Functional heterogeneity in brain reward circuitry: Characterization of a subpopulation of reward neurons linked to energy balance

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ABSTRACT

Functional heterogeneity in brain reward circuitry: Characterization of a subpopulation of reward neurons linked to energy balance

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The neural circuitry that gives rise to the rewarding effects of goal-objects and goal-directed behaviors can be directly studied with the use of brain stimulation reward (BSR). Brief trains of electrical stimulation to some brain regions produces a highly rewarding effect that rats will learn to self-administer. Stimulation of some sites in the lateral hypothalamus (LH) has been linked to the regulation of energy balance, supporting the notion that brain reward circuitry is subdivided along functional lines. Finding from the Master's thesis showed that the rewarding effect of the stimulation is enhanced by chronic food restriction and weight loss in some rats. In contrast, a short-term period of food deprivation was unable to increase BSR in these same subjects. The adipose hormone, leptin, mimicked the effect of fattening by opposing the effect of food restriction on BSR. The present body of work examines whether the neurons supporting BSR comprise functionally distinct subcomponents and characterizes a subset of reward neurons that respond to weight loss and leptin. Alterations in the reward effectiveness of LH stimulation were measured following different manipulations of energy state by examining the willingness of the rat to work (lever-press) for varying strengths of stimulation. The first set of experiments in Chapter Two examines the contribution of some neuropeptides known to mediate the actions of leptin on restriction-sensitive and -insensitive reward circuitry. The divergent effect of corticotropin-releasing hormone (CRH) and agouti-related peptide (AgRP) on BSR obtained at restriction-sensitive versus –insensitive sites further suggests that there are separate subpopulations of reward neurons with distinct neurochemical profiles. That CRH, AgRP, neuropeptide Y and melanin-concentrating

hormone largely fail to alter BSR at restriction-sensitive sites suggest that these peptides are not involved in the modulation of BSR by food restriction. In Chapter Three, the ability of food restriction to enhance BSR in a leptin-resistant rat strain suggests that there may be other peripheral signals mediating the actions of weight loss on restriction-sensitive reward circuitry. Finally, data presented in Chapter Four demonstrate that the effect of food restriction on BSR is contingent on the placement of the electrode amongst a functionally heterogeneous population of directly-activated neurons. This evidence provides a basis for the distinction between restriction-sensitive and -insensitive stimulation sites. Together, these data describe a subcomponent of reward circuitry that carries reward signals relevant to the maintenance of long-term energy balance. Unraveling the identity, function and neurochemistry of these neurons will increase our understanding of the neural mechanisms underlying body weight regulation and may shed light on the development of metabolic disorders such as obesity.

CONTRIBUTION OF AUTHORS

This is a manuscript-based thesis. Below is a description of the contribution of the authors to each manuscript.

Chapter Two:

Section 2.1:

Fulton, S., Richard, D., Woodside, B. & Shizgal, P. (2002) Interaction of CRH and energy balance in the modulation of brain stimulation reward. *Behavioral Neuroscience*, 116(4): 651-9.

- I contributed to the design of the experiment, carried out the research, and performed the principal analysis of the data. I wrote the first draft of the manuscript and contributed to subsequent revisions.
- Denis Richard (Département de Physiologie, Faculté de Médecine, Université Laval, Québec) contributed to the design of the project, supplied the corticotrophin-releasing hormone, and contributed to the revisions of the manuscript.
- Barbara Woodside contributed to the design of the experiment, participated in discussions of the results, and provided feedback on all drafts of the manuscript.
- Peter Shizgal was involved in designing the experiments and contributed to the interpretation of the results. He worked on all versions of the manuscript.

Section 2.2:

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- I drafted the design of the experiment, carried out the research, and performed the principal analysis and interpretation of the data. I wrote the first draft of the manuscript and contributed to subsequent revisions.
- Barbara Woodside participated in the design of the experiment and helped prepare of the neuropeptide Y for central injection. She contributed to the interpretation of the data and the revisions of the manuscript
- Peter Shizgal was involved in designing the experiments and contributed to the interpretation of the results. He worked on all versions of the manuscript.

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- I drafted the design of the experiment, carried out the research, and performed the principal analysis and interpretation of the data. I wrote the first draft of the manuscript and contributed to subsequent revisions.
- Barbara Woodside contributed to data analysis and helped revise earlier drafts of the manuscript.
- Peter Shizgal was involved in designing the experiments, interpreting the results, and revising the manuscript.

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- I drafted the design of the experiment, carried out the research, and performed the principal analysis of the data. I wrote the first draft of the manuscript, contributed to subsequent revisions, and contributed to interpreting the results..
- Denis Richard, from Université Laval, supplied the Zucker rats used in this study. Dr. Richard also provided feedback on an earlier draft of this manuscript.
- Barbara Woodside contributed to the design of the experiment, the interpretation of the results, and helped revise earlier drafts of the manuscript.
- Peter Shizgal was involved in designing the experiments, interpreting the results, and revising the manuscript.

Chapter Four:

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- I contributed to the design of the experiment and the dual-electrode assembly and carried out the research. I performed the principal analysis and interpretation of the data. wrote the first draft of the manuscript, and contributed to subsequent revisions.
- Barbara Woodside contributed to the design of the study and helped revise earlier drafts of the manuscript.
- Peter Shizgal was involved in designing the experiments, interpreting the results, and revising the manuscript.

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DEDICATION

To my loving parents: Joy and Michael Fulton. Their courage, strength and accomplishments are the greatest inspiration for this thesis.

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CHAPTER ONE

INTRODUCTION

We recognize pleasure as the first good innate in us, and from pleasure we begin every act of choice and avoidance, and to pleasure we return again, using the feeling as the standard by which we judge every good.

–Epicurus (342-270 BCE) [Letter to Menoeceus]

Epicurus' advice to Menoeceus provides one of the first documented accounts of the role of pleasure in behavior. This portrayal elegantly captures the idea of pleasure as the experience that shapes our evaluations, guides our actions and influences our future choices.

The degree of pleasure that we experience when coming into contact with an object or when engaging in a particular behavior can dramatically influence our approach or avoidance of that object or behavior in the future. Consider how a delicious morsel of food propels you to take another bite or how a warm encounter with a friend increases your desire to share time with that friend again. Pleasurable feelings play a significant role in behavioral choice. For this reason, it is easy to understand why a public eruption ensued from reports that the neural basis of *pleasure* had been identified. The discovery by Olds and Milner (1954) that rats will self-administer electrical stimulation to some regions of their brain was a remarkable beginning to the study of the neural mechanisms of reward ("brain reward circuitry"). The vigor and avidity displayed by rats when performing a task to obtain the rewarding stimulation demonstrates just how powerful and arousing the effect of the stimulation is. Rats will cross electrified grids (Olds, 1958) and forgo feeding in conditions of starvation (Routtenberg & Lindy, 1965; Spies, 1965) in order to maintain contact with the manipulator that triggers the stimulation. This phenomenon,

also known as brain stimulation reward (BSR), is considered to tap into the neural circuitry that conveys the rewarding properties of natural stimuli and behaviors.

The term *pleasure* can refer to a complex experience that involves emotions such as happiness, delight and enjoyment. These feelings can incorporate cognitive processes that are difficult to evaluate and should not be implied in the study of non-human animals. The term "reward", on the other hand, is commonly used in the scientific literature to refer to a more rudimentary and measurable quality of a stimulus or behavior. Nonetheless, in much the same way that Epicurus discerned the influence of pleasure on behavior, rewards too can be defined by certain temporal features. As used here, rewards are goal-objects or goal-directed behaviors that (1) bias an animal to continue ongoing actions, (2) increase the behaviors that lead to the procurement and/or consumption of the reward (positive reinforcement) and (3) direct future actions to result in further attainment of the reward.

Despite the complexity that surrounds the meaning of pleasure and its relevance to the study of BSR, it is interesting that subjective reports from humans receiving electrical stimulation to subcortical structures indicate that the stimulation can induce feelings of pleasure (Bishop, Elder & Heath, 1963; Heath, 1964). In response to stimulation of the septal area, patients would switch from prestimulation expressions of sadness and despair to those of optimism and cheerfulness (Heath, 1964). Moreover, these early investigations in humans revealed that stimulation at different loci could elicit distinct subjective reports. In one patient, septal stimulation was continually associated with positive reports of sexual experiences, both past and anticipated. In contrast, the same patient reported no sexual motive when stimulation

was delivered to the lateral hypothalamus (LH), instead he reported "this is a good feeling" and asked that the stimulation be repeated several times (Heath, 1964). These rare observations in humans provide some of the first suggestions that brain reward circuitry may be subdivided along functional lines.

What is the meaning of the signal produced by the rewarding stimulation? This is one of the long-standing questions in research on BSR. Since the discovery of BSR, there has been an extensive line of research directed towards understanding the identity, characteristics and function of the neurons that give rise to the rewarding effect of the stimulation. On the basis of these studies, there have been several different hypotheses and speculations with respect to the functional nature of reward circuitry. One fundamental postulate divides researchers in the area into one of two factions: (1) those subscribing to the notion that the reward substrate is composed of a functionally heterogeneous population of neurons and (2) those that view the reward substrate as functionally homogeneous. For researchers that support the first position, the rewarding effect of stimulating some sites is related to energy balance, whereas at other sites, BSR is linked to other homeostatic systems or behaviors. Members of the opposing group, however, believe that the rewarding effect of the stimulation is non-specific and that signals related to physiological and reproductive needs provide input to a uniform reward path at points upstream. Until recently, there has been no direct evidence in support of either of these hypotheses.

The data presented herein build upon a set of findings I obtained for the Master's thesis (published article in Appendix) describing the modulation of BSR by manipulations of energy balance. The first experiment replicates previous findings by

showing that chronic food restriction and body weight loss can enhance the rewarding effect of the stimulation at some sites in the LH (Fulton, Woodside & Shizgal, 2000). In contrast, an acute period of food deprivation had relatively little effect on BSR obtained from the same stimulation sites. Central administration of the adiposity hormone, leptin, attenuated BSR but only at stimulation sites that were sensitive to food restriction and weight loss. These earlier findings suggest that there are at least two, functionally specific subpopulations of reward neurons that can be activated by stimulating the LH and one of these subpopulations is modulated by changes in long-term energy signals.

Whether leptin modulates the neural substrate of BSR directly or by effects on other mediators is not clear. A growing number of signaling molecules have been implicated as downstream mediators of leptin's effects on energy balance, three studies presented in Chapter Two set out to examine the contribution of some of these neuropeptides to this unique component of reward circuitry (Fulton, Richard, Woodside & Shizgal, 2002; Fulton, Woodside & Shizgal, 2002). The results of these studies are compared to what we have learned from research investigating the behavioral and biological basis of energy balance to help provide some functional interpretation of the reward neurons that are sensitive to alterations in long-term energy stores. In Chapter Three, data on the effects of long- and short-term energy signaling on BSR in a genetically obese rat strain are presented (Fulton, Richard, Woodside & Shizgal, 2003). These strains are particularly relevant to the current studies because the obese Zucker rat shows potentiated responses to manipulation of food availability including acute fasts. If the difference in effect on BSR of food

deprivation and food restriction that we observed in lean rats were a reflection of the relative intensity of the two food manipulations then one might expect to see differential effects of food deprivation in the obese and lean Zucker rats. In addition, given the profound resistance to circulating leptin and insulin in the obese Zucker rat, the ability of food restriction to potentiate BSR in this model suggests additional peripheral signals may link fat stores to restriction-sensitive reward circuitry. The final set of data presented in Chapter Four provide direct evidence for functional partitioning amongst the neurons responsible for BSR by showing differing effects of food restriction on BSR obtained at different stimulation sites in the same subject (Fulton, Woodside & Shizgal, 2002). Further, the activity of the restriction-sensitive subset of reward neurons is shown to vary along with changes in body weight. Together, this work lays down an initial framework for a neural network sub serving BSR that comprises functionally specific pathways. Moreover, this research offers interesting insights into the neural mechanisms underlying energy balance and demonstrates how BSR can serve as a useful tool for mapping reward-relevant components of the energy balance system.

Before presenting the findings, I will review some fundamentals of BSR and discuss the background research that has provided the rationale for this body of work.

1.1. **Attributes of BSR**

Rewarding self-stimulation is obtained from electrodes located in many brain regions, ranging from the orbito-frontal cortex to the brainstem. However, particularly

robust self-stimulation behavior can be obtained with stimulating electrodes ranging from the basal septum all along the medial forebrain bundle (MFB) and into the midbrain extension of the MFB (Corbett & Wise, 1979, 1980; Olds & Olds, 1963). The MFB is a dense bundle of fibers with 50 or more components that courses rostrally and caudally through the forebrain (Nieuwenhuys, Geeraedts, & Veening, 1982). The stimulation can activate local cell bodies, fibers of passage and distant cell bodies and terminals, thus it is not known which of the many pathways coursing through the MFB are responsible for the rewarding effect of the stimulation. Nonetheless, much progress has been made in narrowing down the neurons which give rise to BSR. This work will be described in the next section.

To understand how BSR can be used as a tool to investigate reward circuitry, it is essential to know how the neurons surrounding the tip of the electrode are affected by the electrical stimulus. First, it is important to realize that which neurons are recruited by the stimulation depends on their density (Rompre & Miliaressis, 1985) as well as their excitability. Some control over which neurons get activated and to what extent can be exerted by manipulating parameters of the electrical stimulus. In the majority of BSR studies, the stimulation consists of trains of short-duration pulses of cathodal current. With these two parameters (train and pulse duration) held constant, the strength of the stimulation is determined by the current (pulse amplitude) and the number of stimulation pulses (frequency). The number of directly stimulated neuron recruited depends jointly on the current and the pulse duration. Over the range of stimulation frequencies used in most studies, each stimulation pulse evokes one action potential.

These attributes of BSR are captured in the "counter model" (Gallistel, 1978; Gallistel, Shizgal & Yeomans, 1981). Based on the assumption that one pulse evokes one action potential, the counter model states that the aggregate rate of firing produced by stimulating a population of neurons equals the number of times a neuron is fired (frequency) multiplied by the number of neurons being fired (determined by the current). Thus, it does not matter whether 100 neurons are fired 20 times or whether 50 neurons are fired 40 times, the aggregate output of the stimulated neurons is the same. The aggregate firing rate of a sub-population of reward neurons within a fixed time-frame is considered to provide input to an integrator that combines the effects of the action potentials over time and space to provide a subjective magnitude of reward. In the view of Shizgal (1999), it is the output of this integrator that is the sole determinant of "instantaneous utility", which influences an animal to either continue or terminate a particular action.

1.2. The search for the neurons responsible for BSR

1.2.1. Physiology foretold

An enduring goal amongst researchers of BSR is to identify the neurons responsible for the rewarding effect of the stimulation. If we are to understand the neural substrates that underlie motivation for natural rewards, it is crucial that this objective be met. Given that vigorous low-threshold self-stimulation is found along the MFB, there is reason to think that among those activated MFB fibers are those that generate the rewarding signal.

The search for the fibers responsible for BSR has been narrowed by the use of psychophysically-based methods that can characterize these fibers in terms of their refractory periods and conduction velocity (Yeomans,1975; Shizgal et al., 1980). The refractory period of a neuron is determined by measuring the time interval required between two stimulation pulses in order for both pulses to elicit action potentials in a subset of neurons. Specifically, a conditioning (C) stimulation pulse is delivered through the electrode followed by a test (T) pulse from the same electrode, and if the T pulse is delivered while the axons are still refractory then the T pulse does not evoke an action potential. When refractory period information is obtained, one can then use the collision technique to infer the conduction velocity and trajectory of these neurons (Bielajew & Shizgal, 1982; Shizgal, Bielajew, Corbett, Skelton & Yeomans, 1980; Shizgal & Murray, 1994). In short, this is done by delivering pulses from two separate electrodes that are located in different areas and inferring whether the action potentials evoked from the pulse pair meet and cancel each other out. Thus, the signals that are generated in those fibers that are common to both fields of stimulation will collide. The conduction velocity of the fibers can then be estimated by dividing the distance between the two electrodes by the conduction time between electrodes.

On the basis of paired-pulse data estimating refractory periods and conduction velocity, we know that BSR obtained from some sites in the lateral hypothalamic MFB depends upon the direct activation of small-diameter, myelinated fibers (Yeomans, 1975, 1979; (Shizgal, Bielajew, Corbett, Skelton, & Yeomans, 1980); Schenk & Shizgal, 1982; Bielajew & Shizgal, 1982). According to the results of

collision studies, there is evidence for a fiber bundle that projects between the LH and ventral tegmental area (VTA) (Bielajew and Shizgal, 1982, 1986) and a bundle that projects between the lateral preoptic area and LH (Bielajew, Konkle, Fouriezos, Boucher-Thrasher & Schindler, 2001). The direction of conduction in at least some of the LH-VTA fibers has been inferred as rostrocaudal by using a related blocking technique (Bielajew & Shizgal, 1986). In support of these findings, single-unit recording studies have found neurons in the basal forebrain that conduct in a rostrocaudal manner and that have physiological properties corresponding to those inferred psychophysically (Murray & Shizgal, 1996; Rompré & Shizgal, 1986; Shizgal, Schindler & Rompré, 1989).

The above mentioned findings have narrowed down the potential candidate neurons responsible for the rewarding effect of the stimulation. One group of cells that has been sufficiently ruled out as the directly stimulated substrate is the mesolimbic dopamine neurons. Several studies have documented the ability of dopaminergic manipulations to alter thresholds for BSR (Colle & Wise, 1988; Conover, Oda & Shizgal, 1999; Gallistel, Boytim, Gomita & Klebanoff, 1982; Gallistel & Freyd, 1987), and thus it was once thought that direct activation of dopamine neurons plays a role in generating the reward signal. However, the MFB axons of the mesolimbic dopamine system are unmyelinated, have high thresholds, relatively long refractory periods and low conduction velocities and therefore, do not match the criteria established by psychophysical and electrophysiology studies.

1.2.2. Lessons from lesion studies

Evidence from psychophysical and unit recording studies has delineated some physiological properties of neurons giving rise to BSR, however there are likely several candidates that fit the established criteria. For this reason, lesion studies are indispensable for determining the contribution of cell groups to the rewarding effect of the stimulation. In cases where damage to a particular area produces a long-lasting decrease in the rewarding impact of the stimulation we can assume that cell bodies, terminals and/or fibers of passage once located in the lesioned region contribute to the rewarding effect of the stimulation.

As summarized in the previous section, there is evidence that at least some of the directly activated neurons conduct in a rostrocaudal direction. These data have been taken to suggest that some of the cell bodies responsible for the rewarding effect of the stimulation are located in the forebrain and send projections down through the MFB in a rostrocaudal manner. This view has come to be known as the "descending path" hypothesis of MFB self-stimulation.

In some studies, lesions producing extensive damage to forebrain areas have not severely disrupted MFB self-stimulation. For example, in cases where the most of the forebrain rostral to bregma was removed reductions in the rewarding effect of MFB stimulation were only modest (Colle & Wise, 1987). Nonetheless, there are several other lines of evidence supporting the idea that there are neurons in the basal forebrain that generate reward signals. Damage to MFB projection systems between the LPOA and LH can substantially reduce the rewarding effect of MFB stimulation (Arvanitogiannis, Waraczynski & Shizgal, 1996; Murray & Shizgal, 1991, 1996; Waraczynski, 1988). Some of the largest reductions in the reward effectiveness of the

stimulation reported in lesion studies were produced with damage to the LPOA and surrounding regions, including the sublenticular-extended amygdale (SLEA) and anterior LH (Arvanitogiannis et al., 1996). Further evidence implicating components of the extended amygdala (EA) in the rewarding MFB stimulation come from a recent study by Waraczynski (2003). Lidocaine-induced inactivation of central EA structures such as the lateral bed nucleus of the stria terminalis (BNST), central SLEA and the interstitial nucleus of the posterior limb of the anterior commissure substantially reduced the rewarding effect of the stimulation. However, since BSR is elicited by artificial activation of axons, lidocaine-induced impairments of BSR could be due to inactivation of axonal segments. For this reason, it is possible that reward-relevant cell bodies lie at sites caudal to the stimulating electrode, thus supporting an ascending path hypothesis

Given that larger behavioral effects were not seen in the studies mentioned above it is possible that there are additional cell populations that contribute to rewarding MFB stimulation. In support of the idea that multiple cell groups contribute to the rewarding effect of MFB stimulation, Gallistel and coworkers (1996) found that lesions and knife cuts caudal to the site of MFB stimulation, near the VTA, produce impairments in BSR larger than those obtained with lesions to the anterior LH, rostral to the stimulating electrode (Gallistel, Leon, Lim, Sim & Waraczynski, 1996). This finding has been taken to suggest that reward neurons are more concentrated in the caudal hypothalamus and anterior ventral tegmentum than in the rostral hypothalamus (Gallistel et al., 1996). Yet other evidence suggests the possibility of axons arising from cerebellum (Acheson, Waraczynski, & Perkins, 2000) and the midbrain

retrorubral fields (Waraczynski & Perkins, 2000) as contributors to the reward signal elicited by MFB stimulation.

In summary, when reviewing the literature on the impact of lesions to alter the rewarding effect of MFB stimulation, it appears as though damage or inactivation of structures residing in the forebrain, midbrain, hindbrain and cerebellum can impair MFB stimulation. Experiments demonstrating the impact of lesions or inactivation to midbrain or hindbrain structures on MFB self-stimulation do not discount the notion of a descending path because it has not been determined whether cell bodies, axons or terminals of a damaged or inactivated region contribute to the reward signal induced by MFB stimulation. Thus, damage to structures caudal to an MFB stimulating electrode may be destroying the projections or terminal fields of forebrain neurons.

Arvanitogiannis and colleagues (1996) proposed a model for the contribution multiple subpopulations of neurons that are responsible for the rewarding effect of MFB stimulation. In their view, differences in the placement of stimulating electrodes in the MFB may be a large source of variability across studies and subjects with respect to the effectiveness of lesions to impair MFB stimulation. This hypothesis deserves serious consideration in light of some findings to be presented in Chapter Four. Slight variations in the placement of the stimulating electrode in the LH can affect the sensitivity of BSR to food restriction and weight loss (Fulton, Woodside and Shizgal, 2002). These data provide evidence for functionally separate subpopulations of reward-relevant fibers. With this in mind, the degree to which a particular lesion reduces the reward effectiveness of the stimulation would depend on

the alignment of the damaged region to the specific composition of neurons directly activated by the electrode.

As will be discussed in the following section, the notion that the substrate for BSR is functionally subdivided was born out of an early body of work relating the reward signal produced by the electrical stimulus to neural signals governing natural rewards. These studies provided the groundwork for the investigations to be presented here.

1.3. Relationship between BSR and energy balance

It is a wonder that artificial activation of neurons at the tip of the electrode can produce a meaningful signal, but the fact that animals will learn to self-administer the rewarding stimulation is evidence that the exogenous electrical signal can be incorporated and processed in an orderly manner. Since it inception, BSR has been used as a tool to investigate the natural function of the underlying circuitry. Out of the early observations showing that rats would continuously self-stimulate the LH while forgoing physiological needs, such as feeding and drinking, grew the idea that the stimulation tapped into the neural circuitry subserving natural rewards (Olds, 1958; 1962; Spies, 1965; Routtenberg & Lindy, 1965)

A considerable amount of data published around the time that BSR was discovered likely inspired the idea that stimulation of the LH specifically signals the rewarding properties of food. A landmark study by Anand and Brobeck in 1951 showed that bilateral electrolytic lesions of the LH resulted in a profound decrease in

food and water intake and a resulting drop in body weight. Thereafter, similar findings documented the consistent symptomology of LH electrolytic lesions (Oltmans & Harvey, 1972; Teitelbaum and Stellar, 1954), which collectively became known as the LH syndrome. Together, these studies encouraged the notion of the LH as a "feeding center" (Bernardis & Bellinger, 1996).

In addition to the lesion data implicating the LH in feeding, several studies demonstrated that long trains of electrical stimulation at LH sites could elicit a drive-like state by inducing feeding when given in the presence of food (Delgado & Anand, 1954). This phenomenon called "stimulation-induced feeding" was mapped throughout the LH, with the most sensitive sites shown to be located in the perifornical area (Hernandez and Hoebel, 1989; Murzi, Hernandez & Hoebel, 1986; Roberts, 1980). More convincingly, stimulation-induced feeding and self-stimulation could be obtained from the same electrode (Coons & Cruce, 1968; Hoebel & Teitelbaum, 1962). Subsequent research however, failed to support the notion that signal was specific to feeding by showing that the electrical stimulus could also elicit drinking, gnawing or sexual activity (Valenstein, Cox, Kakolewski, 1968; Valenstein & Cox, 1970). The initial response to the stimulation was maintained as long the sensory stimuli relevant to the particular behavior was present (Huang & Mogenson, 1972). These findings support the idea that the signal generated by the stimulation is non-specific and that it produces an active state in which animals learn to respond to objects in their environment (Fray, Koob & Iversen, 1982).

Despite the ambiguous findings in the stimulation-induced feeding literature, the notion that LH stimulation mimics the rewarding properties of food was upheld by

reports that LH self-stimulation can be modulated by manipulation of energy balance. Using conventional rate measures, self-stimulation of the LH was shown to increase in response to acute food deprivation (Carey, Goodall & Lorens, 1970; Deutsch & DiChiara, 1967; Margules & Olds, 1962) and chronic food restriction resulting in weight loss (Blundell & Herberg, 1968). In addition, the potentiating effects of acute food deprivation (Goldstein, Hill & Templer, 1970, Frutiger, 1986) and chronic food restriction (Blundell & Herberg, 1968) have been observed at stimulation sites that elicit stimulation-induced feeding. In a manner resembling its influence on food intake, force feeding and spontaneous meal consumption suppressed LH self-stimulation rates (Hoebel, 1968; Hoebel & Thompson, 1969). On the basis of these results, it was suggested that the reward signal induced by the stimulation is similar to the signal conveying the rewarding properties of food (Hoebel & Thompson, 1969; Olds, 1977).

In the studies described above, changes in the reward effectiveness of the stimulation were inferred from alterations in the rate of self-stimulation. For example, an increase in the self-stimulation rate of the animal was assumed to reflect an increase in the reward effectiveness of the stimulation. By themselves, rate measures are not able to distinguish changes in the performance capability that can occur in response to a manipulation from alterations in the rewarding effect of the stimulation. The curve-shift paradigm, on the other hand, provides just such a dissociation (Edmonds & Gallistel, 1974; Miliaressis, Rompre, Laviolette, Philippe & Coulombe, 1986). The curve-shift approach examines responding over a range of stimulation frequencies that drive response rates from minimal to maximal (asymptotic) levels. A

plot of the resulting data on a logarithmic frequency axis yields a curve roughly sigmoidal in form (Fig. 1). Lateral shifts in the curve are presumed to correspond to a change in the rewarding impact of the stimulation whereas a horizontal shift in the upper asymptote is thought to reflect alterations in factors affecting the performance of the animal.

Studies employing the curve-shift method to measure changes in the reward effectiveness of the stimulation have yielded different results from those obtained using rate measures. An acute period of food deprivation substantially increases food consumption, however, it has been shown to have little or no effect on rate-frequency thresholds for BSR in some cases (Fulton et al., 2000; Fulton et al., 2001; Giovino and Wise, 1986) while having an effect in others (Rossi and Panksepp, 1985). In addition, food intake is elevated in response to a glucoprivic state induced by peripheral 2-deoxyglucose (2-DG) administration (Smith and Epstein, 1969), yet, 2-DG has been reported to suppress BSR obtained at anterior LH sites (Frutiger and Drinkwine, 1992) or have no effect on the rewarding stimulation at posterior LH sites (Cabeza de Vaca et al., 1998). In summary, models that propose that rewarding LH stimulation is analogous to food reward are faced with the paradoxical findings that manipulations that dramatically increase food intake largely fail to alter BSR in a similar manner.

An elegant series of studies from Conover and colleagues (1994) provide compelling evidence that the reward signal induced by LH stimulation is quite different from the signal generated by a rewarding piece of food. In one experiment, they determined the effect of postingestive feedback on the value of intraoral sucrose and LH stimulation

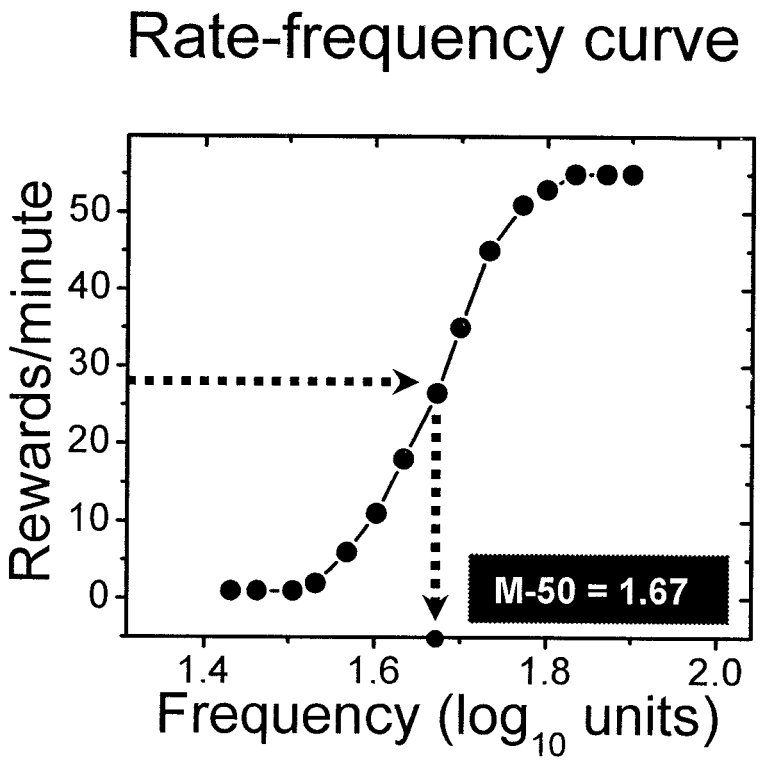


Figure 1.1. Rate-frequency curve. Number of rewards earned (behavioral output) grows as the stimulation frequency (number of pulses in $\log_{10}$ units) is increased over a particular range. A threshold in this case is defined as the stimulation frequency required to maintain half-maximal rewards earned (M-50).

(Conover & Shizgal, 1994b). Rats were given a choice between LH stimulation, which varied in intensity from trial to trial, and either a sucrose reward alone or a compound reward consisting of sucrose plus a fixed train of stimulation. Conover et al. found that BSR remained stable as the sucrose solution accumulated in the gut, whereas responding for the compound reward was substantially suppressed. In a similar study, the impact of sodium depletion on the preference for a saline solution versus LH stimulation was assessed (Conover, Woodside & Shizgal, 1994). The authors found that negative sodium balance did not augment the value of the stimulation as it did the value of the saline reward. These results suggest that gastrointestinal signals that modulate short-term food intake and signals pertaining to negative sodium balance exert their effect upstream from the point where the rewarding effect of LH stimulation converges with the rewarding effect of saline or sucrose.

While Conover and Shizgal's postingestive feedback experiment demonstrated important differences between food and LH stimulation, other evidence derived from their work illustrates that gustatory stimuli and LH stimulation share something in common (Conover and Shizgal, 1994a). In a forced-choice preference paradigm, rats could choose between the two rewards in a manner that implied that the gustatory stimuli and the electrical stimulation were being evaluated along a common dimension. This common scale of evaluation is an essential antecedent of choice among goals with multidimensional properties. It represents a point where the different properties of goals are reduced to a single, common dimension. The "general currency" exists to allow choice among many alternatives, whereas there must also be

local currencies which compute the value of specific goods such as food and water (Shizgal, 1998). As proposed by Shizgal, such a configuration, consisting of multiple stages of local currency evaluations specific to feeding and energy balance, would be an orderly way for the brain to process and evaluate the multiple properties of food stimuli so as to meet energy requirements.

Findings from several studies suggest that the stimulation of certain LH sites activates a stage of reward circuitry that contributes to the long-term regulation of energy balance. Early investigations of Blundell and Herberg (1968) showed that response rates for rewarding LH stimulation were increased by chronic food restriction and body weight loss only when the stimulating electrodes were located in the perifornical region of the LH. Using frequency thresholds to measure changes in BSR over a 3 week period of food restriction, Carr and Wolinsky (1993) found similar results. In rats with stimulating electrodes in the perifornical region of the LH, but not in rats with electrodes in the zona incerta or ventral hypothalamus, the rewarding effect of the stimulation was enhanced by food restriction and weight loss. Moreover, these authors found thresholds for BSR to vary as a function of body weight when the stimulating electrode was located in the perifornical area. Interestingly, in the same study the authors show that the potentiation of BSR by food restriction can be reversed by intraventricular injection of the non-selective opioid antagonist, naltrexone. The modulation of BSR by weight loss at some LH stimulation sites has been replicated several times by Carr and colleagues using frequency threshold (Carr and Papadouka, 1994) and rate-frequency measures (Abrahamsen, Berman & Carr, 1995; Abrahamsen & Carr, 1996; Cabeza de Vaca,

Holiman & Carr, 1998; Carr, 1996, Carr, Kim & Cabeza de Vaca, 2000) to evaluate changes in BSR. These data are consistent with the view that stimulation of the LH can activate at least two subpopulations of reward neurons and that one of these subpopulations is linked to the regulation of body weight.

In summary, studies using frequency threshold or rate-frequency measures largely reveal that the modulation of LH self-stimulation by homeostatic challenges depends on the nature of the energy state being manipulated: Long-term signals related to body weight loss appear to enhance the rewarding stimulation at a subset of stimulation sites. To understand how the modulation of BSR could be linked exclusively to long-term regulatory signals, it is necessary to describe the widely-accepted distinction between short- and long-term energy balance.

1.3.1. Short- and long-term peripheral signals controlling energy balance

Research over recent years has substantially advanced our understanding of the distinct mechanisms regulating short- and long-term energy balance (Havel, 2001; Saper, Chou & Elmquist, 2002; Schwartz, Woods, Porte, Seeley & Baskin, 2000). To ensure that nutritional needs are met, the brain must have circuitry that senses nutrient levels to affect feeding and energy expenditure. During a meal, the ingestion of food induces a suppression of hunger that leads to the termination of eating, this process is referred to as satiation. Signals in the gastrointestinal (GI) tract and liver act primarily to stimulate food intake and limit meal size in the short-run. Cholecystokinin (CCK), glucagons-like peptide-1 (GLP-1), ghrelin and peptide YY (PYY) are released from the GI tract in response to nutrient availability and target mediators in the CNS to

affect appetite and meal size (Saper, Chou & Elmquist, 2002). In addition, stretch receptors and chemoreceptors are activated by nutrients in the stomach and small intestine to reduce meal size by sending signals to the brain via vagal afferents. In accordance with nutrient levels the liver releases glucose and ketones to alter food intake and energy expenditure. These short-term signals themselves do not produce sustained alterations in energy intake and adiposity.

Body weight remains relatively constant over time. One explanation for this is that the adipose mass is sensed by the brain and leads to appropriate alterations in energy intake and expenditure. Early investigations in surgically joined parabiotic rodents revealed that there are circulating factors that affect food intake and body adiposity over a prolonged period of time. In cases where genetically obese mice (*ob/ob*) mice were surgically linked to lean mice, *ob/ob* mice reduced their food consumption and gradually lost weight (Coleman, 1973). This finding implied the existence of a blood-born factor, not present in *ob/ob* mice, that affects food intake and energy expenditure to regulate the size of the adipose mass.

In 1994, Zhang and colleagues identified the mutation responsible for obesity in the *ob/ob* mouse (Zhang, Proenca, Maffei, Barone, Leopold & Friedman, 1994). The long sought-after circulating factor was given the name leptin, derived from the Greek word *leptos,* meaning thin. Leptin is the protein-product of the obesity gene (*ob*). Synthesized and released by white adipose cells, leptin circulates in direct proportion to the amount of white adipose tissue (Maffei, Halaas, Ravussin, Pratley, Lee, Zhang, Fei, Kim, Lallone, Ranganathan & al., 1995). Genetic abnormalities resulting in the lack of leptin (*ob/ob*) or lack of functional leptin signaling at its

receptor in mice (*db/db*) or rats (*fa/fa*) results in hyperphagia and obesity. It was soon discovered that leptin replacement in *ob/ob* mice could correct the behavioral, metabolic and autonomic abnormalities associated with lack of leptin (Campfield, Smith, Guisez, Devos, & Burn, 1995; Halaas, Gajiwala, Maffei, Cohen, Chalt, Rabinowitz, Lallone, Vurley & Friedmen, 1995; Pellymounter, Cullen, Baker, Hecht, Winters, Boone & Collins, 1995). Central or peripheral administration of leptin decreases food intake and increases energy expenditure (Campfield, Smith, Gulsez, Devos & Burn, 1995). Upon accessing the brain, leptin binds to the long-form of its receptor (*Ob-Rb*) which is found in several brain sites, including the hypothalamus and hindbrain (Mercer, Hoggard, Williams, Lawrence, Hannah, Morgan, Trayhurn, 1996; Schwartz, Seeley, Campfield, Burn & Baskin, 1996). Via its actions at hypothalamic and hindbrain neurons, leptin modulates the release of a variety of neuropeptides known to mediate its influence on feeding and energy expenditure (Schwartz, Woods, Porte, Seeley & Baskin, 2000).

Insulin is another hormone that circulates in proportion to body fat and thus fulfills the criterion for an adiposity signal (Bagdade, Bierman & Porte, 1967; Woods & Seeley, 2000). Like leptin, insulin receptors are expressed on neurons implicated in energy balance (Baskin, Wilcox, Figlewicz & Dorsa, 1988) and central insulin administration reduces food intake (Woods, Lotter, McKay, Porte, 1979). In addition to the long-term regulatory control exerted by leptin and insulin, these two hormones also exert meal-to-meal influences on energy balance. In response to increased glucose levels, insulin is secreted from pancreatic β-cells to ensure that normal glucose homeostasis is met. Elevated glucose levels also result in increased adipocyte

glucose metabolism to cause a release of leptin. These transient increases in leptin and insulin are hypothesized to promote satiation and increase energy expenditure. In a converse manner, and more vital to the survival of species during conditions of low food availability, deficiency in leptin and insulin during negative energy balance serves to stimulate food intake and decrease energy expenditure (Ahima, Prabakaran, Mantzoros, Qu, Lowell, Maratos-Flier & Flier, 1996; Spiegelman & Flier, 2001).

In conclusion, research has delineated different mechanisms and signaling molecules controlling short- and long-term energy balance. In the short-run, signals from the gastrointestinal tract, liver, pancreas and adipose tissue are released to affect meal initiation, termination and size. There have been inconsistent findings with respect to the ability of acute energy signals to alter BSR obtained from LH sites. Rather, signals associated with the regulation of long-term energy stores, principally the adipose mass, seem important for the potentiation of BSR by chronic food restriction and weight loss at a subset of stimulation sites. Thus, it stands to reason that understanding the different signaling molecules and processes regulating short- and long-term energy states will shed some light on the neurochemical and functional nature of reward circuitry linked to energy balance.

Still much remains to be elucidated with respect to the regulation of genes, peptides, neurotransmitters and receptor substrates in the CNS by peripheral signals. For instance, little is known about how hormones, such as leptin and insulin, can serve as both short- and long-term energy feedback signals to the brain. More germane to the present body of work is the question of which neural processes give rise to the behaviors that are an integral aspect of energy input and a major

component of energy output? Given that reward signals are an antecedent to behavioral choice, identifying the neural mechanisms that give rise to the rewarding properties of stimuli and behaviors has large implications for understanding energy balance. An important goal in this light is to tease apart the various subdivisions of brain reward circuitry pertaining to different objects and behaviors contributing to energy balance.

1.3.2. Linking fat stores to brain reward circuitry: A role for leptin

The enhancement of BSR by chronic food restriction and weight loss obtained by stimulating some sites in the LH suggests that there are peripheral signals reflecting adiposity that enter the brain to modulate a component of reward circuitry. The adiposity hormone leptin represents an ideal candidate mediator in the effects of weight loss on BSR, since both central and peripheral leptin levels reflect the amount of adipose tissue. During states of negative energy balance leptin release is diminished (Maffei et al., 1995; Ahima, Prabakaran, Mantzoros, Qu, Lowell, Maratos-Flier & Flier, 1996). Deficiency in circulating leptin has been proposed to serve a much more adaptive role when energy stores are low by stimulating food intake and decreasing energy expenditure (Ahima et al., 1996).

A series of experiment described in my Master's thesis (see Appendix) tested the hypothesis that leptin may be contributing to the modulation of BSR by food restriction. As others have reported previously (e.g., Blundell & Herberg, 1968; Carr and Wolinsky, 1993), we found that BSR was enhanced by chronic restriction in a subset of subjects: In 5 out of the 10 subjects, the rewarding effect was "restriction-

sensitive;" it was enhanced by ~25% weight loss. In the remaining rats, the rewarding effect was "restriction-insensitive;" there were no reliable changes in BSR following weight loss (Appendix, Fig.1). Intraventricular administration of leptin attenuated the rewarding impact of the stimulation but only in rats in which the rewarding impact of the stimulation was restriction-sensitive (Appendix, Fig. 2). In contrast, at restriction-insensitive sites central leptin injection had an opposite effect in 3 out the 4 rats tested: it potentiated the rewarding impact of the stimulation. The contrasting actions of leptin on restriction-sensitive and –insensitive reward circuitry suggest that there may be at least two separate subpopulations of reward neurons with distinct neurochemical profiles.

Despite the data describing changes in the rewarding effect of the stimulation by weight loss and the adiposity hormone leptin, it remained unclear whether depletion of long-term energy stores was crucial to the modulation of restriction-sensitive reward circuitry. As rats were fed their daily allotment of food following the completion of the BSR session, it was possible that the influence of food restriction on BSR was due to the actions of a daily acute fast. To explore the contribution of acute fasting to restriction-sensitive reward circuitry we assessed the influence of 48 hour food deprivation on LH-self-stimulation in the same group of rats in which the rewarding effect of the stimulation was either restriction-sensitive or –insensitive. Even though rats were deprived of food for a relatively long time, there was little or no change in the rewarding effect of the stimulation at all sites tested. Thus, it appears as though the facilitation of BSR obtained at restriction-sensitive sites depends on

alterations in long-term energy signals rather than fluctuation in signals relaying information about short-term energy stores.

This earlier body of work introduces several interesting questions: (1) How is leptin altering the rewarding effect of BSR obtained at restriction-sensitive and -insensitive stimulation sites? There are several candidate neuropeptides that are known to mediate leptin's actions on feeding and energy expenditure - could any these neuropeptides be involved in the influence of food restriction and leptin on BSR? (2) Are there additional circulating signals that tie changes in long-term energy stores to restriction-sensitive reward circuitry? The obese Zucker rat develops resistance to circulating leptin and thus, provides a good model for assessing how impairments in central leptin signaling affect the ability of food restriction to modulate BSR. (3) Is reward circuitry subdivided along functional lines or are the variations between rats with respect to the influence of food restriction on BSR solely due to individual differences? If we are to fully comprehend the neurobiological basis of reward, including how and where the rewarding effects of different stimuli and behaviors are processed, it is essential that we understand how brain reward circuitry is organized.

1.4. Summary

The neural circuitry that confers the rewarding properties of goal objects and goal-directed behavior can be activated and studied by means of electrical brain stimulation reward. In one view, the neurons that give rise to the rewarding effect of the stimulation are comprised of multiple subpopulations, each of which is

functionally and anatomically distinct. In another view, the reward substrate is functionally homogeneous, and signals reflecting specific physiological needs exert their impact at a point upstream from the reward substrate. The identity of the neurons that carry the reward signal has yet to be elucidated however, some physiological attributes of a subset of the directly activated neurons have been established. Moreover, lesion studies have identified brain regions that contribute to the rewarding effect of stimulating MFB sites. The results of some of these lesion studies suggest that there may be multiple subpopulations of reward neurons. This notion is also supported by data showing that chronic food restriction and weight loss can potentiate the rewarding impact of the stimulation only at a subset of stimulation sites. In addition, leptin, the protein-product of the obesity gene, was shown to reverse the potentiation of BSR by weight loss. This evidence suggests that there is a subdivision of reward circuitry linked to long-term regulatory signals controlling body weight.

CHAPTER TWO

It is not clear whether leptin alters reward circuitry directly or via its effect on other neural mediators. It is well known that leptin targets several neuropeptides in the hypothalamus and hindbrain to alter food intake and energy expenditure. The next three studies examine the contribution of some of these neuropeptides to the rewarding effect obtained at restriction-sensitive and –insensitive sites. As we have found previously, the effect of leptin was contingent on the whether or not food restriction potentiates the rewarding effect of the stimulation. Thus, the influence of each neuropeptide was assessed in rats that were either sensitive or insensitive to the influence of chronic food restriction on BSR.

2.1. INTERACTION OF CRH AND ENERGY BALANCE IN THE MODULATION OF BRAIN STIMULATION REWARD

Weight loss due to chronic food restriction enhances the rewarding effect produced by electrical stimulation of certain sites in the lateral hypothalamus (LH) (Blundell & Herberg, 1968; Carr & Wolinsky, 1993; Fulton, Woodside & Shizgal, 2000; Fulton, Woodside & Shizgal, 2002) but fails to alter the rewarding effect of stimulating neighboring LH sites. Thus, manipulation of energy stores appears to exert a modulatory influence on signals arising in a sub-population of reward-related neurons. Given that activation of reward-relevant neurons has been implicated in the control of goal-directed behavior, variations in energy states may influence choice among goal objects by modulating the activity of this sub-population of neurons (Shizgal, Fulton & Woodside, 2001). If so, molecules involved in signaling the state of energy reserves to central circuitry would be expected to alter the rewarding impact of these restriction-sensitive neurons.

In support of this hypothesis, we have recently shown that intraventricular administration of leptin, the protein-product of the obesity gene, decreases the reward efficacy of LH stimulation at restriction-sensitive sites but not at restriction-insensitive sites (Fulton et al., 2000). Thus it appears that leptin reduces the impact of stimulating the restriction-sensitive sub-population and may contribute to coupling the state of peripheral fat stores to the sensitivity of the restriction-sensitive pathway. In contrast to the effects of chronic food restriction and leptin administration, reduction of metabolic fuel availability (Cabeza de Vaca, Holiman & Carr, 1998) or

short-term food deprivation (Fulton et al., 2000) fails to change the rewarding effect of stimulating restriction-sensitive neurons. Thus, only a subset of challenges to energy balance, likely those that modify long-term signals, are capable of modulating this pathway.

The identification of leptin looms large among recent discoveries concerning hormones, neuropeptides, and neural pathways that contribute to the regulation of food intake and energy balance. This hormone, which is produced principally in adipocytes, is implicated in the control of feeding, energy expenditure, and reproduction (Campfield, Smith, Guisez, Devos, & Burn, 1995; Halaas, Gajiwala, Maffei, Cohen, Chalt, Rabinowitz, Lallone, Vurley & Friedmen, 1995; Pellymounter, Cullen, Baker, Hecht, Winters, Boone & Collins, 1995). Via its action on hypothalamic and hindbrain neurons and their efferents, leptin modulates the release of a variety of neuropeptides, including corticotropin-releasing hormone (CRH) (Schwartz, Woods, Porte, Seeley & Baskin, 2000; Grill & Kaplan, 2002). Leptin can suppress the synthesis (Huang, Rivest & Richard, 1998) and secretion (Heiman, Ahima, Craft, Schoner, Stephens & Flier, 1997). In contrast, direct stimulation of CRH release and CRH receptor up-regulation in hypothalamic and limbic tissues in response to leptin has been reported (Schwartz, Seeley, Campfield, Burn & Baskin, 1996; Uehara, Shimizu, Ohtani, Sato & Mori, 1998) presumably in non-hypophysiotropic neurons.

In addition to its fundamental role in the stress response, there is abundant evidence tying the actions of CRH to the control of food intake and energy

expenditure (Richard, Huang & Timofeeva, 2000; Watts, Sanchez-Watts, & Kelly, 1999). For example, intraventricular administration of CRH promotes a state of negative energy balance by lowering food intake (Morley & Levine, 1982; Britton, Koob, Riviere & Vale, 1982) and stimulating thermogenesis (LeFeuvre, Rothwell & Stock, 1987). Both CRH and its two known receptor subtypes, CRH_1 and CRH_2, are distributed throughout brain regions implicated in energy balance, including hypothalamic nuclei, such as the paraventricular nucleus (PVN), ventromedial nucleus (VMN) and LH (Grigoriadis, Lovenberg, Chalmers, Liaw & De Souze, 1996) and brainstem nuclei such as the nucleus of the solitary tract (NTS) and the parabrachial nucleus (Bittencourt & Sawchenko, 2000).

The purpose of this study was to explore the role of CRH in the modulation of brain reward circuitry by assessing the effects of central CRH administration on LH self-stimulation. It was of particular interest to determine whether CRH alters self-stimulation of restriction-sensitive sites in a manner similar to the effect of leptin and whether, as is the case with leptin, the influence of this neuropeptide on brain stimulation reward (BSR) differs at restriction-sensitive and restriction-insensitive sites.

2.1.1. Materials and Methods

2.1.1.1. Subjects

Subjects were male Long Evans rats from the Charles River Breeding Farms (St. Constant, Quebec) weighing between 400 and 500 g at the time of surgery. Each rat was housed individually in plastic solid-floor cages with *ad libitum* access to food

and water. All subjects were kept in a temperature-controlled room under a reverse 12 hour dark/12 hour light cycle that switched at 8 am/pm. All behavioral testing was carried out in the middle of the dark phase of the cycle.

2.1.1.2. Surgery

Prior to surgery rats were administered atropine sulfate (0.5 mg/kg, SC) to reduce bronchial secretions and were anesthetized with sodium pentobarbital anesthesia (Somnotol, 65 mg/kg, i.p.). With bregma and lamda at the same horizontal coordinates, bilateral, monopolar electrodes were aimed at the perifornical region of the LH (3 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, 7.8 mm below the dura mater), and a 24- gauge stainless-steel guide cannula (Plastics One, Inc., Roanoke, VA) was aimed at the right lateral ventricle (0.4 mm posterior to bregma; 1.6 mm lateral to the midsagittal sinus; 4 mm below the dura mater). The electrodes were constructed from 00 insect pins and were insulated with Formvar to within 0.5 mm of the tip. A wire twisted around two jeweler's screws implanted in the cranium served as the current return. The electrodes and cannula assembly were bonded to the skull and screw anchors by means of dental acrylic. The intraventricular cannula was then closed with a removable obturator. To reduce postoperative pain, Buprenorphine (Buprenex, 0.05 mg/kg, SC) was administered once following surgery.

2.1.1.3. Apparatus

Subjects were screened for self-stimulation in wooden boxes (25cm x 25 cm 70 cm) with Plexiglas front panels and wire mesh floors. A lever, positioned 3 cm

above the floor, protruded from the middle of one wall, and a key light positioned 5 cm above the lever signaled the availability of the reward. Electrical stimulation was generated by dual constant current amplifiers and controlled by hand-operated circuit pulse generators. Depression of the lever triggered the stimulator. The connector on the rat's head was linked to the stimulator via a flexible lead and a 7-channel slip-ring located at the top of the test chamber.

The experimental sessions took place in a separate room housing computer-controlled stimulators. Test chambers were similar to those used for training except they were constructed entirely of Plexiglas and equipped with a ceiling-mounted white house light. Each test chamber was enclosed in a 50 cm x 50 cm x 90 cm plywood enclosure lined with sound attenuating foam. Stimulation trains were generated by microprocessor-controlled circuitry and monitored on an oscilloscope in an adjoining room. Subjects were monitored during testing by means of a remote-controlled video camera.

2.1.1.4. Design and Procedure

2.1.1.4.1. Brain stimulation reward procedure

Rats were shaped to lever press for a 0.5 s train of cathodal, rectangular, constant-current pulses, 0.1 ms in duration, on a continuous reinforcement schedule. Initially, stimulation parameters were set to low currents and frequencies. If the rat displayed signs of aversion, training was discontinued; otherwise, the subject was shaped to press the lever. Stimulation frequencies (pulses per second) were adjusted to produce optimal levels of responding at a current that remained fixed for each rat ranging between 100 and 400 μA. Self-stimulation of both sites was assessed, and the

electrode that supported the most vigorous lever-pressing in the absence of motoric side-effects was chosen for further testing.

Following initial training, the subjects were trained in the computer-controlled testing setup to respond for a descending series of stimulation frequencies, on a 0.5 sec fixed-interval schedule of reinforcement. The stimulation current was held constant throughout testing, within the range used for initial training. The stimulation frequency was decreased from trial to trial in steps of 0.033 $\log_{10}$ units (~8%) in order to generate curves relating the rate at which rewards were earned to the stimulation frequency ("rate-frequency" curves). Each frequency was available during a single 60 sec trial that was preceded by a 10 sec inter-trial interval during which 5 priming (non-contingent) trains of stimulation were delivered; the priming trains were identical to those available during the trial. Stimulation frequencies were adjusted so as to produce maximal (asymptotic) responding during the first few trials of each frequency sweep. A rate-frequency curve was deemed complete when the rats emitted fewer than 5 responses on each of two consecutive trials. Seven rate-frequency curves were collected per test session. Test sessions lasted anywhere between 1 ¼ to 2 ½ hours. The first curve of the session served as a warm-up and was not included in the data analysis.

2.1.1.4.2. Intraventricular injection procedure.

Cannula placement was verified by administering 50 ng of angiotensin II (Sigma, St. Louis, MO) and determining whether vigorous drinking began within 5 min. All substances were infused in the right lateral ventricle at a rate of 1 µl/min through a stainless steel internal cannula (Plastics One, Roanoke, VA) that extended 1

mm beyond the tip of the guide cannula. The internal cannula was connected to a 5 μl microsyringe (Hamilton Company) by means of flexible polyethylene tubing. After infusion, the internal cannula was kept in place for 15 seconds.

2.1.1.4.3. Design

Training in the self-stimulation paradigm lasted for 1-2 days. As an index of the effectiveness of the rewarding stimulation, we used the stimulation frequency that maintained a half-maximal rate of reward delivery (M-50). Baseline responding was deemed stable when mean M-50 values shifted by ≤ 0.04 $\log_{10}$ units from day to day. Rate-frequency data was collected for 5-7 days following stabilization of baseline responding and then the influence of food restriction on BSR was assessed. Daily food intake was limited to 10 grams/day until body weight reached ~75% of free-feeding values (approx. 2-3 weeks time). During this time BSR testing was carried out every few days to maintain self-stimulation performance. When body weights reached ~75% of normal free-feeding values, the amount of food supplied to each rat was adjusted daily in an attempt to hold body weight constant. Body weights stabilized after 2-4 days. During this period regular BSR testing was carried out until steady M-50 values were achieved.

The influence of a 5 μg dose of CRH (Sigma, St. Louis, MO) on BSR was tested twice in each rat, once at the end of the baseline (free-feeding) condition, when body weight was normal, and again at the end of the restriction condition, when body weight was stabilized at ~ 25% below its normal level. During both conditions vehicle treatment (0.1 mol/l Tris, pH 7.35) was administered for two consecutive days,

followed by CRH treatment on the third day. BSR testing began 15 min after injections.

2.1.1.4.4. Food intake

The effectiveness of CRH (5 μg) to suppress food intake was assessed in a separate group of rats (n = 4). Powdered food was placed in cups attached to aluminum sheets that were molded to hang on the wall of the home cage. A flat-bottomed bowl was fixed underneath each food cup to catch spillage. Following a week of habituation to the powdered food diet, 3-hour food intake was measured after ICV vehicle (0.1 mol/l Tris, pH 7.35) administration and then measured again the following day after administration of 5 μl of a 1 μg/μl CRH solution over a 5 min period. All injections were made in the middle of the dark cycle, in accordance with self-stimulation testing times.

2.1.1.5. Data analysis

Broken-line functions, with a horizontal lower asymptote, a rising linear segment, and a horizontal upper asymptote (Gallistel & Freyd, 1987), were fit to each of the six rate-frequency curves collected daily. To indicate the position of the rate-frequency curve along the frequency axis, the M-50 value was derived from each broken-line function by interpolation. Potentiation of the rewarding effect drives these curves to the left, thus reducing the M-50 value, whereas reductions in reward effectiveness produces rightward shifts and increases the M-50 value. The maximum number of rewards earned (Max-R) was estimated from the upper asymptote of each broken-line function. Changes in Max-R serve as an index of performance capacity

(Miliaressis, Rompré, Laviolette, Philippe, Coulombe, 1986).

By means of a one-way analysis of variance (ANOVA), a set of 12 M-50 values from two consecutive days in the free-feeding condition was compared to a set of 12 values obtained just after body weight stabilized at ~75% of normal values for each subject. Similarly, the ANOVA carried out to assess the effects of food restriction on performance capacity in each rat entailed comparison of a set of 12 Max-R values from the free-feeding condition to a set of 12 Max-R values from the food restriction condition. Delta M-50 was calculated for each rat by subtracting the mean of the 12 M-50 values collected during free-feeding from the mean of the 12 M-50 values obtained under food restriction. Given that only a proportion of the subjects demonstrate a decrease in M-50 values following food restriction ("restriction-sensitive"), a one-way ANOVA was used to compare the delta M-50 values from this group to the delta M-50 values from the remaining rats that were insensitive to the food restriction manipulation (restriction-insensitive").

The CRH test during free-feeding intervened between the gathering of the M-50 values during free-feeding and the food restriction test, whereas the CRH test during food restriction was carried out when desired body weight values became stable. For the analysis of the effects of CRH, a within-subject, one-way ANOVA was employed to compare a set of 12 M-50 values collected during the two vehicle-treatment days to the 6 M-50 values collected on the day of CRH administration. Delta M-50 was calculated for each rat by subtracting the mean of the 12 M-50 values collected during vehicle administration from the mean of the 6 M-50 values obtained

just after CRH administration. By means of a two-way ANOVA, delta M-50 values following CRH administration were compared as a function of restriction-sensitivity and feeding condition (free-feeding and food restriction). Changes in the Max-R parameter were evaluated in an analogous fashion. A level of $p < 0.05$ for a two-tailed test was considered critical for statistical significance.

Differences in total electrical charge between restriction-sensitive and restriction-insensitive rats were assessed by means of a one-way ANOVA that compared total charge per train (μC) in the baseline condition between groups. Total charge was calculated by multiplying the current (μA) x number of pulses x pulse duration (0.0001 sec) for each rat.

2.1.1.5.2. Histology

Following completion of testing, the stimulation sites were marked by means of the Prussian Blue method. With the stimulating electrode serving as the anode, a 100 μA direct current was applied for 15 sec. Rats were then injected with a lethal dose of sodium pentobarbitol (Somnotol, 100 mg/kg, IP) and perfused intracardially with phosphate-buffered saline followed by a mixture of 10% formalin (100 ml), trychloroacetic acid (0.5 g), potassium ferrocyanide (3 g) and potassium ferricyanide (3 g). Brains were removed and stored in 10% formalin. After immersion in a 20% sucrose-formalin solution for at least 24 hr, the brains were frozen, sliced on a cryostat in 30 μm coronal sections, and mounted on pre-coated slides (Fisher Scientific). Sections were stained for Nissl substance using formal thionin. The

location of stimulation sites was identified with the aid of a stereotaxic atlas (Paxinos & Watson, 1998).

2.1.2. Results

2.1.2.1. Food intake

During the 3 hours following a 5μg infusion of CRH, total food intake was 64% lower than following vehicle treatment (Fig 2.1.1).

2.1.2.2. Chronic food restriction

Initial body weights ranged from 455 - 610 g. Following food restriction, body weight fell to 347 - 460 g.

In 8 out of 16 subjects, chronic food restriction reduced M-50 values. Examples of the data obtained from these rats are shown in Figure 2.1.2A and C and show that curves taken during food restriction lie to the left of the curves obtained during free-feeding; the corresponding M-50 values (Fig. 2.1.2E) decreased by 0.03 - 0.27 $\log_{10}$ units. In the remaining 8 subjects, the rate-frequency curves obtained during restriction overlap the curves obtained during free-feeding (e.g., Fig. 2.1.2 B and D), and the corresponding M-50 values remained stable (Fig. 2.1.2F). The results of a one-way ANOVA revealed that delta M-50 values of the restriction-sensitive group (-0.104 ±0.08) were significantly lower than the delta M-50 values of the restriction-insensitive groups (-0.004 ±0.01) ($F(1, 15) = 11.96$, $p = .004$). Table 2.1.1 illustrates changes in the maximum number of rewards earned (Max-R) following the three manipulations. Three subjects in the restriction-insensitive group showed a

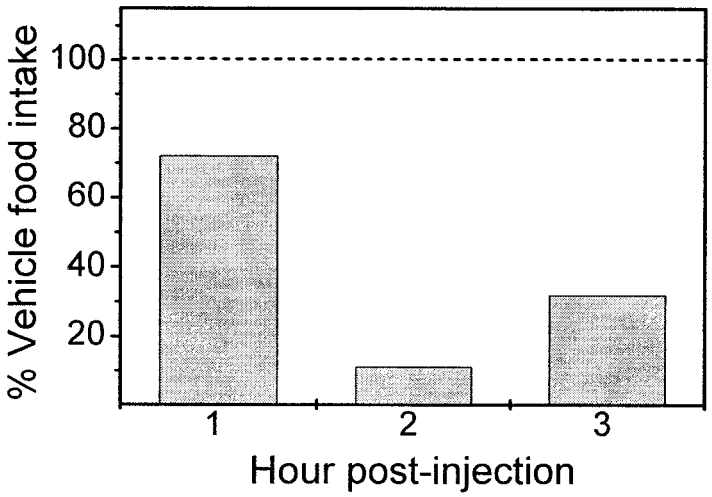


Figure 2.1.1. Effect of an intraventricular infusion of CRH (5 μg) on 3-hr food intake as compared to vehicle treatment (n=4).

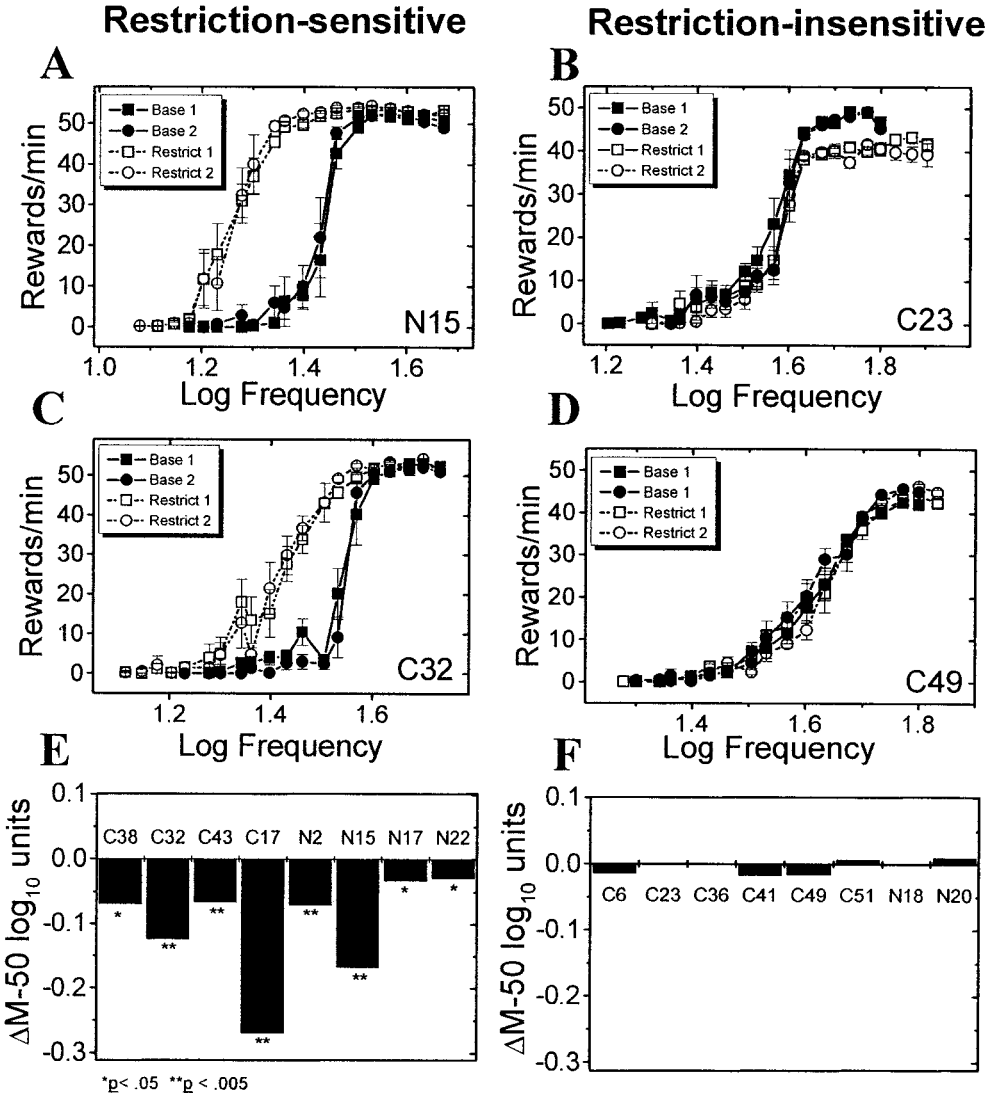


Figure 2.1.2. Effects of chronic food restriction on self-stimulation at LH sites where the rewarding effect of electrical stimulation is sensitive (left panels) or insensitive (right panels) to chronic food restriction. (**A,C**) Rate-frequency curves obtained with stimulation of a restriction-sensitive site are shifted leftwards following chronic food restriction (open symbols) with respect to curves obtained during free-feeding (filled symbols). (**B,D**) Conversely, stimulation of a nearby, restriction-insensitive site generates overlapping rate-frequency curves following chronic food as compared to curves collected during free-feeding. Each data point in panels A, B, C and D is an average of six measures collected on each test day. (**E,F**) Magnitude of the curve shifts produced by chronic food restriction in all subjects. M-50 represents the stimulation frequency required to maintain half the maximal number of rewards earned. (*$p < 0.05$; ** $p < 0.005$)

Table 2.1.1. The effect of food restriction and CRH administration on the maximum number of rewards earned (Max-R) for each rat (means and S.E.M).

			Free-feeding		Restriction	
Rat	**Baseline**	**Restriction**	**Vehicle**	**CRH**	**Vehicle**	**CRH**
R_{ins}						
C6	47.75 ±1.11	48.40 ±2.12	48.74 ±2.42	46.86 ±2.35	45.78 ±0.45	45.27 ±2.91
C23	46.58 ±1.05	40.06 ±2.16*	45.63 ±2.07	36.70 ±2.09*	38.38 ±2.36	34.27 ±0.86*
N18	50.25 ±1.07	40.30 ±3.17*	46.59 ±2.74	40.00 ±1.51*	41.73 ±2.72	26.48 ±2.15*
N20	51.19 ±0.95	52.18 ±0.94*	51.93 ±0.87	51.06 ±1.24	52.65 ±0.85	51.82 ±0.61
C36	31.33 ±2.20	28.46 ±2.66*	32.09 ±2.71	27.38 ±6.38*	28.46 ±2.66	25.66 ±2.07*
C41	50.04 ±2.06	49.07 ±0.98	50.36 ±0.62	48.29 ±1.55*	49.66 ±0.82	42.49 ±2.73*
C49	43.87 ±1.72	43.08 ±2.37	43.87 ±1.72	41.11 ±2.08*	42.91 ±1	36.97 ±1.77*
C51	43.43 ±3.01	44.62 ±0.91	43.02 ±2.24	44.71 ±2.16	46.20 ±1.17	45.90 ±0.97
R_{sen}						
N2	44.27 ±2.70	50.39 ±1.08*	46.47 ±1.47	48.67 ±1.19*	50.78 ±1.22	50.20 ±0.78
N15	50.45 ±0.82	51.79 ±0.93	50.23 ±1.31	48.25 ±2.00*	32.28 ±1.83	33.96 ±3.66
C17	46.46 ±1.94	52.91 ±0.47*	48.45 ±2.37	50.60 ±1.39	52.01 ±1.37	53.16 ±0.95
N17	48.81 ±0.61	51.70 ±0.72*	49.89 ±1.22	47.48 ±0.84*	51.68 ±0.87	49.02 ±0.50*
N22	43.91 ±1.95	46.98 ±0.93*	45.94 ±1.94	44.18 ±3.07	46.30 ±0.88	45.17 ±1.90
C32	50.28 ±1.14	51.31 ±1.53	50.63 ±1.18	51.92 ±0.92*	NA	
C38	46.91 ±1.37	41.03 ±2.17*	42.41 ±1.96	44.82 ±2.03*	48.64 ±1.38	48.04 ±2.77
C43	52.15 ±0.93	52.20 ±0.83	51.94 ±0.79	51.07 ±1.67	52.20 ±0.83	52.82 ±0.57

* $p \leq .05$ Rats with restriction-insensitive stimulation sites (R_{ins}); Rats with restriction-sensitive stimulation sites (R_{sen})

significant decrease in Max-R, whereas another subject in that group displayed a significant increase. In the restriction-sensitive group, Max-R was significantly elevated in 4 rats following food restriction, whereas it decreased in one subject.

The analysis of total charge per train revealed no systematic differences between the restriction-sensitive and –insensitive groups ($F(1, 15) = 0.12$, $p = .73$).

2.1.2.3. Effect of CRH on M-50 values

In 7 of the 8 rats in which M-50 values decreased following chronic food restriction, CRH administration had very little effect on LH self-stimulation (Fig. 2.1.3E). The rate-frequency curves obtained from these subjects following CRH administration overlap those obtained after vehicle administration (Fig. 2.1.3A and C), both during both the free-feeding and food restriction conditions. In contrast, CRH produced a large and significant potentiation of BSR (a decrease in M-50) in subject C17 (Fig. 2.1.3E).

Strikingly different effects were seen in the 8 rats that were unresponsive to chronic food restriction. In these subjects, CRH reduced the rewarding impact of the stimulation. This is illustrated in Figure 2.1.3B and D by the rightward shift of rate-frequency curves obtained after CRH administration with respect to the curves obtained after vehicle administration. The increase in the M-50 values produced by CRH was of similar magnitude during the free-feeding and food restriction conditions (Fig. 2.1.3F).

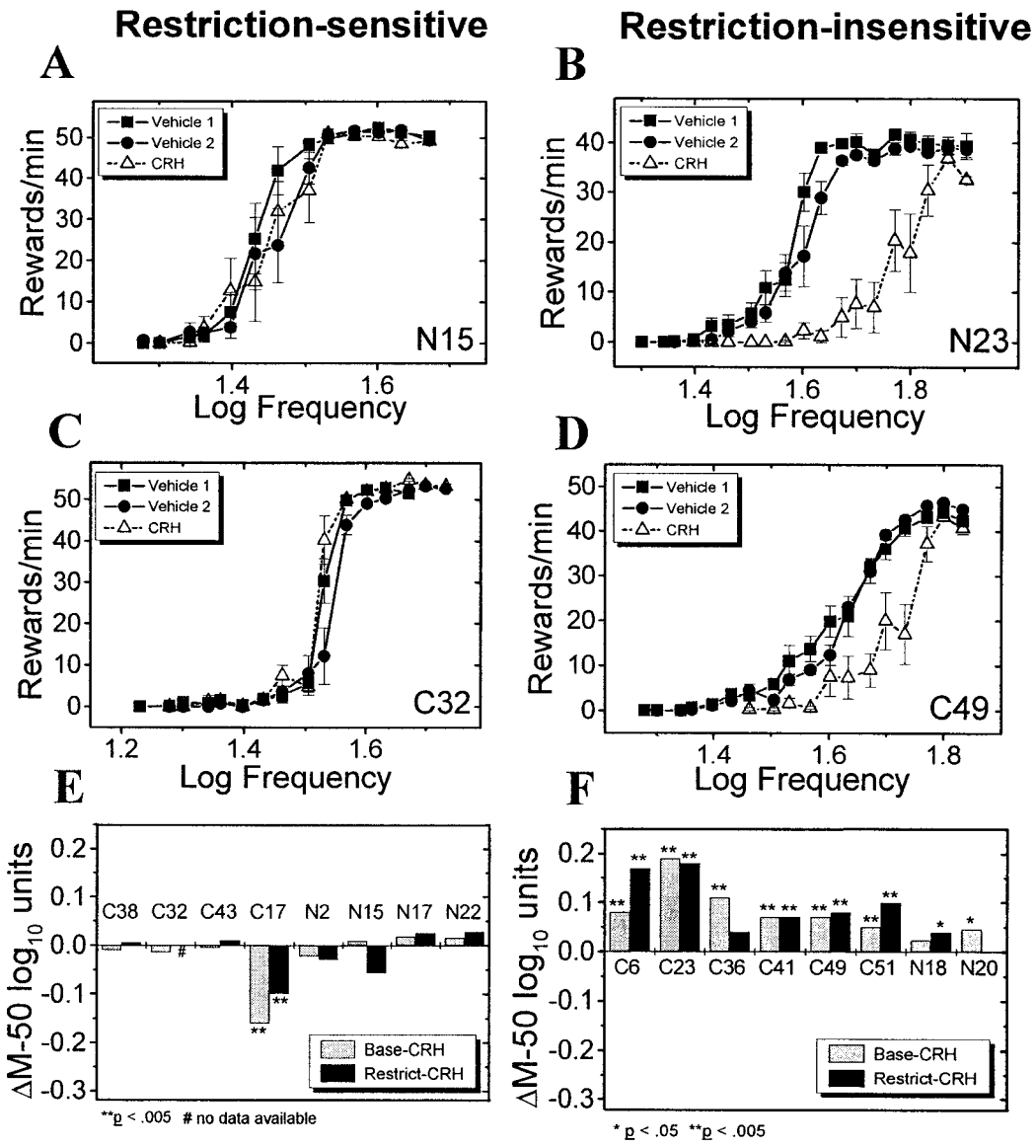


Figure 2.1.3. Divergent effects of CRH on the rewarding impact of LH stimulation at restriction-sensitive sites (left panels) or restriction-insensitive sites (right panels). **(A,C)** At restriction-sensitive stimulation sites, rate-frequency curves obtained following CRH infusion (open symbols) overlap those collected during vehicle infusion (closed symbols). **(B,D)** In contrast, at restriction-insensitive stimulation sites, rate-frequency curves obtained after CRH administration are shifted to the right with respect to curves collected after vehicle administration. Each data point in panels A, B, C and D is an average of six measures collected on each test day. **(E,F)** Magnitude of the curve shifts produced CRH in all subjects. M-50 represents the stimulation frequency required to maintain half the maximal number of rewards earned. (*$p < 0.05$; ** $p < 0.005$)

The results of a two-way ANOVA, assessing delta M-50 values following CRH administration as a function of restriction-sensitivity and feeding condition, show a significant main effect of restriction-sensitivity: delta M-50 values following CRH administration were significantly greater for the restriction-insensitive group (0.082 ±0.056) as compared to the restriction-sensitive group (0.018 ±0.05) ($F(1, 31) = 26.96$, $p < .0001$). There was no difference in delta M-50 values between feeding conditions ($F(1, 31) = 0.09$, $p = 0.76$) and no interaction between restriction-sensitivity and feeding condition ($F(1, 31) < .001$, $p = .96$).

2.1.2.4. Effects of CRH on performance capacity

As shown in Table 2.1.1, five of the eight restriction-insensitive subjects showed significant decreases in Max-R following CRH administration during both the free-feeding and food restriction condition. Three rats from the restriction-sensitive group showed significant increases in Max-R following CRH administration during free-feeding, whereas two rats from this group displayed significant decreases. During food restriction, CRH infusion decreased Max-R in only one restriction-sensitive subject.

The results of a two-way ANOVA show a significant main effect of restriction-sensitivity on Max-R values. Max-R values following CRH administration decreased by an average of 3.27 ±3.36 in restriction-insensitive rats as compared to a mean increase of 0.018 ±0.013 in restriction-sensitive rats ($F(1, 31) = 26.96$, $p < .0001$). There was no difference in delta Max-R values between feeding conditions

($F(1, 31) = 0.09$, $p = 0.76$) and no interaction between restriction-sensitivity and feeding condition ($F(1, 31) < .001$, $p = .96$).

2.1.2.5. Electrode placements

Histological localization of the electrode tips revealed that sites where BSR was enhanced by chronic food restriction and unaffected by CRH were dorsal or dorsolateral to the fornix (Fig. 2.1.4). The remaining sites were sometimes nearby, but tended to lie around the periphery of the region where restriction-sensitive sites have been found in this and previous studies (Carr & Wolinsky, 1993; Fulton et al., 2000; Fulton et al., 2002). The site where BSR was enhanced both by food restriction and by CRH (Rat C17) was the most dorsal of all electrode placements (Plate –3.14). One rat (C15) died prematurely and the brain of another (C6) was subject to histological error, thus the location of their electrode tips could not be determined.

2.1.3. Discussion

In half of the 16 subjects, chronic food restriction produced a significant potentiation of BSR: the rate frequency curves shifted leftwards by varying amounts. In the remaining subjects, the M-50 values remained remarkably stable despite the fact that the weight loss in these subjects was as great as in the subjects with restriction-sensitive electrode placements and was very substantial (~25%). This finding is consistent with previous reports that food restriction and body weight loss lower rate-frequency thresholds at certain stimulation sites in the LH (Abrahamsen et al., 1995; Fulton et al., 2000; Fulton et al., 2002). The simplest explanation for the

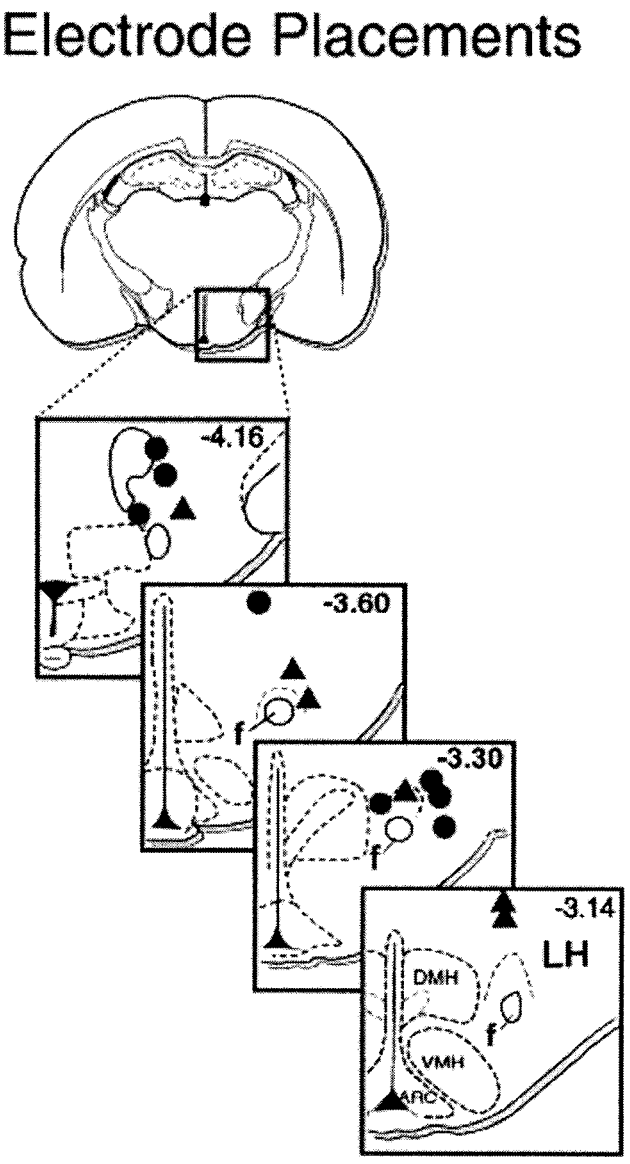


Figure 2.1.4. Location of the tips of the stimulation electrodes. Electrodes producing rewarding effects that were enhanced by chronic food restriction are designated by filled triangles (▲) and electrodes producing rewarding effects that were unaffected by chronic food restriction are designated by filled circles (●). The coronal sections are based on the atlas of G. Paxinos and C. Watson, (Academic Press, New York, Fourth Edition, 1998).

striking contrast in the effect of weight loss is that signals reflecting the state of the fat stores modulate a neural signal that is induced by or dependent on the stimulation of neurons arising, terminating, or coursing through the LH. Consistent with this interpretation is the notion that the rewarding effect of LH stimulation arises from activation of multiple, functionally distinct sub-populations of intertwined neurons (Arvanitogiannis, Waraczynski & Shizgal, 1996).

More support for the existence of multiple sub-populations of reward neurons activated by LH stimulation comes from the different effects of CRH administration we obtained at the two sets of stimulation sites. At all 8 restriction-insensitive sites, CRH significantly increased M-50 values, indicating that the rewarding impact of the stimulation was reduced. In contrast, CRH was without effect at 7 out of the 8 restriction-sensitive sites. In a previous experiment, the modulation of BSR by central administration of the anorexigenic hormone, leptin, was assessed (Fulton et al., 2000). The influence of leptin was found to depend on whether the rewarding effect of the LH stimulation was sensitive to chronic food restriction. Here, we demonstrate that the influence on BSR of another centrally administered anorexigenic substance, CRH, also depends on whether the rewarding effect produced at a given LH site was sensitive to chronic food restriction. Nonetheless, it is unlikely that CRH mediates the influence of leptin on BSR at restriction-sensitive sites: the effects of CRH at these sites differed from the previously reported effects of leptin.

That CRH increased M-50 values at restriction-insensitive sites is consistent with a recent report that CRH and urocortin increase the threshold current required to

support LH self-stimulation, an effect that was reversed by a nonselective CRH receptor antagonist (Macey, Koob & Markou, 2000). Such CRH-induced decreases in BSR are reminiscent of the effects of stressors (Valentino, Dufresne & Riccitelli, 1990; McCutcheon, Rosellini & Bandel, 1991). For example, Valentino and coworkers subjected rats to an inescapable swim and found that BSR thresholds were elevated 3 hours post-swim. CRH-containing neurons in addition to those controlling ACTH release are activated by stressors, and intraventricular administration of CRH produces anxiogenic effects (Koob & Heinrichs, 1999). Thus, CRH-containing neurons could well mediate the attenuation of BSR produced by exposure to stressors. It has also been pointed out (Macey et al., 2000) that CRH is implicated in drug withdrawal and may contribute to the elevation of BSR thresholds during such states.

The stability of the rate-frequency curves obtained from 7 of the 8 restriction-sensitive sites following CRH administration stands in contrast to the results reported by Macey et al. (2000). This contrast could reflect different distributions of electrode placements in the two studies. For example, it is possible that the stimulation sites in the study of Macey et al. were drawn largely or solely from the restriction-insensitive population. If so, the results of the two studies would be in agreement.

The failure of CRH to alter M-50 values at most restriction-sensitive sites could reflect insensitivity to the effects of this neuropeptide in the restriction-sensitive sub-population of reward-related neurons and their efferents. Perhaps stimulation of the restriction-sensitive sub-population antagonizes the anxiogenic effect of CRH. An alternate model also bears consideration. It is likely that even at the restriction-sensitive sites, only a portion of the directly stimulated reward-related neurons are

drawn from the restriction-sensitive sub-population. Perhaps CRH produces two opposing effects that cancel, enhancing the component of the rewarding effect due to the restriction-sensitive neurons and attenuating the component due to the restriction-insensitive neurons. A reason for considering such a view is that a robust reward-enhancing effect of CRH was seen in the 8th subject in the restriction-sensitive group, C17. In this subject, CRH produced substantial leftward curve shifts, both during food restriction and during free-feeding. It is noteworthy that the effect of chronic food restriction in this subject was larger than in any other, halving the M-50 value. Thus, if CRH enhances the rewarding effect of stimulating a restriction-sensitive population of reward-related neurons, such an effect would most likely be seen in this subject. It is not beyond the realm of plausibility that certain CRH-containing neurons promote appetitive behaviors whereas others suppress such behaviors. Indeed, although infusion of CRH into the lateral ventricles does reduce food intake, release of CRH in the amygdala has been noted during and immediately following ingestive behavior (Merali, McIntosh, Kent, Michaud & Anisman, 1998).

There were consistent changes in the Max-R values obtained from the restriction-insensitive sites following CRH administration, reflecting a decrease in asymptotic performance. In 5 of these 8 cases, CRH significantly reduced Max-R values when administered during free feeding. During food restriction, even more pronounced reductions in Max-R were seen in these 5 rats, and there was a trend towards a similar effect in the remaining three rats. Neither chronic food restriction nor CRH administration produced consistent alterations in this measure of asymptotic performance at restriction-sensitive stimulation sites. There were differences between

the two groups of rats such that CRH produced an overall decrease in the Max-R parameter in the restriction-insensitive group that was not observed in the restriction-sensitive group.

Decreases in asymptotic performance may reflect reduction in the capacity of the subjects to perform the operant response (Milliaresis, Rompré, Laviolette, Philippe, & Coulombe, 1986). Alternatively, CRH may potentiate a behavior, such as grooming (Morley & Levine, 1982), that is incompatible with lever pressing. As discussed above, the absence of systematic changes in Max-R values in the restriction-sensitive group could reflect the absence of CRH action on the restriction-sensitive pathway or to an anxiolytic effect of activating these neurons.

In their study of the modulation of BSR by CRH and urocortin, Macey et al. (2000) did not observe changes in the latency to respond or in an index of response forcefulness. They concluded that CRH did not reduce performance capacity. The difference between their results and the changes reported here in the Max-R values obtained at the restriction-insensitive sites may be due to differences between the two testing paradigms. In the discrete-trial paradigm employed by Macey et al., an inter-trial interval averaging 10 sec in duration provides the rats with the opportunity to engage in behaviors other than lever pressing without losing the opportunity to obtain rewarding stimulation; the inter-trial interval is longer than the trial. In the paradigm employed here, the 10-sec inter-trial interval is short in comparison to the 60-sec trial, and thus the tendency to engage during the trial in behaviors incompatible with lever pressing is likely to be greater. Thus, CRH-induced potentiation of behaviors such as

grooming would be more likely to reduce the asymptotic reward rate in the paradigm employed here than in the paradigm employed by Macey et al.

The position set forth in the discussion above to explain the observed differences between restriction-sensitive and -insensitive rats is that the response to chronic food restriction and CRH administration is dependent upon the site of stimulation. A similar distinction between restriction-sensitive and restriction-insensitive sites in the LH has also been drawn by others (Blundell & Herberg, 1968; Carr & Wolinsky, 1993; Carr and Papadouka, 1994; Carr, 1994; Abrahamsen, Berman and Carr, 1995; Abrahamsen and Carr, 1996; Cabeza de Vaca, Holiman & Carr, 1998; Carr, Kim & Cabeza de Vaca, 2000). In this study, as in two others, we have found that the rewarding effect induced via electrode tips dorsal or dorsolateral to the fornix is always restriction-sensitive, whereas we have never seen restriction-sensitivity at sites in the far-lateral hypothalamus or at sites medial to the fornix (Fulton et al., 2000, Fulton et al., 2002). The results are mixed at intervening sites as well as in a region roughly 1 mm dorsal to the fornix at the anteroposterior level of the dorsomedial hypothalamus. Our results are consistent with the reports of others such that sites where BSR is most often enhanced by food restriction lie dorsal or dorsolateral to the fornix (Carr & Wolinsky, 1993).

That small differences in electrode location could produce differential recruitment of neural populations seems plausible when data from moveable electrode mapping studies are taken into account. These studies, carried out using electrode sizes and stimulation parameters similar to those employed in the present study, demonstrate that downward displacement of a stimulation electrode by only 130-

160 μM can produce substantial changes in the BSR threshold (Rompré & Miliaressis, 1985; Forgie & Shizgal, 1993).

An alternative hypothesis that deserves consideration is that the divergent responses to chronic food restriction and CRH are not due to variation in electrode placement but rather reflect individual differences in sensitivity to food restriction or stress. Evidence from related areas of research suggests that such individual differences can be important. For example, only a proportion of rats become obese in response to a high calorie diet ("diet-induced obesity"), and glucoresponsive neurons in these rats show a reduced response to glucose in comparison to diet-resistant rats (Levin, Dunn-Meynell & Routh, 1999). In addition, there are pronounced individual differences in the propensity of rats to self-administer rewarding drugs (Piazza, Deroche-Gamonent, Rouge-Pont, & Le Moal, 2000). By challenging BSR at multiple stimulation sites in the same rat, it will be possible to determine whether differences in electrode placement contribute to the observed variation in the response to chronic food restriction and CRH, above and beyond any contribution of individual differences. Identifying the neurons responsible for such an effect of varying electrode placement would shed light on the functional organization of brain reward circuitry. Similarly, establishing that individual differences render particular subjects more sensitive to the effects of chronic food restriction and CRH would add to a large body of work on individual vulnerability to behavioral and physiological dysregulation.

2.1.4. Summary

In a previous study, the rewarding effect produced by electrical stimulation of restriction-sensitive PFH sites was shown to be reduced by leptin. In contrast, in the

present study, CRH failed to alter BSR at most restriction-sensitive sites. Thus, CRH is unlikely to mediate the influence of leptin on the rewarding effect of stimulating restriction-sensitive sites. At restriction-insensitive sites, CRH produced consistent rightward shifts of rate-frequency curves, suggesting that the rewarding effect of the stimulation was attenuated. These rightward shifts are reminiscent of the effects of stressors on BSR. In addition, asymptotic responding was reduced in most rats with restriction-insensitive stimulation sites, implying that CRH reduces the capacity of rats to perform for BSR at these sites. Taken together, the results add to the evidence that the sensitivity of BSR to chronic food restriction is a crucial variable in determining the influence of hormones and neuropeptides on the rewarding effect of the stimulation. Functional heterogeneity in central CRH pathways may contribute to the contrasting influence of this neuropeptide on the rewarding effect produced by stimulation of restriction-sensitive and restriction-insensitive sites. In turn, the demonstration that the reward-modulating effects of both CRH and leptin differ at these two classes of BSR sites provides further support for the view that central reward-related circuitry is sub-divided along functional lines (Hoebel, 1969).

2.2. DOES NEUROPEPTIDE Y CONTRIBUTE TO THE MODULATION OF BRAIN STIMULATION REWARD BY CHRONIC FOOD RESTRICTION?

Electrical stimulation of certain sites in the lateral hypothalamus (LH) elicits a rewarding effect that can be potentiated by chronic food restriction and body weight loss [Blundell & Herberg, 1968; Carr & Wolinsky, 1993; Fulton, Woodside & Shizgal, 2000]. The modulation of brain stimulation reward (BSR) by chronic food restriction can predict alterations in the state of reward circuitry produced by some hormones involved in energy balance. Thus whereas food restriction can enhance the rewarding effect of stimulating particular sites in the perifornical region of the LH, central administration of leptin (Fulton, Woodside & Shizgal, 2000) and insulin (Carr, Kim & Cabeza de Vaca, 2000) two satiety hormones, have been shown to reduce the rewarding impact of stimulating these sites. Conversely, at adjacent LH sites where BSR is unaffected by food restriction leptin acted primarily to increase the reward effectiveness of the stimulation (Fulton, Woodside & Shizgal, 2000). In addition, we have recently shown that the influence of another anorexigenic agent, corticotropin-releasing hormone (CRH) on BSR, depends upon the sensitivity to food restriction (Fulton, Richard, Woodside & Shizgal, 2001). At restriction-insensitive stimulation sites CRH increased the threshold for BSR whereas it had no effect at most restriction-sensitive sites.

Evidence that chronic food restriction and body weight loss can moderate BSR indicates that the activity of a sub-population of reward-relevant neurons is linked to changes in long-term energy balance (Cabeza de Vaca, Holiman & Carr, 1998; Fulton

et al., 2000). That this effect can be reversed by central administration of leptin and insulin suggests that these peripheral signals are involved in the process whereby food restriction modifies this subset of reward circuitry. Secreted by adipocytes, leptin, the protein-product of the obesity gene, acts as a major link between fat stores and the brain to regulate reproduction and energy balance [Ahima, Prabakaran, Mantzoros, Lowell & Maratos-Flier, Flier, 1996; Halaas, Gajiwala, Maffei, Cohen, Chalt et al., 1995; Pelleymounter, Cullen, Baker, Hecht, Winters, Boone & Collins, 1995). Like leptin, insulin circulates in direct proportion to the amount of adipose tissue and enters the brain to promote a state of negative energy balance (Schwartz, Woods, Porte, Seeley & Baskin, 2000). Upon binding to hypothalamic receptors, both leptin and insulin modulate the release of a variety of neuropeptides, including neuropeptide Y (NPY) (Schwartz et al., 2000). NPY gene expression within the arcuate nucleus (ARC) is inhibited by leptin (Stephens, Basinski, Bristow, Bue-Valleskey, Burgett, Craft, Hale, Hoffman, Hsiung, Kriauciunas et al., 1995) and insulin (Scwartz, Marks, Sipols, Baskin, Woods, Kahn & Porte, 1991). In addition, knocking out the gene for NPY in leptin-deficient *ob/ob* mice reduces obesity, indicating that NPY is necessary for the complete manifestation of the leptin-deficiency syndrome (Erickson, Hollopeter & Palmiter, 1996)

NPY is widely distributed throughout neurons of the central and peripheral nervous system but found in high concentrations in the hypothalamus. A particularly dense population of NPY neurons involved in the regulation of energy balance resides in the ARC (Elmquist, Elias & Saper, 1999). These neurons send projections to hypothalamic nuclei such as the paraventricular nucleus and the LH. Centrally

administered NPY promotes a state of positive energy balance by dramatically increasing food intake and decreasing energy expenditure (Levine & Morley, 1984; Stanley, Kyrkouli, Lampert & Leibowitz, 1986). The most profound feeding effects are produced when NPY is administered directly into the perifornical region of the LH (Stanley, Magdalin, Seirafi, Thomas & Leibowitz, 1993). Furthermore, it is pertinent to the present study that NPY has been reported to increase the rewarding effects of food as shown by its ability to increase break points under a progressive ratio schedule of reinforcement (Jewett, Cleary & Levine, 1995).

In consideration of these findings, the present study was carried out to assess the effects of intraventricular NPY administration on BSR at restriction-sensitive and restriction-insensitive stimulation sites in the LH. Our goal was to establish whether, as is the case with leptin and CRH, the influence of NPY on BSR would differ between the two sub-populations of stimulation sites. Further, if the effects of leptin on BSR are mediated through suppression of NPY release then NPY administration would be expected to decrease thresholds for BSR at restriction-sensitive sites, thereby producing effects opposite to those of leptin and insulin.

2.2.1. MATERIALS AND METHODS

2.2.1.1. Subjects

Male Long Evans rats from the Charles River Breeding Farms (St. Constant, Quebec) were used. Subjects weighed between 400 and 500 g at the time of surgery. Following surgery rats were housed individually in plastic solid-floor cages with *ad*

libitum access to food and water. All subjects were kept in a temperature-controlled room under a reverse 12 hour dark/12 hour light cycle. All behavioral testing was carried out in the middle of the dark phase of the cycle.

2.2.1.2. Surgery

Atropine sulfate (0.5 mg/kg, SC) was administered to each rat to reduce bronchial secretions prior to induction of anesthesia with sodium pentobarbital (Somnotol, 65 mg/kg, i.p.). With bregma and lamda in the same horizontal plane, bilateral, monopolar electrodes were aimed at the perifornical region of the LH (3 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, 7.8 mm below the dura mater), and a 24- gauge stainless-steel guide cannula (Plastics One, Inc., Roanoke, VA) was aimed at the right lateral ventricle (0.4 mm posterior to bregma; 1.6 mm lateral to the midsagittal sinus; 4 mm below the dura mater). The electrodes were constructed from 00 insect pins and were insulated with Formvar to within 0.5 mm of the tip. A wire twisted around two jeweler's screws implanted in the cranium served as the current return. The electrodes and cannula assembly were bonded to the skull and screw anchors by means of dental acrylic. The intraventricular cannula was then closed with a removable obturator. Buprenorphine (Buprenex, 0.05 mg/kg, SC) was administered just following surgery to reduce postoperative pain.

2.2.1.3. Apparatus

Screening for self-stimulation took place in wooden boxes (25cm x 25 cm 70 cm) with Plexiglas front panels and wire mesh floors. A lever, positioned 3 cm above

the floor, protruded from the middle of one wall, and a key light positioned 5 cm above the lever signaled the availability of the reward. Electrical stimulation was generated by dual constant current amplifiers and controlled by hand-operated circuit pulse generators. Depression of the lever triggered the stimulator. The connector on the rat's head was linked to the stimulator via a flexible lead and a 7-channel slip-ring located at the top of the test chamber.

The experimental sessions took place in a separate room housing computer-controlled stimulators. Operant chambers were similar to those used for training except they were constructed entirely of Plexiglas and equipped with a ceiling-mounted white house light. Each chamber was enclosed in a 50 cm x 50 cm x 90 cm plywood enclosure lined with sound attenuating foam. Stimulation trains were generated by microprocessor-controlled circuitry and monitored on an oscilloscope in an adjoining room. Subjects were monitored during testing by means of a remote-controlled video camera.

2.2.1.4. Procedure

Rats were shaped to lever press for a 0.5 s train of cathodal, rectangular, constant-current pulses, 0.1 ms in duration, on a continuous reinforcement schedule. Initially, stimulation parameters were set to low currents and frequencies. If the rat displayed signs of aversion, training was discontinued; otherwise, it was trained to press the lever. Stimulation frequencies (pulses per train) were adjusted to produce optimal levels of responding at a fixed current between 100 and 400 μA. Self-stimulation of both sites was assessed, and the electrode that supported the most

vigorous lever-pressing in the absence of motoric side-effects was chosen for further testing.

Following initial training, the subjects were trained in the computer-controlled testing setup to respond for a descending series of stimulation frequencies, on a 0.5 sec fixed-interval schedule of reinforcement. The stimulation current was held constant throughout testing, within the range used for initial training. The stimulation frequency was decreased from trial to trial in steps of 0.033 $\log_{10}$ units (~8%) in order to generate curves relating the rate at which rewards were earned to the stimulation frequency ("rate-frequency" curves). Each frequency was available during a single 60 sec trial that was preceded by a 10 sec inter-trial interval during which 5 priming (non-contingent) trains of stimulation were delivered; the priming trains were identical to those available during the trial. Stimulation frequencies were adjusted so as to produce maximal (asymptotic) responding during the first few trials of each frequency sweep. A rate-frequency curve was deemed complete when the rats emitted fewer than 5 responses on each of two consecutive trials.

The placement of the intraventricular cannula was verified by administering 50 ng of angiotensin II (Sigma, St. Louis, MO) and examining whether extended drinking began within 5 min. All substances were infused in the right lateral ventricle at a rate of 1 μl/min through a stainless steel internal cannula (Plastics One, Roanoke, VA) that extended 1 mm beyond the tip of the guide cannula. The internal cannula was connected to a 5 μl microsyringe (Hamilton Company) by means of flexible

polyethylene tubing (PE 50). After infusion, the internal cannula was kept in place for 15 seconds.

Rats were tested for BSR during free-feeding in order to obtain stable baseline responding. As an index of the effectiveness of the rewarding stimulation, we used the stimulation frequency that maintained a half-maximal rate of reward delivery (M-50). The influence of chronic food restriction on BSR was assessed by limiting daily food intake to 10 grams/day until body weights reached ~75% of free-feeding values (approx. 2-3 weeks time). During this time BSR testing was carried out every few days to maintain self-stimulation performance. When body weights reached ~75% of normal free-feeding values, the amount of food supplied to each rat was adjusted daily in an attempt to hold body weight constant. During this period regular BSR testing was carried out. At the end of the food restriction phase of the experiment, the influence of a 4 μg dose of NPY on BSR was assessed. To do this, 4 μl of vehicle treatment (0.04 M phosphate buffer containing 0.15 M NaCl, 0.01 % ascorbic acid, and 0.2 % bovine serum albumin [22]) was administered for two consecutive days, followed by 4 μg of NPY in 4 μl of vehicle on the third day. BSR testing began 15 min after injections.

In the same group of rats, the influence of a 4 μg dose of NPY (Sigma, St. Louis, MO) on food intake was assessed during the initial free-feeding period. Rats were habituated to a powdered food diet for at least 1 week prior to food intake measures. Powdered food was placed in jars attached to aluminum sheets that were molded to hang on the wall of the home cage. A flat-bottomed bowl was positioned

underneath each food cup to catch spillage. Food intake was measured each hour for 3 hours following intraventricular administration of vehicle and then measured again the following day after administration of NPY. All injections were made in the middle of the dark phase, in accordance with self-stimulation testing times. Food intake data was not obtained for rat N2 due to illness.

2.2.1.4.1. Data analysis

Seven rate-frequency curves were collected per test session. The first curve served as a warm-up and was not included in the data analysis. Broken-line functions, with a horizontal lower asymptote, a rising linear segment, and a horizontal upper asymptote [12], were fit to each of the six rate-frequency curves collected daily. To indicate the position of the rate-frequency curve along the frequency axis, the M-50 value was derived from each broken-line function by interpolation. Potentiation of the rewarding effect drives these curves to the left, thus reducing the M-50 value, whereas reductions in reward effectiveness produces rightward shifts and increases the M-50 value.

For each subject a one-way analysis of variance (ANOVA) was used to compare a set of 12 M-50 values from two consecutive days at the end of the free-feeding condition with a set of 12 M-50 values from two consecutive days after body weights stabilized at ~75% of free-feeding levels. For the analysis of the effects of NPY, a one-way ANOVA was employed to compare a set of 12 M-50 values collected during the two vehicle-treatment days to the 6 M-50 values collected on the

day of NPY administration. A level of $p < 0.05$ for a two-tailed test was considered critical for statistical significance.

To assess the effects of NPY on hourly food consumption, food intake following vehicle treatment was compared to food intake after NPY treatment for each hour post-injection by means of a one-way ANOVA.

2.2.1.4.2. Histology

Following completion of testing, stimulation sites were marked by means of the Prussian Blue method. With the stimulating electrode serving as the anode, a 100 μA direct current was applied for 15 sec. Rats were then injected with a lethal dose of sodium pentobarbitol (Somnotol, 100 mg/kg, IP) and perfused intracardially with phosphate-buffered saline followed by a mixture of 10% formalin (100 ml), trychloroacetic acid (0.5 g), potassium ferrocyanide (3 g) and potassium ferricyanide (3 g). Brains were removed and stored in 10% formalin. After immersion in a 20% sucrose-formalin solution for at least 24 hr, the brains were frozen, sliced on a cryostat in 30 μm coronal sections, and mounted on pre-coated slides (Fisher Scientific). Sections were stained for Nissl substance using formal thionin. The location of stimulation sites was identified with the aid of a stereotaxic atlas (Paxinos & Watson, 1998).

Visual inspection confirmed the penetration of the cannula into the right lateral ventricle and the absence of physical obstructions in this portion of the ventricle.

2.2.2. Results

2.2.2.1. Effects of chronic food restriction on BSR

Following food restriction, body weight fell by an average of 25.4%. Body weight loss reduced M-50 values in 6 out of the 12 subjects (Fig 2.2.1C). As exemplified in Figure 2.2.1A, rate-frequency curves for these subjects were shifted to the left following food restriction as compared to curves collected during free-feeding. In the remaining subjects, M-50 values remained fairly stable following food restriction (Fig. 2.2.1D), with one exception (N39), in which case M-50 values increased after restriction by a small but significant amount. In the latter group of subjects, the rate-frequency curves taken during food restriction generally overlap those collected during free-feeding (Fig. 2.2.1B).

2.2.2.2. Effects of NPY on food intake

Overall food intake was increased by an average of 61.3% (se±12.9) during the first hour, 26.8% (se±20.51) during the second hour and 84.56% (se±63.78) during the third hour following NPY administration as compared to vehicle-treatment (Fig. 2.2.2A and B). The results of a one-way ANOVA reveal that elevations in food intake during the first hour post-injection were significant ($F(1, 21) = 14.49$, $p = .001$). Analyses of the second ($F(1, 21) = 0.34$, $p = 0.57$) and third hour ($F(1, 21) = 1.73$, $p = 0.2$) were not significant.

2.2.2.3. Effect of NPY on M-50 values

In the 4 out of the 6 rats in which M-50 values decreased following chronic food restriction, NPY administration failed to significantly alter M-50 values (Fig. 2.2.3C). In the remaining two rats, N40 and N43, NPY increased M-50 values by an

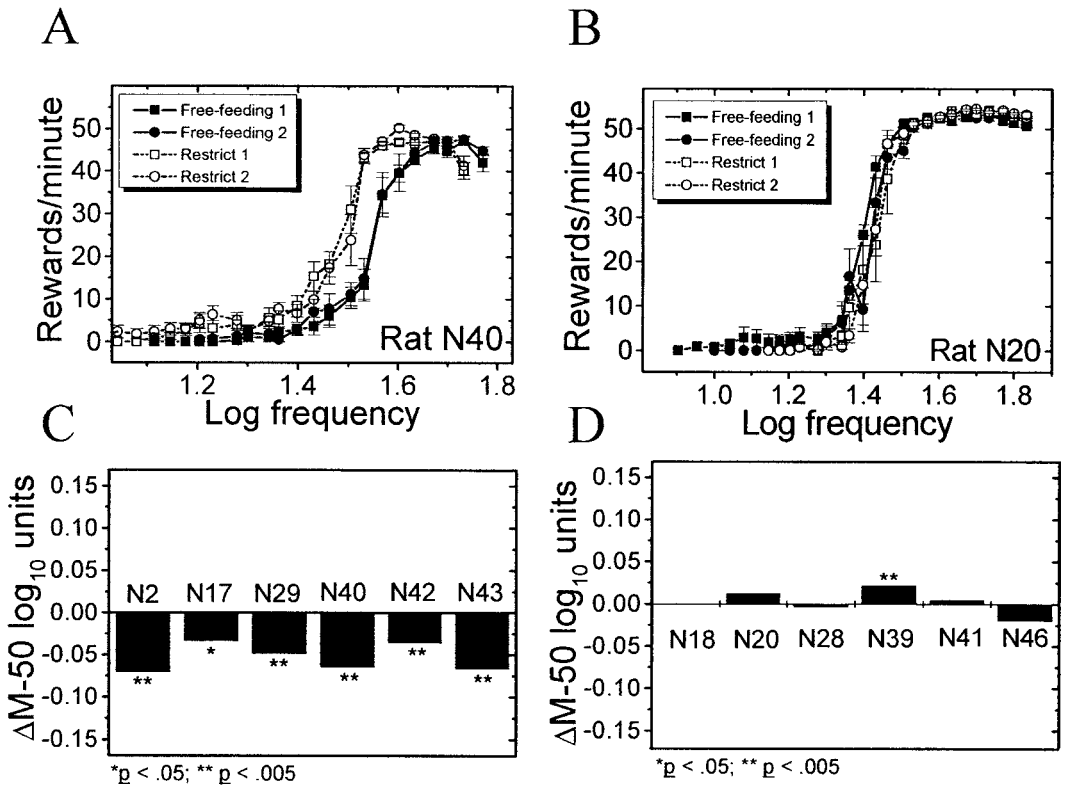


Figure 2.2.1. Effects of chronic food restriction on self-stimulation at LH sites where the rewarding effect of electrical stimulation is sensitive (left panels) or insensitive (right panels) to chronic food restriction. (**A**) Rate-frequency curves are shifted leftwards following chronic food restriction (open symbols) with respect to curves obtained during free-feeding (filled symbols). (**B**) In contrast, stimulation of a nearby, restriction-insensitive site produces overlapping rate-frequency curves following chronic food restriction relative to curves collected during free-feeding. Each data point in panels A and B is an average of six measures collected on each test day. (**C, D**) Magnitude of the curve shifts produced by chronic food restriction in all subjects. M-50 represents the stimulation frequency required to maintain half the maximal number of rewards earned. (*$p < 0.05$; ** $p < 0.005$)

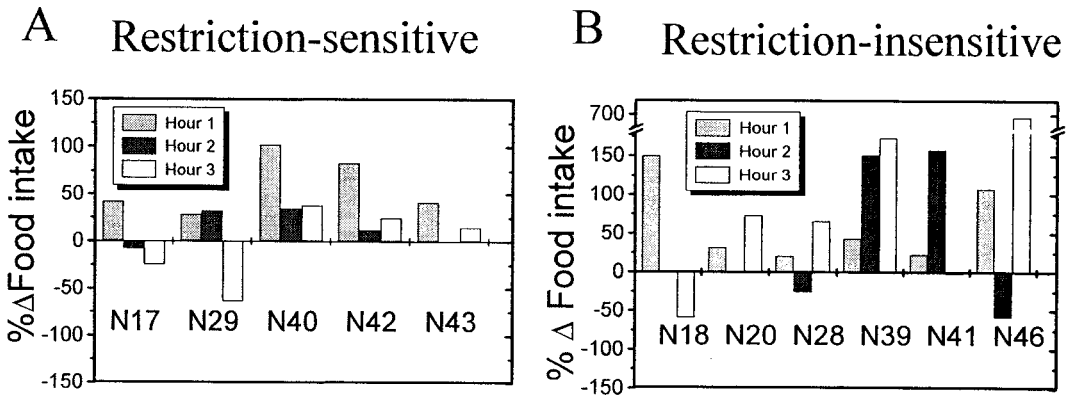


Figure 2.2.2. Effects of an intraventricular infusion of NPY (4 μg) on hourly food intake measured over a period of 3 hours post-injection. Data are expressed as the percent change in food intake produced by NPY relative to vehicle treatment for each subject.

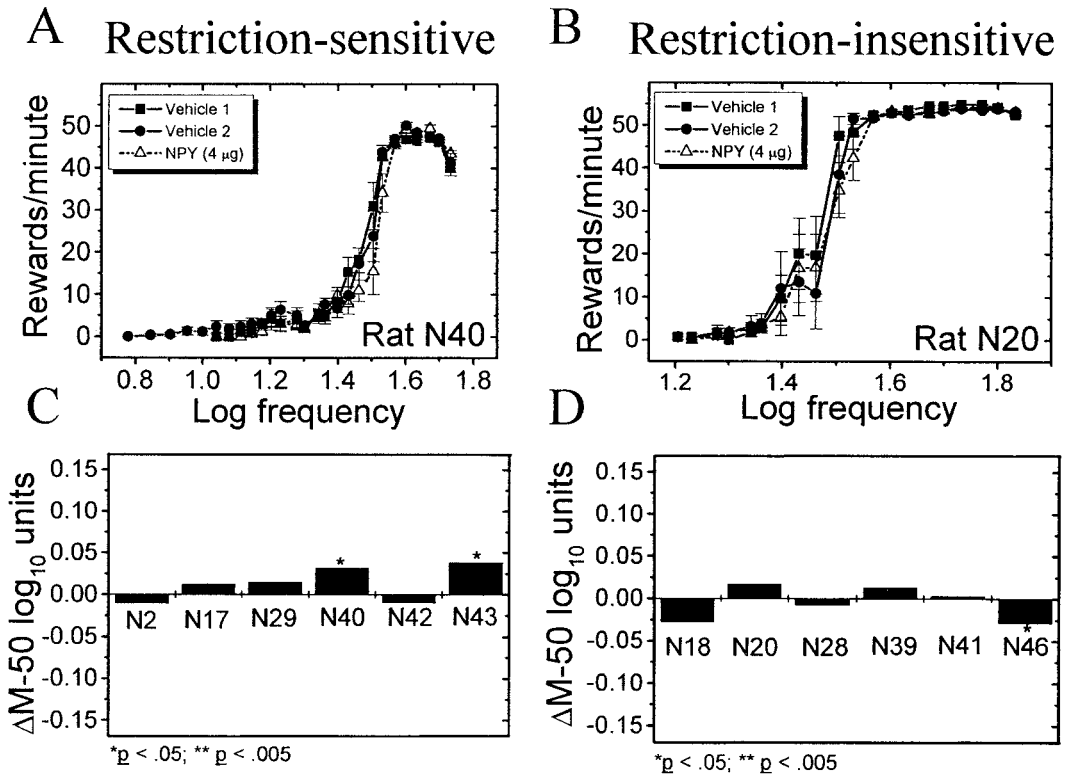


Figure 2.2.3. Effect of NPY on the rewarding impact of LH stimulation at restriction-sensitive sites (left panels) or restriction-insensitive sites (right panels). **(A)** At restriction-sensitive stimulation sites, rate-frequency curves obtained following NPY infusion (open symbols) overlap those collected during vehicle infusion (closed symbols). **(B)** Similarly, at restriction-insensitive stimulation sites, rate-frequency curves collected after NPY administration overlap curves collected after vehicle administration. Each data point in panels A, and B is an average of six measures collected on each test day. **(C, D)** Magnitude of the curve shifts produced NPY in all subjects. M-50 represents the stimulation frequency required to maintain half the maximal number of rewards earned. (*$p < 0.05$; ** $p < 0.005$)

average of 0.032 and 0.039 $\log_{10}$ units, respectively. Rate-frequency data from one of these rats (N40) is shown in Figure 2.2.3A to demonstrate that the curves collected after NPY administration overlap those obtained following vehicle infusion. There were no statistically reliable changes in M-50 values following NPY administration in 5 of the 6 subjects in which BSR was insensitive to the effects of food restriction (Fig. 2.2.3D). The exception is rat N46 in which M-50 values were decreased by a small, yet significant, average of 0.029 $\log_{10}$ units. An example of the rate-frequency curves obtained for one of these subjects is displayed in Figure 2.2.3B.

2.2.2.4. Electrode and cannula placements

Histological analysis revealed that the restriction-sensitive rewarding effects arose from stimulation of sites dorsal and/or lateral to the fornix (Fig. 2.2.4 & Table 2.2.1). The remaining sites were scattered around the periphery of the region containing the restriction-sensitive sites. Visual examination of cannula placement in the right lateral ventricle during slicing did not reveal any displacement of cannulae or obstructions in the ventricle.

2.2.3. Discussion

Chronic food restriction resulting in substantial weight loss decreased M-50 values for LH self-stimulation in half of the 12 subjects. Rate-frequency curves for these subjects were shifted leftward by food restriction indicating that the rewarding effect of the stimulation was enhanced. Despite the considerable weight loss in the

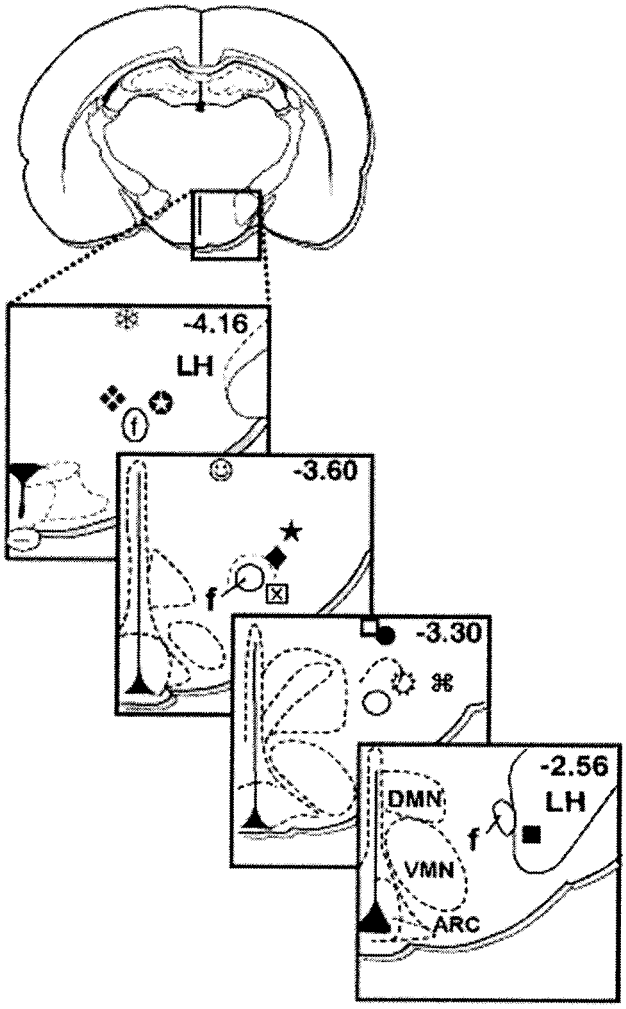


Figure 2.2.4. Location of the tips of the stimulation electrodes. Symbols denote electrode placements for individual rats (see Table 1). The coronal sections are based on the atlas of G. Paxinos and C. Watson, (Academic Press, New York, Fourth Edition, 1998).

Table 2.2.1. Symbols marking electrode placements for individual rats.

Restriction-sensitive		Restriction-insensitive	
Rat	Symbol	Rat	Symbol
N2	☼	N18	❖
N17	✪	N20	❄
N29	●	N28	⌘
N40	■	N39	★
N42	♦	N41	☒
N43	□	N46	☺

remaining subjects, no considerable change was observed in their M-50 values following food restriction. These findings are consistent with previous reports that food restriction and body weight loss lower rate-frequency thresholds only at certain stimulation sites in the LH (Carr & Wolinsky, 1993; Fulton et al., 2000).

The dose of NPY (4 μg) was chosen because it lies near the middle of the dose range shown by Jewett et al to enhance food reward on a progressive ratio schedule of reinforcement (Jewett, Cleary & Levine, 1995). Overall, this dose of NPY produced a 61.3% increase in food intake during the first hour after administration in the present study. Although all rats showed increased food intake during the first hour, the magnitude of the increases varied substantially, from 28.4 to 83.3%. Increases in food intake during the second and third hour were not significant. However, these effects are not unusual as others have found that a 5 μg dose of NPY delivered into the lateral ventricle failed to increase 1-hour food intake in rats (Jolicoeur, Michaud, Rivest, Menard, Gaudin, Fournier & St. Pierre, 1991).

While the 4 μg dose of NPY produced increases in food intake, it did not elicit any consistent changes in BSR. At restriction-sensitive sites NPY did not significantly alter M-50 values, with the exception of two subjects in which M-50 values were slightly increased following NPY administration. Thus, NPY failed to potentiate the rewarding effect of stimulating restriction-sensitive sites. Similarly, NPY had very little impact at restriction-insensitive sites: M-50 values were not significantly changed in 5 out of 6 subjects. In the remaining rat, M-50 values were decreased by an average of 0.029 $\log_{10}$ units, indicating that NPY produced a modest enhancement

of the rewarding effect of the stimulation. Together, the results of the current study suggest that NPY administration does not change thresholds for BSR at either restriction- sensitive or -insensitive sites. Given, however, that the present study used only one dose of NPY it remains possible that higher doses would be effective.

That NPY was unable to modify BSR is consistent with a report by Cabeza de Vaca et al. that frequency thresholds required to support low rates of LH self-stimulation remained stable when challenged with NPY (Cabeza de Vaca, Holiman & Carr, 1998. In this study, sensitivity of BSR to food restriction was not assessed. Rather, the authors assumed, based upon histological verification, that the stimulating electrodes of the four rats examined were positioned in a restriction-sensitive zone of the LH. However, sites where BSR is enhanced by food restriction can be located in close proximity to sites insensitive to this manipulation (Fulton et al., 2000; Fulton et al., 2002). Furthermore, in most cases the influence of leptin on BSR at sites insensitive to food restriction was opposite to the effect produced at sites sensitive to this manipulation. Thus, there are at least two possible interpretations of the failure of NPY to alter BSR in the study by Cabeza de Vaca et al.: either NPY is truly ineffective at all placements, or opposite effects at restriction-sensitive and -insensitive sites cancel out by averaging results across subjects. By testing all placements for restriction sensitivity and analyzing the results within subject, it was possible to distinguish between these interpretations in the current study.

Leptin was previously shown to attenuate the rewarding effect of stimulating sites responsive to food restriction. The increases in M-50 values produced by leptin on the day of injection in this study ranged from 0.045 to 0.09 $\log_{10}$ units, and M-50

values remained elevated in most cases for up to 4 days post-injection. Leptin has been reported to reduce NPY expression (Stephens, Basinski, Bristow, Bue-Valleskey, Burgett, Craft, Hale, Hoffman, Hsiung, Kriauciunus et al., 1995), thus NPY administration would be expected to enhance BSR at restriction-sensitive sites if it participated in the effect of leptin on BSR at these sites. In the present study however, NPY did not produce any consistent alterations in M-50 values at these sites. Instead, in 4 of the 6 rats with restriction-sensitive stimulation sites no change in BSR was observed. In the remaining two subjects there were modest elevations in M-50 values (0.032 and 0.039 $\log_{10}$ units) which are contrary to the hypothesis that NPY mediates the influence of leptin at restriction-sensitive sites: NPY suppressed the rewarding effect of the stimulation instead of enhancing it. These findings suggest that NPY does not contribute to the modulation of restriction-sensitive reward circuitry by leptin.

Whereas there is strong data supporting a role for NPY in the regulation of energy balance by leptin (Schwartz, Woods, Porte, Seeley & Baskin, 2000) there is considerable evidence to suggest that altered NPY signaling is not the only means by which leptin moderates feeding and energy expenditure. Indeed, whereas mice lacking leptin (*ob/ob*) are extremely hyperphagic and obese, NPY-deficient mice have normal food intake and body weight and show sensitivity to leptin (Erickson, Clegg & Palmiter, 1996)..

It is now well understood that, in addition to its interaction with NPY, leptin also influences energy balance via other hypothalamic neuropeptides. Through direct action on the ARC, leptin reduces the expression of agouti-regulated peptide (AgRP),

a potent orexigenic neuropeptide coexpressed with NPY (Arvaniti, Huang & Richard, 2001). Leptin also engages a separate population of ARC neurons coexpressing anorexigenic peptides such as pro-opiomelanocortin (precursor of a-melanocyte-stimulating hormone) and cocaine-and amphetamine-regulated transcript (Elmquist, Elias & Saper, 1999). In addition, leptin reduces the expression of two hormones that stimulate feeding and are produced exclusively in the LH: the orexins and melanin-concentrating hormone (Hakansson, de Lecea, Sutcliffe, Yanagisawa & Meister, 1999; Kokkotou, Tritos, Mastaitis, Slieker & Maratos-Flier, 2001). Thus, the unraveling of an elaborate network of neuropeptides involved in the neural control of energy balance introduces numerous candidates, other than NPY, that could mediate the effects of food restriction and leptin on brain reward circuitry.

2.2.4. Conclusions

Chronic food restriction and body weight loss potentiated the rewarding effect produced by electrical stimulation of sites in the PFH. In a previous study, the rewarding impact of stimulating these sites was shown to be reduced by leptin. In contrast, a 4 ug dose of NPY failed to alter BSR at most restriction-sensitive sites in the present study. Thus, it is unlikely that NPY mediates the influence of food restriction or leptin on the rewarding effect of stimulating restriction-sensitive sites. Similarly, at restriction-insensitive sites the rewarding impact of the stimulation was mostly unchanged when challenged with NPY. Taken together, the results of the present study suggest that NPY does not impinge on brain reward circuitry activated by stimulating restriction-sensitive and restriction-insensitive stimulation sites in the LH.

2.3. THE ROLE OF AGOUTI-RELATED PEPTIDE AND MELANIN-CONCENTRATING HORMONE IN THE POTENTIATION OF BRAIN STIMULATION REWARD BY FOOD RESTRICTION

The neural circuitry that is responsible for the rewarding effects of stimuli and behaviors can be directly activated and studied by means of electrical brain stimulation reward (BSR). Electrical stimulation of some brain areas can elicit a powerfully rewarding effect. The artificial activation of neurons near the electrode tip can induce a meaningful signal such that animals learn to self-administer the rewarding stimulation and evaluate it along a common dimension with natural rewards such as food (Conover and Shizgal, 1994).

Chronic food restriction and body weight loss have been shown to increase the rewarding impact of stimulation delivered to particular stimulation sites in the lateral hypothalamus (LH) (Blundell & Herberg, 1968; Carr & Papadouka, 1993; Abrahamsen et al., 1995; Fulton, Woodside & Shizgal, 2000; 2002, Fulton, Richard, Woodside & Shizgal, 2002). The ability of food restriction to modulate BSR depends on the placement of the stimulating electrode amongst a functionally heterogeneous population of reward-relevant fibers (Fulton, Woodside & Shizgal, 2002) and the size of this effect is contingent on the degree of weight loss (Carr and Wolinsky, 1993; Fulton et al., 2002). Thus, the enhancement of the rewarding effect of the stimulation by weight loss is believed to rely on signals reflecting adipose mass. In support of this hypothesis, the adiposity hormones, leptin and insulin, have been shown to reverse the potentiation of BSR by weight loss (Carr, Kim, & Cabeza de Vaca, 2000; Fulton et al., 2000).

Whether leptin and insulin modulate the neural substrate of BSR directly or by their effects on other mediators is not clear. A plethora of research has contributed to the identification of central signaling molecules that mediate the actions of leptin on food intake and body weight including corticotropin-releasing hormone (CRH) and neuropeptide Y (NPY) (Grill et al., 2002); Jequier, 2002; Schwartz et al., 2000). Previous data suggest however that neither of these peptides is a good candidate for mediating the effects of leptin on BSR. Both Cabeza de Vaca et al. (1998) and Fulton et al. (2002) reported that BSR remains fairly stable following central NPY administration. Further, we have recently shown that BSR was relatively unaltered by CRH at restriction-sensitive stimulation sites although it did increase thresholds at restriction insensitive sites (Fulton, Richard, Woodside & Shizgal, 2002).

Another important target of leptin is the central melanocortin system (Schwartz, Woods, Porte, Seeley, & Baskin, 2000; Seeley et al., 1997). Alpha-melanocyte-stimulating hormone (α-MSH) is the endogenous agonist at the MC3 and MC4 receptors, and central administration of α-MSH or synthetic ligands for these receptors reduce food intake (Fan et al., 1997; Grill et al., 1998; Poggioli et al., 1986). Interestingly, the MC3/4 receptor has an endogenous antagonist, agouti-related peptide (AgRP), that is produced exclusively in the arcuate nucleus. AgRP is upregulated during food restriction (Harold et al., 1999). and leptin deficiency (Ollman et al., 1997), and transgenic mice overexpressing AgRP are hyperphagic and obese (Graham et al., 1997).

A single intraventricular injection of AgRP (83-132) has been shown to increase food intake for as long as 7 days (Grill et al., 1998; Hagan et al., 2001b). Furthermore, preference tests reveal that AgRP induces increased consumption of high fat food over carbohydrate-rich food (Hagan et al., 2001) The long-term actions of AgRP (83-132) on food consumption are accompanied by elevations in Fos expression in a key component of reward circuitry, the nucleus accumbens, and thus it has been speculated that AgRP may contribute to the rewarding properties of food or feeding-related behaviors (Hagan et al., 2001a; Saper et al., 2002). Additional evidence implicating the melanocortin system in reward processes comes from a study by Cabeza de Vaca et al. (2002) demonstrating that MTII, a synthetic agonist to the MC4 receptor, can potentiate the threshold lowering effect of amphetamine on BSR. When administered individually, neither MTII nor an AgRP-like antagonist altered the rewarding effect of the stimulation (Cabeza de Vaca et al., 2002).

Neurons containing melanin-concentrating hormone (MCH) receive innervation from AgRP fibers (Elias et al., 1998), and AgRP administration has been show to increase MCH gene expression (Hanada et al., 2000). With somata residing exclusively in the LH, MCH neurons send widespread projections to many brain regions, including the nucleus accumbens (Bittencourt et al., 1992; Peyron et al., 1998). Central MCH administration stimulates food intake over a much shorter period of time than is typically observed following AgRP administration (ref). Nonetheless, $MCH^{-/-}$ mice are hypophagic and lean (Shimada et al., 1998) suggesting that MCH contributes to the regulation of body weight.

In spite of their efficacy in stimulating food intake, it is not yet known whether AgRP and MCH can modulate the restriction-sensitive component of reward circuitry. To determine this, the present study assessed the ability of AgRP and MCH to modulate the rewarding effect produced by stimulating restriction-sensitive or – insensitive sites in the LH. In light of their orexigenic nature, we hypothesized that administration of AgRP and MCH would enhance the rewarding effect of the stimulation at sites sensitive to changes in body weight.

2.3.1. Materials and methods

2.3.1.1. Subjects

Fifteen male Long Evans rats from the Charles River Breeding Farms (St. Constant, Quebec) were used. Each rat was housed individually in plastic solid-floor cages with *ad libitum* access to food and water. All subjects were kept in a temperature-controlled room under a reverse 12 hour dark/12 hour light cycle that switched at 8 am/pm. All behavioral testing was carried out in the dark phase of the cycle.

2.3.1.2. Surgery

The rats were administered atropine sulfate (0.5 mg/kg, SC) to reduce bronchial secretions and were anesthetized with sodium pentobarbital anesthesia (Somnotol, 65 mg/kg, i.p.). Bilateral, monopolar electrodes were aimed at the perifornical region of the LH (3 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, 7.8 mm below the dura mater), and a 24- gauge stainless-steel guide cannula (Plastics One, Inc., Roanoke, VA) was aimed at the third ventricle (0.8 mm

posterior to bregma; 0 mm lateral to the midsagittal sinus; 6.2 mm below the dura mater). The electrodes were constructed from 000 insect pins and were insulated with Formvar to within 0.5 mm of the tip. A wire twisted around two jeweler's screws implanted in the cranium served as the current return. The electrodes and cannula assembly were bonded to the skull and screw anchors by means of dental acrylic. The intraventricular cannula was then closed with a removable obturator. To reduce postoperative pain, Buprenorphine (Buprenex, 0.05 mg/kg, SC) was administered once following surgery.

2.3.1.3. Apparatus

Subjects were screened for self-stimulation in wooden boxes (25cm x 25 cm 70 cm) with Plexiglas front panels and wire mesh floors. A lever, positioned 3 cm above the floor, protruded from the middle of one wall, and a key light positioned 5 cm above the lever signaled the availability of the reward. Electrical stimulation was generated by dual constant current amplifiers and controlled by hand-operated pulse generators. Depression of the lever triggered the stimulator. The connector on the rat's head was linked to the stimulator via a flexible lead and a 7-channel slip-ring located at the top of the test chamber.

The experimental sessions took place in a separate room housing computer-controlled stimulators. Test chambers were similar to those used for training except they were constructed entirely of Plexiglas and equipped with a ceiling-mounted white house light. Each test chamber was enclosed in a 50 cm x 50 cm x 90 cm plywood enclosure lined with sound attenuating foam. Stimulation trains were

generated by microprocessor-controlled circuitry and monitored on an oscilloscope in an adjoining room. Subjects were monitored during testing by means of a remote-controlled video camera.

2.3.1.4. Design and Procedure

2.3.1.4.1. Brain stimulation reward procedure

Rats were shaped to lever press for a 0.5 s train of cathodal, rectangular, constant-current pulses, 0.1 ms in duration, on a continuous reinforcement schedule. Initially, stimulation parameters were set to low currents and frequencies. If the rat displayed signs of aversion, training was discontinued; otherwise, the subject was shaped to press the lever. Stimulation frequencies (pulses per second) were adjusted to produce optimal levels of responding at a current that remained fixed for each rat, ranging between 100 and 400 μA. Self-stimulation of both sites was assessed, and the electrode that supported the most vigorous lever-pressing in the absence of motoric side-effects was chosen for further testing.

Following initial training, the subjects were trained in the computer-controlled testing setup to respond for a descending series of stimulation frequencies, on a 0.5 sec fixed-interval schedule of reinforcement. The stimulation current was held constant throughout testing, within the range used for initial training. The stimulation frequency was decreased from trial to trial in steps of 0.033 $\log_{10}$ units (~8%) in order to generate curves relating the rate at which rewards were earned to the common logarithm of the stimulation frequency ("rate-frequency" curves). Each frequency was available during a single 60 sec trial that was preceded by a 10 sec inter-trial interval during which 5 priming (non-contingent) trains of stimulation were delivered; the

priming trains were identical to those available during the trial. Stimulation frequencies were adjusted so as to produce maximal (asymptotic) responding during the first few trials of each frequency sweep. A rate-frequency curve was deemed complete when the rats emitted fewer than 5 responses on each of two consecutive trials. Seven rate-frequency curves were collected per test session. Test sessions lasted between 85 to 165 minutes. The first curve of the session served as a warm-up and was not included in the data analysis.

2.3.1.4.2. Drug injections

A 1 nMol dose of AgRP (83-132) (Phoenix Pharmaceutical Inc, CA) was dissolved in 2 μl of saline whereas a 5 μg dose of MCH (Phoenix Pharmaceutical Inc, CA) was dissolved in 3 μl of saline. A 1 nMol dose of AgRP was selected because it was reported to produce a larger increase in body weight than the 0.1 and 0.5 nMol doses (Hagan et al., 2000). The 5μg MCH does has been shown to produce a statistically reliable increase in food intake for two hours following injection (Qu, Ludwig, Gameltoft, Piper, Pelleymounter et al., 1996). Cannula placement in the third ventricle was verified by observing whether vigorous drinking began within 5 min of administering 25 ng of angiotensin II (Sigma, St. Louis, MO). All substances were infused at a rate of 0.2 μl/min through a stainless steel internal cannula (Plastics One, Roanoke, VA) that extended 1 mm beyond the tip of the guide cannula. The internal cannula was connected to a 5 μl microsyringe (Hamilton Company, Reno, NV) by means of flexible polyethylene tubing. After infusion, the internal cannula was kept in place for 15 seconds.

2.3.1.4.3. Design

Training in the self-stimulation paradigm lasted for 1-2 days. Baseline (free-feeding) rate-frequency data were obtained until the mean frequency required to maintain half-maximal response rate from each session was stable (day-to-day variation $\leq 0.04 \log_{10}$ units). To assess the influence of food restriction on BSR, daily food intake was limited to 10 grams/day until body weight reached ~75% of free-feeding values (approx. 3-4 weeks time). During this time BSR testing was carried out every few days to maintain self-stimulation performance. When body weights reached ~75% of normal free-feeding values, the amount of food supplied to each rat was adjusted daily in an attempt to hold body weight constant. Body weights stabilized after 2-4 days. During this period regular BSR testing was carried out until steady rate-frequency data were achieved.

The AgRP and MCH test intervened between the gathering of rate-frequency data during free-feeding and the food restriction test. The effect of AgRP (1 nMol) on BSR was assessed for 4 days following a single injection. Five days later, the influence of MCH (5 μg) on BSR was examined in 13 of the 15 subjects. Both peptide injections were preceded by two days of saline administration. BSR testing began 5 minutes after MCH injections and 15 minutes after AgRP injections.

2.3.1.4.4. Food intake measures

Powdered food was placed in cups attached to aluminum sheets that were molded to hang on the wall of the home cage. A flat-bottomed bowl was fixed underneath each food cup to catch spillage. Rats were habituated to the powdered

food diet for one week prior to testing. In the case of AgRP, food intake measurements coincided with examinations of the influence of AgRP on BSR. Food intake was measured over a 6 hour period following the self-stimulation test session (beginning 3 hours after the injection), corresponding to the last half of the dark phase of the cycle. Given that MCH increases food intake over a period of 2 to 3 hours, the influence of MCH on feeding was assessed apart from BSR testing. The amount of food consumed each hour for a 3-hour period was measured in a separate group of rats (N = 5) with third ventricle cannulas. This 3-hour period coincided with BSR testing times. As a baseline measure, food intake was measured after saline administration on the two days preceding neuropeptide treatment.

2.3.1.4.5. Data analysis

Broken-line functions, with a horizontal lower asymptote, a rising linear segment, and a horizontal upper asymptote (Gallistel & Freyd, 1987), were fit to a maximum of six rate-frequency curves that could be collected each day. As an index of the effectiveness of the rewarding stimulation, we used the stimulation frequency that maintained a half-maximal rate of reward delivery (M-50). M-50 values were derived from each broken-line function by interpolation to indicate the position of the rate-frequency curve along the frequency axis. Potentiation of the rewarding effect drives these curves to the left, thus reducing the M-50 value, whereas reductions in reward effectiveness produces rightward shifts and increases the M-50 value.

The placement of the electrode amongst a compact and heterogeneous population of neurons has been shown to determine the effectiveness of chronic food

restriction to decrease M-50 values (Fulton et al., 2002). Thus, the assessment of sensitivity to food restriction has to be carried out *post hoc* for each rat. To determine the effect of chronic food restriction on thresholds for BSR in each rat a set of 12 M-50 values from two consecutive days from the end of the free-feeding condition was compared to a set of 12 values obtained just after body weight stabilized at ~75% of normal values. The comparison was performed using a one-way within-subjects analysis of variance (ANOVA). Rats that showed a significant decrease in threshold following food restriction were assigned to the "restriction-sensitive" group and those that failed to show such a change were judged restriction-insensitive. To confirm that this distinction was indeed behaviorally relevant a delta M-50 value was then calculated for each rat by subtracting the mean of the 12 M-50 values collected during free-feeding from the mean of the 12 M-50 values obtained under food restriction A one-way ANOVA was then used to compare the delta M-50 values between the two groups

The initial analysis of the effects of AgRP on BSR was carried out on each rat by comparing a set of 12 M-50 values collected during the two vehicle-treatment days to the 6 M-50 values collected on each of the four days following AgRP administration. This comparison was performed using a within-subjects ANOVA followed by a Dunnett test for multiple comparisons. To compare the effects of AgRP on BSR between the restriction-sensitive and -insensitive groups a ΔM-50 value was calculated for each rat on each post-injection day by subtracting the mean of the 12 M-50 values collected during saline administration from the mean of the 6 M-50

values collected on the post-injection day. A two-way ANOVA was used to compare the ΔM-50 values as a function of group (restriction-sensitive vs. –insensitive) and time (post-injection day). Pairwise comparisons were carried out to examine the difference between groups for the influence of AgRP on BSR on each of the four post-injection days by using one-way ANOVAs. The alpha level was adjusted for each subsequent comparison by performing a layered Bonferroni correction. Thus, the alpha levels were adjusted to .05, .025, .0167 and .0125 for post-injection days 0 to 3, respectively. Analyses of the effects of MCH on BSR was carried out in the same way as those performed for AgRP.

The influence of AgRP on food intake was assessed by comparing food consumption for all subjects on the saline day to food intake during each of the four post-injection days using a within-subjects ANOVA followed by a Dunnett test for multiple comparisons. A within-subjects ANOVA was used to compare the influence of MCH on food intake as compared to saline treatment. To examine the effects between treatment days as a function of hour, pairwise comparisons were performed for each of the three hours post-injection. The alpha level was corrected for each subsequent comparison by using the layered Bonferroni technique.

2.3.1.4.5. Histology

Following completion of testing, the stimulation sites were marked by means of the Prussian Blue method. With the stimulating electrode serving as the anode, a 100 μA direct current was applied for 15 sec. Rats were then injected with a lethal dose of sodium pentobarbitol (Somnotol, 100 mg/kg, IP) and perfused intracardially with phosphate-buffered saline followed by a mixture of 10% formalin (100 ml),

trychloroacetic acid (0.5 g), potassium ferrocyanide (3 g) and potassium ferricyanide (3 g). Brains were removed and stored in 10% formalin. After immersion in a 20% sucrose-formalin solution for at least 24 hr, the brains were frozen, sliced on a cryostat in 30 μm coronal sections, and mounted on pre-coated slides (Fisher Scientific). Sections were stained for Nissl substance using formal thionin. The location of stimulation sites was identified with the aid of a stereotaxic atlas (Paxinos & Watson, 1998).

2.3.2. Results

2.3.2.1. Effects of AgRP on food intake and body weight

As shown in Figure 2.3.1, AgRP (83-132) administration resulted in a significant increase in food intake on each of the four post-injection days as compared to food intake after saline treatment ($F(4, 69) = 13.33$; $p=<0.001$) (PI day 0: $F(1, 27) = 6.85$, $p< .05$; PI day 1: $F(1, 27) = 5.33$, $p<.05$; PI day 2: $F(1, 27) =4.29$, $p<.05$; PI day 3: $F(1, 27) = 3.17$, $p<.05$).

2.3.2.2. Effects of MCH on food intake

A 5 μg infusion of MCH in a separate group of rats (N=5) increased food intake during the first two hours (Fig. 2.3.2), however the results did not meet our criterion for statistical reliability ($F(1, 9) = 1.21$; $p=0.3$) (PI hour 1: $F(1, 9) = 1.38$, $p=0.27$; PI hour 2: $F(1, 9) = 1.21$, $p=0.3$; PI hour 3: ($F(1, 9) = 0.08$, $p = 0.78$).

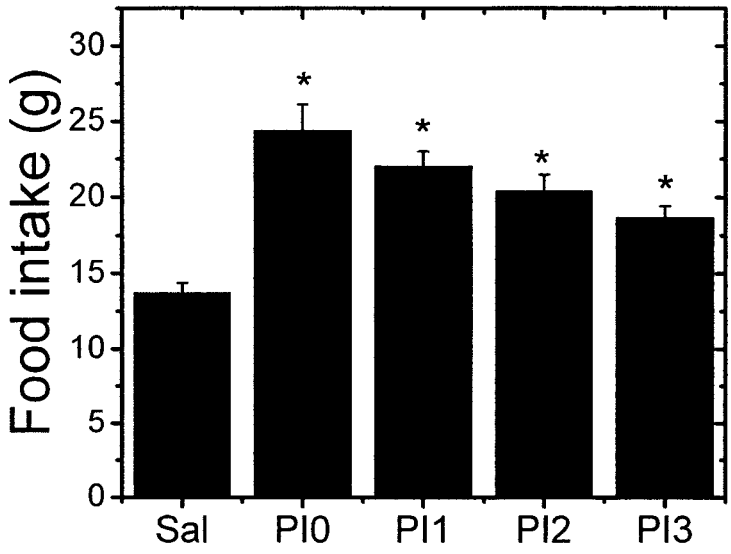


Figure 2.3.1. The influence of a single 1 nMol dose of AgRP (82-132) on food intake over four days post-injection. Data are means and SEM (N=14). PI: post-injection day.

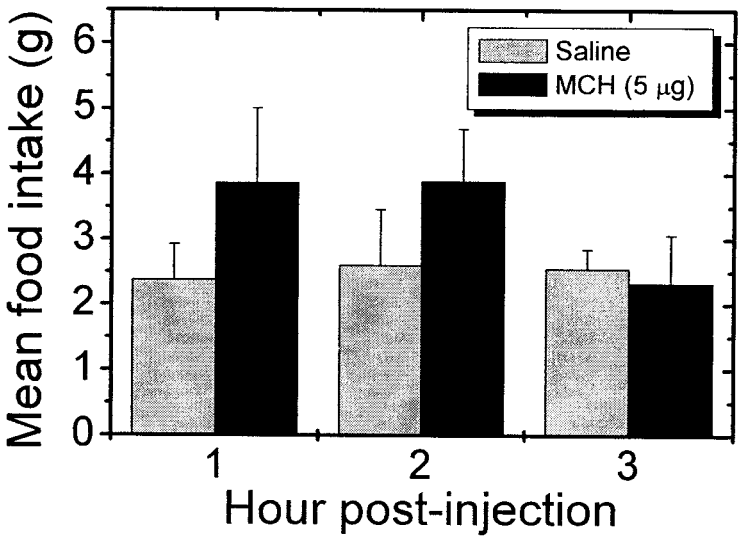


Figure 2.3.2. The influence of single 5 μg dose of MCH on food intake for 3 hours post-injection. Data are means and SEM (N=5).

2.3.2.3. Chronic food restriction

Initial body weights ranged from 445 - 589 g. Following food restriction, body weights fell to 328 - 432 g. In 6 out of 15 subjects, chronic food restriction significantly reduced M-50 values. Examples of the data obtained from these rats are earned. (*$p < 0.05$) shown in Figure 2.3.3A and show that curves taken during food restriction lie to the left of the curves obtained during free-feeding. Figure 2.3.3C shows the corresponding ΔM-50 values following food restriction for rats that were restriction-sensitive. In the remaining 9 subjects, the rate-frequency curves obtained during restriction overlap the curves obtained during free-feeding (e.g., Fig. 2.3.3B), and the corresponding M-50 values remained stable (Fig. 2.3.3D). The results of a one-way ANOVA revealed that delta M-50 values of the restriction-sensitive group (-0.105 ±se0.038) were significantly lower than the ΔM-50 values of the restriction-insensitive groups (-0.001 ±se0.009) ($F(1, 14) = 10.57$, $p = .006$).

2.3.2.4. Effect of AgRP on M-50 values

The results of a two-way ANOVA comparing ΔM-50 values as a function of group and time reveal a significant difference between the influence of AgRP on the rewarding effects produced by stimulating restriction-sensitive and –insensitive sites ($F(1, 59)= 17.2$; $p<0.001$). There was no effect of post-injection day ($F(1, 59)= 1.39$; $p=0.225$). As shown in Figure 2.3.4A, M-50 values obtained from restriction-insensitive sites were increased by AgRP (83-132) on post-injection days 1, 2 and 3 as compared to M-50 values obtained from restriction-sensitive sites (PI day 0: $F(1, 14) = 2.18$, $p=0.163$; PI day 1: $F(1, 14) = 5.73$, $p=0.03$; PI day 2: $F(1, 14) = 5.89$,

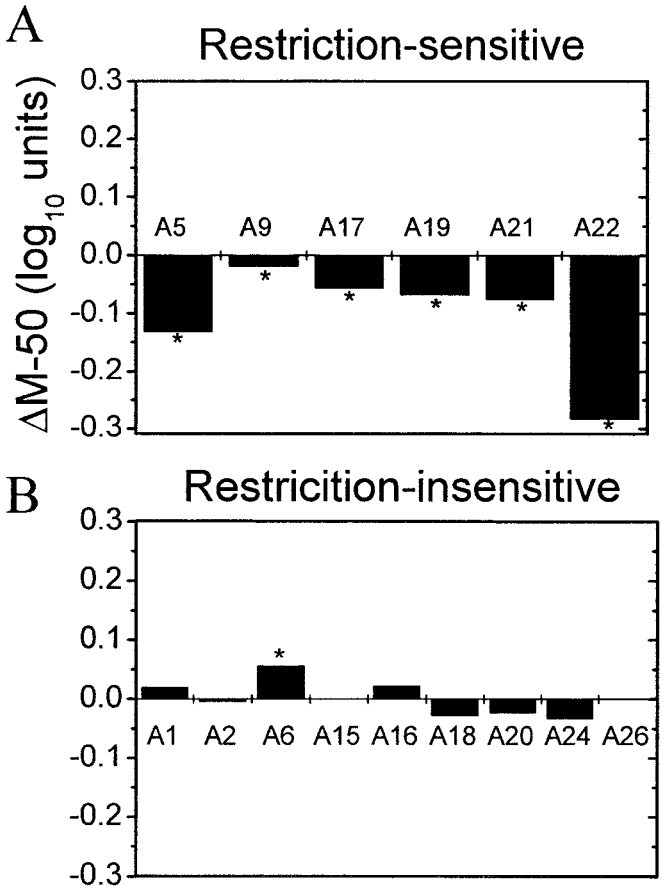


Figure 2.3.3. Effects of chronic food restriction on self-stimulation of LH sites where the rewarding effect of electrical stimulation is sensitive (top panel) or insensitive (bttom panel) to chronic food restriction. Each column represents the mean change in ΔM-50 (log_{10} units) following food restriction and weight loss Magnitude of the curve shifts produced by chronic food restriction in all subjects. M-50 represents the stimulation frequency required to maintain half the maximal number of rewards

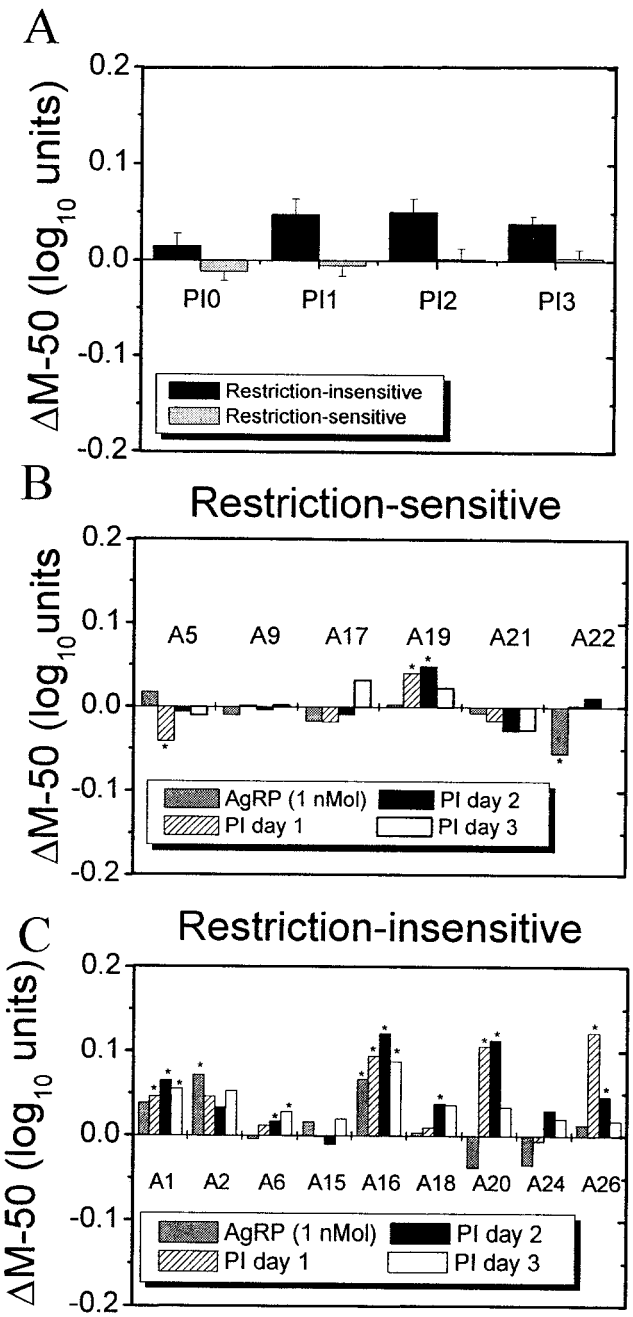


Figure 2.3.4. The influence of a single injection of AgRP on M-50 values for four post-injection days. A) Group data showing the distinct influence of AgRP at restriction-sensitive and –insensitive stimulation sites. B) Individual data for rats from the restriction-sensitive group. C) Individual data for rats from the restriction-insensitive group. M-50 represents the stimulation frequency (log_{10} units) required to maintain half the maximal number of rewards earned. (*$p < 0.05$)

p=0.03; PI day 3: $F(1, 14) = 9.11$, p=0.01). However, the only statistically reliable difference between M-50 values from restriction-sensitive and –insensitive sites was on the last post-injection day due to the Bonferonni correction of the alpha levels. Analyses of individual data revealed that AgRP administration produced significant decreases in M-50 values on one of the first two post-injection days in 2 of the 6 rats that showed decreases in M-50 values following food restriction (A5 and A22) (Fig. 2.3.4B). In another restriction-sensitive subject AgRP increased M-50 values for two consecutive days. For the remaining 3 rats, M-50 values remained relatively stable following AgRP. In contrast, AgRP administration elevated M-50 values for one to four post-injection days in 7 out of the 9 subjects that were insensitive to the effects of food restriction on BSR ("restriction-insensitive") (Fig. 2.3.4C).

2.3.2.5. Effect of MCH on M-50 values

Overall, there was no effect of MCH on BSR in either the restriction sensitive or insensitive group. As illustrated in Figure 5A, there was no difference between restriction-sensitive and –insensitive rats with respect to the influence of MCH on BSR (PI day 0: $F(1, 12) = 2.32$, p=0.16; PI day 1: $F(1, 12) = 3.34$, p=0.09). In 2 of the 6 restriction-sensitive rats there were significant increases in M-50 values (Fig. 2.3.5B), which was also observed in one of the rats from the restriction-insensitive group. In addition, 2 of the 7 rats from the restriction-insensitive group showed significant decreases in M-50 values on the first day of MCH administration (Fig. 2.3.5C).

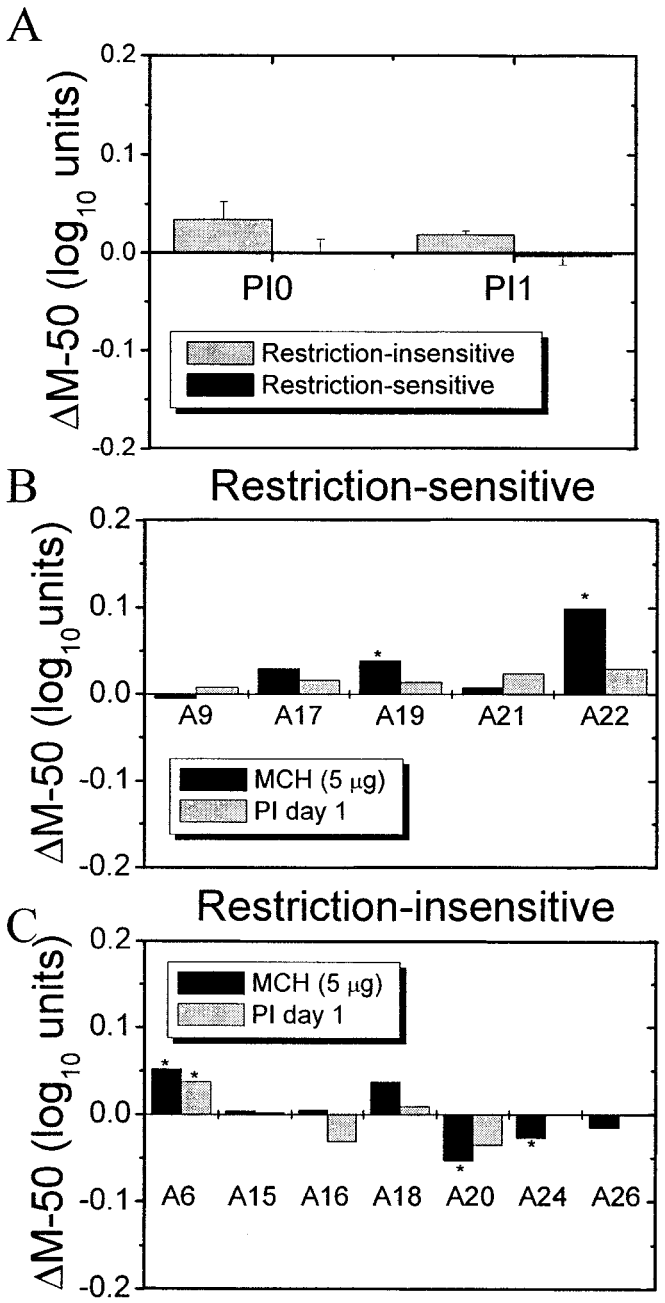


Figure 2.3.5. The influence of a single 5μg injection of MCH on M-50 values for two post-injection days. A) Group data showing the influence of MCH at restriction-sensitive and –insensitive stimulation sites. B) Individual data for rats from the restriction-sensitive group. C) Individual data for rats from the restriction-insensitive group. M-50 represents the stimulation frequency required to maintain half the maximal number of rewards earned. (*$p < 0.05$)

2.3.2.6. Histology

As depicted in Figure 2.3.6, stimulation sites for restriction sensitive rats were located dorsolateral to the fornix in the LH. The restriction-sensitive stimulation sites were positioned in the 1.4 to 1.8 mediolateral range. The stimulation sites for the restriction-insensitive rats were located nearby: dorsal, lateral and dorsolateral to the fornix, and in two cases slightly medial to the fornix. The stimulation site for rat A24 could not be determined due to a histological error.

2.3.3. Discussion

Chronic food restriction resulting in ~25% body weight loss enhanced the rewarding effects of the stimulation in 6 out of the 15 subjects whereas for the remaining 9 subjects BSR was relatively stable following severe weight loss. The influence of AgRP on BSR differed between groups: AgRP suppressed the rewarding impact of the stimulation in the restriction-insensitive group and had no consistent effect in the restriction sensitive group. In contrast, MCH administration failed to produce any consistent changes in BSR in either group. Together, these data suggest that AgRP does modulate brain reward circuitry but that it is unlikely that this effect is mediated through MCH.

The influence of AgRP on the rewarding stimulation observed here stands in contrast to the results obtained by Cabeza de Vaca et al. (2002) assessing the impact of an AgRP-like antagonist, SHU9119, on BSR. In their study, lateral ventricular injection of SHU9119 (0.5 or 1 μg) failed to alter the rewarding effect of LH stimulation at 25 and 60 minutes post-injection. One possible explanation for this discrepancy is that the stimulation sites in the study of Cabeza de Vaca and colleagues

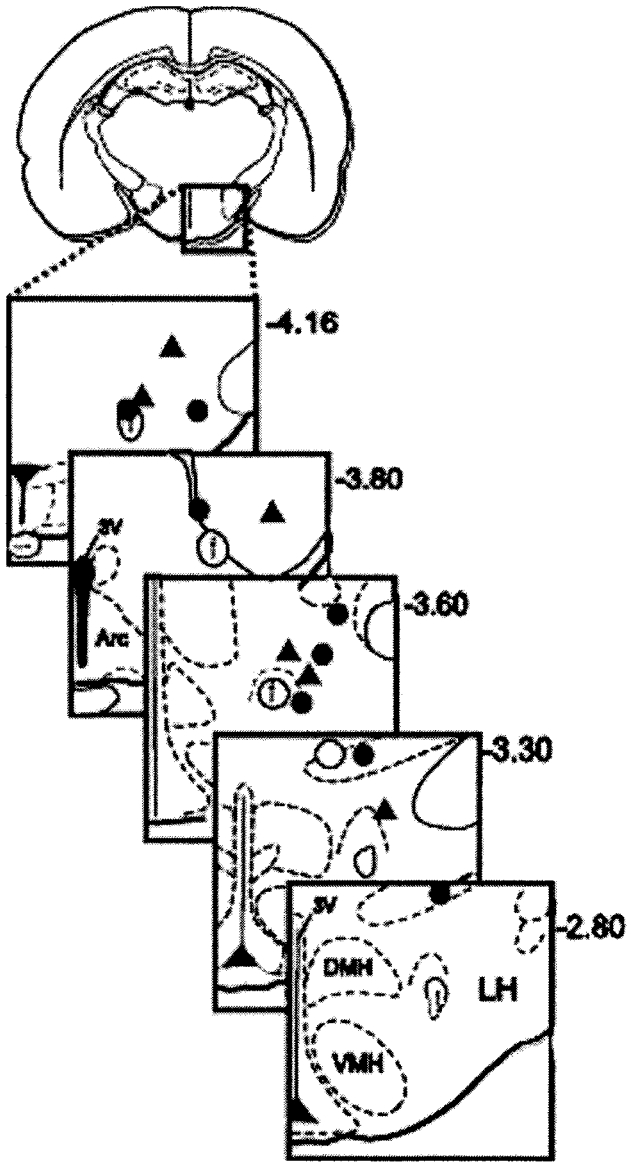


Figure 2.3.6. Location of the tips of the stimulation electrodes. Electrodes producing rewarding effects that were enhanced by chronic food restriction are designated by filled triangles (▲) and electrodes producing rewarding effects that were unaffected by chronic food restriction are designated by filled circles (●). The coronal sections are based on the atlas of G. Paxinos and C. Watson, (Academic Press, New York, Fourth Edition, 1998).

were not distinguished according to their sensitivity to food restriction. Thus, any individual effects of AgRP at functionally distinct sites could have been cancelled out in their analysis of group data. Alternatively, the discrepancy between the two sets of findings may be due to the smaller doses and/or different site of infusion (lateral ventricle) used by these authors.

Seven out of the nine animals in the restriction-insensitive group showed a significant increase in threshold for BSR after AgRP treatment. Interestingly, the ability of AgRP to decrease the rewarding impact of the stimulation at restriction-insensitive sites lasted for as long as four days. This finding is consistent with the long-lasting influence of AgRP on food consumption observed in this and previous studies (Grill et al., 1998; Hagan et al., 2001a)). Long-term elevations in food intake by AgRP is associated with increased Fos expression in the nucleus accumbens (Nacc), central amygdala (CeA) and LH (Hagan et al., 2001). These regions are reciprocally interconnected and are considered important components of reward-relevant circuitry. It is worth speculating whether the ongoing modulation of rewarding LH stimulation by AgRP is linked to neural adaptations occurring at the level of the Nacc, CeA and LH.

Central leptin administration mimicked the effect of fattening at restriction-sensitive stimulation sites by suppressing the rewarding impact of the stimulation. In contrast, leptin either enhanced or had no effect on BSR obtained at sites insensitive to food restriction (Fulton et al., 2000; 2001). Given that leptin deficiency upregulates AgRP expression (Ollman et al., 1997), one would expect AgRP to produce effects

opposite to that of leptin to be considered a potential mediator. However, as we have observed, although, AgRP did enhance BSR in two restriction-sensitive rats, it had no overall effect on the rewarding stimulation in the restriction-sensitive group. In contrast, AgRP suppressed BSR in rats that were insensitive to the effects of restriction. Together, these data do not provide strong support for the notion that AgRP is one of the signaling molecules mediating the actions of leptin on restriction-sensitive reward circuitry.

The ability of AgRP to suppress BSR at restriction-sensitive sites is similar to the influence of CRH on BSR: a single injection of CRH attenuated BSR in restriction-insensitive rats for as long as two days (Fulton et al., 2002). Such CRH-induced decreases in BSR are reminiscent of the effects of stressors on LH self-stimulation (Valentino et al., 1990; McCutcheon et al., 1991). AgRP immunoreactive fibers project to the paraventricular nucleus, a site of CRH synthesis (Broberger et al., 1998). In addition, hypothalamic explants treated with AgRP resulted in a significant increase in CRH release (Dhillo et al., 2002). These findings suggest that AgRP may be inhibiting restriction-insensitive reward circuitry via its stimulatory actions on CRH. Interestingly, α-MSH and AgRP alone stimulate CRH release in vitro, but when combined their effects on CRH release are not additive (Dhillo et al., 2002). This result suggests that AgRP may not be acting by competitive antagonism at MC3/4 receptors to stimulate CRH release and suggest the presence of some unknown receptor or mechanism by which this might occur. In this regards, it would be interesting to determine the contribution of MC3/4 receptors to the rewarding effect

obtained by stimulating restriction-insensitive stimulation sites by assessing the influence of an MC3/4 agonist on BSR.

The effect of AgRP treatment in rats in the restriction-sensitive group was much more variable – two rats showed significant decreases in threshold on at least one day and one showed a significant increase. This within group variability makes it difficult to draw any firm conclusions about the effects of AgRP on the restriction sensitive substrate. Such variability is not surprising given the given that the stimulation can recruit multiple, functionally distinct subpopulations of reward neurons (Fulton et al., 2003). Small variations in the placement of the electrode tip and the path of current flow could alter the relative weights of the subpopulations sampled by different electrodes, thus the probability of obtaining a stimulation site that exclusively activates the restriction-sensitive subpopulation of reward neurons is low. As such, we speculate that in the case of the two rats that showed increases in BSR by AgRP there was a greater recruitment of restriction-sensitive reward neurons. In the remaining four cases in which a more modest reduction in M-50 values were observed following weight loss, it can be assumed that there is a lower ratio of restriction-sensitive to -insensitive reward-relevant fibers recruited by the stimulation. Thus, the inability of AgRP to potentiate BSR in these four rats could be due to actions of AgRP to inhibit restriction-insensitive neurons (as seen in seven subjects), thereby canceling out or opposing (in the case of A19) the facilitation of restriction-sensitive reward circuitry by AgRP. In order to make a more conclusive statement about the impact of AgRP on restriction-sensitive reward circuitry it would be

necessary to test AgRP in a much larger sample of rats with restriction-sensitive electrode placements.

The identity of the neurons that give rise to the rewarding effect produced by stimulating restriction-sensitive and –insensitive sites is unknown. Thus, the effects of AgRP on reward circuitry may be occurring directly via antagonism of MC-3/4 receptors on reward-relevant neurons or it may involve the modulation of other neuropeptides. MCH has been implicated as a downstream signal mediating the actions of AgRP on feeding and body weight regulation. The present data do not support a role for MCH in the modulation of reward circuitry by AgRP. In 7 of the 12 rats tested, the rewarding effect of the stimulation was relatively unchanged following MCH administration. MCH suppressed BSR in two rats from the restriction-sensitive group, an effect that was also observed in one rat from the restriction-insensitive group. Increases in BSR were also observed in two additional rats that were insensitive to the effects of food restriction. Despite the alterations in BSR by MCH in these few cases, there was no overall distinction in the effects of MCH on BSR between restriction-sensitive and –insensitive sites.

As with AgRP, the influence of orexin-A on feeding is mediated by opioid receptors and orexin-A administration preferentially increases intake of high fat over carbohydrate-rich food (Clegg et al., 2002). Moreover, AgRP administration has also been shown to increase fos expression in orexin-A neurons 23 hours after injection (Zheng et al., 2002). Together, these data suggest that AgRP may act through orexin-A to produce its characteristic effects of feeding. In this regard, it would be interesting to examine the ability of orexin-A to alter BSR at restriction-sensitive and –insensitive

sites, especially since orexin-A administration in the pedunculopontine nucleus has been shown to decrease the rewarding effect of LH stimulation (Yeomans, Hood, Koziel, Doesburq, Fletcher, Steidl, 2001).

The disparate effects of AgRP, CRH and leptin on the rewarding effect arising from different subpopulations of neurons may reflect the comparative process believed to underlie behavioral allocation (Shizgal, 1998; Shizgal et al., 2001). The effect of AgRP, leptin and CRH at restriction-insensitive sites suggest that these peptides may contribute to the rewarding effects of stimuli and behavior that are incompatible with feeding, yet still involved in the regulation of energy balance. Reward signaling of restriction-insensitive neurons may be linked to energetically expensive activities, for example. In contrast, the restriction-sensitive component of reward circuitry may tie long-term changes in energy balance to the rewarding effects of high fat food or food hoarding. Determining the function of these reward circuits remains an important goal of future work..

2.3.4. Summary

Chronic food restriction and body weight loss potentiated the rewarding effect of the stimulation at a subset of stimulation sites in the LH. As we have previously shown with leptin and CRH, the modulation of BSR by food restriction can predict the impact of AgRP on BSR. Most notably, AgRP inhibited the rewarding effect of the stimulation at sites insensitive to restriction. In contrast, AgRP had no consistent effect on restriction-sensitive stimulation. Nonetheless, AgRP facilitated BSR in two subjects in which the rewarding impact of the stimulation was substantially enhanced

by food restriction. Alterations in BSR produced by AgRP do not involve MCH as MCH treatment failed to consistently alter BSR at restriction-sensitive and – insensitive sites. Together, this work extends our previous findings regarding the distinct impact of hormones and neuropeptides on restriction-sensitive reward circuitry.

CHAPTER THREE

Leptin and insulin are two adiposity signals that have been shown to reverse the potentation of BSR by food restriction. These data suggest that leptin and insulin may be involved in the process whereby food restriction and weight loss potentiate the rewarding effect produced by stimulating restriction-sensitive sites. How crucial are both of these hormones to the modulation of BSR by food restriction? Could there be other peripheral signals mediating the actions of food restriction on reward circuitry? The next study addresses these questions by examining the impact of food restriction and weight loss on BSR in a rat strain that develops resistance to circulating leptin and insulin: the obese Zucker rat (*fa/fa*). As shown previously, a 48 hour period of food deprivation fails to enhance BSR at restriction-sensitive sites in lean rats. However, the obese Zucker rat shows increased hyperphagia in response to an acute period of food deprivation as compared to lean littermates. Thus, we also determined whether a 48 hour period of food deprivation could enhance BSR at restriction-sensitive sites in this model. Despite the reduced signaling capacity of the leptin receptor resulting from the *fa* mutation, several studies have shown that central leptin administration can still reduce food intake in this model, albeit to a lesser degree than in leans. Thus, to determine whether leptin can also affect reward circuitry in this model, we assessed the effect of central leptin administration on BSR.

4.1. FOOD RESTRICTION AND LEPTIN IMPACT BRAIN REWARD CIRCUITRY IN LEAN AND OBESE ZUCKER RATS

Changes in long-term energy stores are met by alterations in behaviors that contribute to the regulation of energy balance. A highly effective way to modify behavior is to change the rewarding impact of different activities and their consequences. Brain stimulation reward (BSR) has long been used to assess the state of the neural circuitry ("brain reward circuitry") that computes the value of goal objects and goal-directed behaviors. Electrical stimulation of several brain regions can produce a rewarding effect that animals will seek out and work vigorously to obtain. Alterations in physiological state can modulate the rewarding properties of the stimulation of particular brain regions, and thus BSR can be used to tease apart functionally different subsets of neurons.

Stimulating some sites in the lateral hypothalamus (LH) produces a rewarding effect that is potentiated by chronic food restriction and body weight loss, whereas self-stimulation of nearby LH sites is unaffected by this manipulation (Blundell & Herberg, 1968; Carr & Wolinsky, 1993; Fulton, Woodside & Shizgal, 2000). That the effect of food restriction on BSR is contingent upon the position of the stimulating electrode is supported by evidence of contrasting effects of food restriction at different stimulation sites in the same rat (Fulton, Woodside & Shizgal, 2002). Furthermore, the ability of food restriction to enhance BSR at restriction-sensitive stimulation sites is proportional to the degree of weight loss, suggesting that peripheral signals reflecting the status of adipose stores may mediate this process (Carr & Wolinsky, 1993; Fulton et al., 2002). We hypothesize that alterations of long-term energy stores

may influence choice among competing behaviors and goal objects by modulating the activity of the restriction-sensitive sub-population of neurons (Shizgal, Fulton & Woodside, 2001).

How does brain reward circuitry detect the state of peripheral adiposity stores? Putative chemical signals linking adiposity stores to the central control of energy balance include leptin and insulin (Havel, 2001; Woods & Seeley, 2001). Leptin is an adipocyte-derived hormone that is implicated in the control of feeding, energy expenditure, and reproduction (Ahima et al., 1996; Pelleymounter et al., 1995). Circulating levels of leptin are directly proportional to the amount of adipose tissue (Maffei et al., 1995). Upon accessing the brain, leptin targets the long-form its receptor (Ob-Rb) in the hypothalamus and caudal brainstem to decrease energy intake and increase energy expenditure (Elmquist et al., 1998; Grill et al., 2002; Spiegelman & Flier, 2001). Previously, we found that intraventricular leptin mimicked the effect of fattening by attenuating the rewarding impact of restriction-sensitive stimulation (Fulton et al., 2000). Moreover, Carr et al. (2000) have shown that insulin can also attenuate the rewarding impact of restriction-sensitive stimulation. Together, this evidence suggests that leptin and insulin may mediate the process whereby chronic food restriction and weight loss modulates BSR.

If leptin and insulin carry the signal that links peripheral fat stores to the modulation of BSR we should expect differences in the effect of food restriction on BSR in the genetically obese Zucker rat (*fa/fa*), who is profoundly resistant to circulating leptin and insulin (Burguera, Couce, Curran, Jensen, Lloyd, Cleary et al., 2000; Lin, Truett, Levens & York, 1996; Stein, Dorsa, Baskin, Figlewicz, Porte &

Woods, 1987). Obesity in the Zucker rat results from a missense mutation in the gene encoding the leptin receptor ($Lepr^{fa}$) (Chua, Chung, Wu-Peng, Zhang, Liu et al., 1996; Ida, Murakami, Ishida, Mizuno, Kuwajima et al., 1996). As a consequence of impaired leptin receptors, the obese Zucker rat exhibits marked hyperphagia, hyperglycemia, hyperinsulinemia and hyperleptinemia. Thus far the sensitivity of brain reward circuitry to manipulations of energy balance in the obese Zucker rat is unknown. We set out to test the influence of chronic food restriction on BSR in obese Zucker rats to determine whether changes in metabolic signaling associated with weight loss can influence brain reward circuitry in this leptin and insulin resistant model.

In contrast to the effects of long-term food restriction on BSR, an acute period of food deprivation (Fulton et al., 2000) or reductions of short-term metabolic fuel availability (Cabeza de Vaca et al., 1998) fail to alter BSR at sites sensitive to body weight loss. This evidence suggests that the modulation of BSR at restriction-sensitive sites is more directly associated with changes in long-term energy reserves, such as adipose tissue, rather than fluctuations of short-term energy stores. Should we expect this to be so as well in the obese Zucker rat? Relative to their lean littermates, acute food deprivation in obese Zucker rats produces increased hyperphagia (Vasselli, Cleary, Jen & Greenwood, 1980) and neuronal activation (Timofeeva & Richard, 2001). Thus, short-term food deprivation may have a different influence on brain stimulation reward in the obese Zucker rat than in lean rats. To determine this, the effect of acute food deprivation on BSR was assessed in lean and obese Zucker rats.

In contrast to their insensitivity to circulating leptin, obese Zucker rats do show some responsiveness to leptin injected directly into the cerebral ventricles. For example, central leptin administration can reduce food intake and body weight in the Zucker model, but is less effective in the obese rats than in the lean rats. To determine whether central leptin administration can influence the rewarding effect of LH stimulation in the Zucker model, we assessed the effect of this manipulation in both lean and obese rats.

3.2. Materials and methods

3.2.1. Subjects

Subjects were male Zucker rats, 9 obese (*fa/fa*) and 8 lean (*Fa/?*), from the Charles River Breeding Farms (St. Constant, Quebec). At the time of surgery, lean rats weighed between 402 and 480 g, and obese rats weighed between 553 and 641 g. Each rat was housed individually in plastic solid-floor cages with *ad libitum* access to food and water. All subjects were kept in a temperature-controlled room under a reverse 12 hour dark/12 hour light cycle that switched at 8 am/pm. All behavioral testing was carried out in the second half of the dark phase of the cycle.

3.2.2. Surgery

Prior to surgery, rats were pre-treated with atropine sulfate (0.5 mg/kg, SC) to reduce bronchial secretions. Obese *fa/fa* rats were anesthetized with either a ketamine (Ketaset, 80 mg/kg, i.p.) and xylazine (Rompun, 12 mg/kg, i.p.) mixture or with the vaporized gas anesthetic, isoflurane (Aerrane). Lean rats were anesthetized with sodium pentobarbital (Somnotol, 65 mg/kg, i.p.). With bregma and lamda at the same

horizontal coordinates, bilateral, monopolar electrodes were aimed at the perifornical region of the LH (3 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, 7.8 mm below the dura mater), and a 24- gauge stainless-steel guide cannula (Plastics One, Inc., Roanoke, VA) was aimed at the right lateral ventricle (0.4 mm posterior to bregma; 1.6 mm lateral to the midsagittal sinus; 4 mm below the dura mater). The electrodes were constructed from 00 insect pins and were insulated with Formvar to within 0.5 mm of the tip. A wire twisted around two jeweler's screws implanted in the cranium served as the current return. The electrodes and cannula assembly were bonded to the skull and screw anchors by means of dental acrylic. The intraventricular cannula was then closed with a removable obturator. To reduce postoperative pain, Buprenorphine (Buprenex, 0.05 mg/kg, SC) was administered once following surgery.

3.2.3. Apparatus

Subjects were screened for self-stimulation in wooden boxes (25cm x 25 cm 70 cm) with Plexiglas front panels and wire mesh floors. A lever, positioned 3 cm above the floor, protruded from the middle of one wall, and a key light positioned 5 cm above the lever signaled the availability of the reward. Electrical stimulation was generated by dual constant current amplifiers and controlled by hand-operated circuit pulse generators. Depression of the lever triggered the stimulator. The connector on the rat's head was linked to the stimulator via a flexible lead and a 7-channel slip-ring located at the top of the test chamber.

The experimental sessions took place in a room housing computer-controlled stimulators. Test chambers were similar to those used for training except they were constructed entirely of Plexiglas and equipped with a ceiling-mounted, white house

light. Each test chamber was enclosed in a 50 cm x 50 cm x 90 cm plywood enclosure lined with sound attenuating foam. Stimulation trains were generated by microprocessor-controlled circuitry and monitored on an oscilloscope in an adjoining room. Subjects were monitored during testing by means of a remote-controlled video camera.

3.3. Procedure

3.3.1. Brain stimulation reward

Rats were shaped to lever press for a 0.5 s train of cathodal, rectangular, constant-current pulses, 0.1 ms in duration, on a continuous reinforcement schedule. Initially, stimulation parameters were set to low currents and frequencies. If the rat displayed signs of aversion, training was discontinued; otherwise, the subject was shaped to press the lever. Stimulation frequencies (pulses per second) were adjusted to produce optimal levels of responding at a current that remained fixed for each rat, ranging between 150 and 450 μA. Self-stimulation of both sites was assessed, and the electrode that supported the most vigorous lever-pressing in the absence of motoric side-effects was chosen for further testing.

Following initial screening, subjects were trained in the computer-controlled testing setup to respond for a descending series of stimulation frequencies, on a 0.5 sec fixed-interval schedule of reinforcement. The stimulation current was held constant throughout testing, within the range used for initial training. The stimulation frequency was decreased from trial to trial in steps of 0.033 $\log_{10}$ units (~8%) in order to generate curves linking the rate at which rewards were earned to the stimulation frequency ("rate-frequency" curves). Each frequency was available during a single 60

sec trial that was preceded by a 10 sec inter-trial interval, during which 5 priming (non-contingent) trains of stimulation were delivered; the priming trains were identical to those obtained during the trial. Stimulation frequencies were adjusted so as to produce maximal (asymptotic) responding during the first few trials of each frequency sweep. A rate-frequency curve was considered complete when the rats emitted fewer than 5 responses on each of two consecutive trials. Seven rate-frequency curves were collected per test session. Test sessions lasted anywhere between 60 to 150 minutes, approximately. The first curve of the session served as a warm-up and was not included in the data analysis.

3.3.2. Intraventricular injections

Cannula placement was verified by administering 50 ng of angiotensin II (Sigma, St. Louis, MO) and determining whether vigorous drinking began within 5 min. All substances were infused in the right lateral ventricle at a rate of 1 μl/min through a stainless steel internal cannula (Plastics One, Roanoke, VA) that extended 1 mm beyond the tip of the guide cannula. The internal cannula was connected to a 5 μl microsyringe (Hamilton Company) by means of flexible polyethylene tubing. After infusion, the internal cannula was kept in place for 15 seconds.

3.3.3. Food measurement

The ability of a 4 μg dose of leptin (Peprotech, Inc) to suppress food intake was assessed in the same group of lean and obese rats after BSR testing had completed. Rats were habituated to a powdered food diet for one week. Powdered food was placed in cups attached to aluminum sheets that were molded to hang on the

wall of the home cage. A flat-bottomed bowl was fixed underneath each food cup to catch spillage. Food intake was measured for 3 hours following ICV vehicle (3.2 µl, 0.1 mol/l Tris, pH 7.35) administration and then again after administration of 4 µg/3.2 µl leptin solution the next day. The amount of food consumed was measured in this manner for four days following a single injection of leptin. All injections were made during the second half of the dark cycle, in accordance with self-stimulation testing times.

3.3.4. Design

Training in the self-stimulation paradigm lasted for one to two days. As a measure of the rewarding impact of the stimulation, we used the stimulation frequency that maintained a half-maximal rate of reward delivery (M-50). Baseline responding was deemed stable when mean stimulation frequency require to maintain half-maximal responding shifted by ≤ 0.04 $\log_{10}$ units from day to day. Rate-frequency data was collected for 5 to 7 days following stabilization of baseline (free-feeding) responding. At the end of this period, the effect of food deprivation on BSR was assessed by removing all food from the home cage for a period of 48 hours. Subjects were tested immediately after this period of food deprivation and then were provided with *ad libitum* access to food after the test session. Following an additional day of testing, food consumption was limited to 10 grams/day to investigate the influence of chronic food restriction on BSR. Food was restricted for 18 to 28 days until body weight reached ~75% of free-feeding values. During this restriction period BSR testing was carried out every few days to maintain self-stimulation performance.

When body weights reached target values, the amount of food supplied to each rat was adjusted daily in an attempt to hold body weight constant. When body weight stabilized at ~75%, BSR testing was carried out until steady M-50 values were achieved.

The influence of leptin (4 μg) on BSR was examined at the end of the restriction condition, when body weight was stabilized at ~ 25% below its normal level. Vehicle (3.2 μl of 0.1 mol/l Tris, pH 7.35) was administered for two consecutive days, followed by leptin (4 g in 3.2 μl of vehicle) treatment on the third day. BSR testing began 1 hour after injections.

3.3.5. Data analysis

Broken-line functions, with a horizontal lower asymptote, a rising linear segment, and a horizontal upper asymptote (Gallistel & Freyd, 1987), were fit to each of the six rate-frequency curves collected daily. To indicate the position of the rate-frequency curve along the frequency axis, the M-50 value was derived from each broken-line function by interpolation. Enhancement of the rewarding effect shifts these curves to the left, thus reducing the M-50 value, whereas reductions in reward effectiveness produces rightward shifts and increase M-50 values.

To assess the effect of food restriction on each subject, a set of 12 M-50 values from two consecutive days in the free-feeding condition was compared to a set of 12 values obtained just after body weight stabilized at ~75% of normal values with a one way ANOVA. Delta M-50 values were calculated for each rat by subtracting the mean of the 12 M-50 values collected during free-feeding from the mean of the 12 M-50

values obtained under food restriction. To determine whether groups differed in their response to food restriction, ΔM-50 values were compared between each group (restriction-sensitive/obese; restriction-sensitive/lean; restriction–insensitive/obese; restriction-insensitive/lean) with a two-way ANOVA.

To determine the effectiveness of the food deprivation manipulation on each subject, 6 M-50 values taken immediately before food deprivation were compared with 6 M-50 values collected after ~48 hours of food deprivation using a one-way analysis of variance (ANOVA). ΔM-50 was calculated for each rat by subtracting the mean of the 6 M-50 values collected during baseline from the mean of the 6 M-50 values obtained after food deprivation.

For the analysis of the effects of leptin on BSR, a Dunnett test for multiple comparisons was performed. Six M-50 values collected during the second vehicle-treatment day were compared to the 6 M-50 values collected on each of the four days post-administration of leptin for each rat. ΔM-50 was calculated for each rat by subtracting the mean of the 6 M-50 values collected during the second day of vehicle administration from the mean of the 6 M-50 values obtained from each day post-injection of leptin. To determine whether groups differed in their response to leptin treatment, a one-way ANOVA was used to compare delta M-50 values from all four days following leptin administration between restriction-sensitive and –insensitive rats. The same analysis was performed to examine differences between genotypes. A level of $p \leq 0.05$ for a two-tailed test was considered critical for statistical significance.

For the analysis of the effects of leptin on food intake, values representing the ratios of food consumption following leptin treatment to consumption following vehicle treatment were compared to one by means of a single sample t–test. This analysis was performed independently for lean and obese rats for each of the 4 days post-treatment. Differences in the influence of leptin on food intake between genotypes were assessed by means of an independent-measures t-test for each day post-treatment.

3.3.6. Histology

At the end of the experiment, the stimulation sites were marked by means of the Prussian Blue method. With the stimulating electrode serving as the anode, a 100 μA direct current was applied for 15 sec. Rats were then injected with a lethal dose of sodium pentobarbitol (Somnotol, 100 mg/kg, IP) and perfused intracardially with saline followed by a mixture of 10% formalin (100 ml), trychloroacetic acid (0.5 g), potassium ferrocyanide (3 g) and potassium ferricyanide (3 g). Brains were removed and stored in 10% formalin. After immersion in a 20% sucrose-formalin solution for at least 24 hr for cryoprotection, brains were frozen, sliced on a cryostat in 30 μm coronal sections, and mounted on gelatin coated slides. Sections were stained for Nissl substance using formal thionin. The location of stimulation sites was identified with the aid of a stereotaxic atlas (Paximos & Watson, 1998).

3.4. Results

3.4.1. Chronic food restriction

During the free-feeding condition body weights of obese rats ranged from 592 - 761 g and body weights of lean rats ranged from 448 – 564 g. Following food restriction, body weights of obese rats fell to 458 – 585 g and body weights of lean rats dropped to 337 – 411 g.

In 5 out of the 9 obese rats, chronic food restriction reduced M-50 values. Examples of the data obtained from these subjects are shown in Figure 3.1A and show that curves taken during food restriction lie to the left of the curves obtained during free-feeding. The corresponding M-50 values decreased by 0.041 – 0.392 $\log_{10}$ units for the obese rats (Fig. 3.1C). Similarly, M-50 values decreased following food restriction in some lean rats. Rate-frequency curves were shifted leftward by food restriction in 4 out of the 8 lean rats, and the resultant M-50 values decreased by 0.027 - 0.147 $\log_{10}$ units (Fig. 3.1C). In the remaining four obese rats, rate-frequency curves collected during restriction overlap the curves obtained during free-feeding (e.g., Fig. 3.1B), and the corresponding M-50 values were not significantly altered (Fig. 3.1D). Similarly, in 3 out of the 4 remaining lean rats M-50 value were not significantly altered by chronic food restriction as illustrated in Figure 3.1D. In one lean rat, L25, there was a small but significant increase in M-50 values following body weight loss.

Rats were labeled as restriction-sensitive when chronic food restriction produced statistically significant reductions in M-50 values relative to baseline M-50 values. The results of a two-way ANOVA, assessing Δ M-50 values following chronic food

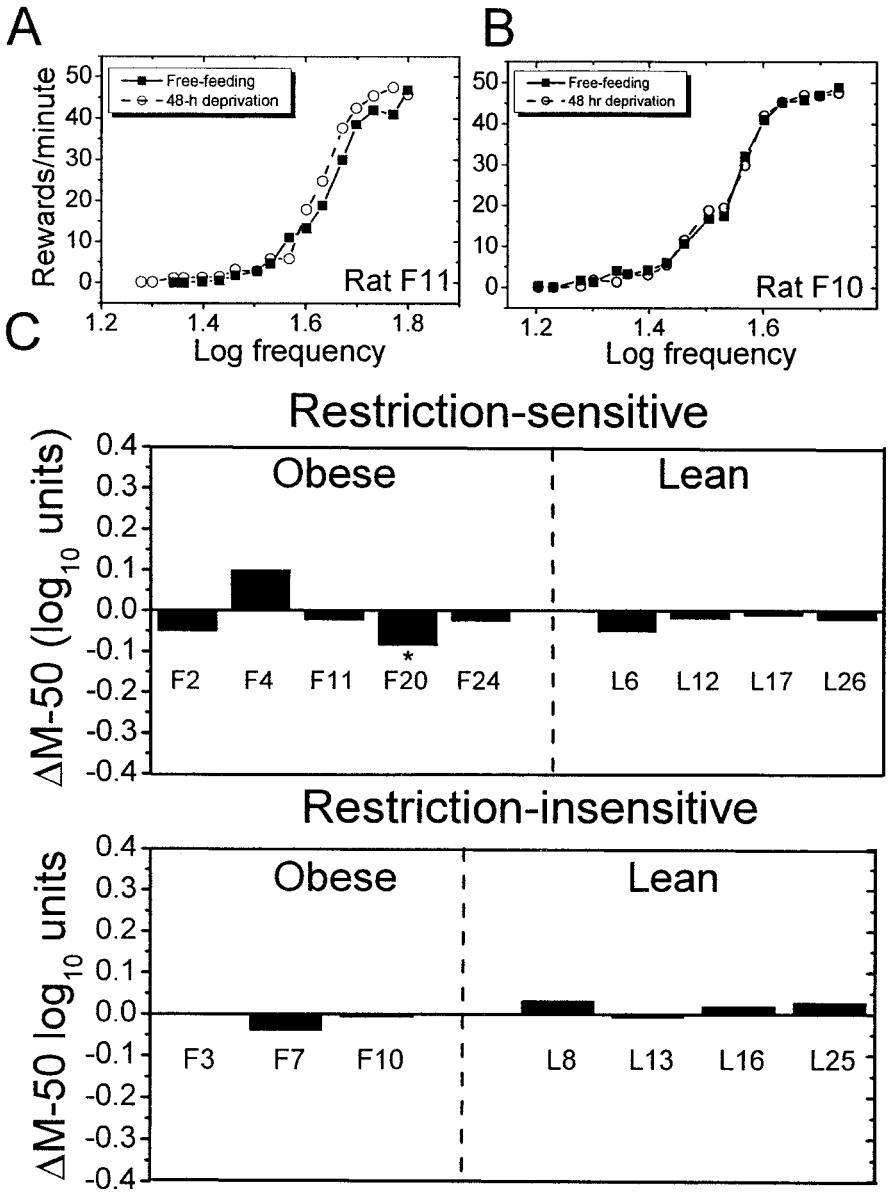


Figure 3.1. Effects of chronic food restriction on self-stimulation at LH sites where the rewarding effect of electrical stimulation is either sensitive or insensitive to chronic food restriction. **A)** Rate-frequency curves are shifted leftwards during chronic food restriction (open symbols) with respect to curves obtained during the free-feeding baseline (filled symbols) in a subset of obese rats. **B)** In contrast, rate-frequency curves remained stable following chronic food restriction in the remaining obese rats. Each data point is an average of six measures collected on each test day. **C)** Magnitude of lateral curve shifts produced by chronic food restriction in all restriction-sensitive lean and obese subjects. **D)** Magnitude of curve shifts produced by food restriction in all restriction-insensitive lean and obese subjects. M-50 refers to the stimulation frequency required to maintain the half-maximal number of rewards earned. *$p < 0.05$; ** $p < 0.005$.

restriction, indicate a significant main effect of restriction-sensitivity: Food restriction resulting in ~25% weight loss lowered M-50 values in restriction-sensitive rats 0.151 ±0.063 $\log_{10}$ units) as compared to the restriction-insensitive rats (-0.007 ±0.016 $\log_{10}$ units) ($F(1, 13) = 7.98$, $p = .014$). There was no difference in ΔM-50 values between genotypes ($F(1, 13) = 1.44$, $p = 0.251$) and no interaction between restriction-sensitivity and genotype ($F(1, 13) = 0.46$, $p = 0.51$).

3.4.2. Acute food deprivation

A sample of the data obtained from restriction-sensitive and restriction-insensitive rats is displayed in Figure 3.2A and B, respectively, and show that curves taken after food deprivation overlap curves collected prior to deprivation. A 48-hour period of food deprivation did not produce any significant changes in M-50 values in 7 out of 8 obese *fa/fa* rats (Fig. 3.2C and D). In the remaining obese rat, F20, M-50 values were decreased by food deprivation (Fig. 3.2C). In a similar manner, M-50 values remained stable in the face of acute food deprivation in all lean *Fa/?* rats (Fig. 3.2C and D). Data for rat F19 is unavailable.

3.4.3. Leptin administration

Intraventricular leptin treatment led to reductions in the effectiveness of the rewarding stimulation (increased M-50 values) on at least one day post-injection in 3 out of the 5 obese rats that showed a potentiation of BSR by food restriction ("restriction-sensitive") (Fig. 3.3C). The rate-frequency curves obtained from these subjects are shifted to the right by leptin administration as exemplified in Figure 3.3A.

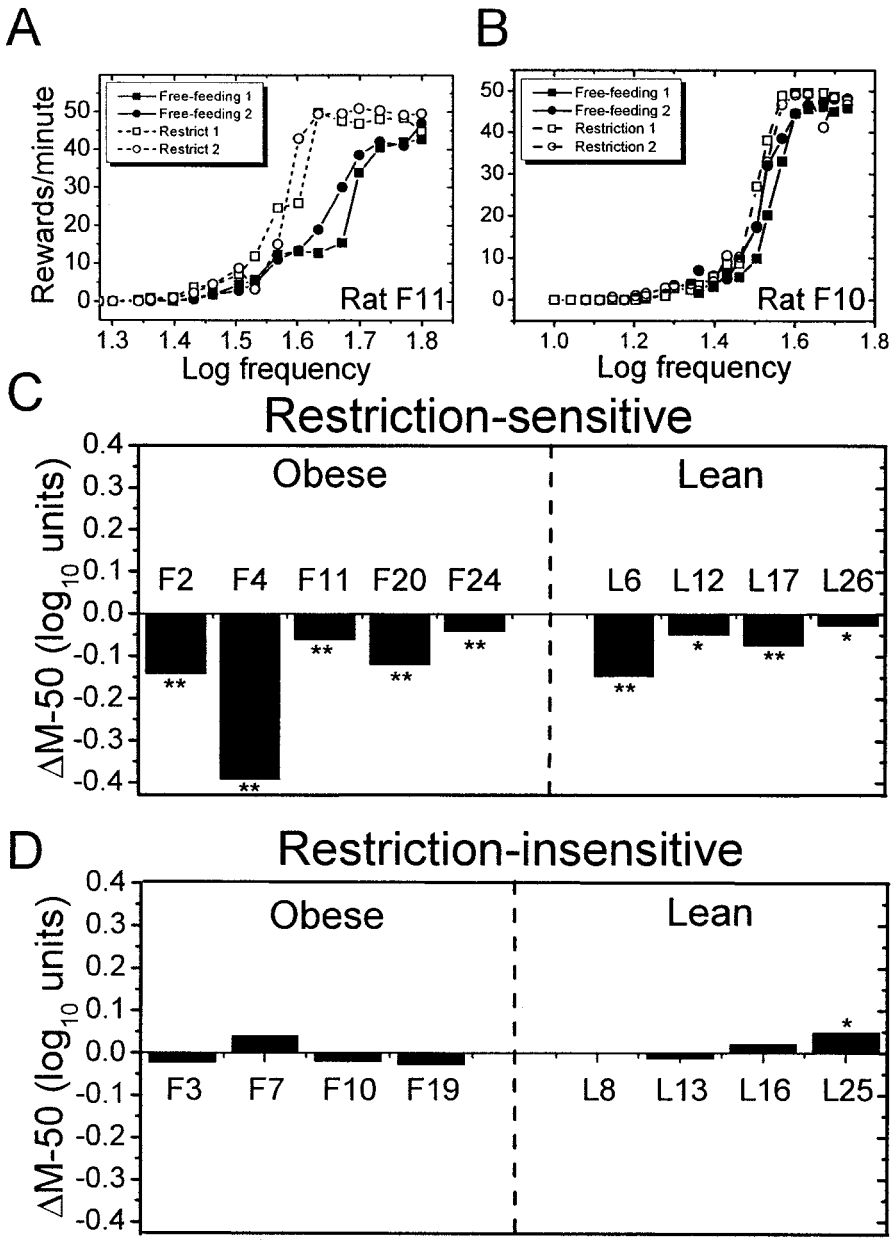


Figure 3.2. Failure of acute food deprivation to alter self-stimulation in lean and obese rats. **A)** Rate-frequency curves obtained following 48-hour food deprivation (open symbols) overlap those collected during free-feeding (closed symbols) in obese Zucker rats. **B)** Similarly, in leans rats food deprivation failed to shift rate-frequency curves. Each data point is an average of six measures collected on each test day. **C)** Magnitude of the curve shifts (ΔM-50) produced by acute food deprivation in all obese and lean subjects. *$p \leq 0.05$; ** $p \leq 0.005$.

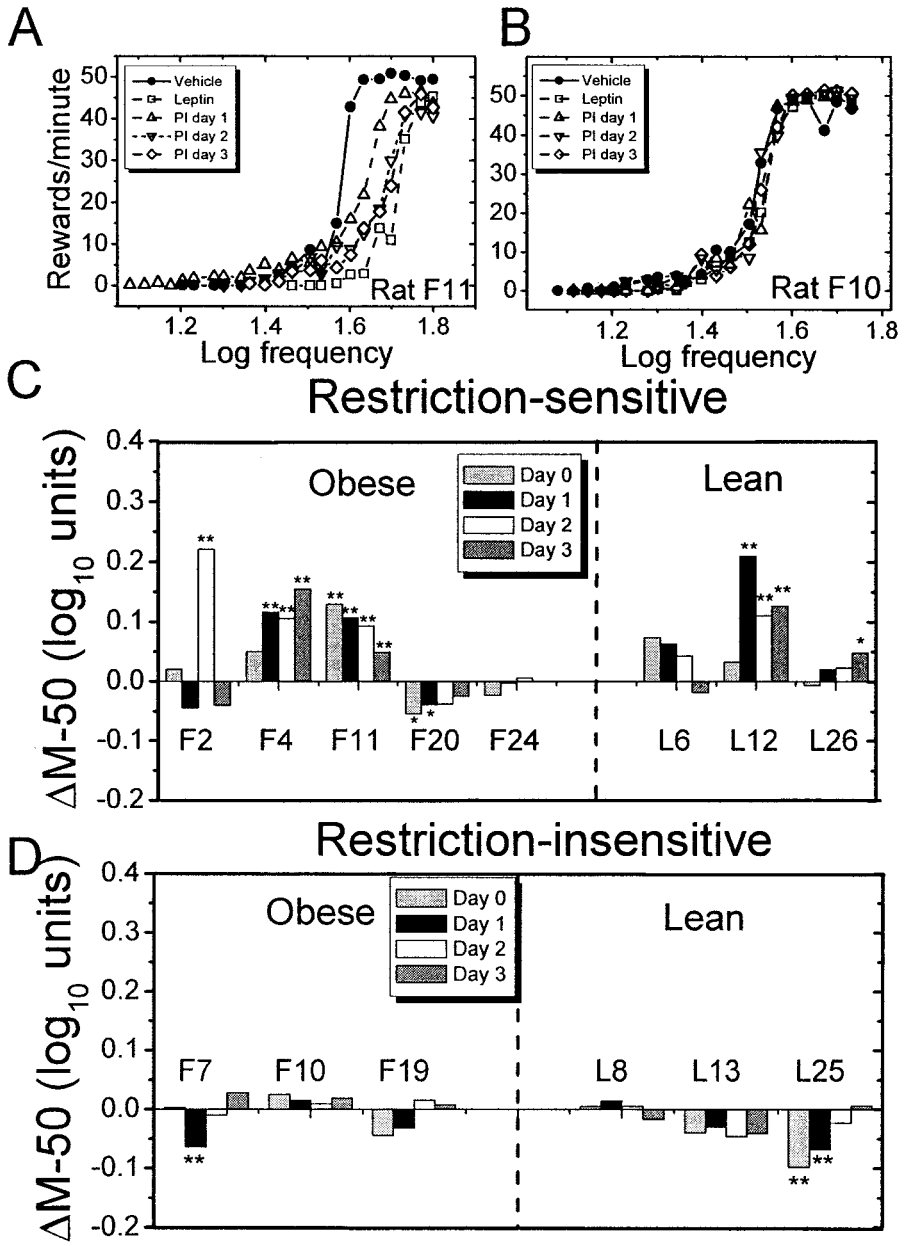


Figure 3.3. Effect of a single ICV injection of leptin (4 μg) on the rewarding effect of LH stimulation. **A)** Leptin shifted rate-frequency curves rightward (leptin: open symbols; vehicle control: filled symbols) at a stimulation site in an obese rat that was sensitive to the effects of weight loss (see Figure 2a). **B)** At a stimulation site in an obese rat where chronic restriction failed to enhance the rewarding effect (see Figure 2b), BSR was unchanged after leptin administration. Each data point is an average of six measures collected on each test day. **C)** Magnitude of curve shifts (ΔM-50) during the 4 days following ICV leptin in all restriction-sensitive lean and obese rats. **D)** Magnitude of curve shifts (ΔM-50) during the 4 days following ICV leptin in all restriction-insensitive rats. ; *$\underline{p} < 0.05$; ** $\underline{p} < 0.005$.

For the remaining 2 rats in this group there was a decrease in M-50 values in one case (F20) and no significant change in M-50 values in the other (F24). Of the 3 lean rats that were sensitive to the effects of food restriction, two showed increased M-50 values on at least one of the days following leptin administration as shown in Figure 3C. For the remaining restriction-sensitive lean rat the M-50 value increases were not statistically significant.

In contrast to the overall effects of leptin at restriction-sensitive sites, leptin treatment at restriction-insensitive sites failed to increase M-50 values in both obese and lean Zucker rats. In 2 of the 3 obese rats that were insensitive to the effects of restriction, leptin administration failed to alter M-50 values on any of the days following injection (Fig 3.3.D). This is illustrated in Figure 3.3B by the overlap of rate-frequency curves obtained after administration of leptin and vehicle. In the remaining restriction-insensitive obese rat there was a significant reduction in M-50 values on one of the days following administration. Similarly, in 2 of the 3 lean rats that were unresponsive to the effects of food restriction, M-50 values following leptin treatment were unchanged, and in the remaining rat M-50 values decreased during the two days following leptin injection (Fig. 3.3B).

A one-way ANOVA revealed a significant difference between restriction-sensitive and -insensitive rats with respect to leptin treatment: over the four days following leptin administration delta M-50 values were significantly elevated in restriction-sensitive rats (0.047 $\pm$0.013 $\log_{10}$ units) relative to restriction-insensitive

rats (-.015 ±0.007 $\log_{10}$ units) ($F(1, 52) = 15.77$, $p < .001$). There was no contrasting effects of leptin on M-50 values between genotypes ($F(1, 31) = 0.44$, $p = 0.62$).

3.4.4. Food intake

As illustrated in Figure 3.4, leptin administration decreased food intake in obese Zuckers on each of the 4 days post-injection (PI 0: $t(7) = 2.93$, $p < .05$; PI 1: $t(7) = 2.6$, $p \leq .05$; PI 2: $t(7) = 2.29$, $p \leq .05$; PI 3: $t(7) = 2.43$, $p \leq .05$). In lean Zuckers leptin produced significant reductions in food intake on the first two days post-treatment (PI0: $t(6) = 8.84$, $p < .005$; PI 1: $t(6) = 3.88$, $p < .005$), whereas reductions in food intake on post-injection day 2 and 3 were not statistically significant. There were no statistically reliable differences in the amount of food consumed between lean and obese rats on any of the four days post-injection.

3.4.5. Electrode placements

Histological localization of the electrode tips revealed that most stimulation sites were located dorsal or dorsolateral to the fornix in the LH (Fig. 3.5). In the remaining rats, one electrode was ventral to the fornix (F24), and the other was in the subincertal nucleus (L8). Table 3.1 shows the symbols that correspond to the electrode placements of individual rats. A clear distinction between the distributions of restriction-sensitive and –insensitive stimulation sites is not apparent. Electrode placements for rats F10 and F4 could not be determined due to histological error.

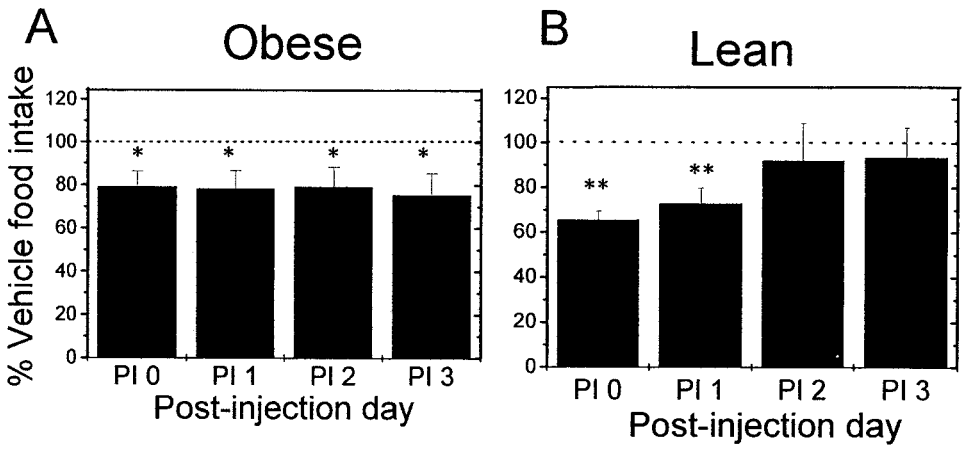


Figure 3.4. Effects of a single ICV injection of leptin (4 μg) on food intake for four days in obese and lean Zucker rats. Data represent means and SEM. *$\underline{p} < 0.05$; ** $\underline{p} < 0.005$.

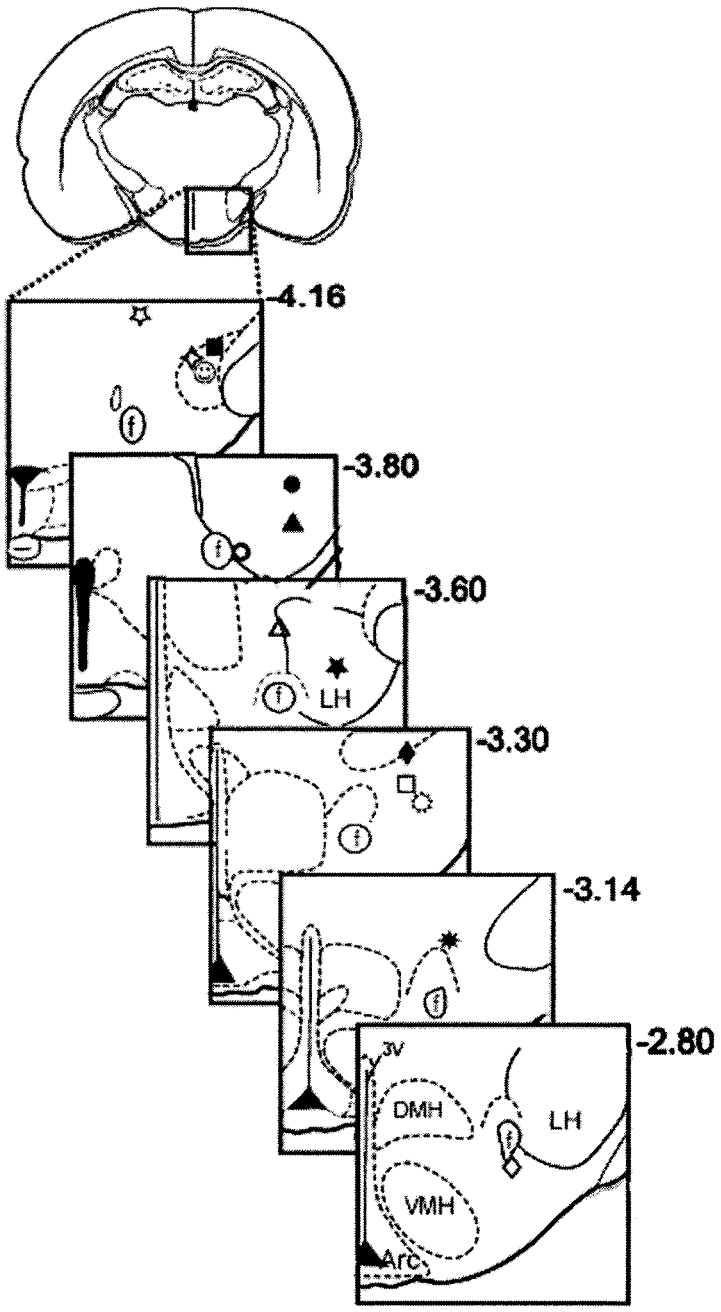


Figure 3.5. Location of the tips of stimulation electrodes for individual rats (see Table 3.1). Coronal plates are derived from the atlas of Paxinos and Watson (1998).

Table 3.1. Symbols marking electrode placements for individual rats.

Restriction-sensitive		Restriction-insensitive	
Rat	Symbol	Rat	Symbol
F2	□	F3	★
F11	✧	F7	◆
F20	○	F19	■
F24	◇	L8	✸
L6	△	L13	●
L12	☺	L25	▲
L17	☼		
L26	☆		

3.5. Discussion

As we found previously, chronic food restriction resulting in significant body weight loss enhanced the reward effectiveness of the stimulation in a subset of lean rats. The key finding of the present study was that BSR was also potentiated by food restriction in a subset of obese Zucker rats despite their known insensitivity to circulating leptin and insulin. Again consistent with our earlier data, BSR was unchanged by acute food deprivation in almost all lean rats, even in those rats that subsequently showed an increase in BSR following chronic food restriction. Similarly, we found that acute food deprivation failed to enhance BSR in obese rats. Finally, in the majority of restriction-sensitive lean and obese rats, the rewarding impact of the stimulation was suppressed by central leptin administration on at least one of the four days post-injection. In contrast, in all restriction-insensitive rats there was no attenuation of BSR produced by leptin; instead, leptin either had no effect or decreased the rewarding impact of the stimulation.

In a subset of the lean and obese Zucker rats, the substantial body weight loss (~25%) produced by chronic food restriction was accompanied by a reduction in M-50 values; in these rats, the rewarding impact of the stimulation was potentiated by chronic restriction. Conversely, BSR remained stable in the face of similar weight loss in the remaining lean and obese rats. These data are consistent with the existence of a subset of reward neurons in both the obese and lean Zucker rat whose signaling can be modified by peripheral signals reflecting body weight. That chronic restriction

enhances BSR only in a subset of subjects replicates previous findings obtained in lean rats (Abrahamsen et al., 1995; Fulton et al., 2000).

Both leptin and insulin have been shown to oppose the reward-enhancing effects of food restriction on BSR and they have been proposed as links between peripheral fat stores and the activity of the restriction-sensitive subpopulation of reward neurons. The current finding that leptin can counter the increases in BSR produced by restriction in some obese Zucker rats supports the notion that leptin acts centrally to modulate the reward-enhancing effects of food restriction. However, the sensitivity of BSR to restriction in some obese Zucker rats does not add support for the notion that leptin and insulin link the state of the peripheral fat stores to central reward circuitry. It is unlikely that food restriction and weight loss would result in reduced central levels of these two hormones in the obese Zucker rat. Uptake of leptin and insulin from the periphery to the cerebrospinal fluid (CSF) is reduced in the obese Zucker rat, suggesting that the receptor-mediated transendothelial transport systems are saturated (Burguera et al., 2000; Stein et al., 1987). It is likely, therefore, that a dramatic reduction in circulating leptin and insulin levels would be required to produce a decrease in central levels. The chronic food restriction regimen used in the present study was not sufficient to reduce the body weights of obese Zucker rats to that of their lean litter mates. In fact, obese Zucker rats with body weights much lower than those of the restricted obese rats in the present study exhibit higher plasma/CSF insulin ratios than lean littermates (Stein et al., 1987). Moreover while it is unknown whether decreasing peripheral leptin levels affects central quantities of the hormone in the obese Zucker rat, there is evidence that lowering plasma insulin concentrations

actually increases CSF insulin levels (Alemzadeh & Holshouser, 1999). Determining the precise role of central insulin and leptin signaling in the potentiation of BSR by chronic food restriction requires the measurement of CSF concentrations of these two hormones following food restriction in both lean and obese Zucker rats. Nonetheless, the evidence available at this point suggests that the facilitation of BSR by food restriction depends on a peripheral factor other than or in addition to leptin and insulin. Several other signals link long-term energy stores to the central control of energy balance, including fatty acid synthase inhibitors (e.g., cerulenin) (Loftus, Jaworsky, Frehywot, Townsend, Ronnett et al., 2000), and could play a critical role in signaling the state of long-term energy stores to restriction-sensitive reward neurons.

There was no statistically reliable difference between lean and obese rats with respect to the magnitude of the restriction-induced reduction in M-50 values. Nonetheless, it is not very meaningful to carry out such comparisons between two groups of rats with different distributions of electrode placements. Thus, this finding could reflect similar sensitivities of lean and obese rats to the effects of restriction on BSR or differences in the sampling of restriction-sensitive reward neurons in the groups of lean and obese rats. Electrodes may be situated so as to recruit more or fewer neurons from the restriction-sensitive subpopulation, resulting in differential potentiation of BSR by food restriction. In order to determine the relative sensitivity of BSR to food restriction in lean and obese Zucker rats, one would need an extremely large group of subjects in order to average out the variations in electrode placement.

Unlike chronic food restriction, a 48 hour period of food deprivation failed to enhance BSR in all but one rat. During chronic food restriction, rats were fed their daily ration immediately after the test session. Given that this relatively small amount of food is consumed rapidly, short-term energy stores are likely depleted by the time of the test session on the following day. Accordingly, it was important to assess whether the facilitation of BSR in obese rats is generated by reductions in long-term energy stores rather than diminished short-term stores. The failure of acute food deprivation to increase the rewarding effect of the stimulation at restriction-sensitive sites in obese rats implies that the potentiation of BSR is contingent upon reducing long-term energy stores as we have shown previously in lean rats (Fulton et al., 2000). Furthermore, these results coincide with reports that acute glucopenia and lipoprivation do not alter BSR at sites sensitive to chronic food restriction(Cabeza de Vaca et al., 1998).

Despite the absence of intact Ob-Rb leptin receptors in the obese Zucker rat, intraventricular infusion of leptin significantly decreased the rewarding effect of the stimulation in 3 out of the 5 restriction-sensitive obese rats, on at least one day post-injection. Similarly, the rewarding impact of the stimulation was suppressed by central leptin administration in 2 out of the 3 lean rats with restriction-sensitive sites, whereas in the remaining rat there was a trend towards this effect.

The reduction in the effectiveness of the rewarding stimulation persisted for as long as four days after leptin administration, an effect that was seen in a previous study of the effects of leptin on BSR (Fulton et al., 2000). These findings are also consistent with the long-lasting suppression of food intake seen in this and a prior

study following a single injection of leptin (Cusin, Rohner-Jeanrenaud, Striker-Kongrad & Jeanrenaud, 1996).

The overall effects of leptin on restriction-insensitive reward circuitry stand in contrast to the actions of leptin at restriction–sensitive stimulation sites: BSR was either unaltered or potentiated by leptin at restriction-insensitive BSR sites in both lean and obese rats. Together, these results lend support to our previous findings in lean rats that leptin can attenuate the rewarding effect of the stimulation only at stimulation sites that are sensitive to restriction. These contrasting effects of leptin may reflect the evaluative process that biases choice between competing behaviors. In this view, the occurrence of a particular goal-directed behavior can be moderated either by decreasing the reward value it generates or by increasing the value of competing behaviors. Thus, the neural circuitry underlying BSR at restriction-sensitive sites may contribute to the regulation of energy balance by altering the rewarding effect of behaviors that promote changes in long-term energy storage, whereas at restriction-insensitive sites, BSR may arise from the activation of neurons subserving incompatible behaviors (Shizgal et al., 2001).

The observation of reward-potentiating effects of leptin at restriction-insensitive sites may help account for the variable effect of leptin at the restriction-sensitive sites. In two obese rats with restriction-sensitive sites, M-50 values were either decreased by leptin (F20) or remained stable (F24). In these cases, it is conceivable that the sampling of restriction-sensitive and insensitive reward neurons was such as to generate two opposing effects of leptin.

Intraventricular leptin reduced food intake both in lean and obese Zucker rats. Decreases in food intake following central administration in the Zucker obese rat have been reported in some cases (Cusin et al,1996, Lin et al., 1996; Wang, Hartzell, Flatt, Martin & Baile, 1998) and not in others (Such effects are not surprising, given that the missense mutation reduces but does not eliminate signaling at the leptin receptor. That the same dose of intraventricular leptin was effective in altering BSR thresholds in several of the obese Zucker rats is another indication that the central leptin receptors retained residual function.

To explain the observed differences between restriction-sensitive and -insensitive rats we have proposed that the response to chronic food restriction, CRH and leptin depends on the placement of the stimulating electrode in the LH. In strong support of this notion, we have recently observed contrasting effects of food restriction at different stimulation sites in the same rat [18]. Previously, we have found that the rewarding effect induced via electrode tips dorsal or dorsolateral to the fornix was restriction-sensitive, whereas we did not see restriction-sensitivity at sites in the far-lateral hypothalamus or at sites medial to the fornix [15, 16, 17]; restriction sensitivity has also been seen in a region approximately 1 mm dorsal to the fornix at the anteroposterior level of the dorsomedial hypothalamus. Not all of the electrode placements in the present experiment are consistent with the prior data. The location of the stimulating electrodes in two obese rats and two lean rats that were sensitive to food restriction were located in the far-lateral hypothalamus, and the electrode in another obese rat was positioned immediately ventral to the fornix. It is unclear to what degree these differences reflect strain differences and to what degree they reflect

variation in the proximity of the restriction-sensitive neurons to the histologically identified position of the electrode tips.

Research over recent years has made substantial progress in describing the neuropeptides and receptors that are triggered downstream from leptin to mediate changes in food intake and energy expenditure [34, 36, 33]. Hypothalamic neurons expressing Ob-Rb also produce one or more of the neuropeptides involved in the regulation of energy balance [4, 10, 42]. These include arcuate neurons synthesizing neuropeptide Y (NPY) and agouti-related protein (AgRP), or □-melanocyte stimulating hormone (a POMC derivative) and cocaine-amphetamine regulated transcript. In addition, Ob-Rb is expressed on hypothalamic neurons secreting corticotropin-releasing hormone (CRH), another neuropeptide implicated in energy balance [32]. There is, as yet, no evidence that any of these neuropeptides link leptin to the modulation of brain reward circuitry. Previous data suggests that neuropeptide Y is not involved in the modulation of BSR by food restriction and leptin as it failed to alter the rewarding effect of the stimulation at restriction-sensitive and –insensitive sites [7, 17]. In addition, we have shown that intraventricular CRH administration fails to alter BSR at most restriction-sensitive sites, but suppresses the rewarding impact of the stimulation at sites insensitive to restriction [16]. It remains to be determined how leptin alters the signaling of restriction-sensitive reward neurons and which neurotransmitters, receptors and neuropeptides are responsible.

3.6. Summary

Chronic food restriction and body weight loss enhanced the rewarding impact of lateral hypothalamic stimulation in a subset of lean and obese Zucker rats. In

contrast, a 48 hour period of food deprivation was unable to influence BSR in all but one of these rats. Thus, as in lean rats, signaling in a subpopulation of reward neurons in obese Zucker rats can be modulated by changes in adiposity. Leptin was able to alter brain reward circuitry in spite of the reduced signaling capacity of Ob-Rb. This observation and the demonstration of reduced food intake following intraventricular administration of leptin show that there is some residual sensitivity to the effects of central leptin administration in the obese Zucker rat. Nonetheless, the resistance to peripheral leptin and insulin manifested by the obese Zucker rat renders rather unlikely the view that leptin levels couple changes in adiposity to BSR thresholds in these subjects. If so, leptin may act in lean rats as only one of several peripheral signals linking the effect of food restriction to brain reward circuitry. Among the other candidates are several factors that are either secreted by adipocytes or that regulate adipocyte metabolism, such as adiponectin and cerulenin. It will be of interest to assess in future studies whether these signals play a role in the modulation of restriction-sensitive reward circuitry.

CHAPTER FOUR

The modulation of BSR by food restriction, leptin, CRH and AgRP appears to depend on the placement of the stimulating electrode amongst a functionally heterogeneous population of reward-relevant neurons. The close proximity of restriction-sensitive and –insensitive sites, however, makes it difficult to differentiate between the two types of sites. It remains possible that individual differences rather than the position of the stimulating electrode might make the major contribution to the between rat variability in the ability of food restriction and peptides to alter the rewarding effect of LH stimulation. This hypothesis deserves consideration in light of the observed individual differences with respect to the propensity of a rat to develop obesity in response to high fat diet and self-administration of rewarding drugs. To determine whether reward circuitry activated via LH stimulation is anatomically and functionally subdivided, the present study assessed the effect of food restriction and weight loss at two stimulation sites in each rat.

4.1. FUNCTIONAL ORGANIZATION OF BRAIN REWARD CIRCUITRY: POTENTIATION OF BRAIN STIMULATION REWARD BY FOOD RESTRICTION

Brain stimulation reward (BSR) has been used extensively to characterize and identify the neural circuitry underlying reward ("brain reward circuitry"). Electrical stimulation of various brain regions can elicit a rewarding effect that powerfully directs the animal to seek out and reinitiate the stimulation. The stimulation at some sites in the medial forebrain bundle (MFB) can produce such a strong control over behavior that rats will self-starve (Routtenberg & Lindy, 1965; Spies, 1965) and cross over an electrified grid (Olds, 1958) in order to maintain contact with the lever that triggers the rewarding stimulation.

The MFB is a densely packed bundle of fibers, comprising at least 50 components comprising ascending and descending components that course through the forebrain (Veening, Swanson, Cowan, Nieuwenhuys & Geeraedts, 1982). This complex anatomical configuration has made it difficult to identify the neurons that give rise to the rewarding effect. Nonetheless, considerable progress has been made in describing the physiological and anatomical features of the neurons responsible for self-stimulation of the MFB (Bielajew & Shizgal, 1982; 1986; Shizgal, Bialejew, Corbett, Skelton & Yeomans, 1980; Shizgal & Murray, 1994).

In spite of these developments the question of whether the reward circuitry is functionally homogeneous is still to be resolved. Heterogeneity in the substrate underlying BSR has been suggested by studies examining the impact of lesions on the rewarding effect of MFB stimulation. Within a given study anatomically similar

damage to a particular region can reduce the effectiveness of rewarding LH stimulation by varying magnitudes (Arvantigiannis et al., 1996; Acheson et al., 2000) One would expect the size of a lesion effect to vary across rats if the neurons directly activated by MFB stimulation were composed of intermixed and anatomically distinct subpopulations. Thus, the size of a lesion effect on BSR would depend on the alignment of the damaged regions to the specific composition of neurons directly activated by the electrode. These data provide support for an anatomically heterogeneous reward substrate, however, it should be noted that they do not speak directly to the idea that different subpopulations of reward neurons responsible for BSR are functionally specialized.

In support of the view that reward circuitry is functionally subdivided, it has been shown that chronic food restriction and body weight loss can potentiate the rewarding effect of the stimulation at some sites in the LH (Carr & Papadouka, 1994; Abrahamsen, Berman & Carr, 1995; Fulton, Woodside & Shizgal, 2000; Fulton, Richard, Woodside & Shizgal, 2002, Fulton, Woodside & Shizgal, 2002). The ability of food restriction to enhance BSR at some sites but not at others is consistent with the view that there are at least two functionally distinct subpopulations of reward neurons that can be activated via stimulation of the LH. However, it could be argued that the variation in the sensitivity of BSR to food restriction is due to individual differences between rats rather than variation in the placement of the stimulating electrode. Indeed, individual differences in the acquisition of drug self-administration (Piazza, Deminiere, LeMoal & Simon, 1989; DeSousa, Bush & Vaccarino, 2000) and the propensity of rats to become obese with a high-fat diet (Levin & Keesey, 1998) have

been documented. If so, between subject analyses of the effect of food restriction and weight loss on rewarding LH stimulation does not provide unambiguous evidence for functional organization in brain reward circuitry.

The present study addresses the question of whether there is functional specificity of brain reward circuitry is investigated by using a multiple electrode design to assess the effect of chronic food restriction and body weight loss at two stimulation sites in each rat. Examination of histological data from multiple studies (Carr and Wolinsky, 1993; Fulton et al., 2000; Fulton et al., 2002; Fulton et al., 2002) employing rewarding LH stimulation suggests that between-site variation in sensitivity to food restriction is most pronounced in the coronal plane. Thus, instead of moveable electrodes that have traditionally been used to examine dorsal-ventral variations in BSR, we assembled fixed dual-electrodes spaced so as to sample differentially from the restriction-sensitive and –insensitive sites observed in prior studies.

4.2. Materials and methods

4.2.1. Subjects

Twelve male Long Evans rats from the Charles River Breeding Farms (St. Constant, Quebec) were used. Subjects weighed between 461 and 603 g at the time of surgery. Following surgery rats were housed individually in plastic solid-floor cages with *ad libitum* access to food and water. Subjects were kept in a temperature-controlled room under a reverse 12 hour dark/12 hour light cycle. All behavioral

testing was carried out in the latter half of the dark phase of the cycle (between 3 and 6 pm).

4.2.2. Surgery

Atropine sulfate (0.5 mg/kg, SC) was administered to each rat to reduce bronchial secretions prior to induction of anesthesia with sodium pentobarbital (Somnotol, 65 mg/kg, i.p.). An assembly of two monopolar electrodes was implanted into each hemisphere. The medial electrode of each assembly was aimed at the perifornical region of the LH (3 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, 7.8 mm below the dura mater). Electrodes were constructed from 000 insect pins (Fine Science, Vancouver, BC) and were insulated with Formvar to within 0.5 mm of the tip. To construct a dual-electrode assembly, Two electrodes were fixed in a custom jig that held them parallel at a separation of 0.5 mm; the tips were offset vertically by 0.5 mm. Electrodes were attached together with epoxy glue. Prior to implantation, a current was passed through each electrode to ensure that the two electrodes of each assembly were not in electrical contact. A wire twisted around two jeweler's screws implanted in the cranium served as the current return. The two electrodes assemblies were bonded to the skull and screw anchors by dental acrylic. Buprenorphine (Buprenex, 0.05 mg/kg, SC) was administered just following surgery to reduce postoperative pain.

4.2.3. Apparatus

Subjects were screened for self-stimulation in wooden boxes (25cm x 25 cm 70 cm) with Plexiglas front panels and wire mesh floors. A lever, positioned 3 cm

above the floor, protruded from the middle of one wall, and a key light positioned 5 cm above the lever signaled the availability of the reward. Electrical stimulation was generated by dual constant current amplifiers and controlled by hand-operated circuit pulse generators. Depression of the lever triggered the stimulator. The connector on the rat's head was linked to the stimulator via a flexible lead and a 7-channel slip-ring located at the top of the test chamber.

The experimental sessions took place in a separate room housing computer-controlled stimulators. Test chambers were similar to those used for training except they were constructed entirely of Plexiglas and equipped with a ceiling-mounted white house light. Each test chamber was enclosed in a 50 cm x 50 cm x 90 cm plywood enclosure lined with sound attenuating foam. Stimulation trains were generated by microprocessor-controlled circuitry and monitored on an oscilloscope in an adjoining room. Subjects were monitored during testing by means of a remote-controlled video camera.

4.2.4. Procedure

Subjects were trained to lever press for stimulation obtained from two of the four implanted electrodes. Rats were shaped to lever press for a 0.5 s train of cathodal, rectangular, constant-current pulses, 0.1 ms in duration, on a continuous reinforcement schedule. Initially, stimulation parameters were set to low currents and frequencies. If the rat displayed signs of aversion (e.g., vocalization, backwards movement, jumping) training was discontinued; otherwise, the rat was trained to press the lever. Stimulation frequencies (pulses per train) were adjusted to produce optimal

levels of responding (>25 presses/minute) at the lowest possible current. The lowest current that could produce optimal levels of responding was used so as to minimize the possibility of recruiting multiple, reward-relevant sub-populations of neurons. The current remained fixed for each electrode and ranged between 200 and 600 μA. Self-stimulation was assessed at the four stimulation sites. The two electrodes that supported the most vigorous lever-pressing in the absence of motoric side-effects were chosen for further testing.

Following initial training, the subjects were trained in the computer-controlled testing setup to respond for a descending series of stimulation frequencies, on a 0.5 sec fixed-interval schedule of reinforcement. The stimulation current was held constant throughout testing, within the range used for initial training. The stimulation frequency was decreased from trial to trial in steps of 0.033 $\log_{10}$ units (~8%) in order to generate curves relating the rate at which rewards were earned to the stimulation frequency ("rate-frequency" curves). Each frequency was available during a single 60 sec trial that was preceded by a 10 sec inter-trial interval during which 5 priming (non-contingent) trains of stimulation were delivered; the priming trains were identical to those available during the trial. Stimulation frequencies were adjusted so as to produce maximal (asymptotic) responding during the first few trials of each frequency sweep. A rate-frequency curve was deemed complete when rats made fewer than 5 responses on each of two consecutive trials. Test sessions comprised seven rate-frequency curves and took 2-3 hours to complete.

One self-stimulation test session was completed per day and the test electrode alternated from day-to-day. As an index of the effectiveness of the rewarding

stimulation, we used the stimulation frequency that maintained a half-maximal rate of reward delivery ("M-50"). Rats were tested for BSR during free-feeding in order to obtain stable baseline responding (day-to-day variation in M-50 $\leq$ 0.04 $\log_{10}$ units). The influence of chronic food restriction on BSR was assessed by limiting daily food intake to ~10 grams/day until body weights reached ~75% of free-feeding values.

4.2.5. Data analysis

A maximum of seven rate-frequency curves could be collected per test session. The first curve served as a warm-up and was not included in the analysis. Data from test sessions in which fewer than three rate-frequency curves were completed were not included in the analysis. Broken-line functions, with a horizontal lower asymptote, a rising linear segment, and a horizontal upper asymptote [12], were fit to each rate-frequency curve plotted along a logarithmic frequency. To indicate the position of the rate-frequency curve along the frequency axis, the stimulation frequency required to maintain half-maximal rewards earned ("M-50") was derived from each broken-line function by interpolation. Enhancing the rewarding effect of the stimulation drives these curves to the left, thus reducing the M-50 value, whereas reductions in reward effectiveness produces rightward shifts and increases the M-50 value. The mean M-50 value was calculated for each session used in the analysis. To determine the magnitude of change in M-50 following ~25% weight loss at each stimulation site, the mean M-50 of the last free-feeding session was subtracted from the mean M-50 of the last food restriction session. To ascertain whether changes in M-50 following weigth loss were statistically reliable, a one-way analysis of variance (ANOVA) was used to

compare the set of M-50 values from the last free-feeding session with the set of M-50 values from the last food restriction session (~25% weight loss). The maximum number of rewards earned (MaxR) was calculated to determine whether food restriction produced any changes in the performance capability of the subject. ΔMaxR was determined by subtracting the mean MaxR of the last free-feeding session from the mean MaxR of the last food restriction session. Changes in MaxR following 25% weight loss were assessed by carrying out an ANOVA to compare MaxR values from the last free-feeding session to the values collected in the last food restriction session.

To determine whether food restriction had different effects as a function of stimulating electrode, a set of ΔM-50 values and corresponding body weight values obtained from one stimulation site were compared with a set of delta M-50 and body weight values from the other stimulation site by means of an analysis of covariance (ANCOVA). The ANCOVA model performs a linear regression of ΔM-50 on body weight for each stimulation site and then compares the two regression lines to determine if the slopes are different. In cases where the slopes of the two lines are significantly different there is an interaction between body weight and stimulation site. A level of $p \leq 0.05$ was used as the criterion for statistical significance.

4.2.6. Histology

Following completion of testing, stimulation sites were marked by means of the Prussian Blue method for 7 of the 13 subjects. With the stimulating electrode serving as the anode, a 100 μA direct current was applied for 15 sec. For the

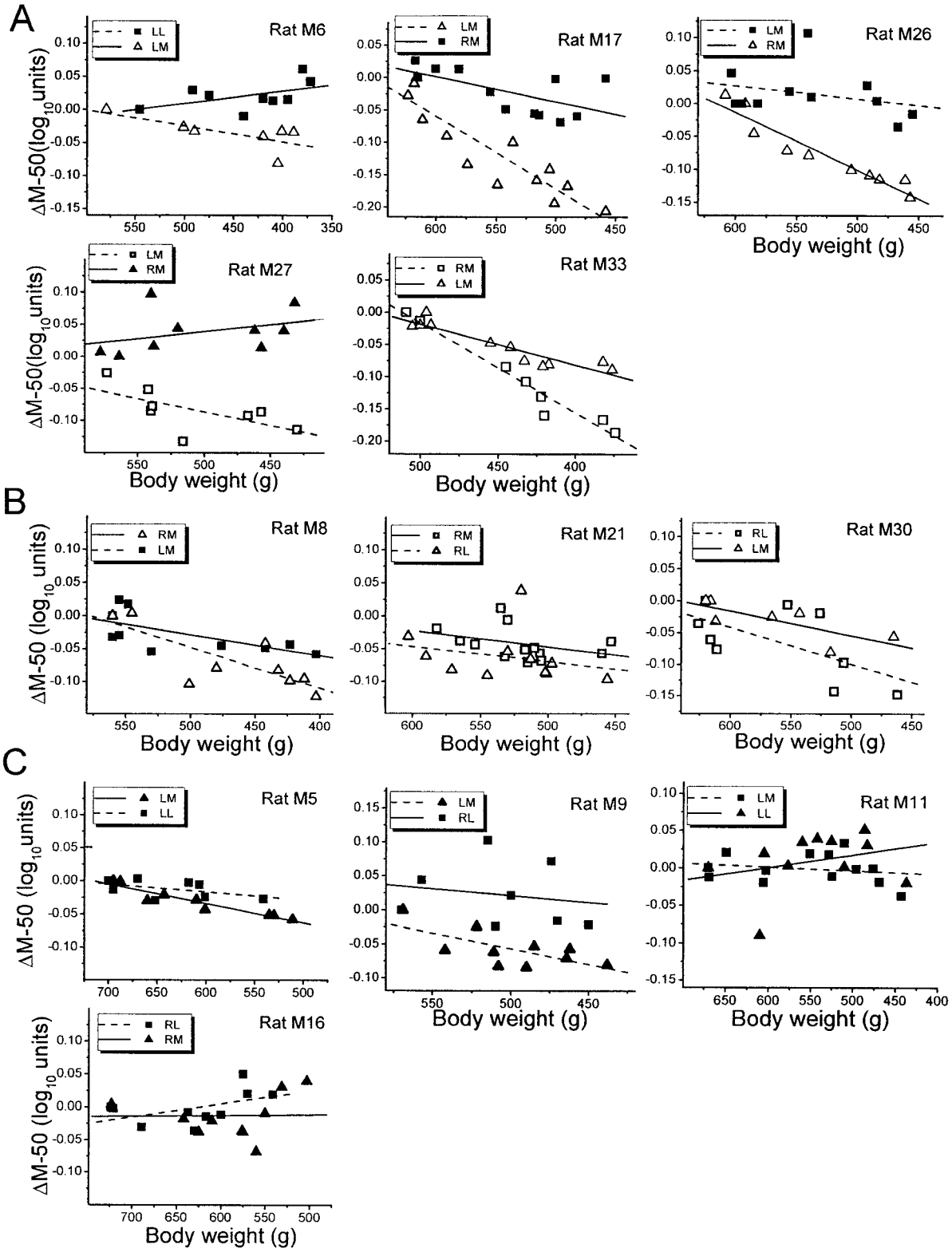
remaining 6 subjects a marking lesion could not be made because it would confound subsequent tissue analyses (for a separate study). Instead, the most ventral part of the lesion left by the electrode was established as the site of stimulation for these rats. Rats were injected with a lethal dose of sodium pentobarbitol (Somnotol, 100 mg/kg, IP) and perfused intracardially with phosphate-buffered saline. To mark the lesion site, saline was followed by a mixture of 10% formalin (100 ml), trychloroacetic acid (0.5 g), potassium ferrocyanide (3 g) and potassium ferricyanide (3 g), whereas in the remaining rats cold saline (300 ml) was followed by 300 ml of 4% paraformaldehyde. Brains were removed and stored in 10% formalin or 4% paraformaldehyde. After immersion in a 20% sucrose-formalin solution or a 30% sucrose-paraformaldehyde solution for at least 24 hr, the brains were frozen, sliced on a cryostat in 30 μm coronal sections, and mounted on pre-coated slides (Fisher Scientific, Nepean, Ontario). Sections from rat brains that received the marking lesion were stained for Nissl substance using formal thionin. The location of stimulation sites was identified with the aid of a stereotaxic atlas (Paxinos & Watson, 1998).

4.3. Results

During the free-feeding condition body weights ranged from 509 - 712 g. Following food restriction body weights fell to 375 – 569 g.

In 5 out of 12 rats, rewarding effect produced by stimulation of the two sites was altered differentially by food restriction. The data obtained from these subjects are shown in Figure 4.1A which shows that the slopes of the two regression lines differ. Thus, in these rats reductions in body weight had a greater impact on M-50 values at one stimulation site compared to the other. Time-course data collected from two of

Figure 4.1. Data illustrating the effect of food restriction and weight loss on M-50 values obtained from two separate stimulation sites in each rat. A. Subjects in which food restriction produced different effects on M-50 values obtained using the two electrodes. B. Subjects in which food restriction decreases M-50 values obtained using one or both stimulation sites, but there were no differences in magnitude of effect between sites. C. Subjects in which food restriction failed to decrease M-50 values at both stimulation sites and there was no difference between sites. Each data point represents a mean of ΔM-50 values. Lines represent the regression for each stimulation site. Open symbols refer to stimulation sites where ~25% weight loss (last data point to the right) significantly decreased M-50 values. LM: left medial electrode; LL: left lateral electrode; RM: right medial electrode; RL: right lateral electrode.


A
Rat M6
LL
LM
Rat M17
LM
RM
Rat M26
LM
RM
Rat M27
LM
RM
Rat M33
RM
LM
ΔM-50(log10units)
Body weight (g)
B
Rat M8
RM
LM
Rat M21
RM
RL
Rat M30
RL
LM
ΔM-50 (log10units)
Body weight (g)
C
Rat M5
LM
LL
Rat M9
LM
RL
Rat M11
LM
LL
Rat M16
RL
RM
ΔM-50 (log10units)
Body weight (g)

these subjects reveal that M-50 values obtained using one of the electrodes decreased as a function of weight loss, whereas those obtained using the second electrode remained relatively stable; in three other cases, M50 values obtained using the two electrodes decreased at different rates as a function of weight loss (Fig. 4.2). In 3 out of the 7 remaining rats there was a significant influence of food restriction on M-50 values at one or both stimulation sites, however there was no interaction between body weight and stimulation site (Figure 4.2B). In the 4 remaining rats weight loss failed to decrease M-50 values at either stimulation site and there was no interaction between body weight and site (Figure 4.2C).

Table 4.1 provides statistical values for the body weight by site interaction, slopes of regression lines, and the magnitude of change in mean M-50 and MaxR following ~25% weight loss. In 12 out of the 24 stimulation sites tested, ~25% body weight loss significantly reduced M-50 values. Changes in the performance of the rat following weight loss are reflected in the ΔMaxR value. Only three subjects showed a significant increase in MaxR (positive values) whereas four subjects showed significant decreases in MaxR. These changes in MaxR varied across the restriction-sensitive and –insensitive stimulation sites.

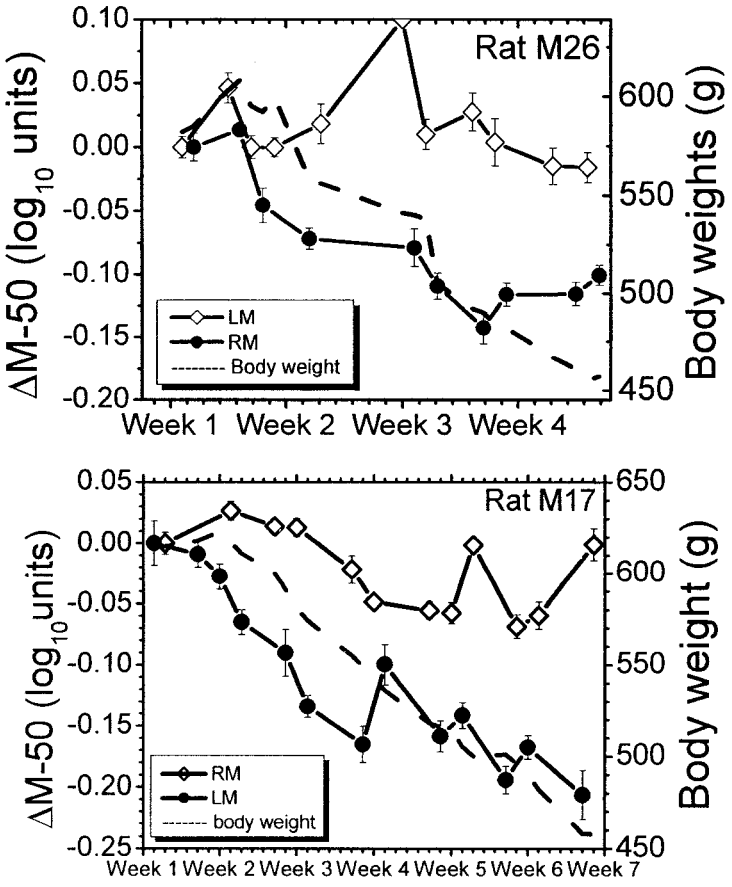


Figure 4.2. Time-course data for two rats. M-50 values obtained at one stimulation site but not at another decrease as a function of weight loss for each rat. Each data point represents a mean of ΔM-50 values.

Table 4.1. Summary statistics for each subject

Rat	BW x site Interaction	Site*	Slope of regression line	ΔM-50 (log_{10} units)	ΔMaxR
M5	$F(1, 13) = 2.81$	LM	-1.98^{-1}	-0.053	0.294
		LL	-1.86^{-1}	-0.030	0.457
M6	$F(1, 12) = 6.93$*	LM	-5.25^{-2}	-0.034	-2.342**
		LL	4.84^{-3}	0.018	3.037**
M8	$F(1, 15) = 1.95$	LM	-2.53^{-1}	-0.117**	3.233**
		RM	-2.77^{-1}	0.034	2.610
M9	$F(1, 14) = 0.34$	LM	-2.25^{-1}	-0.037	5.410**
		RL	-1.48^{-1}	-0.021	2.583
M11	$F(1, 21) = 0.95$	LM	-7.68^{-3}	-0.037	-0.0479
		LL	1.18^{-2}	0.034	1.280
M16	$F(1, 18) = 1.01$	RM	4.84^{-2}	-0.032	-0.030
		RL	3.53^{-2}	0.036	-4.083
M17	$F(1, 21) = 10.62$**	LM	-7.32^{-1}	-0.179**	-1.696
		RM	-2.30^{-1}	-0.058	-2.601**
M21	$F(1, 20) = 0$	RM	-2.07^{-1}	-0.065*	-1.989
		RL	-1.95^{-1}	-0.038*	-0.417
M26	$F(1, 17) = 9.37$*	LM	-9.23^{-2}	-0.062**	0.089
		RM	-5.03^{-1}	-0.114**	-2.873*
M27	$F(1, 13) = 9.30$*	LM	-2.94^{-1}	-0.088**	-0.185
		RM	1.82^{-1}	0.086**	-3.962**
M30	$F(1, 12) = 0.30$	LM	-3.10^{-1}	-0.056*	-1.144
		RL	-3.43^{-1}	-0.112**	-3.787
M33	$F(1, 15) = 37.54$**	LM	-3.28^{-1}	-0.070**	1.266
		RM	-6.95^{-1}	-0.167**	-2.949

* LM: left medial electrode; LL: left lateral electrode; RM: right medial electrode; RL: right lateral electrode.

4.3.1. Histology

Electrode placements in 7 rats was assessed whereas in the remaining 5 rats histological data has not yet been obtained because of analyses being performed for a separate study. Histological analysis revealed that the restriction-sensitive rewarding effects arose from stimulation of sites dorsal and/or lateral to the fornix (Fig. 4.3 & Table 4.2). The remaining sites were scattered around the periphery of the region containing the restriction-sensitive sites.

4.4. Discussion

The present data demonstrate functional heterogeneity in the neural substrate underlying BSR. By employing a multiple electrode design we were able to assess the influence of chronic food restriction at two stimulation sites in each rat to determine whether the ability of food restriction to potentiate BSR is contingent on the placement of the stimulating electrode. In 5 out of 12 subjects (Group A), chronic food restriction and body weight loss differentially altered M-50 stimulation thresholds at the two stimulation sites. In Group B, weight loss potentiated BSR obtained from one or both stimulation sites, however there was no significant difference between sites. As expected, there was another subset of subjects (Group C) that showed no reductions in the rewarding impact of the stimulation with weight loss at either site, and thus there was no discrepancy between stimulation sites with respect to the influence of food restriction on BSR. These findings support and extend previous data showing that chronic food restriction and weight loss can enhance the rewarding effects of LH stimulation in rats with electrodes located in the vicinity of

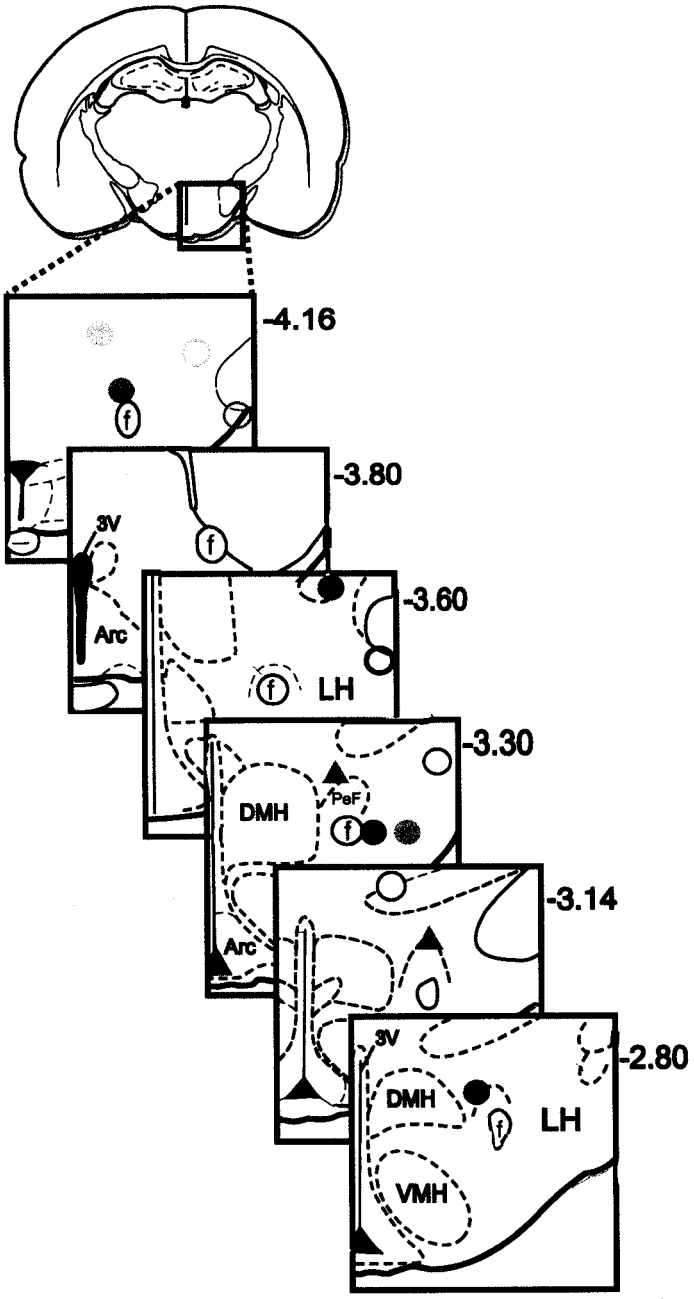


Figure 4.3. Electrode placements for 7 out of the 12 subjects. Individual rats are colour coded. Triangles: restriction-sensitive placements, and circles: restriction-insensitive placements. Open symbols refer to the lateral electrode and closed symbols refer to the medial electrode. See Table 4.2 for further description of symbols.

Table 4.2. Symbols marking electrode placements.

Rat	Site*	Restriction-sensitive	Restriction-insensitive
M5	LM		
	LL		
M6	LM		●
	LL		○
M8	LM	▲	
	RM		
M9	LM		
	RL		
M11	LM		
	LL		
M16	RM		●
	RL		○
M27	LM	▲	
	RM		●

*LM: left medial electrode; LL: left lateral electrode;
RM: right medial electrode; RL: right lateral electrode.

the perifornical area (Blundell & Herberg, 1968; Carr & Wolinsky, 1993; Fulton, Woodside & Shizgal, 2000).

In 12 out of the 24 stimulation sites tested, the reward effectiveness of the stimulation decreased as a function of weight loss. Plotted time-course data from two rats (Rats M17 and M26) illustrate the highly systematic manner by which M-50 thresholds can decline with body weight at one electrode (filled symbols) while not at the other. Our evidence that there is a positive relationship between M-50 values and body weight is consistent with previous findings (Carr and Wolinsky, 1993) and suggests that signals reflecting the status of adiposity stores link chronic food restriction to the modulation of BSR. To this end, we have previously shown that leptin, an adiposity hormone that circulates in direct proportion to the amount of fat tissue, can oppose the reward-enhancing effects of food restriction on BSR, but only at restriction-sensitive stimulation sites (Fulton, Woodside & Shizgal, 2000). Similarly, it has been demonstrated that yet another adiposity signal, insulin, can attenuate the rewarding effect of stimulating restriction-sensitive sites. These findings suggest that leptin and insulin may couple the state of long-term energy stores to restriction-sensitive reward circuitry.

Divergent effects of food restriction on BSR

In subjects of Group A, the effect of food restriction and weight loss on the rewarding impact of the stimulation differed between the two stimulation sites. Thus, the ability of chronic food restriction to potentiate BSR depended on the position of the stimulating electrode. The simplest account for this finding is that there are at least

two functionally distinct sub-populations of reward-relevant neurons that lie in, terminate in or course through the LH. In cases where the influence of food restriction did not differ significantly between electrodes we propose that the relative recruitment of restriction-sensitive and/or –insensitive neurons by each of the two electrodes was similar. This outcome is not surprising given the proximity of restriction-sensitive and restriction-insensitive sites observed in this and previous studies (Carr & Wolinsky, 1993; Fulton, Woodside & Shizgal, 2000; Fulton, Richard, Woodside & Shizgal, 2002, Fulton, Woodside & Shizgal, 2002).

The notion of functional heterogeneity in the substrate for BSR originates from an early body of work investigating the impact of short- and long-term manipulations of energy balance on self-stimulation in the LH. Self-stimulation rates were shown to be increased by acute food deprivation or long-term food restriction and in some cases at stimulation sites that could induce feeding (Blundell & Herberg, 1968; Carey, Goodall & Lorens, 1970; Deutsch & DiChiara, 1967; Margules & Olds, 1962). Moreover, in a manner resembling its influence on food intake, force feeding and spontaneous meal consumption was shown to suppress self-stimulation rates (Hoebel, 1968; Hoebel & Thompson, 1969). On the basis of these and other data, the idea of a separate feeding-related reward circuit that could be triggered via LH stimulation was incorporated into the thinking of the time.

The rate measures used in these studies are not able to factor out changes in performance, thus many of these experiments were subsequently repeated using the rate-frequency measures of reward effectiveness (i.e., curve-shift paradigm). With stimulating electrodes located in the LH, some studies collecting rate-frequency

curves to measure changes in reward effectiveness of the stimulation reported that BSR was relatively unaltered by acute manipulations of energy balance, such as food deprivation (Fulton, Woodside & Shizgal, 2000; Giovino and Wise, 1986) and gastric loading (Conover & Shizgal, 1994). In contrast, other reports suggested that acute deprivation enhances the rewarding impact of LH stimulation (e.g., Rossi & Panksepp, 1986). These inconsistencies called to question the notion of a separate subpopulation of reward neurons related to feeding and thus, the portrait of a heterogeneous reward substrate.

Although acute changes in energy balance cues have been shown to have varied effects on BSR thresholds, several studies have documented the modulation of BSR by food restriction and weight loss. The potentiation of rewarding LH stimulation has remained a highly consistent finding in cases where the stimulating electrode is located in a region close to the fornix (Blundell & Herberg, 1968; Carr & Wolinsky, 1993). In this study, as in three others, we have found that the rewarding effect induced via electrode tips immediately dorsal or dorsolateral to the fornix ($\sim <$ 0.2mm from fornix) is always restriction-sensitive. Nonetheless, the growing literature on the predisposition of certain animals to engage in dysregulatory behaviors, such as over-feeding in response to a high fat diet (Levin & Keesey, 1998) or compulsively self-administering rewarding drugs (Piazza, Deminiere, LeMoal & Simon, 1989; DeSousa, Bush & Vaccarino, 2000), offers an alternative explanation for the effect of food restriction to enhance the rewarding stimulation. This line of reasoning suggests that the ability of food restriction to increase BSR is due to individual differences. While the present data cannot rule out individual variability in the reward circuitry

underlying restriction-sensitivity, they refute the hypothesis that there is no site-specificity with respect to the effect of food restriction on BSR. Thus, the present data provide evidence for a functionally heterogeneous substrate and advance our hypothesis that alterations of long-term energy stores affect choice among competing behaviors and goal objects by modulating the activity of a restriction-sensitive sub-population of neurons (Shizgal, Fulton & Wooside, 2001).

The observation that small variations in the placement of the electrode in the MFB can alter the influence of food restriction on BSR speaks to the complexity of the neuroanatomy of this fibre tract. Such complexity might also help explain between subject and study variability in the effect of lesions, current manipulations or neurochemicals on the rewarding impact of MFB stimulation. For example, the degree to which tissue damage can reduce the reward effectiveness of the stimulation often varies from rat to rat and does not seem to depend on the specific damage caused by the lesion (Arvanitogiannis et al., 1996; Ascheson et al., 2001; Gallistel et al., 1996). One would expect the size of the lesion effect to vary between rats if the fibers recruited by MFB stimulation were from anatomically distinct subpopulations and were juxtaposed through the MFB in a very compact and intermixed manner.

The modulation of BSR by neurochemical manipulations could also be affected by the relative activation of different subpopulations of reward neurons. Alterations in the rewarding effect of the stimulation by neurochemical manipulations is determined by the distinct physiological characteristics of the neurons triggered by the stimulation and their inputs. Accordingly, the effectiveness of neuropeptides to alter BSR has been show to vary across subjects and studies. Indeed, we have

previously demonstrated that the modulation of BSR by the hormones leptin and corticotropin-releasing hormone depends on whether food restriction can potentiate the rewarding stimulation (Fulton, Woodside & Shizgal, 2000; Fulton, Richard, Woodside & Shizgal, 2002). Thus, evidence for functional heterogeneity in brain reward circuitry may not only help elucidate the variable results observed across studies of BSR, they have implications for the interpretation of the effects of lesions, drugs, physiological manipulations, and even changes in stimulation parameters on BSR.

Diverse reward signals

We have proposed a network of functionally specific pathways that determine the reward value of stimuli and activities (Shizgal et al., 2001). At least one of these pathways is linked to the long-term regulation of energy balance. What is the nature of the rewarding signal produced by stimulating restriction-sensitive sites? In consideration of the evidence that food deprivation, a manipulation that dramatically increases the value of food, does not augment BSR obtained from restriction-sensitive sites (Fulton et al., 2000) it is difficult to characterize these neurons as encoding information about hunger *per se*. Nonetheless, it should be noted that feeding can be elicited by stimulation ("stimulation-induced feeding") via electrodes in the perifornical region of the LH and at perifornical sites where self-stimulation is increased by food restriction (Blundell & Herberg, 1968; Carr & Simon, 1984). Alternatively, the potentiating effect of weight loss on perifornical self-stimulation may be tied to the influence of long-term energy stores on non-ingestive behaviors

that defend body weight, such as food hoarding (Shizgal, Fulton & Woodisde, 2001). In this respect, it is worth mentioning that electrical stimulation of sites in the LH that elicit feeding also produces food hoarding behavior (Herberg & Blundell, 1967) . Additional work is required to determine the precise function of this circuitry and the manner in which it contributes to energy balance.

There are likely other neural pathways that generate the reward value of food in a manner that responds to the impact of post-ingestive and short-term energy signals. For example, decreases in BSR thresholds in response to acute deprivation have been reported in the case of some LH sites in the rat (Rossi & Panksepp, 1992) and increases in BSR were observed at orbitofrontal sites in monkeys fed to satiety (Mora, Avrith, Phillips, Rolls, 1979). Along these same lines, there would be additional pathways that govern the reward value of stimuli and activities important for the regulation of other motivational states such as copulation. It has been proposed that the outputs of functionally specific pathways converge at a point downstream so that the value of different goal-objects and goal-directed behaviors could be evaluated along a common measurement scale for behavioral choice (Shizgal, 1999). An important objective for future work is to map the BSR sites that gain access to other specialized reward pathways so that we may identify, characterize and perhaps target pharmacologically the circuitry that confers reward value of other stimuli and activities.

CHAPTER FIVE

CONCLUSIONS

The findings in this thesis extend previous research demonstrating the potentiating effects of chronic food restriction and body weight loss on the rewarding impact of electrical stimulation obtained from a subset of sites in the LH (Abrahamsen et al., 1995; Blundell & Herberg, 1968; Carr & Wolinsky, 1995; Fulton et al., 2000). Earlier findings show that the modulation of BSR by energy restriction depends on the energy store being altered (Cabeza de Vaca et al., 1998; Fulton et al., 2000). The ability of chronic food restriction, but not acute food deprivation, to alter BSR at restriction-sensitive sites was demonstrated by data collected for the Master's thesis (Appendix 1). The most parsimonious explanation for the distinct effects of short- and long-term energy restriction on BSR is that the potentiation of restriction-sensitive stimulation relies on the depletion of adipose stores. Consistent with this view, the adipose hormone, leptin, was shown to attenuate the rewarding effect of the stimulation obtained at restriction-sensitive sites (Fulton et al., 2000). These data further underline the distinction between restriction-sensitive and –insensitive reward circuitry and suggest that leptin may be involved in the process whereby weight loss enhances BSR.

A report from Carr and colleagues shows that insulin may also mediate the actions of food restriction on restriction-sensitive reward circuitry (Carr et al., 2000). Like leptin, central insulin injection reversed the increases in BSR produced by weight loss, however it is not clear whether this effect is specific to restriction-sensitive stimulation. Thus, both leptin and insulin may tie the actions of weight loss to the restriction-sensitive subpopulation of reward neurons.

The hypothesis that leptin and insulin link fat stores to changes in the activity of restriction-sensitive neurons makes sense when considering that plasma and CNS levels of these hormones reflect the amount of adipose tissue (Burguera et al., 2000; Considine et al. 1996; Stein et al., 1987) and that BSR thresholds decrease as a function of body weight (Carr and Wolisky, 1993; Fulton et al., 2002). Nonetheless, if the amount of leptin and insulin alone were crucial to the modulation of BSR at restriction-sensitive sites, then we should expect to see changes in BSR by other manipulations that alter the levels of these two hormones. Because acute changes in energy balance can alter leptin and insulin release, the levels of these hormones can become transiently separated from levels of body fat. For example, a short-term period of food deprivation lowers plasma leptin and insulin concentrations (Sivitz, Bailey & Donahue, 1996), yet as I have shown, an acute period of food deprivation failed to alter thresholds for BSR at restriction-sensitive sites. If leptin and insulin are important signals for the modulation of restriction-sensitive reward circuitry in lean rats then there must be additional mechanisms that contribute to the long-term regulatory control of reward circuitry by these hormones, apart from those that respond to acute fluctuations.

It has yet to be resolved whether leptin and insulin produce their effects on reward circuitry directly or via their influence on neuropeptides known to mediate the actions of these hormones on food intake. Many of the same targets in the hypothalamus are shared by leptin and insulin. Although less well-studied, insulin also appears to regulate AgRP, NPY, α-MSH and CART (Obici et al., 2001; Sipols et al., 1995; Williams et al., 1989). Hypothalamic nuclei, including the PVN and LH, are

richly innervated by the ARC neuropeptidergic systems. The PVN neurons synthesizing CRH and LH neurons containing MCH or orexin have been described as "second-order" neurons involved in the control of food intake (Schwartz et al., 2000). Both leptin and insulin receptors have been identified on these second-order neurons, however, unlike leptin, it is not yet known whether insulin affects the release of CRH, MCH and orexin. The results of experiments in Chapter Two provide little support for the notion that CRH NPY, AgRP and MCH contribute to the modulation of BSR by food restriction, leptin and insulin. The anorexigenic peptide, CRH, had a strikingly dissimilar effect at restriction-sensitive and –insensitive sites, yet CRH largely failed to alter BSR at restriction-sensitive sites. Both NPY and MCH failed to consistently alter BSR at all stimulation sites tested. AgRP, on the other hand, enhanced the rewarding effect of the stimulation in two rats that showed the largest increases in BSR by food restriction. In view of the inconsistent data, further testing of AgRP in subjects that show larger (>0.15 $\log_{10}$ units) reductions in M-50 stimulation thresholds with 25 % weight loss is warranted. Given that AgRP-induced increases in BSR were only seen in these two subjects however, there is insufficient evidence at this moment to suggest a role for AgRP in the process whereby food restriction and leptin modulate BSR.

Other candidate peptide mediators in the effects of leptin at restriction-sensitive stimulation sites include the anorexigenic peptide, CART, and the orexins. However, the influence of leptin on brain reward circuitry need not be restricted to its actions on hypothalamic neurons. Other targets of leptin include the amygdala, thalamic nuclei, the raphe system, pontine nuclei, locus coeruleus, parabrachial

nucleus, nucleus of the solitary tract and the medullary reticular formation (Elias et al., 1998; Hay-Schmidt et al., 2001). The neurons that generate the restriction-sensitive reward signal have not been identified, and it is possible that leptin is modulating restriction-sensitive reward circuitry via its actions at extrahypothalamic sites.

The data presented in Chapter Three call into question whether leptin and insulin are necessary for the regulation of restriction-sensitive reward circuitry. With the onset of obesity, the *fa/fa* Zucker rat develops resistance to circulating leptin and insulin. Specifically, with increasing obesity the CSF-to-plasma ration of these two hormones drops, suggesting that some form of transport saturation is taking place at the level of the blood-brain barrier. Given that food restriction was able to enhance the rewarding effect of the stimulation in a subset of obese rats, signals associated with adiposity appear to influence reward circuitry. Although food restriction and consequent weight loss reduce circulating levels of leptin and insulin in the Zucker rat, central resistance to these hormones appears to decouple their central and peripheral concentrations. Indeed, there is no evidence showing that food restriction or weight loss reduces the CNS concentrations of either of these hormones in this model. Therefore, in the case of the obese Zucker rat there appear to be other peripheral signals that link fat stores to brain reward circuitry. Nevertheless, we cannot conclude from these data that there are additional peripheral signals at work in lean rats since the mutation responsible for obesity in the Zucker rat could lead to some form of developmental compensation.

Data in Chapter Four provide evidence for at least two, functionally separate subpopulations of reward neurons that can be activated by electrical stimulation of the LH. This hypothesis was confirmed by assessing the modulation of BSR by restriction using two stimulation electrodes in each rat. In five of twelve subject, restriction modulated BSR exclusively or more strongly in the case of one of the two stimulation sites. Thus, alterations in long-term regulatory signals exert an influence on a functionally unique component of reward circuitry. On the basis of these data, one would expect rats to choose trains of stimulation that trigger the restriction-sensitive subpopulation over behaviorally-equivalent trains of restriction-insensitive stimulation during weight loss. Indeed, a powerful demonstration of the functional segregation of these reward substrates would be to assess relative preference for their activation under conditions of concurrent availability following weight loss.

Identifying the neurons that give rise to the rewarding effect of restriction-sensitive stimulation remains a crucial goal if we are to fully understand their function, physiology, neurochemistry and anatomical linkage. This will not be an easy task as there are many fiber systems that course through the LH. The histological data collected over several studies show that BSR obtained from stimulation sites immediately dorsolateral to the fornix is almost always sensitive to food restriction (Figure 5.1). On the basis of these data, it is suggested that the directly activated, restriction-sensitive neurons lie in, course through or terminate in this area. it cannot be concluded that these cells are the ones that generate the reward signal.

One method for investigating which neurons are activated by stimulation of restriction-sensitive sites is to examine immunohistochemical labeling of immediate-

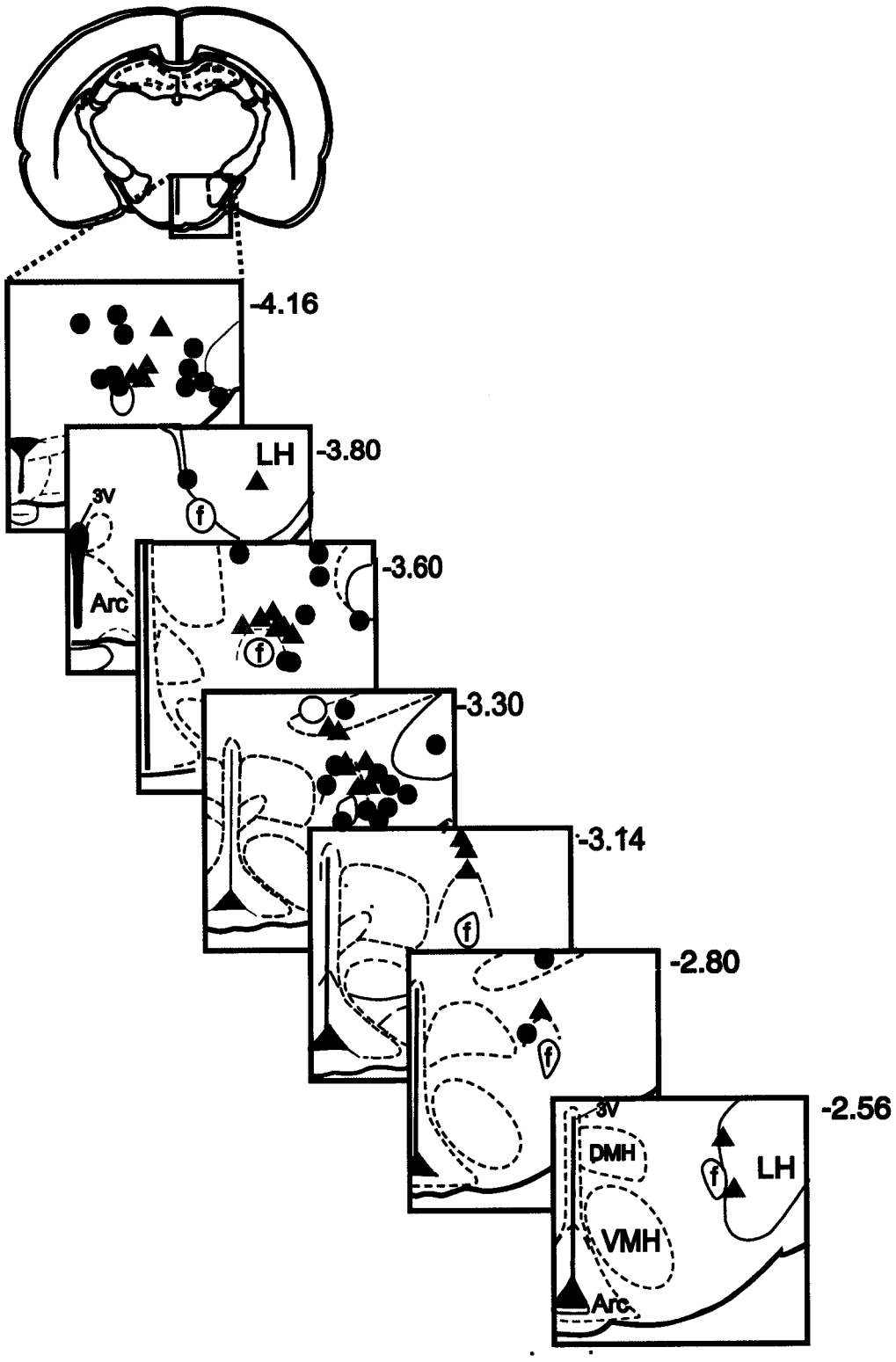


Figure 5.1. Inclusive histology figure. Electrode placements for rats from five experiments (Chapter Two, Chapter Four and Appendix). Red triangles: Restriction-sensitive; Blue circles: Restriction-insensitive. **3v**: third ventricle; **Arc:** arcuate nucleus; **DMH**: dorsal medial nucleus; **f**: fornix; **LH:** lateral hypothalamus; **VMH**: ventral medial hypothalamus.

early genes (IEGs) that serve as markers of neuronal activation. One such IEG commonly used for this purpose if *c-fos*. The use of Fos immunohistochemistry as an effective means to visualize neurons that have been activated by rewarding stimulation has been successfully demonstrated (Arvanitogiannis, Flores, Pfaus & Shizgal, 1996; Arvanitogiannis, Tzschentke, Riscaldino, Wise, & Shizgal, 2000). Given that LH stimulation has been shown to induce widespread Fos labeling, it would be beneficial to determine differences in Fos expression induced by stimulation of restriction-sensitive versus restriction–insensitive sites. Such a study would hopefully elucidate key differences in neural activation uniquely associated with the restriction-sensitive subset of reward circuitry.

Upon visualization of Fos labeling, one could establish the contribution of a labeled region by lesioning or inactivation methods. For example, application of a neurochemical agent that would induce apoptosis while preserving myelin and fibers of passage could provide crucial evidence that cell bodies in a given brain region are important for the generation of reward signals produced by restriction-sensitive stimulation. As described in the Introduction, some studies have identified several regions that play an important role in the reward effectiveness of MFB stimulation. In this regard, it is worth mentioning that BSR produced by stimulating sites immediately dorsolateral to the fornix was substantially reduced by inactivation of the lateral BNST (Waracynski, 2003). The results of lesion and inactivation studies, in combination with Fos data, will likely provide useful information for determining which nuclei to examine first.

What is the function of restriction-sensitive reward circuitry?

Identification of the restriction-sensitive subpopulation of reward neurons would make determining the function of this subset of reward circuitry a less demanding task. Nonetheless, what has been learned to date imposes some constraints on likely functional correlates. It is not viable to suggest that these neurons encode information generally related to hunger given that manipulations that increase the rewarding effects of food, such as food deprivation, 2-DG or NPY administration, fail to alter the rewarding impact of stimulating restriction-sensitive sites. Nonetheless, there is reason to speculate whether this subset of neurons could be, more specifically, signaling the rewarding properties of high-fat foods.

The ability of rats to distinguish and prefer one macronutrient over another has been well-documented in the laboratory (see Sclafani, 2001). The preference for and over-consumption of fat- and sugar-rich foods can be attributed to their palatability (hedonic response to the food stimuli), availability and to their post-ingestive nutritive consequences. Under conditions of low-food availability, animals will eat what is available to them in order to survive. Similarly, in controlled laboratory settings it has been shown that food deprived rats will increase their consumption of carbohydrate-, protein- or fat-rich foods when either of these foods are presented alone (Sclafani, 2001). However, when allowed the option to choose between different macronutrients, rats show clear preferences under certain energy states. Choice tests with calorically dilute sugar and corn oil suspensions reveal that food restriction resulting in 15% body weight loss increases preference for corn oil while reducing preference for sugar (Sclafani & Ackroff, 1993). In a similar study, Sclafani (1996)

examined the influence of food restriction and weight loss on choice between non-nutritive fat and sugar substances. During free-feeding conditions, the percentage of total intake consumed as saccharin was 80% whereas following weight loss preference switched to mineral oil such that 76% of total intake was derived from this source. Given that this preference reversal was observed with non-nutritive substances, these data suggest that food restriction enhances the orosensory response to oily, high-fat foods. Most notably, the shift in preference during food restriction from carbohydrate-rich substances to fatty substances seems to be related to the long-term energy state of the rat. Access to a meal immediately prior to preferences tests or 24 hours of *ad libitum* feeding before the test failed to reverse food restricted rats preference for mineral oil over the saccharin solution (Sclafani, 1996). These results indicate that flavor preferences are influenced by physiological signals related to long-term energy balance.

The macronutrient preference findings described above are consistent with recent evidence implicating separate neural mechanisms driving consumption of high-fat foods. Manipulations that have been shown to elevate intake of high-fat food over sugar-rich substances include central administration of AgRP (Hagan et al., 2001), orexin-A (Clegg et al., 2002), enterostatin (Koizumi et al., 2001) and μ opioid receptor agonists in the ventral striatum (Kelley et al., 2002; Will et al., 2003; Zhang et al., 2000). On the other hand, NPY, a very potent appetite stimulant, increases carbohydrate intake to a much greater extent when offered together with fat (Stanley et al., 1985). Much progress has been made lately in elucidating the neural mechanisms underlying consumption of fatty foods. In particular, recent data suggests

that μ and K opioid receptor activation (Brugman et al., 2002) and orexin-A stimulation (Clegg et al., 2002) may be involved in the initial actions of AgRP to stimulate high fat feeding, suggesting that these peptides may collaborate as part of a system controlling intake of fatty substances.

It is tempting to suggest that the restriction-sensitive subpopulation of reward neurons is responsible for generating signals related to the rewarding effects of high fat food. The relationship between stimulation thresholds and body weight fit well with the data describing the impact of weight loss to increase preference for high fat foods. Moreover, threshold reductions produced by food restriction and body weight can be reversed by μ and K receptor antagonism (Carr and Papadouka, 1994). The verdict is not in with respect to the influence of AgRP on restriction-sensitive reward circuitry, however the data presented here suggest that AgRP should not be ruled out. In addition, the effects of AgRP on restriction-sensitive reward circuitry could very well be mediated at extrahypothalamic sites. It is noteworthy that AgRP immunoreactivty is very dense in all compartments of the BNST, an area shown to contribute the rewarding effects of perifornical self-stimulation (Waraczynski, 2003). Rather than the administering AgRP to the third ventricle as was done in the experiment presented in Chapter Two, lateral ventriclular administration of AgRP would be more efficient at targeting areas outside the hypothalamus. Such an investigation would hopefully provide a more conclusive answer to the question of whether AgRP signaling contributes to restriction-sensitive reward circuitry.

That CRH, NPY and MCH failed to consistently alter restriction-sensitive reward circuitry also correspond with this hypothesis since there is no evidence that any of these peptides can selectively increase consumption of high fat food (Clegg et al., 2002; Stanley et al., 1985). Finally, it should be noted that feeding can be elicited by stimulation via electrodes in the perifornical region of the LH and at perifornical sites where self-stimulation is increased by food restriction (Blundell and Herberg, 1968; Hernandez and Hoebel, 1989). For these reasons, I speculate that restriction-sensitive reward neurons may well carry a signal for the rewarding effects of high fat food.

An alternative explanation is that the modulation of restriction-sensitive reward circuitry is linked to food hoarding (Shizgal et al., 2001). At normal body weight rats do not hoard food, however as body weight decreases the amount of food hoarded grows. In contrast, a short-term period of food deprivation nor pre-feeding alter the amount of food hoarded (Cabanac & Gosselin, 1996; Fantino & Cabanac, 1980). These findings bode well for the hypothesis that the restriction-sensitive neurons are linked to the rewarding effects of hoarding. In this respect, it is worth mentioning that electrical stimulation of sites in the LH that elicit feeding also produces food hoarding behavior (Herberg and Blundell, 1967).

Whether the restriction-sensitive neurons code information about the reward value of fatty foods, food hoarding or some other stimulus or behavior, it is likely that these contribute in some way to long-term energy balance. The findings reported here and the flood of recent findings on the neurobiological basis of energy balance offer exciting new opportunities for achieving a better understanding of the role of brain

reward circuitry in foraging, ingestion and other behaviors that contribute to the regulation of energy balance.

REFERENCES

Abrahamsen, G., Berman, Y., & Carr, K. (1995). Curve-shift analysis of self-stimulation in food-restricted rats: relationship between daily meal, plasma corticosterone and reward sensitization. *Brain Res., 695*(2), 186-194.

Acheson, A., Waraczynski, M., & Perkins, M. (2000). Lesions and inactivation implicate dorsolateral hindbrain in MFB self-stimulation. *Physiol Behav, 71*(1-2), 159-171.

Ahima, R., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., et al. (1996). Role of leptin in the neuroendocrine response to fasting. *Nature, 382*, 250-252.

Alemzadeh, R., & Holshouser, S. (1999). Effect of diazoxide on brain capillary insulin receptor binding and food intake in hyperphagic obese Zucker rats. *Endocrinology, 140*(7), 3197-3202.

Anand, B., & Brobeck, J. (1951). Hypothalamic control of food intake in rats and cats. *Proc. Soc. Exp. Biol. Med., 77*, 323-324.

Arvanitogiannis, A., Waraczynski, M., & Shizgal, P. (1996). Effects of excitotoxic lesions of the basal forebrain on MFB self-stimulation. *Physiol Behav, 59*(4-5), 795-806.

Baskin, D., Seeley, R., Kuiper, J., Lok, S., Weigle, D., Erikson, J., et al. (1998). Increased expression of mRNA for the long form of the leptin receptor in the hypothalamus is associated with leptin hypersensitivity and fasting. *Diabetes, 47*, 538-543.

Baskin, D. G., Breininger, J. F., & Schwartz, M. W. (1999). Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. *Diabetes, 48*(4), 828-833.

Berman, Y., Devi, L., & Carr, K. (1994). Effects of chronic food restriction on prodynorphin-derived peptides in rat brain regions. *Brain Res., 664*(1-2), 49-53.

Bernardis LL, & LL, B. (1996). The lateral hypothalamic area revisited: ingestive behavior. *Neurosci Biobehav Rev, 20*(2), 189-287.

Bielajew, C., & Shizgal, P. (1982). Behaviorally derived measures of conduction velocity in the substrate for rewarding medial forebrain bundle stimulation. *Brain Research, 237*, 107-119.

Bielajew, C., & Shizgal, P. (1986). Evidence implicating descending fibers in self-stimulation of the medial forebrain bundle. *Journal of Neuroscience, 6*, 919-929.

Bielajew, C., Konkle, A. T., Fouriezos, G., Boucher-Thrasher, A., & Schindler, D. (2001). The substrate for brain-stimulation reward in the lateral preoptic area: III. Connections to the lateral hypothalamic area. *Behav Neurosci, 115*(4), 900-909.

Bishop, M., Elder, S., & Heath, R. (1963). Intra-cranial self-stimulation in man. *Science, 140*, 394-396.

Bishop, M. P., Elder, T. M., & Heath, R. G. (1964). Attempted control of operant behavior in man with intracranial self-stimulation. In R. G. Heath (Ed.), *The Role of Pleasure in Behavior* (pp. 55-81). New York: Harper and Row.

Bittencourt, J. C., & Sawchenko, P. E. (2000). Do centrally administered neuropeptides access cognate receptors?: an analysis in the central corticotropin-releasing factor system. *J Neurosci, 20*(3), 1142-1156.

Blundell, J. E., & Herberg, L. J. (1968). Relative effects of nutritional deficit and deprivation period on rate of electrical self-stimulation of lateral hypothalamus. *Nature, 219*, 627-628.

Boyd, E., & Gardiner, L. (1962). Positive and negative reinforcement from intracranial stimulation of a teleost. *Science, 136*, 648-649.

Brady, L. S., Smith, M. A., Gold, P. W., & Herkenham, M. (1990). Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology, 52*(5), 441-447.

Britt, M., & Wise, R. (1981). Kinic acid spars fibers of the dorsal noradrenergic bundle. *Brain Res. Bull., 7*, 437-440.

Britton, D. R., Koob, G. F., Rivier, J., & Vale, W. (1982). Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. *Life Sci, 31*(4), 363-367.

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

Brugman, S., Clegg, D. J., Woods, S. C., & Seeley, R. J. (2002). Combined blockade of both micro - and kappa-opioid receptors prevents the acute orexigenic

action of Agouti-related protein. *Endocrinology, 143*(11), 4265-4270.

Burton, M. J., Rolls, E. T., & Mora, F. (1976). Effects of hunger on the responses of neurons in the lateral hypothalamus to the sight and taste of food. *Experimental Neurology, 51*, 668-677.

Cabeza de Vaca, S., Holiman, S., & Carr, K. (1998). A search for the metabolic signals that sensitize lateral hypothalamic self-stimulation in food restricted rats. *Physiol Behav, 64*(3), 251-260.

Campfield, L., Smith, F., Guisez, Y., Devos, R., & Burn, P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science, 269*(5223), 546-549.

Carr, K. D., & Simon, E. J. (1984). Potentiation of reward by hunger is opioid mediated. *Brain Research, 297*, 369-373.

Carr, K. D., & Wolinsky, T. D. (1993). Chronic food restriction and weight loss produce opioid facilitation of perifornical hypothalamic self-stimulation. *Brain Research, 607*, 141--148.

Carr, K. D., & Papadouka, V. (1994). The role of multiple opioid receptors in the potentiation of reward by food restriction. *Brain Res, 639*(2), 253-260.

Carr, K. D. (1996). Feeding, drug abuse, and the sensitization of reward by metabolic need. *Neurochem Res, 21*(11), 1455-1467.

Carr, K., Park, T., Zhang, Y., & Stone, E. (1998). Neuroanatomical patterns of Fos-like immunoreactivity induced by naltrexone in food-restricted and ad libitum fed rats. *Brain Res., 779*(1-2), 26-32.

Carr, K. D., Kim, G., & Cabeza de Vaca, S. (2000). Hypoinsulinemia may mediate the lowering of self-stimulation thresholds by food restriction and streptozotocin-induced diabetes. *Brain Res, 863*(1-2), 160-168.

Carruba, M., Nisoli, E., Garosi, V., Pizzi, M., Memo, M., & Spano, P. (1991). Characterization of monoaminergic receptors in hypothalamic nuclei mediating feeding behavior in rats. *J Pharmacol Exp Ther, 259*(1), 124-130.

Chen, G., Koyama, K., Yuan, X., Lee, Y., Zhou, Y., O'Doherty, R., et al. (1996). Disappearance of body fat in normal rats induced by adenovirus-mediated leptin gene therapy. *Proc Natl Acad Sci, 93*(25), 14795-14799.

Cheung, C. C., Clifton, D. K., & Steiner, R. A. (1997). Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology, 138*(10),

4489-4492.

Chronwall, B., Dimaggio, D., Massari, V., Pickel, V., Ruggerio, D., & O'Donahue, T. (1985). The anatomy of neuropeptide-Y-containing neurons in rat brain. *Neuroscience, 15*, 1159-1181.

Chua, S. C., Jr., Chung, W. K., Wu-Peng, X. S., Zhang, Y., Liu, S. M., Tartaglia, L., et al. (1996). Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science, 271*(5251), 994-996.

Clark, J., Kalra, P., Crowley, W., & Kalra, S. (1984). Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. *Endocrinology, 115*, 427-429.

Clegg, D. J., Air, E. L., Woods, S. C., & Seeley, R. J. (2002). Eating elicited by orexin-a, but not melanin-concentrating hormone, is opioid mediated. *Endocrinology, 143*(8), 2995-3000.

Conover, K. L., & Shizgal, P. (1994). Competition and summation between rewarding effects of sucrose and lateral hypothalamic stimulation in the rat. *Behavioral Neuroscience, 108*(3), 537--548.

Conover, K. L., Woodside, B., & Shizgal, P. (1994). Effects of sodium depletion on competition and summation between rewarding effects of salt and lateral hypothalamic stimulation in the rat. *Behavioral Neuroscience, 108*(3), 549--558.

Conover, K. L., & Shizgal, P. (1994). Differential effects of postingestive feedback on the reward value of sucrose and lateral hypothalamic stimulation in the rat. *Behavioral Neuroscience, 108*(3), 559--572.

Coons, E., & Cruce, J. (1968). Lateral hypothalamus: food current intensity and maintaining self-stimulation of hunger. *Science, 159*, 1117-1119.

Currie, P., & Coscina, D. (1995). Dissociated feeding and hypothermic effects of neuropeptide Y in paraventricular anf perifornical hypothalamus. *Peptides, 16*(4), 599-604.

Cusin, I., Rohner-Jeanrenaud, F., Stricker-Kongrad, A., & Jeanrenaud, B. (1996). The weight reducing effect of an intracerebroventricular injection of leptin in genetically obese fa/fa rats. Reduced sensitivity compared with lean animals. *Diabetes, 45*(10), 1446-1450.

Delgado, J., & Anand, B. (1953). Increase of food intake induced by electrical stimulation of hte lateral hypothalamus. *J. Comp. Physiol. Psychol., 172*, 162-

168.

Deutsch, J., & DiCiara, L. (1967). Hunger and extiction in intracranial self-stimulation. *J. Comp. Physiol. Psychol., 63*, 344-347.

Dunnett, S., Lane, D., & Winn, P. (1985). Ibotenic acid lesions of the lateral hypothalamus: comparison with 6-hydroxydopamine-induced sensori-motor deficits. *Neuroscience, 14*, 509-518.

Eckert, B., & Lewis, M. (1967). Competition between drives for intracranial stimulation and sodium chloride by adrenalectomized NaCl-deprived rats. *Journal of Comparative and Physiological Psychology, 64*, 349-352.

Edmonds, D. E., & Gallistel, C. R. (1974). Parametric analysis of brain stimulation reward in the rat: III. Effect of performance variables on the reward summation function. *Journal of Comparative and Physiological Psychology, 87*, 876-883.

Eipper, B., & Mains, R. (1980). Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocr Rev, 1*(1), 1-27.

Elias, C. F., Saper, C. B., Maratos-Flier, E., Tritos, N. A., Lee, C., Kelly, J., et al. (1998). Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *J Comp Neurol, 402*(4), 442-459.

Elias, C. F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R. S., Bjorbaek, C., et al. (1999). Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron, 23*(4), 775-786.

Elias, C. F., Kelly, J. F., Lee, C. E., Ahima, R. S., Drucker, D. J., Saper, C. B., et al. (2000). Chemical characterization of leptin-activated neurons in the rat brain. *J Comp Neurol, 423*(2), 261-281.

Elmquist, J. K., Bjorbaek, C., Ahima, R. S., Flier, J. S., Saper, C. B. (1998). Distribution of leptin receptor mRNA isoforms in the rat brain. *J Comp Neurol, 395*, 535-546.

Elmquist, J., Elias, CF., Saper, CB. (1999). From lesions to leptin: Hypothalamic control of food intake and body weight. *Neuron, 22*, 221-232.

Epstein, A., & Teitelbaum, P. (1967). Specific loss of the hypoglycemic control of feeding in recovered lateral rats. *Am. J. Physiol., 213*, 1159-1167.

Epstein, A. (1971). The lateral hypothalamic syndrome: It's implications for the physiological psychology of hunger and thirst. In E. Stellar & J. Sprague (Eds.), *Progress in Phsiological Psychology* (Vol. 4). New york: Academic

Press.

Erickson, J., Hollopeter, G., Palmiter, RD. (1996). Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science, 274*(5293), 1704-1707.

Erickson, J. C., Clegg, K. E., & Palmiter, R. D. (1996). Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature, 381*(6581), 415-421.

Fan, W., Boston, BA., Kesterson, RA., Hruby, VJ., Cone, RD. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature, 385*(6612), 165-168.

Fray, P., Koob, G., & Iversen, S. (1982). Tail-pinch-elicited behavior in rats: preference, plasticity, and learning. *Behav. Neurol. Biol., 36*(2), 108-125.

Friedman, J. M. (1997). Tha alphabet of weight control. *Nature, 385*, 119-120.

Frutiger, S., & Drinkwine, P. (1992). Effect of glucoprivation on self-stimulation rate-frequency functions. *Physiol Behav, 52*(2), 313-319.

Fulton, S., Woodside, B., & Shizgal, P. (2000). Modulation of brain reward circuitry by leptin [published erratum appears in Science 2000 Mar 17;287(5460):1931]. *Science, 287*(5450), 125-128.

Fulton, S. E., Richard, D., Woodside, B., Shizgal, S.E. (2002). Interaction of CRH and energy balance in the modulation of brain stimulation reward. *Society for the Study of Ingestive Behavior.*

Fulton, S., Woodside, B., Shizgal, P. (2002). Does Neuropeptide Y Contribute to the Modulation of Brain Stimulation Reward by Chronic Food Restriction? *Behavioral Brain Research, In Press.*

Fulton, S., Woodside, B., Shizgal, P. (2002). *Restriction-sensitivity of brain stimulation reward: Individual or electrode placement differences?* Paper presented at the Society for Neuroscience, Orlando, Florida.

Gallistel, C. R. (1978). Self-stimulation in the rat: Quantitative characteristics of the reward pathway. *Journal of Comparative and Physiological Psychology, 92*, 977-998.

Gallistel, C. R., Shizgal, P., & Yeomans, J. S. (1981). A portrait of the substrate for self-stimulation. *Psychological Review, 88*, 228-273.

Gallistel, C. R., & Freyd, G. (1987). Quantitative determination of the effects of catecholaminergic agonists and antagonists on the rewarding efficacy of brain stimulation. *Pharmacol. Biochem. Behav., 26*(4), 731-741.

Gallistel, C. R., Leon, M., Lim, B. T., Sim, J. C., & Waraczynski, M. (1996). Destruction of the medial forebrain bundle caudal to the site of stimulation reduces rewarding efficacy but destruction rostrally does not. *Behav Neurosci, 110*(4), 766-790.

Gerald, C., Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, Smith KE, Vaysse P, Durkin MM, Laz TM, Linemeyer DL, Schaffhauser AO, Whitebread S, Hofbauer KG, Taber RI, Branchek TA, Weinshank RL. (1996). A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature, 382*(6587), 168-171.

Glass, M. J., O'Hare, E., Cleary, J. P., Billington, C. J., & Levine, A. S. (1999). The effect of naloxone on food-motivated behavior in the obese Zucker rat. *Psychopharmacology (Berl), 141*(4), 378-384.

Gosnell, B., Morley, J., & Levine, A. (1986). Opioid-induced feeding: Localization of sensitive brain sites. *Brain Res., 369*, 177-184.

Grandison, L., & Guidotti, A. (1977). Stimulation of food intake by muscimol and beta endorphin. *Neuropharmacology, 16*(7-8), 533-536.

Gray, T., O'Donahue, T., & Magnuson, D. (1986). Neuropeptide Y innervation of amygdaloid and hypothalamic neurons that project to the dorsal vagal complex in rat. *Peptides, 7*, 341-349.

Green, L., & Rachlin, H. (1991). Economic substitutability of electrical brain stimulation, food, and water. *Journal of the Experimental Analysis of Behavior, 55*, 133-143.

Grigoriadis, D. E., Lovenberg, T. W., Chalmers, D. T., Liaw, C., & De Souze, E. B. (1996). Characterization of corticotropin-releasing factor receptor subtypes. *Ann N Y Acad Sci, 780*, 60-80.

Grill, H. J., Schwartz, M. W., Kaplan, J. M., Foxhall, J. S., Breininger, J., & Baskin, D. G. (2002). Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology, 143*(1), 239-246.

Hagan, M. M., Rushing, P. A., Schwartz, M. W., Yagaloff, K. A., Burn, P., Woods, S. C., et al. (1999). Role of the CNS melanocortin system in the response to overfeeding. *J Neurosci, 19*(6), 2362-2367.

Hagan, M. M., Rushing, P. A., Pritchard, L. M., Schwartz, M. W., Strack, A. M., Van Der Ploeg, L. H., et al. (2000). Long-term orexigenic effects of AgRP-(83---132) involve mechanisms other than melanocortin receptor blockade. *Am J Physiol Regul Integr Comp Physiol, 279*(1), R47-52.

Hagan, M. M., Benoit, S. C., Rushing, P. A., Pritchard, L. M., Woods, S. C., & Seeley, R. J. (2001). Immediate and prolonged patterns of Agouti-related peptide-(83--132)-induced c-Fos activation in hypothalamic and extrahypothalamic sites. *Endocrinology, 142*(3), 1050-1056.

Hagan, M. M., Rushing, P. A., Benoit, S. C., Woods, S. C., & Seeley, R. J. (2001). Opioid receptor involvement in the effect of AgRP- (83-132) on food intake and food selection. *Am J Physiol Regul Integr Comp Physiol, 280*(3), R814-821.

Hakansson, M., Brown, H., Ghilardi, N., Skoda, R., & Meister, B. (1998). Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *Neuroscience, 18*(1), 559-572.

Hakansson, M., de Lecea, L., Sutcliffe, J. G., Yanagisawa, M., & Meister, B. (1999). Leptin receptor- and STAT3-immunoreactivities in hypocretin/orexin neurones of the lateral hypothalamus. *J Neuroendocrinol, 11*(8), 653-663.

Halaas, J., Gajiwala, K., Maffei, M., Cohen, S., Chalt, B., Rabinowitz, D., et al. (1995). Weight reducing effects of the plasma protein encoded by the obese gene. *Science, 269*, 543-546.

Halaas, J., & al., e. (1997). Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proceedings of the Natioanl Academy of Science USA, 94*, 8878-8883.

Havel, P. J. (2001). Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis. *Exp Biol Med (Maywood), 226*(11), 963-977.

Heath, R. G. (1964). Pleasure response of human subjects to direct stimulation of the brain: Physiologic and psychodynamic considerations. In R. G. Heath (Ed.), *The Role of Pleasure in Behavior* (pp. 219-243). New York: Harper and Row.

Heiman, M. L., Ahima, R. S., Craft, L. S., Schoner, B., Stephens, T. W., & Flier, J. S. (1997). Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. *Endocrinology, 138*(9), 3859-3863.

Heinrichs, S. C., & Richard, D. (1999). The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. *Neuropeptides, 33*(5), 350-

359.

Herberg, L. J., & Blundell, J. E. (1967). Lateral hypothalamus: hoarding behavior elicited by electrical stimulation. *Science, 155*(760), 349-350.

Hernandez, L., & Hoebel, B. G. (1989). Food intake and lateral hypothalamic self-stimulation covary after medial hypothalamic lesions or ventral midbrain 6-hydroxydopamine injections that cause obesity. *Behavioral neuroscience., 103(2)*, 412-422.

Heymsfield, S. B., Greenberg AS, Fujioka K, Dixon RM, Kushner R, Hunt T, Lubina JA, Patane J, Self B, Hunt P, McCamish M. (1999). Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA, 282*(16), 1568-1575.

Himmi, T., Boyer, A., & Orsini, J. (1988). Changes in lateral hypothalamic neuronal activity accompanying hyper- and hypoglycemias. *Physiol. Behav., 44*, 347-354.

Hodos, W., & Valenstein, E. S. (1962). An evaluation of response rate as a measure of rewarding intracranial stimulation. *Journal of Comparative and Physiological Psychology, 55*, 80-84.

Hoebel, B. G. (1968). Inhibition and disinhibition of self-stimulation and feeding: hypothalamic contol and postingestional factors. *Journal of Comparative and Physiological Psychology, 66*, 89-100.

Hoebel, B. G., & Thompson, R. D. (1969). Aversion to lateral hypothalamic stimulation caused by intragastric feeding or obesity. *Journal of Comparative and Physiological Psychology, 68*, 536-543.

Hoebel, B. G. (1969). Feeding and self-stimulation. *Annals of the New York Academy of Sciences, 157*, 758-778.

Hokfelt, T., Fuxe, K., Goldstein, M., & Johannsen, O. (1974). Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain. *Brain Res., 66*, 235-251.

Horvath, T., Diano, S., van den Pol, AN. (1999). Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: A novel circuit implicated in metabolic and endocrine function. *J Neurosci, 19*(3), 1072-1087.

Huang, Y., & Mogenson, G. (1972). Neural pathways maintaining drinking and feeding in rats. *Exp. Neurol., 37*, 269-286.

Huang, X., Lin, S., & Zhang, R. (1997). Upregulation of leptin receptor mRNA expression in obese mouse brain. *Neuroreport, 8*(4), 1035-1038.

Huang, Q., Rivest, R., & Richard, D. (1998). Effects of leptin on corticotropin-releasing factor (CRF) synthesis and CRF neuron activation in the paraventricular hypothalamic nucleus of obese (ob/ob) mice. *Endocrinology, 139*(4), 1524-1532.

Huszar, D., Lynch, CA., Fairchild-Huntress, V., Dunmore, JH., Fanq, Q., Berkemeier, LR., Gu, W., Kesterson, RA., Boston, BA., Cone, RD., Smith, FJ., Campfield, LA., Burn, P., Lee, F. (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell, 88*(1), 131-141.

Iida, M., Murakami, T., Ishida, K., Mizuno, A., Kuwajima, M., & Shima, K. (1996). Substitution at codon 269 (glutamine --> proline) of the leptin receptor (OB-R) cDNA is the only mutation found in the Zucker fatty (fa/fa) rat. *Biochem Biophys Res Commun, 224*(2), 597-604.

Iversen, I. (1993). Techniques for establishing schedules with wheel running as reinforcement in rats. *J Exp Anal Behav, 60*(1), 219-238.

Jacobpwitz, D., & Palkovits, M. (1974). Topographic atlas of catecholamine and acetylcholinesterase-containing neurons in the rat brain. *J. Comp. Neurol., 157*, 13-28.

Jequier, E., Tappy L. (1999). Regulation of body weight in humans. *Physiol Rev, 79*(2), 451-480.

Jewett, D. C., Cleary, J., Levine, A. S., Schaal, D. W., & Thompson, T. (1995). Effects of neuropeptide Y, insulin, 2-deoxyglucose, and food deprivation on food-motivated behavior. *Psychopharmacology (Berl), 120*(3), 267-271.

Keesey, R., Mitchell, J., & Kemnitz, J. (1979). Body weight and body composition of male rats following hypothalamic lesions. *Am J Physiol, 237*, R68-R73.

Kelley, A. E., Bakshi, V. P., Haber, S. N., Steininger, T. L., Will, M. J., & Zhang, M. (2002). Opioid modulation of taste hedonics within the ventral striatum. *Physiol Behav, 76*(3), 365-377.

Kennedy, G. (1953). The role of depot fat in the hypothalamic control of food intake in the rat. *Proc R Soc Lond B Biol Sci, 611*, 221-235.

Kokkotou, E. G., Tritos, N. A., Mastaitis, J. W., Slieker, L., & Maratos-Flier, E. (2001). Melanin-concentrating hormone receptor is a target of leptin action in

the mouse brain. *Endocrinology, 142*(2), 680-686.

Koob, G. F., & Heinrichs, S. C. (1999). A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res, 848*(1-2), 141-152.

Korbonits, M., Trainer, P., Little, J., Edwards, R., Kopelman, P., Besser, G., et al. (1997). Leptin levels do not change acutely with food administration in normal or obese subjects, but are negatively correlated with pituatary-adrenal activity. *Clin. Endocrinol., 46*(6), 751-757.

Kotz, Grace, M., Billington, C., & Levine, A. (1993). The effect of norbinaltophimine, beta-funaltrexamine and naltrindole on NPY-induced feeding. *Brain Res., 631*(2), 325-328.

Kotz, C., Grace, M., Briggs, J., Levine, A., & Billington, C. (1995). Effects of opioid antagonists naloxone and naltrexone on neuropeptide Y-induced feeding and brown fat thermogenesis in the rat. Neural site of action. *J Clin Invest, 96*(1), 163-170.

Kristensen, P., Judge, M. E., Thim, L., Ribel, U., Christjansen, K. N., Wulff, B. S., et al. (1998). *Hypothalamic CART is a new anorectic peptide regulated by leptin.*

Lambert, P., Wilding, J., al-Dokhayel, A., Gilbey, S., Ghatei, M., & Bloom, S. (1994). Naloxone-induced anorexia increases neuropeptide Y concentrations in hte dorsomedial hypothalamus: evidence for neuropeptide Y-opioid interactions in the control of food intake. *Peptides, 15*(4), 657-660.

Larsen, P. J., Hay-Schmidt, A., & Mikkelsen, J. D. (1994). Efferent connections from the lateral hypothalamic region and the lateral preoptic area to the hypothalamic paraventricular nucleus o rat. *J Comp Neurol, 342*, 299-319.

Larsen, P., Hay-Schmidt, A., & Mikkelson, J. (1994). Efferent connections from the lateral hypothalamus region of the lateral preoptic area to the pariventricular nucleus of the rat. *J. Comp. Neurol., 342*, 299-319.

LeFeuvre, R. A., Rothwell, N. J., & Stock, M. J. (1987). Activation of brown fat thermogenesis in response to central injection of corticotropin releasing hormone in the rat. *Neuropharmacology, 26*(8), 1217-1221.

Leibowitz, S. (1975). Catecholaminergic mechanisms of the lateral hypothalamus: Their rols in the meditation of amphetamine anorexia. *Brain Res., 98*, 529-545.

Leibowitz, S., & Rossakis, C. (1979). Pharmacological chracterization of perifornical hypothalamic dopamine receptors mediating feeding inhibition in the rat. *Brain Res., 172*, 115-130.

Levin, B. E., Dunn-Meynell, A. A., & Routh, V. H. (1999). Brain glucose sensing and body energy homeostasis: role in obesity and diabetes. *Am J Physiol, 276*(5 Pt 2), R1223-1231.

Levine, A. S., & Morley, J. E. (1984). Neuropeptide Y: a potent inducer of consummatory behavior in rats. *Peptides, 5*(6), 1025-1029.

Lin, L., Truett, G.E., Levens, N., York, D.A. (1996). Central but not peripheral administration of leptin reduced food intake and body weight in Zucker fatty (fa/fa) and lean (Fa/?) rats. *Obesity Research, 4(Suppl. 10)*, 2s.

Loftus, T. M., Jaworsky, D. E., Frehywot, G. L., Townsend, C. A., Ronnett, G. V., Lane, M. D., et al. (2000). Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors. *Science, 288*(5475), 2379-2381.

Lopez, M., Seoane, L., Garcia, M. C., Lago, F., Casanueva, F. F., Senaris, R., et al. (2000). Leptin regulation of prepro-orexin and orexin receptor mRNA levels in the hypothalamus. *Biochem Biophys Res Commun, 269*(1), 41-45.

Louis-Sylvestre, J., & Le Magnen, J. (1980). A fall in blood glucose level precedes meal onset in free-feeding rats. *Neurosci. Biobehav. Res., 4*, 13-16.

Macey, D. J., Koob, G. F., & Markou, A. (2000). CRF and urocortin decreased brain stimulation reward in the rat: reversal by a CRF receptor antagonist. *Brain Res, 866*(1-2), 82-91.

MacLeod, P. (1971). Structure and function of higher olfactory centers. In L. Beidler (Ed.), *Handbook of Sensory Physiology* (Vol. IV, pp. 182-204). Berlin: Springer Verlag.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R., Lee, G., Zhang, Y., et al. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight reduced subjects. *Nat Med, 1*(11), 1155-1161.

Margules, D., & Olds, J. (1962). Identical "feeding" and "rewarding" systems in the lateral hypothalamus of rats. *Science, 135*, 374-375.

Marshall, J., & Teitelbaum, P. (1974). Further analysis of sensory inattention following lateral hypothalamic damage in rats. *Comp. Physiol. Psychol., 87*, 375-395.

McCutcheon, N. B., Rosellini, R. A., & Bandel, S. (1991). Controllability of stressors and rewarding brain stimulation: effect on the rate-intensity function. *Physiol Behav, 50*(1), 161-166.

Menendez, J. A., McGregor, I. S., Healey, P. A., Atrens, D. M., & Leibowitz, S. F. (1990). Metabolic effects of neuropeptide Y injections into the paraventricular nucleus of the hypothalamus. *Brain Res, 516*(1), 8-14.

Merali, Z., McIntosh, J., Kent, P., Michaud, D., & Anisman, H. (1998). Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. *J Neurosci, 18*(12), 4758-4766.

Mercer, J. G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T., Morgan, P. J., Trayhurn, P. (1996). Localization of leptin receptor mRNA and the long splice variant (O-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett, 387*, 113-116.

Mercer, J. G., Moar, K. M., & Hoggard, N. (1998). Localization of leptin receptor (Ob-R) messenger ribonucleic acid in the rodent hindbrain. *Endocrinology, 139*(1), 29-34.

Milliaresis, E., Rompre, P., Laviolette, P., Philippe, L., & Coulombe, D. (1986). The curve-shift paradigm in self-stimulation. *Physiol. Behav., 37*, 85-91.

Mizuno, T. M., Kleopoulos SP, Bergen HT, Roberts JL, Priest CA, Mobbs CV. (1998). Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes, 47*(2), 294-297.

Moon, B. C., & Friedman, J. M. (1997). The molecular basis of the obese mutation in ob2j mice. *Genomics, 42*, 152-156.

Morley, J. E., & Levine, A. S. (1982). Corticotrophin releasing factor, grooming and ingestive behavior. *Life Sci, 31*(14), 1459-1464.

Murray, B., & Shizgal, P. (1996) Behavioral measures of conduction velocity and refractory period for reward-relevant axons in the anterior LH and VTA. *Physiol Behav 59*, 643-652

Murzi, E., Hernandez, L., & Baptista, T. (1986). Lateral hypothalamic sites eliciting eating affect medullary taste neurons in rats. *Physiol. Behav., 36*(5), 829-834.

Myer, R., & Martin, G. (1973). 6-OHDA lesions of the hypothalamus: Interaction of aphagia, food palatability, set point for weight regulation, and recovery of feeding. *Pharmacol. Biochem. Behav., 1*, 329-345.

Mystkowski, P., Seeley, R. J., Hahn, T. M., Baskin, D. G., Havel, P. J., Matsumoto, A. M., et al. (2000). Hypothalamic melanin-concentrating hormone and

estrogen-induced weight loss. *J Neurosci, 20*(22), 8637-8642.

Nadler, J. (1979). Kainic acid: Neurophysiological and neurotoxic actions. *Life sci., 24*, 289-299.

Nieuwenhuys, R., Geeraedts, L. M., & Veening, J. G. (1982). The medial forebrain bundle of the rat. I. General introduction. *J Comp Neurol, 206*(1), 49-81.

Nishino, H., Ono, T., Sasaki, K., Fukuda, M., & Muramoto, K.-I. (1984). Caudate unit activity during operant feeding behavior in monkeys and modulation by cooling prefrontal cortex. *Behavioural brain research., 11*, 21-33.

Novin, D., Rogers, R., & Hermann, G. (1981). Visceral afferent and efferent connections of the brain. *Diabetologia, 20*, 331-336.

Olds, J., & Milner, P. M. (1954). Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *Journal of Comparative and Physiological Psychology, 47*, 419-427.

Olds, J. (1958). Self-stimulation of the brain. *Acta. Physiol. Scand., 127*(3294), 315-324.

Olds, M. E., & Olds, J. (1962). Approach-escape interactions in rat brain. *American Journal of Physiology, 203(5)*, 803-810.

Oomura, Y. (1983). Glucose as a regulator of neuronal activity. In A. J. Szabo (Ed.), *Advances in Metabolic Disorders* (pp. 31-65). New York: Academic.

Paxinos, G., & Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates* (Fourth Edition ed.). New York: Academic Press.

Pellymounter, M. A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., & Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science, 269*, 540-543.

Piazza, P. V., Deroche-Gamonent, V., Rouge-Pont, F., & Le Moal, M. (2000). Vertical shifts in self-administration dose-response functions predict a drug-vulnerable phenotype predisposed to addiction. *J Neurosci, 20*(11), 4226-4232.

Pierroz, D. D., Gruaz, N. M., d'Alieves, V., & Aubert, M. L. (1995). Chronic administration of neuropeptide Y into the lateral ventricle starting at 30 days of life delays sexual maturation in the female rat. *Neuroendocrinology, 61*(3), 293-300.

Qu, D., Ludwig, D., Gammeltoft, S., Piper, M., Pelleymounter, M., Cullen, M., et al.

(1996). A role for melanin-concentrating hormone in the central regulation of feeding behavior. *Nature, 380*(6571), 243-247.

Ricardo, J. A., & Tongju Koh, E. (1978). Anatomical Evidence of Direct Projections from the Nucleus of the Solitary Tract to the Hypothalamus, Amygdala, and other Forebrain Structures in the Rat. *Brain Research, 153*, 1-26.

Richard, D., Huang, Q., & Timofeeva, E. (2000). The corticotropin-releasing hormone system in the regulation of energy balance in obesity. *Int J Obes Relat Metab Disord, 24 Suppl 2*, S36-39.

Roberts, W., Steinberg, M., & Means, L. (1967). Hypothalamic mechanism for sexual, aggressive and other motivational behaviors in hte opossum didelphis virginiana. *J. Comp. Physiol. Psychol., 64*, 1-15.

Roberts, W. W. (1980). C-Deoxyglucose mapping of first-order projections activated by stimulation of lateral hypothalamic sites eliciting gnawing, eating and drinking in rats. *The Journal of Comparative Neurology, 194*, 617-638.

Rogers, R., Kita, H., Butcher, L., & Novin, D. (1980). Afferent projections to dorsal motor nucleus of the vagus. *Brain Res. Bull., 5*, 365-374.

Rohner-Jeanrenaud, F., Cusin, I., Sainsbury, A., Zakrewska, K., & Jeanrenaud, B. (1996). The loop between neuropeptide Y and leptin in normal and obese rodents. *Horm Metab Res, 28*, 642-648.

Rolls, E. T., Burton, M. J., & Mora, F. (1976). Hypothalamic neuronal responses associated with the sight of food. *Brain research., 111*, 53-66.

Rolls, E. T., Sanghera, M. K., & Roper-Hall, A. (1979). The latency of activation of neurones in the lateral hypothalamus and substantia innominata during feeding in the monkey. *Brain research., 164*, 121-135.

Rolls, E., Burton, M., & Mora, F. (1980). Neurophyiological analysis of brain-stimulation reward in the monkey. *Brain Res, 194*(2), 339-357.

Rompré, P. & Shizgal, P. (1986) Electrophysiological characteristics of neurons in forebrain regions implicated in self-stimulation of the medial forebrain bundle in the rat. *Brain Res, 364*(2), 338-49.

Rossi, R., & Stutz, R. (1978). The self-deprivation phenomenon: Competition between appetitive rewards and electrical brain stimulation of hte brain. *Physiol. Psychol., 6*, 204-208.

Rossi J, I. I. I., & Panksepp, J. (1992). Analysis of the relationships between self-

stimulation sniffing and brain-stimulation sniffing. *Physiol Behav, 51*, 805-813.

Routtenberg, A., & Lindy, J. (1965). Effects of the availability of rewarding septal and hypothalamic stimulation on bar pressing for food under conditions of deprivation. *Journal of Comparative and Physiological Psychology, 60*, 158-161.

Sahu, a., Kalra, P., & Kalra, S. (1988). Food deprivation and ingestion induce reciprocal changes in neurpeptide Y concentrations in the paraventricular nucleus. *Peptids, 9*(1), 83-86.

Sakurai, T., Akira, A., Ischii, M., Matsuzaki, I., Chemalli, R., Tanaka, H., et al. (1998). Orexin and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell, 92*, 573-585.

Saladin, R., De vos, P., Guerre-Milo, M., Leturque, A., Girard, J., Staels, B., et al. (1995). Transient increase in ob gene expression after food intake or insulin administration. *Nature, 377*(6549), 527-529.

Saper, C., Loewy, A., Swanson, L., & Cowan, W. (1976). Direct hypothalamo-autonomic connections. *Brain Res., 117*(2), 305-312.

Saper, C. B., Swanson, L. W., & Cowan, W. M. (1979). An autoradiographic study of the efferent connections of the lateral hypothalamic area in the rat. *Journal of Comparative Neurology, 183*, 689-706.

Saper, C. B., Chou, T. C., & Elmquist, J. K. (2002). The need to feed: homeostatic and hedonic control of eating. *Neuron, 36*(2), 199-211.

Sasaki, K., Ono, T., Muramoto, K., Nishino, H., & Fukada, M. (1984). The effects of rewarding brain stimulation on lateral hypothalamic unit activity in freely moving rats. *Brain Res, 322*(2), 201-211.

Satoh, N., Ogawa, Y., Katsuura, G., Hayase, M., Tsuji, T., Imagawa, K., et al. (1997). the arcuate nucleus as a primary site of satiety effect of leptin in rats. *Neurosci. Lett., 224*(3), 149-152.

Schwartz, M. W., Marks, J. L., Sipols, A. J., Baskin, D. G., Woods, S. C., Kahn, S. E., et al. (1991). Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. *Endocrinology, 128*(5), 2645-2647.

Schwartz, M., Seeley, R., Campfield, L., Burn, P., & Baskin, D. (1996). Identification

of targets of leptin action in rat hypothalamus. *Clin Invest, 98*, 1101-1106.

Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J., & Baskin, D. G. (2000). Central nervous system control of food intake. *Nature, 404*(6778), 661-671.

Seeley, R., Matson, C., Chuvez, M., Woods, S., Dallman, M., & Schwartz, M. (1996). Behavioral, endocrine, and hypothalamic responses to involuntary overfeeding. *Am J Physiol, 271*(3 pt 2), R819-R823.

Seeley, R. J., Yagaloff, K. A., Fisher, S. L., Burn, P., Thiele, T. E., van Dijk, G., et al. (1997). Melanocortin receptors in leptin effects. *Nature, 390*(6658), 349.

Shizgal, P., Bielajew, C., Corbett, D., Skelton, R., & Yeomans, J. (1980). Behavioral methods for inferring anatomical linkage between rewarding brain stimulation sites. *Journal of Comparative and Physiological Psychology, 94*, 227-237.

Shizgal, P., Schindler, D., & Rompre, P.-P. (1989). Forebrain neurons driven by rewarding stimulation of the medial forebrain bundle in the rat: comparison of psychophysical and electrophysiological estimates of refractory periods. *Brain Research, 499*, 234-248.

Shizgal, P. (1999). *On the neural computation of utility: implications from studies of brain stimulation reward.* New York: Russell Sage Foundation.

Shizgal, P., Fulton, S., & Woodside, B. (2001). Brain reward circuitry and the regulation of energy balance. *Int J Obes Relat Metab Disord, 25 Suppl 5*, S17-21.

Skofitsch, G., Jacobowitz, D., & Zamir, N. (1985). Immunohistochemical localization of a melanin concnetrating hormone-like peptide in the rat brain. *Brain Res Bull, 15*(6), 635-649.

Smith, F., Campfield, L., Moschera, J., & Bailon, P. (1996). Feeding inhibition by neuropeptide Y. *Nature, 381*, 415-418.

Sokoloff, L., Reivich, C., Kennedy, M., Desrosiers, C., Patlak, O., Sakaruda, O., et al. (1977). The deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J Neurochem, 23*, 897-916.

Spanswick, D., Smith, M., Groppi, V., Logan, S., & Ashford, M. (1997). Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature, 390*(6659), 521-525.

Spiegelman, B. M., & Flier, J. S. (2001). Obesity and the regulation of energy

balance. *Cell, 104*(4), 531-543.

Spies, G. (1965). Food versus intracranial self-stimulation reinforcement in food deprivaed rats. *J. Comp. Physiol. Psychol., 60*, 153-157.

Stanley, B., & Leibowitz, S. (1984). Neuopeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. *Life Sci, 35*(26), 2635-2642.

Stanley, B., Lanthier, D., Chin, A., & Leibowitz, S. (1989). Suppression of neuropeptide Y-elicited eating by adrenalectomy or hypophysectomy: reversal with corticosterone. *Brain Res., 501*(1), 32-36.

Stanley, B., Ha, H., Spears, L., & Dee, M. (1993). Lateral hypothalamic injection of glutamate, kainic acid, D,L-alpha-amino-3-hydroxy-5-methyl-isoxazole propionic acid or N-methyl-D-aspartic acid rapidly elicit intense transient eating in rats. *Brain Res., 613*, 88-95.

Stanley, B., Magdalin, W., Seirafi, A., Thomas, W., & Leibowitz, S. (1993). The perifornical area: the major focus of (a) patchily distributed hypothalamic neuropeptide Y-sensitive feeding system(s). *Brain Res, 604*(1-2), 304-317.

Steffens, A. (1975). Influence of reversible obesity on eating behavior, blood glucose and insulin in the rat. *Am. J. Physiol., 228*, 1738-1744.

Stein, L. J., Dorsa, D. M., Baskin, D. G., Figlewicz, D. P., Porte, D., Jr., & Woods, S. C. (1987). Reduced effect of experimental peripheral hyperinsulinemia to elevate cerebrospinal fluid insulin concentrations of obese Zucker rats. *Endocrinology, 121*(5), 1611-1615.

Stenbaum, E., & Miller, N. (1965). Obesity from eating elicited by daily stimulation of the hypothalamus. *Am. J. Physiol., 208*, 1-5.

Stephens, T., Basinski, M., Bristow, P., Bue-Valleskey, J., Burgett, S., Craft, L., et al. (1995). The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature, 377*(6549), 530-532.

Stephens, T. W., Basinski, M., Bristow, P. K., Bue-Valleskey, J. M., Burgett, S. G., Craft, L., et al. (1995). The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature, 377*(6549), 530-532.

Tartaglia, L., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., et al. (1995). Identification and expression cloning of a leptin receptor, OB-R. *Cell, 83*, 1263-1271.

Teitelbaum, P., & Stellar, E. (1954). Recovery from the failure to eat produced by hypothalamic lesions. *Science, 120*, 894-895.

Teitelbaum, P., & Epstein, A. (1962). The lateral hypothalamic syndrome. *Psychol. Rev., 69*, 74-90.

Thomas, T., Burguera, B., Melton, L. J., 3rd, Atkinson, E. J., O'Fallon, W. M., Riggs, B. L., et al. (2000). Relationship of serum leptin levels with body composition and sex steroid and insulin levels in men and women. *Metabolism, 49*(10), 1278-1284.

Timofeeva, E., & Richard, D. (2001). Activation of the central nervous system in obese Zucker rats during food deprivation. *J Comp Neurol, 441*(1), 71-89.

Uehara, Y., Shimizu, H., Ohtani, K., Sato, N., & Mori, M. (1998). Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin. *Diabetes, 47*(6), 890-893.

Ungerstedt, U. (1970). Is interruption of the nigrostriatal dopamine system producing the "lateral hypothalamic syndrome"? *Acta. Physiol. Scand., 80*, 35A-36A.

Valenstein, E. S., & Valenstein, T. (1964). Interaction of positive and negative reinforcing neural systems. *Science, 145*, 1456-1458.

Valenstein, E., Cox, V., & Kakolewski, J. (1968). Modification of motivated behavior elicited by electrical stimulation of the hypothalamus. *Science, 159*, 1119-1121.

Valenstein, E., & Cox, V. (1970). Influence of hunger, thirst and previous experience in the test chamber on stimulus-bound eating and drinking. *J. Comp. Physiol. Psychol., 70*, 189-199.

Valentino, D. A., Dufresne, R. L., & Riccitelli, A. J. (1990). Effects of a single inescapable swim on long-term brain stimulation reward thresholds. *Physiol Behav, 48*(2), 215-219.

Vasselli, J. R., Cleary, M. P., Jen, K. L., & Greenwood, M. R. (1980). Development of food motivated behavior in free feeding and food restricted Zucker fatty (fa/fa) rats. *Physiol Behav, 25*(4), 565-573.

Veening, J., Swanson, L., Cowan, W., Nieuwenhuys, R., & Geeraedts, L. (1982). The medial forebrain bundle of the rat. II. An autoradiographic study of the topography of the major descending and ascending components. *J Comp Neurol, 206*(1), 82-108.

Veening, J. G., Swanson, L. W., Cowan, W. M., Nieuwenhuys, R., & Geeraedts, L.

M. (1982). The medial forebrain bundle of the rat. II. An autoradiographic study of the topography of the major descending and ascending components. *J Comp Neurol, 206*(1), 82-108.

Wang, Q., Bing, C., Al-Barazanji, K., Mossakowaska, D., Wang, X., McBay, D., et al. (1997). Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. *Diabetes, 46*(3), 335-341.

Waraczynski, M. (2003). Lidocaine inactivation demonstrates a stronger role for central versus medial extended amygdala in medial forebrain bundle self-stimulation. *Brain Res, 962*(1-2), 180-98.

Waraczynski, M., & Perkins, M. (2000). Temporary inactivation of the retrorubral fields decreases the rewarding effect of medial forebrain bundle stimulation. *Brain Res, 885*(2), 154-165.

Wildman, H. F., Chua, S., Jr., Leibel, R. L., & Smith, G. P. (2000). Effects of leptin and cholecystokinin in rats with a null mutation of the leptin receptor Lepr(fak). *Am J Physiol Regul Integr Comp Physiol, 278*(6), R1518-1523.

Wilson, B. D., Bagnol, D., Kaelin, C. B., Ollmann, M. M., Gantz, I., Watson, S. J., et al. (1999). Physiological and anatomical circuitry between Agouti-related protein and leptin signaling. *Endocrinology, 140*(5), 2387-2397.

Woods, S. C., & Seeley, R. J. (2000). Adiposity signals and the control of energy homeostasis. *Nutrition, 16*(10), 894-902.

Woods, S. C., Schwartz, M. W., Baskin, D. G., & Seeley, R. J. (2000). Food intake and the regulation of body weight. *Annu Rev Psychol, 51*(2), 255-277.

Woods, S. C., & Seeley, R. J. (2001). Insulin as an adiposity signal. *Int J Obes Relat Metab Disord, 25 Suppl 5*, S35-38.

Yeomans, J.S. (1975) Quantitative measurement of neural post-stimulation excitability with behavioral methods. *Physiol Behav 15,* 593-602.

Yeomans, J.S. (1979) Absolute refractory periods of self-stimulation neurons. *Physiol Behav 22*, 911-919.

Yeomans, J. S., Hood, S., Koziel, N., Doesburg, S., Fletcher, P., Steidl, S. (2001). Amphetamine and orexin-A in the pedunculopontine tegmental nucleus inhibit lateral hypothalamic brain stimulation reward in rats. *Scoiety for Neuroscience Abstracts*, 539.538.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. (1994). Positional cloning of the mouse obese gene and it's human homologue. *Nature, 372*, 425-432.

Zhang, F., Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, DiMarchi RD, Furman TC, Hale JE, Hsiung HM, Schoner BE, Smith DP, Zhang XY, Wery JP, Schevitz RW. (1997). Crystal structure of the obese protein leptin-E100. *Nature, 387*(6629), 206-209.

APPENDIX

The manuscript presented in this appendix is based on data presented for the Master's thesis. As presented here, the text and figures are a copy of an article published in *Science* (2000; 287(5450), 125-128), with one exception: Figure 2A is from the published erratum (Science 2000; 287(5460):1931).

Modulation of Brain Reward Circuitry by Leptin

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Abstract

Leptin, a hormone secreted by fat cells, suppresses food intake and promotes weight loss. To assess the action of this hormone on brain reward circuitry, changes in the rewarding effect of lateral hypothalamic stimulation were measured following leptin administration. At five stimulation sites near the fornix, the effectiveness of the rewarding electrical stimulation was enhanced by chronic food restriction and attenuated by intracerebroventricular infusion of leptin. In contrast, the rewarding effect of stimulating neighboring sites was insensitive to chronic food restriction and was enhanced by leptin in three of four cases. These opposing effects of leptin may mirror complementary changes in the rewarding effects of feeding and of competing behaviors.

Research on the regulation of feeding and energy balance has been galvanized by the sequencing of the *obese* (ob) gene and the expression of its protein product, leptin, a circulating hormone secreted by adipocytes *(1)*. Circulating leptin levels reflect the size of the fat mass *(2)* and thus, this hormone has been considered a signal that regulates long-term energy balance. Rodents with homozygous mutations in the *ob* gene (the *ob/ob* mouse) or in the gene for the leptin receptor (the *db/db* mouse or the *fa/fa* rat) manifest profound hyperphagia and obesity. Central or peripheral administration of leptin reverses the obesity syndrome found in *ob/ob* mice, stimulates metabolism, and reduces food intake in lean mice or rats *(3)*.

Among the many ways in which leptin could alter food intake is by reducing the appetitive value of food. Such changes could ensue if leptin were to alter the state of brain reward circuitry. Self-administration of rewarding electrical brain stimulation ("self-stimulation") has long been used to assess the state of this circuitry. Rats and a wide range of other vertebrates will actively seek out electrical stimulation of certain brain regions, including the lateral hypothalamus (LH) *(4)*. The effect that induces the subject to re-initiate the stimulation is called "brain stimulation reward" (BSR). Weight loss resulting from chronic food restriction has been shown to enhance the rewarding effect of stimulating LH sites close to the fornix *(5)*; this perifornical region has been implicated in the control of feeding and energy balance *(6)*. Thus, one might expect that the rewarding effect produced by stimulation of this region would be influenced by leptin. We tested this hypothesis by measuring leptin-induced changes in self-stimulation of the perifornical hypothalamus.

In the demonstrations by Carr and his co-workers that perifornical self-stimulation is modulated by chronic food restriction *(7)*, the rate at which the rats harvested the electrical rewards was measured as a function of the stimulation frequency. Chronic food restriction shifted the resulting rate-frequency function leftwards, towards weaker stimulation strengths; the lower the body weight, the weaker the stimulation required to entice the rats to earn a given number of rewards. We adopted an analogous approach to determine whether leptin modulates the rewarding effect of perifornical stimulation.

Male Long Evans rats bearing chronic stimulating electrodes and cerebro-ventricular cannulas *(8)* self-stimulated by pressing a lever that triggered a 1 sec train of rectangular, constant-current pulses, 0.1 msec in duration. The stimulation frequency was varied across trials over a range that drove the number of rewards earned from maximal to minimal levels *(9)* (see Fig. 1, A and B). The measure of the effectiveness of the rewarding stimulation was the frequency that produced a half-maximal rate of reward delivery ("M-50") *(10)*. Manipulations that potentiate BSR decrease the M-50 value.

Prior to leptin treatment, BSR data were obtained under the influence of chronic food restriction. Daily food intake was limited to 10 grams/day until body weight reached ~75% of the weight of age-matched controls. Rate-frequency curves collected during this period of restriction were compared to those obtained during a later stage of the experiment, when body weight had returned to normal levels following a period of free-feeding *(11)*.

In 5 subjects, chronic food restriction enhanced BSR. As illustrated in Figure 1A, rate-frequency curves obtained in these rats during food restriction lie to the left of the curves obtained following subsequent re-feeding, and the M-50 values (Fig. 1C) declined by 0.07 - 0.33 $\log_{10}$ units. In contrast, re-feeding following food restriction had little effect in the remaining 5 subjects. The rate-frequency curves obtained during restriction in these rats overlap the curves obtained during subsequent free-feeding (e.g., Fig. 1B), and the M-50 values remained relatively stable (Fig. 1D). Taken together, the results are consistent with previous reports that food restriction facilitates self-stimulation only at certain LH sites.

The effects of leptin on self-stimulation were examined at the end of the period of chronic food restriction, when body weight was ~75% of control values. One hour prior to the test sessions, 2 μg of recombinant murine leptin (Peprotech Inc., Roanoke, VA) dissolved in 1.6 μl of water was infused into the right lateral cerebral ventricle over a 2 min period. In a separate group of rats, this dose produced a reliable reduction in dark-cycle food intake over a period of 4 hours (*12*). In the 5 rats that had shown enhancement of BSR during chronic food restriction, leptin decreased the effectiveness of the rewarding stimulation (13). Whereas chronic food restriction produced leftward shifts in the rate-frequency curves obtained from these subjects, leptin produced rightward shifts (Fig. 2, A and C) (*14*). These leptin-induced rightward shifts persisted for as long as 4 days following a single infusion. Intriguingly, leptin had the opposite effect in 3 of 4 rats in which the rewarding effect of LH stimulation was unresponsive to food restriction. In these 3 rats, leptin

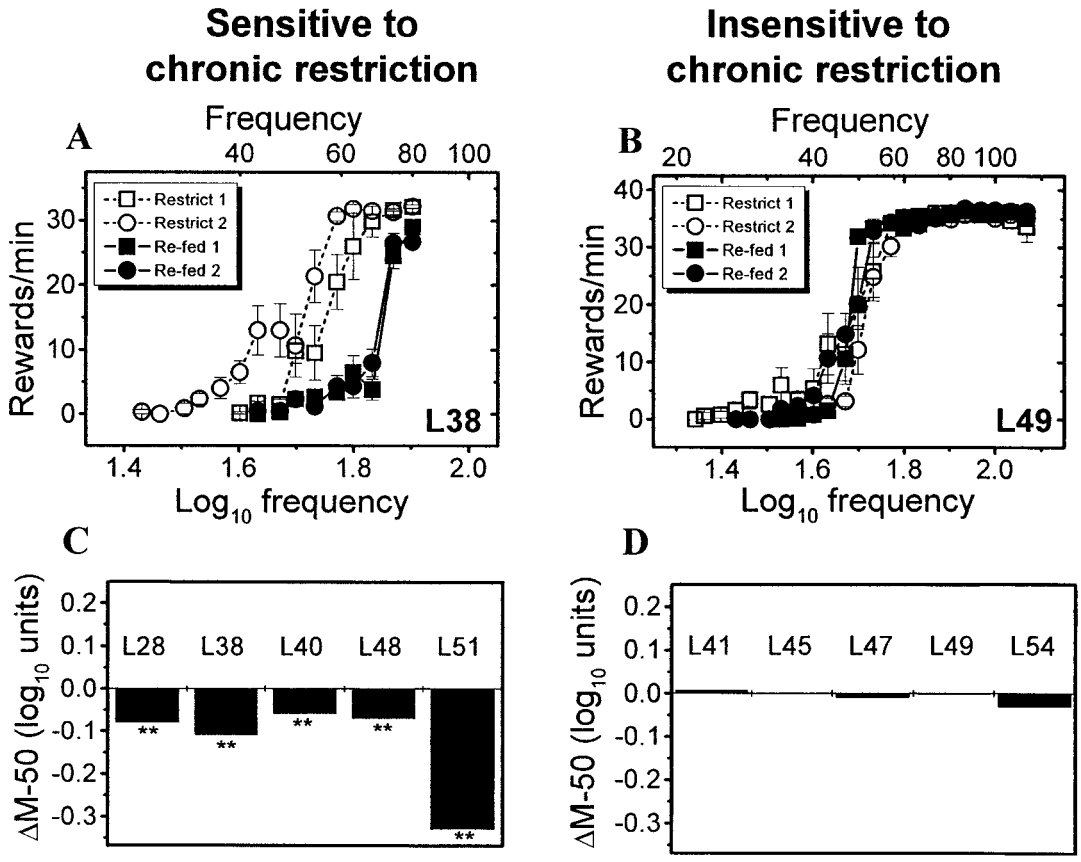

Figure 1. Effects of chronic food restriction on self-stimulation at LH sites where the rewarding effect of electrical stimulation is sensitive (left panels) or insensitive (right panels) to chronic food restriction. (**A**) Rate-frequency curves obtained with stimulation of a perifornical site are shifted leftwards during chronic food restriction (open symbols) with respect to curves obtained following subsequent re-feeding (filled symbols). (**B**) In contrast, stimulation of a neighboring site yields overlapping rate-frequency curves during chronic food restriction and following re-feeding. Each data point in panels A and B is an average of six measures collected on each test day. (**C, D**) Magnitude of the curve shifts produced by re-feeding following chronic food restriction in all subjects. M-50 represents the stimulation frequency required to induce the rat to earn half the maximal number of rewards available per trial. (** $p < 0.005$)

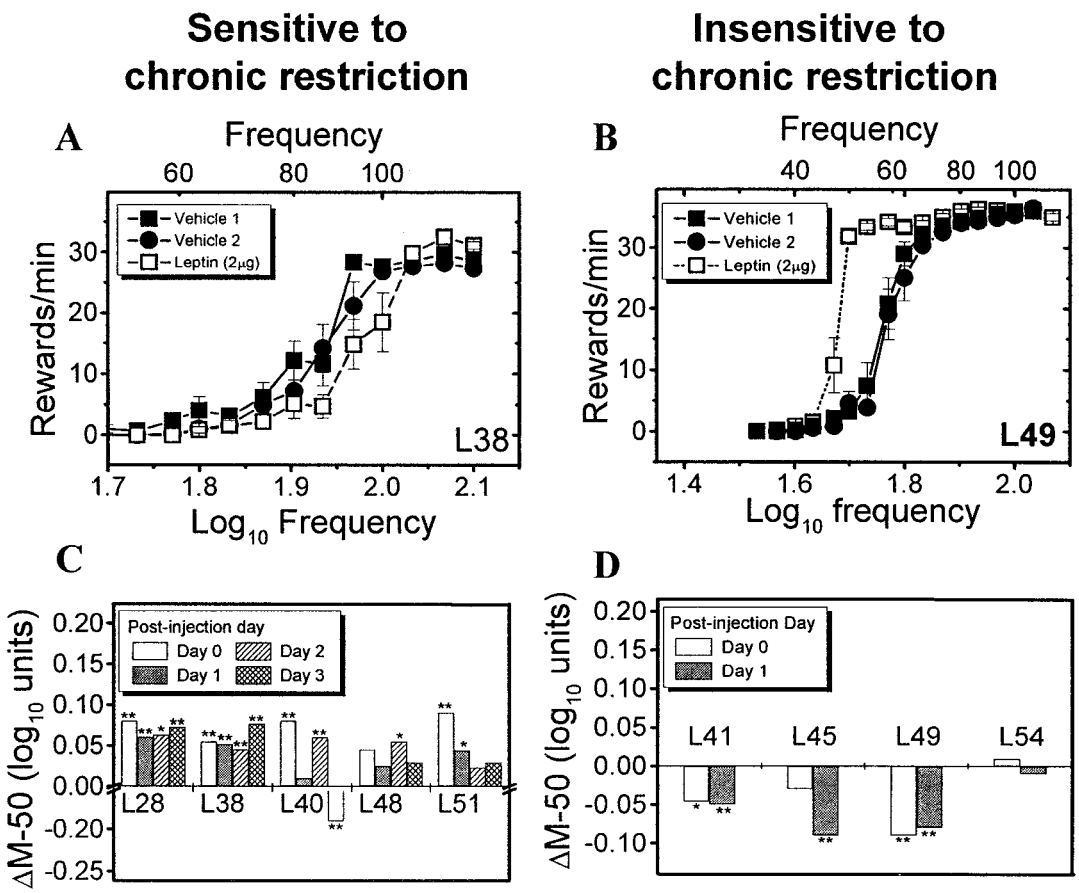

Figure 2. Opposite influence of ICV leptin on rewarding effects of LH stimulation. (**A**) At a site where chronic restriction enhanced the rewarding effect (Fig. 1A), leptin shifted the rate-frequency rightward (leptin: open symbols; vehicle control: filled symbols). (**B**) At a site where chronic restriction failed to enhance the rewarding effect (Fig. 1B), leptin shifted the rate-frequency leftward. (**C**) Magnitude of curve shifts (ΔM-50) during the 4 days following ICV leptin at 5 sites where the rewarding effect was enhanced by chronic food restriction. (Note the break in the y-axis between 0 and -0.155.) (**D**) Magnitude of curve shifts during the 2 days following ICV leptin at 4 sites where the rewarding effect was not altered by chronic food restriction. (* $p < 0.05$; ** $p < 0.005$)

increased the effectiveness of the rewarding stimulation: the rate-frequency curves were shifted leftwards (Fig. 2, B and D). In one rat in which BSR was unresponsive to chronic food restriction, the rate-frequency curves were not altered significantly by leptin administration; the effect of leptin could not be tested in the remaining restriction-insensitive subject (*15*).

Following completion of testing, the LH stimulation sites were marked by means of the Prussian Blue method *(16)*. Sites where BSR was enhanced by chronic food restriction and diminished by leptin were located dorsal or dorsolateral to the fornix (Fig. 3). The remaining sites were nearby but non-overlapping. Such a distribution is consistent with the notion that the rewarding effect of LH stimulation arises from activation of multiple, functionally different, subpopulations of inhomogeneously intertwined neurons *(17)*. In this view, small differences in the location of the electrode tip and the path of current flow could alter the relative weights of the subpopulations sampled by different electrodes. In the simplest account of the results reported here, one of the stimulated subpopulations consists of neurons that arise in, terminate in, or course through the perifornical hypothalamus; activation of these cells produces a rewarding effect that is enhanced by chronic restriction and attenuated by leptin.

The chronic character of the food-restriction regimen, which was in force long enough to produce substantial weight loss (~25%), was crucial to the enhancement of BSR. In contrast to the effects of the chronic regimen, acute food deprivation was ineffective in altering BSR, even when imposed for 48 hours (*18*). As shown in Figure

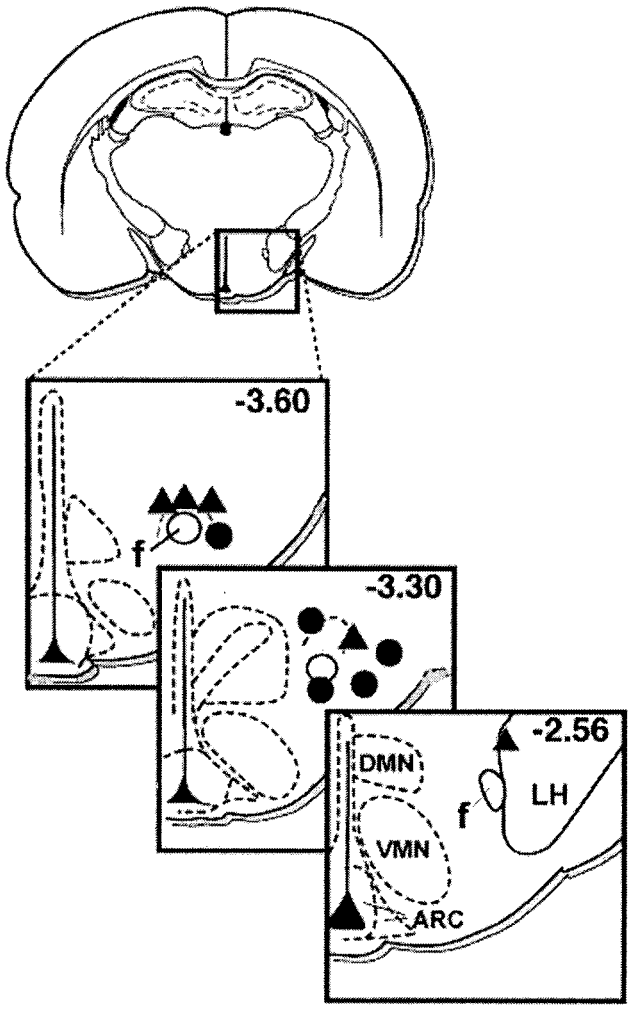

Figure 3. Location of the tips of the stimulation electrodes. Electrodes producing rewarding effects that were enhanced by chronic food restriction are designated by filled triangles (▲), and electrodes producing rewarding effects that were unaffected by chronic food restriction are designated by filled circles (●). The coronal sections are based on the atlas of G. Paxinos and C. Watson, (Academic Press, New York, Fourth Edition, 1998).

4, most rate-frequency curves obtained during acute deprivation overlap curves obtained during free feeding, even in the subjects in which BSR was enhanced by chronic food restriction (Fig. 1, A and C). Thus, the enhancement of BSR by food restriction appears to depend on signals that contribute to the regulation of long-term energy balance.

The notion that BSR is modulated by signals related to the long-term rather than the short-term regulation of energy balance is consistent with previous findings. For example, at LH sites where BSR is enhanced by chronic food restriction, the rewarding effect is not altered during acute glucopenia induced by 2-deoxy glucose (2-DG) or during acute lipoprivation induced by nicotinic acid *(19)*. It has also been shown that BSR is insensitive to acute accumulation of sucrose in the gut *(20)*.

The reduction in the effectiveness of the rewarding stimulation persisted for as long as 4 days after a single injection of leptin. The long duration of this effect is consistent with a report of body weight changes lasting up to 6 days after a single injection of leptin *(21)*.

Leptin attenuated BSR at restriction-sensitive sites but facilitated self-stimulation of 3 of the 4 sites where BSR was unresponsive to chronic food restriction. These opposite effects of leptin may reflect the comparative process believed to underlie behavioral allocation (*22*). In such views, the prevalence of a particular behavior, such as feeding, can be reduced either by decreasing the reward value it generates or by increasing the value of competing activities. If so, leptin could make complementary contributions to energy balance by reducing food reward while

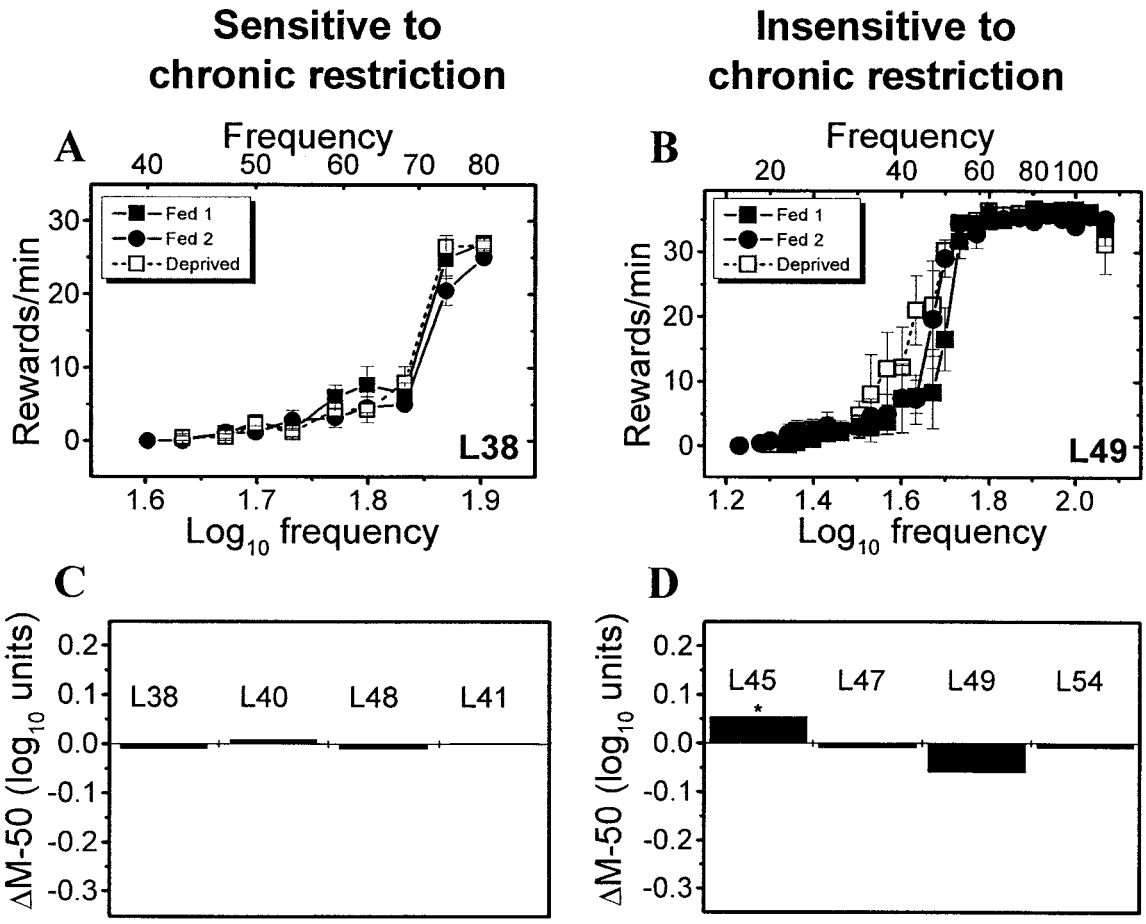

Figure 4. Failure of acute food deprivation to alter self-stimulation. Data from sites where the rewarding effect of electrical stimulation was enhanced or unchanged by chronic restriction are in the left and right panels, respectively. (**A, B**) Neither in the case of a site where BSR was enhanced by chronic restriction (Fig. 1A) nor in the case of a site where BSR was insensitive to chronic restriction (Fig. 1B) did rate-frequency curves obtained after 48 hours of food deprivation differ systematically from the free-feeding baseline. (**C, D**) Magnitude of the curve shifts (ΔM-50) produced by acute food deprivation (4 restriction-sensitive and 4 restriction-insensitive sites). In the one case in which a significant effect was observed (L45), deprivation produced a small rightward shift, suggesting that the rewarding effect was attenuated. (* $p < 0.05$)

enhancing the value of behaviors incompatible with feeding. At restriction-sensitive sites, neurons that link long-term changes in energy balance to the rewarding effect of food may be prominent in generating BSR, whereas at the remaining sites, BSR may arise primarily from the activation of neurons subserving behaviors incompatible with the ingestion of energy-rich substances.

The results reported here tie the actions of leptin to modulation of brain reward circuitry. A rich basis for linking these effects to specific populations of cells has been provided by recent progress in describing the receptors, neurotransmitters, and interconnections of hypothalamic neurons. For example, the perifornical area and other regions of the LH receive projections from leptin-sensitive cells that contain neuropeptides, such as neuropeptide Y, α-melanocyte-stimulating hormone, agouti-related protein and cocaine-amphetamine-regulated transcript, that are implicated in the control of feeding and energy balance (*23*). The perifornical LH includes neurons that express the long form of the leptin receptor *(24)*. Orexin or melanin-concentrating hormone *(25)*, neuropeptides that promote food intake and weight gain *(26)* have been found in LH neurons, and LH neurons containing corticotropin releasing hormone have been implicated in dehydration-induced anorexia (*27*). Working out the contribution of such cells to the rewarding effects of electrical brain stimulation and feeding could prove important to understanding energy balance. Conversely, progress in understanding the neural control of food intake and energy expenditure may well shed light on the structure and function of brain reward circuitry.

References and Notes

1. Y. Zhang et al., *Nature* **372**, 425 (1994).

2. M. Maffei et al., *Nat. Med.* **1**, 1155 (1995).

3. M. A. Pellymounter et al., *Science* **269**, 540 (1995); L. A. Campfield, F. Smith, Y. Guisez, R. Devos, P. Burn, *Science* **269**, 546 (1995); J. Halaas et al., *Science*4 **269**, 543 (1995); R. J. Seeley et al., *Horm. Metab. Res.* **28**, 664 (1996).

4. J. Olds and P. M. Milner, *J. Comp. Physiol. Psych.* **47**, 419 (1954); M. E. Olds and J. Olds, *J. Comp. Neurol.* **120**, 259 (1963).

5. J. E. Blundell and L. J. Herberg, *Nature* **219**, 627 (1969); K. D. Carr and T. Wolinsky, *Brain Res.* **607**, 141 (1993).

6. B. Stanley, W. Magdalin, A. Seirafi, W. Thomas, S. Leibowitz, *Brain Res.* **604**, 304 (1993); E. Gillard et al., *J. Neurosci.* **18**, 2646 (1998); S. Leibowitz, and C. Rossakis, *Brain Res.* **172**, 115 (1979); S. Leibowitz, *Brain Res.* **98**, 529 (1975).

7. G. Abrahamsen, Y. Berman, K. D. Carr, *Brain Res.* **695**, 186 (1995).

8. With skull landmarks bregma and lamda positioned on the same horizontal plane, bilateral, monopolar electrodes were aimed at the perifornical LH (3 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, 7.8 mm below the dura mater), and a 24- gauge stainless-steel guide cannula (Plastics One, Inc., Roanoke, VA) was aimed at the right lateral ventricle (0.4 mm posterior to bregma; 1.6 mm lateral to the midsagittal sinus; 4 mm below the dura mater). Following recovery from surgery and prior to leptin administration, cannula placement was verified by injecting 2 μg of angiotensin II and determining whether vigorous drinking began

within 5 min. For further details concerning electrodes and surgery, see A. Arvanitogiannis, L. Riscaldino, P. Shizgal, *Physiol. Behav.* **65**, 805 (1999).

9. The current was held constant throughout testing and ranged from 200-450 μA across subjects. The stimulation frequency was decreased from trial to trial by 0.033 $\log_{10}$ units. All testing was conducted towards the end of the dark phase of the light/dark cycle.

10. Broken-line functions, with a horizontal lower asymptote, a rising linear segment and a horizontal upper asymptote (C. R. Gallistel and G. Freyd, *Pharmacol. Biochem. Behav.* **26**, 731 (1987)) were fit to each of the six rate-frequency curves collected daily. The frequency required to maintain a half-maximal rate of reward delivery earned (M-50) was derived from each of the broken-line functions. An M-50 value was retained for further analysis when the broken-line function accounted for at least 75% of the variance of the rate-frequency data. Use of this goodness-of-fit criterion eliminated less than 3% of the data.

11. By means of within-subject, one-way analyses of variance, a set of 12 M-50 values from the food-restriction condition was compared to 12 values obtained following return to normal body weight, as determined by comparison to the body weights of age-matched control subjects. The leptin test intervened between the gathering of the M-50 values during food restriction and the onset of the period of re-feeding.

12. Data not shown.

13. A Dunnett test for multiple comparisons was employed to compare the 12 M-50 values from two vehicle treatment days to the 6 values obtained on each day

following leptin administration.

14. In a subsequent test of the restriction-sensitive placements under free-feeding conditions, leptin again produced significant increases ($p < .005$) in M-50 values in rats L38 and L51. Smaller increases seen on 2 of 4 days in rat L28 fell short of the statistical criterion, and little change was seen in the remaining two rats. (Data posted on *Science Online* www site.) It is not surprising that in several rats, the effect of a fixed amount of exogenous leptin was stronger during restriction than following re-feeding. Body weight overshot pre-restriction levels during re-feeding, and hence, circulating levels of leptin were likely much higher.
15. The cannula in rat L47 became clogged prior to leptin testing.
16. With the stimulating electrode serving as the anode, a 100 μA current was applied for 15 sec. Rats were then injected with a lethal dose of sodium pentobarbitol (100 mg/kg, IP) and perfused intracardially with phosphate-buffered saline followed by a mixture of 10% formalin (100 ml), trychloroacetic acid (0.5 g), potassium ferrocyanide (3 g) and potassium ferricyanide (3 g). Brains were removed and stored in 10% formalin. After a 24 hr immersion in a 20% sucrose-formalin solution, the brains were frozen, sliced on a cryostat into 30 μm coronal sections, and mounted on gelatin-coated slides (Fisher Scientific). Sections were stained for Nissl substance using formal thionin. The location of stimulation sites was identified with the aid of a stereotaxic atlas (G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York, Fourth Edition, 1998).
17. A. Arvanitogiannis, M. Waraczynski, P. Shizgal, *Physiol. Behav.* **59**, 795 (1996).

18. Acute-deprivation data for rat L28 did not meet the stability criterion, and rat L51 died prior to acute-deprivation testing.

19. S. Cabeza de Vaca, S. Holiman, K. D. Carr, *Physiol. Behav.* **64**, 251 (1998).

20. K. L. Conover and P. Shizgal, *Behav. Neurosci.* **108**, 559 (1994).

21. I. Cusin, F. Rohner-Jeanrenaud, A. Stricker-Kongrad, B. Jeanrenaud, *Diabetes* **45**, 1446 (1996).

22. R. Herrnstein, *J. Exp. Anal. Behav.* **13**, 243 (1970); P. Shizgal, *Curr. Opin. Neurobiol.* 7 198 (1997).

23. C. Elias et al., *J. Comp. Neurol.* **402**, 442 (1998); T. Horvath, S. Diano, A. N. van den Pol, *J. Neurosci.* **19**, 1072 (1999).

24. J. G. Mercer et al., *FEBS Lett.* **387**, 113 (1996); J. K. Elmquist, C. Bjorbaek, R. S. Ahima, J. S. Flier, C. B. Saper, *J. Comp. Neurol.* **395**, 535 (1998);

25. C. Elias et al., *J. Comp. Neurol.* **402**, 442 (1998). C. Broberger, L. de Lecea, J. G. Sutcliffe, T. J. Hokfelt, *J. Comp. Neurol.* **402**, 460 (1998);

26. D. Qu et al., *Nature* **380**, 243 (1996); T. Sakurai et al., *Cell* **92**, 573 (1998).

27. A. G. Watts, G. Sanchez-Watts, A. B. Kelly, *J. Neurosci.***19,** 6111 (1999).

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