NOTE TO USERS

Effects of Estradiol on Brain Stimulation Reward and Energy Balance in Male Rats

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ABSTRACT

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The rewarding effect produced by electrical stimulation of the perifornical region of the lateral hypothalamus (LH), is modulated by energy balance. At this site of stimulation, reward effectiveness is increased by chronic food-restriction and decreased by leptin and insulin. Like leptin and insulin, estrogen decreases body weight and food intake in male rats; levels of these three hormones vary as a function of fat stores. Thus, it was of interest to determine whether the effect of estrogen on brain stimulation reward (BSR) mimics that of leptin and insulin. The results did not support this prediction: estradiol increased the reward effectiveness of the stimulation in all subjects. In two cases, this increase in reward effectiveness was greater than would be expected as a result of the estradiol-induced weight loss alone. Thus, the influence of estradiol on BSR differs sharply from that of leptin and insulin, and the mechanisms underlying the reward-modulating effects of these hormones are unlikely to be related to their common physiological actions. The potentiation of BSR during estradiol treatment could be due either to synergy between the direct effect of the hormone and those of weight loss or to sensitization caused by the prior episode of food restriction. One way to overcome the interpretation problems associated with this experimental design would be to test the effect of estradiol implantation in food-restricted subjects. However, the effect of estradiol on the energy balance of food-restricted male subjects had not been reported previously. The second experiment investigated this relationship by measuring the effect

of estradiol implantation on body weight and food intake in ad libitum fed and food-restricted male rats. It was found that estrogen further decreased body weight in subjects that had been food-restricted to a level below that of ad libitum fed subjects treated with estrogen. This decrease in body weight is likely due to an increase in energy expenditure. Once ad libitum access to food was restored, the estrogen-treated rats increased their food intake and body weight. This implies that it would be possible to hold constant the weight of food-restricted, estrogen-treated, male rats by adjusting their daily ration. Thus, it should be feasible to investigate the effect of estrogen on BSR in food-restricted male rats whose body weight is held constant. In this way, a direct action of estrogen on the neural substrate for BSR could be distinguished from an indirect influence due to changes in body weight.

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DEDICATION

I dedicate this thesis to my devoted and loving parents: Mumtaz and Nuruddin Ismail. Their accomplishments, hard work and determination are the greatest inspiration for this thesis.

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α-MSH	α-melanocyte-stimulating hormone
AgRP	agouti-related peptide
ARC	arcuate nucleus
BSR	brain stimulation reward
CART	cocaine-and-amphetamine-regulated transcript
CCK	cholecystokinin
LH	lateral hypothalamus
LHSS	lateral hypothalamic self-stimulation
M-50	half-maximal rate of reward
MCH	melanin-concentrating hormone
NAcc	nucleus accumbens
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
PBN	parabrachial nucleus
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
SEM	standard error of the mean
VMH	ventromedial hypothalamus
VTA	ventral tegmental area

Introduction

The central nervous system contains mechanisms that coordinate the animal's behavioral and physiological responses to achieve and maintain a balance between energy input and expenditure. According to Schwartz and colleagues (2003): "the brain senses and interprets neural, endocrine and metabolic signals and activates distinct effector pathways to regulate energy intake and expenditure". These energy balance signals can be divided into two main categories: short-term and long-term signals, which act through distinct but interacting mechanisms (Leibowitz & Wortley, 2004). Short-term signals act primarily as determinants of satiety to limit the size of individual meals and circulate as a function of the energy consumed over a short period of time. On the other hand, long-term signals of energy balance are released in proportion to both body adipose stores and to the amount of energy consumed over a more prolonged period of time (Havel, 2001). Examples of such long-term energy balance signals are insulin and leptin. Both are circulating signals that are sensed by the brain and are known to affect energy balance. Certain changes in energy balance have been shown to modulate the value of reward as measured by brain stimulation reward (Carr, Kim, & Cabeza, V, 2000; Fulton, Woodside, & Shizgal, 2000). The experiments presented in this thesis investigated the effects of estrogen, another long-term signal of energy balance, on body weight and food intake and on thresholds for brain stimulation reward in male rats. The introduction of this thesis begins by reviewing examples of long-term signals of energy balance and their action. Then, the concept of brain stimulation reward and examples of modulation of brain stimulation reward by changes in long-term signals of energy balance are described.

The role of estrogen as a potential modulator of brain stimulation reward is then discussed.

Long-Term Signals of Energy Balance

Insulin is a hormone produced by the pancreas. Its receptors are widely distributed in peripheral tissues and in the brain with significant concentrations in hypothalamic and limbic forebrain structures (Schulingkamp, Pagano, Hung, & Raffa, 2000). Although insulin's primary role is to control blood glucose level by altering glucose uptake in adipose, hepatic or muscle cells (Brown, 1994), insulin also acts in the brain to modulate energy balance (Cancello, Tounian, Poitou, & Clement, 2004). Insulin enters the brain via a saturable transport mechanism where it acts on arcuate neurons in the hypothalamus to increase the activation of peptides that suppress food intake, such as pro-opiomelanocortin (POMC), the precursor of the anorectic α-melanocyte-stimulating hormone (α-MSH), and decrease the activation of peptides that stimulate food intake, such as neuropeptide Y (NPY) and agouti-related peptide (AgRP) in hypothalamic arcuate neurons (Benoit, Clegg, Seeley, & Woods, 2004). Research has shown that central insulin administration results in a dose-dependent reduction in food intake and body weight (Air, Benoit, Clegg, Seeley, & Woods, 2002). Furthermore, insulin has been shown to circulate in the body in proportion to the energy content of the fat stores; thus insulin levels in the blood increase after feeding and fall when animals are food-deprived (Porte & Woods, 1981). Based on this, insulin is considered to be a circulating long-term signal of energy balance in the brain.

Another peptide that is often characterized as a circulating long-term energy balance signal is leptin. Leptin is a protein encoded by the *ob* gene and is produced by

white adipose cells. Like insulin, the amount of leptin in the circulation is positively correlated with the amount of fat in the body; thus the leptin level in the blood increases when animals are fed and falls when animals are food-deprived (Maffei, Halaas, Ravussin, Pratley, Lee, Zhang et al., 1995). Exogenous leptin administration causes dose-dependent reductions in food intake, body weight and fat deposits of normal weight and obese rodents and augments energy expenditure in lean and leptin deficient rodents (Halaas, Gajiwala, Maffei, Cohen, Chait, Rabinowitz et al., 1995; Simpson & Davis, 2001). Therefore, one way to view leptin is as a circulating long-term signal of energy balance that acts on the brain.

Leptin, like insulin, enters the brain via a saturable transport mechanism (Banks, Kastin, Huang, Jaspan, & Maness, 1996). Its actions on energy balance are mediated by the activation of Ob-Rb receptors found in many brain areas such as the caudal brainstem (Grill, Schwartz, Kaplan, Foxhall, Breininger, & Baskin, 2002), but predominantly in the hypothalamus (Velkoska, Morris, Burns, & Weisinger, 2003). There, leptin increases the activation of peptides that suppress food intake such as POMC and cocaine- and amphetamine- regulated transcript (CART) and decreases the activation of peptides that increase food intake such as NPY, AgRP and indirectly that of melanin-concentrating hormone (MCH). During a period of food restriction, when leptin concentration is decreased due to fat loss, central mechanisms responding to decreased leptin act to coordinate systems that increase food intake, providing a possible explanation for why most individuals and rodents re-gain their weight after dietary restraints are removed (Keesey & Hirvonen, 1997).

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Steroid Hormone (Estrogen)

Estrogen, a steroid hormone, also regulates energy balance. In fact, Blaustein, Gentry, Roy, & Wade (1976) demonstrated that ovariectomy increases both food intake and body weight in adult mice and that estrogen replacement decreases food intake and body weight in ovariectomized animals.

Estrogen is derived through the process of aromatization from testosterone, a hormone produced by testes and ovaries and is present in males as well as in females (Simpson & Davis, 2001).

Recently, Mystkowski and colleagues (2000) showed that estrogen has potent effects on energy balance in males. They found that 17β-estradiol implanted in the periphery over a wide range of doses decreased daily food intake by 20% and body weight by 25% after 29 days. Thus, estrogen, like insulin and leptin decreases food intake in male rats over a long period of time.

Interestingly, increased adipose mass causes an increase in the conversion of testosterone to estrogen in proportion to fat mass in males (Mystkowski & Schwartz, 2000). In addition, there is powerful evidence showing that estrogen influences energy expenditure (Mystkowski, Seeley, Hahn, Baskin, Havel, Matsumoto et al., 2000; Roy & Wade, 1977). Thus, just like insulin and leptin, estrogen in males circulates in the body proportionally to the level of energy stores, influences energy expenditure and could act as a long-term signal of energy balance (Bronson, 1986).

One way that energy balance is maintained is by increasing and decreasing the rewarding property of food (Davis, Strachan, & Berkson, 2004). For example, when hungry, food is more rewarding than when satiated. The word "reward" can be defined in

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many different ways. One definition of reward is any stimulus, such as a drug or food that an animal will work to obtain (Yoemans, 1990). Electrical stimulation of certain brain sites, also known as brain stimulation reward (BSR), is a technique that has often been used to study changes in the reward values.

Brain Stimulation Reward

The term BSR was coined following the observation that electrical brain stimulation at particular brain sites can produce a rewarding effect that will make the experimental subjects work vigorously to obtain the stimulation. BSR is believed to result from the activation of neural circuits that are also activated by natural rewards (Olds & Milner, 1954). It has been proposed that rewarding stimulation is meaningful because it can mimic a neural signal that is evaluated in the same currency as natural goal objects (Shizgal, 1999). In other words, the rewarding effect of the stimulation is comparable to the rewarding effect caused by natural stimuli though it produces no apparent physiological benefits.

Electrical stimulation of the LH is considered particularly vigorous and stable. Rats will choose LH stimulation over food and water in conditions of severe deprivation (Rossi & Stutz, 1978). The rewarding effect of lateral hypothalamic self-stimulation (LHSS) can summate with the rewarding properties of food (Conover, Woodside, & Shizgal, 1994; Conover & Shizgal, 1994). Also, certain energy balance manipulations have been shown to modulate LHSS at particular stimulation sites. Experiments demonstrating changes in LHSS following energy balance manipulation employed the rate-frequency method and derived M-50 values to represent their results.

Derivation of M-50 Values

This rate-frequency method is an efficient way to distinguish between treatment effects on reward effectiveness from treatment effects on non-specific performance factors (Miliaressis, Rompre, Laviolette, Philippe, & Coulombe, 1986). Rate-frequency curves are obtained by exposing the subjects to a range of stimulation frequencies and recording the rate of reward delivery at each frequency. The ensuing plot yields a sigmoidal function relating the rate of reward delivery to the logarithm of pulse frequency. The stimulation frequency required to maintain a half maximal rate of reward earned (M-50) is interpolated from the curve to define its position along the abscissa. If an experimental manipulation results in an increase in M-50 values, it implies a decrease in the reward efficacy of self-stimulation because higher stimulation frequencies are now required to maintain the same level of responding as before the manipulation. On the other hand, a decrease in M-50 values implies that there has been an increase in the reward efficacy of self-stimulation because lower stimulation frequencies are now required to maintain the same level of responding as before the manipulation.

Energy Balance Manipulations

Energy balance can be manipulated both acutely and chronically. Chronic food restriction is a dietary restraint technique that limits access to food until a target body weight level has been reached. This is considered to be a long-term manipulation of energy balance. In contrast, acute food deprivation, a procedure that provides the subject with no food whatsoever during a limited time period, is considered to be a short-term manipulation of energy balance. Interestingly, acute food deprivation has been shown to increase the reward effectiveness of the stimulation (Abrahamsen, Berman, & Carr, 1995)

but this effect failed to be replicated by other researchers (Fulton et al., 2000). This discrepancy is probably due to differences in electrode placements. Chronic food restriction, on the other hand, is known to increase the reward effectiveness of the stimulation in only a subset of rats (Carr & Wolinsky, 1993; Fulton et al., 2000).

Site-Specificity

Histological procedures have revealed that the tips of the stimulating electrodes in rats that demonstrated an increase in the reward value of LHSS following chronic food restriction were positioned in the perifornical area of the lateral hypothalamus. The perifornical area is said to contain cells and/or fibers of passage whose output is modulated by the status of long-term signals of energy balance. These sites, given our current state of knowledge, are called "restriction-sensitive sites" of stimulation in the hypothalamus (Carr & Papadouka, 1994; Fulton et al., 2000; Fulton, 2003). This leads to the notion that it is the manipulations of long-term energy stores that modulate LHSS at these sites rather than short-term manipulations. It is thus of interest to see whether manipulations of naturally present circulating long-term signals of energy balance, such as insulin and leptin, could also modulate LHSS.

Modulation of BSR by Long-Term Signals of Energy Balance

Carr, Kim and Cabeza de Vaca (2000) tested the effect of central insulin administration on LHSS in ad libitum fed and food-restricted rats. They found that, in ad libitum fed subjects, insulin increased the LHSS M-50 values at stimulation sites sensitive to chronic food restriction resulting in a decrease in reward efficacy. On the other hand, in subjects that underwent chronic food-restriction, insulin administration reversed the threshold-lowering effect due to chronic food-restriction. Based on the

commonalities between insulin and leptin regarding energy balance modulation, could leptin mimic insulin's effect on LHSS?

Fulton and colleagues (2000) investigated the effects of central leptin administration on LHSS in ad libitum fed and food-restricted subjects. Like insulin, leptin increased LHSS M-50 values in ad libitum fed subjects and attenuated the potentiation of LHSS by chronic food-restriction. Based on this finding, the authors suggested that the modulation of M-50 values at these sites was sensitive to long-term energy balance manipulations involving fat stores and that leptin may be one of the signals that play a role in this mechanism. Thus, it can be seen that insulin and leptin, two long-term energy balance signals, similarly modulated the reward efficacy of LHSS. To gain a better understanding of the signals that sensitize LHSS to changes in energy balance, it is of interest to see the effect of other long-term signal of energy balance such as estrogen on LHSS.

Modulation of BSR by Estrogen?

Experiment 1 was designed to investigate the effect of estrogen implantation on LHSS M-50 values in ad libitum fed animals. Based on the numerous similarities between insulin, leptin and estrogen, it was hypothesized that estrogen would increase LHSS M-50 values in ad libitum fed rats at stimulation sites that are responsive to chronic food-restriction.

Results from the above experiment indicated that estrogen's effect on BSR is opposite from that of leptin and insulin. In order to facilitate the interpretation of these data, it is important to remove the effect of estrogen-induced body weight loss. This can be achieved by testing the effect of estrogen on BSR in food-restricted subjects.

However, it is first necessary to better understand the effect of estrogen on body weight and food intake of food-restricted subjects.

Experiment 2 was designed to examine the effect of estrogen on food intake and body weight in ad libitum fed and food-restricted male rats.

The two experiments were carried out to gain a better understanding of: (1) the ability of long-term metabolic signals to affect LHSS and (2) the anorectic action of estrogen in food-restricted subjects.

EXPERIMENT 1

The goal of this experiment was to investigate the effects of subcutaneous estradiol implantation on BSR in ad libitum fed male rats. Previous research has shown that changes in long-term energy balance, such as chronic food restriction, increases the reward effectiveness of the stimulation in subjects in which the stimulating electrode is located in a particular quadrant of the perifornical region of the lateral hypothalmus (Carr & Papadouka, 1994, Fulton, Woodside and Shizgal; Fulton, 2003). This leads to the notion that a sub-population of reward-related neurons is sensitive to the manipulation of long-term energy stores. Insulin and leptin, two circulating signals of long-term energy balance, have shown to decrease the reward effectiveness of the stimulation at restriction-sensitive sites of stimulation (Carr et al., 2000; Fulton et al., 2000). Based on these findings, estrogen, another signal of long-term energy balance was hypothesized to attenuate BSR, like leptin and insulin.

Method

Subjects

Eight male Long Evans rats, weighing between 540 and 602 g at the start of the experiment, were obtained from Charles River Breeding Farms (St-Constant, Quebec). Rats were housed individually, in plastic solid floor cages (20.5 cm x 20.5 cm x 44 cm), with free access to food and water, and maintained on a 12-hour dark/light cycle (lights on at 8 pm) in a temperature-controlled room (21 ± 2°C). Behavioral testing was conducted in the dark phase of the cycle.

Materials.

Electrodes. Electrodes were constructed from stainless steel insect pins, insulated to within 0.5 mm of the tip and soldered onto a copper wire to which a male Amphenol pin was attached.

Hormones. Estrogen pellets containing 7.5 mg of 17-β estradiol (Innovative Research of America, Sarasota, Florida), manufactured to release their content over 21 days, were used as the exogenous hormone in this study. This dose was chosen based on the findings of Mystkowski et al (2000). Cholesterol pellets were made in the laboratory by filling Silastic tubing (1.5 cm) with cholesterol powder and sealing the ends with Type A silicone medical adhesive. Cholesterol was used as a control treatment because it is a precursor of estrogen but has no known activity at estrogen receptors (Wade, 1986).

Apparatus

Screening. Subjects were screened for self-stimulation in a wooden box (25 cm x 25 cm x 70 cm). This wooden box consisted of a Plexiglas front panel, a wire mesh floor

and a lever, which was positioned 3 cm above the floor in the middle of one wall. Five centimeters above the lever, a light was placed to signal the availability of the reward. Electrical stimulation was generated by a constant current amplifier and was controlled by hand-operated pulse generators. To allow the rats to circle without tangling the leads, the output of the constant-current amplifier was routed to the electrodes through a slip-ring, mounted at the top of the box.

Testing. Testing took place in a room with computer-controlled test chambers. The test chambers were similar to those used for self-stimulation screening except they were made entirely of Plexiglas. To ensure sound attenuation, each testing chamber was enclosed in a 50 cm x 50 cm x 90 cm plywood box lined with Styrofoam. Stimulation parameters for each test cage were monitored on an oscilloscope in an adjoining room and were controlled by a microprocessor.

Surgery

Electrode Implantation. Atropine Sulfate (0.5 mg/kg s.c.) was administered ten minutes before the anesthetic to reduce mucous accumulation in the airway. Surgery was performed under pentobarbital anesthesia (Somnotol 65mg/kg i.p.). Before surgery, the level of anesthesia was assessed by determining the response to tail pinch. Once pinching the tail failed to elicit a leg twitch, the rat was mounted in a stereotaxic device. The skull landmarks bregma and lambda, were positioned on the same horizontal plane. Monopolar electrodes were aimed bilaterally 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). Four jewellers screws were screwed into the skull and served as anchors for the assembly. A wire was wrapped around two of the screws and served as the current return. After

implantation of the electrode, dental acrylic was applied to bond the electrodes to the skull and screw anchors. The male Amphenol pins attached to the electrodes and current return were inserted into a nine-pin connector (ABS nine-pin plug, Ginger Scientific, Ottawa, Ontario), which was then fixed onto the animal's head with dental acrylic. The incision was closed with surgical clips. At the end of the surgery, subjects received an analgesic (Buprenorphine 0.05 mg/kg s.c.) to reduce pain and a topical antibiotic (Polysporin, TM/MC Warner-Lambert Canada inc.) to prevent infection around the incision. Subjects were given 4 days to recover before behavioral testing began.

Hormone Implantation. Prior to the implantation of estrogen and cholesterol capsules, rats were anesthetized with isoflurane and an incision was made in the skin behind the neck. Implants were placed just below the skin. The incision was closed with surgical clips. At the end of the surgery, subjects were given an analgesic (Buprenorphine 0.05 mg/kg s.c.) to reduce pain and the same topical antibiotic as above to prevent infection around the incision.

Procedure

The training was carried out in two phases. First, rats were trained to press a lever on a 0.5 s fixed interval schedule: responses were rewarded except when they occurred during a stimulation train, with a 0.5 s train of cathodal, rectangular, constant-current pulses, 0.1 ms in duration. Initially, the current and frequency were set to low values, for example 200 μA and 40 pulses. If the rats displayed any sign of aversion (e.g., vocalizations, freezing, jumping) or disruptive stimulation-evoked movement, stimulation at that particular electrode was stopped, and the second electrode was tested. If rats still displayed signs of aversion or disruptive stimulation-evoked movement, testing was

discontinued. Otherwise, the current was increased (within a range of 200-400 μA) and frequency was adjusted (within a range of 40-200 pulses) so as to produce maximal levels of responding. Thus, the rat was shaped to self-stimulate via both electrodes, but only the electrode that produced the most visible vigorous lever pressing was chosen for further testing.

The second phase of the training session took place in the computer-controlled test chambers. The current was held constant during this phase of training. A 0.5 s fixed interval schedule was again used. Each trial was preceded by a 10 s inter-trial interval when 5 priming trains of stimulation were delivered; the parameters of the priming trains were identical to those of the trains available during the trial. Each trial lasted 60 s. The frequency decreased from trial to trial by 0.033 log10 unit steps from values that produced maximal response to values that produced minimal response until the rats earned fewer than 5 rewards on two consecutive trials. This was repeated 6 times, thus yielding 7 rate frequency curves. This phase of the training period lasted approximately two weeks.

The experiment itself consisted of five phases. Self-stimulation data were collected during each of these phases. In the first phase, the rats were fed ad libitum. In the second phase, rats underwent chronic food-restriction, receiving about 9 grams of standard lab chow daily until their body weights stabilized at 75% of the original values. Subjects were maintained at the stabilized weight during three days. In the third phase, the rats were given ad libitum access to food and body weight was allowed to climb back to its free-feeding level. Once the body weight stabilized, the fourth phase of the experiment began. During this phase, a cholesterol pellet was implanted subcutaneously

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in all the rats. Five days later, the pellet was removed. In the fifth phase of the experiment, a 7.5 mg pellet of 17-β estradiol was implanted subcutaneously in all the rats. Twenty-one days later, the implant was removed and behavioral data were collected for the following four days.

Data analysis

The raw data consisted of the number of rewards earned during each 60 s trial. Out of the 7 determinations collected, the first determination was considered as a warm-up and was not taken into account in statistical analyses. The remaining 6 determinations were each summarized by fitting a "broken line" function consisting of three segments: a lower left horizontal line linked to an upper horizontal line by an ascending middle segment. The stimulation frequency required to maintain a half maximal rate of reward earned (M-50) was interpolated from the fitted broken-line function to define its position along the abscissa.

For the analysis of the effects of chronic food-restriction on BSR thresholds, daily change in M-50 values from baseline was plotted as a function of daily change in body weight from baseline. RS/Explorer, a statistical expert program designed by Bolt Beranek and Newman Inc., was used to fit a linear regression line to this plot. The progression of the subjects through the experiment depended on their data during this phase of the experiment. Only subjects that showed a systematic relationship, as revealed by the regression analysis, between the decrease in M-50 values and body weight loss during chronic food-restriction were allowed to progress through the experiment. Testing was discontinued for subjects that did not show any systematic decrease in M-50 values during this phase.

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The same procedure was used to evaluate the effects of cholesterol and estrogen on BSR. Daily change in M-50 values from the day before implantation was plotted as a function of daily change in body weight from the day before implantation and a linear regression line was fitted to these values.

An additional analysis was used to see whether the change in M-50 values observed following estradiol implantation is accounted for by estradiol's action on body weight alone or by a more direct influence of the hormone on the reward substrate. An analysis of covariance was carried out, using the same software as above, to see whether the slope of the regression lines, representing the change in M-50 values as a function of body weight loss during the chronic food-restriction and estradiol conditions, are significantly different from one another. If the regression lines were not significantly different, it would mean that the change in M-50 values obtained during the estradiol condition is accounted for by estradiol-induced body weight loss. However, if the regression lines were significantly different from one another and there is a significant interaction, it would indicate that the change in M-50 values obtained during the estradiol condition cannot be attributed entirely to estradiol-induced body weight loss; such a result would imply a more direct influence of the hormone on the reward substrate.

Histology

Following completion of testing, the location of the electrode tips was marked by means of the Prussian Blue technique. With the stimulating electrode serving as the anode, a 100 µA current was delivered for 15 sec. Then, rats were injected with sodium pentobarbitol (Somnotol, 100 mg/kg, IP) and perfused intracardially with phosphate-buffered saline followed by a mixture of 10% formalin (50 ml), trychloroacetic acid (0.25

g), potassium ferrocyanide (1.5 g) and potassium ferricyanide (1.5 g). Brains were removed and stored in 10% formalin. After immersion in a 20% sucrose-formalin solution for 24 hr, the brains were frozen and sliced on a cryostat in 30 μm coronal sections. Each section was then mounted on pre-coated slides (Fisher Scientific). Once the sections were dry, formal thionin was used to stained the sections for Nissl substance. A stereotaxic atlas (Paxinos & Watson, 1998) was used to identify the location of the stimulation sites.

Results

Phase 1: Effects of Chronic Food Restriction on Brain Stimulation Reward.

Figure 1 shows body weights (open circles) and M-50 values (solid squares) for each session during baseline and chronic food-restriction. In this figure, the portion to the left of the first dotted line represents the data from the baseline phase, and the portion between the two dotted lines represents the data from the chronic food-restriction phase. As can be seen in Figure 1, initial body weights for the 8 subjects ranged from 525 - 650 g. By the end of the food-restriction period, body weights had fallen to 380 - 460 g. Thus, subjects lost an average 27 % of their baseline body weight during chronic food-restriction.

Figure 1 also shows that food-restriction produced different effects on M-50 values across animals. Inspection of these data suggests that whereas some animals showed a decrease in M-50 values as their body weight decreased, others did not. A regression analysis was carried out to determine if there was a significant linear relationship between body weight loss and the change in M-50 values during chronic food-restriction for each rat. Figures 2 and 3 show the scatter plots, the regression lines and the corresponding equation for each animal during the food-restriction phase. As can be seen in Figure 2, all these rats showed a systematic decrease in M-50 values with body weight loss. Table 1 depicts the result of the regression analysis for each rat. The regression analysis generates an F-ratio and a t-value, which indicate the significance of the relationship between the change in body weight and the change in M-50 values. It can be seen in Table 1 that the slope of the regression line met the criterion for statistical reliability ($p < 0.05$) for subjects E57, E59, E60 and E64.
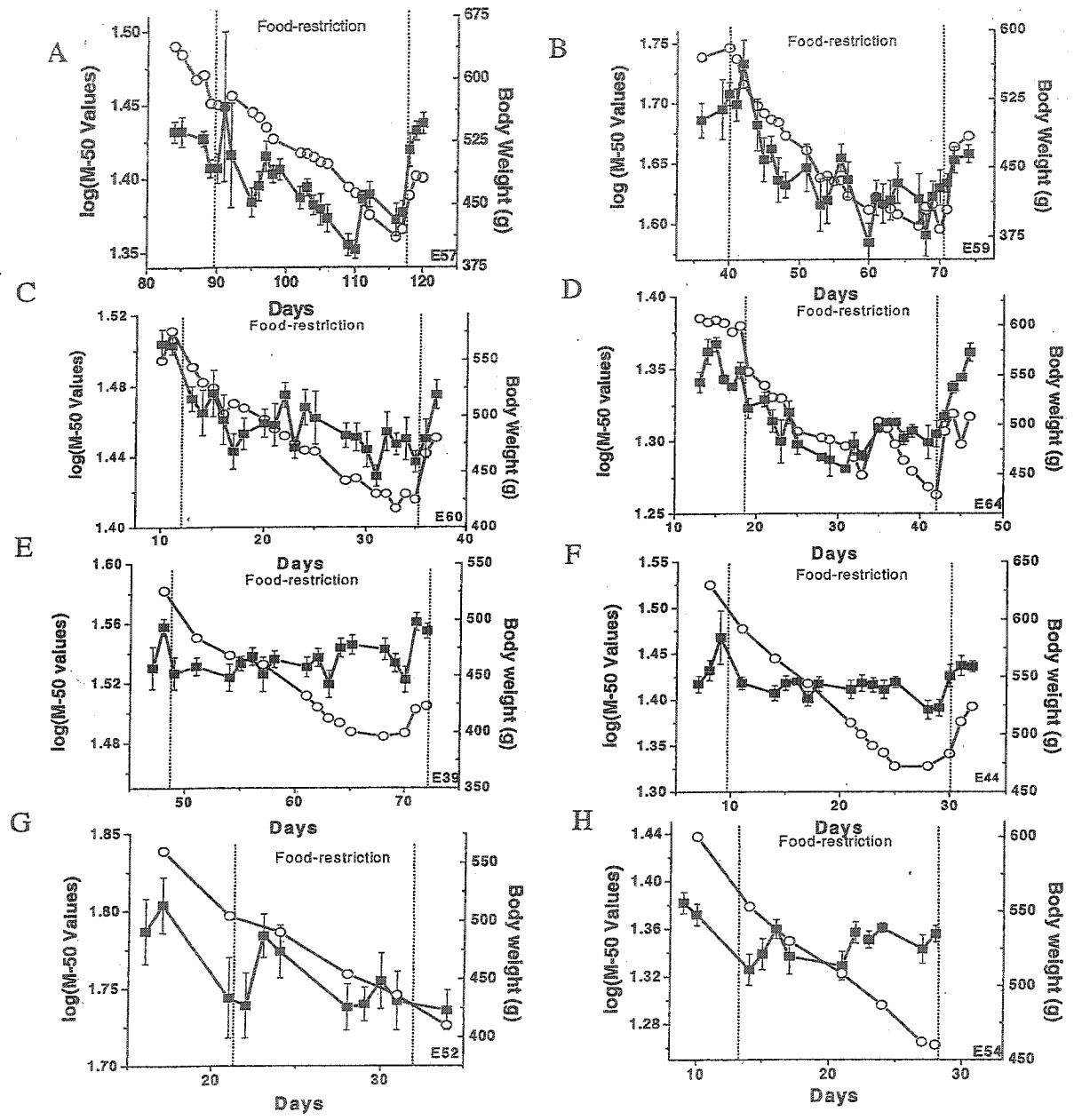
Figure 1. Effect of chronic food-restriction on daily M-50 values (filled squares/mean ±
SEM) and body weight measures (open circles) for all subjects.
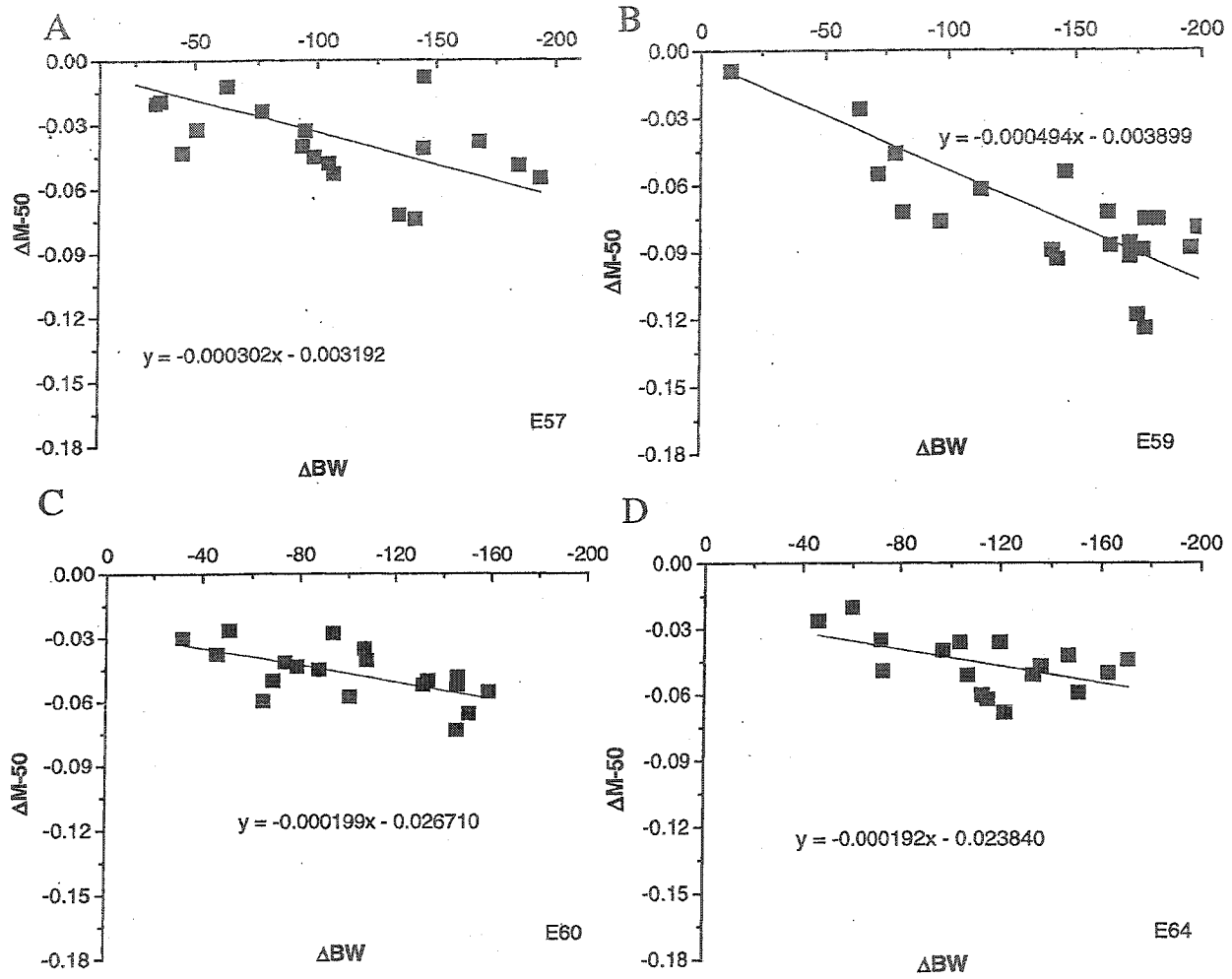
Figure 2. The regression of the change in M-50 values (ΔM-50) on the change in body weight (ΔBW) during chronic food-restriction for subjects E57, E59, E60 and E64.

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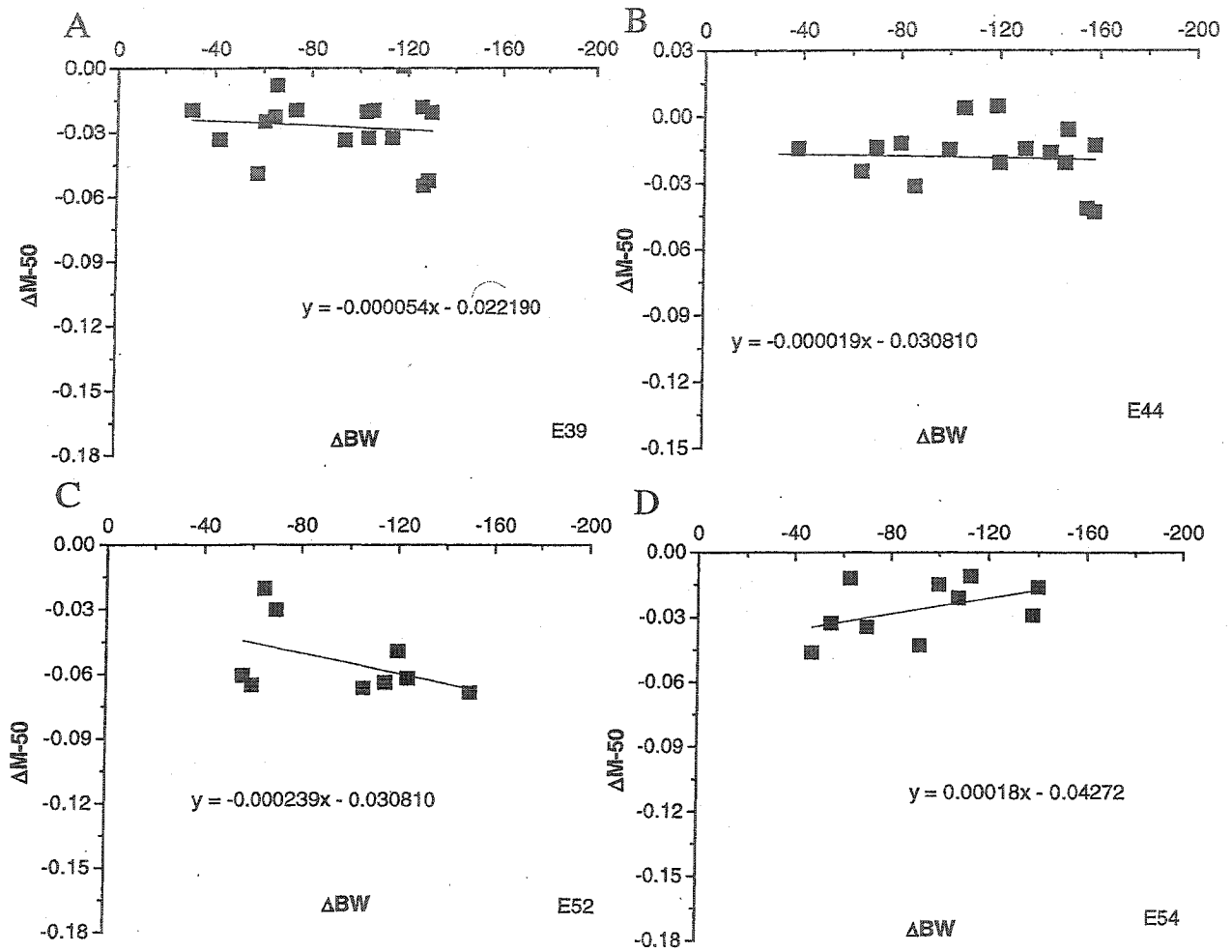
Figure 3. The regression of the change in M-50 values (ΔM-50) on the change in body weight (ΔBW) during chronic food-restriction for subjects E39, E44, E52 and E54.

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Table 1

Regression analysis on the change in M-50 values and the change in body weight during chronic food-restriction

Rat	dfs	F-ratio	slope ($\times 10^{-4}$)	t-value
E57	1,19	11.259**	-3.02	-3.36**
E59	1,21	36.249**	-4.94	-6.02**
E60	1,18	10.899**	-1.99	-3.30**
E64	1,18	6.040*	-1.92	-2.46*
E39	1,16	0.209	-0.54	-0.46
E44	1,14	0.056	-0.19	-0.24
E52	1,8	1.956	-2.39	-1.40
E54	1,9	2.170	1.80	1.47

* p < 0.05, ** p< 0.01
Error!

In contrast to the effects seen in the above subjects, the relationship between body weight and M-50 values for the remaining subjects appears less systematic (Figure 1). Note that in all these four rats there are fewer data points than the previous four subjects (E57, E59, E60 and E64). One reason is that subjects E39, E44, E52 and E54 were not weighed as regularly. A statistical analysis restricted to the data from days on which weights were measured would be limited in power. Thus, body weight data were interpolated for subjects E39, E44, E52 and E54, to provide weight estimates for each day on which a self-stimulation session was carried out. The interpolation was achieved by averaging the body weight measures from the days immediately before and after the day for which a weight value was missing. The regression analysis, carried out on the data set that included interpolated weights (Figure 3), indicates that for rats E39 and E44, the slope of the regression line does not meet the statistical criterion (Table 1). Note that even after interpolation of body weight measures, subjects E52 and E54 had a lower number of data points than the other rats. Although visual inspection of the data from subjects E52 and E54, in Figure 3, suggests that there may be a trend in the change in M-50 values, the regression analysis failed to provide evidence of a statistically reliable relationship due to lack of power (Table 1). Thus, in these four subjects, the regression analysis failed to demonstrate that the reward effectiveness in these subjects is modulated by body weight, and hence failed to provide evidence for their sensitivity to chronic food-restriction. Because the goal of this experiment was to determine the effect of estrogen implantation on the rewarding effect sensitive to chronic food-restriction, testing was discontinued for subjects E39, E44, E52 and E54.

Phase 3: Effect of cholesterol implantation on brain stimulation reward.

 After chronic food-restriction, the four rats that showed a significant relationship between the decrease in M-50 values and weight loss were given ad libitum access to food. Following stabilization of their body weight, they were implanted with a cholesterol pellet. The effect of cholesterol implantation on M-50 values could only be assessed in 3 out of the four rats. The data from subject E57 are missing due to equipment failure. Figure 4 illustrates daily M-50 values (solid squares) and body weight (open circles) during the cholesterol condition, which is the portion in the graph between the two dotted lines. In subject E59 and E60 there appears to have been no systematic change in body weight during the cholesterol treatment, whereas the body weight of subject E64 had decreased by 11g at the end of this condition.

 As shown in Figure 4, there also appears to have been little or no systematic change in M-50 values following cholesterol implantation in these subjects. The M-50 values of subject E59 decreased slightly, but the M-50 values of subjects E60 and E64 appear unchanged following cholesterol implantation. It is important to note here that there are very few data points for each rat during the cholesterol condition. In such a situation, it is pointless to conduct a regression analysis because there is insufficient power for the analysis to be meaningful.

Phase 4: Effects of estradiol on Brain Stimulation Reward.

 Figure 5 shows the body weights (open circles) and M-50 values (solid squares) for each session during estradiol implantation, which corresponds to the portion of the graph between the two dotted lines. As can be seen in Figure 5, body weights prior to estradiol implantation for the four subjects in which the reward effectiveness of the
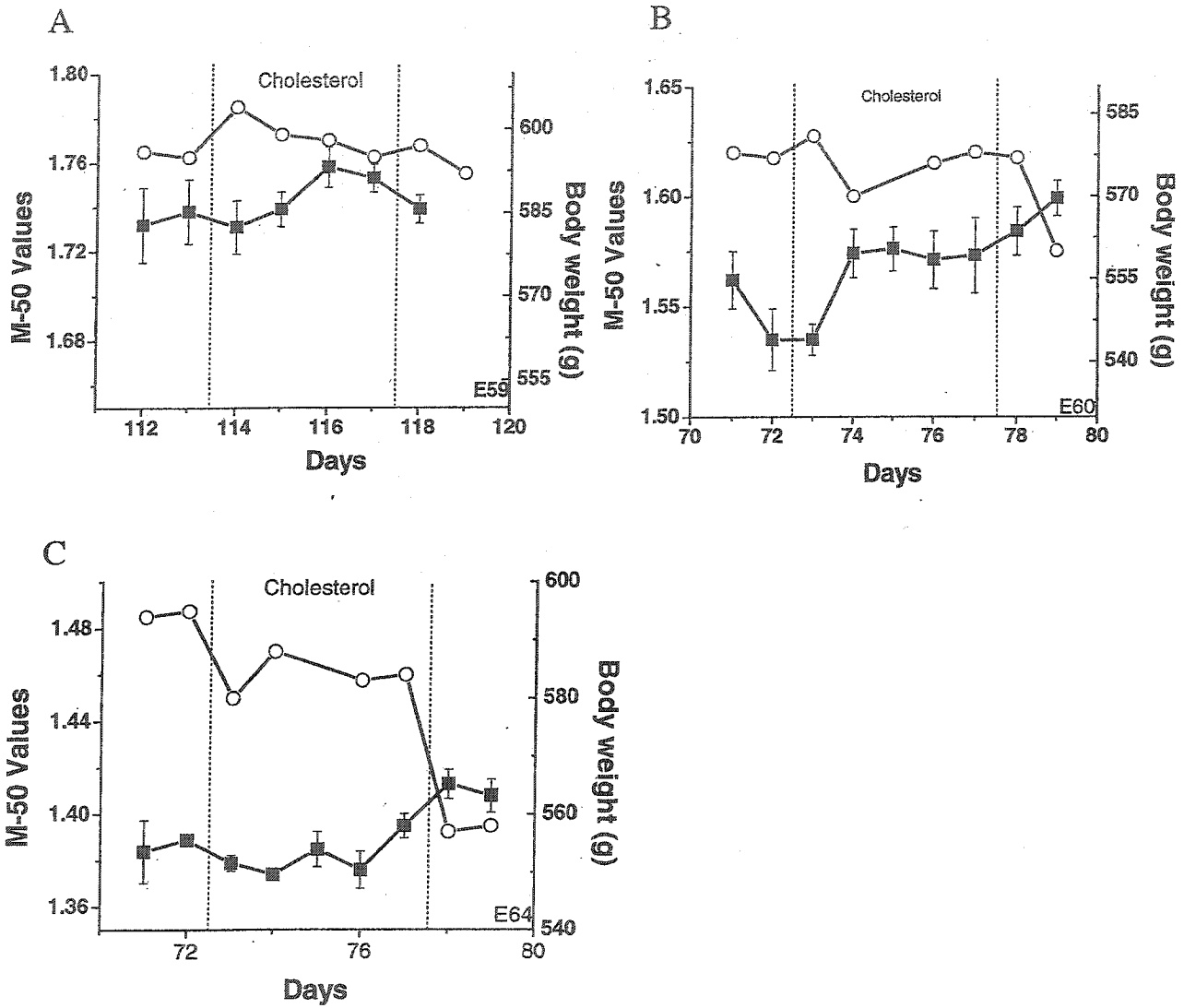
Figure 4. Effect of cholesterol implantation on daily M-50 values (filled squares/mean ± SEM) and body weight measures (open circles) for subjects E59, E60 and E64.
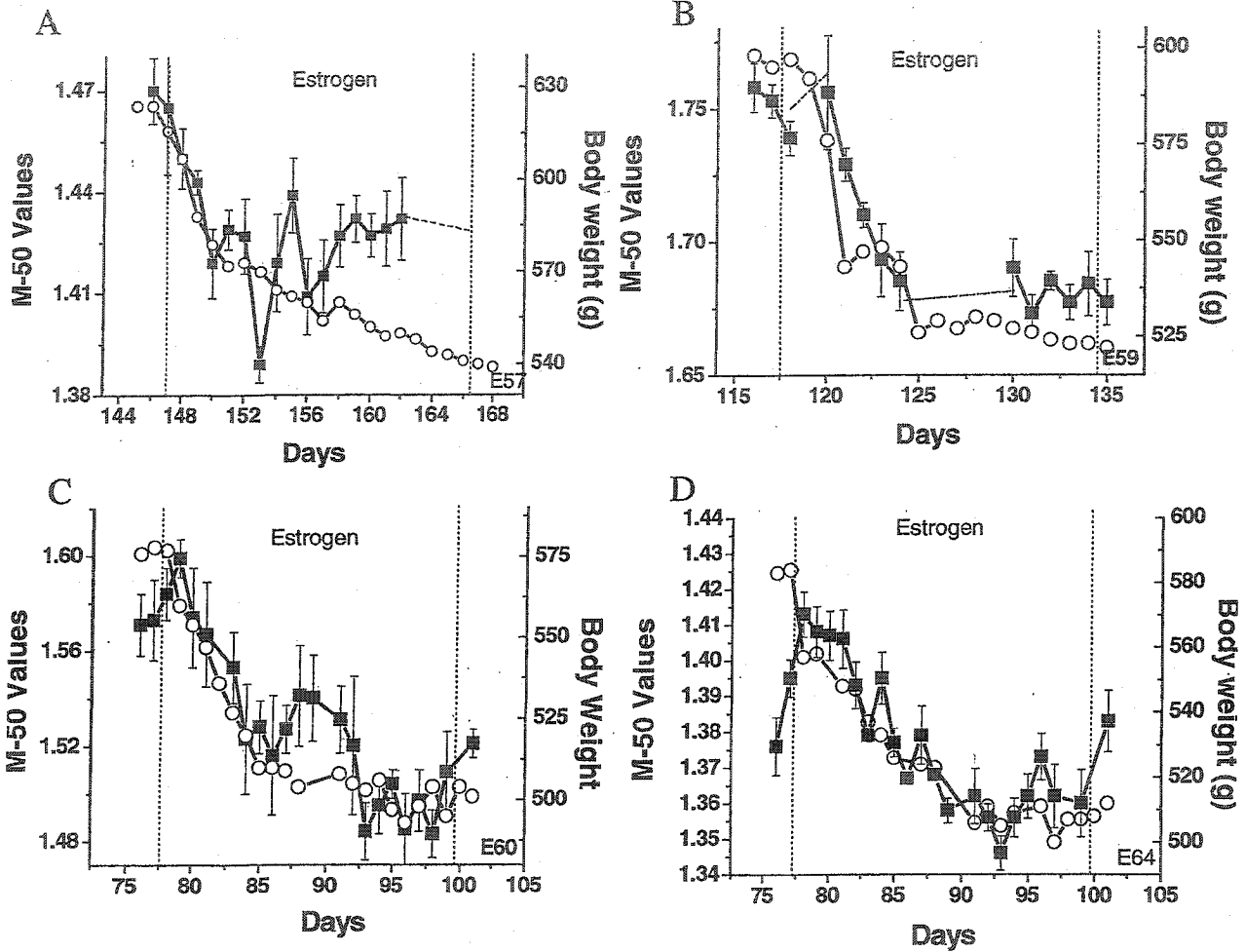
Figure 5. Effect of estradiol implantation on daily M-50 values (filled squares/mean ± SEM) and body weight measures (open circles) for subjects E57, E59, E60 and E64.

stimulation was sensitive to chronic food restriction ranged from 576 - 625 g. By the end of the estradiol treatment, body weights had fallen to 480 - 525 g. Thus these subjects lost on average 17% of their body weight following estradiol treatment.

Figure 5 also illustrates that estradiol implantation decreased M-50 values in all four subjects. Some of the M-50 values from rat E57 are missing at the end of the estradiol treatment due to a short-circuit in the return wire. These missing values are represented by a dashed line in the graph. Some of the M-50 values from subject E59 are also missing during the estradiol treatment due to equipment failure and are also represented by a dashed line in the graph. Nonetheless, inspection of these data suggests that all four rats (E57, E59, E60 and E64) demonstrated a decrease in M-50 values and in body weight following estradiol implantation. A regression analysis was carried out to see if there is a systematic relationship during this condition between body weight loss and the change in M-50 values from the day before implantation. Figure 6 shows the scatter plots, regression lines and corresponding equations for each animal during the estradiol condition. As can be seen from this figure, all these rats showed a decrease in M-50 values and in body weight. The regression analysis shows that, in all these subjects, the slope of the regression lines is statistically significant (Table 2).

An additional statistical analysis was used to differentiate between the decrease in M-50 values due to estradiol induced body weight loss and the direct effect of estrogen on the reward substrate. An analysis of covariance was carried out to see whether the slope of the regression lines during the chronic food-restriction and estradiol conditions were significantly different from one another. Figure 7 shows the body weight (open circles) and M-50 values (filled squares) for each session throughout the whole
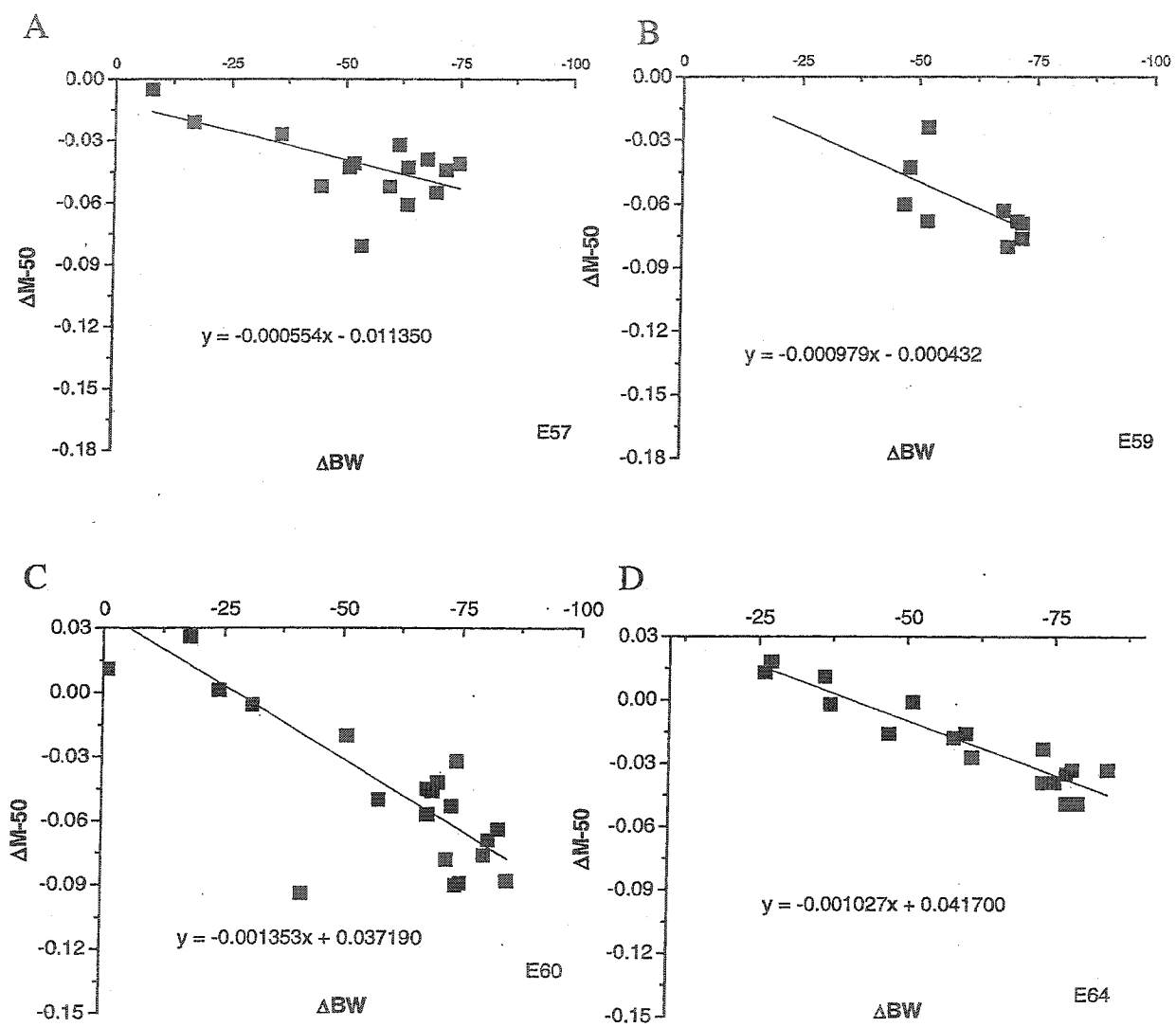
Figure 6. The regression of the change in M-50 values (ΔM-50) on the change in body weight (ΔBW) during estradiol implantation for subjects E57, E59, E60 and E64.

Table 2

Regression analysis on the change in M-50 values and the change in body weight during estradiol implantation

Rat	*dfs*	*F*-ratio	slope	*t*-value
E57	1,14	9.516**	-0.000554	-3.08**
E59	1,10	23.362**	-0.000979	-4.83**
E60	1,17	27.564**	-0.001353	-5.25**
E64	1,16	105.63**	-0.001027	-10.28**

** $p < 0.01$
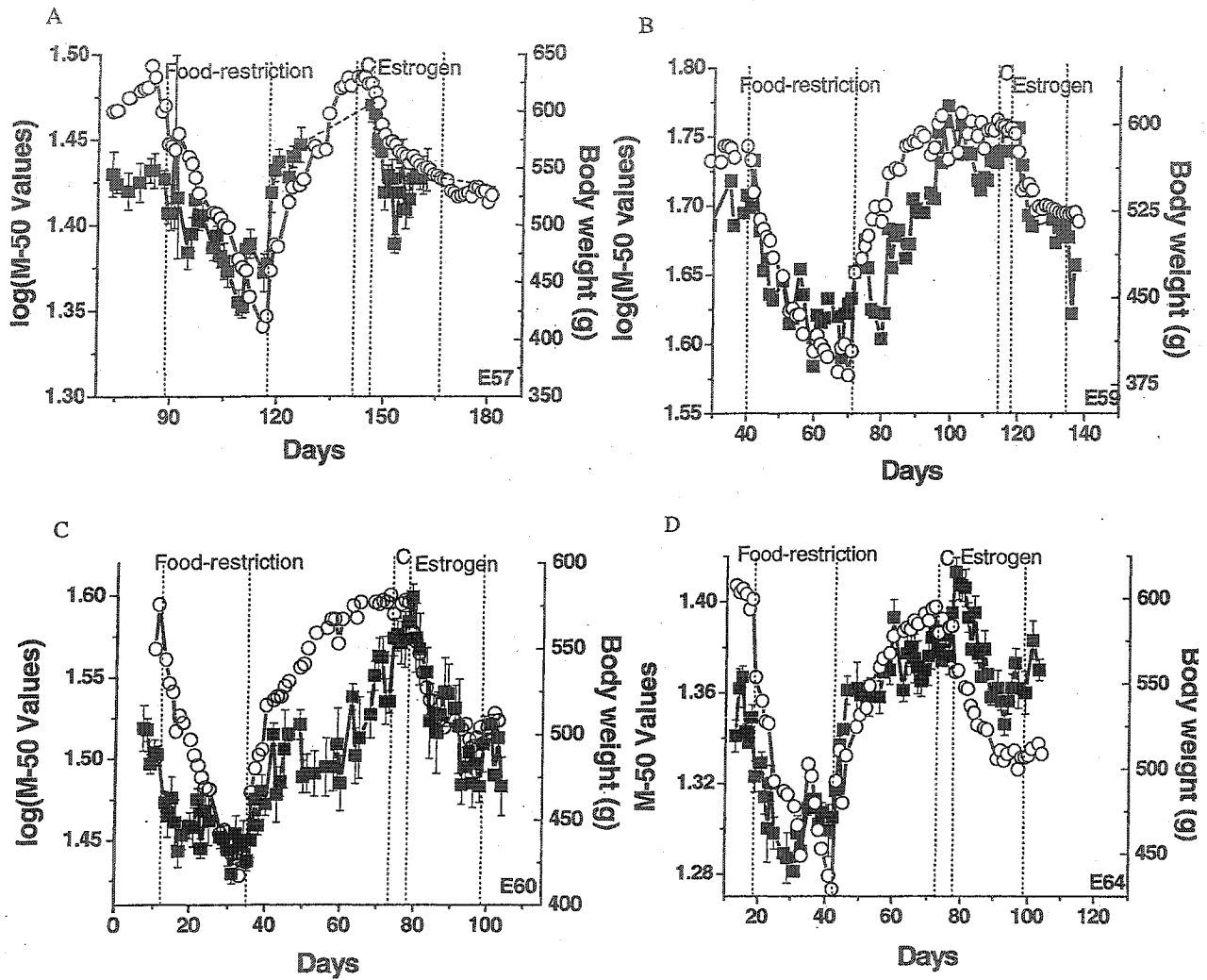
Figure 7. Body weight (open circles) and M-50 values (filled squares/mean ± SEM) for
subjects E57, E59, E60 and E64 over the course of the entire experiment.

experiment. Visual inspection of this figure suggests that in all four subjects, M-50 values and body weight decreased during chronic food-restriction, which is the portion of the graph between the first two dotted lines, and during estradiol treatment, which is the portion between the fourth and fifth dotted lines. In all four subjects, the body weight loss during the estradiol treatment was half of that during chronic food-restriction but the M-50 values decreased by the same amount or more during estradiol treatment than during chronic food-restriction. Figure 8 depicts the relationship between the change in M-50 values and the change in body weight during chronic food-restriction (open circles) and estradiol treatment (filled squares) for each subject. The analysis of covariance shows that the interaction for subjects E57 and E59 was not significant, indicating that the influence of food restriction and estradiol on the M-50 values could not be distinguished statistically (Table 3). However, for subjects E60 and E64, the analysis of covariance detected a significant interaction, indicating that the change in M-50 values differed during these two conditions (Table 3). In these subjects, the effect of estradiol on the M-50 values exceeds that expected due to estradiol-induced weight loss alone.

Histological Results

The four subjects that were responsive to the effects of chronic food-restriction (E57, E59, E60 and E64) had electrode tips located immediately dorsal and/or lateral to the fornix (Figure 9). Three of the remaining subjects (E39, E44 and E52) had the electrode tips scattered ventral or medial to the fornix; however, the electrode tip in rat E54 was dorso-lateral to the fornix, adjacent to the tips of the subjects in which the rewarding effect was sensitive to long-term restriction.

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A

$y = -0.000302x - 0.003192$

$y = -0.000554x - 0.011350$

ΔBW

ΔM-50

E57

B

$y = -0.000494x - 0.003899$

$y = -0.000979x - 0.000432$

ΔBW

ΔM-50

E59

C

$y = -0.001353x + 0.037190$

$y = -0.000199x - 0.026710$

ΔBW

ΔM-50

E60

D

$y = -0.001027x + 0.041700$

$y = -0.000192x - 0.023840$

ΔBW

ΔM-50

E64

Figure 8. Comparison of the regression of the change in M-50 values (ΔM-50) on the change in body weight (ΔBW) during chronic food-restriction (open circles) and estrogen implantation (filled squares) for subjects E57, E59, E60 and E64.

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Table 3

Analysis of covariance on the change in M-50 values and the change in body weight during chronic food-restriction and during estradiol implantation

Rat	dfs	F-ratio (interaction)
E57	1,32	3.366
E59	1,35	3.933
E60	1,35	30.635**
E64	1,31	35.474**

** $p < 0.01$
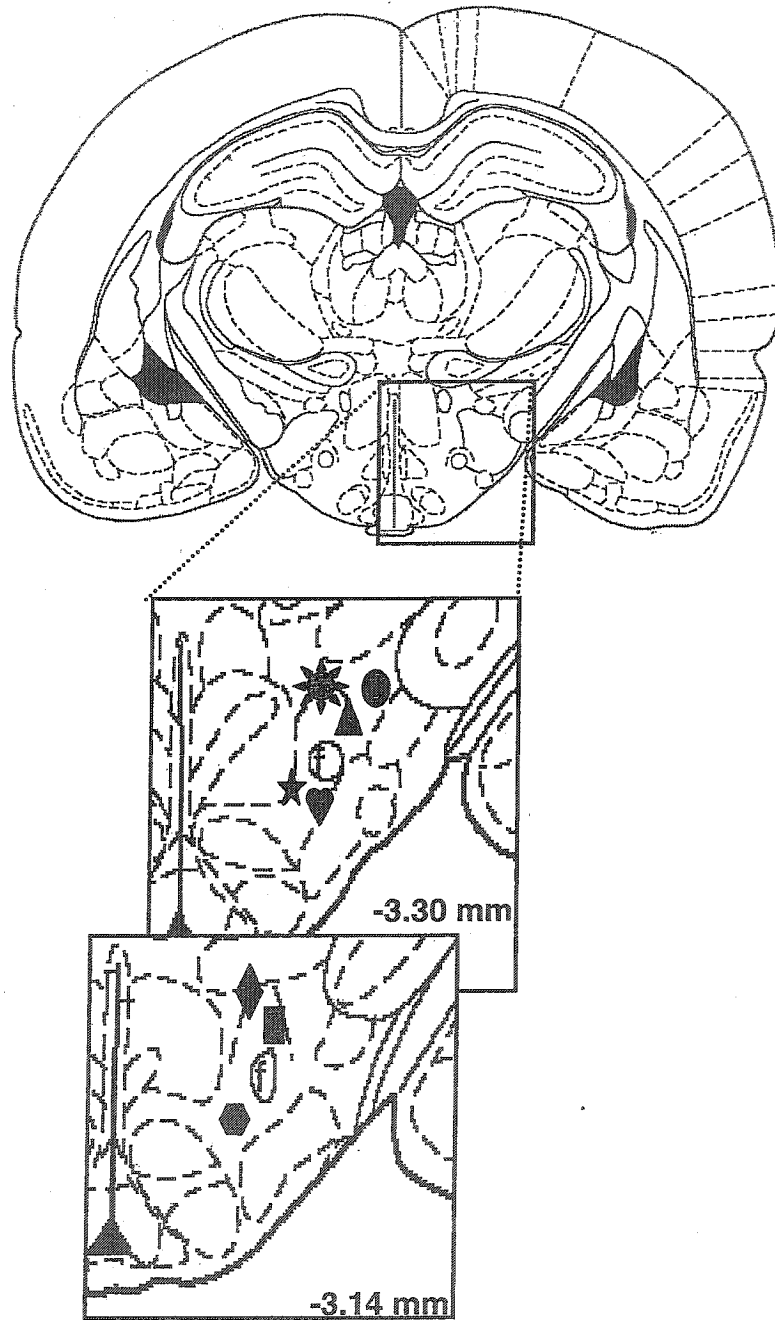
Figure 9. Location of the tips of the stimulation electrodes. Symbols denote electrode placements for individual rats (see Table 4). The coronal sections are based on the atlas of G. Paxinos and C. Watson (1998).

Table 4

Symbols marking electrode placements for individual rats.

Restriction-sensitive		Restriction-insenstive	
Rat	Symbol	Rat	Symbol
E57	◆	E39	⬡
E59	▮	E44	★
E60	✳	E52	♥
E64	▲	E54	●

The objective of this experiment was to investigate the effect of estradiol on BSR. Like leptin and insulin, estrogen reduces food intake in male rats. Leptin and insulin both reduce the reward effectiveness of LH stimulation. Thus, it was expected that estrogen would produce a similar effect. Surprisingly, estrogen did the opposite. Indeed, the potentiation of BSR by estrogen was greater, in some subjects, than would have been expected solely on the basis of estrogen induced weight loss.

Effect of chronic food-restriction on BSR

Changes in M-50 values track changes in the effectiveness of the rewarding stimulation; the lower the M-50 value, the smaller the amount of stimulation required to produce a reward of a given strength and thus the greater the effectiveness of the stimulation. In the current experiment, chronic food-restriction decreased LHSS M-50 values in half of the 8 subjects. The fact that this manipulation potentiated the reward effectiveness of the stimulation in only a subset of the subjects could be a result of the placement of the stimulating electrode (Fulton, 2003). Histological procedures in these rats revealed that the four subjects that were responsive to the effects of chronic food-restriction (E57, E59, E60 and E64) had electrode tips located dorsal and/or lateral to the fornix whereas three of the remaining subjects (E39, E44 and E52) had the electrode tips scattered ventral or medial to the fornix. However, the electrode tip of subject E54 was dorso-lateral to the fornix, adjacent to the tips of the subjects in which the reward effectiveness was sensitive to long-term restriction.
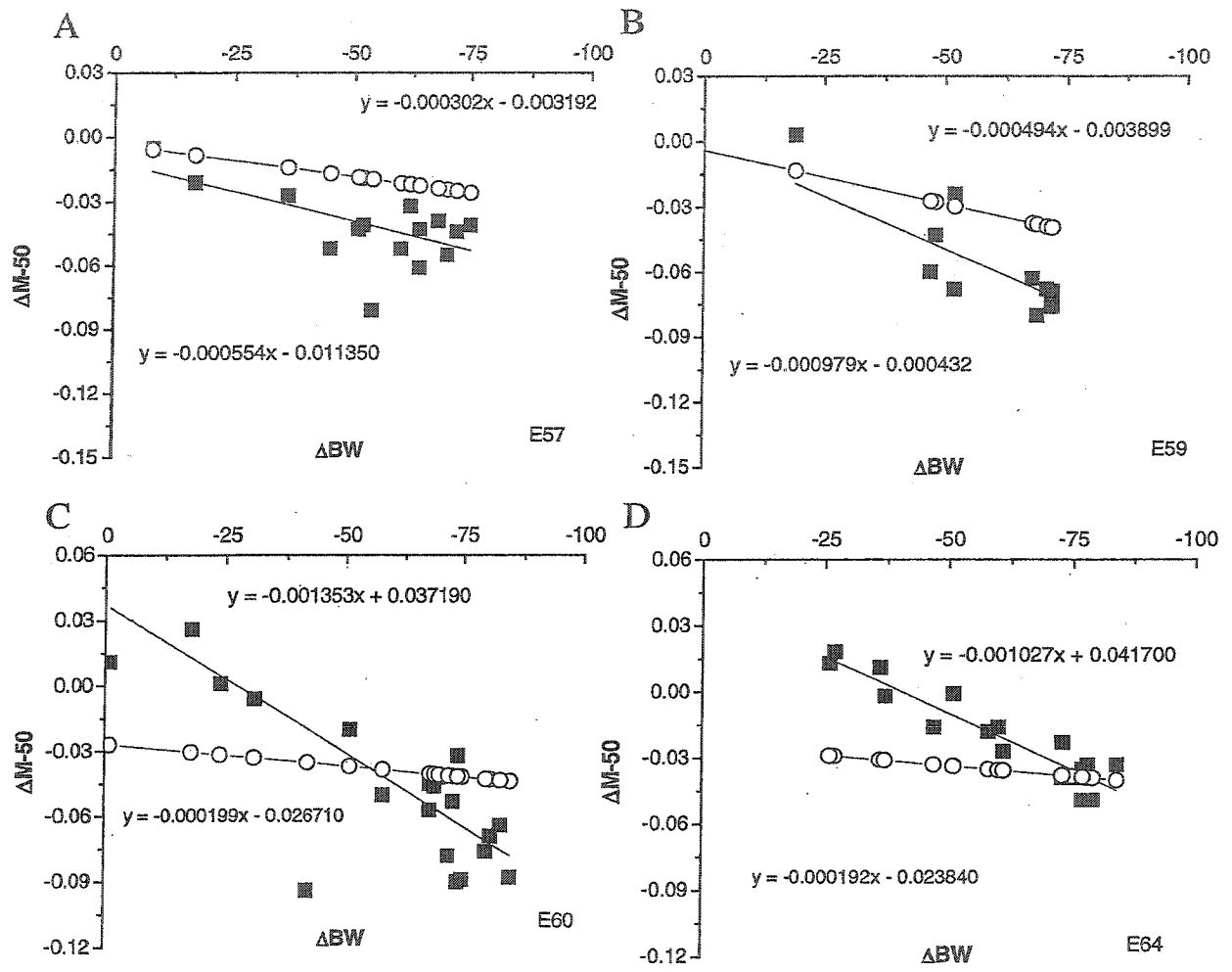
The LHSS data collected during chronic food-restriction are consistent with previous reports that have shown that the distribution of the electrode tip of subjects responsive and unresponsive to chronic food-restriction differ but overlap (Abrahamsen et al., 1995; Carr & Wolinsky, 1993; Carr & Papadouka, 1994; Fulton et al., 2000; Fulton, 2003). An explanation for this contrast in the effect of body weight loss could be that the substrate for LHSS is functionally heterogeous. On this view, the LH electrode samples neurons from at least two different populations, only one of which is embedded in a circuit that is sensitive to long-term energy signals (Fulton et al., 2000; Fulton, 2003). The effects of lesions on LHSS have also been interpreted in terms of the activation of multiple sub-populations of intertwined neurons (Arvanitogiannis, Waraczynski, & Shizgal, 1996).

Effect of estrogen on BSR

This experiment assessed the influence of estradiol on LHSS in those cases in which the reward effectiveness was sensitive to chronic food-restriction. All four rats showed a decrease in LHSS M-50 values, indicating a potentiation of the reward effectiveness of the stimulation following subcutaneous estradiol implantation. Two out of the four subjects demonstrated a potentiation of LHSS that was greater than that expected by estrogen-induced weight loss alone. In essence, the effect of estrogen on LHSS mimicked that of chronic food restriction, except that during estrogen treatment subjects had ad libitum access to food but chose to restrict themselves. This finding is not consistent with previous reports on the effect of other long-term signals of energy balance on LHSS. Research has shown that the administration of insulin and leptin, long-term signals of positive energy balance, increases LHSS M-50 values at stimulation sites that

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are responsive to chronic food-restriction (Carr et al., 2000; Fulton et al., 2000). An explanation for this contrast in the effect of estrogen on LHSS as compared to that of leptin and insulin could be that estrogen modulates energy balance through a different mechanism than that of leptin and insulin. The potentiation of BSR during estradiol treatment could be due either to a synergy between the direct effect of the hormone and that of weight loss or to a sensitization of the stimulated neurons caused by the prior episode of food-restriction. These two possibilities are discussed below.

The observation that estradiol treatment increased the reward effectiveness of the stimulation, beyond what was expected on the basis of weight-loss alone, could be due to a direct effect of the hormone on brain reward circuitry. One way that this could be achieved is through its action on dopaminergic neurons. Estrogen has been shown to modulate the response of the mesolimbic dopamine system to naturally rewarding stimuli. For example, in female rats, dopamine release in the nucleus accumbens (NAcc) varies across the estrous cycle in response to highly palatable food; such that the largest release in dopamine in response to food occurs on the day of proestrus and the lowest release on the day of diestrus (Bless, McGinnis, Mitchell, Hartwell, & Mitchell, 1997). The actions of estrogen on midbrain dopaminergic systems are receptor mediated. Mapping studies have shown that, among many other areas, estrogen receptors can be found in the ventral tegmental area (VTA) (Shughrue, Scrimo, Lane, Askew, & Merchenthaler, 1997), a site of cell bodies of dopaminergic neurons and an area known to be involved in the neural circuit mediating reward (Hoebel, Hernandez, Schwartz, Mark, & Hunter, 1989). According to this line of argument, it is possible that estrogen potentiates BSR by influencing the mesolimbic dopamine activity. If this is true, then

estrogen should potentiate the reward effectiveness of the stimulation not only in subjects in which the reward effectiveness of the stimulation is sensitive to chronic food-restriction, but also in subjects in which the reward effectiveness of the stimulation is insensitive to chronic food-restriction. However, the effect of estrogen on BSR in subjects in which the reward effectiveness of the stimulation is insensitive to chronic food-restriction remains yet to be investigated.

The potentiation of BSR following estradiol implantation for subjects in which the LHSS demonstrated sensitivity to chronic food-restriction could also be due to a sensitization of the stimulated neurons caused by prior episode of food restriction. The concept of sensitization is best illustrated in drug research. The term sensitization was coined following the observation that locomotor activity induced by psychostimulant drugs increases following repeated exposure to these drugs (Kalivas, Taylor, & Miller, 1985; Vezina & Stewart, 1984). Similarly, it is possible that repeated periods of weight loss produce sensitization in the brain reward circuitry. Based on this, the potentiation of BSR following estradiol implantation, in subjects in which the reward effectiveness of the stimulation was sensitive to chronic food restriction, could be due to an increased sensitivity of the neural substrate to estradiol-induced body weight loss caused by the previous episode of food restriction. Subjecting the rats to two periods of chronic food-restriction each followed by a period of re-feeding could test this proposal. If the effect of chronic food-restriction is greater during the second period of food-restriction, then sensitization occurred.

In summary, it was surprising to see in this study that estrogen decreased LHSS thresholds. Like estrogen, leptin and insulin reduce body weight, but unlike estrogen,

both hormones increased BSR thresholds. The potentiation of BSR seen following estrogen implantation could either be due to estrogen's action on the mesolimbic dopamine system or to a sensitization of the stimulated neurons caused by repeated periods of weight loss. The data would be clearer and easier to interpret if the effect of estrogen on BSR was tested in food-restricted subjects. Doing this may remove the effect of estrogen-induced body weight loss, and hence reduce the interpretation problems associated with the current experimental design. However, before conducting such an experiment, it is necessary to better understand how estrogen influences food intake and body weight in food-restricted male rats. The next experiment was designed with this purpose in mind.

EXPERIMENT 2

The goal of this experiment was to investigate the effect of estradiol on food intake and body weight in food-restricted male subjects. It was challenging to interpret the results of Experiment 1 because estrogen could have influenced BSR both by means of a direct action on brain reward circuitry and indirectly by means of a decrease in body weight. It would be easier to interpret the effect of estrogen on BSR if these two effects could be dissociated. This would require testing the effect of estrogen on BSR in food-restricted subjects whose body weight was held constant by adjustment of the daily ration. However, the influence of estrogen on the food intake and body weight of food-restricted male rats has not been reported previously. Thus, Experiment 2 was carried out in order to determine these effects.

Subjects

Twenty-eight male Long Evans rats obtained from Charles River Breeding Farms (St-Constant, Quebec), weighing between 495 and 570 g at the start of the experiment were tested in this experiment. Rats were housed in the same conditions as animals from Experiment 1. Hormone implantation and body weight recording were performed as in Experiment 1. Body weight and food intake data were recorded in the dark phase of the cycle.

Apparatus

Food intake measurement. Powdered food was placed in cups attached to aluminum sheets that were molded to hang on the wall of the home cage. A bowl was positioned underneath each food cup to catch spillage. Daily food intake was measured using a Mettler PJ360 DeltaRange scale.

Procedure

All subjects were first habituated to the powdered food. Following habituation, pre-baseline body weight and food intake measures were recorded until food intake levels varied within 5 g or less on 7 consecutive days. Then, rats were assigned to one of 4 groups: ad libitum fed estrogen, ad libitum fed cholesterol, food-restricted estrogen and food restricted cholesterol, so that mean weight and range of weights were similar across all 4 groups. Then, the rats in the food-restricted cholesterol and estrogen groups were chronically food-restricted by providing them with only 8 g of food per day until their body weights had fallen to 75 % of the initial value. The baseline phase began once the target weight was reached. During baseline, which lasted 11 days, rats were given 14 to
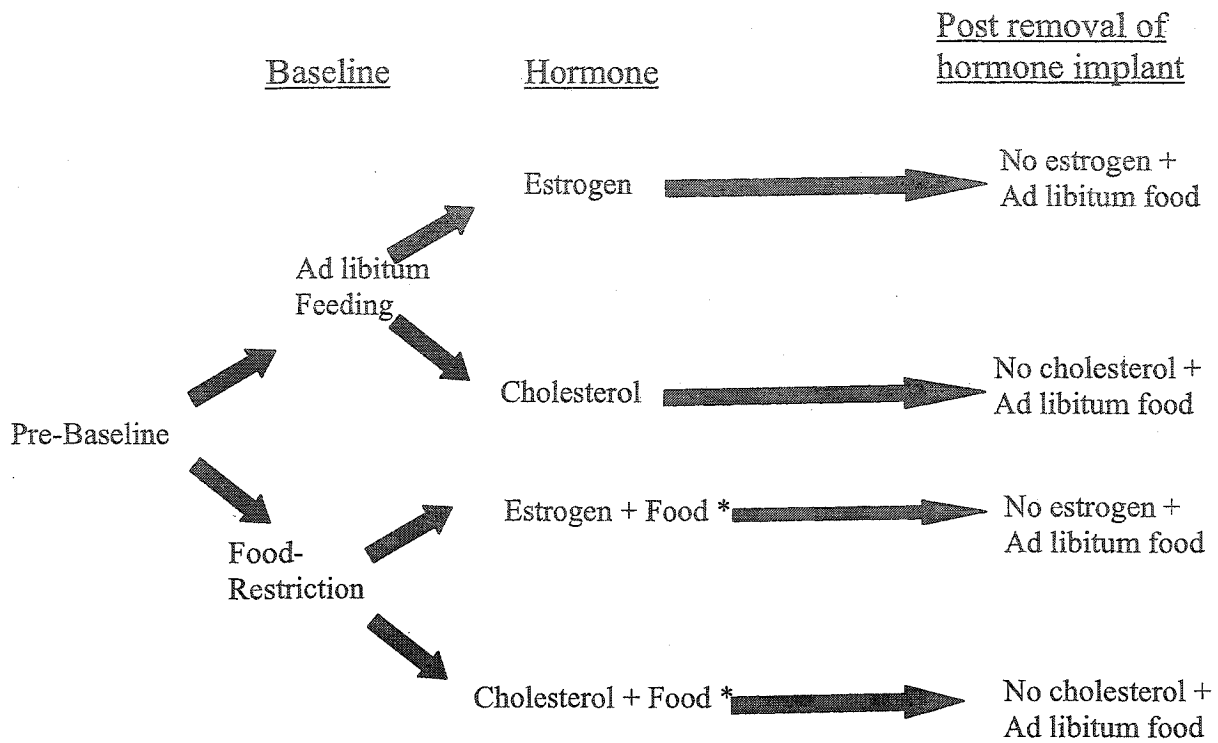
16 g of powdered food. This ration was consumed entirely by all rats. Following baseline, a cholesterol pellet was implanted in rats in the ad libitum fed and food-restricted cholesterol groups for 21 days, and a 7.5 mg pellet of 17-β estradiol was implanted in subjects in the ad libitum fed and food-restricted estrogen group for 21 days. Ten days following implantation, rats from both of the groups were given ad libitum access to food. Then, 11 days later, the implant was removed and post-implant food intake and body weight measures were collected for the following 20 days.

Data Analysis

The raw data collected consisted of daily body weight and food intake. The latter was calculated by subtracting the amount of powdered food left in the cup from the amount of powdered food placed in the cup at the end of the previous day. Daily food intake and body weight data were averaged across subjects within each of the 4 groups.

A three-way mixed analysis of variance was carried out, using the SPSS software designed by SPSS inc. In this design, the three independent variables were diet condition (ad libitum fed and food-restricted), hormonal state (estrogen and cholesterol) and phase (the first 10 days of hormone treatment, the following 11 days, the first 10 days following removal of the hormone implant and the remaining 10 days). Phase was the within-subjects factor. To determine the sources of the interactions, a two-way analysis of variance (ANOVA) was carried out, using the same software as above, to compare the body weight and food intake of all four groups on the last day of each of the phases of the experiment. First, average body weight and food intake were compared across all four groups at baseline to verify homogeneity across the groups. Then, the average change in body weight and average food intake were compared across all four groups on the first 10

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days of hormone treatment, the following 11 days, the first 10 days following removal of

the hormone implant and the remaining 10 days.

* Food was given to the rats after the 10th day of hormone treatment.

Results

Body weight and food intake did not differ among the four groups at the start of the experiment: ad libitum fed estrogen (aE) ($M = 534.8$, $SEM = 11.39$ and $M = 30.24$, $SEM = 1.0$, respectively), ad libitum fed cholesterol (aC) ($M = 530$, $SEM = 10.63$ and $M = 32.57$, $SEM = 1.34$, respectively), food-restricted estrogen (rE) ($M = 534.4$, $SEM = 11.30$ and $M = 30.24$, $SEM = 1.34$, respectively) and food-restricted cholesterol (rC) ($M = 530$, $SEM = 4.48$ and $M = 30.8$, $SEM = 0.98$, respectively) groups.

During the preimplantation phase, rats in rE and rC groups were restricted to 75% of their body weights so that, at the end of that phase, average body weights for the 4 groups were: aE ($M = 529.83$, $SEM = 10.56$), aC ($M = 528.17$, $SEM = 8.89$), rE ($M = 397.38$, $SEM = 6.18$) and rC ($M = 398.13$, $SEM = 5.58$). Food intake remained similar in the two ad libitum fed groups (aE ($M = 32.60$, $SEM = 1.46$) and aC ($M = 32.26$, $SEM = 1.13$)) and similar amounts of food were required to maintain the low body weight of the two food-restricted groups (rE ($M = 16.16$, $SEM = 0.64$) and rC ($M = 15.39$, $SEM = 0.46$)).

Food intake

Figure 10 shows the average food intake for all four groups across the four experimental phases: the first 10 days following hormone implantation (during which rats in groups rE and rC were maintained on the same restriction schedule), the following 11 days, the first 10 days after hormone removal and the remaining 10 days. During the latter 3 phases all rats had ad libitum access to food. To facilitate the statistical analysis, an overall three-way analysis of variance was carried out only on the data from the last
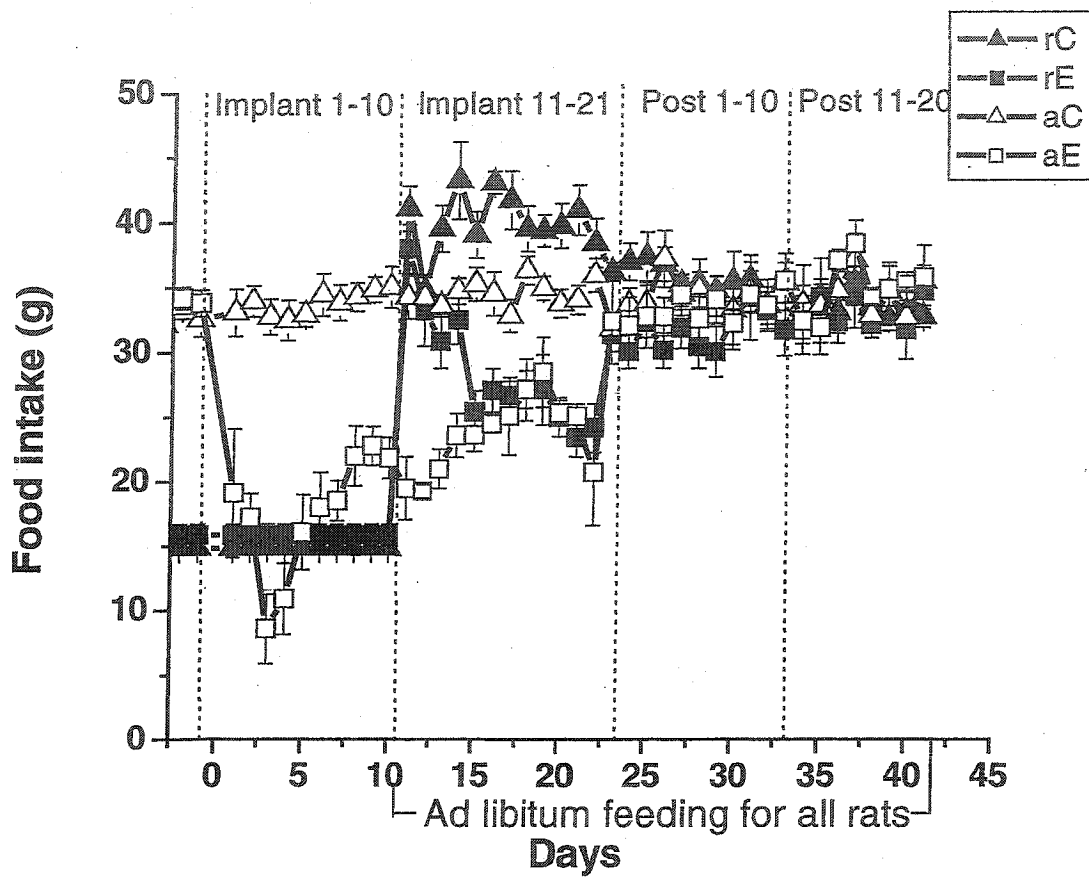
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Figure 10. Average food intake (± SEM) of the four groups of rats across the four phases of the experiment (Filled triangles: food-restricted cholesterol-treated group (rC); filled squares: food-restricted estradiol-treated group (rE); open triangles: ad libitum fed cholesterol-treated group (aC); open squares: ad libitum fed, estrogen-treated group (aE)).

day of each of the four phases.

The analysis showed that there were significant main effects of hormone condition and of phase on food intake ($F_{(1, 24)} = 68.81$, $p < 0.05$ and $F_{(1, 22)} = 14.54$, $p < 0.05$, respectively). In addition, there were significant interactions between phase and both diet condition and hormonal state as well as a significant interaction between hormonal state and diet condition ($F_{(1, 24)} = 162.60$, $p < 0.05$, $F_{(1, 24)} = 5.53$, $p < 0.05$ and $F_{(1, 24)} = 10.50$, $p < 0.05$, respectively).

Body weight

Figure 11 shows the average body weight across the four experimental phases for all four groups. To ease the statistical analysis a three-way analysis of variance was carried out on the change in body weight between the first and last day of each of the four phases of the experiment.

As expected, both food-restriction and estrogen treatment reduced body weight, and both refeeding and removal of hormone implants increased it. These effects were reflected in the significant main effects of phase, diet condition and hormonal state on body weight ($F_{(1, 24)} = 417.79$, $p < 0.05$, $F_{(1, 24)} = 194.68$, $p < 0.05$ and $F_{(1, 24)} = 67.11$, $p < 0.05$, respectively). There were also significant interactions between phase and both diet condition and hormonal state ($F_{(1, 24)} = 42.43$, $p < 0.05$ and $F_{(1, 24)} = 464.90$, $p < 0.05$, respectively). As can be seen in Figure 11, changes in body weight varied as subjects progressed through the different phases of the experiment, such that the aC group gained weight throughout the whole experiment, whereas the aE group lost weight during the first two phases of the experiment and then started gaining weight; the two food-restricted (rE and rC) groups lost weight during the first phase of the experiment
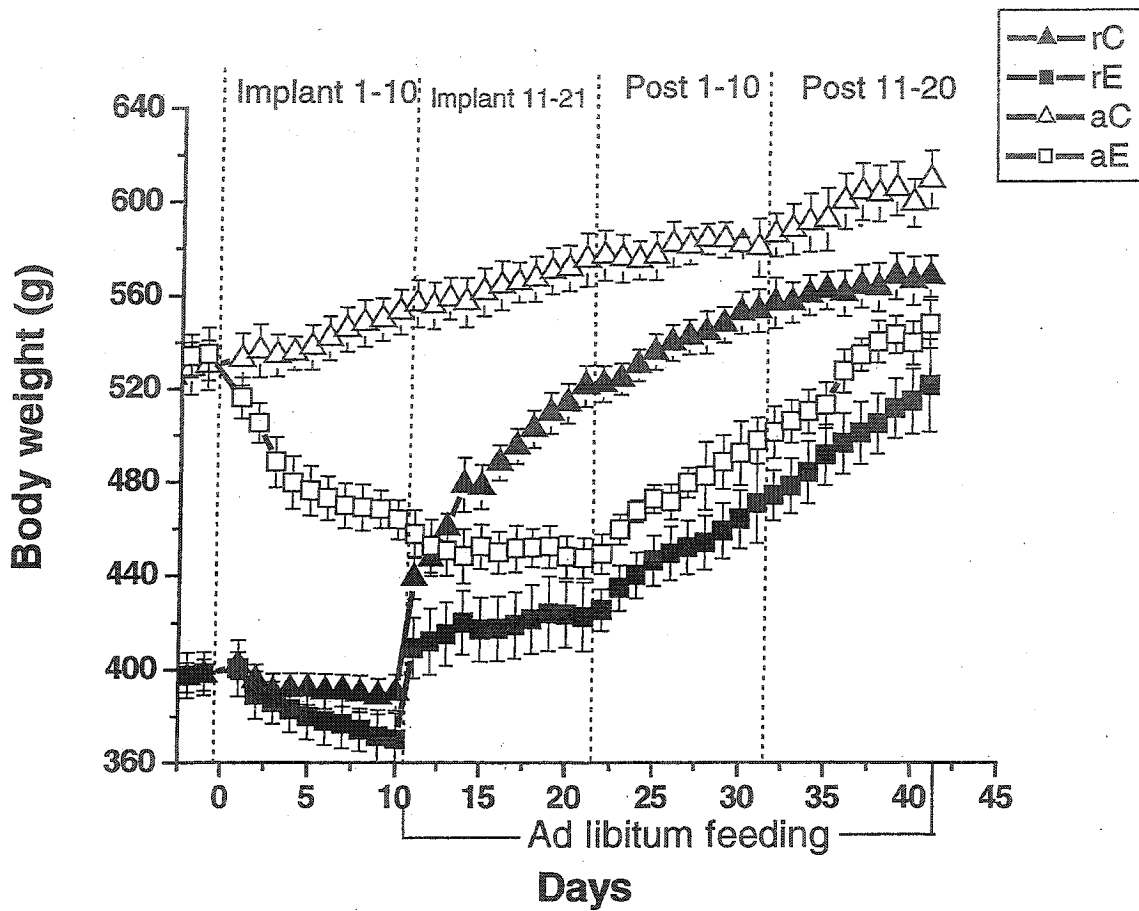
Figure 11. Average body weight (± SEM) of the four groups of rats across the four phases of the experiment (Filled triangles: food-restricted cholesterol-treated group (rC); filled squares: food-restricted estradiol-treated group (rE); open triangles: ad libitum fed cholesterol-treated group (aC); open squares: ad libitum fed, estrogen-treated group (aE)).

and started gaining weight at different rates during the second phase.

To further explore the effects of hormone treatment under ad libitum and food restricted conditions, simple effects were analyzed by running a two-way ANOVA on the food intake and the change in body weight at the end of each of the four phases of the experiment.

<u>Effects of estrogen implants on the food intake and body weight of ad libitum fed and food-restricted rats from days 1 to 10 following hormone implantation.</u>

<u>Food intake</u>

As shown in Figure 12, estrogen implantation reduced food intake in ad libitum fed rats to a level similar to those seen in the food-restricted groups but did not further reduce the food intake of rats in the food-restriction condition.

A two-way ANOVA on the data shown in Figure 12 during phase 1 showed a significant main effect of hormonal state and a significant interaction between diet condition and hormonal state on food intake ($F(1, 28) = 130.83$, $p < 0.05$ and $F(1, 28) = 16.08$, $p < 0.05$, respectively).

<u>Body weight</u>

In the case of body weight, estrogen reduced the body weight of both ad libitum fed and food-restricted rats, although the effect was much larger in the ad libitum fed condition (Figure 13).

A two-way ANOVA performed on these data showed a significant main effect of hormonal state and a significant interaction between diet condition and hormonal state ($F(1, 28) = 199.796$, $p < 0.05$ and $F(1, 28) = 87.178$, $p < 0.05$, respectively).
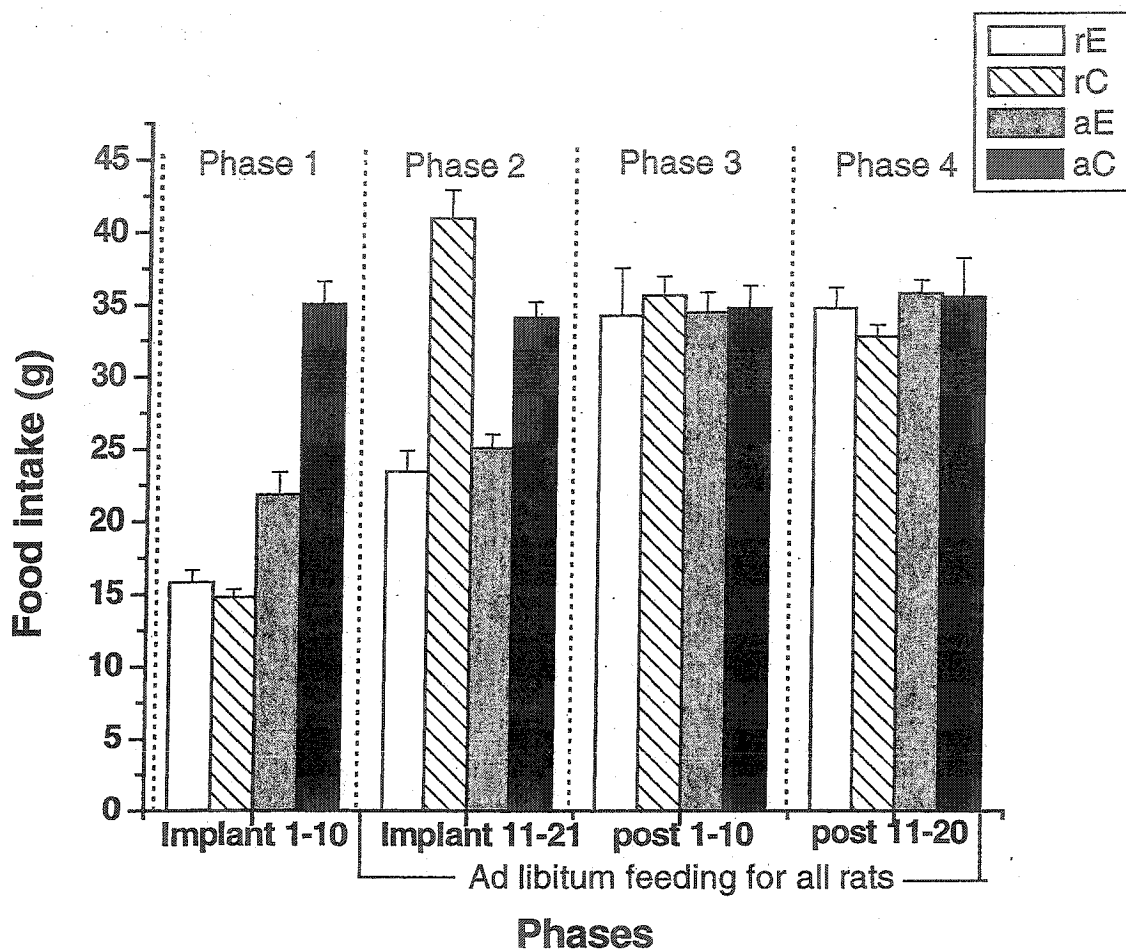
Figure 12. Average food intake (± SEM) of the four groups of rats on the last day of each the four phases of the experiment (white bars: food-restricted estradiol-treated group (rE); striped bars: food-restricted cholesterol-treated group (rC); gray bars: ad libitum fed estradiol-treated group (aE); black bars: ad libitum fed, cholesterol-treated group (aC)).

Figure 13. Change in body weight (± SEM) between the first and last day of each of the four phases of the experiment (white bars: food-restricted estradiol-treated group (rE); striped bars: food-restricted cholesterol-treated group (rC); gray bars: ad libitum fed estradiol-treated group (aE); black bars: ad libitum fed, cholesterol-treated group (aC)).

<u>Effects of estrogen implants on the food intake and body weight of ad libitum fed and</u>
<u>food-restricted rats from days 10 to 21 following hormone implantation.</u>

<u>Food intake</u>

All rats were fed ad libitum as of the beginning of this phase of the experiment. As expected and as can be seen in Figure 12, rats in the rC groups had higher levels of food intake than any other group. This post-restriction increase in food intake was not seen in the estrogen-treated food-restricted group which had similar levels of food intake as the estrogen-treated ad libitum fed group.

A two-way ANOVA carried out on these data showed a significant main effect of hormonal state and a significant interaction between diet condition and hormonal state on food intake ($F_{(1, 28)} = 51.86$, $p < 0.05$ and $F_{(1, 28)} = 8.60$, $p < 0.05$, respectively).
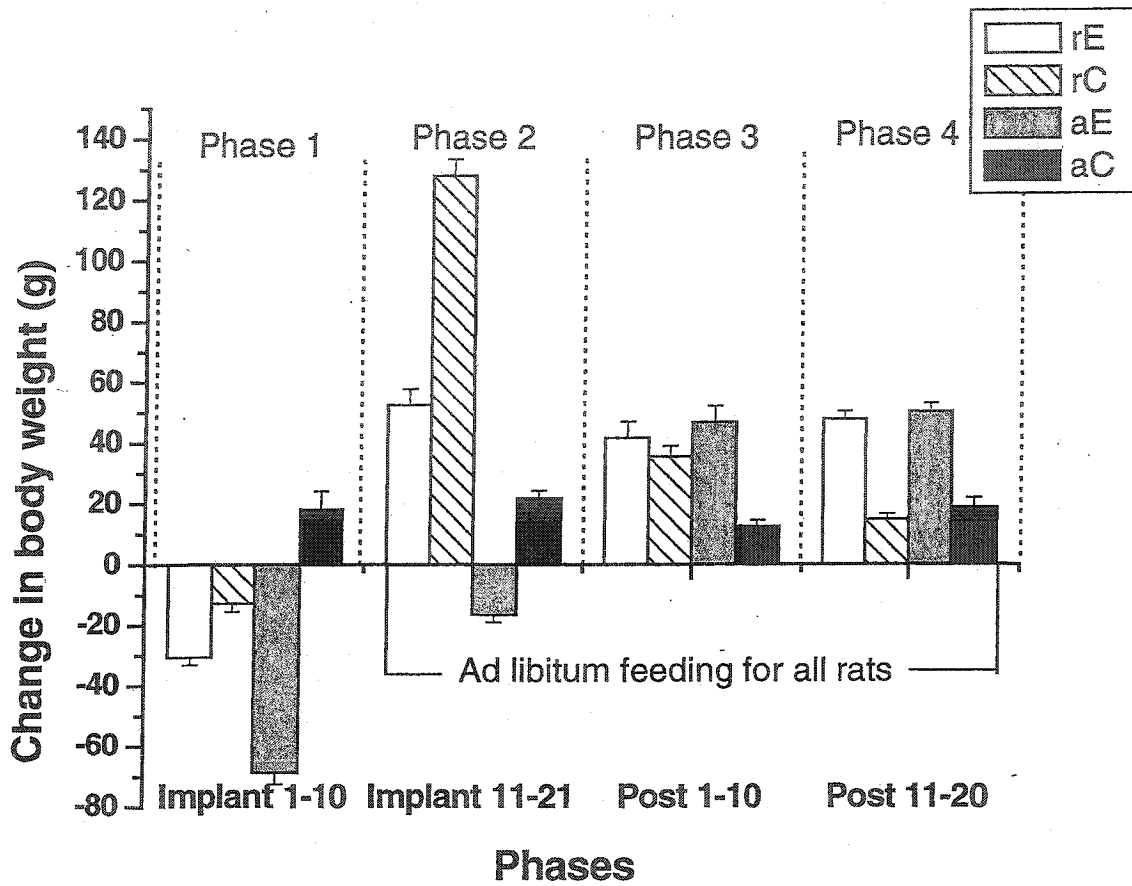
<u>Body weight</u>

The differences in body weight change among groups in this phase were similar to those in food intake. Rats in the rC groups showed a large increase in body weight after refeeding and this effect was attenuated in the rats in the rE group. Furthermore, estrogen treatment continued to reduce body weight in ad libitum fed rats.

A two-way ANOVA of these data revealed significant main effects of diet condition and hormonal state ($F_{(1, 28)} = 363.08$, $p < 0.05$ and $F_{(1, 28)} = 153.03$, $p < 0.05$, respectively) and a significant interaction between diet condition and hormonal state ($F = 16.509$, $p < 0.05$).

Effects of estrogen implants on the food intake and body weight of ad libitum fed and food-restricted rats from days 1 to 10 post hormone implant removal.

Food intake

Removal of the estrogen implants resulted in a rapid increase in food intake with the result that there was no difference in food intake between the groups at the end of this phase.

The two-way ANOVA of these data indicates that there are no main effects of diet condition or hormonal state ($F = 0.154$, $p > 0.05$ and $F = 0.95$, $p > 0.05$, respectively) and no significant interaction between diet condition and hormonal state ($F = 0.507$, $p > 0.05$).

Body weight

Figure 13 shows that rats in the rC, rE and aE groups all gained more weight than those in the aC group during this phase of the experiment. However, as can be seen in Figure 11, the rats previously treated with estrogen remained lighter than the cholesterol treated rats throughout this period.

Two-way ANOVA also indicates that there is a marginal main effect of diet condition ($F(1, 28) = 4.16$, $p = 0.053$) and a significant main effect of hormonal state ($F(1, 28) = 71.06$, $p < 0.05$) on body weight. There is also a significant interaction between diet condition and hormonal state ($F(1, 28) = 10.43$, $p < 0.05$) on body weight.

Effect of estrogen implants on the food intake and body weight of ad libitum fed and food-restricted rats from days 10 to 20 post hormone implant removal.

Food intake

All four groups continued to eat similar amounts during this phase of the experiment.

Again, the two-way ANOVA indicates that there are no main effects of diet condition and hormonal state ($F_{(1, 28)} = 0.254$, $p > 0.05$ and $F_{(1, 28)} = 0.005$, $p > 0.05$, respectively) and no significant interaction between diet condition and hormonal state ($F_{(1, 28)} = 0.007$, $p > 0.05$).

Body weight

Rats that had previously been treated with estrogen continued to show a greater weight gain than cholesterol treated rats during this phase of the experiment.

This pattern of results was reflected in a significant main effect of hormonal state on body weight ($F_{(1, 28)} = 151.83$, $p < 0.05$), and there were no other significant effects.

Discussion

This experiment was designed to gain a better understanding of the effect of estrogen treatment on the body weight and food intake of ad libitum fed and food-restricted male rats. As expected, and consistent with previous findings, (e.g. Mystkowski et al., 2000), estrogen treatment decreased both food intake and body weight in ad libitum fed rats and this effect was maintained throughout the treatment period. Data such as these have been interpreted to suggest that estrogen treatment leads to defense of a lower body weight. However, the fact that estrogen treatment also produced a decrease in the body weight of food-restricted rats that had a body weight at or below that reached by ad libitum fed estrogen-treated rats suggests that changes in defended body weight are not the best way to describe the effects of estrogen. Furthermore, although estrogen treatment produced a pronounced reduction in food intake in the ad libitum fed rats, it had no effect on food intake in the restricted group suggesting that the decrease in body weight seen in this group resulted from increased energy expenditure. Once ad libitum feeding was reinstated for all groups the food intake of the two estrogen treated groups stabilized at similar levels but the body weight of the food-restricted group remained lower than that of the ad libitum fed group. These data also argue against interpreting the effect of estrogen on energy balance as simply a change in defended body weight. Rather, these data suggest that estrogen is able to activate both pathways: those that increase energy expenditure and those that decrease energy intake (in the case of ad libitum fed rats).

There are numerous points at which estrogen could modulate one or both of these systems. For example, estrogen could increase energy expenditure by increasing

sympathetic outflow or through it's action on MCH neurons and it could decrease energy intake by activating satiety mechanisms.

One way that estradiol could increase energy expenditure is by increasing sympathetic outflow. Research has shown that there are estrogen receptors in brain areas involved in the regulation of the sympathetic nervous system such as, the preoptic area (POA), the paraventricular (PVN) and suprachiasmatic (SCN) nucleus and the nucleus of the solitary tract (Pelletier, Liao, Follea, & Govindan, 1988). The location of estrogen receptors in these areas suggest that estrogen perhaps acts directly on the autonomic nervous system and increases sympathetic activity, which results in an increase in energy expenditure.

Estradiol could also increase energy expenditure through its action on MCH neurons. Mystkowski and colleagues (2000) conducted an experiment on estrogen-induced weight loss in male rats. Some systems that normally favor the recovery of lost weight were activated. For example, NPY and AgRP levels increased and POMC levels decreased. Nonetheless, there was a decrease in MCH mRNA and an increase in energy expenditure. These latter findings suggest that the inhibition of MCH neurons induced by estrogen may contribute to its influence on food intake and energy expenditure. However, while investigating the role of MCH in estrogen-induced anorexia, Tritos and colleagues (2004) observed that MCH knockout mice express hypophagia and body weight loss when treated with estrogen. These findings suggest that the anorectic effect of estrogen is independent of MCH. However, results from such knockout experiments need to be interpreted with caution as the absence of MCH throughout neural development could

have been compensated through some unkown mechanism. Thus, the role of MCH in the anorectic effect of estrogen in normal rats is not yet clear.

Estrogen treatment could also decrease body weight and food intake by increasing satiation. In fact, Blaustein & Wade (1976) showed that in female rats the decrease in feeding observed during estrus is entirely due to a decrease in meal size and not to a decrease in meal frequency. Meal size is known to be modulated by signals that act to maintain and terminate eating and hence produce satiation. One signal that controls meal termination is cholecystokinin (CCK). CCK is released from the small intestine during meals and acts as a negative feedback signal. Interestingly, several reports have shown that chronic estrogen treatment increases the satiating action of CCK (Butera, Doerflinger, & Roberto, 2002; Geary, Trace, McEwen, & Smith, 1994; Linden, Uvnas-Moberg, Forsberg, Bednar, & Sodersten, 1989). Thus, estrogen treatment could be decreasing energy intake by increasing satiation.

In summary, the observed body weight loss in estradiol treated subjects could be due to the fact that estradiol may be increasing energy expenditure and decreasing energy intake by increasing sympathetic outflow and increasing the action of satiating signals such as CCK. There is conflicting evidence concerning the possible contribution of MCH.

General Discussion

The primary objective of this work was to enhance our understanding of the sensitivity of LHSS to the manipulation of fat stores by examining the influence of chronic estradiol administration. Like insulin and leptin, estradiol decreases body weight and food intake while increasing energy expenditure. Unlike what was expected based on previous reports on the effects of insulin and leptin on LHSS, the results from Experiment 1 demonstrated that in ad libitum fed male rats, subcutaneous estradiol implantation potentiated BSR in subjects in which the reward effectiveness was sensitive to chronic food-restriction. These findings suggest that the influence of estradiol on BSR differs sharply from that of leptin and insulin. Thus, the mechanisms underlying the reward-modulating effects of these hormones are unlikely to be related to their common physiological actions. The potentiation of BSR during estrogen implantation could be due to a synergy between the direct effect of the hormone and those of weight loss on mesolimbic dopamine neurons. The potentiation of BSR could also be due to a sensitization of the stimulated neurons caused by the repeated periods of weight loss.

To clarify whether the potentiation of BSR is caused by a sensitization of the restriction-sensitive circuitry, it is of interest to investigate the effect of multiple cycles of restriction and re-feeding and to see whether the influence of restriction grows as a function of the number of prior periods of restriction. If the influence of restriction on BSR grows as the rats are repeatedly food-restricted, then this may explain why the effect on BSR of the estrogen-related weight loss was greater in two subjects that the previously measured effect of food restriction. However, if the influence of restriction on BSR does

not grow, then this would mean that the potentiation of BSR during estrogen treatment is due to a more direct effect of the hormone on the brain reward circuitry.

Estrogen levels have been shown to influence mesolimbic dopamine release in response to natural appetitive stimuli (Bless et al., 1997). According to this line of argument, it is possible that estrogen potentiates BSR by potentiating the stimulation-induced increase in dopamine tone (Doherty & Gratton, 1991; Ranaldi & Beninger, 1994). However, it is as yet unknown whether the influence of dopamine on BSR is the same at both restriction-sensitive and insensitive sites. Thus, it would be of interest to determine whether stimulation-induced changes in dopamine tone differ at restriction-sensitive and insensitive sites. This could then be followed-up by investigating the effect of estradiol on BSR in ad libitum fed subjects at stimulation sites where reward effectiveness is insensitive to chronic food-restriction. Such an experiment would enable us to determine whether the effect of estrogen on BSR is specific to particular stimulation sites. If the influence of dopamine is similar at restriction-sensitive and insensitive sites, then estrogen would be expected to produce at least some potentiation of BSR at restriction-insensitive sites. However, this potentiation might be smaller than that seen at the restriction-sensitive sites, if the effect of weight loss on BSR is mediated by non-dopaminergic neurons and summates with the contribution of estrogen-induced enhancement of dopamine release.

Furthermore, it would be of interest as well to compare changes in dopamine release in the nucleus accumbens (NAcc) following chronic food-restriction and estradiol implantation during electrical stimulation of sites where the reward effectiveness is sensitive and insensitive to chronic food-restriction. The NAcc is a good site for

dopamine measurements because it receives dopaminergic projections from the VTA, a site of cell bodies of dopaminergic neurons and also an area known to contain estrogen receptors. Furthermore, research has shown that the dopamine neurons originating in the VTA play an important role in ingestive behavior and self-stimulation (Hoebel et al., 1989; Wise, 1997; Wise, 2002). This would help to clarify the mechanism underlying any direct influence of estradiol on the neural circuitry underlying BSR.

The likelihood that the observed effect of estrogen on BSR was due, at least in part, to estrogen-induced weight loss complicated the interpretation of the findings. This problem might be minimized by testing the effect of estrogen on BSR in food-restricted male rats in which body weight is held constant by adjustment of the daily ration. However, the effect of estrogen implantation on the body weight and food intake of food-restricted male rats is not yet known. Experiment 2 was carried out in order to obtain this information, comparing the effects of estradiol in ad-libitum fed and food-restricted subjects.

The results of Experiment 2 showed, as expected, that estrogen treatment decreased both food intake and body weight in ad libitum fed rats. After the end of the restriction period, the food intake of the restricted subject increased. This implies that it would be possible to hold constant the weight of food-restricted, estrogen-treated, male rats by adjusting their daily ration. In other words, the modest decrease in body weight observed during the period of restriction could be eliminated by increasing the amount of food provided. Thus an experiment on the effect of estrogen on BSR in "weight-clamped" subjects is feasible. By eliminating the influence of estrogen-induced weight loss, such an experiment should more clearly reveal any direct effect of estrogen on BSR.

It was striking that following chronic food-restriction and stabilization of body weight to a level below that of ad libitum fed rats treated with estradiol, the subcutaneous estrogen implantation further decreased mean body weight in food-restricted estrogen-treated subjects. The decrement of body weight during estradiol implantation, in food-restricted subjects could be due to increased energy expenditure, which, in turn, could stem from increased sympathetic outflow and perhaps from inhibition of MCH neurons.

One way to determine whether MCH neurons contribute to the estrogen-induced anorexia is to administer centrally an MCH antagonist in the ventricule during the duration of the estrogen treatment and to measure body weight and food intake. If estrogen-induced anorexia remains in these conditions, than the effect of estrogen on body weight and food intake is independent of MCH neurons.

It would also be of interest to investigate whether the decrease in body weight during estrogen-treatment in food-restricted subjects is due to an increase in sympathetic outflow. One way to test this would be to measure energy expenditure before and after estrogen-treatment by measuring oxygen consumption and the respiratory quotient (Hwa, Ghibaudi, Gao, & Parker, 2001), and to see whether there is a correlation between weight loss under estrogen in food-restricted rats and oxygen consumption and the respiratory quotient.

Due to the fact that, in both experiments, estradiol was implanted in the periphery, there is no means of knowing whether the suppression of food intake and body weight was due to estradiol's action on central mechanisms. Thus, one potential experiment could be to chronically administer estradiol centrally and observe if this mimics the

suppression of food intake and body weight obtained following subcutaneous estradiol implantation.

Taken together, the findings of the two experiments add to our knowledge of the effects of estrogen on brain reward circuitry, ingestive behaviour, and energy expenditure in male rats. The effect of estrogen on BSR contrasts sharply with those of other anorexic agents, such as insulin and leptin. The results of the experiments point to future avenues for exploring the mechanisms underlying the action of estrogen on brain reward pathways.

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