiveness of leptin to alter *in vitro* NPY and CRH secretions (within 20 min) from hypothalamic

preparations obtained from these intact mice. Possibly, the presence of opposing actions of endogenous glucocorticoids in intact mice might have masked or inhibited hypothalamic NPY and/or CRH responses to administered leptin. Consistent with this hypothesis, leptin (30 nM) rapidly, i.e. within 20 min, decreased NPY release and rapidly increased CRH release from hypothalamic blocks obtained from ADX mice, and dexamethasone (DEX, 0.5 μM), a synthetic glucocorticoid, had opposite actions on secretion of NPY (stimulatory) and CRH (inhibitory) from these tissue preparations. Actions of DEX on NPY and CRH secretion predominated when it was added to the tissue 20 min prior to addition of leptin. Failure of leptin to reverse DEX effects on NPY and CRH secretion from the hypothalamic blocks from ADX mice is consistent with the ineffectiveness of leptin administration to alter *in vitro* NPY and CRH secretion from the hypothalamus obtained from intact mice in the present studies.

In conclusion, my present studies suggest that leptin and glucocorticoids exert opposing actions on NPY and CRH secretion within the hypothalamus. The presence of glucocorticoids may restrain actions of leptin on secretion of these neuropeptides within the hypothalamus.

ACKNOWLEDGMENTS

I would like to express my great appreciation to my advisor Dr. Dale Romsos for his guidance and support. Without his continuous encouragement, I couldn't have accomplished this work. I would like to thank my committees, Dr. Bennink, Dr. Lookingland, and Dr. Song for their willingness to serve on my committee and for their support. My special thanks go to Dr. Lookingland for his valuable input to my study. I thank my colleagues, Dr. Anahita Mistry, Joo-Won Lee, Soo-Young Kang, and Sangjin Chung for their friendship. I also thank Shelli Pfeifer for her friendly assistance during my study. I deeply appreciate my mother and family in Korea for their love and support, which made me move on. Most of all, I would like to thank my husband Seung-Jae Song and my lovely son Christopher for their love, understanding, and support.

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LIST OF ABBREVIATIONS

ACTH ..... Adrenocorticotropic hormone

ADX ..... Adrenalectomy

AH ..... Anterior hypothalamus

ARC ..... Arcuate nucleus of hypothalamus

BAT ..... Brown adipose tissue

cAMP ..... 3',5'-Cyclic adenosine monophosphate

CNS ..... Central nervous system

CRH ..... Corticotropin releasing hormone

DEX ..... Dexamethasone

DMH ..... Dorsomedial nucleus of hypothalamus

ICV ..... Intracerebroventricular injection

IL-6 ..... Interleukin 6

IP ..... Intraperitoneal injection

IV ..... Intravenous injection

LH ..... Lateral hypothalamus

LSD ..... Least significant difference

MPOA ..... Medial preoptic area of hypothalamus

mRNA ..... Messenger ribonucleic acid

NPY .....Neuropeptide Y

OB-R .....Leptin receptor

PEPCK.....Phosphoenolpyruvate carboxykinase

PKC .....Protein kinase C

PKA .....Protein kinase A

PP .....Pancreatic peptide

PVN .....Paraventricular nucleus of hypothalamus

PYY .....Peptide YY

SC .....Subcutaneous injection

SCN .....Suprachiasmatic nucleus of hypothalamus

SPO .....Supraoptic nucleus of hypothalamus

VMH .....Ventromedial nucleus of hypothalamus

CHAPTER I. INTRODUCTION

Obesity is the result of a disorder in body energy balance that occurs when energy intake chronically exceeds energy expenditure. The high prevalence of obesity in the United States of America continues to be a public health concern since obesity is closely related to non-insulin dependent diabetes, hypertension, cardiovascular disease, and certain cancers (Flegal, 1996). Animal models of genetic obesity, including obese (ob/ob), diabetes (db/db), tub, and yellow mice, and Zucker fa/fa rats, have been widely used to understand the mechanisms underlying development of obesity (Coleman, 1978). Parabiosis experiments of normal mice with db/db mice, and ob/ob mice with db/db mice and normal mice, suggested that ob/ob mice may lack a circulating satiety factor but possess a normal satiety center while db/db mice may produce a circulatory satiety factor but possess an abnormal satiety center (Coleman and Hummel, 1969, Coleman, 1973).

Cloning and identification of the ob gene (Zhang et al. 1994) has greatly accelerated obesity research. Leptin, the product of the obese (ob) gene, is a 16-KDa hormone produced by adipocytes (Zhang et al. 1994). Leptin has been shown to decrease food intake and increase energy expenditure in rodents, which leads to weight loss (Campfield et al. 1995, Cusin et al. 1996, Halaas et al. 1995, Pelleymounter et al. 1995, Stephens et al. 1995).

A nonsense mutation at codon 105 (from arginine to stop codon) of the ob gene is found in ob/ob mice (Zhang et al. 1994). This defect in the ob gene leads to production of

a nonfunctional truncated leptin in ob/ob mice. The absence of leptin (ob gene product) in ob/ob mice is the primary cause of severe obesity in these mice. Their obesity was corrected when exogenous leptin was given to ob/ob mice (Campfield et al. 1995, Halaas et al. 1995, Pelleymounter et al. 1995, Stephens et al. 1995). The effects of leptin on food intake and body weight were more effective when leptin was injected centrally than when administered peripherally in mice and rats (Campfield et al. 1995, Stephens et al. 1995). These observations indicate that actions of leptin likely occur within the central nervous system (CNS), the hypothalamus would be a likely target for leptin action on food intake.

To understand the sites and mechanism of leptin action, efforts were made to identify the leptin receptor. Leptin receptor (OB-R), the product of the db gene, was first identified in the choroid plexus where cerebrospinal fluid is made (Tartaglia et al. 1995, Malik and Young, 1996). OB-R is a single transmembrane protein composed of three domains; an 816 amino acid long-extracellular domain next to a predicted 23 amino acid, transmembrane domain and a variably sized cytoplasmic domain (Tartaglia et al. 1995). Different subtypes of OB-R, which differ in the length of intracellular domain (either short or long), are produced by alternative splicing (Chen et al. 1996, Chua et al. 1996a, Lee et al. 1996). Among the different subtypes of OB-R, only the long form of the OB-R was found to be active in signal transduction through the *Jak*/STAT (*Janus* kinase/signal transducers and activators of transcription) pathway (Ghilardi et al. 1996, Tartaglia et al. 1997).

A nonsense mutation in the intracellular domain of the db gene results in the production of truncated form of leptin receptor in db/db mice (Tartaglia et al. 1995). This short form of OB-R is inactive in signal transduction via *Jak*/STAT pathway (Ghilardi et

al. 1996). Unresponsiveness to leptin action on food intake and body weight in db/db mice is explained by this defect in OB-R (Campfield et al. 1995, Halaas et al. 1995, Stephens et al. 1995).

Several lines of evidence support the notion that leptin acts in the brain, especially in the hypothalamus (Campfield et al. 1995, Ghilardi et al. 1996, Schwartz et al. 1996a, Vassie et al. 1996). First, the marked effects of leptin on food intake after ICV injection suggest that the brain is a target tissue for leptin action (Campfield et al. 1995, Stephens et al. 1995). Leptin, produced by fat cells, can cross the blood-brain barrier by a saturable transport mechanism with an influx constant (K_i) of (5.87) 10^{-4} ml/g-min to exert its action within the CNS (Banks et al.1996). The relative abundance of long form OB-R mRNA (% of L to S+L) is the highest (36 %) within the hypothalamus among all the tissues that have been examined (Ghilardi et al. 1996). Leptin receptor mRNA is densely concentrated in the ARC and VMH, with lower levels present in the PVN and DMH in rats (Elmquist et al. 1998, Hakansson et al. 1998, Schwartz et al. 1996a). This anatomical evidence of high expression of leptin receptor within the hypothalamus also supports the hypothesis that leptin acts within this region to regulate food intake and energy metabolism (Ghilardi et al. 1996, Schwartz et al. 1996a).

Glucocorticoids have also been linked to the etiology of obesity in many animal models of obesity including ob/ob mice. Removal of glucocorticoids by adrenalectomy (ADX) reverses the symptoms of obesity such as hyperphagia and hyperinsulinemia in ob/ob mice (Johnson et al. 1991, Okuda and Romsos, 1994, Walker and Romsos, 1992). The mechanism by which ADX attenuates obesity is not yet clearly understood. Replacement of glucocorticoids into the CNS of ADX ob/ob mice reverses these effects of ADX,

suggesting a major role for glucocorticoids within the CNS in the development of obesity (Tokuyama and Himms-Hagen, 1987, Walker and Romsos,1992).

Possible mechanisms for leptin and glucocorticoid action have not yet been elucidated. Leptin and glucocorticoids may act on neurotransmitters to exert their actions on food intake and body weight (**Fig. 1**). Neuropeptide Y (NPY), a stimulator of feeding, and corticotropin-releasing hormone (CRH), an inhibitor of feeding, have been postulated as the candidates for mediating the actions of leptin or glucocorticoids within the hypothalamus (Campfield et al. 1996, Rohner-Jeanrenaud et al. 1996).

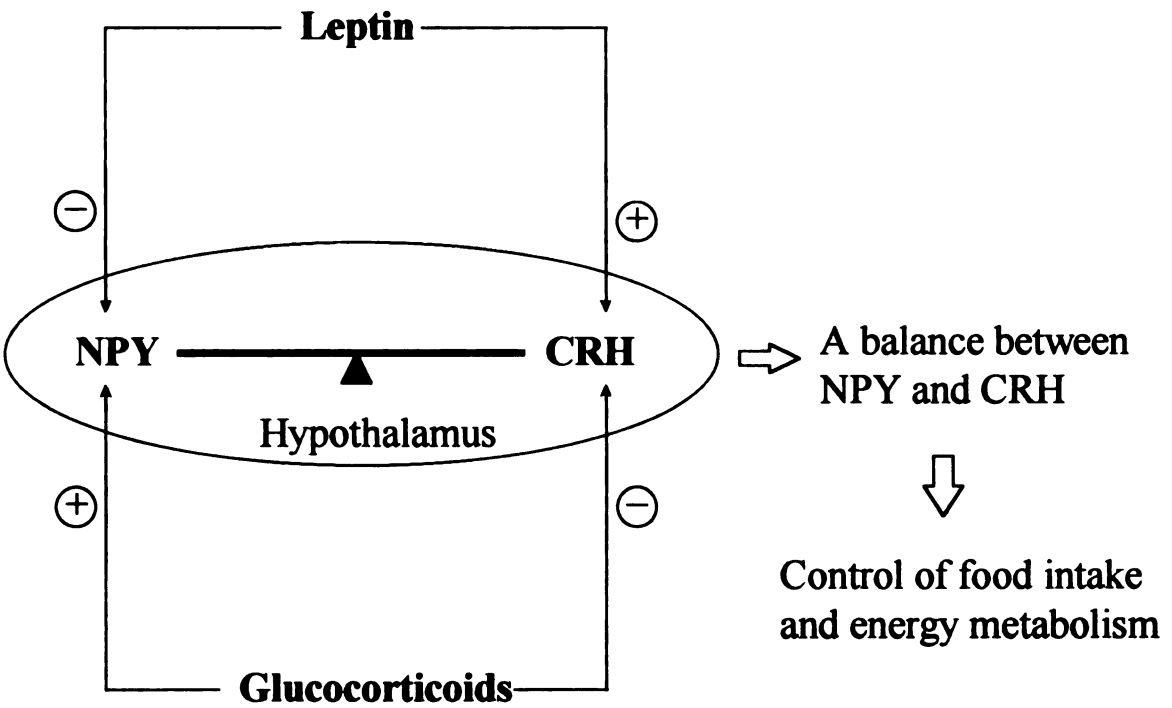

FIGURE 1. Possible interactions between leptin and glucocorticoids within the hypothalamus. Leptin and glucocorticoids may act in opposite ways to maintain a balance between NPY and CRH within the hypothalamus.

NPY, a 36-amino acid peptide of the pancreatic polypeptide family, is widely distributed throughout the CNS with dense NPY-containing neurons especially in the hypothalamus (Hendry, 1993). Chronic NPY injections into the PVN of hypothalamus causes increases in food intake as well as dramatic weight gain (Stanley et al. 1986). Brown adipose tissue (BAT) thermogenesis which contributes substantially to total energy expenditure in rodents, was suppressed ~ 50 % by central injection of NPY (Billington et al. 1991). It has been reported that NPY decreases BAT thermogenesis by suppressing sympathetic nerve activity to BAT (Egawa et al. 1991). NPY is also known to increase lipoprotein lipase activity in white adipose tissue, resulting in increased fat storage and body weight (Billington et al, 1991). These accumulated evidences suggest that NPY administration produces positive energy balance.

It has been reported that hypothalamic NPY mRNA and contents are higher in obese fa/fa rats and diet-induced obese rats than in lean controls (Beck et al. 1990a, McKibbin et al. 1991). NPY secretion from the PVN was 2-fold higher in fa/fa rats than lean controls as measured by push-pull cannula *in vivo*. (Dryden et al. 1995).

These observations suggest that NPY may possibly contribute to development of obesity in rodents by enhancing food intake as well as by increasing metabolic efficiency. Thus, I examined the concentrations and/or release of NPY in the hypothalamus of lean and ob/ob mice to help understand the possible role of NPY in development of obesity in ob/ob mice.

CRH, synthesized in the PVN of hypothalamus, is a neurotransmitter responsible for the inhibition of food intake and body weight (Arase et al. 1989a and 1989b, Hotta et al. 1991, Hulsey et al. 1995b, Krahn et al. 1988, Rohner-Jeanrenaud et al. 1989). Chronic ICV injections (5 ug/day for 7 days) of CRH prevented excessive body weight gain

present even in pair-fed Zucker fa/fa rats (Rohner-Jeanrenaud, 1989). This finding suggests that CRH increases energy expenditure as well as decreases food intake. Effects of CRH on energy expenditure are linked to regulation of BAT thermogenesis.

CRH (5 ug) injected ICV increased BAT thermogenesis by 20 ~ 30 % after ICV injection in normal rats and in VMH-lesioned obese rats (Arase et al. 1988 and 1989b). Increased BAT thermogenesis after ICV CRH administration in rats may be due to stimulation of sympathetic nerve activity to the BAT (Egawa et al. 1990). It is possible that the action of CRH is suppressed in ob/ob mice, resulting in obesity. I thus examined the concentrations and secretion of CRH from the hypothalamus of lean and ob/ob mice to determine if any alterations in CRH are observed in ob/ob mice.

Glucocorticoids might oppose leptin actions (Ur et al. 1996, Zakrzewska et al. 1997). Inhibitory actions of leptin on food intake and body weight were recently reported to be more pronounced in ADX rats and mice than in intact ones (Mistry et al. 1997, Zakrzewska et al. 1997). This observation and the localization of leptin and glucocorticoid receptors within the PVN (a site of NPY release/CRH synthesis) and ARC (a site of NPY synthesis/CRH release) support the hypothesis that interactions between leptin and glucocorticoids may contribute to the regulation of NPY and CRH within the hypothalamus (Mercer et al. 1996, Tempel and Leibowitz, 1994). It is possible that the absence of leptin in ob/ob mice leads to a dysregulation of glucocorticoid action that may result in development of obesity.

Numerous studies demonstrate that the inhibitory actions of leptin on food intake are rapid in onset, i.e. within 30 min to 2 hr (Campfield et al. 1995, Flynn et al. 1998, Mistry et al. 1997, Rentsch et al. 1995, Seeley et al. 1996). These rapid-onset actions of leptin on

food intake may more likely occur via mechanisms other than altered gene expression, given the time typically required for modulation of protein synthesis. Leptin is able to rapidly, within seconds, depolarize rat PVN neurons and activate ATP-sensitive potassium channels within the hypothalamus (Glaum et al. 1996, Powis et al. 1998, Spanswick et al. 1997). These leptin-induced changes in membrane potential and ion channels within the hypothalamus may lead to rapid changes in neurotransmitter secretion.

Glucocorticoids via yet to be fully defined mechanisms cause rapid-onset changes in secretion of hypothalamic NPY (stimulatory) and CRH (inhibitory) in addition to their slower genomic actions (Beato 1989, Calogero et al. 1988, Chen and Romsos, 1995, Stephens et al. 1995, Suda et al. 1985). These actions of glucocorticoids on hypothalamic NPY and CRH secretion are opposite those proposed for leptin.

Whether acute administration of leptin was associated with changes in hypothalamic NPY and CRH secretion in lean and ob/ob mice were subjects of great interest in this study. The possible counter-regulatory actions of leptin and glucocorticoids on hypothalamic NPY and CRH secretion were also determined in this study. The overall focus of my research was to examine the acute effects of leptin on secretion of NPY and CRH within the hypothalamus and possible inhibitory actions of glucocorticoids on the actions of leptin. The specific objectives of the present study were to determine:

1) if NPY and CRH are altered in the hypothalamus of ob/ob mice by measuring the concentrations of NPY and CRH within the specific regions of hypothalamus in both lean and ob/ob mice, and by examining release of NPY and CRH from the hypothalamus of both lean and ob/ob mice *in vitro*.
2) the acute effects of leptin on the concentrations of NPY and CRH within the discrete

regions of hypothalamus of lean and ob/ob mice.

3) if acute action of leptin on NPY and CRH in the hypothalamus is more pronounced in the absence of glucocorticoids by using ADX mice.

4) if leptin and glucocorticoids act in opposite ways to regulate NPY and CRH secretion within the hypothalamus.

CHAPTER II. REVIEW OF LITERATURE

A. Leptin

1. Discovery and actions of leptin

Animal models of genetic obesity including obese (ob/ob), diabetes (db/db), tub, and yellow mice, and the Zucker fa/fa rat have been widely used to understand the mechanisms underlying the development of obesity (Coleman, 1978). Single gene mutations cause obesity in these animal models with the characteristics of hyperphagia, hyperinsulinemia and high plasma corticosterone. Parabiosis experiments of normal mice with db/db mice, ob/ob mice with db/db mice, and ob/ob mice with normal mice, suggested that ob/ob mice may lack a circulating satiety factor with a normal satiety center while db/db mice may have an abnormal satiety center (Coleman and Hummel, 1969, Coleman, 1973).

The ob gene was first cloned and identified by the Friedman group with positional cloning techniques (Zhang et al. 1994). The ob gene product, now named leptin, is a 16-kDa protein (167 amino acid) primarily secreted by adipocytes (Zhang et al. 1994). Leptin has been shown to decrease food intake and increase oxygen consumption, and thus decrease body weight in mice and rats (**Table 1**, Campfield et al. 1995, Cusin et al. 1996, Halaas et al. 1995, Pelleymounter et al. 1995, Stephens et al. 1995).

A nonsense mutation at codon 105 (arginine to stop codon) of ob gene, which leads to the production of a truncated protein, was found in ob/ob mice (Zhang et al. 1994). This

TABLE 1. The effects of leptin on food intake, body weight, and oxygen consumption

Animals and leptin treatment	Findings	References
Lean, ob/ob, and db/db mice; 5 ug/g, ip injection daily for 33 d.	Decreased FI and BW in lean and ob/ob, but not in db/db	Halaas et al. 1996
Lean and ob/ob mice; Different doses for 28 d. (0.1, 1, & 10 ug/g).	A dose-dependent decrease in FI and BW in lean and ob/ob mice. Increased oxygen consumption in ob/ob mice.	Pelleymounter et al. 1995
Lean and ob/ob mice; Single injection iv (3 ug/mouse) or ICV (1 ug/mouse).	Decreased FI during 7 h. More effective with ICV injection.	Campfield et al. 1995
Lean, ob/ob, and db/db mice; Different doses, sc or ICV, for 2 d.	Marked reduction in FI with ICV injection at lower doses (10 ng/g). No effect of leptin in db/db mice.	Stephens et al. 1995
Lean and fa/fa rats 3 ug/rat, ICV.	Higher doses (2-10 times) was used to decrease FI in fa/fa rat than in lean rats.	Cusin et al. 1996

Abbreviations: FI, food intake; BW, body weight; ip, intraperitoneal; iv, intravenous; ICV, intracerebroventricular; sc, subcutaneous.

defect in the ob gene leads to the severe obesity in ob/ob mice with the characteristics of hyperphagia, hyperinsulinemia, and high plasma corticosterone. When leptin was given peripherally or centrally, food intake and body weight were decreased in ob/ob mice without any effects in db/db mice (Campfield et al. 1995, Halaas et al. 1995, Pelleymounter et al. 1995, Stephens et al. 1995). This finding supports the hypothesis made by the Coleman group that ob/ob mice lack a satiety factor (leptin) whereas db/db mice may have an abnormal satiety center.

Several lines of evidence support the notion that leptin acts in the brain, especially in the hypothalamus (Campfield et al. 1995, Ghilardi et al. 1996, Shwartz et al. 1996a, Vassie et al. 1996). First, the marked effects of leptin on food intake after ICV injection suggest that the brain is a target tissue for leptin action (Campfield et al. 1995). Anatomical evidence of high expression of leptin receptor mRNA within the hypothalamus also support the hypothesis that leptin acts within the hypothalamus to regulate food intake and energy metabolism (Ghilardi et al. 1996, Schwartz et al. 1996a). Leptin, produced by fat cells, crosses the blood-brain barrier by a saturable transport mechanism with an influx constant (K_i) of (5.87) 10^{-4} ml/g-min as measured by intravenously injected ^{125}I-leptin (Banks et al.1996).

2. Leptin receptor (OB-R) and signal transduction

The db gene was suggested to encode a leptin receptor since db/db mice, which have a defect in db gene, were resistant to the actions of leptin when recombinant leptin was administered either peripherally or ICV (Halaas et al. 1995, Stephens et al. 1995). Leptin receptor (OB-R) was first identified in the choroid plexus where cerebrospinal fluid is

made (Malik and Young, 1996, Tartaglia et al. 1995). OB-R is a single membrane-spanning receptor most related to class I cytokine receptors, the gp 130 signal-transducing component of the cytokine, interleukin-6 (IL-6) receptor, the granulocyte colony-stimulating factor (G-CSF) receptor, and the leukemia inhibitory factor (LIF) receptor (Tartaglia et al. 1995). OB-R is composed of three domains; an 816 amino acid long-extracellular domain is followed by a predicted 23 amino acid transmembrane domain and a variably sized cytoplasmic domain. Five different subtypes of OB-R (a-e), which differ in the length of intracellular domain (either short or long), are produced by alternative splicing (Chen et al. 1996, Chua et al. 1996a, Lee et al. 1996).

Mouse OB-R mRNA coding short form (OB-Ra, 34 amino acid intracellular domain) is highly abundant in the choroid plexus, lymph nodes, lung, liver, uterus, and is also present in nearly all tissues in the body in a smaller amount (Ghilardi et al. 1996, Tartaglia et al. 1995). The functions of OB-Ra have not yet been identified (Baumann et al. 1996, Ghilardi et al. 1996). Presence of OB-Ra in nearly all tissues may imply its physiological importance in the body. The OB-Ra have been suggested to serve a leptin transport or clearance function in the body (Tartaglia et al. 1995). Whether or not OB-Ra functions as a transporter of leptin into the brain, as predicted by its high abundance in the choroid plexus needs further investigations (Tartaglia et al. 1995).

Mouse OB-Rb mRNA coding long form (OB-Rb, 304 amino acid intracellular domain) is also detected in nearly all tissues at very low levels, < 10 % of total OB-R mRNA (Ghilardi et al. 1996). The relative abundance of this OB-Rb mRNA (% of L to S+L) is the highest (36 %) in the hypothalamus (Ghilardi et al. 1996). This OB-Rb mRNA is densely localized in the ARC and VMH with lower levels present in the PVN and DMH in

rats as identified by *in situ* hybridization using a probe complementary to OB-Rb mRNA **(Fig. 2)**(Elmquist et al. 1998, Hakansson et al. 1998, Schwartz et al. 1996a).

The homology of leptin receptor to a class I cytokine receptor family has greatly accelerated a search for possible mechanisms of leptin actions within the hypothalamus (Tartaglia et al. 1997). The class I cytokine receptors are known to act through *Jak*/STAT (*Janus* Kinase/signal transducers and activators of transcription) pathway **(Fig. 3)**(Ihle, 1995). Activation of cytokine receptors induces the tyrosine phosphorylation and activation of one or more *Jak* associated with the membrane-proximal sequence of the receptor intracellular domain (Ihle, 1995). Activated *Jak* then phosphorylates cytoplasmic tyrosine residues of receptor. The phosphorylated intracellular domain of receptor then provides a binding site for the Src homology 2 (SH2) domain containing cellular signalling proteins such as STATs and the SH2 containing protein tyrosine phosphatase 2 (SHP-2) (Darnell et al. 1994, Darnell 1996). Tyrosine phosphorylation of carboxy-terminal domains mediates homo- or heterodimerization of STAT proteins through SH2 domains, triggering translocation to the nucleus and DNA binding (Ihle, 1996). STAT proteins then stimulate gene transcription. The OB-Rb is known to be active in signal transduction through *Jak*/STAT pathway because of homology with the cytokine receptor family (Baumann et al. 1996, Ghilardi et al. 1996). Ghilardi et al. (1996) reported that the long form of OB-R was able to activate *Jak* associated with STAT-3, STAT-5, and STAT-6 while the short form of OB-R seen in db/db and wild-type mice failed to activate the *Jak*/STAT pathway in human hepatoma cell lines. OB-R activated STAT-3 in HepG2 cells in the presence of monoclonal antibodies against human gp130, which are known to prevent signaling by all IL-6-type cytokine receptors. The expression of STAT-3 by IL-6

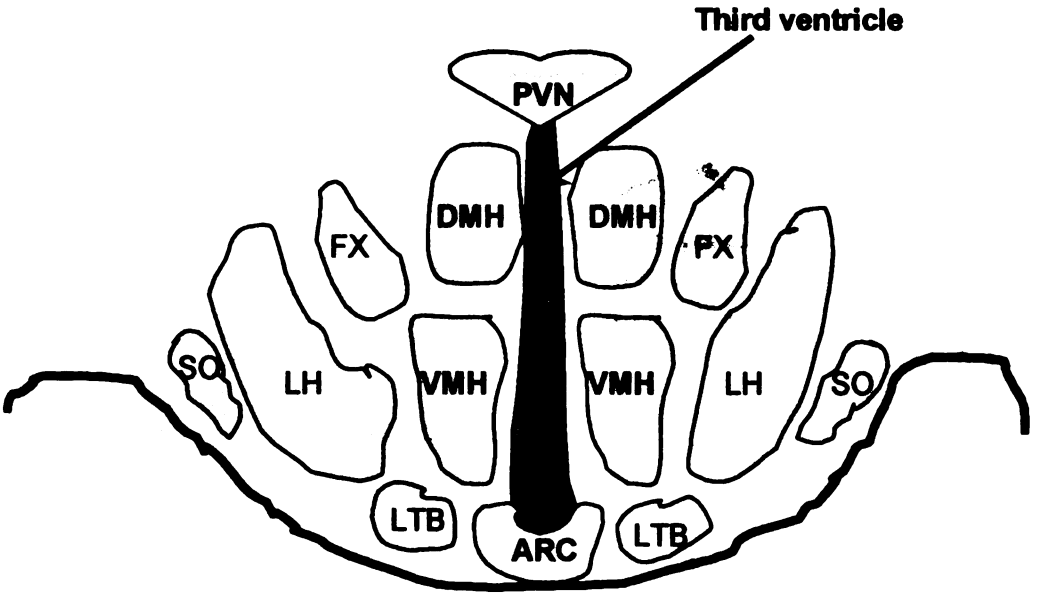

FIGURE 2. Location of long-form leptin receptors within the hypothalamus. Long-form leptin receptors are localized in the ARC, VMH with lower levels in the PVN and DMH. Abbreviations: ARC, arcuate nucleus; DMH, dorsomedial nucleus; FX, fornix; LH, lateral hypothalamic area; LTB, lateral tuberal nucleus; PVN, paraventricular nucleus; SO, supraoptic nucleus; VMH, ventromedial nucleus.

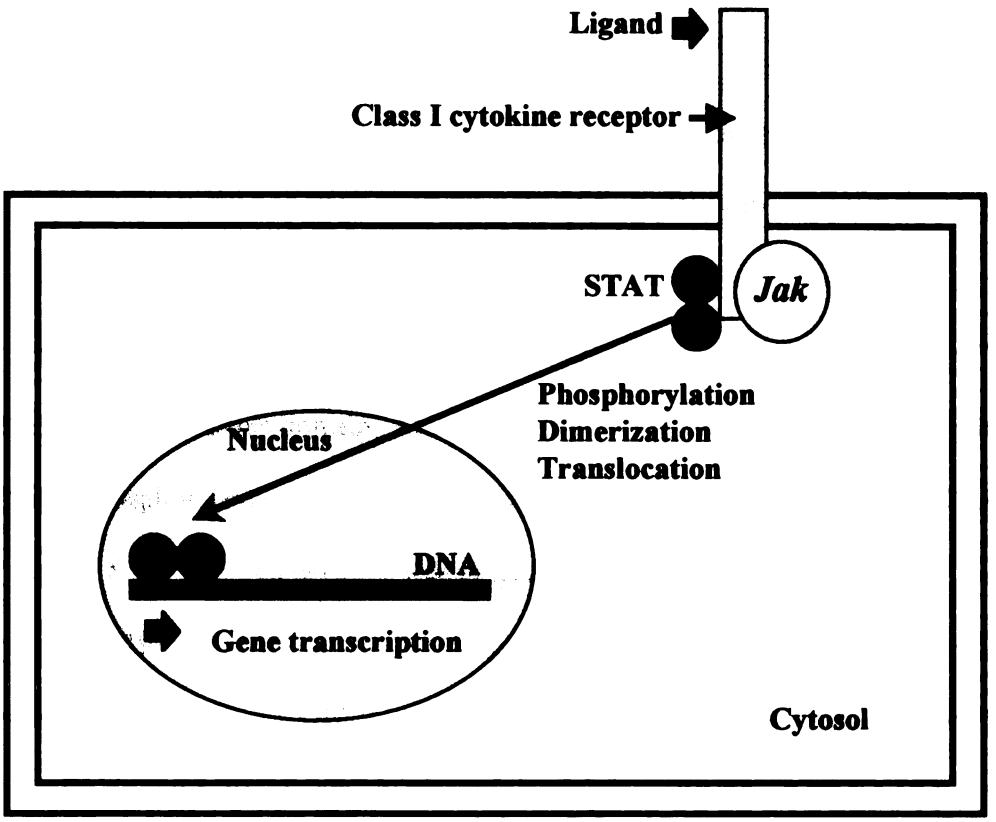

FIGURE 3. Signal transduction of class I cytokine receptors via *Jak*/STAT pathway. Activation of class I cytokine receptors induces tyrosine phosphorylation of one or more *Jak* associated with the membrane-proximal sequence of the receptor intracellular domain. Activated *Jak* then phosphorylates cytoplasmic tyrosine residues of receptor. The phosphorylated intracellular domain of receptor then provides a binding site for the SH2 domain containing cellular signaling proteins such as STATs. Tyrosine phosphorylation of receptor carboxy-terminal domains mediates homo- or heterodimerization of STAT proteins through SH2 domains, triggering translocation to the nucleus and DNA binding. STAT proteins then regulate gene transcription. Abbreviations: STAT, signal transducers and activators of transcript; *Jak*, *Janus* kinase.

was completely abolished under the same conditions (Baumann et al. 1996). This finding suggests that OB-R may function independently of gp 130 unlike the IL-6-type cytokine receptors.

Binding of leptin to OB-Rb phosphorylated *Jak* 1 and *Jak* 2, and subsequently caused phosphorylattion of STAT 3 in COS-7 cell lines (Bjørbæk et al. 1999, Carpenter et al. 1998). Carpenter et al. (1998) reported that activation of OB-R by leptin mediates tyrosine phosphorylation of SHP-2, and that this phosphorylation is dependent on Tyr^{986} within the OB-R cytoplasmic domain. Mutation of Tyr^{986} to Phe within the OB-Rb, which block SHP-2 phosphorylation, dramatically increased gene induction mediated by STAT 3, suggesting that SHP-2 plays a negative role in STAT3-mediated gene induction (Carpenter et al. 1998). It was also recently reported that leptin binding to OB-Rb leads to tyrosine phosphorylation of SHP-2 in 293T cells (Li and Friedman, 1999). This SHP-2 was also isolated from mouse hypothalamus, suggesting that SHP-2 is a component of the leptin signal transduction pathway within the hypothalamus. In the absence of SHP-2 in the transfected cells, *Jak* 2 was highly phosphorylated in response to leptin after 30 and 60 min of treatment, suggesting that SHP-2 dephosphorylates *Jak* 2. This observation also supports the notion that activation of SHP-2 by binding of leptin to leptin receptor may act to attenuate leptin signal transduction. The identification of SH2 domain containing proteins, SHP-2, activated by the leptin receptor is likely to have important implications for an understanding of the leptin signal transduction pathway and molecular mechanisms that control body weight. It is not clear yet whether SHP-2 plays a negative role in leptin signal transduction *in vivo*.

A nonsense mutation within the intracellular domain of OB-R in db/db mice results in a

truncated form of the receptor with a 34 amino acid long cytoplasmic domain (Chen et al. 1996, Lee et al. 1996). As speculated, unresponsiveness to leptin action in db/db mice is due to this defect in OB-R. The OB-R found in db/db mice is the same as the short form of leptin receptor which is defective in signal transduction in normal mice and rats (Chen et al. 1996, Ghilardi et al. 1996, Lee et al. 1996).

A missense mutation (from glutamine to proline at codon 269) within the extracellular domain of the OB-R leads to the production of an abnormal leptin receptor in obese Zucker fa/fa rats (Chua et al. 1996, Crouse et al. 1998, Iida, et al. 1996, Phillips et al. 1996, Takaya et al. 1996, White et al. 1997). Unlike the total unresponsiveness seen in db/db mice, food intake and body weight were decreased in fa/fa rats after ICV administration of high doses (> 18 ug/rat) of leptin (Cusin et al. 1996), suggesting less expression of receptors on the membrane or decreased binding affinity of leptin receptors to leptin. It is now known that a missense mutation in the extracellular domain of leptin receptor results in less expression of receptors on the membrane without affecting binding affinity to leptin (Crouse et al. 1998, White et al. 1997). The fatty mutation (leptin receptors with a single extracellular domain missense mutation) caused constitutive activation of STAT1 and 3, and failed to activate STAT 5B in cell cultures after leptin administration (Crouse et al. 1998, White et al. 1997). Whether constitutive activation of STAT1 and STAT3 by fatty receptor causes desensitization to leptin and development of obesity, or whether impaired activation of STAT5 leads to obesity in fatty Zucker rats remains to be fully resolved.

3. Genomic actions of leptin

Cohen et al. (1996) reported that phosphoenolpyruvate carboxykinase (PEPCK), a rate-limiting enzyme in gluconeogenesis, was affected by leptin in rat hepatoma cell line, H4-II-E. The amount of PEPCK mRNA in H4-II-E cells first treated with dibutyryl-cAMP and then with 10 nM insulin for 2 h was reduced by about 80 % compared to cells treated with dibutyryl-cAMP alone. Preincubation of cells with 60 nM leptin for 2 h prior to the addition of insulin partially reversed the down-regulating effect of insulin on PEPCK gene expression. The mechanism by which leptin affected PEPCK gene expression *in vitro* is uncertain yet.

Several lines of evidence support the hypothesis that leptin regulates body fat balance by influencing neuropeptides, including NPY and CRH, involved in regulation of food intake and energy expenditure (Campfield et al. 1995, Rohner-Jeanrenaud et al. 1996, Schwartz et al. 1996a and 1996b, Stephens et al. 1995, Wang et al. 1997). Central administration of leptin, either a single injection or repetitive injections, has been shown to decrease NPY mRNA in the ARC of rats and mice (Cusin et al. 1996, Sahu et al. 1998, Schwartz et al. 1996b. Stephens et al. 1995), and also to increase CRH mRNA in the PVN of rats (Schwartz et al. 1996a). These leptin-induced changes in hypothalamic NPY and CRH gene expression have been noted between 6 h and 5 days post leptin administration. Concomitant with the lowering of NPY mRNA, NPY peptide concentrations were also lowered in the ARC, PVN, and DMH of rats 6 h after administration of leptin (Wang et al. 1997), suggesting less synthesis of NPY. These results provide one possible explanation of how a single injection of leptin might cause relatively long-lasting (i.e. 12 - 24 h) effects on food intake. The mechanisms by which leptin influences NPY and CRH gene

expression in the ARC and PVN, respectively, are not yet understood. It is postulated that leptin may affect NPY and CRH gene expression by *Jak*/STAT pathway (**Fig. 4**)

Accumulating evidence suggests that other neuronal feeding-regulatory factors including alpha-melanocyte stimulating hormone (α-MSH), cocaine- and amphetamine-regulated transcript (CART), and agouti-related protein (AGRP) present within the hypothalamus are also regulated by leptin (Cheung et al. 1997, Ebihara et al. 1999, Kristensen et al. 1998, Wilson et al.1999). Alpha-MSH is a peptide cleaved from pro-opiomelanocortin gene in the ARC, and is known to reduce food intake in animals (Cheung et al. 1997, Schwartz et al. 1997). Repeated intraperitoneal (IP) or ICV injections of leptin increased POMC mRNA in the ARC of ob/ob mice and rats (Mizuno et al. 1998, Schwartz et al. 1997). Hypothalamic neuropeptide CART has been recently shown to reduce food intake in rats (Kristensen et al. 1998). Daily IP injections of leptin to ob/ob mice for 10 days increased CART mRNA expression in the DMH and ARC (Kristensen et al. 1998). AGRP, an endogenous antagonist of the hypothalamic melanocortin receptor, is known to increase food intake and body weight in rats and mice (Ebihara et al. 1999, Wilson et al. 1999). Repeated IP or single ICV injection of leptin to rats and mice decreased AGRP in the ARC. The hypothalamic effects of leptin are likely to involve several pathways that might act in parallel to regulate food intake. ICV leptin injection activated STAT protein (STAT-3) within 15 –30 min after administration in the hypothalamus of wild-type and ob/ob mice and rats, as was seen *in vitro* (McCowen et al. 1998, Vaisse et al. 1996). Whether activation of STATs by leptin influences gene

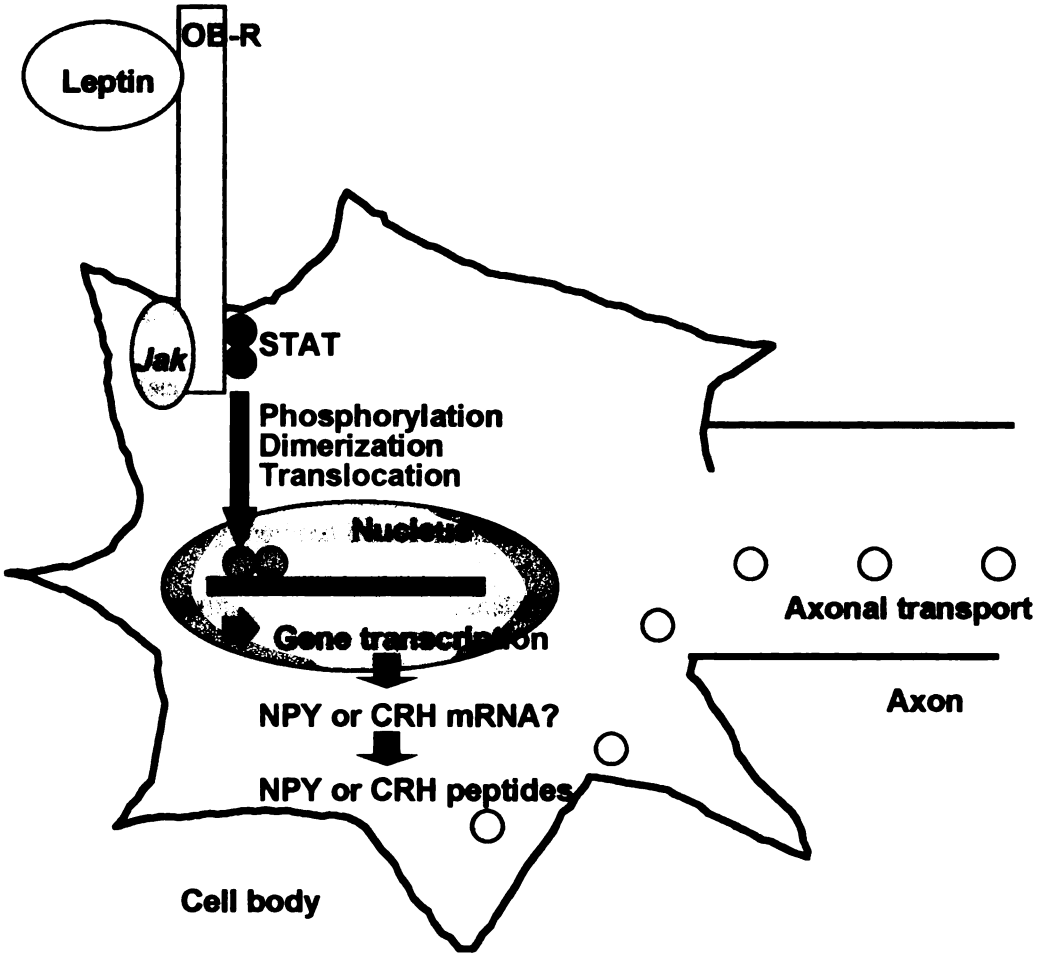

FIGURE 4. Possible mechanisms of leptin actions on hypothalamic NPY or CRH gene expression via *Jak*/STAT pathway within the cell bodies of NPY (ARC) and CRH (PVN). Abbreviations: CRH, corticotropin-releasing hormone; NPY, neuropeptide Y; OB-R, leptin receptor.

expression of feeding-regulatory neuropeptides α-MSH, CART, AGRP as well as NPY and CRH within the hypothalamus is not yet known.

4. Rapid actions of leptin in the CNS

Numerous studies demonstrate that the inhibitory actions of leptin on food intake are rapid in onset, i.e. within 30 min to 2 hr (Campfield et al. 1995, Flynn et al. 1998, Mistry et al. 1997, Rentsch et al. 1995, Seeley et al. 1996). These rapid-onset actions of leptin on food intake may more likely occur via mechanisms other than altered gene expression, given the time typically required for modulation of protein synthesis.

Leptin, by decreasing secretion of hypothalamic NPY and/or by increasing secretion of hypothalamic CRH, would be well-positioned to influence rapid-onset actions on food intake. Several attempts have been made to examine the acute effects of leptin on hypothalamic NPY and CRH secretion, with inconsistent outcomes. Leptin (1 uM) inhibited NPY release (204 ± 35 versus130 ± 18 ng/ml for control and leptin-treated hypothalamic preparations, respectively) that was first induced by a 2 h exposure of hypothalamic preparations from rats to 0.6 uM corticosterone (Stephens et al. 1995). In the absence of corticosterone, NPY secretion from this preparation was undetectable. Thus, it was not possible to test potential effects of leptin on hypothalamic NPY secretion in the absence of added glucocorticoid. IP administration of leptin to rats failed to influence *in vivo* NPY release from the PVN, as measured by a push-pull cannula, during a 2 h time period (Beck et al. 1998). The conditions under which leptin might acutely affect NPY secretion remain to be resolved. Leptin increased CRH secretion, within 20 min, from hypothalamic preparations of rats and mice incubated in 3 - 5.5 m M glucose (Costa

et al. 1997, Raber et al. 1997), but in another report addition of leptin to hypothalamic preparations from rats blocked potentiation of CRH secretion induced by low (1.1 mM) glucose (Heiman et al. 1997). It is also possible that NPY is affected by leptin and this action alters the activity of CRH neurons or vice versa, as predicted by co-presence of both NPY and CRH neurons within the many regions of the hypothalamus (Whitnall et al. 1993).

The mechanisms whereby leptin causes rapid changes in NPY and CRH secretion are not understood yet. Perfusion of hypothalamic slices with 100 ng/ml leptin produced a robust inhibition of the excitatory postsynaptic current within 1 min in the ARC of lean (Fa/?) Zucker rats (Glaum et al. 1996). This finding indicates that leptin may act on the membrane to affect postsynaptic current (Glaum et al. 1996). Leptin is able to rapidly, within seconds, depolarize rat PVN neurons and activate ATP-sensitive potassium channels within the hypothalamus (Glaum et al. 1996, Powis et al. 1998, Spanswick et al. 1997). Leptin, 30 ng/ml, increased the intracellular concentrations of Ca^{2+} in isolated rat ARC neurons *in vitro* (Glaum et al. 1996). These leptin-induced changes in membrane potential, ion channels, and calcium influx within the hypothalamus may lead to rapid changes in neurotransmitter secretion.

Leptin has been also reported to affect cAMP concentrations via stimulation of phosphodisterase 3 B (Zhao et al. 1998) as well as affect nitric oxide generation (Yu et al. 1997), and PKC activity (Chen et al. 1997). Changes in cAMP, nitric oxide and PKC also may influence the secretion of NPY and CRH. These signal transducers are thus potential candidates to mediate leptin-induced changes in neuropeptide secretion (**Fig. 5**).

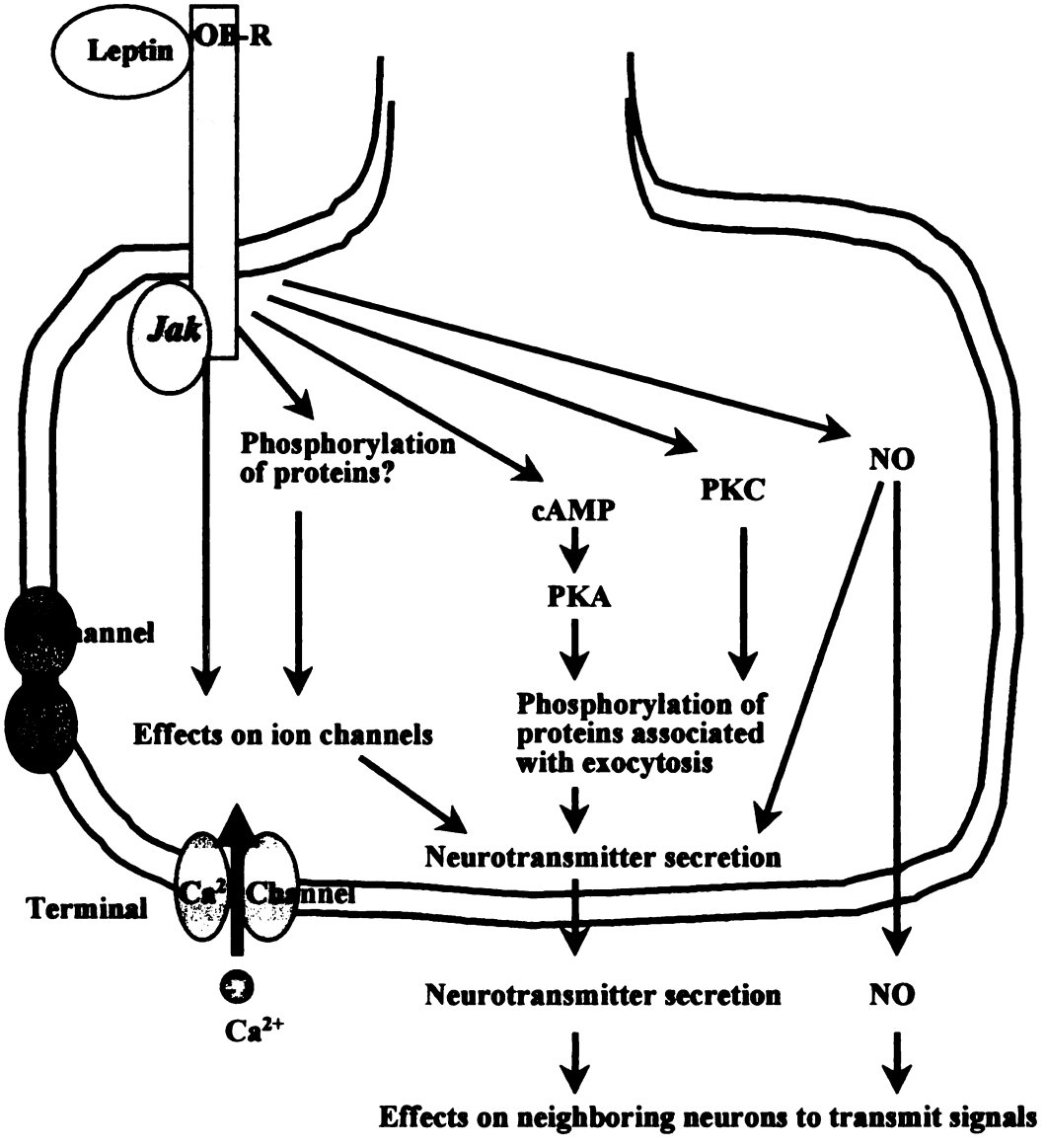

FIGURE 5. Possible rapid-onset actions of leptin on neurotransmitter secretion at neuron terminal. Leptin may affect neurotransmitter secretion by rapidly affecting ion channels, cAMP concentrations, PKA, PKC as well as nitric oxide production. Abbreviation: NO, nitric oxide.

B. Neuropeptide Y

1. Distribution of NPY neurons in the CNS

NPY, a 36 amino acid-peptide, is one of the most abundant neurotransmitters in the CNS, with high abundance in the cerebral cortex, caudate putamen, hippocampus (Tatemoto et al. 1982, Hendry, 1993). NPY-containing neurons are also densely located in the hypothalamus that is involved in regulation of food intake and energy metabolism (Hendry, 1993).

NPY is abundant in many specific regions of hypothalamus including ARC, PVN, VMH, DMH, and SCN (Chronwall et al. 1985). The ARC contains cell bodies of NPY neurons, which project to the PVN, VMH, DMH, and elsewhere (sites of NPY release) in the hypothalamus (Hendry, 1993). There are two sources of NPY fibers innervating the PVN, one from a short projection that originates in the ARC, and the other from the brainstem (Bai et al. 1985, Everitt et al. 1984, Sawchenko et al. 1985). Projections of NPY neurons from the ARC to the PVN appear to be very important in the regulation of feeding and energy metabolism by NPY (Akabayashi et al. 1993, Egawa et al. 1991, Jhanwar-Uniyal et al. 1993). Sawchenko and coworkers (1985) reported that NPY neurons in the PVN arise from adrenergic cell groups in the medulla (C1, C2, and C3), which contain NPY as well as epinephrine, and from noradrenergic somata in the A1 cell group, which is immunoreactive for both NPY and norepinephrine. The hypothalamic innervation by NPY axons originating from adrenergic or noradrenergic neurons in the brainstem has also been implicated in feeding behavior, and in the control of fluid and blood pressure (Sahu et al. 1988a). Experimental studies have shown that NPY axons innervating the rat SCN arise from the ventral lateral geniculate (intrageneculate leaflet)

nucleus of the thalamus (Card and Moore, 1989, Harrington et al. 1987). NPY neurons originating in the ventral lateral geniculate nucleus and terminating in the SCN may be a key component in the regulation of circadian rhythm in rats since destruction of these NPY neurons affects circadian function (Moore and Card, 1990, Moore and Eichler, 1972).

2. NPY receptors in the CNS

Binding sites for NPY are found in a variety of areas in the brain, particularly in the limbic system, hippocampus, cortex, some thalamic nuclei, hypothalamus, and the brainstem (Dumont et al. 1992, Gerald et al. 1996). There are currently 5 identified subtypes of receptors for NPY (i. e. Y1, Y2, Y3, Y4, and Y5) in the CNS. Four of these subtypes (except Y3) have been identified from human, porcine and rat brain (Bard et al. 1995, Gerald et al. 1996, Rose et al. 1995). NPY receptors are G-protein-coupled receptors with 7 transmembrane domains (Gerald et al. 1996, Grundemar et al. 1993). Forskolin-stimulated cAMP production was suppressed by more than 60 % in rat Y1 or Y2-transfected LMTK$^-$ or Y5-transfected 293 cells by human NPY (Gerald et al. 1996). Suppression of forskolin-stimulated cyclic AMP (cAMP) production by rat pancreatic peptide (PP) was suppressed by ~ 70 % in rat Y4-transfected LMTK$^-$ cells in the same study. These findings indicate that NPY may have an inhibitory action on adenylate cyclase through its receptors (i.e. Y1, Y2, Y4, and Y5). Several studies showed that NPY receptor subtypes (i.e. Y1, Y2, and Y4) are also related to the rise of intracellular calcium concentration (Bard et al. 1995, Nakamura et al. 1995, Rose et al. 1995). Enhanced intracellular calcium concentration upon NPY application may be linked to the activation

of PLC since NPY-induced intracellular Ca^{2+} increase was markedly suppressed by pretreatment of cells with a PLC inhibitor U-73122 (Nakamura et al. 1995).

The subtypes of NPY receptors have usually been distinguished by their different response profiles to fragments of the NPY molecule. The Y1 receptor subtype requires a tyrosine residue at the N-terminal for complete activation while the Y2 subtype (31 % homology to Y1) is relatively insensitive to N-terminal amino acid deletion, with NPY fragment 13-36 (NPY_{13-36}) as effective as the entire NPY in Y2 receptor-dependent events (Sheikh et al. 1989). Peptide YY (PYY), structurally related to NPY, activates both Y1 and Y2 receptor subtypes. The Y3 subtype receptor, in contrast to Y1 and Y2 receptors, does not respond to PYY. The Y3 receptor also requires the N terminus for full activation since NPY_{13-36} has 20-40 time lower affinity than the intact NPY molecule (Grundemar et al. 1993). PP and PYY are more potent agonists than NPY for Y4 subtype receptor (Y4/PP1) that is 42 % homologous to Y1 receptor (Bard et al. 1995).

More recently a new NPY receptor subtype has been cloned from rat brain (Gerald et al. 1996). The amino acid sequence of the rat Y5 cDNA clone shows only 30-33% identity to other NPY receptors, including Y1, Y2, and Y4/PP1. The Y5 mRNA is localized in SCN, PVN, LH, ARC of the hypothalamus, hippocampus, and some medial thalamic nuclei, which project to the PVN and to the amygdala, as measured by *in situ* mRNA hybridization (Gerald et al. 1996).

3. Effects of NPY on food intake and energy metabolism

The wide distribution of NPY within the CNS and the highly conserved amino acid sequence of NPY across species imply an important physiological function for this

neuropeptide (Blomqvist et al. 1992, Leibowitz, 1990 and 1991, Morley et al. 1987). NPY is known to decrease blood pressure and heart rate, stimulate anterior pituitary hormone release (luteinizing hormone, growth hormone, and adrenocorticotropic hormone) and shift circadian rhythms (Hanson and Dallman, 1995, Leibowitz, 1991, Morley et al. 1987, Wahlestedt et al. 1987, White, 1993). One of the most pronounced actions of NPY is its effect on food intake.

The stimulatory effect of NPY on feeding in the rat was first noted by Clark et al. (1984), and confirmed by other researchers in the rats and mice (**Table 2**, Stanley and Leibowitz, 1984, Stanley et al. 1986, Walker and Romsos, 1993). ICV injection of NPY increased food intake more than 3 fold with a shorter latency to initiate eating than in rats injected with saline (Clark et al. 1984). Direct injection of NPY to the PVN, with 1/20th dose of NPY used for ICV injection, elicited an immediate feeding response even in satiated rats (Stanley et al. 1984). The PVN, as predicted by much lower dose required to observe a response, might be one of the important sites of NPY actions within the hypothalamus.

ICV administration of anti-NPY antisense oligonucleotides significantly reduced food intake 2 h after injection, and food intake remained suppressed by 15 % for 24 h, suggesting an important role for endogenous NPY in food intake regulation (Hulsey et al. 1995a). Injection of antisense NPY oligonucleotides to the ARC also decreased food intake by ~ 65 % during the 90 min post-injection period and also concomitantly decreased (40 %) NPY contents in the ARC compared to the saline injected rats (Akabayashi et al. 1994). This finding also provides evidence for a role for endogenous NPY within the CNS on food intake regulation.

BIBLIOGRAPHY

TABLE 2. Stimulatory actions of NPY on food intake and body weight

Animals and NPY treatment		Findings	References
Female rats adult	ICV NPY 480 pmol, 2400 pmol	Increased food intake Short latency (15 min)	Clark et al. 1984
Female rats adult	Injection to PVN 35 pmol 3 times/day for 10 days	Increased food intake (35 g/day vs. 16 g/day) Increased weight gain (11 g/day vs. 2 g/day) Increased body fat in carcass (about 2.5 times increase)	Stanley et al. 1986
Male rats adult	Injection to PVN 24 pmol 78 pmol	Increased food intake in dose-dependent manner Short latency (10 min) Sustained response (4 hrs)	Stanley & Leibowitz. 1984
Male mice	ICV NPY 480 pmol	Increased food intake 0.5, 1, 1.5 hr post-injection	Walker & Romsos. 1993

Surprisingly, food intake and body weight of mice lacking NPY by gene knock-out were not altered compared to those of control mice during a 12 wk feeding study (Erickson et al. 1996). It is uncertain whether other compensatory mechanisms exist or whether actions of PP or PYY for example are enhanced in NPY knock-out mice. The mutant mice lacking NPY showed greater decreases in food intake and body weight than control mice within 2 days after i.p. leptin (45 ug) administration. This observation dose not rule out the notion that endogenous NPY is important in the regulation of food intake and body weight.

Gerald et al. (1996) examined the NPY receptor subtypes that are responsible for mediating NPY effects on feeding. ICV injection of human PYY 3-36, porcine NPY 2-36, porcine NPY and human PP, at the dose of 300 pmol, markedly increased (> 6-fold) food intake 4 h after injection. These peptides are potent Y5 agonists *in vitro*. However, porcine C2-NPY and rat PP were ineffective at this dose to promote feeding response, suggesting Y2 and Y4 are not involved in feeding response since C2-NPY and rat PP are potent agonists for Y2- and Y4-receptors, respectively. In addition, food intake induced by porcine NPY at the dose of 300 pmol was not reduced by the Y1 selective antagonist BIBP 3226, suggesting no involvement of Y1 subtype in the regulation of feeding behavior. Food intake and body weight of NPY Y1 receptor-deficient mice were not altered compared to those of control mice during a 20 wk feeding study (Kushi et al. 1998). This observation also supports the notion that NPY Y1 receptor in the hypothalamus is not a key receptor mediating NPY effects on feeding control. It is, however, possible that other compensatory mechanisms exist. For example, actions of NPY Y5 receptors might be enhanced in NPY Y1 receptor-deficient mice.

Location of Y5 receptors within the hypothalamus and effects of its agonists on feeding, taken together, indicate that Y5 receptor may be important for mediating the effects of NPY on feeding (Gerald et al. 1996). If this is true, production of synthetic Y5 receptor antagonist which would block NPY actions on feeding would provide a new approach for the treatment of obesity.

Stanley et al.(1986) showed that chronic PVN injection of NPY, 3 times/day for 10 days, doubled daily food intake and increased daily weight gain 6-fold compared to the vehicle-injected group. This observation suggests that marked weight gain with ICV NPY administration may not be only due to increased food intake, but also due to decreased energy expenditure. Effects of NPY on energy expenditure were thus explored by measuring thermogenesis in the interscapular BAT after repeated ICV injection of NPY during a 24 h period, since energy expended for the thermogenesis in BAT can contribute substantially to total energy expenditure (Billington et al.1991, Foster and Frydman, 1979). BAT thermogenesis was suppressed by more than 50 % both in NPY and NPY-yoked (food intake controlled equal to that of control rats) group. Decreased BAT thermogenesis in both NPY-treated groups was not secondary to a change in insulin since plasma insulin concentrations were not increased in the NPY-yoked group. These accumulated evidences suggest that administration of NPY to the CNS produces positive energy balance by increasing food intake and by decreasing energy expenditure.

Regulation of energy expenditure in BAT is believed to be controlled mainly by sympathetic nerve fibers from the CNS (Bray, 1991). Effects of NPY on the sympathetic nerve activity to BAT, therefore, were investigated by Egawa et al.(1991). ICV injection of NPY suppressed sympathetic nerve activity in a dose- and time-dependent manner.

Doses ranging from 250 pmol to 1 nmol of NPY significantly decreased sympathetic nerve activity, with the lowest sympathetic nerve activity recorded 20-30 min postinjection followed by a gradual recovery. Further studies identified targets within the hypothalamus responsible for this action of NPY. Sympathetic nerve activity was suppressed by 60 % when NPY was injected to PVN, but increased by 33 % when NPY was applied to medial preoptic area, and no response was observed after NPY injection to the other nuclei (AH, VMH, and LH). Inhibitory effects of NPY induced in PVN predominated over the stimulatory effect of NPY acting on the medial MPOA since NPY given ICV suppressed sympathetic nerve activity in this study. It thus appears that NPY may modulate metabolic events in a site specific manner to meet different functional needs in the body. The suppression of sympathetic nerve activity after ICV NPY injection support decreased BAT thermogenesis in NPY injected rats reported by Billington et al. (1991).

4. NPY in relation to obesity

Bilateral destruction of the VMH region leads to rapid onset of hyperphagia and excessive body weight gain, resulting in obesity (Magnen et al. 1983). Dube et al. (1995) reported that ICV administration of NPY antibodies abolished the hyperphagia in VMH-lesioned rats as compared to the sham-operated rats, suggesting a physiological role for NPY in regulation of hyperphagia. It is speculated that NPY may be linked to the etiology of obesity since ICV administration of NPY produces positive energy balance in the body by enhancing food intake as well as by increasing metabolic efficiency in animals (Billington et al. 1991, Billington and Levine, 1992, Stanley et al. 1986).

Only a few studies have examined hypothalamic NPY in ob/ob mice, and to my

knowledge none have examined CRH in these mice (Mistry et al. 1994, Qu et al. 1996, Stephens et al. 1995, Wilding et al. 1993, Williams et al. 1991). Hypothalamic NPY mRNA abundance is elevated in ob/ob mice (Qu et al. 1996, Stephens et al. 1995, Wilding et al. 1993), with no reported alterations in NPY protein in the whole hypothalamus of these mice compared to lean controls (Wilding et al. 1993, Williams et al. 1991). Since specific hypothalamic regions are involved in regulation of metabolism by NPY (Morley 1987, Stanley et al. 1986), measurements utilizing the whole hypothalamus may mask regional differences.

Disturbances in hypothalamic NPY have also been observed in leptin-resistant db/db mice (Chua Jr. et al. 1991, Mizuno et al. 1997), and considerable evidence has accumulated to demonstrate abnormal regulation of NPY and CRH in leptin-resistant fa/fa rats (Bchini-Hooft van Huijsduijnen et al. 1993, Beck et al. 1990a and 1993, Fukushima et al. 1992, McKibbin et al. 1991, Nakaishi et al. 1990 and 1993, Pesonen et al. 1992). Hypothalamic NPY mRNA abundance is higher in db/db mice and fa/fa rats than in the respective lean controls (Chua Jr. et al. 1991, Mizuno et al. 1997, Pesonen et al. 1992). Hypothalamic NPY mRNA abundance was increased (> 90 %) in obese fa/fa rats after weaning when they start to exhibit hyperphagia, suggesting that NPY may function as a mediator in development of hyperphagia in obese rats (Huijsduijnen et al. 1994). NPY protein in specific hypothalamic nuclei including the ARC, PVN, VMH, DMH, and SCN is also elevated in fa/fa rats compared to lean controls (Beck et al. 1990a and 1993, McKibbin et al. 1991). These observations indicate that NPY may have an important role in obesity, and its actions may occur in a site-specific manner within the hypothalamus.

High concentrations of NPY in the hypothalamus are not always observed in obese

rats. In the study of Beck et al.(1992), NPY concentrations in the regions of the hypothalamus examined (ARC, PVN, VMH, and DMH) were not different between 10-wk-old obese fa/fa rats and lean controls at the any feeding status (fed ad lib., or food-deprivation for 48 h or refeeding for 6 h after food-deprivation) examined. Abe et al. (1991) also reported no difference in NPY contents within the hypothalamic nuclei except in the PVN between Wistar fatty and lean rats.

It is possible that secretion of NPY within the hypothalamus is higher in obese rats, and thus differences in NPY contents in the discrete regions of hypothalamus between two phenotypes are not noticeable at the time of measurement. Dryden et al. (1995) recently reported that NPY secretion from the PVN was 2-fold higher in fa/fa rats than lean controls, as measured by push-pull cannula *in vivo* during a 3 h period of perfusion. Whether NPY secretion from hypothalamus is higher in ob/ob mice than lean mice is of interest to help understand the role of NPY in development of obesity in ob/ob mice.

5. Regulation of NPY

5.1. Regulation of NPY mRNA and peptide concentration by feeding status

Hypothalamic NPY and CRH are reported to change with the feeding status of the animals (Beck et al. 1990b, Brady et al. 1990, Sahu et al. 1988, Schwartz et al. 1993), conditions that are now known to alter plasma leptin concentrations (Hardie et al. 1996, Saladin et al. 1995, Trayhurn et al. 1995). Food-deprivation increases NPY mRNA abundance in the ARC or in the whole hypothalamus of rats and mice including ob/ob and db/db mice (Brady et al. 1990, Chua Jr. et al. 1991, Mizuno et al. 1997, Qu et al. 1996, Schwartz et al.1993), but not in leptin-resistant fa/fa rats (Sanacora et al. 1990). NPY

protein is also known to increase with food-deprivation and decrease with refeeding in site-specific manners within the hypothalamus of control rats (Beck et al. 1990b, Sahu et al. 1988), but not in leptin-resistant fa/fa rats (Beck et al. 1992, Sanacora et al. 1990). It appears that elevated NPY in the ARC and PVN with food-deprivation may be due to increased synthesis and transport at ARC-PVN projection system. NPY mRNA in the ARC, and peptide contents in the PVN and ARC returned to normal after refeeding rats > 6 h after 2-3 day food-deprivation (Beck et al. 1990b, Davies and Marks, 1994, Sahu et al. 1988b). The normalization of NPY contents in the PVN and ARC after refeeding could be due to increased secretion of NPY in the PVN and/or decreased synthesis of NPY in the ARC after feeding is completed. The observation of site-specific changes in NPY contents in the PVN or ARC further support the notion that PVN-ARC are one of the important sites within the CNS for mediating NPY actions on food intake. NPY secretion from PVN, as measured by push-pull cannula *in vivo*, increased with the increased appetite created in rats that were maintained on 4 h of scheduled feeding as compared to the rats fed ad lib (Kalra et al. 1991). Food-deprivation decreases CRH mRNA in the PVN of rats (Brady et al. 1990). I am not aware of any reports on food intake-induced regulation of hypothalamic NPY and CRH concentrations in leptin-deficient ob/ob mice.

5.2. Regulation of NPY mRNA and protein by metabolites, insulin and glucocorticoids

Under different nutritional metabolic status, the profiles of many metabolites or hormones change simultaneously. It has been postulated that glucose, insulin and glucocorticoids may affect metabolism of NPY within the hypothalamus (Kalra et al.

1991, Sahu et al. 1988).

NPY concentrations in the ARC and SCN in Sprague-Dawley rats increased by more than 50 % 2 h after IP administration of 2-deoxy-glucose, which is known to block intracellular glucose utilization (Akabayashi et al. 1993). However, administration of mercaptoacetate, an inhibitor of fatty acid β-oxidation, did not influence the NPY contents within the hypothalamus, suggesting a possible important role for glucose on the regulation of NPY. The precise mechanism by which 2-deoxy glucose elevates hypothalamic NPY contents remains to be elucidated.

The abundance of NPY mRNA increased in the ARC in rats 7 days after intravenous (iv) injection of streptozotocin (50 mg/kg) to produce diabetes characterized by a 50 % reduction in plasma insulin, and a 3-fold increase in serum glucose (Marks et al. 1993). When streptozotocin-induced diabetic rats were treated with insulin for 20 h, the abundance of NPY mRNA in the ARC decreased. Consistent with the observed changes in NPY mRNA the contents of NPY increased in the ARC, PVN, VMH (among the 5 regions examined) after iv administration of streptozotocin at a dose of 55 mg/kg. Likewise insulin treatment, 32 days after streptozotocin-induced diabetes in rats, decreased the contents of NPY in the ARC and PVN. NPY neurons in the ARC-PVN projection system are known to be implicated in feeding behavior and energy metabolism (Jhanwar-Uniyal et al. 1993). This action of insulin on NPY in the ARC and the PVN might be due to direct inhibitory actions of insulin on NPY neurons as predicted by the localization of insulin receptors in the ARC (Corp et al. 1986).

Chronic subcutaneous administration of DEX (0.4 mg/kg/day) increased hypothalamic NPY mRNA and NPY contents in the PVN and ARC in rats (Wilding et al. 1993).

Coadministration of DEX (0.4 mg/kg/day) and insulin (60 U/kg/day) for 28 days completely abolished the stimulatory effects of DEX on NPY in the PVN and ARC, suggesting an antiregulatory actions of insulin on the stimulatory actions of DEX on hypothalamic NPY contents. The mechanisms responsible for inhibitory actions of insulin and stimulatory actions of glucocorticoids on NPY in the CNS needs to be elucidated.

C. Corticotropin Releasing Hormone

Numerous studies have shown that ICV administration of CRH, either acute or chronic, decreases food intake and body weight in normal or genetically obese rats (fa/fa) or VMH lesioned rats (Arase et al. 1989a and 1989b, Hotta et al. 1991, Hulsey et al. 1995b, Krahn et al. 1988, Rohner-Jeanrenaud et al, 1989). These actions of CRH are opposite to those observed when NPY is injected. Potential interactions between CRH and NPY are thus likely to occur within the hypothalamus to regulate food intake and energy metabolism.

1. Distribution of CRH neurons and receptors in the CNS

CRH, a 41 amino acid peptide, is widely distributed throughout the CNS, and has multiple biological effects (Owens and Nemeroff,1991, Rothwell, 1990, Whitnall et al. 1993). The abundance of CRH mRNA is highest in the brain stem followed by olfactory bulb > hypothalamus > midbrain > cerebral cortex as measured by northern blot analysis (Imaki et al. 1991). CRH neurons within the hypothalamus seem to be implicated in the regulation of food intake (Krahn et al. 1988).

Several sources of CRH neuron projections to the hypothalamus exist in the CNS

(Whitnall et al. 1993). Projections from both the hippocampus and the amygdala to the parvocellular division of the PVN are known to be involved in regulation of the hypothalamo-pituitary-adrenal (HPA) axis (Herman et al. 1994). CRH neurons in the parvocellular division of the PVN also arise from catecholaminergic neurons in the brainstem (Whitnall 1993). Stimulation of CRH neurons via ascending catecholaminergic pathways contributes to the regulation of HPA axis in response to stress (Whitnall 1993). CRH synthesized in the medial parvocellular division of the PVN mainly projects to the median eminence, the site of the plexus of the hypothalamohypophysial portal system (Liposits et al. 1983, Liposits and Paull, 1985). Some CRH containing fibers from the PVN also project to other hypothalamic nuclei and to extrahypothalamic brain regions. CRH-immunoreactive cell bodies are also found in supraoptic, suprachiasmatic, preoptic, premammilary, magnocellular PVN, and ARC, these neurons project to the median eminence or to other hypothalamic nuclei (Kawata et al. 1983). But the location of the majority of their projections is unknown (Kawata et al. 1983).

Two subtypes of CRH receptors, CRH_1 and CRH_2, have been cloned from pituitary and brain of rats (Lovenberg et al. 1995, Wong et al. 1994). The CRH_2 receptor has ~70 % amino acid homology to the CRH_1 receptor. A number of studies have localized the sites of these CRH receptors throughout the pituitary and brain by *in situ* hybridization or by Northern blot analysis (Chalmers et al. 1995, Potter et al. 1994, Wong et al. 1994). CRH_1 mRNA is known to be highly expressed in the intermediate lobe and anterior pituitary, this CRH receptor mediates CRH actions to stimulate ACTH secretion (Potter et al. 1994, Wong et al. 1994). CRH_1 receptor mRNAs are widespread in many regions of the brain: neo-, olfactory, and hippocampal cortices, subcortical limbic structures in the

septal region, amygdala, and brainstem. CRH_1 receptors found in the brain are 91 % identical in the coding region with 97 % amino acid homology to CRH_1 receptors located in the pituitary (Perrin et al. 1993).

Expression of CRH_1 receptor mRNA is low or undetectable in the hypothalamus, a central site of CRH actions on feeding and energy metabolism (Potter et al. 1994). The CRH_2 receptor mRNA is abundant in the VMH of the hypothalamus, the lateral septum, the amygdala, and entorhinal cortex (Lovenberg et al.1995). Whether this CRH_2 receptor is responsible for the actions of CRH on the food intake needs to be further investigated.

The heterogeneous anatomical distribution patterns of CRH_1 and CRH_2 receptor mRNA expression suggest distinct functional roles for each receptor in CRH-related CNS functions. CRH_1 receptors are the primary neuroendocrine pituitary CRH receptor and play a major role in the cortical, cerebellar, and sensory functions of CRH while CRH_2 receptors are involved in the hypothalamic neuroendocrine, autonomic, and behavioral actions of CRH in the brain (Chalmers et al. 1995, Lovenberg et al. 1995, Wong et al. 1994).

Both CRH_1 and CRH_2 receptor subtypes are shown to be linked to the simulation of adenylate cyclase with different pharmacological profiles with respect to the CRH-related peptides (Lovenberg et al. 1995). Cells transfected with CRH_1 receptor responded to rat/human CRH (r/h CRH), ovine CRH (oCRH), and CRH-related peptides sauvagine and urotensin I with the same potency (EC_{50} values of 3 ~ 10 nM) at stimulating cAMP accumulation. However, sauvagine and urotensin I were 10 times more effective in stimulating cAMP accumulation than r/h CRH or oCRH in cells transfected with CRH_2 receptor. Further research is needed to identify the possible roles of CRH_2 receptors in

mediating CRH actions within the hypothalamus as predicted by its abundance within the hypothalamus.

2. Effects of CRH on food intake and energy metabolism

CRH is best known as a stimulant of ACTH release via the hypothalamo-pituitary axis (Brown, 1986, Lenz et al. 1987, Plotsky et al. 1992, River et al. 1984). But numerous studies have shown that ICV administration of CRH, either acute or chronic, also decreases food intake and body weight in normal or genetically obese rats (fa/fa) or VMH lesioned rats (Arase et al. 1989a and 1989b, Hotta et al. 1991, Hulsey et al. 1995b, Krahn et al. 1988, Rohner-Jeanrenaud et al, 1989).

ICV administration of CRH (5 ug) decreased food intake, with a maximum effect within an hour after CRH injection, in rats induced to eat by 21 h food-deprivation (Arase et al. 1988). The inhibitory effect of CRH on food intake during 2 h after ICV CRH injection was observed only when CRH was injected into the PVN, but not into the other brain regions (i.e. striatum, VMH, LH of the hypothalamus, or globus pallidus), suggesting inhibitory actions of CRH on food intake might occur in a site-specific manner in the CNS (Krahn et al. 1988).

The excessive body weight gain present even in pair-fed Zucker fa/fa rats was prevented by chronic ICV injections (5 ug/day for 7 days) of CRH (Rohner-Jeanrenaud, 1989). This finding indicates that CRH increases energy expenditure as well as decreases food intake. These inhibitory actions of CRH on body weight may be independent of the activity of HPA axis since the ICV dose of CRH used to decrease body weight was well below the dose (40 ug/day) required to stimulate the secretion of ACTH and

corticosterone (Rohner-Jeanrenaud, 1989).

Effect of CRH on energy expenditure may be linked to regulation of BAT thermogenesis. CRH (5 ug) increased BAT thermogenesis by 20 ~ 30 % after ICV injection in normal rats or in VMH-lesioned obese rats (Arase et al. 1988 and 1989b). Increased BAT thermogenesis after ICV CRH administration in rats was due to the increased sympathetic nerve activity to the BAT (Egawa et al. 1990). ICV CRH increased sympathetic nerve activity in a dose-dependent manner (1 nmol > 500 pmol > 250 pmol). ICV CRH also increased plasma concentrations of epinephrine and norepinephrine possibly due to the increased sympathetic nerve input since the effects of CRH on these hormone were completely abolished by preinjection of the ganglionic blocker chlorisondamine (Brown et al. 1982). The stimulatory effects of CRH on these hormones were not mediated via the pituitary since ICV CRH also increased plasma epinephrine and norepinephrine in hypophysectomized rats. These increases in catecholamines would be expected to enhance lipolysis and thereby help provide fuel for BAT thermogenesis.

3. Interactions between NPY and CRH

NPY increases food intake and decreases energy expenditure whereas CRH has opposite actions. Anatomical evidence of NPY-containing cell bodies and terminals located in close proximity to the CRH-containing cell bodies and terminals within the hypothalamus suggests that NPY and CRH may interact to regulate food intake and energy metabolism, and thus help maintain homeostasis in the body . If the regulatory feedback loop present between CRH and NPY is disturbed, this may lead to abnormalities in food intake and energy metabolism.

Preadministration of the CRH antagonist α-helical CRH $_{9-41}$ to the PVN potentiated the feeding induced by NPY injected 15 min later to the PVN of rats (Heinrichs et al. 1993). This additive effect of α-helical CRH on NPY-stimulated eating was site-specific since injection of α-helical CRH to the VMH or to the amygdala did not further stimulate NPY-induced feeding. Impairment of CRH neurons in the PVN by injecting a ricin A chain toxin (an inhibitor of protein synthesis) combined with a monoclonal antibody to CRH (CRH-MAb) also further increased food intake induced by NPY in male Wistar rats (Menzaghi et al. 1993). This finding also demonstrates that stimulated feeding induced by NPY is further enhanced by impairment of CRH actions within the hypothalamus.

Hypothalamic NPY mRNA contents are high in adult obese (fa/fa) rats (Huijsduijnen et al. 1993). When 5 ug CRH was given ICV to adult obese rats, weight gain was suppressed, and hypothalamic NPY mRNA contents returned to that of pre-obese (13-day-old) rats (Huijsduijnen et al.1993). This finding suggests that CRH may inhibit NPY synthesis. NPY increased CRH release from rat hypothalamus in a dose-dependent manner (10^{-7} - 10^{-5}) with the maximal effect at a concentration of 10^{-6} M *in vitro* (Tsagarakis et al. 1989). This stimulatory action of NPY on CRH secretion from rat hypothalamus was not observed at a concentration of 10^{-8} M *in vitro* (Tizabi and Calogero, 1992, Tsagarakis et al. 1989). If CRH inhibits NPY synthesis as suggested by decreased hypothalamic NPY mRNA contents after ICV CRH administration (Huijsduijnen et al. 1993), stimulatory actions of NPY on CRH secretion from hypothalamus would be a feedback mechanism to maintain NPY and CRH balance within the hypothalamus. A recent study (Morris and Pavia, 1998) showed that CRH, at 1 or 5 ug/ml, increased *in vivo* NPY release from the rat PVN as measured by push-pull cannula.

Stimulatory effect of CRH on NPY was observed when CRH was administered into the PVN, but not by ICV administration, providing evidence for an interaction between CRH and NPY at the PVN, site important for feeding regulation (Morris and Parvis, 1998). This stimulatory action of CRH on NPY secretion from the hypothalamus would also be a feedback mechanism to maintain NPY and CRH balance within the hypothalamus.

It is speculated that a dysregulation in neuroendocrine system maintaining balance between NPY and CRH within the hypothalamus may lead to development of obesity, and resultant obesity may be corrected when the balance between these neuropeptides is achieved. Whether the absence of leptin causes development of obesity in ob/ob mice through its impact on these neuropeptides was of great interest in my research.

D. Glucocorticoids

1. Glucocorticoids in relation to obesity

Glucocorticoids are linked to the etiology of obesity in many animal models of obesity including ob/ob mice. Removal of glucocorticoids by ADX reverses the symptoms of obesity such as hyperphagia and hyperinsulinemia in ob/ob mice (Johnson et al. 1991, Okuda and Romsos, 1994, Walker and Romsos,1992). The mechanism by which ADX attenuates obesity is not yet clearly understood. Replacement of glucocorticoids into the CNS of ADX ob/ob mice reverses these effects of ADX, suggesting major role for glucocorticoids within the CNS in the development of obesity (Tokuyama and Himms-Hagen, 1987, Walker and Romsos, 1992).

Glucocorticoids might oppose leptin actions (Ur et al. 1996, Zakrzewska et al. 1997). Glucocorticoids via yet to be fully defined mechanisms cause rapid-onset changes in

secretion of hypothalamic NPY (stimulatory) and CRH (inhibitory) (Calogero et al. 1988, Chen and Romsos, 1995, Stephens et al. 1995, Suda et al. 1985). These actions of glucocorticoids on hypothalamic NPY and CRH secretion are opposite those proposed for leptin. Inhibitory actions of leptin on food intake and body weight were recently reported to be more pronounced in ADX rats and mice than in intact ones (Mistry et al. 1997, Zakrzewska et al. 1997). This observation and the colocalization of leptin and glucocorticoid receptors within the PVN and ARC, important sites for NPY and CRH actions, support the hypothesis that interactions between leptin and glucocorticoids may contribute to the regulation of NPY and CRH secretion within the hypothalamus (Mercer et al. 1996, Tempel and Leibowitz, 1994). It is possible that the absence of leptin in ob/ob mice may lead to a dysregulation of glucocorticoid actions, resulting in development of obesity in these mice (**Fig. 6**).

2. Actions of glucocorticoid on food intake and energy metabolism

The ability of NPY injections into the PVN to stimulate feeding is markedly reduced in ADX rats, and subcutaneous administration of corticosterone reverses the effects of ADX on NPY-induced food intake (Stanley et al. 1989). Replacement of corticosterone in hypophysectomized rats after NPY injection to the PVN results in a robust feeding response, suggesting that corticosterone actions on promoting NPY-induced food intake are independent of ACTH (Stanley et al. 1989).

Replacement of corticosterone 5 days after ADX decreased BAT thermogenesis in a dose-dependent manner as assessed by mitochondrial GDP binding (Strack et al. 1995). Lipid storage in BAT and white adipose tissue was also increased with increasing plasma

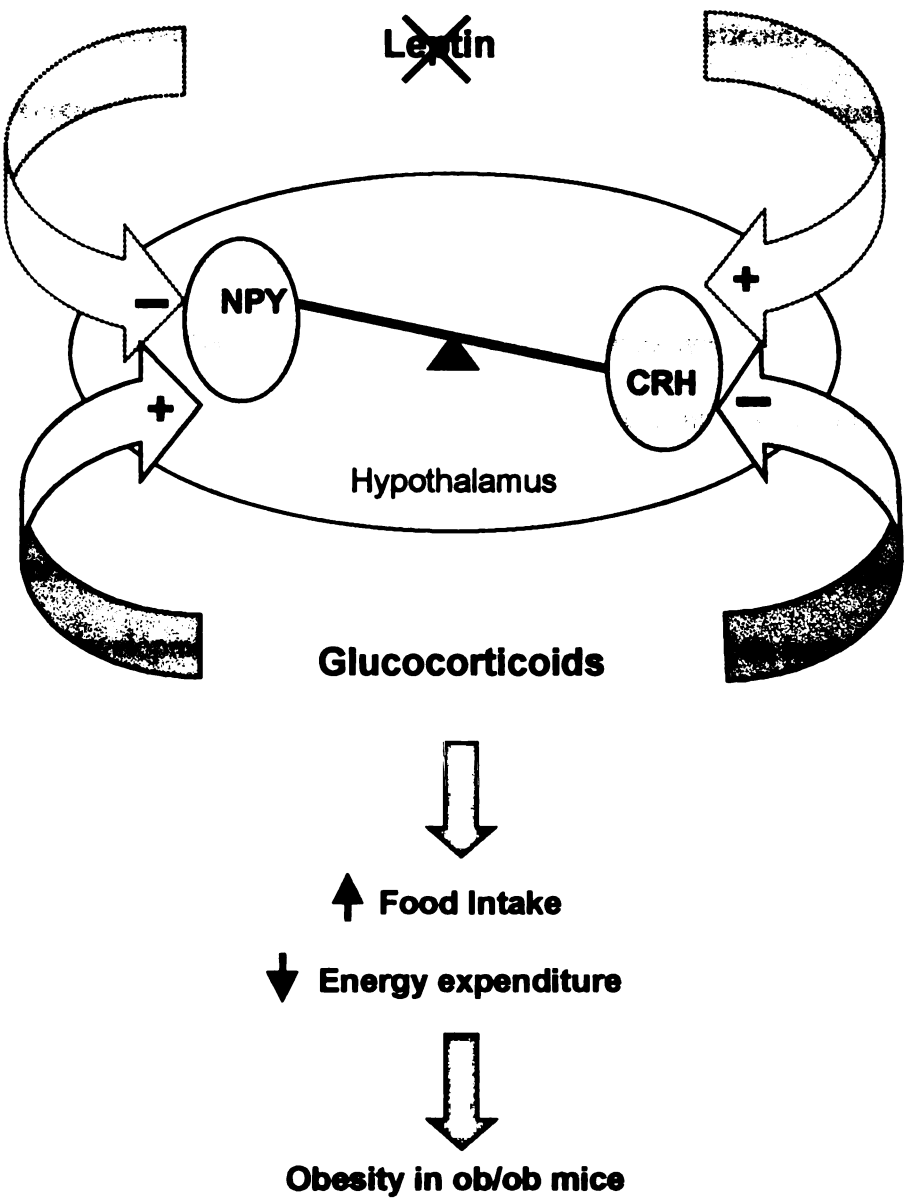

FIGURE 6. Actions of glucocorticoids on hypothalamic NPY and CRH in ob/ob mice. Lack of leptin leads to a dysregulation in neuroendocrine system maintaining a balance between NPY and CRH within the hypothalamus, resulting in development of obesity in ob/ob mice.

corticosterone after replacement of the hormone to ADX rats. In terms of lipid storage, white adipose tissue was more responsive to small increases in plasma corticosterone than BAT, indicating possible regulation of lipid storage by glucocorticoids in white adipose tissue. Decreased BAT thermogenesis and increased lipid storage in adipose tissues in ADX rats with administration of corticosterone may partly explain the functions of glucocorticoids on development of obesity in ob/ob mice. Decreased BAT thermogenesis after corticosterone replacement may result from decreased sympathetic nerve input to the BAT that is caused by increased NPY or decreased CRH within the hypothalamus (Egawa et al. 1990). This finding may support the hypothesis that high glucocorticoids in ob/ob mice results in an imbalance between NPY and CRH tones within the hypothalamus, and thus leads to development of obesity. Whether the absence of leptin leads to high plasma glucocorticoids in ob/ob mice needs to be further explored.

3. Glucocorticoid receptors in the CNS

Traditional actions of corticosteroids are mediated by intracellular receptors (Beato, 1989, McEwen, 1991). There are two types of intracellular receptors for adrenal steroids in the rat brain. Type I receptors (mineralocorticoid receptors) predominant in the lateral septum and hippocampus while type II receptors (glucocorticoids receptors) are widely distributed in the brain including striatum, cerebellum, cortex, hippocampus, hypothalamus and brainstem (Chao et al. 1989, Eekelen et al. 1987, Reul and Kloet, 1985). Type I receptors display a high affinity for both the naturally occurring mineralocorticoid aldosterone and the glucocorticoids corticosterone and cortisol (Ratka et al. 1989, Reul and Kloet, 1985). Type II receptors have a higher affinity for DEX than for

corticosteorne (Chao et al. 1989, Eekelen et al. 1987, Ratka et al. 1989, Reul and Kloet, 1985).

Within the hippocampus of rats, type I receptors were more than 80 % occupied by corticosterone while only 10 % of the type II receptors were occupied in the morning trough in plasma corticosterone (Reul and Kloet, 1985). With stress-induced increases in plasma corticosterone (>20 ug/100 mL) type II receptors are > 70 % occupied by corticosterone in hippocampal cytosol as measured by a [^{3}H]corticosterone binding assay (Reul and Kloet, 1985). Stimulation of type II receptor with the its agonists RU-28362 and RU-28318 increased the abundance of NPY mRNA in basomedial hypothalamus of ADX rats while type I receptor stimulation by aldosterone did not affect NPY mRNA in the same region, suggesting the role for type II receptors in NPY gene expression (White et al. 1994).

Glucocorticoids exert some rapid (i.e. within minutes) responses in the CNS (Liu and Chen, 1995, Sze and Iqbal, 1994a and 1994b). These rapid responses to the glucocorticoids cannot be explained by this genomic effect of glucocorticoids because of the longer time it takes for the steroid hormones to affect protein synthesis. The underlying mechanisms for rapid-onset actions of glucocorticoids have not been clearly understood. Mounting evidence suggests that membrane-bound steroid receptors mediate the rapid actions of glucocorticoids (Liu and Chen, 1995, McEwen, 1991, Moore, 1994, Orchinik et al. 1991, Sze and Iqbal, 1994a and 1994b).

4. Genomic actions of glucocorticoids in the CNS

Once steroids bind to the cytosolic receptors, this steroid-receptor complex is translocated to the nucleus where it is involved in the regulation of genes (**Fig. 7**)(Beato, 1989, McEwen, 1991). This genomic actions take a few hours or days to appear, and is involved in the actions of glucocorticoids on the synthesis of neuropeptides (Sawchenko, 1987a, White et al. 1990). Actions of glucocorticoids on food intake and energy metabolism may be mediated through their effects on neuropeptides within CNS. Possible interactions between glucocorticoids and NPY or CRH within the hypothalamus have been suggested by the neuroanatomical evidence of abundant glucocorticoid receptors in the PVN and ARC which also contain dense NPY/CRH neurons (Eekelen et al. 1987, Kiss et al. 1988, Reul and Kloet, 1985, White et al. 1990).

ADX decreases NPY mRNA levels in the ARC, and subcutaneous replacement of corticosterone in ADX rats increased the abundance of NPY mRNA in the ARC comparable to those of sham-operated rats (White et al. 1990). The stimulatory effects of corticosterone on NPY gene expression are site-specific within the hypothalamus since NPY mRNA contents in the whole hypothalamus or brainstem did not change with the ADX or with corticosterone replacement (White et al. 1990). This finding suggests that brainstem containing high density of NPY neurons as well as glucocorticoid receptors may not be an important site for the actions of corticosterone on the NPY within the CNS. This observation is also supported by the finding that acute DEX administration affects NPY contents in the hypothalamus without any detectable changes in NPY contents in brainstem of ADX ob/ob mice (Chen and Romsos, 1996).

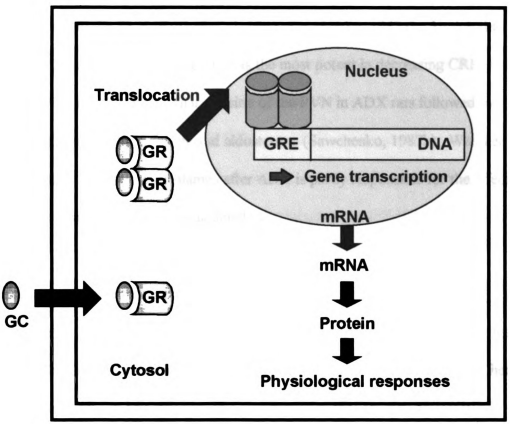

FIGURE 7. Glucocorticoid genomic actions. Glucocorticoids bind to intracellular receptors upon entry into the cell. A glucocorticoid-receptor complex is translocated to the nucleus after dimerization. This complex then binds to glucocorticoid response element of DNA to regulate transcription. Abbreviations: GC, glucocorticoids; GR, glucocorticoid receptor; GRE, glucocorticoid response element.

ADX increases CRH gene expression and CRH immunoreactivity in the PVN of rats (Imaki et al. 1991, Kovacs et al. 1986, Sawchenko, 1987a and 1987b). Implantation of DEX (70 ug) to the PVN attenuated ADX-induced enhancement of CRH immunoreactivity in the PVN (Kovacs et al. 1986, Sawchenko, 1987a). Among the steroids tested, DEX (> 20 ug/100 g wt) was the most potent in decreasing CRH immunoreactivity in the parvocellular division of the PVN in ADX rats followed by corticosterone, deoxycorticosterone and aldosterone (Sawchenko, 1987b). Whether increased CRH within the hypothalamus after ADX is partly responsible for the effects of ADX in ob/ob mice remains to be elucidated.

5. Rapid actions of glucocorticoids in the CNS

In addition to the genomic actions of glucocorticoids, rapid effects of glucocorticoids on neurotransmitters have also been reported (Chen and Romsos, 1996, Liu and Chen, 1995). The acute effects of corticosterone on energy metabolism may be mediated through the type II receptors in the hypothalamus (Chen and Romsos, 1994, White et al. 1994). ICV administration of a potent type II agonist DEX, but not a type I agonist aldosterone, suppressed BAT thermogenesis and increased plasma insulin within 30 min in ADX ob/ob mice (Chen and Romsos, 1994). A type II receptor antagonist, RU-486, completely abolished the effects of DEX in ADX ob/ob mice and rats (Chen and Romsos, 1994, Hardwick et al. 1989), suggesting an important role for the type II receptor in energy metabolism. The effect of DEX on BAT thermogenesis and plasma insulin levels were also blocked by a use of NPY receptor antagonist PYX-2, suggesting a possible role for NPY as a mediator of DEX actions within the CNS (Chen and Romsos, 1996).

DEX decreased NPY contents in the whole hypothalamus within 30 min in ADX ob/ob mice compared to the vehicle-injected ob/ob mice (Chen and Romsos, 1996). This rapid action of DEX within the hypothalamus is unlikely through its stimulatory effects on the synthesis of NPY at the ARC since rapid actions of DEX on the energy metabolism was not abolished by co-administration of a protein synthesis inhibitor (Chen and Romsos, 1994). DEX may have potentiated NPY release from the hypothalamus as predicted by lower levels of NPY in the ARC, DMH, SCN, and MPO within a rapid time-frame (30 min) after ICV DEX injection into ADX ob/ob mice (Chen and Romsos, 1996). DEX, indeed, potentiated NPY secretion from the hypothalamus of ADX ob/ob mice as measured by static incubation *in vitro* (Chen and Romsos, 1996).

In addition to the genomic effects of DEX on CRH in the PVN of rats (Imaki et al. 1991, Kovacs et al. 1986, Sawchenko, 1987a and 1987b), DEX (at 2.5 and 25 uM) is reported to decrease basal CRH release from hypothalami of rats *in vitro* (Suda et al. 1985). This inhibitory effects of DEX (at 25 uM) occurred within 16 min, and hypothalamic CRH release was increased after withdrawal of DEX in the incubation. Rapid and easily reversible suppression of CRH release by DEX supports the presence of a fast feedback mechanism within the hypothalamus. DEX is also able to block or attenuate stimulation of CRH release induced by many other substances including acetylcholine and norepinephrine (Bernadini et al. 1989, Calrogero et al. 1988). The mechanism whereby DEX inhibits CRH release from the hypothalamus is not yet understood.

Corticosterone conjugated with BSA inhibited release of the arginine vasopressin (AVP) from rat hypothalamic slices containing PVN within 20 min of incubation in a dose

dependent manner from 10^{-7} to 10^{-4} M (Liu and Chen, 1995). This rapid inhibitory effect of corticosterone on AVP release may have occurred via its action on the plasma membrane since B-BSA is membrane-impermeable. Rapid inhibitory action of corticosterone on AVP release (within 20 min) also can not be explained by its slow genomic actions (Beato, 1989, McEwen et al. 1986).

There are three main ways membrane receptors transmit signals. These receptors may be coupled to ligand-gated ion channels, ligand-regulated tyrosine kinases, or G-proteins (Moore, 1994). Although the rapid, non-genomic actions of the glucocorticoids have been widely recognized, membrane receptor types for glucocorticoids and the intracellular mechanisms, i.e. the possible second messenger systems and the signal transduction pathway, involved remains unclear.

A study using purified rat brain synaptosomes showed that corticosterone (1 uM) potentiated high K^+-induced Ca^{2+} uptake within 15 min, and this stimulatory effect was sustained for 60 min of incubation (Sze and Iqbal, 1994a). The influx of Ca^{2+} through voltage-dependent Ca^{2+} channels also can be stimulated by veratridine, which depolarizes brain synaptosomes indirectly by inducing Na^+ influx. Preincubation of brain synaptosomes with 1 uM corticosterone increased the uptake of $^{45}Ca^{2+}$ within 60 s after induction of depolarization by high K^+ or veratridine (Sze and Iqbal, 1994a). The use of Ca^{2+} channel antagonists, nitrendipine and nifedipine, resulted in a blockade of the corticosterone action on Ca^{2+} uptake, suggesting that the actions of glucocorticoids take place by affecting voltage-dependent Ca^{2+} channels on the membrane (Sze and Iqbal, 1994a).

Possible actions of glucocorticoids on the Ca^{2+} channels are further supported by the findings that corticosterone (10^{-6} M) increases the binding of [^{125}I]calmodulin to purified synaptic plasma membrane from rat brain (Sze and Iqbal, 1994b). Since a number of biochemical events in plasma membranes are dependent on calmodulin for their activation, increased binding of calmodulin to the voltage-dependent membrane Ca^{2+} channels by corticosterone could activate other enzymes to mediate the actions of glucocorticoids (Sze and Iqbal, 1994b). These observations imply that glucocorticoids may exert rapid actions within the CNS via its impact on Ca^{2+} channels on the membrane since calcium entry into neurons affects many cellular activities including neuronal firing patterns, neurotransmitter release, gene expression as well as a series of Ca^{2+}-dependent enzyme-mediated effects (**Fig. 8**)(Bajjalieh and Scheller, 1995, Sihra and Nichols, 1993).

Recent studies have shown that PKC and PKA play important roles in rapid, nongenomic actions of glucocorticoids (Lou and Chen, 1998, Shipstont et al. 1996, Qiu et al. 1998). Corticosterone, in a dose-dependent manner (ranging 10 nM to 10 uM), rapidly (i.e. within 5 min) inhibited Ca^{2+} increase induced by high KCl (55 mM) in PC12 cells (Lou and Chen, 1998). BSA conjugated-corticosterone, impermeable to membrane, was also able to rapidly (within 5 min) block high KCl-induced Ca^{2+} increase in these cells, suggesting that rapid-onset actions of corticosterone occur via membrane-associated receptors. Preincubation of the PC12 cells with G-protein inhibitor, pertusis toxin, or PKC inhibitors for 5 min diminished the inhibitory effects of corticosterone on high KCl-induced Ca^{2+} increase, indicating that G-protein, especially PKC pathway, is involved in intracellular signal transduction of corticosterone. Qiu et al. (1998) recently reported that corticosterone and bovine serum conjugated-corticosterone were able to rapidly increase

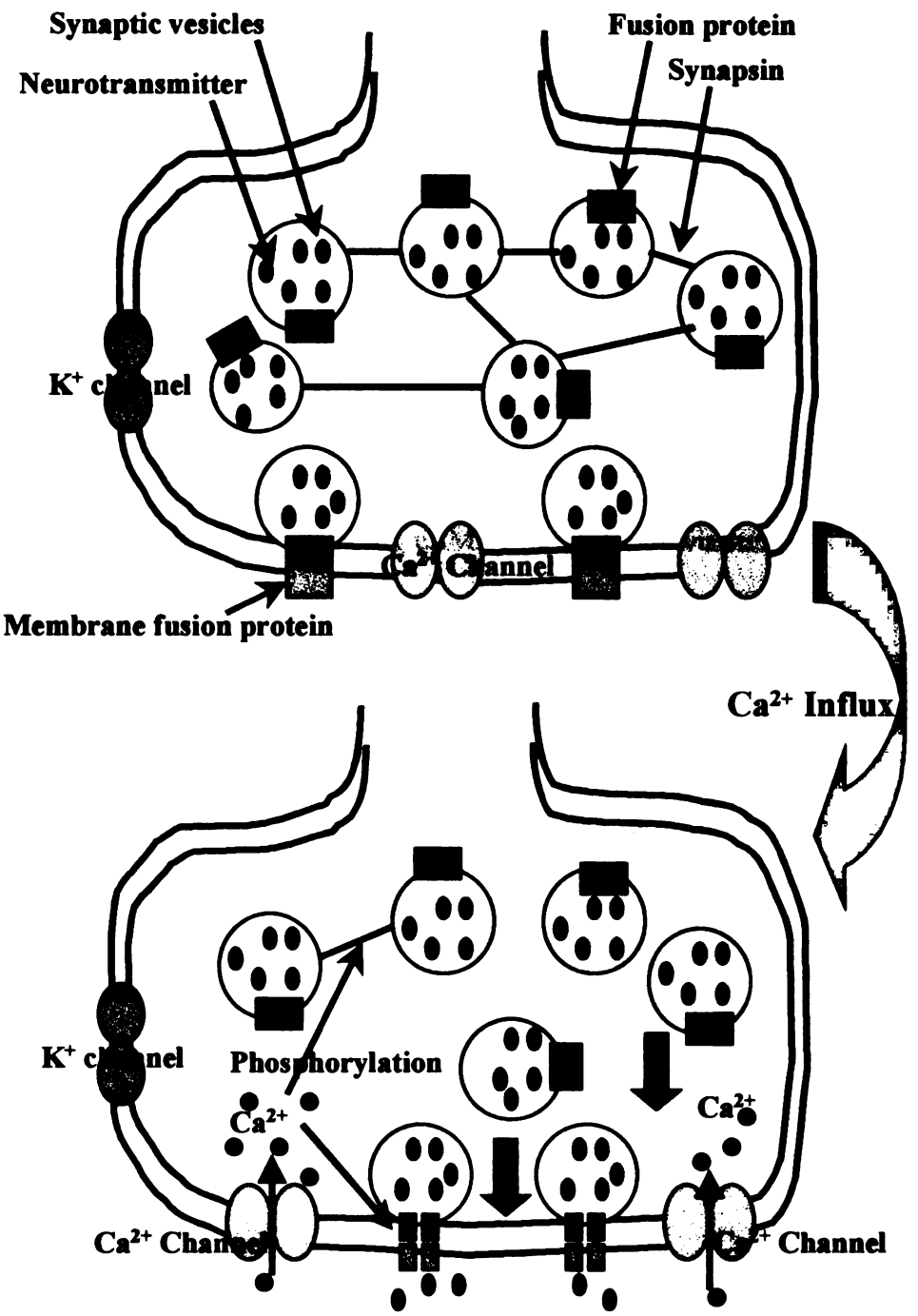

FIGURE 8. Neurotransmitter secretion at neuron terminal after Ca^{2+} entry into the cell. Synaptic vesicles containing neurotransmitters are anchored by synapsin at the neuron terminal at resting state. Calcium entering the cell causes phosphorylation of synapsin, leading to free of synaptic vesicles. Synaptic vesicles then move to the plasma membrane and fuse with membrane fusion protein for exocytosis. Calcium entry also aids fusion of the vesicles with the plasma membrane. From Kelly, 1988.

PKC activity in PC12 cells. CRH and cAMP stimulates ACTH release by activating PKA pathway, and thus by inhibiting calcium-activated potassium channels in the mouse anterior pituitary corticotrope (AtT20) cell lines (Shipston et al. 1996). Pretreatment of cells with 1 uM DEX for 2 h blocked CRH or cAMP-mediated inhibition of potassium channels, resulting in inhibition of ACTH release.

Glucocorticoids-induced changes in calcium influx, ion channels, PKA and PKC pathway may rapidly influence secretion of neurotransmitters within the hypothalamus (**Fig. 9**). Leptin has been also reported to affect membrane potential and/or ion channels (Powis et al. 1998, Spanswick et al. 1997), cAMP concentrations via stimulation of phosphodiesterase 3 B (Zhao et al. 1998), and PKC activity (Chen et al. 1997, Poitout et al. 1998). Glucocorticoid- or leptin-induced changes in membrane potential/ion channels, cAMP, and PKC may influence the secretion of NPY and CRH within the hypothalamus. Whether or not glucocorticoids regulate the actions of leptin at the level of plasma membrane needs further investigation (**Fig. 10**).

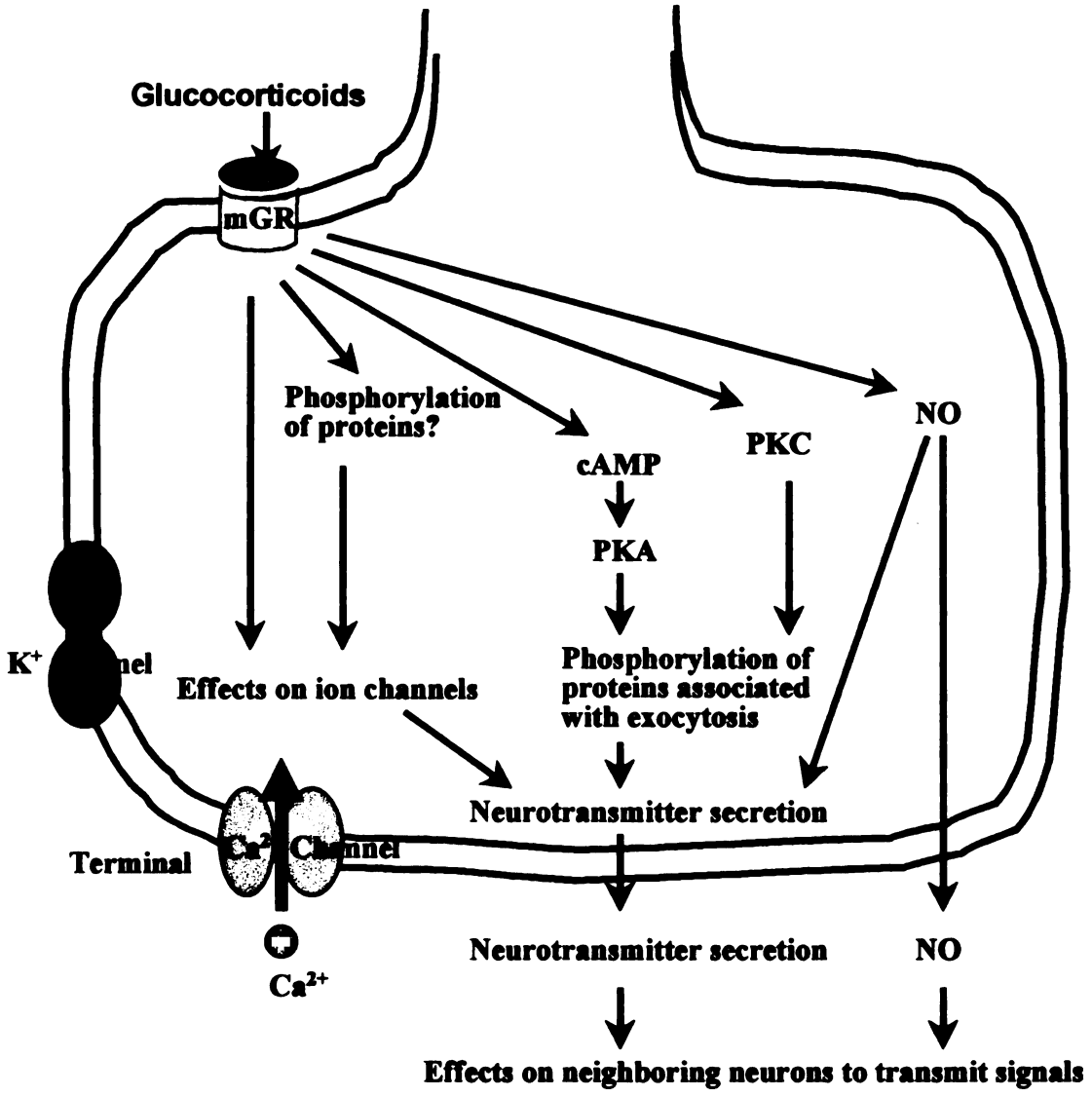

FIGURE 9. Rapid-onset actions of glucocorticoids on neurotransmitter secretion at neuron terminal. Glucocorticoids may affect neurotransmitter secretion by rapidly affecting ion channels, cAMP concentrations, PKA, PKC as well as nitric oxide production via membrane-bound receptors. Abbreviation: mGR, membrane-bound glucocorticoid receptor.

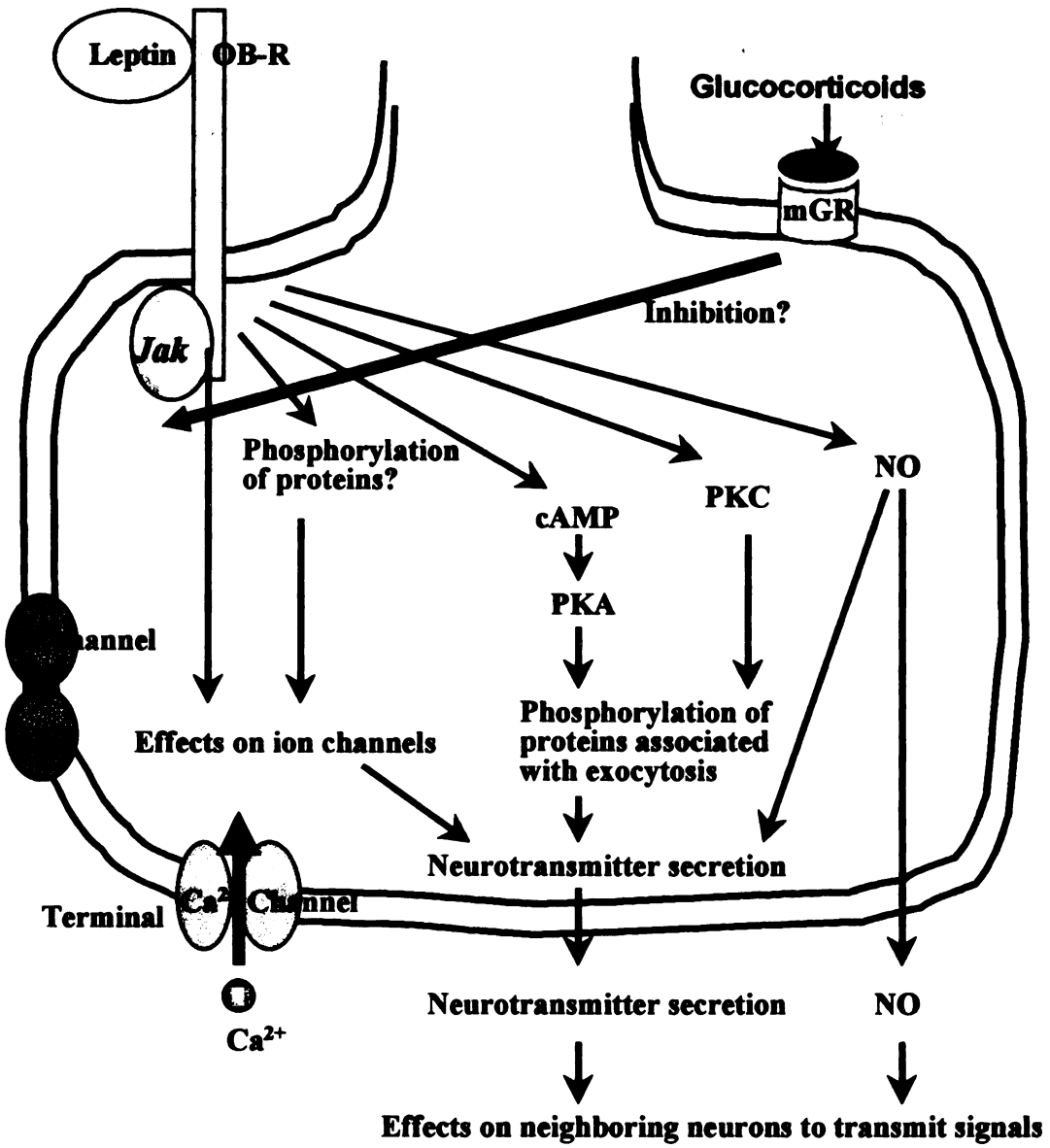

FIGURE 10. Glucocorticoids may oppose leptin actions on neurotransmitter secretion at neuron terminal.

CHAPTER III. NEUROPEPTIDE Y AND CORTICOTROPIN-RELEASING HORMONE CONCENTRATIONS WITHIN SPECIFIC HYPOTHALAMIC REGIONS OF LEAN MICE, BUT NOT OB/OB MICE, RESPOND TO FOOD-DEPRIVATION AND REFEEDING

(Published in the Journal of Nutrition 128: 2520-2525, 1998)

A. ABSTRACT

Leptin is proposed to control food intake in part at least by regulating hypothalamic NPY, a stimulator of food intake, and CRH, an inhibitor of food intake. Ob/ob mice are leptin-deficient and would thus be expected to exhibit alterations in hypothalamic NPY and CRH. We therefore measured concentrations of NPY and CRH in discrete regions of hypothalamus (i.e. ARC, PVN, VMH, DMH, and SCN) of 6.5 - 7 wk old ob/ob and lean mice with free access to stock diet, 24 h after food-deprivation, and 1 h after refeeding. Fed ob/ob mice had 55 – 75 % higher concentrations of NPY in the ARC, VMH, and SCN than lean mice. Food-deprivation increased NPY concentrations ~ 70 % in the ARC, PVN, and VMH of lean mice, and refeeding lowered NPY concentrations ~ 70 % in the PVN of these mice. NPY in these hypothalamic regions of ob/ob mice was unresponsive to food-deprivation or refeeding. The most pronounced change in CRH concentrations within the regions examined (i.e. ARC, PVN, and VMH) occurred in the ARC of lean mice where refeeding lowered CRH concentrations by 75 %, without influencing ARC CRH concentrations in ob/ob mice. The hypothalamic concentrations of two neuropeptides involved in body weight regulation (i.e. NPY and CRH) in leptin-deficient ob/ob mice respond abnormally to abrupt changes in nutritional status.

B. Introduction

A nonsense mutation in the ob gene in ob/ob mice (Zhang et al. 1994) causes severe obesity in these mice. Administration of the ob gene product leptin to these leptin-deficient ob/ob mice decreases their food intake as well as increases their energy expenditure, resulting in a restoration of normal body weight regulation (Halaas et al. 1995, Hwa et al. 1996, Pelleymounter et al. 1995). Leptin likely acts within the CNS, in particular the hypothalamus, to regulate body weight since central administration of leptin produced pronounced effects on food intake with lower doses than those required during peripheral injection (Campfield et al. 1995, Halaas et al. 1995, Stephens et al. 1995).

NPY increases food intake and decreases energy expenditure (Billington et al. 1994, Clark et al. 1984, Stanley et al. 1986) whereas CRH has opposite actions (Arase et al. 1988, Krahn et al. 1988, Rohner-Jeanrenaud et al. 1989). NPY and CRH have thus logically emerged as candidates to mediate leptin actions within the hypothalamus (Mercer et al. 1996, Schwartz et al. 1996a and 1996b, Stephens et al. 1995, Wang et al. 1997). Centrally administered leptin decreases NPY mRNA and increases CRH mRNA within the hypothalamus of rats and mice (Schwartz et al. 1996a and 1996b, Stephens et al. 1995, Wang et al. 1997). Leptin has also been reported to decrease *in vitro* secretion of NPY (Stephens et al. 1995) and increase CRH release from the rat hypothalamus (Costa et al. 1997, Raber et al. 1997). These actions of leptin on hypothalamic NPY and CRH support the hypothesis that leptin, NPY and CRH are linked in the regulation of food intake and energy expenditure.

If leptin regulates food intake and energy expenditure via hypothalamic NPY and/or

CRH, leptin-deficient ob/ob mice would be expected to exhibit alterations in NPY and CRH within the hypothalamus. Only a few studies have examined hypothalamic NPY in ob/ob mice, and to our knowledge none have examined CRH in these mice (Mistry et al. 1994, Qu et al. 1996, Stephens et al. 1995, Wilding et al. 1993, Williams et al. 1991). Hypothalamic NPY mRNA abundance is elevated in ob/ob mice (Qu et al. 1996, Stephens et al. 1995, Wilding et al. 1993), with no reported alterations in NPY protein in the whole hypothalamus of these mice compared to lean controls (Wilding et al. 1993, Williams et al. 1991). Since specific hypothalamic regions are involved in regulation of metabolism by NPY (Morley 1987, Stanley et al. 1986), measurements utilizing the whole hypothalamus may mask regional differences.

Disturbances in hypothalamic NPY have also been observed in leptin-resistant db/db mice (Chua Jr. et al. 1991, Mizuno et al. 1997), and considerable evidence has accumulated to demonstrate abnormal regulation of NPY and CRH in leptin-resistant fa/fa rats (Bchini-Hooft van Huijsduijnen et al. 1993, Beck et al. 1990a and 1993, Fukushima et al. 1992, McKibbin et al. 1991, Nakaishi et al. 1990 and 1993, Pesonen et al. 1992). Hypothalamic NPY mRNA abundance is higher in db/db mice and fa/fa rats than in the respective lean controls (Chua Jr. et al. 1991, Mizuno et al. 1997, Pesonen et al. 1992). NPY protein in specific hypothalamic nuclei including the ARC, PVN, VMH, DMH, and SCN is also elevated in fa/fa rats compared to lean controls (Beck et al. 1990a and 1993, McKibbin et al. 1991). Similar measurements have not been reported for ob/ob or db/db mice. Hypothalamic CRH mRNA abundance is unaltered in fa/fa rats (Pesonen et al. 1992), but CRH protein is lower in these rats than in lean controls (Nakaishi et al. 1990 and 1993). Together these results are consistent with a linkage between leptin and the

regulation of NPY and CRH.

Hypothalamic NPY and CRH are reported to change with the feeding status of the animals (Beck et al. 1990b, Brady et al. 1990, Sahu et al. 1988b, Schwartz et al. 1993), conditions that are now known to alter plasma leptin concentrations (Hardie et al. 1996, Saladin et al. 1995, Trayhurn et al. 1995). Food-deprivation increases NPY mRNA abundance in the ARC or in the whole hypothalamus of rats and mice including ob/ob and db/db mice (Brady et al. 1990, Chua Jr. et al. 1991, Mizuno et al. 1997, Qu et al. 1996, Schwartz et al.1993), but not in leptin-resistant fa/fa rats (Sanacora et al. 1990). NPY protein is also known to increase with food-deprivation and decrease with refeeding in site-specific manners within the hypothalamus of control rats (Beck et al. 1990b, Sahu et al. 1988b), but not in leptin-resistant fa/fa rats (Beck et al. 1992, Sanacora et al. 1990). Food-deprivation decreases CRH mRNA in the PVN of rats (Brady et al. 1990). We are not aware of any reports on food intake-induced regulation of hypothalamic NPY and CRH concentrations in leptin-deficient ob/ob mice. The present study was thus designed to determine (1) whether any alterations are present in NPY and CRH concentrations within specific hypothalamic nuclei of ob/ob mice, and (2) if hypothalamic NPY and CRH concentrations of ob/ob mice change in response to food-deprivation and refeeding.

C. MATERIALS AND METHODS

Animals and diet. Male obese (ob/ob) and lean mice (ob/+ or +/+) were obtained from our breeding colony of C57BL/6J ob/+ mice. The Guide for the Care and Use of Laboratory Animals (NRC 1985) and local institutional guidelines were followed for the care and treatment of the mice. Mice were weaned at 3 - 3.5 wk of age, group-housed in

solid-bottom plastic cages with wood shavings for bedding and fed a nonpurified diet (Teklad Rodent Diet 8640; 22 % protein, 5 % fat and 4.5 % crude fiber; Harlan, Bartonville, IL). Room temperature was 23 - 25 °C and lights were on from 07:00 to 19:00 h.

Reagents. Coating antiserums (rabbit anti-guinea pig Ig G for insulin ELISA and goat anti-rabbit Ig G for NPY and CRH ELISA) were purchased from EY Lab. Inc. (San Mateo, CA). Guinea pig anti-rat insulin was from Linco Research Inc. (St. Louis, MO). Rabbit anti-NPY (human, rat) Ig G, rabbit anti-CRH (human, rat) Ig G, biotinyl-NPY (human, rat), and biotinyl-CRH were obtained from Peninsula Lab. Inc. (Belmont, CA). NPY (human) and CRH (human, rat) were purchased from Bachem (Torrance, CA). Avidin-peroxidase, 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and glucose diagnostic kits were obtained from Sigma Chemical Inc. (St. Louis, MO).

Experimental design. At 6 - 6.5 wk of age, ob/ob and lean mice were individually housed for 3 days and then randomly distributed into 1 of 3 groups; group 1 was fed, group 2 was food-deprived for 24 h, and group 3 was food-deprived for 24 h and then refed for 1 h. Food intake and body weight were recorded. Mice were killed by decapitation between 10:00 and 11:00 h. Blood was collected, and plasma was separated and stored at - 20 °C for measurement of glucose and insulin. The brains were quickly removed from the skulls. A cut was made perpendicular to the midline of the mid-hind brain to prepare the tissue for mounting on the specimen holder. The brain was then immediately frozen on dry ice and stored at - 80 °C.

Brain dissection and micropunch. The frozen brain was glued (Tissue-Tek, 1988 Miles Inc. Elkhart, IN) to a specimen holder and placed in a cryostat (Cryocut 1800, Leica

Inc. Deerfield, IL) at - 10 °C. After equilibration for about 30 min, the brain was repeatedly sectioned until the anterior commissure was clearly visible. From this reference point serial sections of 400 to 500 μm were made according to the stereotaxic atlas of the albino mouse forebrain (Slotnick and Leonard, 1975). The brain sections were placed on glass slides and kept frozen on dry ice. Discrete hypothalamic nuclei were micropunched under a microscope by the technique of Palkovits (1973). Hypothalamic nuclei sampled included the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), and suprachiasmatic nucleus (SCN)(Slotnick and Leonard, 1975). A 20-gauge, oval- shaped needle was used to micropunch the ARC and PVN, and a round 24-gauge needle was used for the other nuclei.

Bilateral tissue samples were immediately placed in 100 μL of HCl (0.1 mol/L) containing a protease inhibitor (aprotinin, 900,000 KIU/L). The tissue samples were sonicated for 15 s and centrifuged at 10,000 x g for 15 min at 4 °C. Supernatants were then lyophilized and stored at - 20 °C until measurement for NPY and CRH. Tissue pellets were dissolved in 200 μL of NaOH (0.1 mol/L) and protein was determined by a modified method of Lowry (Bio-Rad DC protein kit, Hercules, CA).

Assays. Plasma glucose was measured with a glucose oxidase-peroxidase kit (Sigma Chemical Inc., St. Louis, MO). Plasma insulin and NPY and CRH in discrete regions of hypothalamus were measured by competitive ELISAs as described below. Immulon 4 polystyrene microtiter plates with flat-bottomed wells (Dynatech Lab. Inc., Chantilly, VA) were used for these assays. Preparation of coating buffer (0.05 mol/L carbonate bicarbonate, pH 9.6), washing buffer (0.15 mol/L PBS, pH 7.2) and citrate buffer (0.1 mol/L, pH 4.0) used in these ELISAs and other buffers used in the insulin ELISA followed

procedures described by Kekow et al. (1988).

Insulin was measured as described by Kekow et al. (1988) with some modifications. Rabbit anti-guinea pig Ig G (10 mg/L of coating buffer) in a volume of 150 µL was added to each well and dried at 37 °C overnight. Wells were washed thrice with washing buffer. Hundred µL of anti-insulin antibody (1000 radioimmunoassay-tube quantity/L of insulin incubation buffer) was added to each well and incubated overnight at 4 °C. Rat insulin standards or plasma samples in 100 µL of sample buffer [incubation buffer with 60 g BSA/L] were added and incubated at 37 °C for 50 min. Hundred µL of peroxidase-labelled insulin (1.2 mg/L sample buffer) was added to each well and incubated at 37 °C for 50 min followed by three washings. Hundred µL of citrate buffer (0.1 mol/L, pH 4.0) containing substrate for peroxidase, H_2O_2 (8 mmol/L) and a chromogen, ABTS (660 µmol/L), were then added to the wells. Color was developed at room temperature and optical density was measured at 405 nm. Intra- and inter-assay variations were 4 % and 10 %, respectively.

NPY and CRH assays were developed in our laboratory. Wells were coated with goat anti-rabbit Ig G (12.5 mg/L for NPY or 6.25 mg/L for CRH) in 200 µL of coating buffer at 37 °C overnight. Plates were then washed thrice with washing buffer. Rabbit anti-NPY Ig G (2 mg/L) or rabbit anti-CRH Ig G (0.5 mg/L) in 100 µL of sample buffer (0.15 mol/L PBS with 1 mL/L Tween-20 and 10 g/L BSA, pH 7.3) were added and plates were incubated at 37 °C for 3 - 3.5 hrs. After three additional washings, standards (NPY or CRH) or brain samples in 100 µL sample buffer were added and held at 4°C overnight. Biotinyl-NPY (220 nmol/L) or biotinyl-CRH (80 nmol/L) in 50 µL of sample buffer was subsequently added and incubated for 2.5 h at 4°C. After six washings, 100 µL of avidin

peroxidase (2 mg/L sample buffer) was added and the plate was incubated for 1 hr at room temperature, followed by six washings. Citrate buffer (100 µL, pH 4.0) containing H_2O_2 and ABTS was then added (same as for insulin ELISA). Optical density was subsequently measured at 405 nm. The sensitivity of the NPY and CRH ELISA was 1 fmol/well. Intra- and inter-assay variations were less than 5 % and 10 %, respectively, for both NPY and CRH ELISA.

Statistics. Data are expressed as means ± SE and were analyzed with SAS/STAT(V. 6.11, SAS Institute, Carry, NC). Body weight and food intake comparisons were analyzed with Student's t test. Comparisons of insulin, glucose, NPY and CRH during the different feeding states in ob/ob and lean mice were performed by two-way ANOVA with the least-significant difference (LSD) test used for post-hoc comparisons. Data for insulin were log transformed before two-way ANOVA because of unequal variances. Differences were considered significant at $P < 0.05$.

D. RESULTS

Food intake, body weight, plasma insulin and glucose. As expected, the 6.5 - 7 wk old ob/ob mice weighed more (30 ± 1 g body weight versus 22 ± 1 g, n=10 for each group) and consumed more food than the lean mice; daily food intake for the 3-day period before the experiment averaged 6.5 ± 0.3 g/day for ob/ob mice and 3.9 ± 0.1 g/day for lean mice (n=10 for each group). Both ob/ob and lean mice lost ~ 3 g body weight during 24 h of food-deprivation. Food intake during the 1 h refeeding period was similar in ob/ob and lean mice, 0.69 ± 0.04 g/h and 0.67 ± 0.03 g/h (n=10 for each group), respectively.

Plasma insulin concentrations, as expected, were elevated in ob/ob mice (**Table 3**).

Food- deprivation lowered plasma insulin concentrations in ob/ob ($P < 0.05$) and lean mice ($P = 0.17$), although not statistically significant in lean mice, and refeeding elevated plasma insulin. Plasma glucose concentrations were unaffected by phenotype, lowered by food-deprivation and elevated by refeeding.

Hypothalamic NPY. Ob/ob mice with free access to food had ~ 55 - 75 % higher NPY concentrations in the ARC ($P = 0.11$), VMH, and SCN than lean mice, although differences in the ARC were not statistically different (**Fig. 11**). NPY concentrations changed with food-deprivation and refeeding in site-specific manners in ob/ob and lean mice. Only in the SCN of ob/ob mice did food-deprivation influence NPY contents; food-deprivation lowered NPY contents in this nucleus of ob/ob mice. Food-deprivation elevated NPY contents in the ARC, PVN, and VMH of lean, but not ob/ob, mice. Refeeding lean mice, but not ob/ob mice, significantly lowered (- 71 %) NPY concentrations in the PVN.

Hypothalamic CRH. CRH concentrations in the 3 regions examined of fed mice were not affected by phenotype even though lean mice tended ($P = 0.18$) to have higher (about double) CRH concentrations in the ARC than ob/ob mice (**Fig. 12**). CRH concentrations were not modified by food-deprivation or refeeding in any region of the hypothalamus of ob/ob mice examined. CRH contents in the ARC of lean mice were lowered by 75 % with refeeding.

TABLE 3. Plasma insulin and glucose in lean and ob/ob mice

Phenotype	Fed	Food-deprived	Refed
		Insulin, nmol/L	
Ob/ob	10.2 ± 1.4^{P}	$0.8 \pm 0.2^{P,D}$	$6.8 \pm 1.4^{P,R}$
Lean	0.12 ± 0.02	0.08 ± 0.01	1.27 ± 0.16^{R}
		Glucose, mmol/L	
Ob/ob	17 ± 1	8 ± 1^{D}	18 ± 1^{R}
Lean	15 ± 1	8 ± 1^{D}	18 ± 1^{R}

Values are means ± SE for 6.5 - 7 wk old mice (n = 8 - 10). Comparisons of plasma insulin and glucose were performed by two-way ANOVA with the least significant difference (LSD) test used for post-hoc comparisons. Data for insulin were log transformed before two-way ANOVA. ^{P} indicates significant difference between phenotypes within feeding state ($P < 0.05$). ^{D} indicates significant effect of food-deprivation, and ^{R} indicates significant effect of refeeding within phenotype ($P < 0.05$)

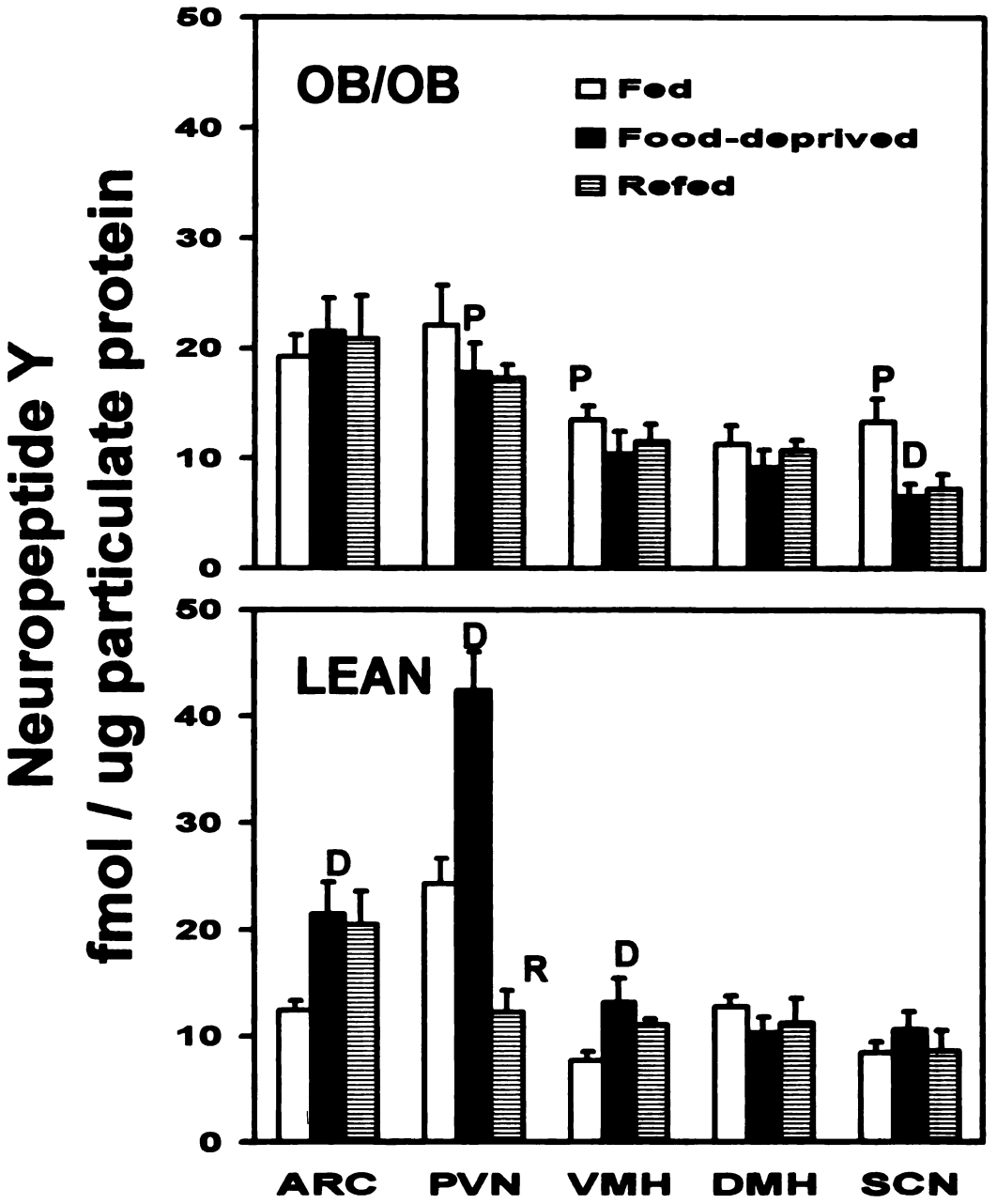

FIGURE 11. NPY concentrations in specific hypothalamic regions of fed, food-deprived, or refed ob/ob and lean mice. Each bar represents means ± SE of 6 – 9 mice per group. Mice were given free access to food, food-deprived for 24 h, or refed for 1 h after 24 h food-deprivation. Two-way factorial ANOVA was used to determine significant effects of phenotype, feeding status, and interaction. Significant effects of phenotype, feeding status, and phenotype-feeding status interaction were observed in the PVN ($P < 0.05$). Significant phenotype-feeding status interactions were also observed in the VMH and SCN ($P < 0.05$). ^{P} significant phenotype differences within the same feeding states with the least significant difference (LSD) test ($P < 0.05$). ^{D} significant effect of food-deprivation, and ^{R} significant effect of refeeding within phenotype as determined by LSD test ($P < 0.05$).

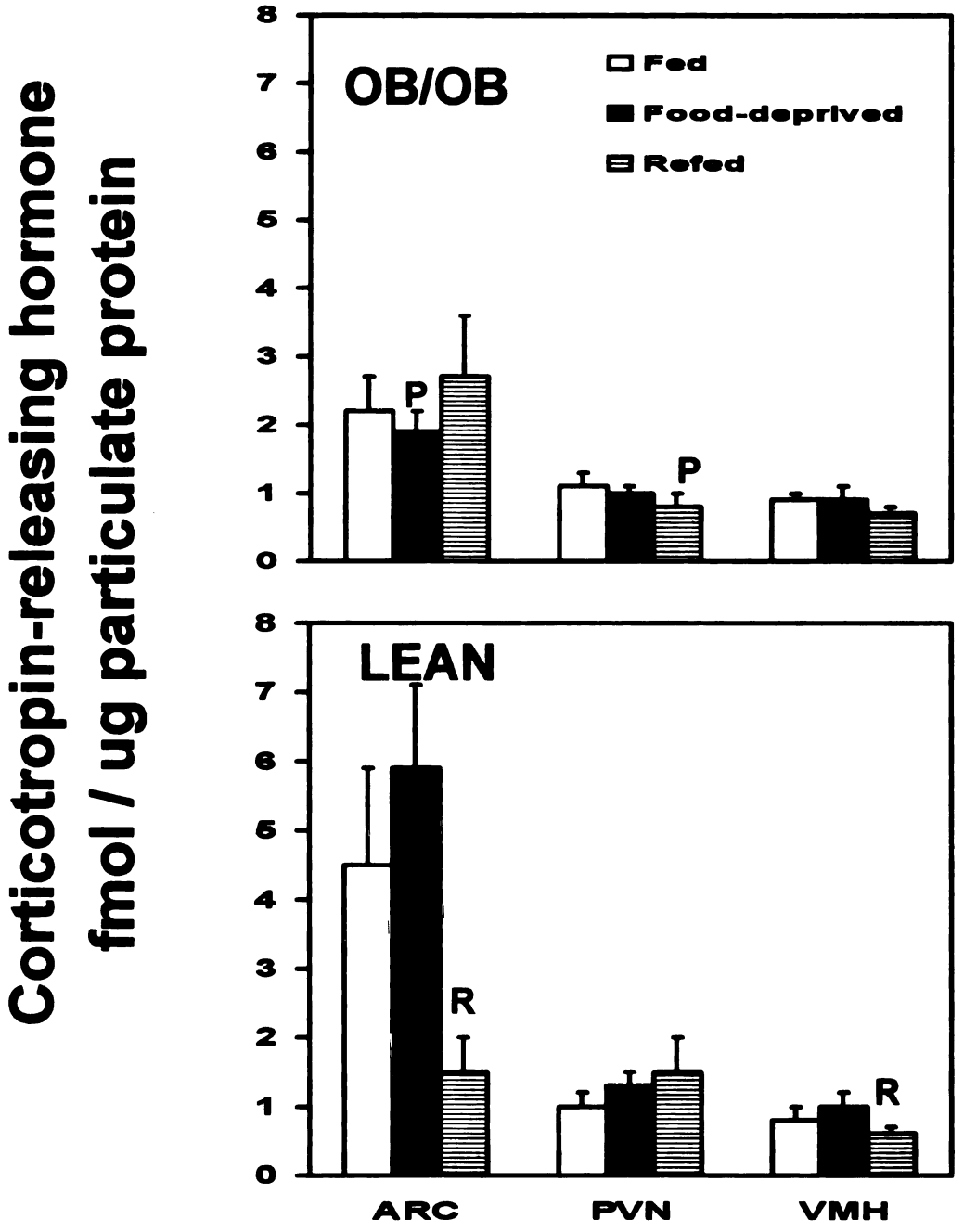

FIGURE 12. CRH concentrations in specific hypothalamic regions of fed, food-deprived, or refed ob/ob and lean mice. Values are means ± SE of 6 – 9 mice per group. Mice were given free access to food, food-deprived for 24 h, or refed for 1 h after 24 h food-deprivation. Two-way factorial ANOVA was used to determine significant effects of phenotype, feeding status, and interaction. A significant phenotype and feeding-status interaction was observed in the ARC ($P < 0.05$). ^{P}indicates significant phenotype differences within the same feeding state with LSD test ($P < 0.05$). ^{R}indicates significant effect of refeeding within phenotype as determined by LSD test ($P < 0.05$).

E. DISCUSSION

Results of the present study may be summarized as follows. First, concentrations of NPY in selected hypothalamic nuclei of leptin-deficient ob/ob mice were elevated. Second, food deprivation and short-term refeeding caused less dramatic changes in hypothalamic NPY concentrations in these ob/ob mice than in lean mice. And third, hypothalamic CRH concentrations were less affected by phenotype and feeding status than NPY concentrations.

Our measurements of neuropeptide concentrations in specific hypothalamic nuclei reflect the balance of peptide synthesis and transport/release/degradation rates. NPY mRNA abundance and presumably NPY synthesis is elevated in the hypothalamus of fed leptin-deficient ob/ob mice (Qu et al. 1996, Stephens et al. 1995, Wilding et al. 1993), consistent with their hyperphagia and hypometabolism (Erickson et al. 1996b, Halaas et al. 1995, Pelleymounter et al. 1995). The ARC is enriched in NPY-containing neuron cell bodies (Chronwall et al. 1985), suggesting that the trend for elevated NPY concentrations in the ARC of fed ob/ob mice (**Fig. 11**) was secondary to elevated rates of NPY synthesis in this nucleus of these mice. Interestingly, NPY was not elevated in the PVN of ob/ob mice (**Fig. 11**). This would be consistent with increased release of NPY from this region of ob/ob mice, in agreement with the well established role of NPY in the PVN to promote food intake (Kalra et al. 1991, Stanley and Leibowitz, 1984). It is difficult to interpret the physiological importance of elevated NPY concentrations in the VMH and SCN of fed ob/ob mice. These findings however agree with observations in leptin-resistant fa/fa rats (Beck et al. 1990a and 1993, McKibbin et al. 1991). NPY concentrations in selected hypothalamic regions appear to respond similarly to leptin deficiency and leptin resistance.

Food-deprivation and refeeding are well-characterized modulators of NPY concentrations in selected hypothalamic regions of rats (Beck et al. 1990b, Brady et al. 1990, Sahu et al. 1988b, Schwartz et al. 1993). Our lean mice responded similarly. The increases in NPY concentrations in the ARC, PVN, and VMH of these lean mice after food-deprivation (**Fig. 11**) are consistent with reported elevations in hypothalamic NPY mRNA, and presumably NPY synthesis, during food-deprivation (Brady et al. 1990, Schwartz et al. 1993). In contrast, NPY concentrations in these hypothalamic regions of ob/ob mice were unchanged during food-deprivation. Consequently, food-deprived lean and ob/ob mice had similar NPY concentrations in the various hypothalamic nuclei examined, except for the PVN where NPY concentrations were now even higher in lean mice than in ob/ob mice (**Fig. 11**). There is now considerable evidence that food-deprivation lowers plasma leptin concentrations (Hardie et al. 1996, Saladin et al. 1995, Trayhurn et al. 1995). This may contribute to the observed changes in hypothalamic NPY in lean mice with food-deprivation. Ob/ob mice, however, are in a chronic state of leptin deficiency. Consistent with this, NPY concentrations in the ARC, VMH, and SCN of fed ob/ob mice were as high as in these regions of food-deprived lean mice, and food-deprivation failed to further influence hypothalamic NPY concentrations in ob/ob mice.

The PVN is an important site for NPY regulation of food intake (Kalra et al. 1991, Stanley and Leibowitz 1984). Within 1 h of food consumption following 24 h of food-deprivation the NPY concentration in the PVN of lean mice declined by about 70 % (**Fig. 11**). In contrast, NPY concentrations in the PVN of similarly treated ob/ob mice failed to change. This occurred even though both groups of mice consumed similar amounts of food and had similar changes in plasma glucose during this refeeding period. Thus, within

this time frame the observed phenotype differences in NPY within the PVN did not translate to differences in food intake. It is not clear whether refeeding the lean mice slowed transport of NPY from cell bodies to terminal regions within the PVN, or accelerated NPY release and degradation within the PVN.

We expected that ob/ob mice might contain lower hypothalamic CRH concentrations than lean mice since corticosterone, high in ob/ob mice, possesses inhibitory actions on CRH synthesis and release within the hypothalamus (Beyer et al. 1988, Sawchenko 1987a and 1987b). Though CRH concentrations tended to be lower in the ARC of fed ob/ob mice than in lean mice, no significant alterations were observed in any hypothalamic nuclei of ob/ob mice examined (**Fig. 12**). This finding is consistent with the observation that leptin-resistant fa/fa rats and control rats also had similar CRH concentrations in many regions of hypothalamus, except in the median eminence (Nakaishi et al. 1993). Refeeding lean mice for only 1 h lowered CRH concentrations in the ARC by 75 %, without influencing CRH in this nucleus of ob/ob mice, findings analogous to changes in NPY within the PVN of these mice upon refeeding.

Leptin is proposed to be a sensor of nutritional status (Flier and Maratos-Flier, 1998). The leptin-deficient ob/ob mice exhibited less pronounced changes in hypothalamic concentrations of NPY and CRH in response to food-deprivation and abrupt refeeding than did lean mice. These results are consistent with a role for leptin-NPY-CRH interaction in the regulation of body weight. Other factors undoubtedly participate in this complex regulatory system. For example, leptin still exerts effects on food intake in NPY-knockout mice (Erickson et al. 1996a), and leptin-deficient ob/ob mice regulate food intake within the normal range when glucocorticoids are removed by ADX (Feldkircher et

al. 1996). An understanding of how the various factors contributing to food intake regulation are integrated remains a formidable challenge.

CHAPTER IV. LEPTIN RAPIDLY INHIBITS HYPOTHALAMIC NEUROPEPTIDE Y SECRETION AND STIMULATES CORTICOTROPIN-RELEASING HORMONE SECRETION IN ADRENALRECTOMIZED MICE

A. ABSTRACT

Leptin may rapidly inhibit food intake by rapidly altering the secretion of hypothalamic neuropeptides such as NPY, a stimulator of food intake, and/or CRH, an inhibitor of food intake. We measured concentrations of NPY and CRH in specific hypothalamic regions (i.e. ARC, PVN, VMH, and DMH) of 7 – 8 wk old lean and ob/ob mice 1 h or 3 h after ICV leptin administration. No rapid onset effects of leptin on hypothalamic NPY and/or CRH concentrations were observed in intact mice, consistent with the ineffectiveness of leptin to also alter *in vitro* NPY and CRH secretions from hypothalamic preparations obtained from intact mice. Glucocorticoids appear to oppose leptin actions. Thus, ADX mice were used to determine whether leptin affects secretion of hypothalamic NPY and CRH in the absence of corticosterone. Leptin administration markedly reduced CRH concentrations in the ARC of ADX mice 3 h post-injection. This rapid reduction in CRH concentration (i.e. within 3 h) in the ARC after leptin administration is more likely due to stimulated CRH release from this region rather than due to decreased synthesis/transport from the PVN because leptin stimulates, not depress, CRH synthesis in the PVN. Within 20 min after exposure to leptin, NPY secretion from hypothalamic preparations obtained from ADX mice was lowered by 27 %, and CRH secretion was elevated by 51 %. The

present study clearly demonstrates that leptin rapidly influences secretion of hypothalamic NPY and CRH, and that these actions of leptin within the hypothalamus are restrained by the presence of endogenous corticosterone.

B. INTROCDUCTION

Several lines of evidence support the hypothesis that leptin acts within the CNS, especially the hypothalamus, to influence neuropeptides, including NPY and CRH, involved in regulation of food intake and energy expenditure (Campfield et al. 1995, Rohner-Jeanrenaud et al. 1996, Schwartz et al. 1996b, Stephens et al. 1995, Wang et al. 1997). NPY increases food intake and decreases energy expenditure (Billington et al. 1994, Clark et al. 1984) whereas CRH has opposite actions (Krahn et al. 1988, Rohner-Jeanrenaud et al. 1989). Leptin, by decreasing the synthesis and/or secretion of hypothalamic NPY and by increasing the synthesis and/or secretion of hypothalamic CRH, would be well-positioned to modulate energy balance.

Central administration of leptin, either a single injection or repetitive injections, has been shown to decrease NPY mRNA in the ARC of rats and mice (Cusin et al. 1996, Sahu et al. 1998, Schwartz et al. 1996b. Stephens et al. 1995), and also to increase CRH mRNA in the PVN of rats (Schwartz et al. 1996b). These leptin-induced changes in hypothalamic NPY and CRH gene expression have been noted between 6 h and 5 days post leptin administration. Concomitant with the lowering of NPY mRNA, NPY peptide concentrations were also lowered in the ARC, PVN, and DMH of rats 6 h after administration of leptin (Wang et al. 1997), suggesting less synthesis of NPY. These results provide one possible explanation of how a single injection of leptin might cause

relatively long-lasting (i.e. 12 - 24 h) effects on food intake. Numerous studies, however, demonstrate that the inhibitory actions of leptin on food intake are also rapid in onset, i.e. within 30 min to 2 hr (Campfield et al. 1995, Flynn et al. 1998, Mistry et al. 1997, Rentsch et al. 1995, Seeley et al. 1996). These rapid-onset actions of leptin on food intake may more likely occur via mechanisms other than altered gene expression, given the time typically required for modulation of protein synthesis. Leptin is able to rapidly, within seconds, depolarize rat PVN neurons and activate ATP-sensitive potassium channels within the hypothalamus (Glaum et al. 1996, Powis et al. 1998, Spanswick et al. 1997). These leptin-induced changes in membrane potential and ion channels within the hypothalamus may lead to rapid changes in neurotransmitter secretion.

Several attempts have been made to examine the acute effects of leptin on hypothalamic NPY and CRH secretion, with inconsistent outcomes. Leptin inhibited NPY release that was first induced by a 2 h exposure of hypothalamic preparations from rats to 0.6 uM corticosterone (Stephens et al. 1995). In the absence of corticosterone, NPY secretion from this preparation was undetectable. Thus, it was not possible to test potential effects of leptin on hypothalamic NPY secretion in the absence of added glucocorticoid. IP administration of leptin to rats failed to influence *in vivo* NPY release from the PVN, as measured by a push-pull cannula, during a 2 h time period (Beck et al. 1998). The conditions under which leptin might acutely affect NPY secretion remain to be resolved. Leptin increased CRH secretion, within 20 min, from hypothalamic preparations of rats and mice incubated in 3 - 5.5 m M glucose (Costa et al. 1997, Raber et al. 1997), but in another report addition of leptin to hypothalamic preparations from rats blocked potentiation of CRH secretion induced by low (1.1 mM) glucose (Heiman et al. 1997).

Glucocorticoids via yet to be fully defined mechanisms cause rapid-onset changes in secretion of hypothalamic NPY (stimulatory) and CRH (inhibitory) (Calogero et al. 1988, Chen and Romsos, 1995, Stephens et al. 1995, Suda et al. 1985). These actions of glucocorticoids on hypothalamic NPY and CRH secretion are opposite those proposed for leptin. Inhibitory actions of leptin on food intake and body weight were recently reported to be more pronounced in ADX rats and mice than in intact ones (Mistry et al. 1997, Zakrzewska et al. 1997). This observation and the colocalization of leptin and glucocorticoid receptors within the PVN and ARC, important sites for NPY and CRH actions, support the hypothesis that interactions between leptin and glucocorticoids may contribute to the regulation of NPY and CRH secretion within the hypothalamus (Mercer et al. 1996, Tempel and Leibowitz, 1994).

The present study was conducted to determine if leptin administration to mice causes rapid changes in NPY and/or CRH concentrations within specific hypothalamic sites. Changes in hypothalamic NPY and CRH concentrations within a rapid time frame, i.e. within 1-3 h, may largely reflect changes in transport/secretion rather than in synthesis of these neuropeptides because of the time lag typically required for modulation of gene expression and subsequent protein synthesis. To more directly assess effects of leptin on secretion of these neuropeptides, hypothalamic preparations were incubated with and without added leptin. The presence of endogenous leptin and/or glucocorticoids might mask/inhibit response to administered leptin. Therefore, leptin-deficient ob/ob mice and ADX mice were used in selected studies.

C. MATERIALS AND METHODS

Animals and diet. Male lean (ob/+ or +/+) and obese (ob/ob) mice were obtained from our breeding colony of C57BL/6J ob/+ mice. The Guide for the Care and Use of Laboratory Animals (NRC 1985) and local institutional guidelines were followed for the care and treatment of the mice. Mice were weaned at 3 - 3.5 wk of age, group-housed in solid-bottom plastic cages with wood shavings for bedding and fed a nonpurified diet (Teklad Rodent Diet 8640, Harlan, Bartonville, IL). Room temperature was 23 - 25 °C and lights were on from 07:00 to 19:00 h. Mice were studied at 7- 8 wk of age.

Reagents. Coating antiserums (rabbit anti-guinea pig Ig G for insulin ELISA, and goat anti-rabbit Ig G for NPY and CRH ELISA) were purchased from EY Lab., Inc. (San Mateo, CA). Guinea pig anti-rat insulin was from Linco Research Inc., (St. Charles, MO). Rabbit anti-NPY (human, rat) Ig G, rabbit anti-CRH (human, rat) Ig G, biotinyl-NPY (human, rat), and biotinyl-CRH were obtained from Peninsula Lab., Inc. (Belmont, CA). NPY (human) and CRH (human, rat) were purchased from Bachem (Torrance, CA). Tissue glue for mounting the hypothalamus was from 1988 Miles Inc (Elkhart, IN). ABTS, aprotinin, ascorbic acid, avidin-peroxidase, bacitracin, HEPES, and glucose diagnostic kits were obtained from Sigma Chemical Inc. (St. Louis. MO). BSA was from Amresco (Solon, OH), dextrose was from J.T. Baker Inc (Phillipsburg, NJ), and protein assay kit was obtained from BioRad (Hercules, CA). Murine leptin was prepared as previously described (Mistry et al. 1997).

Experimental design. To test the acute actions of leptin on food intake and on hypothalamic NPY and CRH concentrations, lean and ob/ob mice were food-deprived for 24 h, injected ICV with vehicle or 60 pmol leptin, and refed for 1 h. Some of the vehicle-

injected mice were food-deprived after injection to serve as controls for the vehicle-injected, refed mice. Other lean and ob/ob mice were food-deprived for 3 h after leptin injection to avoid potential confounding effects of leptin-induced differences in food intake on the neuropeptides. More chronic effects of leptin on food intake and on hypothalamic NPY and CRH concentrations were determined in non-food-deprived lean and ob/ob mice killed 24 h after administration of vehicle or 60 pmol leptin in 2 uL. Mice were killed by decapitation between 1500 and 1700 h to avoid circadian variation.

Glucocorticoids might interact with leptin to help regulate body weight (Ur et al. 1996, Zakrzewska et al. 1997). Effects of leptin on hypothalamic NPY and CRH concentrations in ADX lean and ob/ob mice were thus examined. ADX mice were injected ICV with vehicle or leptin (60 pmol), food-deprived, and killed 3 h later (between 1500 and 1700 h). Measurements of neuropeptide concentrations in specific hypothalamic nuclei reflect the balance of peptide synthesis and transport/release/degradation rates. To obtain more direct measures of peptide secretion, hypothalamic preparations were incubated *in vitro*. *In vitro* hypothalamic secretion of NPY and CRH were compared in lean and ob/ob mice to see if the leptin deficiency in ob/ob mice leads to alterations in hypothalamic NPY and CRH secretion. Effects of leptin on *in vitro* hypothalamic NPY and CRH secretions were also examined in intact and ADX lean and ob/ob mice. Mice were killed between 1300 and 1400 h without food-deprivation.

ADX. Mice were ADX through dorsal incisions under ether anesthesia at 5 wk of age. Incisions were closed with suture clips. Dexamethasone sodium phosphate (40 umol/kg body weight) was given IP at surgery (Feldkircher et al. 1996). ADX mice were given free access to food and physiological saline (155 mmol NaCl / L water). Experiments

were performed 2 wk after surgery.

ICV injection. Mice were lightly anesthetized with ether before ICV injection. Leptin (60 pmol) in 2 μL saline was injected into the lateral ventricle as described previously (Walker and Romsos, 1992)

Blood sampling. Blood was collected, and plasma was separated and stored at - 20 °C for measurement of glucose and insulin.

Brain sectioning and hypothalamic micropunches. The brains were quickly removed from the skulls. A cut was made perpendicular to the midline of the mid-hind brain to prepare the tissue for mounting on the specimen holder. The brain was then immediately frozen on dry ice and stored at - 80 °C. The frozen brain was glued to a specimen holder and placed in a cryostat (Cryocut 1800, Leica Inc. Deerfield, IL) at - 10 °C. After about 30 min, the brain was repeatedly sectioned until the anterior commissure was clearly visible. From this reference point, serial sections of 400 to 500 μm were made according to the stereotaxic atlas of the albino mouse forebrain (Slotnick and Leonard, 1975). The brain sections were placed on glass slides and kept frozen on dry ice. Discrete hypothalamic nuclei were micropunched under a microscope by the technique of Palkovits (1973). Hypothalamic nuclei sampled included the ARC, PVN, VMH, and in selected trials the DMH (Slotnick and Leonard, 1975). A 20-gauge, oval- shaped needle was used to micropunch the ARC and PVN, and a round 24-gauge needle was used for the VMH and DMH. These regions were examined because leptin receptors are located within these regions of hypothalamus, and are also important sites for NPY and CRH action (Frankish et al. 1995, Krahn et al. 1988, Tartaglia et al. 1997).

Bilateral tissue samples were immediately placed in 100 μL of 0.1 mol/L HCL

containing the protease inhibitor aprotinin (90 KIU). The samples were sonicated for 15 s and centrifuged at 10,000 x g for 15 min at 4 °C. Supernatants were then lyophilized and stored at - 80 °C until measurement for NPY and CRH. Tissue pellets were dissolved in 200 μL of 0.1 mol/L NaOH and protein was determined by a BioRad DC protein kit.

Measurement of hypothalamic NPY and CRH release *in vitro*. After rapid removal of the brain from lean and ob/ob mice, the hypothalamus was dissected out along the posterior border of the optic chiasm, the anterior border of the mammillary bodies and the lateral hypothalamic sulci, to a depth of ~ 2 mm. A static incubation system was used to measure neuropeptide release (Kalra et al. 1992).

Krebs-Ringer bicarbonate (KRB) buffer supplemented with 10 mM N-2-hydroxylethyl-piperazine-N′-2-ethanesulfonic acid (HEPES), 1 g BSA / L, 5.5 mM glucose, 0.3 mM ascorbic acid, 30 mg bacitracin / L, and 270,000 KIU aprotinin / L buffer was made fresh and was gassed with 95 % O_2-5% CO_2 for 10 min. The pH was adjusted to 7.4. Dissected hypothalami were immediately placed into polystyrene tubes (12x75 mm, Becton Dickson Labware, Lincoln Park, NJ) containing 750 uL of ice-cold KRB incubation buffer (2 hypothalami/tube). Hypothalami were pre-incubated at 37 ° C with gentle shaking (30/oscillation/min) for 1 h, the medium was replaced at 30 min intervals. Hypothalami were then incubated for 1 h (with medium changed at 20 min intervals) for measurements of basal release of NPY and CRH , followed by depolarization with 50 mM KCl to maximize neropeptide release. The incubation was continued for an additional 20 min with 5 mM KCl to see if neuropeptide release induced by 50 mM KCl returned to the basal release level. To test effects of leptin on the secretion of NPY and CRH, hypothalami were incubated with 30 nM leptin for 40 min (with medium changes at 20

min intervals), after measurement of basal secretion for 1 h.

Assays. Plasma glucose was measured with a glucose oxidase-peroxidase kit (Sigma Chemical Inc., St. Louis, MO). Plasma insulin was measured as described by Kekow et al. (1988) with some modifications (Jang and Romsos, 1998). NPY and CRH were measured by competitive ELISAs described previously (Jang and Romsos, 1998).

Statistical analysis. Data were expressed as means " SE and were analyzed with SAS/STAT (V.6.11, SAS institute, Carry, NC) and SigmaStat (V 2.0, Jandel Scientific, San Rafael, CA). Differences were considered significant at $P < 0.05$.

D. RESULTS

Food intake and plasma insulin and glucose concentrations. Leptin-treated lean and ob/ob mice consumed ~ 60 % less food during a 1 h period, following a 24 h period of food-deprivation, than vehicle-treated mice (0.24 ± 0.03 versus 0.59 ± 0.05 g/h for leptin-treated versus control lean mice, n = 9, and 0.25 ± 0.02 versus 0.64 ± 0.03 g/h for leptin-treated versus control ob/ob mice, n = 8). Lean and ob/ob mice, 7-8 wk old, weighed 23 ± 0.4 g (n = 10) and 37 ± 2g (n = 10), respectively. Vehicle-treated lean mice (n = 10) consumed less food in a 24 h period than ob/ob mice (n = 8), 3.5 ± 0.2 and 5.4 ± 0.3 g/24 h, respectively. Administration of a single ICV dose of leptin markedly lowered food intake for the next 24 h to 2.2 ± 0.3 g/24 h (-38 %) in lean mice (n = 10) and to 1.5 ± 0.3 g/24 h (- 72 %) in ob/ob mice (n = 8).

Plasma insulin concentrations were higher in vehicle-treated ob/ob mice than their lean counterparts (**Tables 4 and 5**). Feeding mice for 1 h, following 24 h of food deprivation and ICV administration of either vehicle or leptin, increased plasma insulin and glucose

concentrations, although plasma insulin concentrations were not increased as much in leptin-treated as in vehicle-treated counterparts (**Table 4**). This effect of leptin on plasma insulin was secondary to a lowered food intake induced by leptin, since plasma insulin concentrations in vehicle-treated mice pair-fed to leptin-treated mice also increased less than in mice with free access to food (0.04 ± 0.01 and 0.44 ± 0.1 nmol/L in lean and ob/ob mice, respectively). Plasma insulin and glucose concentrations in ad libitum fed lean and ob/ob mice were unaffected by leptin 24 h post-injection (**Table 4**). ICV leptin administration in the absence of food also failed to influence plasma insulin 3 h post-injection either in intact or ADX lean and ob/ob mice (**Table 5**). ICV leptin lowered plasma glucose in food-deprived intact, but not in ADX, lean and ob/ob mice. ADX, as expected, lowered plasma insulin to a greater extent in ob/ob mice than in lean mice (**Table 5**). Plasma glucose concentrations, however, were unaffected by ADX.

Effects of ICV leptin administration on hypothalamic NPY and CRH concentrations of lean and ob/ob mice. Intact mice. NPY concentrations in the ARC and PVN of ob/ob mice were lower than in lean mice (**Table 6**). Consumption of food for 1 h lowered NPY concentrations by 36 % in the PVN of vehicle-treated lean mice, but not in ob/ob mice. Leptin administration failed to influence the concentrations of NPY in any of the specific hypothalamic regions of these intact lean and ob/ob mice 1h after refeeding. Twenty four h after leptin administration, NPY concentrations were lowered by 40 % only in the ARC of ob/ob mice, and tended to be lower in the ARC of lean mice (- 21 %, $P = 0.055$). Leptin administration also failed to influence NPY concentrations in any of the specific hypothalamic regions of intact lean and ob/ob mice examined even when food was not provided for a 3 h period, to avoid confounding effects of leptin-induced differences in

food intake on neuropeptide release (**Fig. 13, left panels**).

CRH concentrations in the hypothalamus of intact mice were not influenced by phenotype, food-deprivation, and refeeding, or by leptin treatment, with one exception (**Table 7 and Fig 14, left panels**). CRH concentrations in the VMH of vehicle-treated lean mice tended to increase during 1 h refeeding following 24 h fasting. Leptin-treated mice, however, did not increase CRH concentrations during 1 h refeeding, resulting in 56 % lower CRH concentration in the VMH region of these mice than in vehicle-treated, refed mice ($P < 0.05$, **Table 7**).

ADX mice. ADX significantly lowered NPY concentrations 35 – 47 % in the ARC, PVN, and VMH of ob/ob mice, and only in the DMH of lean mice (**Fig. 13**). ADX significantly elevated CRH concentrations 3 - 4 fold in the ARC of lean and ob/ob mice, but lowered CRH concentrations by 48 % in the VMH of ob/ob mice (**Fig. 14**). ICV leptin administration failed to influence NPY concentrations in any of the regions examined in ADX mice, except in the DMH of lean ADX mice where leptin administration increased the NPY concentration by 70 % (**Fig. 13**). Leptin also failed to change CRH concentrations in the PVN or VMH of ADX lean and ob/ob mice (**Fig. 14**). But, leptin markedly lowered CRH concentrations by 70 – 80 % within the ARC of ADX lean and ob/ob mice, in contrast to the ineffectiveness of leptin to affect CRH concentrations in similarly treated intact mice (**Fig. 14**).

***In vitro* hypothalamic NPY and CRH secretion of lean and ob/ob mice.** Hypothalamus weights did not differ between lean (24 ± 1 mg, n = 10) and ob/ob mice (23 ± 1 mg, n = 6). Likewise, phenotype did not influence hypothalamic NPY and CRH concentrations, 8 ± 1 and 7 ± 1 pmol NPY, and 234 ± 18 and 226 ± 20 fmol CRH, respectively, for lean (n

= 7) and ob/ob mice (n = 5). Release of NPY averaged 11 ± 1 (n = 7 incubations) and 10 ± 1 fmol/20 min/hypothalamus (n = 5 incubations) from lean and ob/ob mice, respectively (**Fig. 15**). Release of CRH averaged 17 ± 4 and 13 ± 3 fmol/20 min/hypothalamus from lean and ob/ob, respectively (**Fig. 15**). Secretion of NPY and CRH in response to 50 mM KCl (to depolarize the neurons) increased 2-3 fold in both lean and ob/ob mice, and returned to basal release when the KCl concentration was returned to 5 mM.

Effects of leptin on *in vitro* NPY and CRH secretion from the hypothalamus. Intact lean and ob/ob mice. Leptin (30 nM) failed to influence *in vitro* NPY secretion from the hypothalamus of intact lean mice (7 ± 1 versus 7 ± 1 fmol NPY released/hypothalamus/20 min in the absence and presence of leptin, respectively, n = 7 incubations) or intact ob/ob mice (13 ± 1 versus 12 ± 1 fmol NPY released/hypothalamus/20 min in the absence and presence of 30 nM leptin, respectively, n = 5 incubations)(**Fig. 16**). Leptin also failed to influence *in vitro* CRH secretion from the hypothalamus of intact lean mice (12 ± 1 versus 11 ± 1 fmol CRH released/hypothalamus/20 min in the absence and presence of 30 nM leptin, respectively, n = 4 - 6 incubations) or ob/ob mice (11 ± 1 versus 10 ± 1 fmol CRH released/hypothalamus/20 min in the absence and presence of 30 nM leptin, respectively, n = 5 incubations) (**Fig. 16**).

ADX lean mice. ADX significantly lowered hypothalamic NPY (9 ± 1 and 6 ± 1 pmol in intact and ADX lean mice, n = 10, respectively) and elevated CRH concentrations (389 ± 25 and 691 ± 63 fmol in intact and ADX mice, n = 10, respectively). ADX did not affect basal secretion of hypothalamic NPY (12 ± 1 and 11 ± 1 fmol NPY released / hypothalamus/20 min in intact and ADX mice, respectively, n = 5 incubations, **Fig. 17**). ADX, as expected (Suda et al. 1985), stimulated CRH release 2-fold from 7 ± 0.4 to 16 ±

1 fmol CRH released/hypothalamus/20 min (n = 5 incubations, $P < 0.05$, **Fig 17**).

As observed in Fig 16, NPY release from the hypothalamus of intact lean mice was not influenced by leptin administration (13 ± 1 and 11 ± 1 fmol NPY released /hypothalamus/20 min in the absence and presence of 30 nM leptin, n = 5 incubations, **Fig. 17**). Leptin (30 nM) administration, however, decreased NPY release from the hypothalamus of ADX lean mice by 27 % (from 11 ± 1 to 8 ± 1 fmol NPY released/hypothalamus/20 min, n = 5 incubations, $P< 0.05$, **Fig. 17**).

Leptin again failed to influence CRH secretion from the hypothalamus of intact lean mice (7 ± 0.4 versus 8 ± 1 fmol CRH released/hypothalamus/20 min in the absence and presence of leptin, respectively, n = 5 incubations, **Fig. 17**). Leptin administration, however, increased CRH secretion by 51 % from the hypothalamus of ADX lean mice (from 16 ± 1 to 25 ± 1 fmol CRH released/hypothalamus/20 min, n = 5 incubations, $P<0.05$, **Fig. 17**).

These leptin actions on NPY and CRH secretion from the hypothalamus of ADX mice occurred rapidly, within 20 min of incubation, and remained constant during 40 min of leptin treatment. These data suggest that leptin and glucocorticoids interact to influence the secretion of hypothalamic NPY and CRH.

TABLE 4. Plasma insulin and glucose concentrations in intact mice fed for 1 h or 24 h after ICV leptin administration

Groups	Insulin	Glucose
	nmol/L	mmol/L
1h post-injection		
Lean mice		
Vehicle-treated, food-deprived	0.03 ± 0.01	7 ± 1
Vehicle-treated, refed	0.22 ± 0.03 ^{R}	18 ± 1 ^{R}
Leptin-treated, refed	0.05 ± 0.01 ^{L}	18 ± 1
Ob/ob mice		
Vehicle-treated, food-deprived	0.19 ± 0.05	9 ± 1
Vehicle-treated, refed	2.47 ± 0.58 ^{R}	18 ± 2 ^{R}
Leptin-treated, refed	0.99 ± 0.17 ^{L}	16 ± 1
ANOVA	**P, P*T**	
24 h post-injection		
Lean mice		
Vehicle-treated	0.09 ± 0.02	12 ± 1
Leptin-treated	0.1 ± 0.03	12 ± 1
Ob/ob mice		
Vehicle-treated	1.65 ± 0.32	19 ± 2
Leptin-treated	1.73 ± 0.37	18 ± 1
ANOVA	**P**	**P**

Values are means ± SE for 6-9 mice. Mice were food-deprived for 24 h, injected ICV with vehicle or 60 pmol leptin, and refed for 1 h. Other fed mice were injected ICV with vehicle or leptin, and killed 24 h later. Comparisons of plasma insulin and glucose were performed by two-way ANOVA (2 x 3 factorial design for 1 h post-injection data, and 2 x 2 factorial design for 24 h post-injection data). P indicates significant effect of phenotype and T indicates significant effect of treatment (either refeeding or leptin administration) at $P< 0.05$. The LSD test was used for post-hoc comparisons with ^{R} indicating a significant effect of refeeding the vehicle-treated mice and ^{L} indicating a significant effect of leptin at $P< 0.05$.

TABLE 5. Plasma insulin and glucose concentrations in intact or ADX mice food-deprived for 3 h after ICV leptin administration

Groups	Insulin	Glucose
	nmol/L	mmol/L
Lean mice		
Intact		
Vehicle-treated	0.10 ± 0.03	12 ± 1
Leptin-treated	0.14 ± 0.03	10 ± 1
ADX		
Vehicle-treated	0.02 ± 0.002 ^{A}	12 ± 1
Leptin-treated	0.02 ± 0.001 ^{A}	11 ± 1
Ob/ob mice		
Intact		
Vehicle-treated	1.28 ± 0.49 ^{P}	16 ± 0.2 ^{P}
Leptin-treated	1.56 ± 0.49 ^{P}	9 ± 1 ^{L}
ADX		
Vehicle-treated	0.09 ± 0.03 $^{P, A}$	12 ± 0.3 ^{A}
Leptin-treated	0.10 ± 0.03 $^{P, A}$	11 ± 1 ^{A}
ANOVA	**P, A, P*A**	**L, L*A**

Values are means ± SE for 6-10 intact or ADX mice. Mice were ADX at 5 wk of age, and used 2 wk later. Intact or ADX lean and ob/ob mice were injected ICV with vehicle or leptin (60 pmol) and killed 3 h post-injection without food provided after injection. Comparisons of plasma insulin and glucose concentrations were performed by three-way ANOVA. P indicates significant effect of phenotype; A indicates significant effect of ADX; L indicates significant effects of leptin administration at $P < 0.05$.

TABLE 6. Hypothalamic NPY concentrations in intact lean and ob/ob mice fed for 1 h or 24 h after ICV leptin administration

	Hypothalamic regions		
Groups	ARC	PVN	VMH
1 h post-injection			
Lean mice			
Vehicle-treated, food-deprived	48 ± 4	55 ± 5	6 ± 1
Vehicle-treated, refed	38 ± 7	35 ± 3 ^{R}	8 ± 1
Leptin-treated, refed	41 ± 4	36 ± 3	10 ± 2
Ob/ob mice			
Vehicle-treated, food-deprived	24 ± 6	21 ± 2	11 ± 1
Vehicle-treated, refed	28 ± 3	30 ± 4	12 ± 2
Leptin-treated, refed	32 ± 3	23 ± 4	12 ± 2
ANOVA	**P**	**P, P*T**	
24 h post-injection			
Lean mice			
Vehicle-treated	39 ± 3	45 ± 4	8 ± 2
Leptin-treated	30 ± 4	48 ± 4	10 ± 2
Ob/ob mice			
Vehicle-treated	33 ± 4	24 ± 4	10 ± 2
Leptin-treated	20 ± 2 ^{L}	32 ± 4	8 ± 1
ANOVA	**P, L**	**P**	

Neuropeptide Y (NPY) concentration (fmol NPY / μg particulate protein) of each region represents mean ± SE of 6 - 10 mice. Mice were food-deprived for 24 h, injected ICV with vehicle or 60 pmol leptin, and refed for 1 h. Some vehicle-injected mice were food-deprived for 1 h post-injection to serve as a control to vehicle-injected, refed mice. Other ad libitum fed mice were injected with vehicle or leptin and killed 24 h later. Mice were killed by decapitation between 15:00 and 17:00. Abbreviations of hypothalamic nuclei punched are: ARC, arcuate nucleus; PVN, paraventricular nucleus; and VMH, ventromedial nucleus. Comparisons of NPY concentrations were performed by two-way ANOVA (2 x 3 factorial design for 1 h post-injection data, and 2 x 2 factorial design for 24 h post-injection data). P indicates significant effect of phenotype and T indicates significant effect of treatment (either refeeding or leptin administration) at $P< 0.05$. The LSD test was used for post-hoc comparisons with ^{R} indicating a significant effect of refeeding the vehicle-treated mice and ^{L} indicating a significant effect of leptin at $P< 0.05$.

TABLE 7. Hypothalamic CRH concentrations in intact lean and ob/ob mice fed for 1 h or 24 h after ICV leptin administration

	Hypothalamic regions		
Groups	ARC	PVN	VMH
1 h post-injection			
Lean mice			
Vehicle-treated, food-deprived	1.9 ± 0.6	0.7 ± 0.2	0.7 ± 0.1
Vehicle-treated, refed	3.8 ± 1.6	0.7 ± 0.1	1.5 ± 0.6
Leptin-treated, refed	4.2 ± 0.8	0.8 ± 0.2	0.7 ± 0.1^{L}
Ob/ob mice			
Vehicle-treated, food-deprived	5.9 ± 1.4^{P}	0.9 ± 0.2	1.1 ± 0.2
Vehicle-treated, refed	4.3 ± 1.1	0.7 ± 0.2	0.8 ± 0.2
Leptin-treated, refed	5.4 ± 0.7	0.4 ± 0.1	0.6 ± 0.1
ANOVA	**P**		
24 h post-injection			
Lean mice			
Vehicle-treated	2.6 ± 1.0	0.5 ± 0.1	0.4 ± 0.04
Leptin-treated	5.3 ± 1.3	0.4 ± 0.04	0.4 ± 0.02
Ob/ob mice			
Vehicle-treated	2.5 ± 0.8	0.4 ± 0.1	0.3 ± 0.04
Leptin-treated	2.4 ± 0.6	0.6 ± 0.2	0.4 ± 0.1

Corticotropin-releasing hormone(CRH) concentration (fmol CRH / μg particulate protein) of each region represents mean ± SE of 6 - 10 mice. Values are means ± SE of 6 - 10 mice. Mice were food-deprived for 24 h, injected ICV with vehicle or 60 pmol leptin, and refed for 1 h. Some vehicle-injected mice were food-deprived for 1 h post-injection to serve as a control to vehicle-injected, refed mice. Other ad libitum fed mice were injected with vehicle or leptin and killed 24 h later. Mice were killed by decapitation between 15:00 and 17:00. See Table 5 for abbreviations. Comparisons of CRH concentrations were performed by two-way ANOVA (2 x 3 factorial design for 1 h post-injection data, and 2 x 2 factorial design for 24 h post-injection data). P indicates significant effect of phenotype at $P< 0.05$. The LSD test was used for post-hoc comparisons with ^{L} indicating a significant effect of leptin at $P< 0.05$.

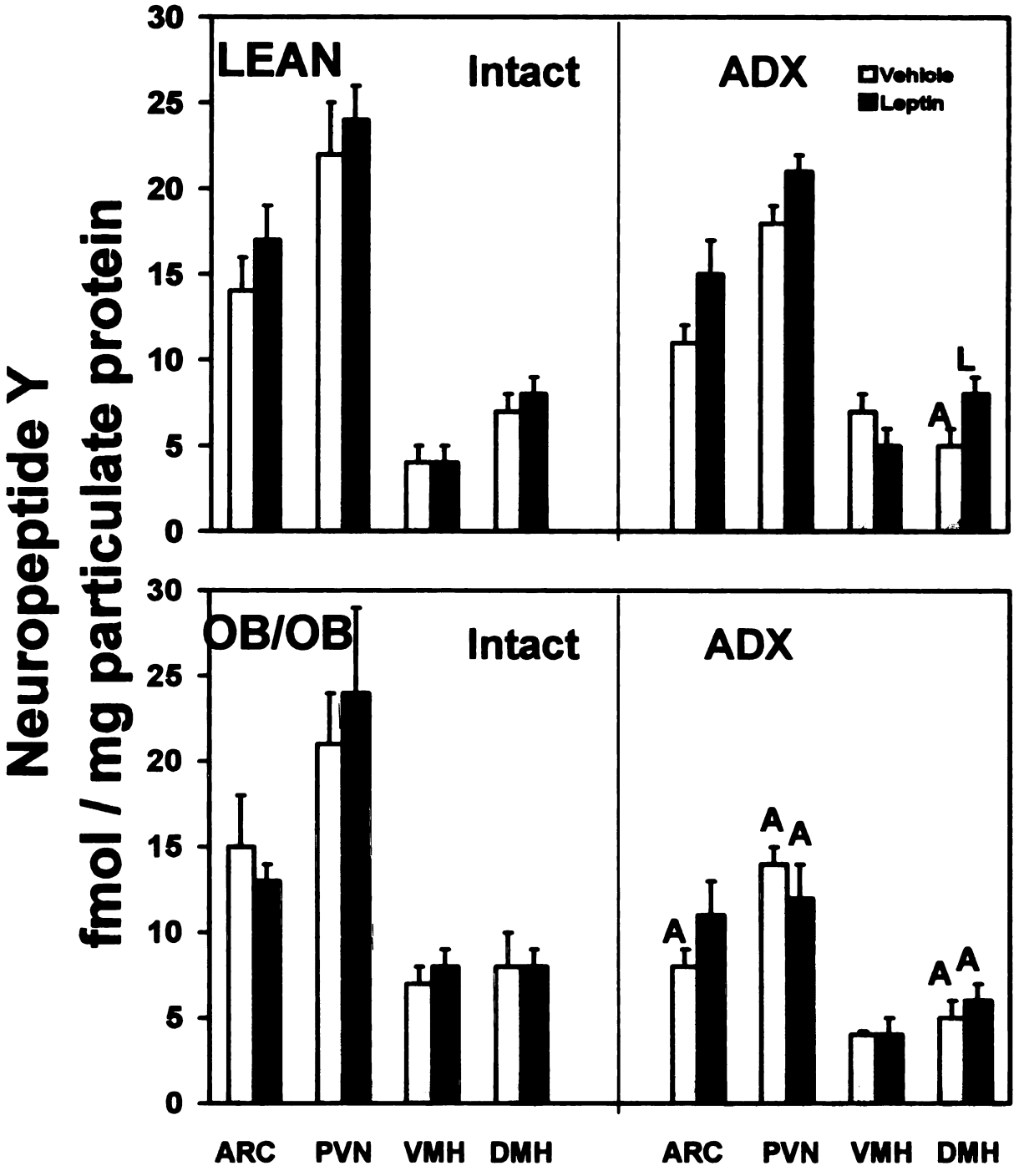

FIGURE 13. Effects of icv leptin administration on NPY concentrations in the hypothalamus of intact or ADX lean and ob/ob mice. Values are means ± SE for 6-10 mice. Mice were ADX at 5 wk of age and used at 7 wk of age. Mice were injected icv with vehicle or leptin (60 pmol) and killed 3 h post-injection, without access to food. Comparisons of NPY concentrations were performed by three-way ANOVA. P indicating a significant effect of phenotype; A indicating a significant effect of ADX; and L indicating a significant effects of leptin administration. Significant effects were: ARC, P, A; PVN, P, A; VMH, P*A; and DMH, A at $P < 0.05$.

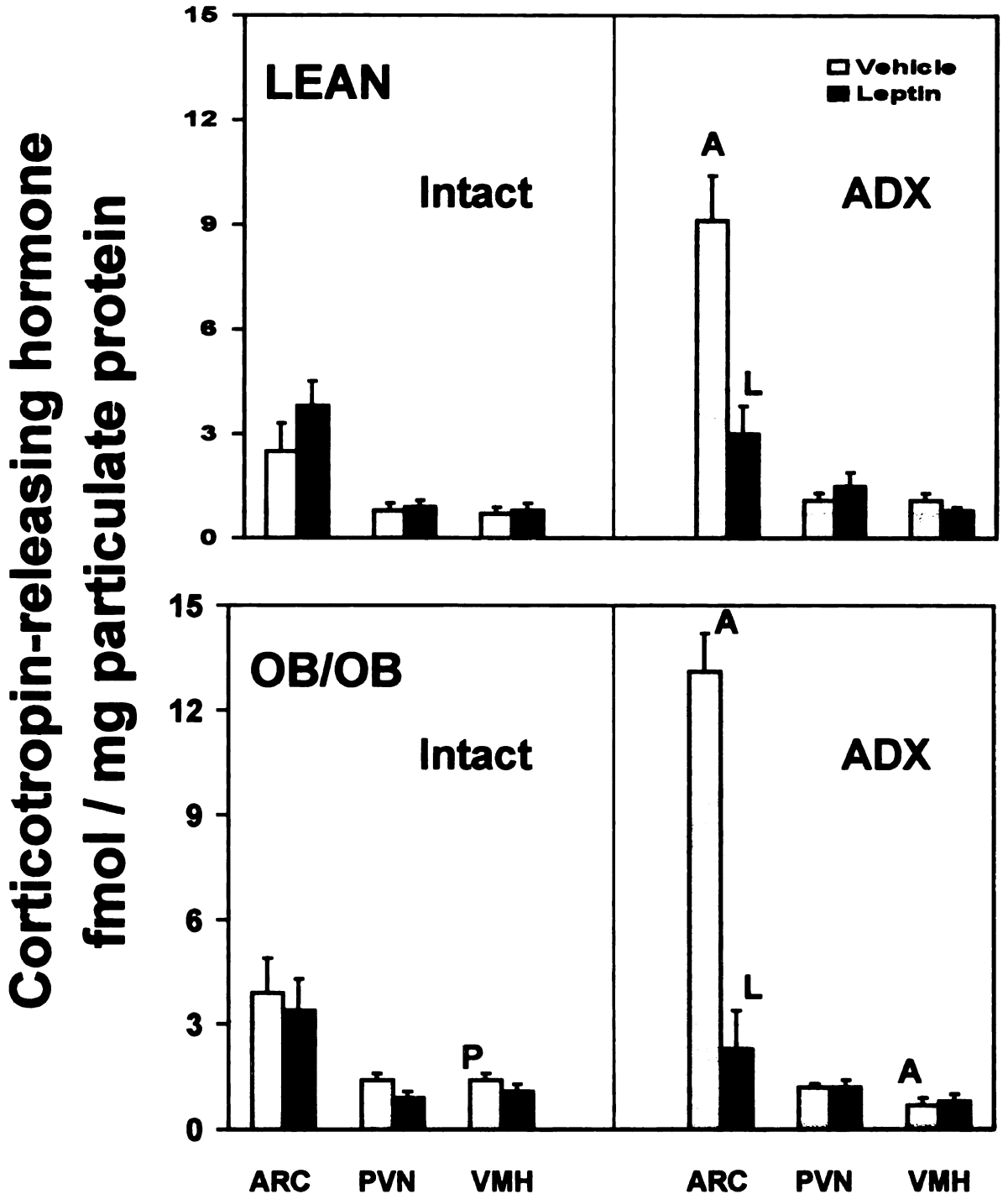

FIGURE 14. Effects of icv leptin administration on CRH in hypothalamus of intact or ADX lean and ob/ob mice. Values are means ± SE for 6 –10 mice. Mice were ADX at 5 wk of age and used at 7 wk of age. Mice were injected icv with vehicle or leptin (60 pmol) and killed 3 h post-injection without access to food. Comparisons of CRH concentrations were performed by three-way ANOVA. A indicating a significant effect ADX; L indicating a significant effect of leptin administration. Significant effects were ARC, A, L, P*L, A*L; VMH, P*A at $P < 0.05$.

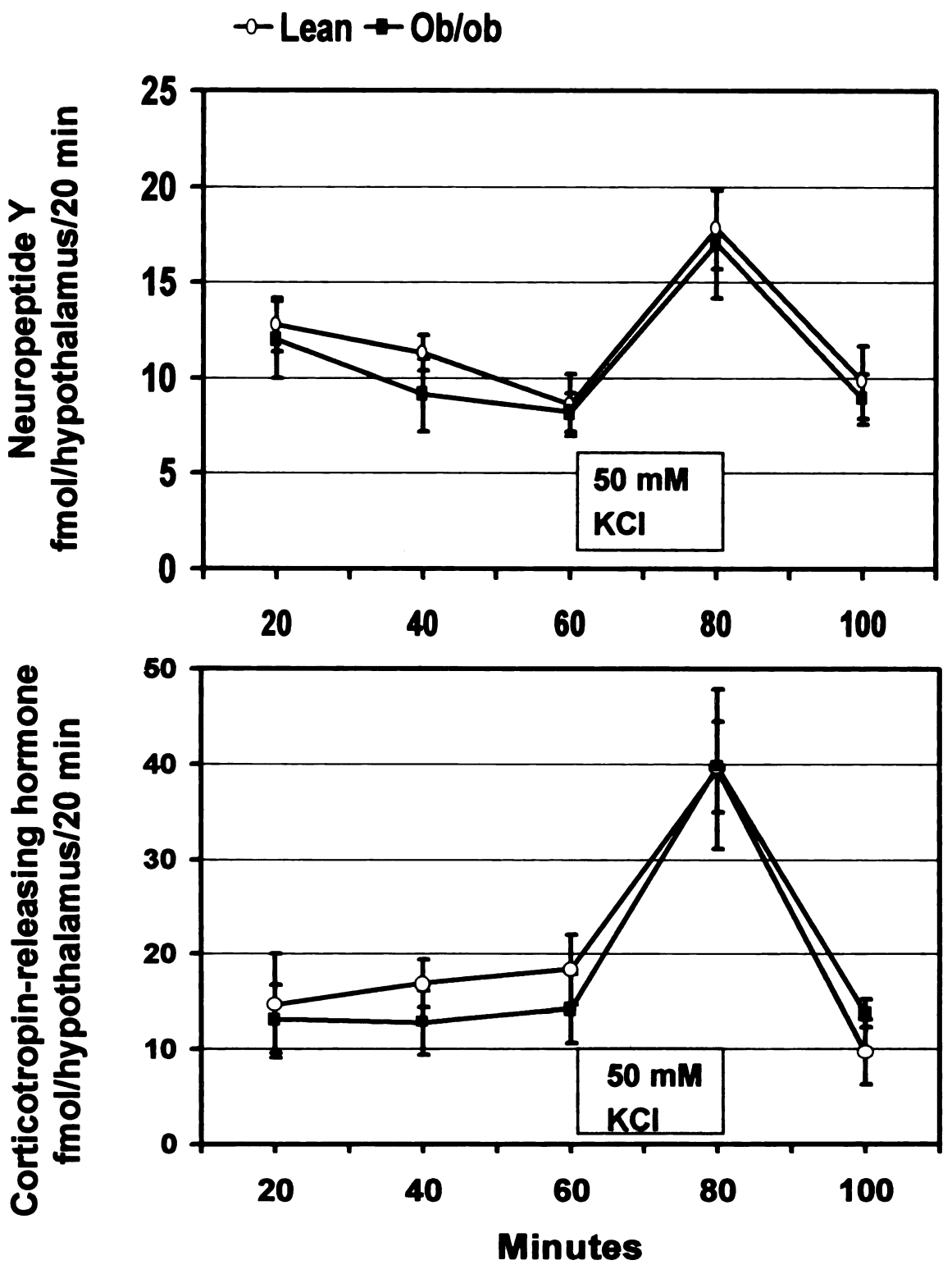

FIGURE 15. *In vitro* release of NPY and CRH from the hypothalamus of lean and ob/ob mice. Data points represent NPY or CRH released per 20 min-period per hypothalamus, and are means ± SE of 5 incubations with two hypothalami blocks per chamber. Exposure of the tissue to 50 mM KCl significantly increased NPY or CRH release versus the average basal release as determined by one way repeated measures analysis of variance (ANOVA) with Bonferroni test as post hoc comparisons ($P < 0.05$).

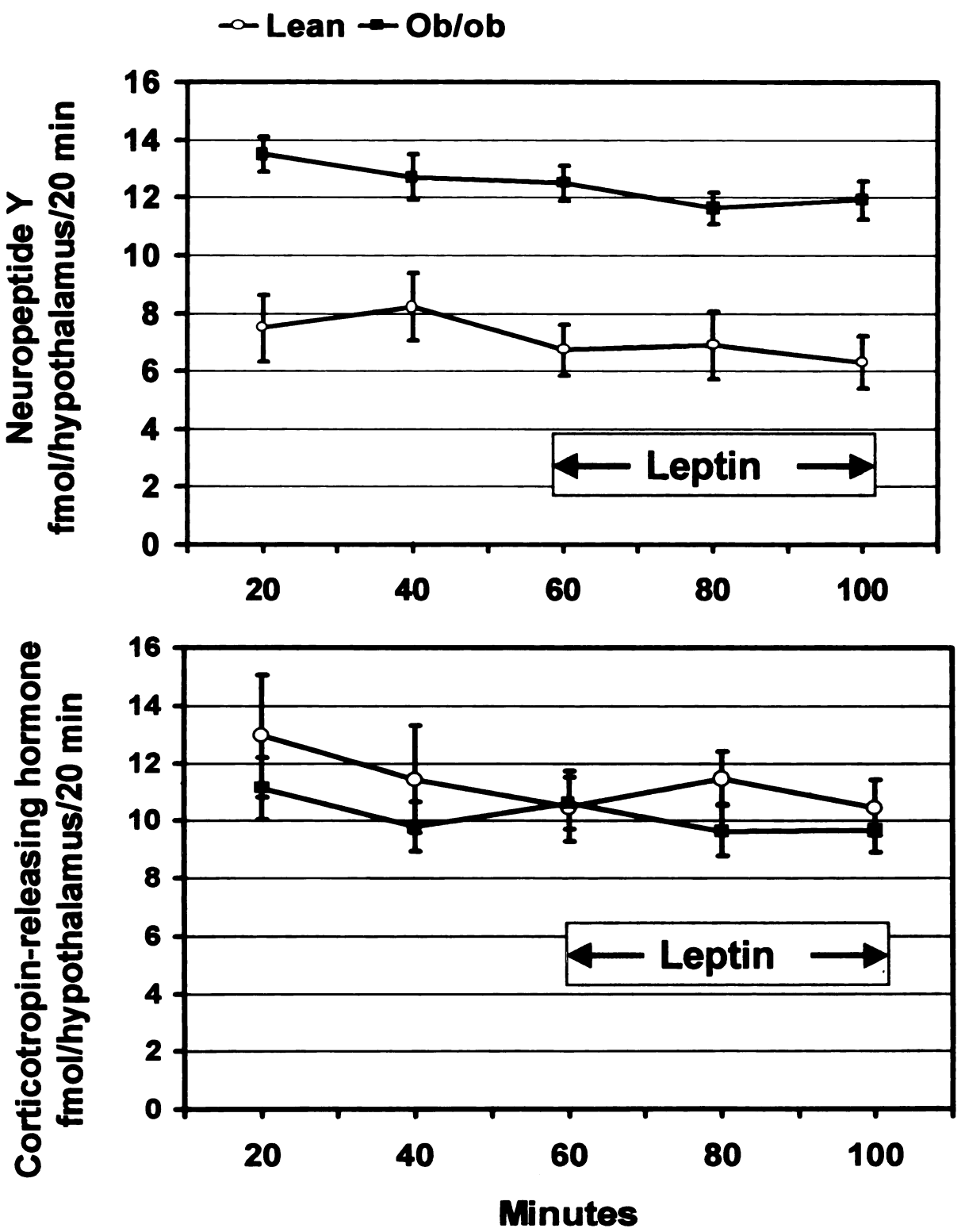

FIGURE 16. Effects of leptin on *in vitro* release of NPY or CRH from hypothalamic blocks of lean and ob/ob mice. Data points represent NPY or CRH released per 20 min-period, and are means ± SE of 4-7 incubations with two hypothalamic blocks per chamber. No significant leptin effects on secretion of either NPY or CRH as determined by one way repeated measures ANOVA with Bonferroni test as post hoc comparisons ($P < 0.05$). Lean and ob/ob mice preparations were obtained on different days, and thus comparisons between lean and ob/ob mice were not made.

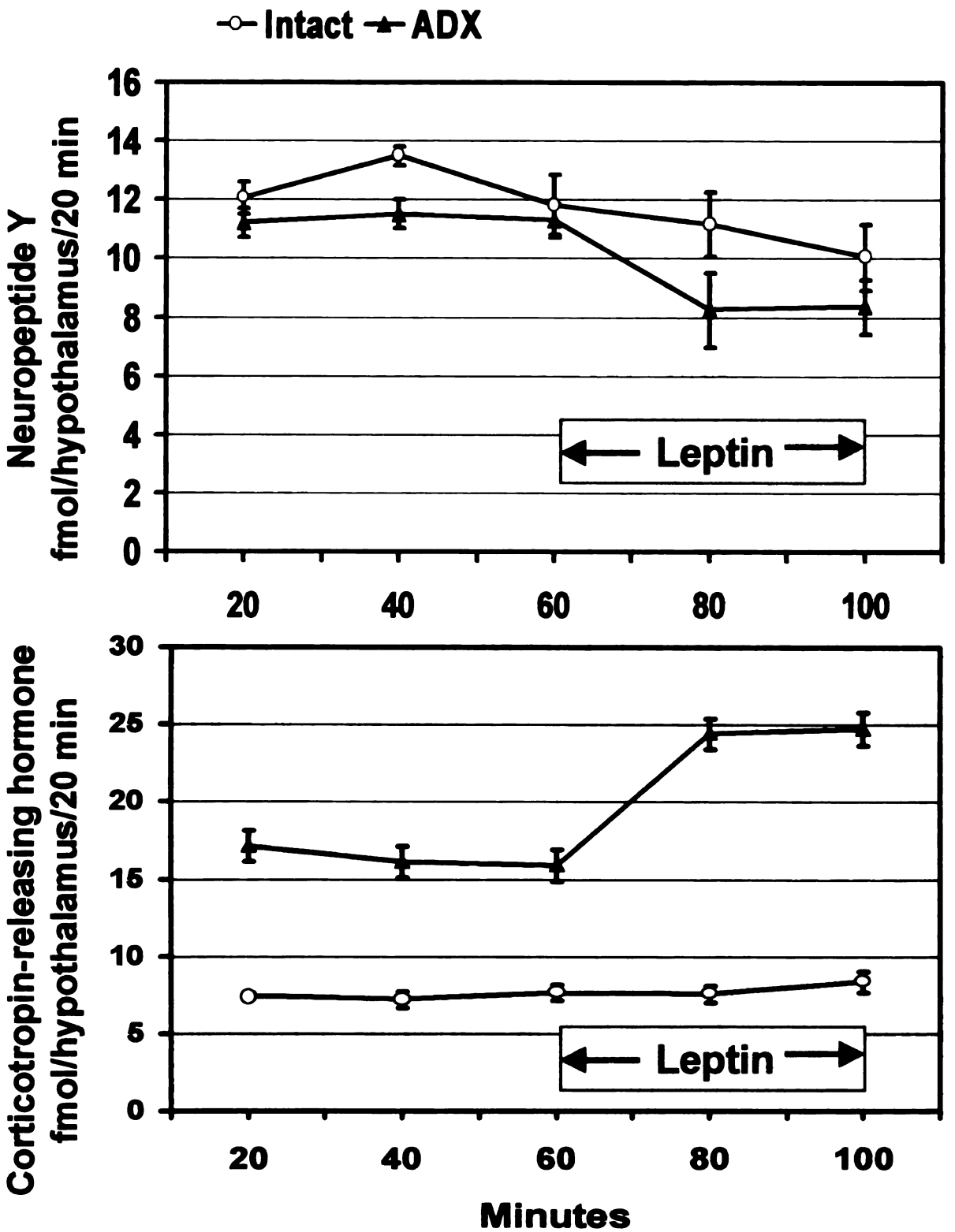

FIGURE 17. Effects of leptin on *in vitro* release of NPY or CRH from hypothalamic blocks of intact and ADX lean mice. Data points represent NPY or CRH released per 20 min per hypothalamus, and are means ± SE of 5 incubations with two hypothalamic blocks per chamber. There was a significant decrease in NPY release and increase in CRH release after leptin treatment as determined by one way repeated measures ANOVA with Bonferroni test as post hoc comparisons ($P < 0.05$).

E. DISCUSSION

Results from the present study may be summarized as follows. First, leptin administration did not rapidly, i.e. within 1-3 h, influence NPY and CRH concentrations in specific hypothalamic regions of intact mice, or *in vitro* secretion of NPY or CRH from hypothalamic preparations from intact mice. Second, regulation of hypothalamic NPY and CRH secretion was not inherently altered in leptin-deficient ob/ob mice. And third, in the absence of glucocorticoids, leptin rapidly (i.e. within 20 min) decreased NPY secretion and increased CRH secretion.

Mounting evidence suggests that leptin exerts very rapid actions within the hypothalamus, in addition to its slower genomic actions (Banks et al. 1996, Elmquist et al. 1997, Powis et al. 1998, Spanswick et al. 1997). We hypothesized that the rapid actions of leptin on food intake in rats and mice were associated with an inhibition of NPY release and/or a stimulation of CRH release within the hypothalamus. Our measurements of changes in NPY and CRH concentrations in specific hypothalamic nuclei within 1-3 h after leptin administration were used as an index of changes in transport/release of these neuropeptides, assuming that minimal effects of leptin on protein synthesis would be evident within this time frame. No rapid onset effects of leptin on hypothalamic NPY and/or CRH concentrations were observed in intact mice, consistent with the ineffectiveness of leptin to alter *in vitro* NPY and CRH secretions from hypothalamic preparations from these intact mice (**Table 6 and 7, and Fig. 13, 14 and 16**).

The possibility that the presence of endogenous leptin in lean mice masked the ability of exogenous leptin treatment to rapidly alter NPY or CRH secretion was tested by administering leptin to leptin-deficient ob/ob mice, and by adding leptin to hypothalamic

preparations from ob/ob mice. Acute leptin administration did not influence NPY and CRH concentrations within specific hypothalamic nuclei of intact ob/ob mice (**Table 6 and 7, and Fig. 13 and 14**). Likewise, leptin did not affect secretion of NPY or CRH from hypothalamic preparations from intact ob/ob mice (**Fig. 16**). Longer term exposure to leptin (i.e. 24 h) did lower NPY concentrations in the ARC, a site of NPY synthesis, of intact ob/ob mice (**Table 6**), suggesting a decrease in NPY synthesis in this region secondary to a lowering of NPY mRNA after longer term exposure to leptin (Sahu et al. 1998, Schwartz et al. 1996b, Stephens et al. 1995). Thus, even though it was possible to detect changes in NPY concentrations within specific hypothalamic nuclei after longer term (i.e. 24 h) exposure to leptin, we did not obtain any evidence for acute, leptin-induced effects on neuropeptide secretion. Possibly, measurements of NPY and CRH concentrations in specific hypothalamic regions after acute leptin administration were not sensitive enough to detect subtle changes in neuropeptide secretion/transport. Baskin et al. (1999) have, for example, shown that not all NPY containing neuron with the ARC have detectable leptin receptor mRNA. It is also possible that *in vitro* measurements using the whole hypothalamus may have masked regional differences in neuropeptide secretion/transport. Alternatively, leptin-induced suppression of food intake in these intact mice may have occurred without direct effects on hypothalamic release of NPY or CRH.

Glucocorticoids might oppose leptin actions (Ur et al. 1996, Zakrzewska et al. 1997). This suggests that leptin-induced effects on neuropeptide secretion, if evident, would be more likely detected in ADX mice than in intact mice. Indeed, leptin administration markedly reduced ARC CRH concentrations in ADX mice (**Fig. 14**). This rapid reduction in CRH concentration (i.e. within 3 h) in the ARC after leptin administration is more likely

due to stimulated CRH release from this region rather than due to decreased synthesis/transport from the PVN, a site of synthesis, because leptin stimulates, not depress, CRH synthesis in the PVN (Schwartz et al. 1996a). Within 20 min after exposure to leptin, NPY secretion from hypothalamic preparations from ADX mice was lowered and CRH secretion was elevated (**Fig. 17**). These hypothalamic responses to leptin are consistent with the more dramatic effects of leptin on food intake in ADX rats and mice than in intact ones (Mistry et al. 1997, Zakrzewska et al. 1997).

Our studies with intact mice were conducted between 1500 and 1700 h, a time of day when corticosterone concentrations are high (Leibowitz, 1992), and mice are consuming food. Based on the results of our studies with ADX mice, it would be interesting to examine effects of leptin on NPY and CRH secretion in intact mice at a time of the day when plasma corticosterone concentrations would be low (i.e. shortly after the lights are turned on in the morning).

The mechanisms whereby leptin causes rapid changes in NPY and CRH secretion are not understood yet. Leptin has been reported to affect membrane potential and/or ion channels (Powis et al. 1998, Spanswick et al. 1997), cAMP concentrations via stimulation of phosphodisterase 3 B (Zhao et al. 1998), nitric oxide generation (Yu et al. 1997), and PKC activity (Chen et al. 1997). Changes in membrane potential/ion channels, cAMP, nitric oxide and PKC also influence the secretion of NPY and CRH. These signal transducers are thus potential candidates to mediate leptin-induced changes in neuropeptide secretion (**Fig. 5**).

The present study demonstrates that leptin exerts rapid onset actions on the hypothalamic NPY and CRH neuroendocrine system, and that these actions of leptin

maybe restrained by the presence of endogenous corticosterone. Other neuronal feeding-regulatory factors including α-MSH and AGRP present within the hypothalamus are also reported to be influenced by leptin (Flier and Maratos-Flier, 1998, Satoh et al. 1998, Wilson et al. 1999). It will be important to consider both the rapid onset signal transduction actions of leptin as well as the longer term effects of leptin on gene transcription to fully understand the role for leptin in regulation of body energy balance.

CHAPTER V. LEPTIN AND GLUCOCORTICOIDS EXERT OPPOSING ACTIONS ON HYPOTHALAMIC NEUROPEPTIDE Y AND CORTICOTROPIN-RELEASING HORMONE SECRETION WITHIN THE HYPOTHALAMUS

A. ABSTRACT

Leptin and glucocorticoids exert major counter-regulatory effects on control of body fat balance. NPY, a stimulator of food intake and/or CRH, an inhibitor of food intake, are candidates for mediating the counter-regulatory effects of leptin and corticosterone within the hypothalamus. The present study was conducted to determine acute effects of leptin and glucocorticoids on hypothalamic NPY and CRH secretion *in vitro*. ADX mice were used to remove endogenous corticosterone. Addition of 30 nM leptin to the hypothalamic preparations decreased NPY secretion by 30 % and increased CRH secretion by 35 % within 20 min. Effects of 0.5 uM DEX on hypothalamic NPY (+ 62 %) and CRH (- 46 %) secretion were opposite those of leptin. To further investigate interactions between leptin and DEX in the regulation of NPY and CRH secretion, hypothalamic blocks were incubated with DEX alone for 20 min, prior to co-incubation with leptin. DEX alone increased NPY secretion and decreased CRH secretion. Addition of leptin following exposure of the tissue to DEX for 20 min failed to further change either NPY or CRH secretion. Leptin and glucocorticoids exert opposing actions on secretion of hypothalamic NPY and CRH. The presence of glucocorticoids may restrain actions of leptin on secretion of these neuropeptides within the hypothalamus.

B. INTRODUCTION

Leptin and glucocorticoids exert major counter-regulatory effects on control of body fat balance. These actions are clearly illustrated in leptin-deficient ob/ob mice where the presence of endogenous corticosterone leads to massive obesity. Removal of endogenous corticosterone from these mice by ADX to create leptin/corticosterone-deficient mice, or administration of leptin to intact ob/ob mice to create leptin/corticosterone-sufficient mice, restores their body composition to near normal (Bray 1991, Muzzin et al. 1996, Pelleymounter et al. 1995, Saito and Bray, 1994). NPY, a stimulator of food intake and/or CRH, an inhibitor of food intake, are candidates for mediating the counter-regulatory effects of leptin and corticosterone within the hypothalamus (Baskin et al. 1999, Luo et al. 1995, Schwartz et al. 1996a and 1996b, Wang et al. 1997, White et al. 1990).

Leptin and glucocorticoids, via their respective receptor-signal transduction pathways alter gene expression (Ahima et al. 1992, Baumann et al. 1996, Beato 1989, Vaisse et al. 1996). This provides one mechanism where leptin and glucocorticoids are able to directly or indirectly modulate NPY and CRH, as well as other neuropeptides, to control body fat balance. But some of the actions of administered leptin and glucocorticoids on food intake are so rapid in onset, i.e. within 30 – 60 min (Chen and Romsos, 1995, Rentsch et al. 1995, Seeley et al. 1996, Walker and Romsos 1992), that mechanisms other than altered gene expression are also likely involved. Leptin is able to rapidly, within seconds, depolarize rat PVN neurons and activate ATP-sensitive potassium channels within the hypothalamus (Powis et al. 1998, Spanswick et al. 1997). Rapid-onset, i.e. within

seconds, effects of glucocorticoids on ion channels and calcium influx have also been reported (Lou and Chen 1998, Qiu et al. 1998, Shipston et al. 1995, Tian et al. 1998). Leptin or glucocorticoids might thus mediate rapid-onset changes in neurotransmitter release to influence food intake and metabolic rate.

A synthetic glucocorticoid DEX rapidly (i.e. within 20 min) inhibits CRH release from hypothalamic blocks from ADX rats and mice, and blocks acetylcholine or norepinephrine-stimulated CRH release (Calogero et al. 1988, Suda et al. 1985). Glucocorticoids also rapidly, within 20 min, increase NPY secretion from hypothalamic preparations from ADX ob/ob mice (Chen and Romsos, 1995). Leptin has been reported in some studies to rapidly stimulate CRH release and to inhibit NPY release (Costa et al. 1997, Raber et al. 1997, Stephens et al. 1995), but not in other studies (Beck et al. 1998, Heiman et al. 1997). We observed that leptin rapidly decreased NPY secretion and stimulated CRH secretion from hypothalamic preparations of ADX mice but not from preparations from intact mice. These studies indicate that glucocorticoids and leptin may interact as they rapidly modulate NPY and CRH secretion within the hypothalamus. ADX mice were thus used in the present study to avoid possible confounding effects of endogenous glucocorticoids on NPY and/or CRH secretion. Acute effects of leptin and glucocorticoids on secretion of NPY and/or CRH from hypothalamic preparations were examined.

C. MATERIALS AND METHODS

Animals. Male lean mice (ob/+ or +/+) were obtained from our breeding colony of C57BL/6J ob/+ mice. The Guide for the Care and Use of Laboratory Animals (NRC

1985) and local institutional guidelines were followed for the care and treatment of the mice. Mice were weaned at 3 - 3.5 wk of age, group-housed (5 mice/cage) in solid-bottom plastic cages with wood shavings for bedding and fed a nonpurified diet (Teklad Rodent Diet 8640; Harlan, Bartonville, IL). Room temperature was 23 - 25 °C and lights were on from 07:00 to 19:00 h. Mice were ADX through dorsal incisions under ether anesthesia at 5 wk of age. Incisions were closed with suture clips. ADX mice were given free access to food and physiological saline (9 g NaCl / L water). Mice were killed at 7 – 8 wk of age.

Measurement of hypothalamic NPY and CRH release *in vitro*. After rapid removal of the brain from lean and ob/ob mice at ~ 1300 h, the hypothalamus was dissected out along the posterior border of the optic chiasm, the anterior border of the mammillary bodies and the lateral hypothalamic sulci, to a depth of ~ 2 mm. A static incubation system was used to measure neuropeptide release (Kalra et al. 1992).

Krebs-Ringer bicarbonate (KRB) buffer supplemented with 10 mM N-2-hydroxylethyl-piperazine-N′-2-ethanesulfonic acid (HEPES), 1 g BSA / L, 5.5 mM glucose, 0.3 mM ascorbic acid, 30 mg bacitracin / L, and 270,000 KIU aprotinin / L buffer was made fresh and was gassed with 95 % O_2-5% CO_2 for 10 min. The pH was adjusted to 7.4. Dissected hypothalami were immediately placed into polystyrene tubes (12x75 mm, Becton Dickson Labware, Lincoln Park, NJ) containing 750 uL of ice-cold KRB incubation buffer (2 hypothalami/tube). Hypothalami were pre-incubated at 37 ° C with gentle shaking (30/oscillation/min) for 1 h, the medium was replaced at 30 min intervals. Hypothalami were then incubated for 1 h for measurements of basal secretion of NPY and CRH. The incubation was continued for an additional 40 min (2 incubations of 20 min)

with or without test substances, (i.e. 30 nM leptin or 0.5 uM DEX or leptin and DEX together). To test possible antiregulatory actions of glucocorticoids on leptin action, hypothalami exposed to 0.5 uM DEX for 20 min were subsequently co-incubated with 0.5 uM DEX and 30 nM leptin for 40 min. Media samples were stored at – 80 °C until measurement of NPY and CRH by competitive ELISA as described previously (Jang and Romsos, 1998).

Statistics. Data are expressed as mean ± SE and were analyzed with SigmaStat (V. 2.0, Jandel Scientific, San Rafael, CA). Hypothalamic NPY and CRH secretion after administration of leptin, DEX or leptin and DEX together, were compared with basal NPY or CRH release using paired Student's t-test. Effects of DEX pre-treatment followed by leptin and DEX co-administration were analyzed by one way repeated measures analysis of variance (ANOVA) with the Bonferroni test used in post hoc comparisons. Differences were considered significant at $P < 0.05$.

D. RESULTS

Leptin lowered NPY release and elevated CRH release from hypothalamic blocks from ADX mice. Basal release of NPY (49 ± 2 pg/hypothalamus/20 min) and CRH (79 ± 3 pg/hypothalamus/20 min) from the hypothalamus of ADX mice remained constant for 1 h (**Fig. 18**). Addition of 30 nM leptin decreased NPY secretion by 30 % (to 34 ± 4 pg/hypothalamus/20 min, $P < 0.05$) and increased CRH secretion by 35 % (to 106 ± 6 pg/hypothalamus/20 min, $P < 0.05$) (**Fig. 18**). These actions of leptin in hypothalamic preparations from ADX mice were rapid-onset, i.e. within the first 20 min, and were sustained for the 40 min of incubation. However, when hypothalamic blocks were

obtained from intact mice, leptin failed to influence either NPY release (32 ± 4 versus 28 ± 4 pg NPY released/hypothalamus/20 min in the absence and presence of leptin, respectively, n = 7 incubations) or CRH release (55 ± 7 versus 52 ± 4 pg CRH released/hypothalamus/20 min in the absence and presence of 30 nM leptin, respectively, n = 4 - 6 incubations).

DEX elevated NPY release and lowered CRH release from hypothalamic blocks from ADX mice. Effects of DEX on NPY and CRH secretion from the hypothalamus of ADX lean mice were opposite those of leptin. Addition of 0.5 uM DEX increased NPY secretion by 62 %, from 30 ± 4 to 47 ± 4 pg/hypothalamus/20 min ($P < 0.05$) and lowered CRH secretion by 46 %, from 81 ± 7 to 43 ± 4 pg/hypothalamus/20 min ($P < 0.05$) (**Fig. 19**). These actions of DEX were also rapid-onset, i.e. within 20 min, and were sustained for the 40 min of incubation. Consistent with the failure of leptin to influence NPY or CRH secretion from hypothalamic preparations from intact mice in the present study, DEX (0.5 uM) also did not influence secretion of NPY (from 32 ± 4 in the absence of DEX to 29 ± 4 pg/hypothalamus/20 min in the presence of DEX, n = 5 – 6 incubations) or CRH (from 43 ± 7 in the absence of DEX to 51 ± 7 pg/hypothalamus/20 min in the presence of DEX, n = 5 – 6 incubations) from the hypothalamic blocks of intact mice.

Effects of Leptin and DEX co-incubation on hypothalamic NPY and CRH. Simultaneous addition of leptin and DEX to the hypothalamic preparations decreased NPY secretion by 52 % ($P < 0.05$) from 49 ± 3 to 23 ± 2 pg/hypothalamus/20 min (**Fig. 20**). This response was similar to the effects of leptin alone on NPY secretion (**Fig. 18**), and suggests that leptin actions predominate when the hypothalamus is simultaneously exposed to leptin and DEX. But a different response occurred when CRH secretion was

examined. Simultaneous administration of leptin and DEX negated effects of either leptin or DEX on CRH secretion (**Fig. 20**). CRH secretion averaged 76 ± 4 and 70 ± 3 pg/hypothalamus/20 min in the absence and simultaneous presence of leptin and DEX, respectively ($P > 0.05$).

Effects of DEX alone followed by leptin and DEX co-incubation on hypothalamic NPY and CRH. To further investigate possible interactions of DEX and leptin in the control of NPY and CRH secretion, hypothalami were incubated with DEX alone for 20 min, prior to co-incubation with leptin for 40 min. As observed in Fig. 19, DEX alone increased NPY secretion by 40 % ($P < 0.05$) and decreased CRH secretion by 48 % ($P<0.05$) within 20 min (**Fig. 21**). Addition of leptin after prior exposure to DEX failed to further change either NPY or CRH secretion ($P > 0.05$) (**Fig. 21**).

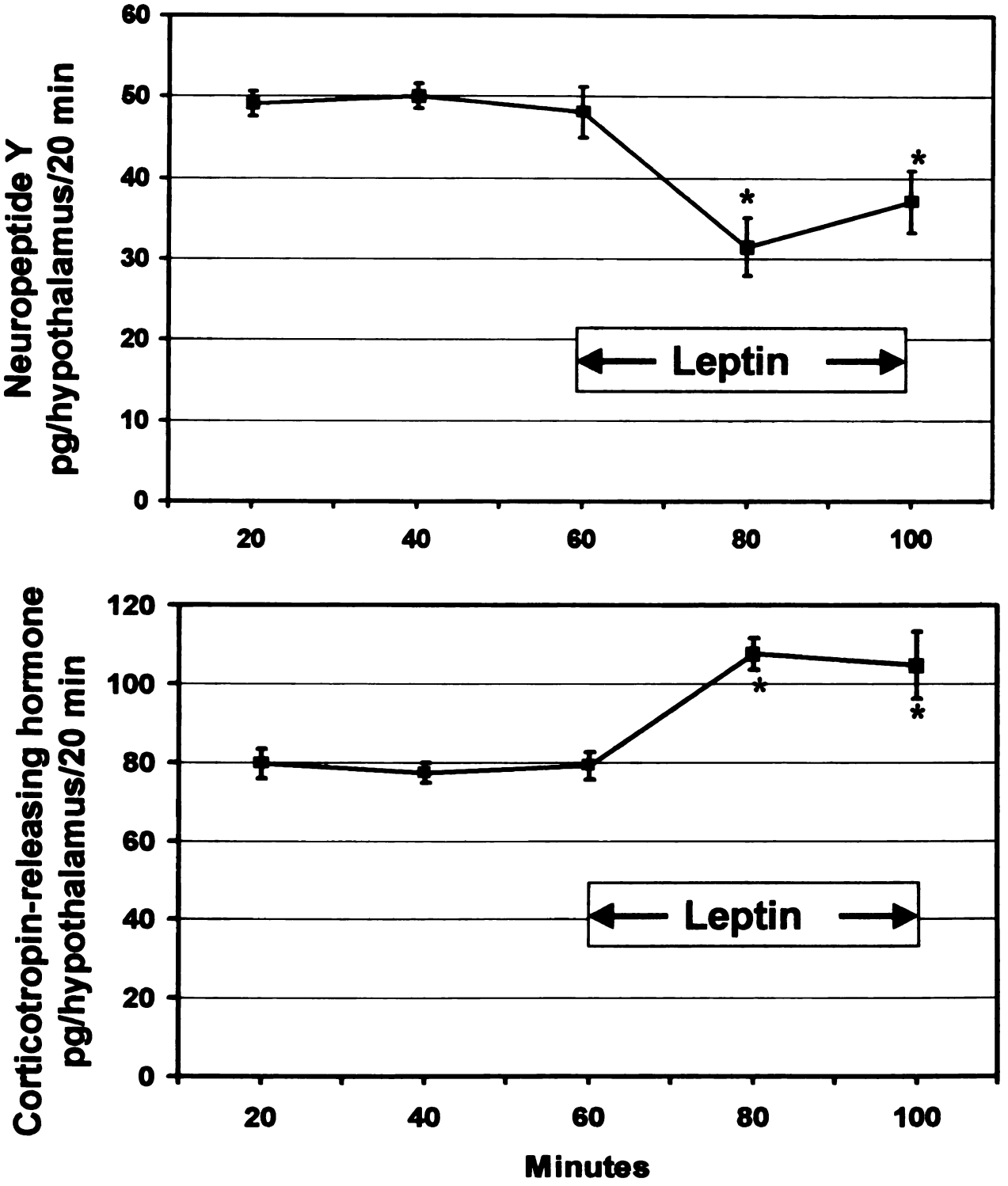

FIGURE 18. Leptin altered in vitro release of NPY and CRH from hypothalamic blocks obtained from ADX lean mice. Data points are means of 4 chambers ± SE (two hypothalami per chamber), and represent NPY or CRH released per 20 min-period. * Significant change after leptin treatment compared with average basal release as determined by one way repeated measures ANOVA with Bonferroni test as post hoc comparisons ($P < 0.05$).

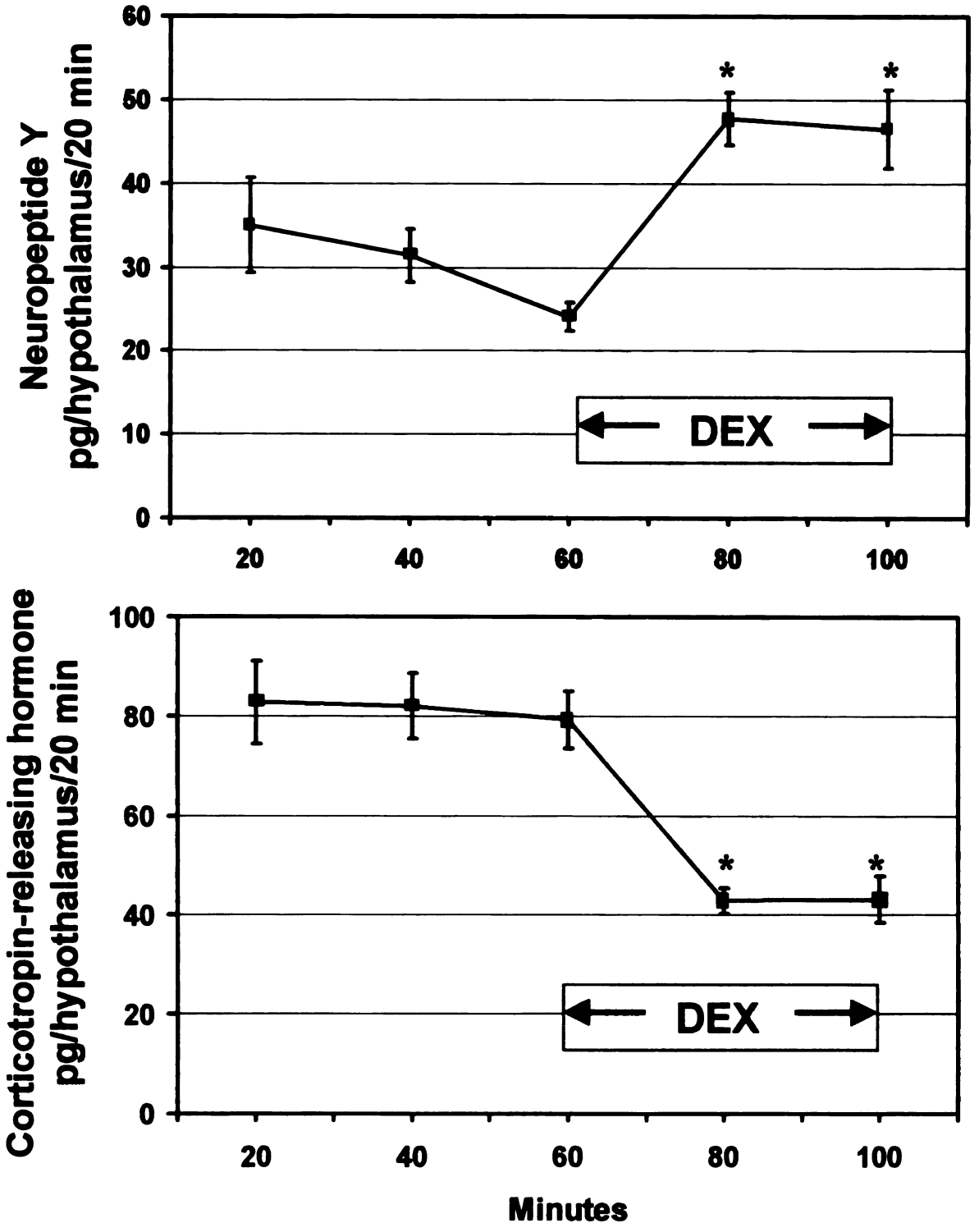

FIGURE 19. DEX altered in vitro release of NPY and CRH from hypothalamic blocks obtained from ADX lean mice. Data points are means of 6 chambers ± SE (two hypothalami per chamber), and represent NPY or CRH released per 20 min-period. * Significant change after DEX treatment compared with average basal release as determined by one way repeated measures ANOVA with Bonferroni test as post hoc comparisons ($P < 0.05$).

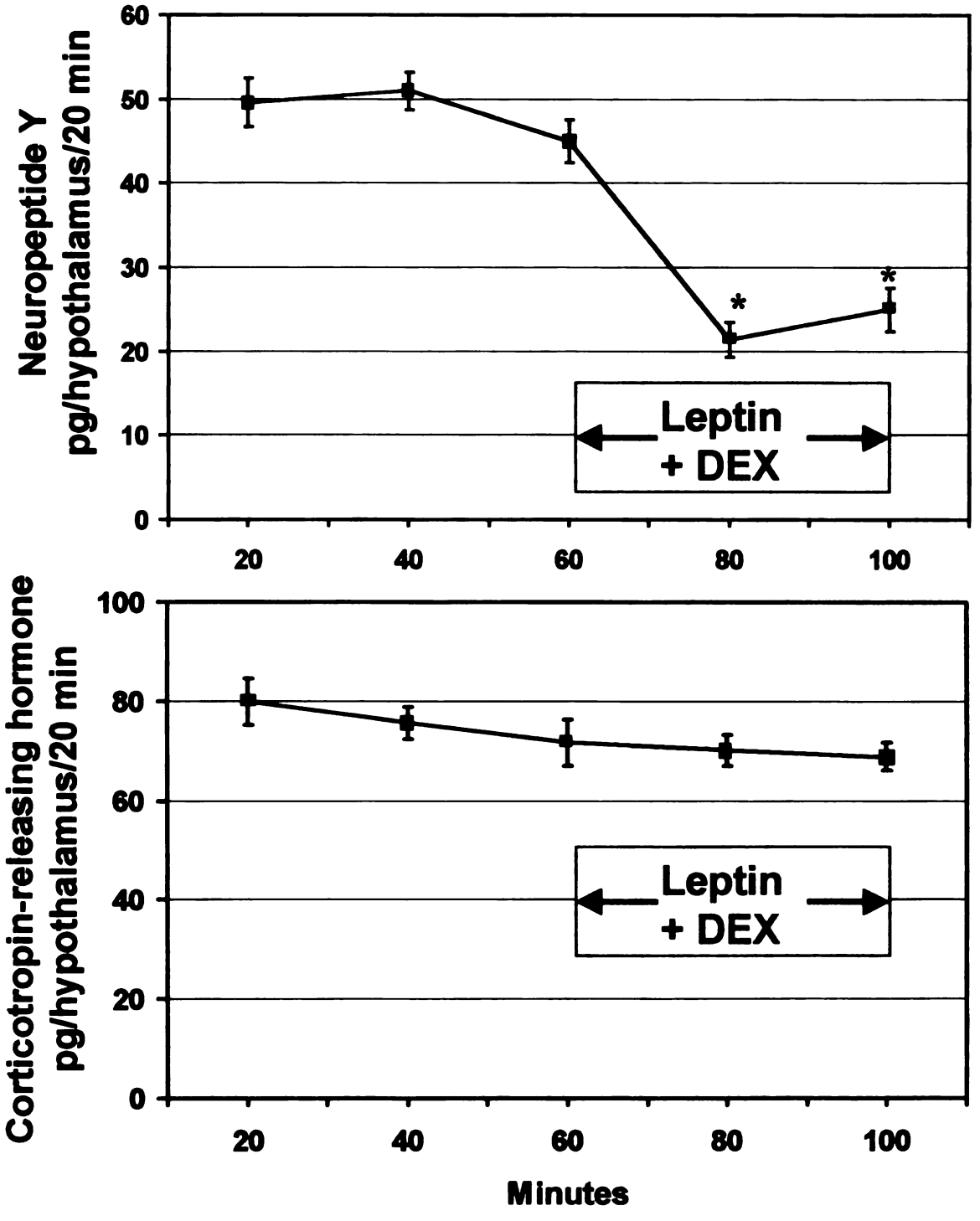

FIGURE 20. Effects of leptin and DEX co-incubation on in vitro release of NPY and CRH from hypothalamic blocks obtained from ADX lean mice. Data points are means of 7-8 chambers ± SE (two hypothalami per chamber), and represent NPY or CRH released per 20 min-period. * Significant decrease in NPY secretion after leptin + DEX treatment compared with average basal release as determined by one way repeated measures ANOVA with Bonferroni test as post hoc comparisons ($P < 0.05$).

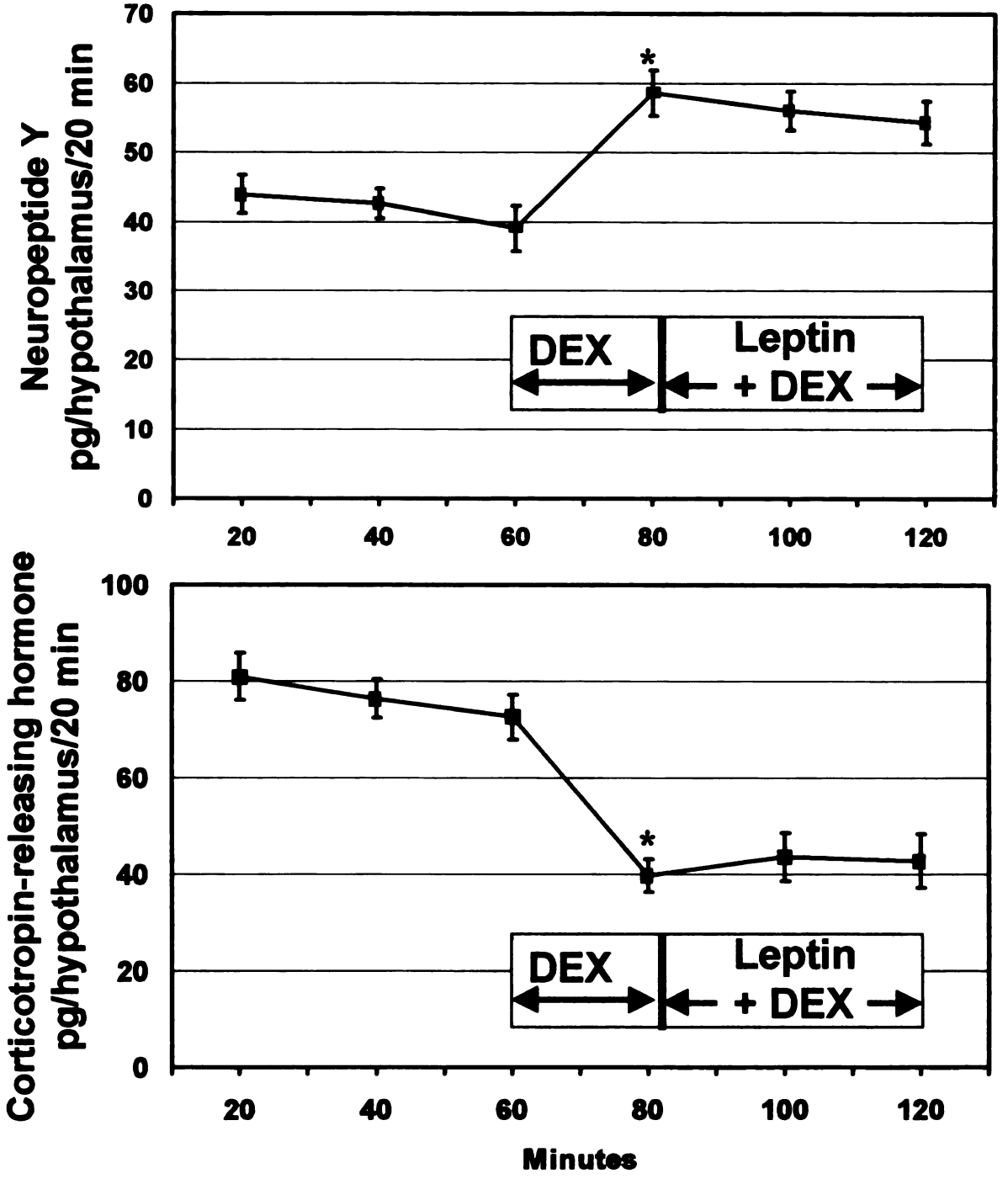

FIGURE 21. Effects of DEX incubation followed by leptin plus DEX co-incubations on in vitro release of NPY and CRH from hypothalamic blocks obtained from ADX lean mice. Data points are means of 6 chambers ± SE (two hypothalami per chamber), and represent NPY or CRH released per 20 min-period. * Significant change after DEX treatments compared with average basal release as determined by one way repeated measures ANOVA with Bonferroni test as post hoc comparisons ($P < 0.05$).

E. DISCUSSION

The present study was conducted to examine acute effects of leptin and glucocorticoids on NPY and CRH secretion, and to determine if interactions between these two hormones exist to regulate secretion of NPY and CRH within the hypothalamus. Our findings may be summarized as follows. First, leptin rapidly, i.e. within 20 min, decreased NPY release and rapidly increased CRH release from hypothalamic blocks obtained from ADX mice, and glucocorticoids had opposite actions on NPY (stimulatory) and CRH (inhibitory) secretion (**Fig. 18 and 19**). Second, simultaneous addition of leptin and DEX to the hypothalamic blocks essentially abolished these actions, except that leptin appeared to predominate over DEX to decrease NPY secretion (**Fig. 20**). Third, actions of DEX on NPY and CRH secretion predominated when it was added to the tissue 20 min prior to addition of leptin (**Fig. 21**).

We expected that leptin-induced changes in NPY and/or CRH secretion would be more likely detected in hypothalamic blocks obtained from ADX mice than from intact mice because the possible counter-regulatory influence of corticosterone would not be present in ADX mice. Consistent with this hypothesis, rapid-onset effects of leptin (30 nM), i.e. within 20 min, on NPY (inhibitory) and CRH (stimulatory) secretion from the hypothalamus of ADX mice, but not from intact mice, were observed (**Fig. 18**). These marked effects of leptin on NPY and CRH secretion from the hypothalamic blocks obtained from ADX mice provide at least one mechanism to explain the more pronounced effects of leptin on food intake in ADX rats and mice than in intact animals (Mistry et al. 1997, Zakrzewska et al. 1997). The ineffectiveness of leptin to alter NPY secretion from the hypothalamus of intact mice in the present study is consistent with a previous report

that showed unaltered *in vivo* NPY release from the PVN of rats within 2 h after IP administration of leptin (Beck et al. 1998). But in contrast, Stephens et al. (1995) reported that leptin decreased NPY secretion from hypothalamic blocks from intact rats. They, however, needed to expose the tissue to 0.6 uM corticosterone for 2 h to observe detectable release of NPY and, thus were unable to test effcts of leptin on NPY secretion in the absence of added glucocorticoid. Ineffectiveness of leptin to alter CRH secretion from the hypothalamic blocks obtained from intact mice in the present study is inconsistent with previous reports, in which leptin stimulated CRH release from (Costa et al. 1997, Raber et al. 1997). Species or *in vitro* incubation system was different in the present study from other studies. Whether use of different species or *in vitro* systems leads to discrepancies in the results remains unclear.

The mechanisms whereby leptin and glucocorticoids rapidly influence NPY and CRH secretion within the hypothalamus have not yet been elucidated. Rapid-onset actions of leptin and glucocorticoids likely occur via mechanisms other than effects on gene expression (**Fig. 5 and 9**). There is a family of leptin receptors (Tartaglia et al. 1997). The receptor thought to be involved in signal transduction has similarities to members of the class I cytokine receptor family which act via the *Jak*/STAT pathway to regulate gene transcription (Baumann et al. 1996, Tartaglia et al. 1997). But these receptors may also transduce signals that cause rapid-onset changes in cell metabolism. Activation of the prolactin receptor, a member of the class 1 cytokine receptor family, causes phosphorylation of *Jak* 2 and subsequent phosphorylation and activation of calcium-activated potassium channels (within 6 min) in excised membrane patches of Chinese hamster ovary cells (Prevarskaya et al. 1995). Leptin-induced phophorylation of *Jak* 2

(Bjørbæk et al. 1997, Li and Friedman, 1999) may similarly regulate ion channels in the hypothalamus. Accumulating evidence suggest that membrane-bound glucocorticoid receptors mediate rapid-onset actions of glucocorticoids (**Fig. 9**)(Lou and Chen, 1998, Qiu et al. 1998, Watson and Gametchu, 1999). Glucocorticoids have been shown to activate potassium channels by facilitating phosphatase action in the mouse anterior pituitary corticotrope cells (Shipston et al. 1996, Tian et al. 1998). Leptin and glucocorticoids have also been reported to rapidly affect membrane potential (Glaum et al. 1996, Lou and Chen, 1998, Powis et al. 1998, Qiu et al. 1998, Spanswick et al. 1997), cAMP concentrations (Zhao et al. 1998), nitric oxide generation (Yu et al. 1997, Sandi et al. 1996), and PKC activity (Chen et al. 1997. Qiu et al. 1998)(**Fig. 5 and 9**). Changes in ion channels/membrane potential and other signal transducers are potential mediator of the observed leptin and glucocorticoid-induced changes in NPY and CRH secretion within the hypothalamus. Whether leptin and glucocorticoids exert their actions on ion channels and/or other signal transducers remains to be fully resolved.

The ability of leptin (and glucocorticoids) to simultaneously increase secretion of one neuropeptide and decrease secretion of another neuropeptide from the hypothalamus suggests involvement of a complex regulatory system. Leptin has been shown to act in site-specific manners to rapidly influence membrane potential and modulate synaptic transmission within the hypothalamus (Glaum et al. 1996, Powis et al. 1998, Spanswick et al. 1997). It rapidly, i.e. within 2-8 sec, depolarized > 67 % of the neurons in the PVN and rapidly, i.e. within 15 min, hyperpolarized neurons in the ARC of hypothalamus (Powis et al. 1998, Spanswick et al. 1997). Leptin also rapidly (< 1 min) increased intracellular Ca^{2+} induced by 50 mM K^+ in some cells isolated from ARC of rats, and

decreased intracellular Ca^{2+} in other cells, without effects on cells obtained from the VMH (Glaum et al. 1996). Glucocorticoids have also been reported to rapidly (within 15 min) increase Ca^{2+} uptake by synaptosomes from rat brain (Sze and Iqbal et al. 1994a) and to rapidly (within 15 min) decrease intracelluar Ca^{2+} induced by 50 mM K^+ by possibly activating PKC pathway in the PC12 cells (Lou and Chen, 1998, Qiu et al. 1998). These leptin and glucocorticoid-induced, site-specific changes in membrane potential and intracellular Ca^{2+} concentrations may contribute to their simultaneous inhibitory and stimulatory actions on secretion of NPY and CRH within the hypothalamus.

It is not clear from the present study whether leptin and glucocorticoids directly influence NPY and CRH secretion within the hypothalamus, or whether the actions are indirect. The long form of the leptin receptor, which is capable of signal transduction via *Jak*/STAT pathway, is located in the ARC, PVN, VMH, and DMH, important synthesis/release sites for NPY and CRH (Bray and York, 1998, Elmquist et al. 1998, Hakansson et al. 1998). Leptin receptors are co-localized on NPY neurons in the ARC (Mercer et al. 1996, Schwartz et al. 1999). Glucocorticoid receptors are also present in the ARC and PVN (Eekelen et al. 1987). These anatomical observations suggest that NPY and CRH may be direct targets of leptin and glucocorticoid actions within the hypothalamus. However, NPY is reported to stimulate CRH secretion and vice versa within the hypothalamus, possibly as a feedback mechanism to maintain a balance between these two neuropeptides (Tsagarakis et al. 1989, Morris and Pavia, 1998). Thus we cannot rule out cross-talk among various pathways to modulate effects of leptin and glucocorticoids on NPY and CRH secretion.

The failure of leptin to reverse DEX effects on NPY and CRH secretion (**Fig. 21**) is consistent with the ineffectiveness of leptin to alter NPY and CRH secretion from the hypothalamic blocks obtained from intact mice in the present study. It is speculated from this observation that leptin and glucocorticoids likely activate a common signal transduction pathway. Leptin may prevent activation of effector system, but can not reverse activated signaling that was induced by glucocorticoids. If this is true, it would be harder to detect any changes in NPY and CRH secretion from the hypothalamus of intact mice than from that of the ADX after leptin administration. Similar modes of leptin actions (ineffectiveness of leptin to alter activated system) were also observed peripherally in pancreatic islets of ob/ob mice (Chen et al. 1997). Leptin rapidly, within 3 min, blocked stimulation of acetylcholine-induced insulin secretion from the pancreatic islets of ob/ob mice when leptin and acetylcholine were simultaneously added to the tissue. Leptin, however, failed to reverse acetylcholine effects on insulin secretion when leptin was added following acetylcholine activation of PLC-PKC pathway. The failure of leptin to reverse DEX actions may also imply glucocorticoid-induced, via as yet unknown mechanism, leptin resistance that is well observed in human obesity (Ur et al. 1996).

In summary, the present study clearly demonstrates that leptin exert rapid-onset actions on NPY (inhibitory) and CRH (stimulatory) secretions within the hypothalamus, and glucocorticoids act opposite to leptin action as a counter-regulatory mechanism. An understanding of the mechanisms by which leptin and glucocorticoids interact to modulate feeding-regulatory neurotransmitters within the hypothalamus would provide a new approach for the treatment of obesity.

CHAPTER VI. SUMMARY AND CONCLUSIONS

Obesity is the result of a disorder in body energy balance that occurs when energy intake chronically exceeds energy expenditure. Cloning and identification of the ob gene (Zhang et al. 1994) has greatly accelerated obesity research during the past 5 years. A nonsense mutation in the ob gene in ob/ob mice (Zhang et al. 1994) causes severe obesity in these mice. Administration of leptin, ob gene product, to these leptin-deficient ob/ob mice decreases their food intake as well as increases their energy expenditure, resulting in a restoration of normal body weight regulation (Halaas et al. 1995, Hwa et al. 1996, Pelleymounter et al. 1995).

Leptin likely acts within the CNS, in particular the hypothalamus, to regulate body weight (Campfield et al. 1995, Halaas et al. 1995, Stephens et al. 1995). NPY, a stimulator of food intake (Billington et al. 1994, Clark et al. 1984, Stanley et al. 1986) and CRH, an inhibitor of food intake (Arase et al. 1988, Krahn et al. 1988, Rohner-Jeanrenaud et al. 1989) have been suggested as candidates to mediate leptin actions within the hypothalamus (Mercer et al. 1996, Schwartz et al. 1996a and 1996b, Stephens et al. 1995, Wang et al. 1997).

If leptin regulates food intake and energy expenditure by influencing hypothalamic NPY and/or CRH, leptin-deficient ob/ob mice would be expected to exhibit alterations in NPY and CRH within the hypothalamus. Hypothalamic NPY and CRH are reported to change with the feeding status of the animals (Beck et al. 1990b, Brady et al. 1990, Sahu

et al. 1988, Schwartz et al. 1993), conditions that are now known to alter plasma leptin concentrations (Hardie et al. 1996, Saladin et al. 1995, Trayhurn et al. 1995).

I therefore first examined whether any alterations were present in NPY and CRH concentrations within specific hypothalamic nuclei of ob/ob mice, and if hypothalamic NPY and CRH concentrations of ob/ob mice change in response to food-deprivation and refeeding. I also determined if leptin deficiency in ob/ob mice leads to alterations in secretion of NPY and/or CRH in the hypothalamus.

Fed ob/ob mice had 55 – 75 % higher concentrations of NPY in the ARC, VMH, and SCN than lean mice (**Fig. 11**) with CRH concentrations unaltered in the 3 regions examined (ARC, PVN, and VMH) even though lean mice tended ($P = 0.18$) to have higher (about double) CRH concentrations in the ARC than ob/ob mice (**Fig. 12**). Food-deprivation increased NPY concentrations ~ 70 % in the ARC, PVN, and VMH of lean mice, and refeeding lowered NPY concentrations ~ 70 % in the PVN of these mice. NPY in these hypothalamic regions of ob/ob mice was unresponsive to food-deprivation or refeeding. Refeeding lowered CRH concentrations by 75 % in ARC of lean mice, without influencing ARC CRH concentrations in ob/ob mice. These findings suggest that leptin deficiency in ob/ob mice may lead to alterations in concentrations of NPY and CRH, and in regulatory mechanisms for these neuropeptides within the hypothalamus.

Basal and high KCl-induced release of NPY and CRH from hypothalamic preparations of ob/ob mice were not altered in ob/ob mice, suggesting that regulation of hypothalamic NPY and CRH secretion was not inherently altered in leptin-deficient ob/ob mice. Rather, the *in vivo* environment likely causes any dysregulations of hypothalamic NPY and CRH release that may be present in ob/ob mice.

Mounting evidence suggests that leptin exerts very rapid actions within the hypothalamus, in addition to its slower genomic actions (Banks et al. 1996, Elmquist et al. 1997, Powis et al. 1998, Spanswick et al. 1997). I hypothesized that the rapid actions of leptin on food intake in rats and mice were associated with changes in secretion of NPY and/or CRH within the hypothalamus. Measurements of changes in NPY and CRH concentrations in specific hypothalamic nuclei within 1-3 h after leptin administration were used as an index of changes in transport/release of these neuropeptides I assumed that minimal effects of leptin on protein synthesis would be evident within this time frame. To more directly assess effects of leptin on secretion of NPY and CRH, *in vitro* hypothalamic NPY and CRH secretion were also determined with and without added leptin.

No rapid onset effects of leptin on hypothalamic NPY and/or CRH concentrations were observed in intact mice, consistent with the ineffectiveness of leptin to alter *in vitro* NPY and CRH secretions from hypothalamic preparations from these intact mice (**Table 6 and 7, and Fig. 13, 14 and 16**). Glucocorticoids might oppose leptin actions (Ur et al. 1996, Zakrzewska et al. 1997). Inhibitory actions of leptin on food intake and body weight were recently reported to be more pronounced in ADX rats and mice than in intact ones (Mistry et al. 1997, Zakrzewska et al. 1997). Possibly, the presence of endogenous glucocorticoids in intact mice might have masked or inhibited hypothalamic NPY and/or CRH responses to administered leptin in the present study.

I thus hypothesized that glucocorticoids oppose the actions of leptin on NPY and CRH secretion within the hypothalamus. Consistent with this hypothesis, leptin administration markedly reduced ARC CRH concentrations in ADX mice, but not intact lean and ob/ob mice (**Fig. 14**). This rapid reduction in CRH concentrations (ie, within 3 h) in the ARC

after leptin administration is more likely due to stimulated CRH release from this region rather than due to decreased synthesis/transport from the PVN, a site of synthesis, because leptin stimulates, not depress, CRH synthesis in the PVN (Schwartz et al. 1996a).

This observation led me to further examine acute effects of leptin and glucocorticoids on NPY and CRH secretions from the hypothalamic preparations obtained from ADX mice to determine if these two hormones exert opposing actions on NPY and/or CRH secretion within the hypothalamus. Leptin rapidly, i.e. within 20 min, decreased NPY release and rapidly increased CRH release from hypothalamic blocks obtained from ADX mice (**Fig. 17 and 18**). These marked effects of leptin on NPY and CRH secretion from the hypothalamic blocks obtained from ADX mice provide at least one mechanism to explain the more pronounced effects of leptin on food intake in ADX rats and mice than in intact animals (Mistry et al. 1997, Zakrzewska et al. 1997). DEX (0.5 uM) was also able to rapidly, within 20 min, influence secretion of NPY (stimulatory) and CRH (inhibitory) from the hypothalamic blocks from ADX mice (**Fig 19**). These rapid-onset actions of glucocorticoids on NPY and CRH secretion, as hypothesized, were opposite to those of leptin.

To better understand interactions between leptin and glucocorticoids on NPY and CRH secretion, I further examined effects of leptin and glucocorticoids in the same incubation. Simultaneous administration of leptin and DEX to the hypothalamic preparations decreased NPY secretion by 52 % (ie, leptin actions predominated) within 20 min while CRH secretion was unchanged (ie, leptin and DEX actions negated each other)(**Fig. 20**). Addition of leptin following exposure of the tissue to DEX for 20 min failed to further change either NPY or CRH secretion (**Fig. 21**). Failure of leptin to

reverse DEX effects on NPY and CRH secretion is consistent with the ineffectiveness of leptin administration to alter NPY and CRH secretion (either *in vitro* or *in vivo* as measured by changes in concentrations) from the hypothalamus obtained from intact mice in the present study.

In conclusion, my present study suggests that leptin and glucocorticoids exert opposing actions on NPY and CRH secretion within the hypothalamus. The presence of glucocorticoids may restrain actions of leptin on secretion of these neuropeptides within the hypothalamus.

CHAPTER VII. RECOMMENDATIONS FOR FUTURE STUDIES

To better understand the present results, and to continue searching for possible mechanisms whereby leptin causes rapid-onset changes in NPY and CRH secretion, I suggest the following studies.

1. Determine whether leptin administration influences hypothalamic NPY and CRH in intact mice at a time when plasma corticosterone concentrations are low.

In vivo studies with intact mice were conducted between 1500 and 1700 h, a time of day when corticosterone concentrations are high (Leibowitz, 1992), and mice are consuming food. Based on the results of our studies with ADX mice, glucocorticoids appear to oppose leptin actions. Therefore it would be interesting to examine effects of leptin on NPY and CRH secretion in intact mice at a time of the day when plasma corticosterone concentrations would be low (i.e. shortly after the lights are turned on in the morning). We used ADX mice to test leptin effects on NPY and CRH since possible counter-regulatory actions of glucocorticoids would not be present in ADX mice. To simplify the experiments, one could determine acute leptin effects in the presence of a glucocorticoid antagonist RU 486 to see if acute blockage of glucocorticoid actions causes pronounced changes in NPY and CRH secretion from the hypothalamus obtained from intact mice.

2. Determine the primary sites of leptin actions within the hypothalamus.

Measurements of NPY and CRH concentrations in specific hypothalamic regions after acute leptin administration in the present study may not have been sensitive enough to detect subtle changes in neuropeptide secretion/transport. Baskin et al. (1999) have recently shown that not all NPY containing neurons within the ARC have detectable leptin receptor mRNA, suggesting a heterogeneous distribution of leptin receptors within specific nuclei of the hypothalamus. The PVN is a highly differentiated structure that contains anatomically discrete regions of neurons containing specific peptides and combination of peptides (Whitnall et al. 1993). I therefore suggest to examine changes in NPY and CRH concentrations after acute leptin administration in more specific regions of nuclei, for example subdivisions of ARC and PVN, by using the techniques of immunohistochemistry.

I also suggest to determine *in vitro* NPY and CRH secretion from the specific sites of hypothalamus, i.e. ARC and PVN, by using the techniques described by of Sahu et al. (1992). If some effects of leptin on secretion of NPY or CRH from these specific sites of hypothalamus are observed, this outcome may at least partly explain ineffectiveness of leptin to alter *in vitro* NPY ad CRH secretion from the hypothalamic blocks obtained from intact mice observed in the present study.

3. Determine the mechanisms to explain rapid actions of leptin on NPY and CRH secretion.

We observed rapid leptin actions on secretion of NPY and CRH from the hypothalamus of ADX mice. Once the system in which leptin influences NPY and CRH

secretion is determined, one could further investigate the mechanisms responsible for rapid-onset leptin actions. There are three main ways membrane receptors transmit signals: direct agonist effect on the channel, second messenger participation in channel modulation, and regulation by kinases and phosphates through channel phosphorylation and dephosphorylation (Moore, 1994). The leptin receptor has similarities to members of the class I cytokine receptor family which act via the *Jak*/STAT pathway (Baumann et al. 1996, Tartaglia et al. 1997). Activation of the prolactin receptor, a member of the class 1 cytokine receptor family, causes phosphorylation of *Jak* 2 and subsequent phosphorylation and activation of calcium-activated potassium channels (within 6 min) in excised membrane patches of Chinese hamster ovary cells (Prevarskaya et al. 1995). Leptin-induced phophorylation of *Jak* 2 (Bjørbæk et al. 1997, Li and Friedman, 1999) may similarly regulate ion channels in the hypothalamus. I suggest to determine if acute leptin administration causes changes in activity of ion channels and also to determine if these changes in ion channels (if observed) are associated with changes in NPY and/or CRH secretion.

One could also determine changes in the activity of the second messenger systems, for example PKA- and PKC- mediated pathways after acute leptin administration. Nitric oxide has been recognized as a messenger molecule in the CNS, and is known to rapidly affect secretion of neurotransmitters including CRH within the hypothalamus (Brann et al. 1997, Costa et al. 1996). Yu et al. (1997) have recently shown that leptin rapidly (within 30 min) increased secretion of luteinizing hormone-releasing hormone from the hypothalamus of rats via production of nitric oxide. It would be interesting to see if leptin modulates NPY and CRH secretion via its effects on nitric oxide production within the

hypothalamus. Nitric oxide synthase inhibitor, N-monomethyl-L-arginine, can be co-administered with leptin (either *in vivo* or *in vitro*) to test if nitric oxide mediates leptin actions on NPY and CRH secretion. To attempt to understand possible counter-regulatory role of glucocorticoids in the present study, one could also examine whether administration of RU 486 alters the effects of leptin on these suggested signaling pathways.

4. Does leptin directly influence NPY and CRH secretion?

NPY is reported to stimulate CRH secretion and vice versa within the hypothalamus, possibly as a feedback mechanism to maintain a balance between these two neuropeptides (Tsagarakis et al. 1989, Morris and Pavia, 1998). Leptin influenced secretion of both NPY (inhibitory) and CRH (stimulatory) from the hypothalamic blocks obtained from ADX mice in the present study. One should examine whether leptin directly influences NPY and CRH or whether the actions are indirect. To test this, *in vitro* NPY and CRH secretion can be determined in the presence of receptor antagonists. If changes in NPY secretion after leptin administration occur in the presence of CRH receptor antagonist, α-helical CRH, it would suggest that the actions of leptin on NPY are not at least influenced by CRH.

5. Determine whether leptin effects on food intake are associated with changes in secretion of other feeding-regulatory neuropeptides.

Rapid (< 1 h) suppression of food intake in intact lean and ob/ob mice after ICV leptin administration was not associated with changes in NPY and CRH within specific nuclei of

hypothalamus in the present study, inconsistent with our hypothesis. Alternatively, leptin-induced suppression of food intake in intact mice may have occurred without direct effects on hypothalamic release of NPY or CRH. To test this, one could measure secretions of other suggested mediators of leptin actions such as α-MSH, CART (inhibitors of food intake) and AGRP (a stimulator of food intake) present within the hypothalamus (Flier and Maratos-Flier, 1998, Satoh et al. 1998, Wilson et al. 1999) as well as NPY and CRH. Measurements of changes in secretion of several peptides after leptin administration would help understand complex feeding-regulatory systems present within the hypothalamus.

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