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I am submitting herewith a thesis written by Harriet E Bergeron entitled "Leptin phase-advances the suprachiasmatic circadian pacemaker *in vitro* " I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology

Rebecca A Prosser, Major Professor

We have read this thesis
and recommend its acceptance

Jim C. Hall, Associate Professor

John Koontz, Department Head

Accepted for the Council

Interim Vice Provost and
Dean of The Graduate School

LEPTIN PHASE-ADVANCES THE SUPRACHIASMATIC CIRCADIAN PACEMAKER *IN VITRO*

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Harriet Elizabeth Bergeron
December 2000

DEDICATION

This thesis is dedicated to my grandmothers, who raised me,

Elizabeth Louise Lister,

for all her love and devotion

and

Chanyce Barnes Bergeron,

who has always supported and encouraged me in my education

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ABSTRACT

The primary circadian clock in mammals is located in the suprachiasmatic nucleus (SCN). Two rhythms controlled by the SCN clock are feeding and metabolic rate. The hormone leptin is a potent regulator of food intake and energy metabolism. Immunocytochemical and *in situ* hybridization studies have indicated the presence of leptin receptors in several hypothalamic nuclei. Also, rodents with mutations in the leptin receptor or leptin protein exhibit abnormal circadian rhythms. These data raised the possibility that leptin may modulate SCN clock activity. Therefore, we investigated the effects of leptin on SCN neuronal activity rhythms *in vitro*. Leptin was applied at various times to rat brain slices containing the SCN. Extracellular recordings of SCN neurons were used to calculate the time-of-peak in SCN activity. Phase shifts of the SCN clock were determined by comparing the time-of-peak in treated slices to that of control slices. Results showed that leptin phase-advances the SCN clock by about 2h when applied during the subjective day and into the early subjective night. To investigate whether these phase shifts might be occurring through stimulation of leptin receptors, we performed leptin receptor immunocytochemistry using two different antibodies to the leptin receptor. The K-20 antiserum is immunoreactive with the amino-terminus of all leptin receptor isoforms and B-3 is specific for the transmembrane forms. We found positive leptin receptor immunoreactivity in the SCN using both antibodies, supporting the hypothesis that the observed phase shifts are the result of a direct action of leptin in the SCN.

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2DG	2-deoxyglucose
ARC	arcuate nucleus
DMH	dorsomedial nucleus of the hypothalamus
GABA	γ-aminobutyric acid
JAK	Janus kinase
K_{ATP}	ATP-sensitive potassium channel
LHA	lateral hypothalamic area
MAPK	mitogen-activated protein kinase
NPY	neuropeptide Y
OB-R	leptin (obese) receptor
POA	preoptic area
PVH	paraventricular nucleus of the hypothalamus
RT-PCR	reverse transcription-polymerase chain reaction
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
STAT	signal transducers and activators of transcription
SUR	sulfonylurea receptor
VMH	ventromedial nucleus of the hypothalamus

Chapter I

Background and Significance

The Suprachiasmatic Nucleus and Circadian Rhythms

Circadian rhythms are biologically important across many species as an adaptation to the solar cycle and other environmental factors that fluctuate over a 24-hour period. The primary circadian clock in mammals is located in the suprachiasmatic nucleus (SCN) (1). The SCN is a set of paired nuclei located in the anterior basal hypothalamus directly above the optic chiasm. Cells of the SCN contain an internal clock mechanism that responds to cues from both the environment and the internal state of the animal.

The SCN is roughly divided into two main divisions, ventrolateral and dorsomedial (2). The SCN is composed of a heterogeneous population of cells. The majority of these cells contain the inhibitory neurotransmitter γ-aminobutyric acid (GABA), which is usually co-localized with various peptides (3). For example, arginine-vasopressin and neurophysin cell bodies are found mostly in the dorsomedial SCN (1,3,4). Somatostatin is found throughout the nucleus (1). Vasoactive-intestinal peptide is found mainly in the ventrolateral subdivision. Other peptides found in smaller quantities include peptide histidine-isoleucine, gastrin-releasing peptide and bombesin (1).

The major afferents to the SCN terminate in the ventrolateral area. The SCN receives photic information from the retina, primarily via the retinohypothalamic tract (5). The major neurotransmitter in this pathway is the

excitatory amino acid glutamate (6). A second, indirect photic input comes from the intergeniculate leaflet. This afferent uses primarily GABA and neuropeptide Y (NPY) (6). Another major afferent input comes from the raphe nuclei, which uses serotonin as its primary neurotransmitter (7). The SCN receives a variety of smaller projections from hypothalamic nuclei such as the ventromedial nucleus (VMH), paraventricular nucleus (PVH), and arcuate nucleus [ARC, (8)].

SCN efferents arise mainly out of the dorsomedial division. The SCN projects mainly to other hypothalamic nuclei including the PVH, the region surrounding the VMH, posterior hypothalamic area, dorsomedial nucleus (DMH), periventricular nucleus, and preoptic area [POA; (9,10)].

The SCN is part of a network of hypothalamic nuclei that regulate body homeostasis through both the endocrine and autonomic nervous systems. These nuclei include the VMH and lateral hypothalamic area (LHA), DMH, ARC, PVH, POA, and supraoptic nucleus (SON). The role of the SCN in this network is believed to be that of a 'master oscillator'. SCN efferents drive the activity rhythms and outputs of these other nuclei, leading to the observed circadian rhythms of body temperature, sympathetic nerve activity, thermogenesis, blood glucose concentration, feeding and drinking behavior, as well as neuroendocrine regulation of pituitary hormone release (11,12)

Circadian Rhythms of Food Intake And Energy Metabolism: Involvement of The SCN and Other Hypothalamic Nuclei

The brain uses glucose as its primary source of energy. Therefore, blood glucose levels are kept under tight homeostatic control by the central nervous system. In both nocturnal and diurnal animals there exists a day/night difference in the way blood glucose levels are maintained During an animal's active period, food intake and catabolism, and release of liver glycogen stores are the main sources of blood glucose. During the rest period, glycogenolysis has to be supplemented with gluconeogenesis in the liver to maintain blood glucose levels. Along with regulating food intake, the hypothalamus also regulates liver glycogen breakdown, release of free fatty acids, and gluconeogenesis through sympathetic nervous system innervation of the pancreas, liver, adipose tissue, and the adrenal glands.

Wild rats (13,14) and laboratory rats housed in a seminatural environment (15) or in a laboratory setting with a normal light/dark cycle (16) exhibit circadian rhythms of activity and food intake. The animals are nocturnal and 80-90% of their food intake also occurs during the night (17). Ingestion of the largest meal is initiated soon after the onset of the dark phase and a second large feeding bout occurs just before lights on. Lesions of the SCN eliminate both eating and drinking circadian rhythms, so that feeding occurs in small bouts throughout the light and dark periods (17,18).

In rodents, plasma glucose, free fatty acids, insulin and glucagon levels also show a circadian rhythm (19,20). Plasma glucose levels are lowest during the light phase and increase to their maximum levels during the dark phase after the onset of feeding (20,21) Basal insulin levels are low during most of the light phase, but begin to rise shortly before lights off (21,22). The glucagon rhythm is the inverse of the insulin rhythm, with the highest levels occurring toward the end of the light phase and the animals' resting period (22). Free fatty acids are low during the early light phase and rise to a peak at the light-dark transition. The levels drop after food intake, perhaps indicative of the shift from reliance on gluconeogenesis to carbohydrate intake for maintenance of blood glucose levels (22).

SCN lesions abolish most of these rhythms, probably due to the loss of circadian feeding patterns (18,20). However, the circadian rhythm of plasma insulin persists, although the rhythm is dampened (20). The insulin rhythm is also not dependent on the temporal distribution of feeding as unlesioned animals exposed to food deprivation or restricted feeding schedules do not show a phase change in the insulin peak (21).

Research has shown the SCN to have a prominent role in the circadian regulation of blood glucose levels. The SCN contains both glucose-sensitive neurons and insulin-sensitive neurons (23,24). Glucose-sensitive neurons show a dose-dependent increase in activity with increasing bath glucose concentration (23), while bath application of insulin mostly inhibits SCN neuronal activity (24).

Insulin infusion into the rat SCN causes hypoglycemia and increases food intake during the light period (12,25), and causes hyperglycemia when infused during the dark period (12).

Glucose tolerance is higher during the night period than during the day period (26) An oral glucose load causes prolonged hyperglycemia only during the day. The hyperglycemia caused by injection of 2-deoxyglucose (2DG), a non-hydrolyzable form of glucose, is also much greater during the day than the night and SCN lesions abolish both the time-dependency and the hyperglycemic response (27,28) 2DG injections also alter feeding behavior in a time-dependent manner Food intake increases following injection of 2DG at the beginning of the light period, and decreases when administered at the beginning of the dark period (26). SCN lesions eliminate the hyperphagic response (29).

Electrical stimulation of the SCN leads to hyperglycemia, hyperglucagonemia, increased liver glycogen phosphorylase α activity (a glycogenolytic enzyme), and decreased liver glycogen content (30). These effects are thought to be caused by SCN modulation of sympathetic nervous system activity Rats with SCN lesions do not show increased adrenal nerve activity associated with 2DG injection (30). These findings suggest that SCN stimulation causes increased sympathetic stimulation of the liver, either through direct innervation or increased plasma epinephrine levels.

The role of the SCN in the control of food intake, maintenance of blood glucose levels, and modulation of sympathetic nervous system outflow is likely

mediated through its connections with other hypothalamic nuclei. The VMH and LHA have been implicated in the central control of feeding and metabolic rate. VMH lesions cause hyperphagia, hyperinsulinemia, and obesity (31). LHA lesions cause hypophagia and lesioned animals can starve themselves to death. Both areas have glucose-responsive neurons and have been implicated as regulators of plasma glucose and free fatty acid levels through autonomic nervous system innervation of the liver, pancreas, adipose tissue, and adrenal medulla. Electrophysiological experiments *in vitro* have shown that the SCN exerts an excitatory effect on VMH neurons and an inhibitory effect on LHA neurons (32).

Electrical stimulation of VMH neurons causes increases in liver phosphoenolpyruvate carboxykinase activity (PEPCK, a key gluconeogenic enzyme), and glycogen phosphorylase a (a rate-limiting enzyme of glycogenolysis), decreases liver glycogen content, and increases blood glucose levels (32). VMH stimulation also increases plasma glucagon levels and decreases plasma insulin levels, increases plasma epinephrine, and increases plasma free fatty acids and glycerol (32). Epinephrine is a potent stimulator of lipolysis and also suppresses insulin release (11). VMH stimulation has also been shown to increase activity in efferent sympathetic fibers to the liver and pancreas, suggesting a direct effect on these target organs (32) However, trans-neuronal tract-tracing studies using pseudorabies virus have found no connections between the VMH and pancreas or white adipose tissue and very few VMH

neurons were labeled after injection of the virus into the adrenal medulla or the liver (33,34). The VMH is known to project to the autonomic portion of the PVH (34), and the PVH, in turn, has been shown to with the viral-tracing technique to project to both the adrenal medulla and liver (34,35). Viral tract-tracing experiments in white and brown adipose tissue have shown neuronal staining in the PVH, SCN, and DMH (33,35)

Stimulation of the LHA increases hepatic glycogen synthase and decreases PEPCK activity, and also increases plasma insulin levels. This results in increased glycogenesis and hypoglycemia presumably through activation of hepatic and pancreatic parasympathetic efferents (32) Tract-tracing experiments with pseudorabies virus in the liver showed a large number of labeled neurons in the LHA (34).

The time-dependency of glucose tolerance and the hyperglycemic response to 2DG injections may be due to a variation in SCN neuronal output to autonomic centers in the hypothalamus in the day period versus the night. Spontaneous SCN neuronal activity is high during the day and low during the night (36,37). It has been proposed that the SCN actively suppresses insulin secretion, and increases plasma epinephrine and glucagon levels during the day by increasing sympathetic nervous system outflow (38) This would allow maintenance of blood glucose levels through increased liver glycogen breakdown and gluconeogenesis and decreased glucose uptake by peripheral tissues during the time when a nocturnal animal is at rest and does not feed. At night, when

SCN neuronal activity is low, sympathetic nervous system outflow is not augmented During this period the animal is active and food intake supplies the brain and peripheral tissues with glucose and allows replenishment of liver glycogen and adipose tissue stores and. A simplified schematic of SCN regulation of metabolism during the day and night periods is shown in Figure 1

Intrinsic Rhythms of The SCN And Its Modulatory Inputs

In vitro brain-slice experiments have shown that the SCN displays a near 24-hour rhythm in neuronal activity (36). Spontaneous neuronal activity in the SCN peaks at midday and is low during the nighttime. This activity rhythm persists for several days *in vitro*, with the peak of activity occurring at the same time each day. The basis for the spontaneous activity rhythm is not completely understood, but recent research suggests that the internal timing mechanism is molecular in nature. For example, both the mRNA and protein products of such genes such as *period* and *bmal* have been shown to oscillate in a circadian manner in the SCN, and light pulses can change the phase of these oscillations [for a recent review see (39)]

The phase of the SCN pacemaker, as shown by both *in vivo* and *in vitro* experiments, can be altered by internal and environmental zeitgebers (time-givers). Light is the primary environmental zeitgeber (40). Light pulses given to an animal during the night cause large phase shifts in circadian rhythms (7).

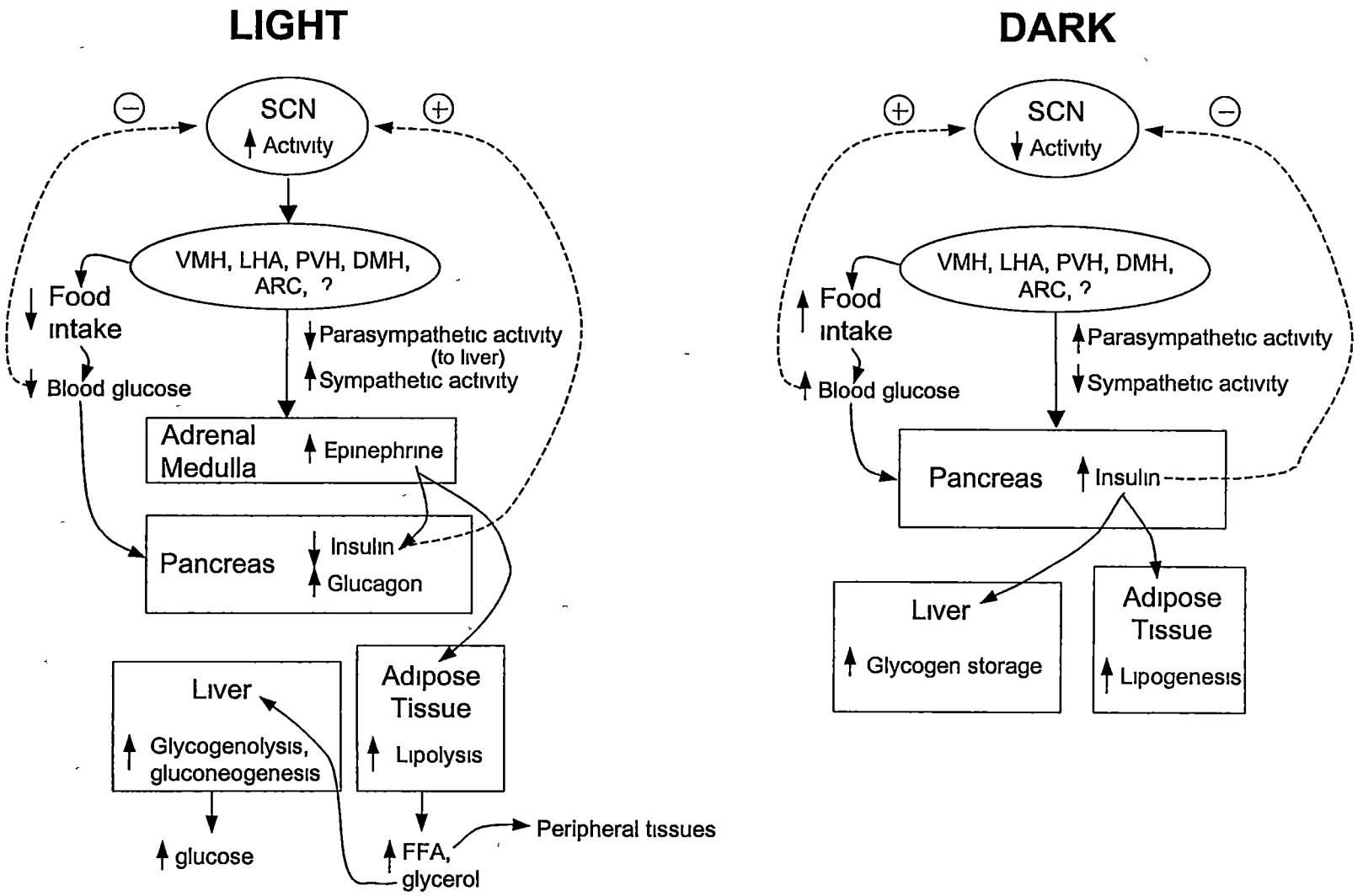


Figure 1. Schematic Showing SCN Modulation of Autonomic Nervous System Activity

Nutritional Status as a Zeitgeber

Nutritional status has been implicated as a potential non-photic zeitgeber. While food-restricted schedules are not generally thought to modulate diurnal rhythmicity through affecting the SCN pacemaker (41), recent studies indicate that both food deprivation (hypocaloric feeding) and decreased glucose availability can alter the phase of SCN driven rhythms (42-44). Mice kept on a hypocaloric restricted feeding schedule show a phase advance in the onset of nocturnal locomotor activity compared to mice fed a normal calorie diet on a 12:12 LD schedule (42). Injections of 2DG into rats kept in constant darkness decreased the amount of phase shifting in locomotor activity rhythms induced by light pulses (44)

Restricted feeding schedules, either *ad libitum* feeding during the light period only or 1-4h food presentation during the light period, on the other hand, do not alter the phase of the SCN pacemaker or the normal onset of locomotor activity at lights off (42,45). When animals are exposed to restricted feeding schedules, an activity bout separate from the normal nocturnal activity rhythm develops 1-4h before the food presentation time. This is called food-anticipatory activity and is not believed to be regulated by the SCN as it persists after SCN lesions (46,47). Instead, it has been postulated that a second food-entrainable oscillator exists outside the SCN, but is coupled to the cycle of the SCN under normal conditions (47,48). The locus of this food-entrainable oscillator has not

been determined, although both central and peripheral locations have been explored.

Other metabolic disturbances can also cause disruptions in the normal feeding pattern of rats. Lesions in the VMH cause hyperphagia, hyperinsulinemia, and obesity. Nocturnal rodents with VMH lesions also show a loss of circadian feeding behavior during the early dynamic phase of weight gain (31,49,50) Genetically obese rodents, such as Zucker (*fa/fa*) rats and diabetic (*db/db*) mice, show behavioral and endocrinological disruptions similar to VMH lesioned animals. Both the *fa/fa* rats and *db/db* mice have a mutation in the gene coding for the leptin receptor (51,52). These animals have elevated blood glucose levels and hyperinsulinemia, are hyperphagic and develop severe obesity (53). The nocturnal feeding pattern of the fatty rats is lost with the progression of obesity (54-57). However, in obese (*ob/ob*) mice, which lack functional leptin protein (58), circadian feeding patterns and activity rhythms are not as disrupted as they appear to be in fatty rats (59)

Leptin

Leptin is a hormone secreted by adipose tissue that both informs the brain about adipose stores and is involved in regulating feeding behavior and metabolic rate (58,60,61). Leptin protein production has been localized primarily to adipose tissue, although leptin mRNA has also been found in the stomach (62), placenta, and brain (63,64). Areas of the brain where leptin

immunoreactivity is found include the PVH, the nucleus of the solitary tract, and the anterior pituitary. The potential role leptin might have as a neurotransmitter is not known, but conceivably could be quite distinct from its role as an indicator of satiety or nutritional status

Leptin administration to rats and mice, both intraperitoneal and intracerebroventricular, causes reduced food intake and weight loss, indicating that leptin acts as a satiety signal (65,66). Leptin has been shown to increase sympathetic activity outflow from the hypothalamus. Injection of leptin into the VMH increases blood glucose uptake in peripheral tissues (67), and both inhibits insulin and augments glucagon release after a glucose load (35,68). Leptin also increases the rate of lipolysis in brown and white adipose tissue (69,70).

Some of these effects of leptin originate from leptin binding to hypothalamic neurons, although leptin also has direct effects on skeletal muscle, pancreatic β-cells, and adipose tissue (71-78). Reported effects of leptin application to neurons in some hypothalamic areas have been conflicting. Leptin has an inhibitory (hyperpolarizing) effect on some glucose-responsive neurons in the arcuate nucleus (79-81), but an excitatory (depolarizing) effect on neurons in the lateral arcuate nucleus, an area that is known to project to sympathetic preganglionic neurons in the thoracic spinal cord (82). Leptin depolarizes rat hypothalamic parvicellular PVH neurons that project to parasympathetic and sympathetic preganglionic neurons (80,83,84). Glucose-responsive VMH neurons were activated by leptin in two experiments (80,81), but inhibited by

leptin in another (79). The activity of some glucose-responsive LHA neurons was inhibited by leptin application (80,81)

These conflicting results are difficult to explain. One possible explanation could be the recording and/or drug application techniques used. In the VMH experiments, Shirashi, *et. al.* (80) used electrophoretic application of leptin and extracellular recordings, while Spanswick, *et.al.* (79) used whole-cell and perforated patch-clamping techniques with bath application of leptin. The bath-applied leptin may have affected neurons in the slice preparation outside the VMH that made synaptic contacts with VMH neurons, so that the effects of leptin in this experiment may not have been direct.

Leptin levels in mice and rats also show a circadian fluctuation where plasma levels are low during most of the light phase, when the animals are at rest, and rise steadily after the onset of the dark phase (85-87). A recent experiment in mice showed that the circadian variation in plasma leptin is controlled by food-intake rhythms, rather than the light/dark cycle. Under *ad libitum* feeding conditions, plasma leptin increases after the onset of the dark phase, reaching a peak during the late dark/early light phase, followed by a decrease shortly after lights on. When *ad libitum* feeding is restricted to the light cycle, the nadir of plasma leptin is shifted to the beginning of the light period (85). Leptin mRNA levels in adipocytes have been also shown to rise acutely after food intake (88).

The circadian rhythm of plasma leptin in rats has been reported in two studies. For rats kept on a 14h:10h light/dark cycle adipocyte leptin mRNA levels peaked 4 hours after lights off (ZT18), and the plasma leptin peaked at the same time (86). In a second study, plasma leptin levels also peaked at ZT18. However, these animals were kept on a 12h:12h light/dark cycle, and so the plasma leptin peak in occurred 6 hours after lights off (87).

Plasma leptin levels have typically been reported as averages over the entire 24h cycle with values ranging from 1-8 ng/ml in rats (89,90). However, for leptin to exert its effects in the hypothalamus it has to cross the blood-brain barrier and there is very little data showing what the leptin concentration is in the central nervous system of rodents. A recent experiment reported the cerebrospinal fluid leptin levels in lean Zucker rats (*Fa/Fa*) to be about 1/400 of the plasma leptin level, or 0.18 ng/ml (91). Interestingly, this experiment found little correlation between serum leptin and cerebrospinal fluid leptin levels. Leptin levels in cerebrospinal fluid were not statistically significant between lean and obese Zucker rats despite significant differences in plasma leptin, which suggests there is a saturable system of leptin transport into the central nervous system

The Leptin Receptor

The leptin receptor is a single transmembrane domain receptor belonging to the class I cytokine receptor family. There are six known isoforms created by alternative splicing - OB-R_a through OB-R_f (52,92-94). OB-R_{a-d} and OB-R_f

possess a putative 20 amino acid transmembrane domain. OB-R_a has a short cytoplasmic domain and is the most common form found in peripheral tissues such as the liver, kidneys, and lungs (95,96). In the brain, the greatest quantity of OB-R_a occurs in brain microvessels and choroid plexus. Here it is thought to form a saturable system of leptin transport between these structures and the brain circulation (97,98). OB-R_c, OB-R_d, and OB-R_f also possess a short cytoplasmic domain, but their importance in leptin function has so far been controversial and undefined (93,94,99). OB-R_d has only been described in the mouse (100), and OB-R_f has only been found in the rat (99). OB-R_b possesses a long cytoplasmic domain that contains sequence motifs similar to other cytokine receptors, including Janus kinase (JAK) and STAT (**S**ignal **T**ransducers And **A**ctivators Of **T**ranscription) protein interaction domains. This isoform is thought to be the primary signaling form of the receptor and is found in greatest quantities in hypothalamic nuclei known to be involved in regulating feeding behavior and energy homeostasis (95,101,102). The OB-R_e isoform lacks the transmembrane domain and is a secreted, soluble protein found in plasma. Proposed functions for this isoform are that it acts as a transport system or inhibitory chelator of the leptin protein in blood (92)

Leptin receptors have been localized in both the rat and mouse brain through leptin receptor immunoreactivity, reverse-transcription/polymerase chain reaction (RT-PCR), and *in situ* hybridization studies to many hypothalamic areas known to be associated with feeding behavior and autonomic nervous system

outflow including the DMH, VMH, ARC, PVH, and LHA (103,104). While some of these studies indicate that the SCN contains positive leptin receptor immunoreactivity (105,106), most do not mention the SCN.

Leptin Signaling

The long form of the leptin receptor, OB-R_b, is thought to be the primary isoform mediating the effects of leptin in the hypothalamus effects of leptin. Most studies of leptin signaling have been done *in vitro*, using transfected cells, cell cultures, and hypothalamic slice preparations. Leptin has been shown to activate Janus kinase 2 (JAK-2), STAT1, and STAT3 proteins in transfected cells (107-109), and mitogen-activated protein kinase (MAPK) in a cultured cell line (110). Leptin has also been shown to activate STAT proteins in hypothalamic slices (103,111). JAK2 and STAT3 were both activated by leptin in cultured myotubes (73,112). *In vivo*, leptin has been shown to activate STAT3 (111,113).

Based on the results of the above experiments, and the known signal transduction pathways of other cytokine receptors, a general scheme for leptin signal transduction via the long form of the receptor (OB-R_b) has been proposed. Leptin receptors undergo homo-oligomerization upon ligand binding (114,115), and then activate JAKs through membrane recruitment to the dimerized receptor. The activated JAKs autophosphorylate and then phosphorylate STAT proteins. Phosphorylated STAT proteins form homo- or heterodimers and translocate to the nucleus where they act as transcription factors (103,116). Receptors cloned

from the *db/db* mice have a truncated intracellular domain and are unable to activate STAT proteins, which may be a major cause of the observed phenotype of these animals (108,113).

The signal transduction pathway for the acute effects of leptin has been worked out in the most detail in pancreatic β-cells In β-cells leptin appears to act in part through activation of ATP-sensitive potassium channels (K_{ATP}) (75). K_{ATP} channels in pancreatic β-cells are composed of two subunits, an inner core of an inwardly rectifying potassium channel subunit ($K_{ir}6.2$) and an outer sulfonylurea-1 (SUR1) channel subunit (117). In resting β-cells, the K_{ATP} channel is open, keeping the cell hyperpolarized. Leptin application increases the probability that this channel will be open, causing increased hyperpolarization of the cell and a corresponding decrease in insulin release (74,75). Leptin is thought to activate K_{ATP} channels by phosphoinositide-3 kinase activation of phosphodiesterase-3B and subsequent depression of cAMP levels (118,119). Both tyrosine kinase inhibitors and non-hydrolyzable cAMP analogs reverse the hyperpolarizing effect of leptin on pancreatic β-cells (118-120). Leptin was also shown to increase STAT protein binding to DNA in isolated islets and cultured insulinoma cells (121,122).

It may be that the glucose-responsive neurons in the hypothalamus are affected by leptin in the same manner. Hypothalamic glucose-sensitive neurons have also been shown to contain K_{ATP} channels (79,123,124). In the VMH; these channels contain an inner core of an inwardly rectifying potassium channel

subunit ($K_{IR}6.1$) and an outer SUR1 channel subunit, similar to pancreatic β-cells (123). Glucose-sensitive SCN cells also contain sulfonylurea-sensitive and calcium-dependent K^+ channels that are inhibited by ATP, although the channel subunits have not been determined (23). These data suggest that leptin may modulate neurons in the hypothalamus in a manner similar to that shown for pancreatic β-cells.

Rationale For This Study

Because the SCN controls circadian rhythms of feeding and sympathetic nervous system activity, and SCN output rhythms are affected by metabolic status, we hypothesized that leptin may modulate the phase of the SCN pacemaker as a feedback from adipose stores. Therefore, we investigated whether leptin application phase-shifts the circadian pacemaker *in vitro*. Furthermore, since reports of leptin receptor localization in the SCN are inconsistent, we used immunocytochemical techniques to investigate whether leptin receptors are localized in the SCN.

Chapter II

Materials and Methods

Single-Unit Recording Experiments

Coronal brain slices (500μm) containing the SCN were prepared from adult male Sprague-Dawley rats housed in a 12:12 LD cycle. The slices do not contain any of the hypothalamic areas known to be a target of leptin signaling such as the ARC, PVH, or SON. In some experiments, the slices were physically reduced such that only the SCN and optic chiasm were present. All slices were prepared during the lights-on period. The slices were placed in a Hatton-style interface brain-slice chamber and perfused continuously with warm (37°C), oxygenated (95% O_2/5% CO_2) Earle's Balanced Salt Solution (Sigma) supplemented with glucose and bicarbonate, and brought to pH 7.4 (125).

Leptin (R & D Systems, Minneapolis, MN) was bath-applied on day 1 *in vitro* for 1 hour. During drug application the normal perfusion was stopped and the medium in the chamber was replaced with medium containing leptin. After the treatment period, the treated medium was exchanged with normal medium and perfusion was resumed.

Extracellular recordings of single-unit activity were obtained on day 2 *in vitro* using glass microelectrodes filled with 3M NaCl. The activity of each neuron was recorded for five minutes and the data stored using a Data*Wave* system. Five to fifteen cells were recorded each hour. Individual firing rates were grouped into two-hour running averages to obtain a measure of population neuronal

neuronal activity, as in previous studies (125,126). The time of peak activity was defined as the time of symmetrically highest activity over the entire recording period. Phase shifts were calculated as the difference in time-of-peak seen in leptin-treated slices from that seen in untreated controls (6.05 ± 0.2, n=5). Student's *t* tests were used to test for significant differences in time-of-peak between treatment and control experiments. A one-way ANOVA was used to test for significant differences in amount of phase shifting between different applied leptin concentrations.

Leptin Receptor Immunohistochemistry

Male Sprague-Dawley rats (300-360g) were housed on a 12:12 LD schedule. Animals were perfused intraventricularly at ZT8.5-9 with 75ml heparinized normal saline, followed by 200ml of fixative containing 4% paraformaldehyde, 0.08% glutaraldehyde, and 15% saturated picric acid in 0.1M phosphate buffer (PB), pH 7.4. The animals were then perfused with 100ml 5% sucrose in PB. The brains were removed and incubated overnight in glutaraldehyde-free fixative and 10% sucrose in PB. The tissue was cryoprotected further in 20% sucrose and 0.01% sodium azide (NaN_3) in PB for 1 day, then 30% sucrose in 0 01% NaN_3 and PB for 4 days.

30μm coronal sections were cut with a cryostat and collected in ice-cold PB. Sections were permeabilized 30 min in 0.1% Triton X-100 (TX-100) and 0.01% NaN_3 in PB. Sections were rinsed briefly in PB, then incubated in 5% normal rabbit serum (NRS) in 0.1% TX-100 and 0 01% NaN_3 in PB for 30 min to

block non-specific binding. Endogenous peroxidase activity was blocked by a 30 min incubation in 0.3% H_2O_2 in 0.1% methanol, 0.1% TX-100, and 0.01% NaN_3 in PB. Sections were rinsed two times in PB for 10 min each, then incubated in primary antibody. Two leptin receptor antibodies were used, K-20 and B-3 (sc-1835 and sc-8391; Santa Cruz Biotechnology, Santa Cruz, CA). The polyclonal K-20 antiserum epitope is common to all splice variants of OB-R and is contained within the extracellular domain of the receptor (amino acids 32-51). B-3 is monoclonal and its epitope lies within the cytoplasmic region of the membrane-bound receptors (OB-R_{a-d}, OB-R_f), close to the transmembrane domain (within amino acids 877-894). Both antibodies were used at a 1:500 dilution in 3% NRS, 0 1% TX-100, and 0.01% NaN_3 in PB. As a control, some sections were incubated in the same solution without the primary antiserum.

Following incubation with agitation for 24h at room temperature, then at 4°C overnight, sections were rinsed three times in PB and then were further processed using the avidin-biotin-peroxidase (ABC) method (Vectastain ABC kit, Vector Labs, Burlingame, CA). Histochemical visualization of peroxidase was done using 0.01% 3,3'-diaminobenzidine (DAB) as a chromogen. Sections were rinsed in PB, mounted on gelatin-coated slides and dried overnight. Slides were serially dehydrated in increasing ethanol concentrations, cleared, and mounted in Permount. Sections were photographed with an Olympus BH2 microscope and images were scanned from negatives to a digital TIFF format.

Chapter III

Results

Single-Unit Recording Experiments

Application of leptin (10nM) at ZT10 induced a phase advance of approximately 2 hours as compared to control experiments (Figure 2). These phase advances persisted as shown by recording neuronal activity on day 3 (data not shown), suggesting that the underlying circadian pacemaker was shifted by these treatments. Application of leptin (10nM) at other time points during the subjective day and early to mid-subjective night also induced phase advances of approximately 2h (Figure 3). However, leptin had no significant effect on the phase of the neuronal activity rhythm when applied at ZT22. Table 1 summarizes the data from these experiments

The phase advances induced by leptin (10nM) application at ZT10 on reduced slice preparations were not statistically different ($p>0.5$) from those obtained with the normal slice preparation (3.25±0.0, n=2). This further narrowed the site of leptin action to the SCN. These results are included in the data shown in Figure 3 and Table 1.

We also investigated the dose-dependence of leptin application on the phase of the SCN neuronal activity rhythm. Application of leptin at a concentration of 1nM did not induce significant phase advances. Application of

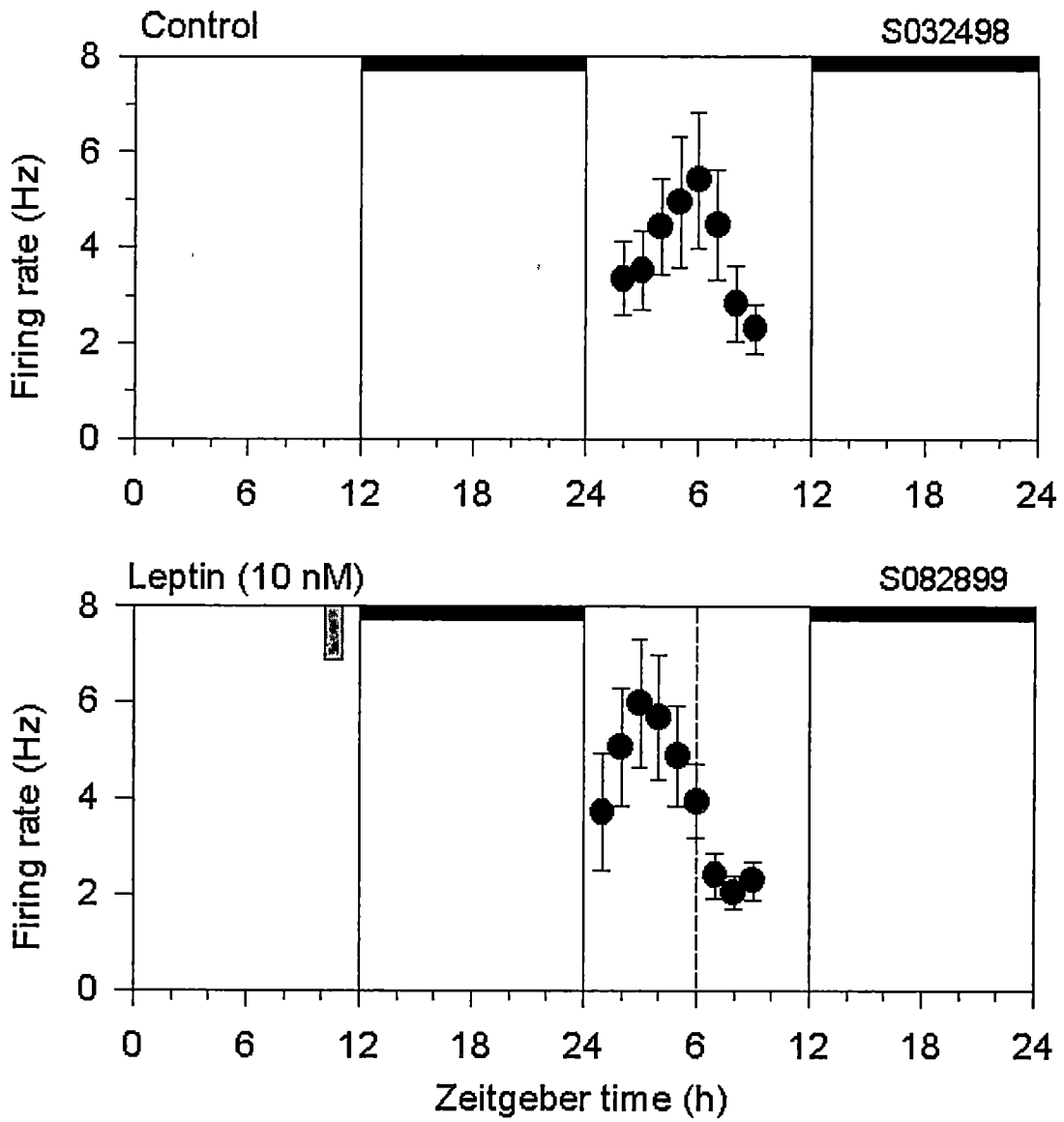


Figure 2 Leptin phase-advances the SCN pacemaker at ZT10 Plotted are the two-hour means ±SEM for two experiments Top A control experiment performed without any drug treatment shows a peak in neuronal activity at ZT6 Bottom Application of leptin (10nM) at ZT10 causes a phase-advance of approximately 2 75h so that neuronal activity peaks near ZT3 Horizontal bar time of lights-off in animal colony Vertical bar time of drug application Dashed line mean time-of-peak in control experiments

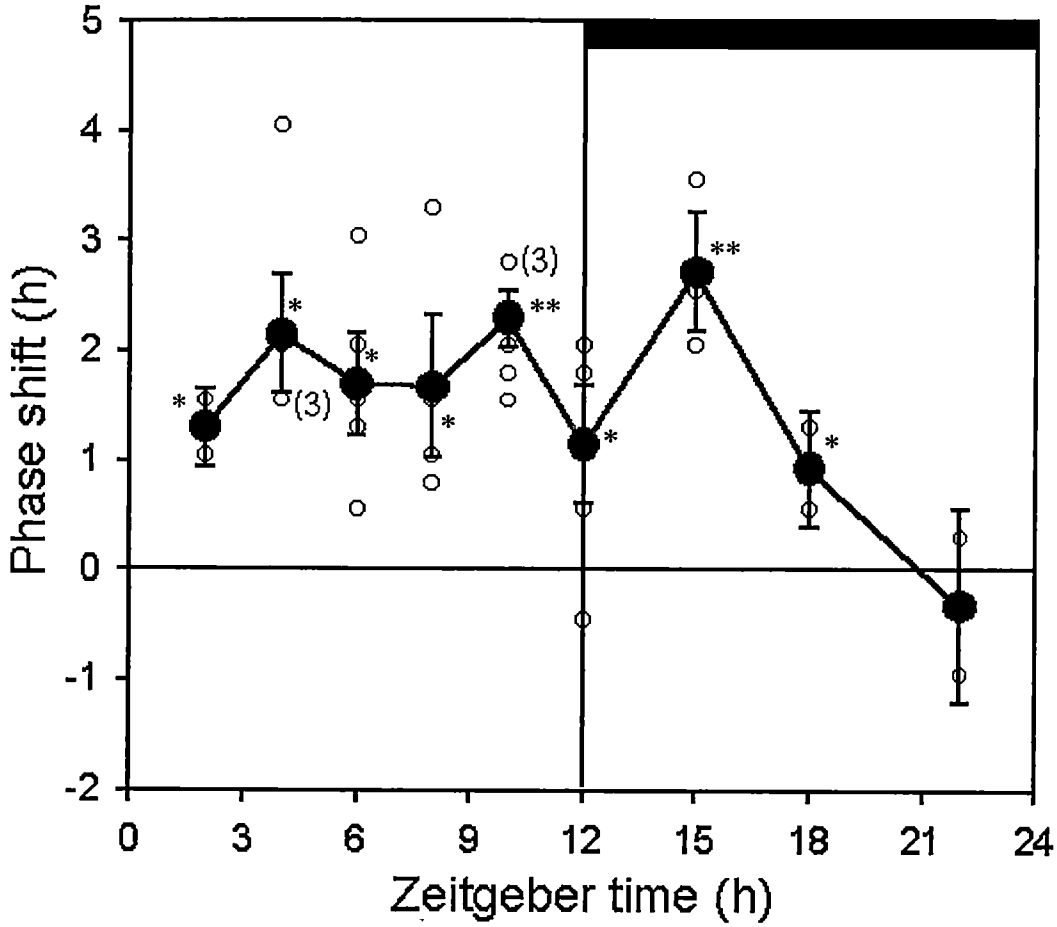


Figure 3 Phase response curve for leptin (10nM) The *in vitro* phase-shifting effects of leptin applied at different time points are shown as mean phase-shift ± SEM from control experiments (closed circles) The results of individual experiments are shown in open circles Numbers in parentheses indicate the number of experiments represented by one data point Mean phase advances of 1-3h occurred in response to leptin administration at all times except ZT22, when no consistent phase-shifting occurred Black bar time of lights-off in animal colony

* - $p < 0.05$ relative to control, ** - $p < 0.001$ relative to control

Table 1. Phase-shifting effects of leptin bath applied to SCN brain slices for 1 h

Treatment	Time of drug application (ZT)	Number of Experiments	Change in time-of-peak (h)*
Leptin (10nM)	2	2	1.30 ± 0.35
	4	5	2.15 ± 0.54
	6	5	1.70 ± 0.46
	8	4	1.68 ± 0.65
	10	6	2.30 ± 0.25
	12	5	1.15 ± 0.53
	15	3	2.72 ± 0.54
	18	2	0.93 ± 0.53
	22	2	-0.33 ± 88
Leptin (1nM)	10	3	0.05 ± 0.18
Leptin (100nM)	10	3	2.38 ± 0.82

* - relative to control (6.05±0.2h, n=5)

100nM leptin did not increase the amount of the phase advance relative to the 10nM concentration (Figure 4)

Leptin Receptor Immunohistochemistry

Leptin receptor immunoreactive (LR-IR) neurons were found widely distributed throughout the hypothalamus. Both antisera gave similar results The B-3 monoclonal antibody to the membrane-bound receptors showed intense staining in the arcuate nucleus (Figure 5a), the magnocellular and parvicellular parts of the paraventricular nucleus (Figure5e), and supraoptic nucleus (not shown). Lighter staining was seen in the dorsomedial nucleus, and ventromedial nucleus (Figure 5a), periventricular nucleus, and lateral hypothalamic area (not shown). Positive LR-IR was also clearly seen in the suprachiasmatic nucleus, although the staining was less intense than that seen in the ARC and PVH (Figure 5c).

The K-20 polyclonal antibody gave essentially the same results, showing positive LR-IR in the SCN, PVH, and ARC (Figure 5a, d, f). These results are consistent with previously published experiments using this antibody (96,105,127). The staining pattern with this antiserum was more diffuse, suggesting a greater cytoplasmic component.

Control sections for experiments with both the K-20 and B-3 antisera did not show any positive leptin receptor immunoreactivity (data not shown).

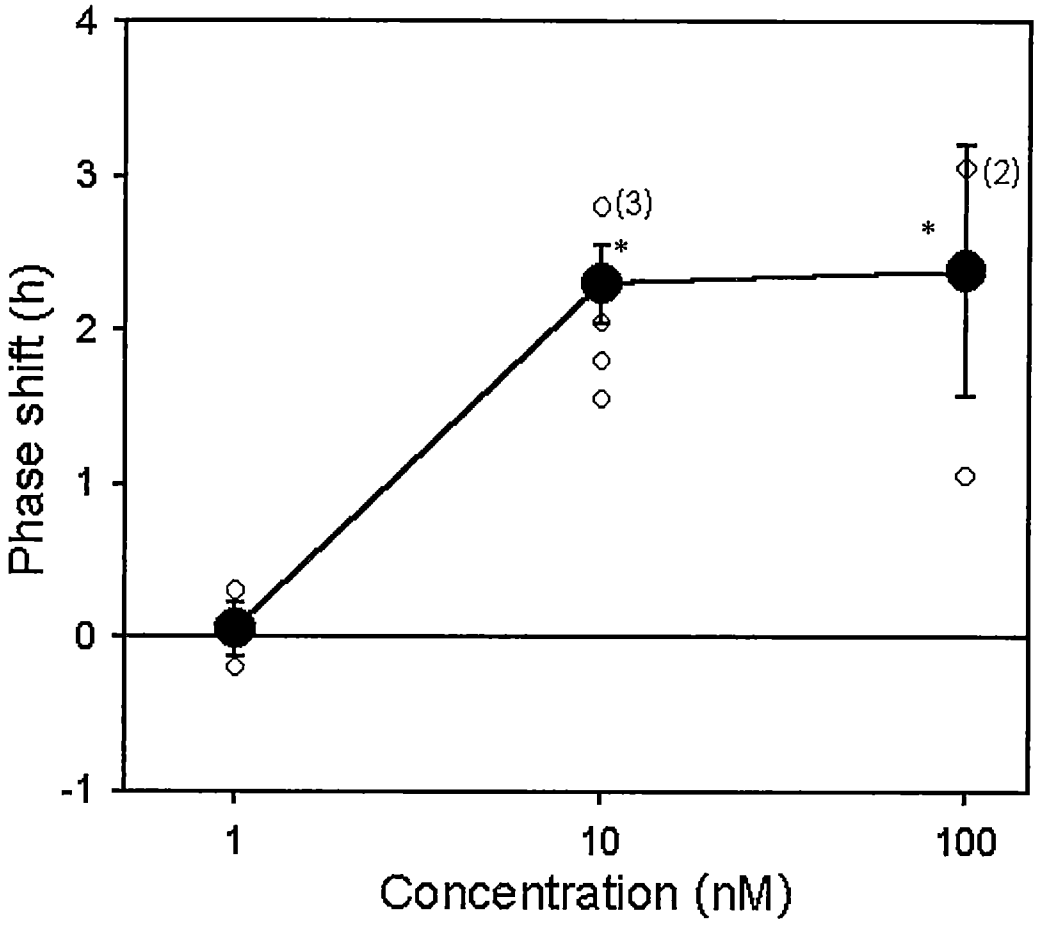
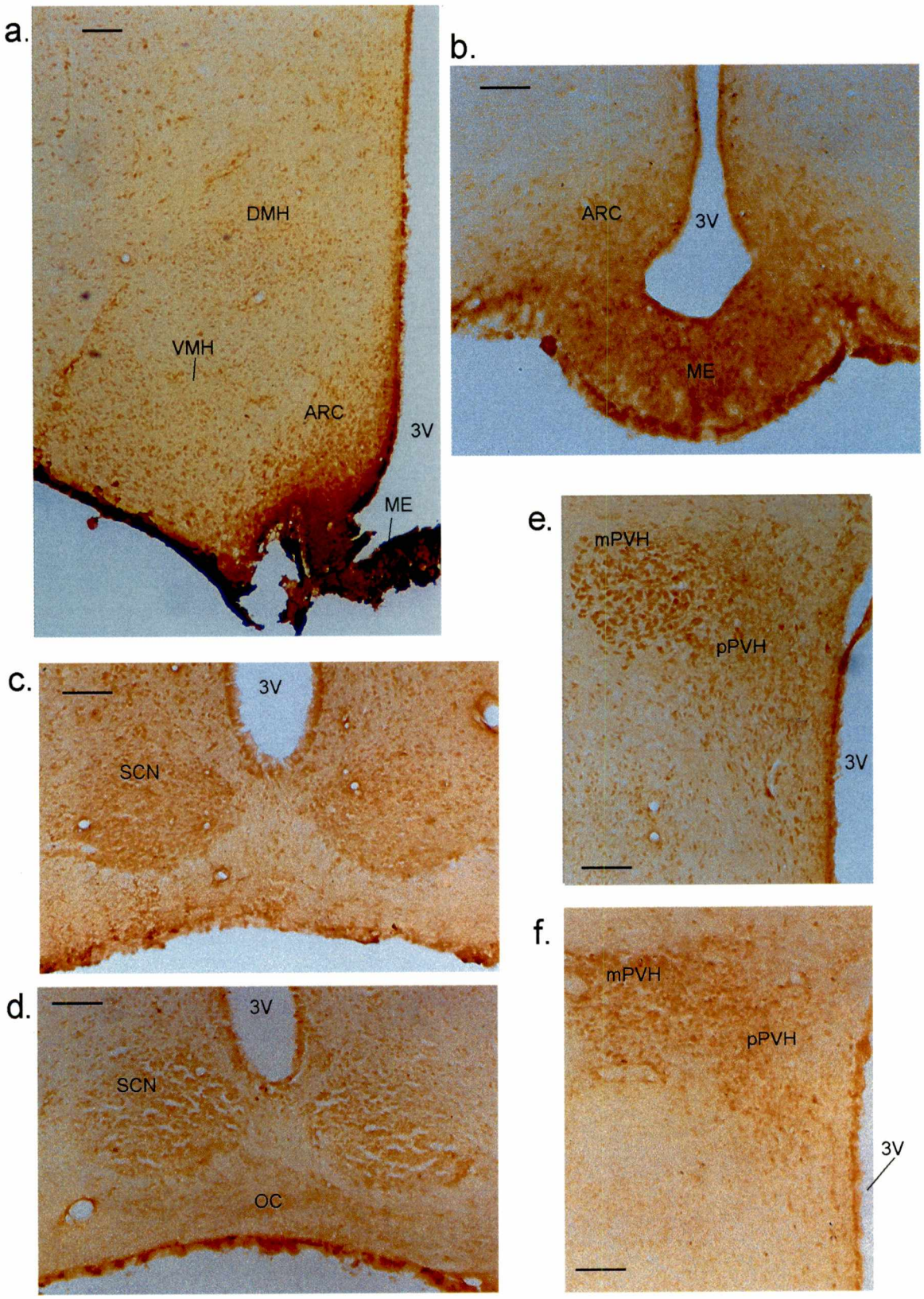


Figure 4 Dose response curve for leptin Experiments were performed with 1nM, 10nM, and 100nM leptin applied at ZT10 Black circles show mean phase-shifts ± SEM Individual experiments are shown in open circles Numbers in parentheses indicate the number of experiments at that data point 1nM leptin did not induce phase-advances at ZT10 The 100nM leptin did not induce phase-advances significantly different from those induced by 10nM leptin ($p>0\ 05$)

*- $p < 0\ 05$ vs 1nM concentration

Figure 5. Leptin receptor immunoreactivity with B-3 (a, c, e) and K-20 (b, d, f) antibodies. Leptin receptor immunoreactivity was seen in the arcuate nucleus (ARC, a, b), suprachiasmatic nucleus (SCN; c, d), and magnocellular PVH (mPVH) and parvocellular PVH (pPVH; e, f). The K-20 antibody shows more diffuse staining in the PVH and ARC, suggesting a greater cytoplasmic component (b, f) DMH, dorsomedial nucleus; VMH, ventromedial nucleus; 3V, third ventricle; OC, optic chiasm. Scale bar: 100μm.


a.
DMH
VMH
ARC
3V
ME
b.
ARC
3V
ME
c.
3V
SCN
d.
3V
SCN
OC
e.
mPVH
pPVH
3V
f.
mPVH
pPVH
3V

Chapter IV

Discussion and Future Directions

The results of the *in vitro* phase shifting experiments show that leptin phase-advances the rhythm of suprachiasmatic neuronal activity during the subjective day and during the early portion of the subjective night and that these phase shifting effects show a dose-dependency at ZT10. Therefore, the phase of the SCN circadian pacemaker appears to be sensitive to modulation by leptin.

Our leptin phase response curve is unusual in that it consists solely of phase advances. The only time there is no effect of leptin *in vitro* is near the time when endogenous leptin should be at its highest level. This suggests that the rhythm in endogenous leptin levels may be driving the rhythm in leptin sensitivity in the SCN. In rats, plasma leptin levels rise steadily after the large feeding bout that begins at lights off (ZT12) and peak 4-6h later at ZT18 (86,87). Comparison of reported leptin levels in rats and our results suggests a slight disparity between the highest plasma leptin levels (ZT18) and the time of leptin insensitivity (ZT22). Part of this difference may be due to the time that it takes for leptin to cross the blood-brain barrier and reach effective levels in cerebrospinal fluid. Measurement of plasma leptin levels and/or cerebrospinal leptin levels in our animal colony may help to clear this discrepancy.

It would be interesting to test whether the plasma leptin rhythm is driving the rhythm in SCN sensitivity to leptin. This could be tested by putting the animals on different restricted-feeding schedules, which should shift the rhythm

in plasma leptin, but not shift the SCN circadian pacemaker. We could then generate a leptin phase response curve in order to see whether the SCN sensitivity to leptin is phase shifted. Concurrent measurement of plasma leptin levels and/or cerebrospinal fluid leptin levels in the animal colony would be important in order to compare the rhythms of plasma leptin and SCN pacemaker sensitivity to leptin.

Previous studies have shown that the circadian rhythm of plasma leptin is entrained to feeding, such that plasma leptin levels increase to maximum levels 3-4h after feeding (85). When food is only available during the day, endogenous leptin levels peak during the day instead of at night. Our results suggest that an early peak in leptin levels in response to an early bout of feeding could act to reinforce a daytime feeding schedule by shifting the SCN circadian pacemaker to a slightly more advanced phase, thereby allowing the animal to change its normal activity pattern.

However, Inouye (41), using *in vivo* multi-unit activity SCN recordings found that restricted feeding schedules do not change the phase of the SCN pacemaker. Other studies also indicate that the phase of the SCN circadian pacemaker is not modulated by restricted feeding schedules (42,128). The differences between our findings and those of previous studies may result from a greater sensitivity of the pacemaker to *in vitro* phase-shifting stimuli due to the loss of modulatory SCN inputs that are severed in the *in vitro* brain-slice preparation. Also, since the amount of phase shifting seen in our experiments is

relatively small (approximately 2h) compared to phase shifts induced *in vitro* by other stimuli, it is likely that leptin signaling plays a minor role in modulating SCN pacemaker activity *in vivo*. *In vivo* experiments with leptin microinjection into the SCN and measurement of behavioral, neuroendocrine, and metabolic rhythms would help to elucidate the role of leptin signaling in the SCN.

Experiments using animals with impaired leptin signaling may also provide some insight in the role of leptin signaling in the SCN Zucker fatty rats lack a functional long form of the leptin receptor (OB-R_b), which is considered to be the primary signaling isoform in the hypothalamus (129) and show a loss of circadian locomotor activity and food intake rhythms with the development of obesity (56). It would be interesting to determine if this behavioral arhythmicity reflects a loss of function of the SCN pacemaker itself, or if the hyperphagia merely obscures the underlying rhythmic output of the clock. To answer this question, *in vitro* single-unit activity recordings of brain-slices from Zucker fatty rats, diabetic (db/db) mice, which lack a functional OB-R_b isoform, or obese (*ob/ob*) mice that lack functional leptin protein could be performed. If the hyperphagia is a masking effect, we would not expect the underlying SCN neuronal activity to be altered in these animals. If the hyperphagia and food intake in the light period is a product of impaired SCN output, we might possibly see phase changes in the neuronal activity rhythm, dampened rhythms, or no rhythmicity at all. *In vivo* multi-unit activity recordings would also show if these animals have altered SCN neuronal

activity rhythms. *In vivo* multi-unit activity recordings would also show if these animals have altered SCN neuronal activity rhythms.

In addition to the effects on the circadian clock, leptin signaling in the SCN might also be involved in modulating sympathetic nervous system activity. If this is the case, then impaired leptin signaling in the SCN may contribute to the observed phenotype seen in fatty rats or diabetic mice. SCN lesions in normal rats produce higher basal plasma insulin levels, greatly reduced glucagon levels, and glucose levels are maintained at a minimum level over the 24 hour circadian cycle (20). SCN lesions in animals with disrupted leptin signaling may help elucidate what role leptin signaling in the SCN has in the development of the observed hyperinsulinemia, hyperglycemia, and obesity.

It is also possible that the actions of leptin in the SCN may be unrelated to nutritional status or metabolic regulation. If the brain produces its own leptin, then it may function in neural pathways not directly associated with feeding. For example, leptin has been found to play a role in neuroendocrine regulation, immune function, and hematopoiesis (53,130). Although several reports have demonstrated leptin-immunoreactivity in the brain, the SCN has not been described as a potential site for leptin production or neurotransmitter input. Ultra-structural histological experiments would have to be done to determine if the SCN receives inputs from any axon terminals containing leptin.

Whatever the physiological role leptin may have in its effects on the SCN, the *in vitro* SCN slice preparation could be an excellent model system for

examining the leptin-signaling pathway(s) in the brain. Thus far, no patch-clamp or intracellular recording experiments of leptin application on SCN cells have been done, but it would be interesting to discover whether leptin depolarizes or hyperpolarizes SCN neurons. If SCN cells are hyperpolarized by leptin, experiments similar to those performed on isolated β-cells and insulin-secreting cells could be performed to determine if similar signal transduction pathways are stimulated by leptin in the SCN. The SCN is known to contain glucose-sensitive neurons that contain ATP-sensitive K^+ channels (23). The involvement of the JAK/STAT or other signal transduction pathways in leptin's phase-shifting effects in the SCN could also be explored *in vitro* with the use of selective protein inhibitors.

Our immunocytochemistry results with the K-20 antibody are consistent with those seen in previous experiments in rats (104,105). We saw strong positive immunoreactivity in the arcuate, paraventricular, and supraoptic nuclei and lighter staining in the suprachiasmatic, ventromedial, and dorsomedial nuclei, lateral hypothalamic area and periventricular nucleus. While our results clearly demonstrate that leptin receptors are located within the SCN, they are not definitive as to which isoforms are present. In the rat, only the isoforms OB-R_a, OB-R_b, OB-R_e, and OB-R_f have been found. The K-20 antibody should react with all of these isoforms. The B-3 antibody, on the other hand, should only recognize isoforms a, b, and f. RT-PCR and *in situ* hybridization experiments have shown that the largest levels of OB-R_f mRNA are found in the spleen and liver and

relatively low levels are found in the hypothalamus (99,131), while OB-R_b and OB-R_a mRNA levels are both high in the hypothalamus (131,132). From these data, we could speculate that the immunoreactivity seen with the B-3 antibody reflects the presence of the a and/or b isoforms.

Differential staining with the two antibodies could point to locations where only the OB-R_e isoform is present. In the arcuate nucleus and paraventricular nucleus, the staining pattern of the K-20 antibody appeared more diffuse than the B-3 antibody, suggesting more cytoplasmic staining and the possible presence of OB-R_e. However, there was no clear difference in staining between the two antibodies in the SCN. From the positive staining seen with B-3, we know that the a and/or b isoforms are likely present. Whether e is present in the SCN cannot be determined. RT-PCR or *in situ* hybridization experiments and western blotting of SCN proteins using the same antibodies would give a clearer picture of the isoforms present in the SCN. There is also now an antibody specific for OB-R_b available and immunocytochemistry using this antibody could determine if this isoform is present.

In conclusion, we have shown that leptin phase-advances the SCN circadian pacemaker. Our immunocytochemistry results confirm the presence of leptin receptors in the SCN, providing further support for leptin acting directly upon SCN cells to produce these phase advances. Additional experiments will be necessary to determine the role of leptin signaling in the SCN, how leptin acutely

affects SCN neurons, what leptin receptor isoforms are present, and what signal transduction mechanisms underlie these effects

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