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Peripubertal, leptin-deficient *ob/ob* female mice were used in an investigation of exogenous estradiol (E_2) and BIBP3226, a neuropeptide Y (NPY) antagonist acting at the Y1 receptor, on food consumption, body mass and sexual receptivity. The absence of leptin in the *ob/ob* model has been proposed to result in chronic hyperphagia due to high levels of NPY signaling. Research findings suggest that NPY Y1 receptors, whether located in the CNS or PNS, are likely candidates for mediating the orexigenic actions of NPY. Moreover, the lack of reproductive organ development characteristic of *ob/ob* mice is proposed herein to be consequent of excessively elevated NPY levels activating peripheral Y1 receptors. Reproductive organ development and function were restored by exogenous estradiol administration as well as by a combined treatment with BIBP3226 and E_2, suggesting a role of E_2 in mediating NPY's effects on caloric intake, reproductive development and sexual receptivity.

THE EFFECTS OF PERIPHERALLY ADMINISTERED 17-β ESTRADIOL AND BIBP3226, A NPY Y1 RECEPTOR ANTAGONIST, ON FOOD INTAKE, BODY MASS, REPRODUCTIVE DEVELOPMENT AND BEHAVIOR IN FEMALE LEPTIN-DEFICIENT *OB/OB* MICE.

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CHAPTER I

INTRODUCTION

Heightened awareness of metabolic syndromes has sparked aggressive research to identify the underlying mechanisms of obesity. Researchers have learned that fat deposition in mammals is regulated by a complex signaling network that continually communicates energy status between the central (CNS) and peripheral nervous systems (PNS) [1, 2]. To find new targets for pharmaceutical therapies, researchers are now exploring a multitude of CNS and PNS signals that influence metabolism.

The hypothalamus has long been known to be the central integration center that responds to signals related to the availability of fuel molecules in the body by effecting changes in behavior (appetite) and physiology (metabolic rate) [3, 4]. Hypothalamic regions, especially the arcuate nuclei (ARC), are richly innervated with both anorexigenic and orexigenic neuronal projections [5, 6]. Organismal energy balance is maintained by signals from these counter-regulatory pathways that stimulate or inhibit metabolism via alterations in caloric intake and in energy expenditure. Colocalization of the known key appetite-regulating pathways, including the appetite-stimulating signals, Neuropeptide Y (NPY) and Agouti-related protein (AgRP), as well as the appetite-inhibiting signals, pro-opiomelanicortin (POMC) and melanocyte-stimulating hormone (MSH), highlight the ARC as a key regulatory site of energy balance and metabolism [1-6] . In addition, leptin, a hormonal peptide that has major anorexigenic actions, functions in both the CNS

and PNS to signal satiety and to attenuate caloric intake [7-9]. In contrast, NPY is a protein that works as a neuronal transmitter or modulator to exert potent orexigenic actions: it stimulates hunger sensations and increases energy intake [11, 12].

The dynamic relationship between the leptin and the NPY systems maintains energy homeostasis and helps to effect the long-term maintenance of a relatively constant body mass (Figure 1) [2, 3]. Manipulation of the opposing actions of these networks in the CNS has been the focus of extensive research toward pharmaceutical attempts to treat obesity. However, the roles of these energy-regulating signals in the PNS are not well understood.

Adequate nutrition is a prerequisite for normal reproductive development. Energy deficits or excess, in the form of adipose tissue, typically halt reproductive functions, especially in female mammals, where the estrous cycle and sexual behavior can be disrupted by malnutrition [14, 15]. Metabolic hormones and neuronal signals, including leptin and NPY, and steroid hormones, such as estradiol, dynamically regulate metabolic changes, alter food intake, energy expenditure, adiposity, fertility and reproductive behavior [4-6]. Sufficient energy reservoirs, in the form of adipose tissue, are necessary to ensure adequate energy supply and reproductive success [10-12]. Limited energy supplies in the form of adipose tissue require physiological alterations to support organismal survival [14]. In times of severely limited energy stores (fasting), fertility and reproductive behavior are eliminated in favor of energy utilization for basic organismal needs and survival [14-15].

Reproductive behavior has a large cost in energy transfer. Adequate stores of chemical energy are thus critical to support reproductive behavior. Correspondingly, reproductive functions are minimized during energetic challenges [14, 21]. Extreme metabolic conditions are characterized by minimal adipose tissue, as seen in patients with anorexia nervosa. Surprisingly, excess adipose tissue, as seen in obese individuals, can also induce a similar stress response resulting in a state of nutritional infertility [13]. Extreme metabolic stress, whether due to abnormal leanness or obesity, inhibits the hypothalamic-pituitary-gonadal axis in female mammals: the resulting decrease of gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus suppresses luteinizing hormone (LH) release, halting ovulation and ultimately inhibiting the synthesis of ovarian steroid hormones, thus halting the expression of female sexual behavior [11,13, 22].

Due to the continual interaction between metabolic and reproductive cues, a minimal alteration in one system parallels significant changes in the other. Continual crosstalk exists between these dynamic signaling systems [23]. In times of fasting, elevated circulating leptin levels attenuates estrogen signaling via target receptors in the arcuate nucleus of the hypothalamus, thus reducing the secretion of luteinizing hormone and follicle-stimulating hormones (LH & FSH) release, thus preventing estrous cycling [23], clearly supporting the effects of metabolic cues on reproductive ability (in mammals) [24, 25].

Sexual receptivity in female rodents is characterized by lordosis, an estrogen-dependent behavior that signals copulatory readiness to males [4]. Lordosis frequency

increases with estrogen dose [27]. Estrogens, in part, contribute to sexual behavior by stimulating specific hypothalamic brain regions, especially the ventromedial nucleus (VMN) of the hypothalamus and ARC [27, 28]. The area of specific interest in this study is the ARC, which is richly innervated with estrogen- and leptin- sensitive neuronal projections. Estrogen and leptin elicit behavioral changes in sexual behavior as a consequence of binding to their target receptors in these hypothalamic regions [5].

The presence of anorexigenic leptin inhibits appetite and enhances the expression of sexual behavior in female hamsters. In times of fasting, sexual behavior is absent thus preventing fertility [13]. Similar to wild-type mice in a severely fasted state, *ob/ob* mice are sexually non-responsive and are infertile [27]. As expected, leptin treatments alleviate reproductive impairments [23], thus implicating a critical role for leptin not only in fat deposition but also in regulating reproductive behavior and therefore evolutionary success. Previous studies have revealed that leptin treatments will promote sexual behavior in fed but not fasted rodents, suggesting a role of leptin as a potential sex signal [9]. In further support of a role for leptin as a reproductive signal, inhibition of lordosis was found to be intensified in food deprived, fasting rodents [9, 10, 29, 30]. Since leptin levels have a positive correlation with body fat mass, reproductive behavior and sexual responsiveness may be in part heightened by increased caloric stores in the form of adipose [9, 10,30]. When female mammals have inadequate or excess calories, reproductive behavior and fertility are impaired.

The mechanistic interactions of leptin with other metabolic cues, including estrogen and NPY, to alter reproductive behavior are highly complex and remain poorly

understood. Previous work by Wade et al. identified a significant reduction in lordosis duration in estrogen-treated, food-deprived wild-type rodents [9, 30]. Similarly, combined treatments of leptin and estrogen did not alter lordosis behavior in food-deprived rodents [9]. However, leptin treatment alone has been found to significantly shorten lordosis duration in food-deprived animals [9, 30]. Based on these findings, food deprivation or inadequate energy stores appears to override the influence of estrogen and leptin to increase lordosis duration.

Estrogen

The ovarian sex steroid, estradiol (E_2), has a crucial role in normal reproductive organ development and puberty onset [32]. Moreover, E_2 exerts an anorexigenic, leptin-like effect on energy homeostasis via estrogen receptors that are broadly distributed throughout the ARC, in parallel with receptors for leptin and NPY (described below, Figure 2) [9, 33]. The exact anorexigenic actions of E_2 in the CNS and PNS remain unclear, but an interaction with the leptin-dependent melanocortin system appears likely, as described below (Figure 3) [9, 26]. Estradiol is known to have anorectic effects on food intake and it can reduce adiposity [33, 34]. Administration of exogenous estradiol in ovariectomized rodents as well as rodents with intact ovaries reduces food intake and body mass [29]. Work by Gao et al. suggested that E_2 substantially increases excitatory inputs onto POMC neurons of the ARC in normal phenotype rodents [34]. Synaptic rearrangement has been found to occur independently of leptin in wild-type, *ob/ob* mice

and leptin receptor deficient *db/db* mice [29]. Moreover, body weight reductions induced by E_2 treatments were linked to Stat 3 activation in the brain [34].

Hormonal changes in the ARC may determine body weight regulation and could be a potential pharmaceutical target for obesity. Intracerebroventricular (icv) administration of estradiol into regions of the ARC, including the medial preoptic nucleus (MPN) and paraventricular nuclei (PVN), caused decreased caloric intake and food mass [3, 9]. These hypothalamic regions are heavily innervated with neuronal networks sending orexigenic and anorexigenic signals that maintain energy homeostasis. E_2 treatments have been found to increase *c-fos* expression in POMC neurons in the ARC [13]. E_2 appears to have an anti-obesity effect that can be expressed independently of leptin-based pathways, as evident by results showing a significant decrease in body weight in leptin-deficient (*ob/ob*) and leptin-receptor deficient (*db/db*) mice over a 24 day treatment [13, 29]. Moreover, a fifty percent reduction in weight gain was evident after 4 weeks in male and female mice treated with estradiol [13].

Administration of E_2 to rodents reduces their caloric intake and body mass by enhancing energy expenditure [4-6, 36]. Disruption of the aromatase enzyme gene, thus blocking the conversion of androgens to estrogens, results in an obese phenotype in rodent models [16, 17]. Further supporting the hypothesis that estrogen resistance results in adiposity, estrogen alpha-receptor (ER-α) knock-out mice have been reported to exhibit an obese phenotype [13, 35, 37]. Previous studies of the estrogen receptors, ER-α and ER-β, using estrogen receptor knockout-mice, have linked ER-α gene deletion to increased body weight and insulin resistance [37]. Inactivation of the ER-α receptor in

rodent models results in a hundred-fold increase in fat mass, thus suggesting a role for ER-α in maintaining a lean phenotype [13]. In the absence of ER-α, estrogen does not reduce food intake or body weight [13]. Estrogen mediation or activation of melanocortin pathways via ER-α may explain its anorectic effects [38].

Similar to the ER-α knockout phenotype, leptin-deficient *ob/ob* mice exhibit insulin resistance partnered with extreme obesity, and develop huge adipose stores [37]. Both models implicate a critical role for estrogen signaling, likely through the ER-α receptor, in mediating its anorexigenic actions to maintain energy homeostasis and a normal body mass [17, 35]. It is evident that in the absence of estrogen ER-α signaling, as seen in the ER-α knock-out mouse, leptin signaling alone is insufficient to maintain a normal body mass. The fact that leptin-deficient *ob/ob* mice exhibit impaired estrogen signaling, suggests an interaction between leptin and estrogen pathways in maintaining a normal body mass phenotype. Similarities between the anorectic actions of leptin and estrogen suggest they both may affect the same neuronal targets in the hypothalamus, and in peripheral organs [9]. Theoretically, exogenous estradiol administration would have a restorative effect in leptin-deficient mice in attenuating appetite and reducing body mass, as well as stimulating estrus and mating. This question is a key objective of this study (see Chapter II).

Several studies suggest that E_2 has direct effects on hypothalamic neurons, specifically leptin- and NPY-modulated neurons [39, 40]. Hypothalamic NPY concentrations significantly increase in number after ovariectomy in rodents, followed by significant body weight increase over 10 weeks, suggesting that E_2 inhibits NPY

synthesis and/or release and thereby mediate the effects of NPY on energy homeostasis [29]. Similarly, researchers have demonstrated that estrogen deficiency leads to hyperleptinemia, thus E_2 is implicated in the excitatory and inhibitory signaling actions of leptin and NPY, respectively [9]. Ovariectomized rodents, in the absence of estrogens, require extremely high leptin doses before they will begin to reduce body mass [29]. These findings suggest that estrogens are potent mediators of the metabolic signals leptin and NPY, and suggest the basis for a molecular mechanism by which E_2 exerts strong influences on both metabolism and food intake.

A dual function of estrogen as a modulator of reproductive organ development and sexual behavior ties in with its anorexigenic actions to maintain energy homeostasis (Figure 4). Thus, estrogens might be a tool used to identify issues in nutritional infertility. Though estrogen does not solely dictate metabolic and reproductive ability, its interactions with other major endocrine/hormonal signals requires clarification to identify and treat physiological disruptions that hinder metabolism and reproductive ability.

Leptin

Leptin, a protein secreted from adipocytes in amounts proportionate to the amount of adipose tissue, decreases caloric intake and stimulates energy expenditure by interactions with its receptors in the ARC of the hypothalamus [7]. Leptin receptors, in five receptors, Ob-Ra through Ob-Re, are widely distributed throughout the CNS and PNS, but are heavily concentrated in the ARC and PVN, where they exert anorexigenic actions by two proposed pathways [9]. Leptin receptors are co-localized in the

hypothalamus with NPY, AgRP, and POMC neurons [7]. Leptin-based modulations of food intake and body mass are partially due to its actions in the hypothalamus, where it interacts with OB-Ra to stimulate the synthesis of anorexigenic peptides (Figure 3) [7]. High circulating concentrations of leptin secreted from active adipocytes act in the ARC to stimulate anorexigenic neurons to ultimately increase synthesis and release of POMC cleavage products, including α-melanocyte-stimulating hormone (α-MSH) [2, 18]. Alpha-MSH binds to its receptors, MC3R and MC4R, to signal satiety, decrease caloric intake and activate pathways that increase energy expenditure [41]. Leptin's stimulation of α-MSH also inhibits orexigenic NPY-signaling in the hypothalamus, via leptin receptors distributed on NPY-containing neurons within the ARC [9, 42].

Further support of the counter-regulatory roles of leptin and NPY comes from the observation that exogenous leptin can reduce the quantity of NPY mRNA *in vivo* and NPY release *in vitro* [43]. NPY neuronal firing is increased threefold during fasting, and, as expected, it is inhibited by leptin administration [7]. Considering the impact that leptin exerts on NPY production and release, it is reasonable to believe that the hyperphagic, obese and infertile phenotype of the leptin-deficient *ob/ob* mouse is the consequence of excessive, chronic NPY signaling due to the chronic lack of leptin's inhibitory actions on the NPY signaling.

Neuropeptide Y

NPY is the most potent orexigenic peptide identified to date. NPY activity leads to conservation of the body's energy reservoirs by reducing energy expenditure and by

enhancing appetite [19, 20, 49]. NPY acts on at least six receptor subtypes (Y1throughY6) distributed centrally within the ARC of the hypothalamus and in peripheral tissues including adipose, ovaries, small intestine and liver [12, 20]. Continual intracerebroventricular (icv) administration of NPY or an NPY analog results in a perceived sense of starvation and induces hyperphagia. Even in animals that have fed to satiation, exogenous NPY administration induces hyperphagia and reduces the activity of the sympathetic nervous system, thereby reducing the overall metabolic rate [42, 45, 46]. In addition, the extremely obese phenotype of the *ob/ob* mouse model is associated with the over-expression of hypothalamic NPY [20]. The mating of male *ob/ob* mice with female NPY knockout mice produces progeny that exhibit a 50% reduction in adipose tissue compared to the *ob/ob* parents [9]. Moreover, increases in hypothalamic NPY RNA and decreases in POMC RNA are normalized following leptin treatment in the *ob/ob* model [7]. Under chronically elevated NPY signaling, increased food consumption and decreased energy expenditure promote positive energy balance and lead to increased body mass, as seen in the *ob/ob* phenotype.

Identification of the specific NPY receptor that mediates its orexigenic effects is relevant for developing effective drugs to treat obesity. Experiments suggest that hyperphagia in the *ob/ob* mice might be due to continual stimulation of NPY on the Y1 receptors [12]. Genetic disruption of the Y1 receptor allele in *ob/ob* mice reduced appetite and initiated activity in the gonadotrope axis [25, 39]. Both wild-type and Y1 receptor knockout mice start life with equivalent birth weights, but Y1 receptor knockout mice weigh less than wild-type controls by the time of weaning (21 days of age) [10].

The Y1 receptor proposed to mediate orexigenic NPY actions is the target of this experimental design and supported by previous findings suggesting a potential dual function of the NPY-Y1 receptor activation in both energy homeostasis and reproductive organ development [47]. The reduced weights of seminal vesicles and reduced levels of pituitary LH contents in *ob/ob* male mice are restored to normal in offspring of *ob/ob* mice mated to NPY Y1 receptor knockout mice [25]. These findings further implicate an inhibitory role for Y1 receptor activation on the HPG axis [25].

The presence of sex steroids might be a prerequisite for NPY mediation of reproductive organ development. In the absence of sex steroids, icv administration of NPY inhibits LH release in the wild-type phenotype, suggesting a sex-steroid dependent action of NPY on the HPA axis to modulate reproductive development [39]. A direct mechanism by which sex steroids alter NPY gene expression is not yet established, but likely changes NPY and/or NPY receptor synthesis and release [39]. In food restricted conditions, Y1 receptor knockout mice appear to be protected against gonadotrope axis disruptions unlike wild-type controls that exhibit delayed puberty and low circulating levels of LH, further supporting the role of Y1 receptor activation in the inhibition of reproductive development [10, 48].

With increased attention towards the obesity epidemic, characterizing the peripheral role of NPY in energy homeostasis is critical for the development of pharmaceutical interventions in this system. There is strong evidence suggesting that NPY modulates metabolism by stimulating energy intake, and alters reproductive development and behavior, via an unidentified NPY receptor subtype [49]. The role of

NPY taken together with evidence that Y1 receptor activation leads to impaired metabolism and reproductive development, focuses attention on the Y1 receptor as the primary target for understanding the orexigenic effects of NPY.

Ob/ob Model

Ob/ob mice are characterized by chronic hyperphagia, a continuous sense of perceived starvation, morbidly obese phenotype and lack of reproductive maturation. Not only do they remain infertile, adults continue to consume twice the calories and weigh up to twice the body mass of wild type mice [27]. This model provides insights for identifying the underlying regulators in the complex networks that control energy homeostasis/metabolism and reproduction.

Continual crosstalk exists between neuroendocrine regulators of energy homeostasis and reproduction. Furthermore, several regulating hormones, including estrogen, leptin and NPY, have dual functions in communicating energy and reproductive ability as well as similar neural signaling targets (ARC) [3]. Knowing that food availability directly affects metabolism and reproductive behavior, it is reasonable to conclude that excess calories, stored in the form of adipose, could also affect fertility and reproductive ability. However, the impaired reproductive organ development (maturation) and absence of estrus in *ob/ob* female mice alludes to endocrine signaling disruptions as a possible underlying mechanism in infertility (Figure 5).

Food availability is a general indicator/predictor of reproductive success, especially in female mammals where pregnancy and lactation can require a doubling (or

more) of metabolic rate. However, the leptin-deficient *ob/ob* model presents the opposite case, i.e., where energy excess impairs reproductive development and functioning. More likely, disruption of one or more of the major hormones, leptin, estrogen and/or NPY, halts network communication and reduces reproductive signaling [13].

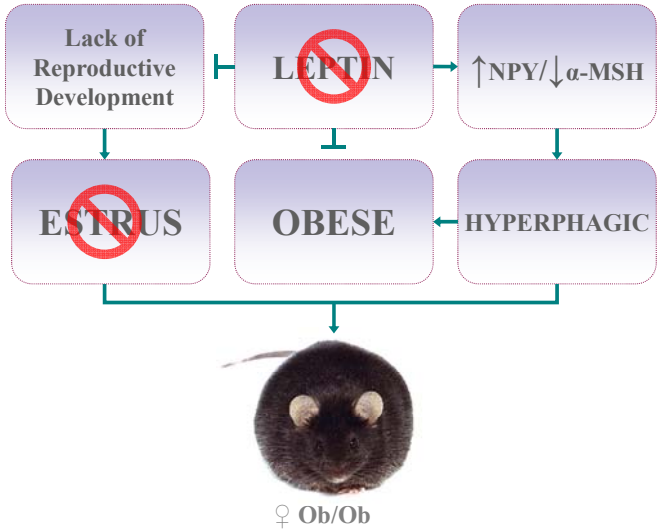

FIGURE 1: Overview of the leptin-deficient *ob/ob* mouse model.

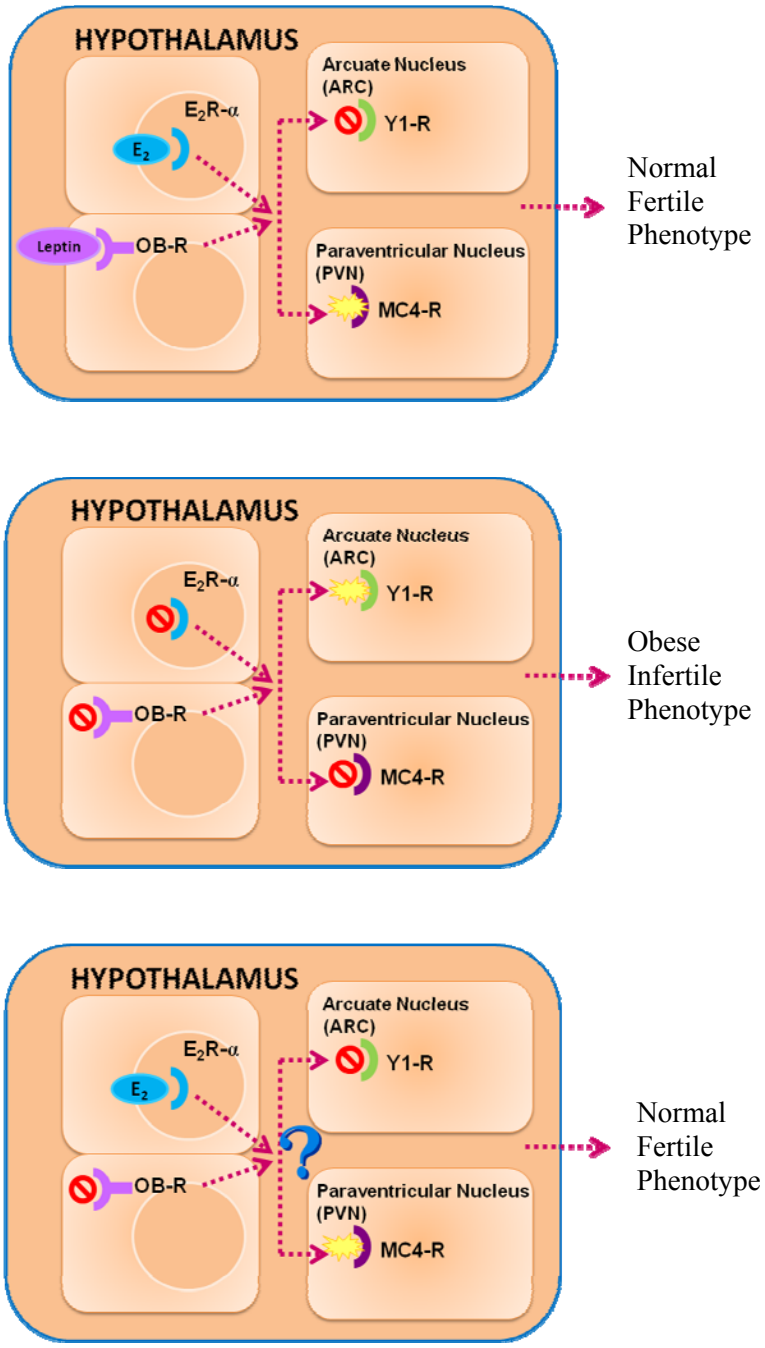

FIGURE 2: E_2 and leptin presence or absence on phenotype.

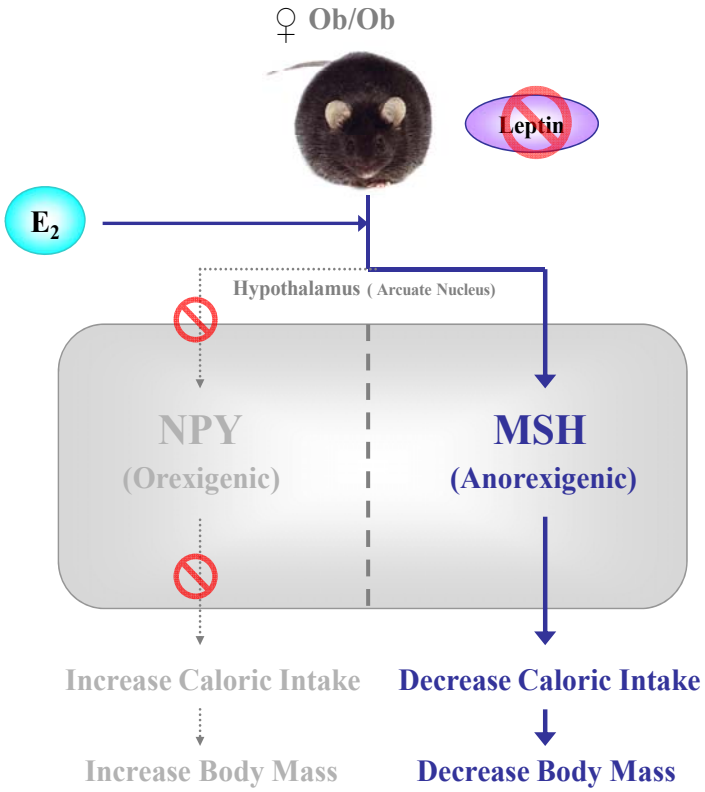

FIGURE 3: Expected physiological changes in the *ob/ob* mouse when administered exogenous E_2.

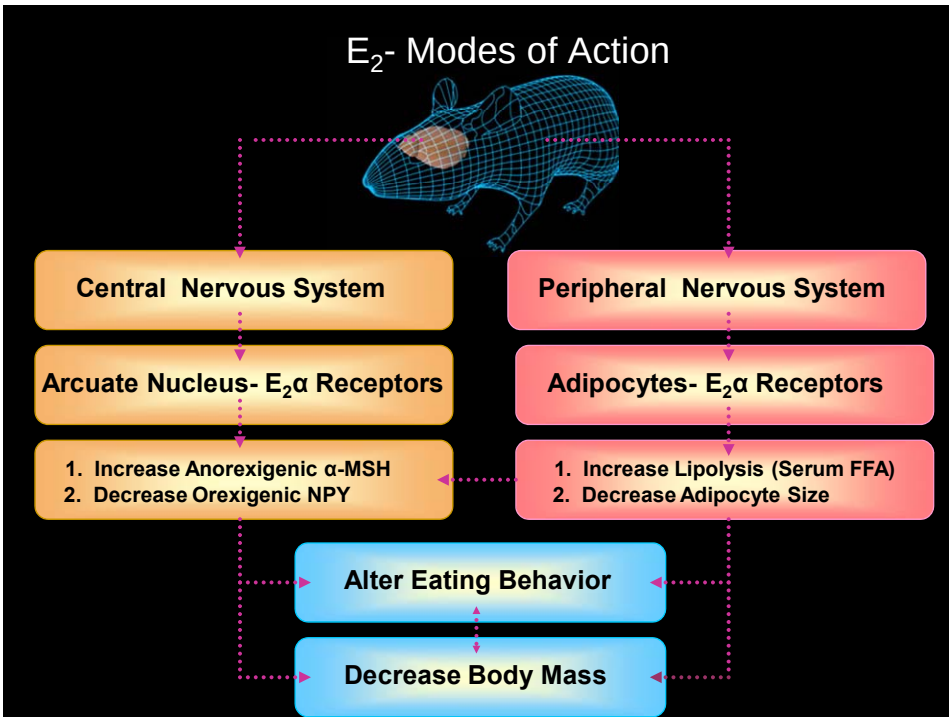

FIGURE 4: E_2 modes of action in the central and peripheral nervous system.

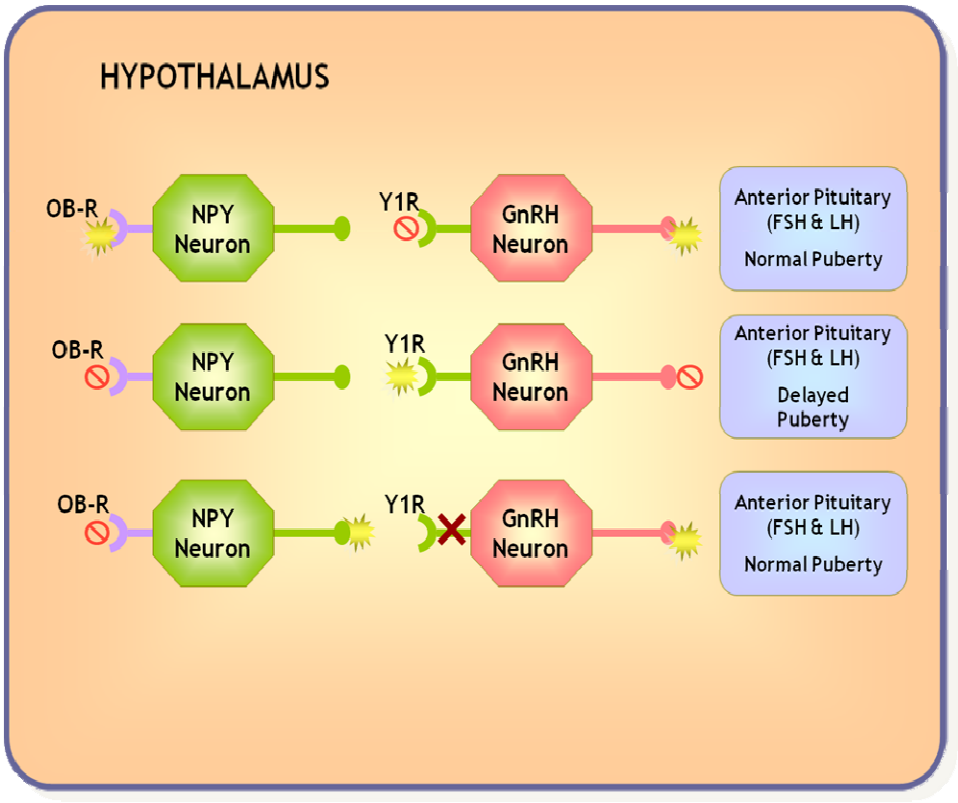

FIGURE 5: Hypothalamic changes that influence puberty.

Hypothesis

This study on female mice lacking leptin, a major anorexigenic signal in mammals, examined the role of NPY signaling in the periphery. A fundamental assumption underlying the proposed work is that the absence of leptin in the *ob/ob* model results in NPY-mediated chronic hyperphagia, an obese phenotype and reproductive impairments. I manipulated NPY and estrogen signaling in the leptin-deficient mice to evaluate the interrelationship between leptin, NPY and estrogen. Previous research suggests that NPY Y1 receptors, whether located in the CNS or PNS, are likely candidates for mediating the orexigenic action of NPY [25]. Moreover, the lack of reproductive development in *ob/ob* mice is hypothesized to be the consequence of elevated NPY levels. If so, reproductive development might occur after inhibition of NPY Y1 receptors in combination with E_2 administration. The role of E_2 in mediating NPY effects on caloric intake and reproduction were also assessed.

Specific Aims

1. I propose that elevated NPY signaling in fasted wild-type mice parallels elevated NPY signaling in leptin-deficient mice, such that increase appetite in both cases is the response to increased Y1 receptor activation by NPY [25, 39, 42]. To determine whether or not hyperphagia and obesity in the *ob/ob* mouse model could be ameliorated by blocking NPY signaling, the NPY Y1 receptor antagonist, BIBP3226, was administered to prepubertal female *ob/ob* mice. Female *ob/ob* mice received BIBP3226 via intraperitoneal (ip) injections twice daily at 12 hour intervals for 14 consecutive days,

while body mass and food consumption were measured twice daily. If injecting the antagonist reduces food intake and body mass, these data will support the hypothesis that high levels of NPY signaling contribute to hyperphagia and the resultant obesity in leptin-deficient mice (Table 2, Box 2).

2. I propose that E_2 exerts anorexigenic effects in leptin-deficient mice. Preliminary findings from my previous experiment support the hypothesis that the leptin-independent actions of exogenous E_2 reduce caloric intake and body mass in female *ob/ob* mice. To confirm the anorexigenic actions of E_2, 17β-estradiol was administered to prepubertal female *ob/ob* mice. Female *ob/ob* mice received 17β-estradiol dissolved in sesame-oil vehicle via subcutaneous injections twice daily. Injections occurred every 12 hours for 14 consecutive days, while body mass and food consumption were measured twice daily. At the end of 14 days of treatment, ovarian and uterine measurements were taken. If exogenous E_2 reduces food intake and body mass, my previous work suggesting an anorexigenic action of E_2 will be confirmed (Table 2, Box 3).

3. I propose that exogenous E_2 administration and Y1R inhibition in leptin-deficient mice each have additive effects in reducing food intake and body mass. To characterize the relationship between E_2 and NPY, both 17β-estradiol and NPY Y1R antagonist were administered, as the "combination treatment," to prepubertal female *ob/ob* mice. 17β-estradiol and BIBP3226 were administered peripherally via subcutaneous and intraperitoneal injections, respectively. Each treatment (17β-estradiol & BIBP3226) was

administered twice daily, totaling 4 injections per day per animal, every 12 hours for 14 consecutive days. If the combined treatment of E_2 and BIBP3226 reduces food intake and body mass, then these data will support the hypothesis that there is an interaction between E_2 and the Y1 receptor subtype in mediating energy balance in the leptin-deficient model (Table 2, Box 4).

4. I propose that elevated NPY signaling inhibits the reproductive axis in leptin-deficient female mice by means similar to the reproductive inhibition that has been observed in fasted wild-type mice. To determine whether or not reproductive development in the *ob/ob* mouse model results from leptin absence and/or from increased NPY signaling, the NPY Y1 receptor antagonist was administered to female *ob/ob* mice. Female *ob/ob* mice received BIBP3226 via intraperitoneal injections twice daily at 12 hour intervals for 14 consecutive days. Following 14 days of treatment, ovarian and uterine weights were compared between treatment groups. If treatment with the Y1R antagonist is associated with increased uterine and ovarian mass, then these data will support the hypothesis that high levels of Y1R activation impairs reproductive development (Table 2, Box 2).

5. I propose that E_2 exerts uterotrophic effects in leptin-deficient mice. Theoretically, leptin absence and/or elevated NPY signaling results in impaired reproductive organ development by inhibiting endogenous E_2 signaling in leptin-deficient mice. My previous findings demonstrated that exogenous E_2 administration to female *ob/ob* mice was associated with reproductive organ development that was comparable to that of wild-

type mice. To confirm the role of E_2 on reproductive development, 17β-estradiol was administered to prepubertal female *ob/ob* mice at the dose indicated in aim 3. Ovarian and uterine measurements were taken as described previously. If exogenous E_2 administration is associated with increased uterine and ovarian mass, these data will support the hypothesis that E_2 signaling influences hypothalamic peptide levels, leptin and NPY, and contributes to reproductive development in *ob/ob* mice (Table 2, Box 3).

6. I propose that exogenous E_2 administration and Y1R inhibition will contribute in additive fashion to promote uterine and ovarian development in leptin-deficient mice. To characterize the combined effects of E_2 and NPY blockade on reproductive development in the absence of leptin, both 17β-estradiol and NPY Y1R antagonist were administered to prepubertal female *ob/ob* mice. 17β-estradiol and BIBP3226 were administered peripherally via subcutaneous and intraperitoneal injections, respectively. Each treatment (17β-estradiol or BIBP3226) was administered twice daily, totaling 4 injections per day per animal, every 12 hours for 14 consecutive days. Following euthanasia, ovaries and uteri were collected for analysis. If the combined treatment of E_2 and BIBP3226 enhances reproductive development to the extent that uterine masses exceed that observed in response to either E_2 or the Y1 receptor antagonist, then these data support the hypothesis that combined E_2 signaling and Y1 receptor blockade is a more potent signal for reproductive development in the leptin-deficient model than either treatment by itself (Table 2, Box 4).

7. I propose that Y1R activation inhibits reproductive behavior in leptin-deficient mice. To understand the impact of Y1R activation on reproductive behavior, female *ob/ob* mice were tested for sexual receptivity following a twelve day treatment of Y1R antagonist. Following treatment on day thirteen, each female *ob/ob* mouse was tested for sexual receptivity in a 10 minute test with a sexually active wild-type male, followed by an extended sexual behavior test occurring on treatment day 14 where each female *ob/ob* mouse was paired with the same stud male for 12 hours. Following the 12 hour behavioral testing, all female mice were euthanized via CO_2 asphyxiation and the presence or absence of a vaginal seminal plug was noted. If Y1R antagonist administration restores sexual receptivity in female *ob/ob* mice, this would support the hypothesis that high levels of Y1R activation are responsible for the absence of sexual receptivity in the *ob/ob* mouse (Table 2, Box 2).

8. I propose that elevated NPY signaling impairs the effects of endogenous E_2 on reproductive behavior in leptin-deficient mice. To identify the role of E_2 on reproductive behavior, female *ob/ob* mice were tested for sexual receptivity following a twelve day 17β-estradiol treatment. Following treatment on day thirteen, each female *ob/ob* mouse was tested for sexual receptivity in a 10 minute test with a sexually active wild-type male. Extended sexual behavior testing occurred on treatment day 14. Each female *ob/ob* mouse was paired with the same stud male for 12 hours. Following 12 hour behavioral testing, as described in aim 7, all female mice were euthanized via CO_2 asphyxiation and the vaginal opening was assessed for the presence or absence of seminal plug, an

indicator of copulation. If exogenous E_2 administration restores reproductive behavior in female *ob/ob* mice, these data will support the hypothesis that endogenous E_2 signaling causes sexual receptivity in *ob/ob* females (Table 2, Box 3).

9. I propose that the combination treatment of E_2 and BIBP3226 will work in additive fashion to enhance sexual receptivity in leptin-deficient mice. To determine the significance of exogenous E_2 and Y1R activation on reproductive behavior, female *ob/ob* mice were tested for sexual receptivity following a twelve day treatment of 17β-estradiol and BIBP3226. Following treatment on day thirteen, each female *ob/ob* mouse will was tested for sexual receptivity in a 10 minute test with a sexually active wild-type male. Extended sexual behavior testing occurred on treatment day 14. Each female *ob/ob* mouse was paired with the same stud male for 12 hours. As mentioned above, following 12 hour behavioral testing, all female mice were euthanized via CO_2 asphyxiation and vaginal opening was assessed for the presence or absence of seminal plug, an indicator of copulation. If the combined treatment of E_2 and Y1R antagonist increases sexual receptivity in female *ob/ob* mice more than either treatment by itself, then these results would support the hypothesis that E_2 signaling andY1R inhibition interact to promote higher levels of reproductive behavior in *ob/ob* mice (Table 2, Box 4).

TABLE 1: Specific Aims

Specific Aims
1. NPY Y1 receptor blockade decreases food intake and decrease body mass in the *ob/ob* mouse model .
2. Exogenous E_2 reduces food intake and reduces body mass in the *ob/ob* mouse model.
3. The combination treatment, BIBP3226 and $E_{2,}$ has an additive effect to reduce food intake and body mass.
4. NPY Y1 receptor blockade restores reproductive development (uterine hypertrophy) in the *ob/ob* mouse model.
5. Exogenous E_2 restores reproductive development in the *ob/ob* mouse model.
6. The combination treatment, BIBP3226 and $E_{2,}$ has an additive effect to restore reproductive development in the *ob/ob* mouse model.
7. NPY Y1 receptor blockade restores reproductive behavior in the *ob/ob* mouse model.
8. Exogenous E_2 restores reproductive behavior in the *ob/ob* mouse model.
9. The combination treatment, BIBP3226 and $E_{2,}$ has an additive effect to restore reproductive behavior in the *ob/ob* mouse model.

TABLE 2: Proposed effects of 17-β estradiol (E_2) and Y1R-selective NPY antagonist (BIBP3226) on behavior and physiology of leptin-deficient female mice.

	Y1R Antagonist Absent	**Y1R Antagonist Present**
E_2 Absent	BOX 1 <u>Abnormal Phenotype of Ob/Ob Mice:</u> Hyperphagic Obese Underdeveloped Reproductive Organs Absence of Reproductive Behavior	BOX 2 Less Hyperphagic vs. Box 1 Reduced Body Mass vs. Box 1 Increased Reproductive Organ Mass vs. Box 1 Presence of Reproductive Behavior vs. Box 1
E_2 Present	BOX 3 Less Hyperphagic vs. Box 1 Reduced Body Mass vs. Box 1 Increased Reproductive Organ Mass vs. Box 1 Presence of Reproductive Behavior vs. Box 1	BOX 4 Less Hyperphagic vs. Box 2 or 3 Reduced Body Mass vs. Box 2 or 3 Increased Reproductive Organ Mass vs. Box 1 Presence of Reproductive Behavior vs. Box 2 or 3

CHAPTER II

MATERIALS AND METHODS

Animals

Physiological studies were conducted on 60 female mice to investigate the relationship between sex hormones, hypothalamic neuropeptides, obesity and reproductive fertility. Female leptin-deficient (*ob/ob*) mice (B6.V-Lep^{ob}) were obtained from Jackson Laboratories (Chicago, IL). All animals were housed in the UNCG -ACUC approved animal facility with 12 hour light/12 hour dark cycles and maintained on ad libitum food and water (Purina Lab Pellets). At age 28 days, mice were housed individually and were randomly assigned to one of the four treatment groups described below (Table 3). Food intake and body mass were monitored twice daily until age 42 days. Prior to experiment, all procedures were approved by the UNCG-ACUC committee.

TABLE 3: Treatment Groups- see Table 4 for doses

Treatment Group	Number
Vehicle	15
E_2	15
BIBP3226	15
Combination	15

Treatment

This study included four treatment groups with fifteen female leptin-deficient mice in each group (Tables 3 and 4). Mice in the control treatment group received intraperitoneal injections of saline vehicle and subcutaneous injections of sesame-oil vehicle. Mice in the estradiol group received intraperitoneal injections of saline vehicle and subcutaneous injections of 17-β estradiol. Mice in the NPY antagonist group received intraperitoneal injections of BIBP3226, an antagonist to NPY at Y1 receptors, and subcutaneous injections of oil vehicle. Mice in the combination group received intraperitoneal injections of BIBP3226 and subcutaneous injections of 17-β estradiol. All mice received two intraperitoneal and two subcutaneous injections per day, at 12 hour intervals, for 14 consecutive days (Table 4 & Figure 6).

TABLE 4: Treatment dose by group.

Treatment Group	**Treatment Dose**
Vehicle	0.05 ml saline vehicle
	0.05 ml sesame oil vehicle
E_2	0.05 ml saline vehicle
	0.05 mg estradiol benzoate in 0.05 ml sesame oil
BIBP3226	0.10 mg of BIBP3226 in 0.05 ml saline
	0.05 ml sesame oil vehicle
Combination	0.10 mg of BIBP3226 in 0.05 ml saline
	0.05 mg estradiol benzoate in 0.05 ml sesame oil

FIGURE 6: Treatment group dosing schedule. Behavioral testing took place on days 40 & 41.
SQ = Subcutaneous injection, IP= Intraperitoneal injection

Behavioral Testing

On the last two days (days 13 and 14) of the experiment, female mice were behavioral tested with sexually-experienced (stud) males. At the onset of the scotophase on day 13, each female mouse was placed into the cage of a stud male for 10 minutes (Figure 7). The presence or absence of reproductive behavior was monitored for duration of 10 minutes; however, no mating occurred during this 10 minute interval on day 13. Following the final injections on day 14, each female mouse was housed for 12 hours with its stud male. After euthanasia, using carbon dioxide inhalation, each female was assessed for copulatory behavior as evidenced by an intravaginal seminal plug. At necropsy, uteri were dissected and cleaned of fat and weighed to the nearest 0.01mg. For data analysis uterine mass was standardized to mg uterus/25 g body mass.

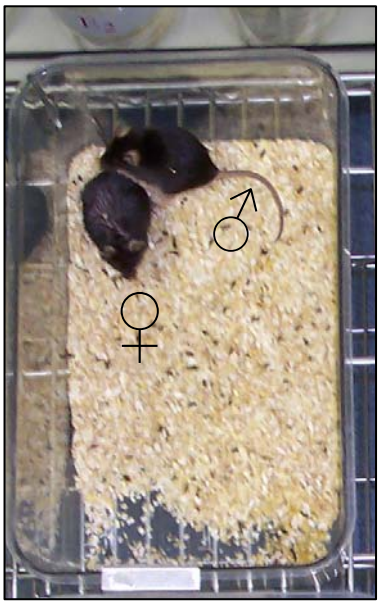

FIGURE 7: Behavioral test to monitor sexual receptivity of female leptin-deficient mice with stud male.

Statistics

All statistical analyses were performed with SPSS 15.0 software. Kolmogorov-Smirnov tests were used on all data sets to test for normality. Three variables met the criterion for normal distribution: daily food intake, daily body mass and uterine mass. Average daily food intakes, terminal food intake, average daily body mass and terminal body mass were calculated for each week and the data were analyzed using repeated measures ANOVA to account for time effects among the treatment groups. Treatment effects on food intake and body mass were analyzed by one-way ANOVA with *post hoc* comparisons using Tukey's test. Uterine mass was expressed as mg of uterine tissue mass/ 25g body mass. Adjusted uterine mass was analyzed using a univariate ANOVA with *post hoc* comparisons by the Duncan's Multiple Range test. The results are expressed as mean ± S.E.M. For all statistical tests, a p value < 0.05 was considered statistically significant.

CHAPTER III

RESULTS

Food Intake Analysis

Daily Food Intake

One-way ANOVA of food intake indicated statistically significant treatment effects across the full duration (14 days) of the experiment (p-value<0.001, Tables 3 and 4; Figure 8). Subsequently, *post hoc* comparisons using Tukey's test resulted in the distinction of two groups of mice. In the first group, the average food intakes of mice which received either vehicle or BIBP3226 were statistically equivalent (Table 5, Figure 9). *Ob*/ob mice in the vehicle group consumed an average of 1.32 g of food per day, and mice in the BIBP3226 consumed an average of 1.46 g of food daily. The average food intakes of mice in the second group, those which received E_2 or combination treatment, were significantly lower than those of the first group (Table 5, Figure 9). The mice who received E_2 treatments consumed 0.83 g of food on average per day, and the mice in the combination treatment group consumed and average of 0.78 g of food per day.

TABLE 5: F and *p* values for time and treatment effects on average food intake.

Effect	F-value	*p*-value
Time	$F_{(1,55)}$= 57.529	<0.001 (see Figure 7, Table 6)
Treatment	$F_{(3,55)}$=19.495	<0.001 (see Figure 8, Table 7)
Time x Treatment	$F_{(3,55)}$=21.127	<0.001 (see Figure 9, Table 8)

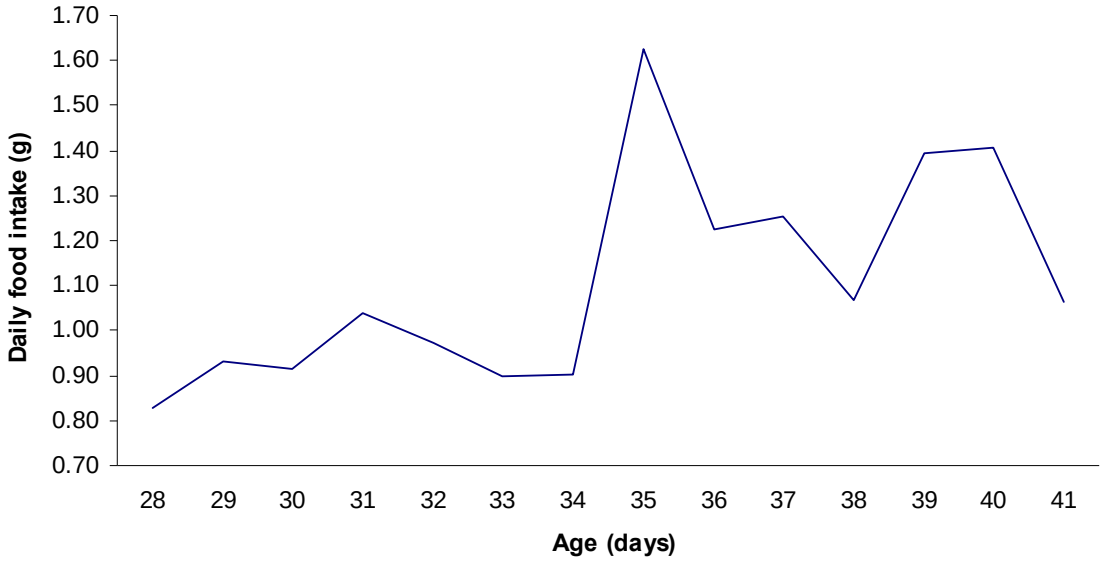

FIGURE 8: Daily food intake of female leptin-deficient mice.

TABLE 6: Average weekly food intake for week 1 and week 2 (*p*-value <0.001).
[a,b] Means with different superscript letters are significantly different.

Week	n	Average Food Intake; Mean (±SEM) g/day
1	59	0.93 (±0.03)[a]
2	59	1.29 (±0.04)[b]

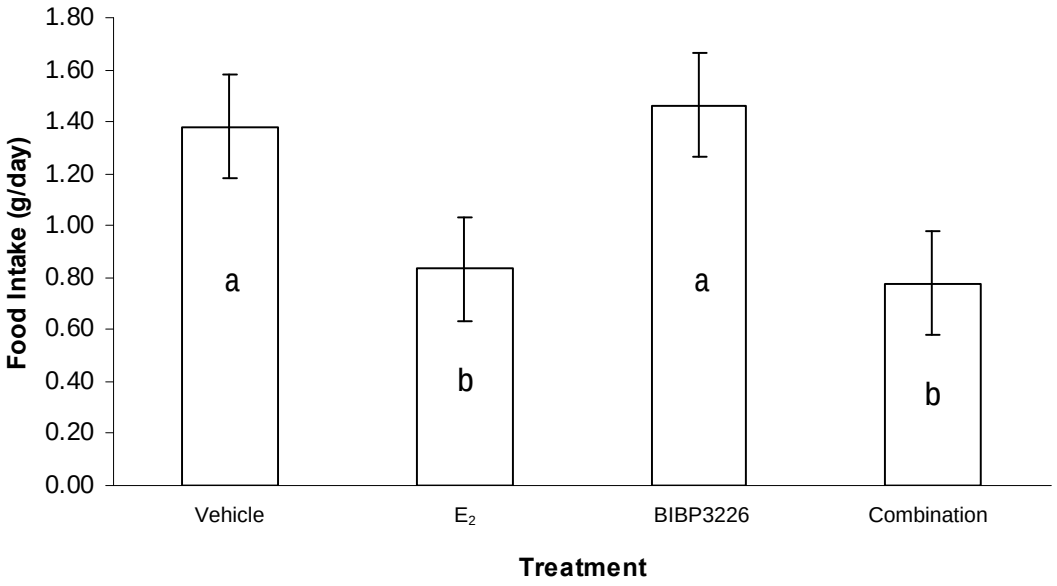

FIGURE 9: The effects of drug treatment on daily food intake (average of all days) in leptin-deficient mice. Different letters indicate statistically significant differences.

TABLE 7: Mean (±SEM) daily food intake by treatment group for leptin-deficient mice. [a,b] Means with different superscript letters are significantly different

Treatment	n	Daily Food Intake (Total Average); Mean (±SEM) g/day
Vehicle	14	1.32 (±0.05)[a]
E_2	15	0.83 (±0.03)[b]
BIBP3226	15	1.46 (±0.08)[a]
Combination (17-β Estradiol & BIBP3226)	15	0.78 (±0.03)[b]

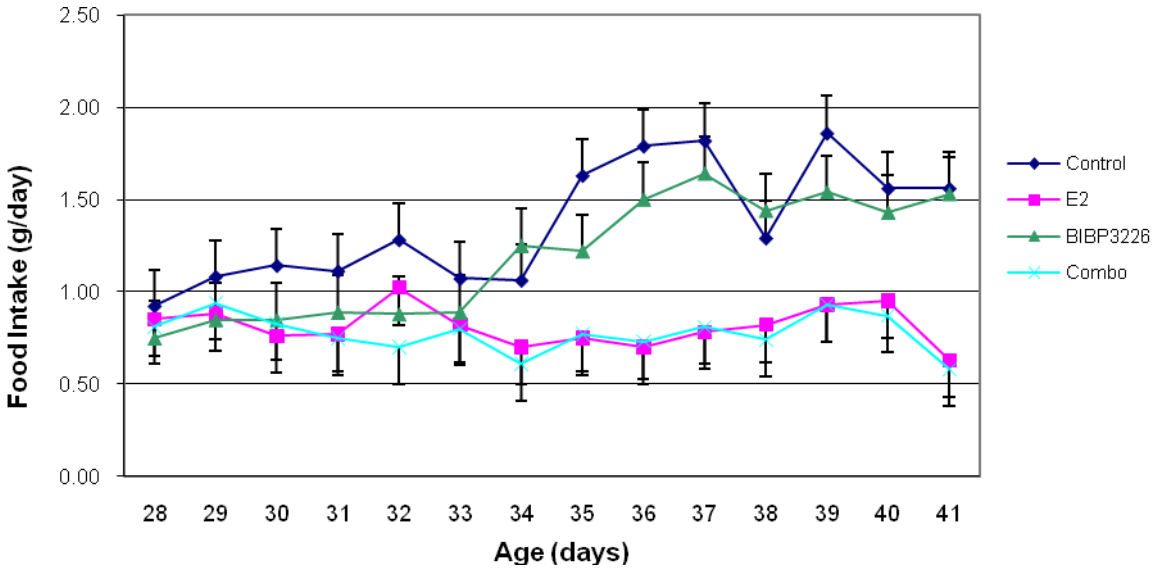

FIGURE 10: Food intake by age for each treatment group.

TABLE 8: Average weekly food intake by treatment group.
[a,b,c] Means with different superscript letters are significantly different.

Treatment	n	Week 1 Average Food Intake; Mean (±SEM) g/day	Week 2 Average Food Intake; Mean (±SEM) g/day
Vehicle	14	1.08 (±0.05)[a]	1.66 (±0.07)[c]
E_2	15	0.83 (±0.04)[b]	0.84 (±0.04)[b]
BIBP3226	15	1.01 (±0.08)[a]	1.92 (±0.12)[c]
Combination	15	0.78 (±0.04)[b]	0.78 (±0.04)[b]

To discern more precisely the time course of response to these treatments, an additional analysis was conducted. An inspection of Figure 10 suggested that it would be productive to compare week 1 with week 2. Accordingly, two different comparisons were made. The first statistical analysis compared the data averages of week one with the averages of week two. The second comparison compared data from the end of week one with data from the end of week two.

Average Weekly Food Intake of Week 1 and Week 2

Repeated measures ANOVA was used to analyze the effects of treatment (vehicle, E_2, BIBP3226, or combination) and two time points corresponding to the averages of each of the two weeks during which food intake was measured, as well as the interaction between these two factors (Table 5). As reported previously (in *Daily Food Intake*), mice in the vehicle and BIBP3226 groups had significantly higher food intakes than those in the E_2 and the combination treatment groups (same result as that shown in Table 7): 1.32 g of food for the vehicle group, 1.46 g of food for the BIBP3226 group, 0.83 g of food for the E_2 group, and 0.78 g for the combination treatment group.

There was significant time by treatment interaction effect (p-value< 0.001, Table 9), indicating that the treatment group averages were not shifting uniformly from week 1 and week 2 (Table 7; Figures 9, 10). Results from week 1 averages of food intake reveal that mice in the vehicle and BIBP3226 treatment groups consumed significantly more than mice in the E_2 and combination treatment groups (results shown in Table 8). Similarly, week 2 averages of food intake indicate that mice in the vehicle and BIBP3226

treatment groups consumed significantly more than mice in the E_2 and combination treatment groups. Mice in the vehicle and BIBP3226 groups consumed significantly more between week 1 and week 2: mice in the vehicle group consumed an average of 1.08 g of food in week 1 and 1.66 g in week 2, and mice in the BIBP3226 treatment group consumed an average of 1.01 g of food in week 1 and 1.92 g in week 2. There was no significant change in average daily food intake for mice in the E_2 and combination treatment groups from week 1 to week 2 (Table 8).

TABLE 9: F and *p* values for time and treatment effects on average food intake. Same results as in Table 3.

Effect	***p*-value**
Time	<0.001 (see Figure 7, Table 6)
Treatment	<0.001 (see Figure 8, Table 7)
Time x Treatment	<0.001 (see Figure 9, Table 8)

End of Week Food Intake for Week 1 and Week 2

A comparison of mean food intakes on day 7 versus those on day 14 generated results similar to those for weekly averages (Table 10). Time and treatment effects were statistically significant (*p*-value= 0.003, Table 10). As in the analysis of overall weekly averages, mice ate more food (approximately 0.16 g) on day 14 than they did on day 7 (Table 11). Moreover, as before, food intake of mice in the vehicle and BIBP3226 groups ate more than mice in the E_2 and combination treatment groups (Table 12).

The between-subjects analysis revealed a significant treatment effect, i.e., end of week food intake was not uniform for the 4 groups (*p*-value<0.001, Table 10). A strong within-subject effect was evident for end of week food intake comparing week 1 to week 2 indicating food intake shifted significantly between weeks (*p*-value=0.006, Table 11). End of week average food intake of *ob/ob* mice given vehicle or BIBP3226 were statistically equivalent (Table 12): 1.24 g of food per day for the vehicle group and 1.39 g of food per day for the BIBP3226 group. Food intakes by estradiol-treated mice were statistically equivalent to that of mice in the combination treatment (Table 12): 0.67 g of food per day for the estradiol group versus 0.60 g of food per day for the combination group. There was a significant time by treatment interaction (*p*-value=0.003, Table 12) indicating that the end of week food intake averages were not shifting uniformly from week 1 and week 2. Statistical results support the formation of two homogenous subsets, one subset including vehicle and BIBP3226 treatments, where mice consumed more from the end of week 1 to the end of week 2, and the other subset including E_2 and combination treatments, where food consumed by mice did not change significantly from end of week 1 to end of week 2 (Table 13, Figure 10).

TABLE 10: F and *p* values for end of week food intake for week 1(days 28 to 34) and week 2 (days 35 to 41).

Effect	**F-value**	***p*-value**
Time	$F_{(1,55)}$= 8.200	0.006 (Table 9)
Treatment	$F_{(3,55)}$=32.627	<0.001 (Table 10)
Time x Treatment	$F_{(3,55)}$=5.196	0.003 (Table 11)

TABLE 11: End of week food intake for week 1 and week 2.
[a,b] Means with different superscript letters are significantly different.

Week	n	End of Week Food Intake; Mean (±SEM) g/day
1	59	0.90 (±0.06) [a]
2	59	1.06 (±0.07) [b]

TABLE 12: Food intake averages for end of week 1 and end of week 2, by treatment group. [a,b] Means with different superscript letters are significantly different.

Treatment	n	Food Intake for Total (End of Both Weeks); Mean (±SEM) g/day
Vehicle	14	1.24 (±0.17)[a]
E_2	15	0.67 (±0.07) [b]
BIBP3226	15	1.39 (±0.13) [a]
Combination	15	0.60 (±0.05) [b]

TABLE 13: End of week food intake for weeks 1 & 2 by treatment group.
[a,b,c] Means with different superscript letters are significantly different.

Treatment	n	End of Week 1 Food Intake; Mean (±SEM) g/day	End of Week 2 Food Intake; Mean (±SEM) g/day
Vehicle	14	1.05 (±0.10) [a]	1.44 (±0.06) [c]
E_2	15	0.70 (±0.07) [b]	0.63 (±0.07) [b]
BIBP3226	15	1.25 (±0.15) [a]	1.53 (±0.11) [c]
Combination	15	0.61 (±0.08) [b]	0.58 (±0.07) [b]

Body Mass Analysis

Repeated Measures ANOVA of body mass indicated a statistically significant time effect on body mass over the 14 day experiment (Figure 11, Table 14). As expected, all mice gained weight over the 14 day experiment. To discern more precisely the time course of response to these treatments, additional analyses were conducted. An inspection of Figure 12 suggested that it would be productive to compare week one with week two. Similar to analyses in *Daily Food Intake*, two different comparisons were made. The first comparison included a statistical analysis comparing data from the averages of week one with the averages of week two. In the second comparison, body mass data from the end of week one was compared with data from the end of week two.

Average Body Mass of Week 1 with Week 2

A repeated measures ANOVA was used to analyze the effects of treatment (vehicle, E_2, BIBP3226, or combination) on body mass over time. Test of within-subjects effects indicate significant time effect where body mass was not uniform for the 4 groups. A within-subject effect was evident for average body mass over week 1 and week 2 indicating body mass shifted significantly (p-value<0.001, Table 14). Time (Tables 14, 15; Figure 11) had a significant effect on average body mass: comparing week 2 to week 1, the average body mass of week 2 (30.10 g) was greater than that of week 1(26.75 g) across all treatment groups (p-value<0.001). This suggests that treatment did not interfere with weight gain expected as the young animals matured. Drug treatment did not result in a significant effect on average body mass between

treatment groups between week 1 and week 2, though mice in the vehicle and BIBP3226 treatment groups weighed more than mice in the E_2 and combination treatment groups (*p*-value=0.328, Tables 14, 16; Figure12). Moreover, the interaction of time and treatment was not statistically significant for average body mass (*p*-value=0.721, Tables 14, 17; Figure 13).

TABLE 14: F and *p* values for average body mass.

Effect	**F-value**	***p*-value**
Time	$F_{(1,55)}$= 44.198	<0.001 (see Figure 10, Table 13)
Treatment	$F_{(3,55)}$=1.173	0.328 (see Figure 11, Table 14)
Time x Treatment	$F_{(3,55)}$=0.446	0.721(see Figure 12, Table 15)

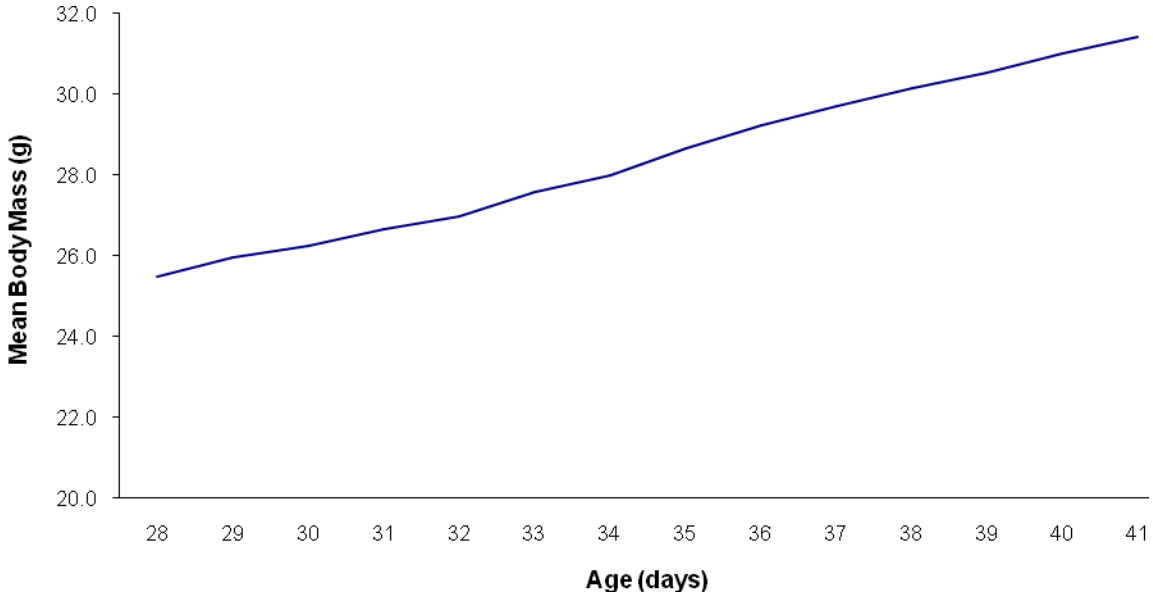

FIGURE 11: Change in body mass by age for all mice. Across the duration of the experiment all animals increased body mass

TABLE 15: Average body mass for week 1 and week 2.
[a,b] Means with different superscript letters are significantly different.

Week	n	Average Body Mass
1	59	26.75 (±0.16) [a]
2	59	30.10 (±0.18) [b]

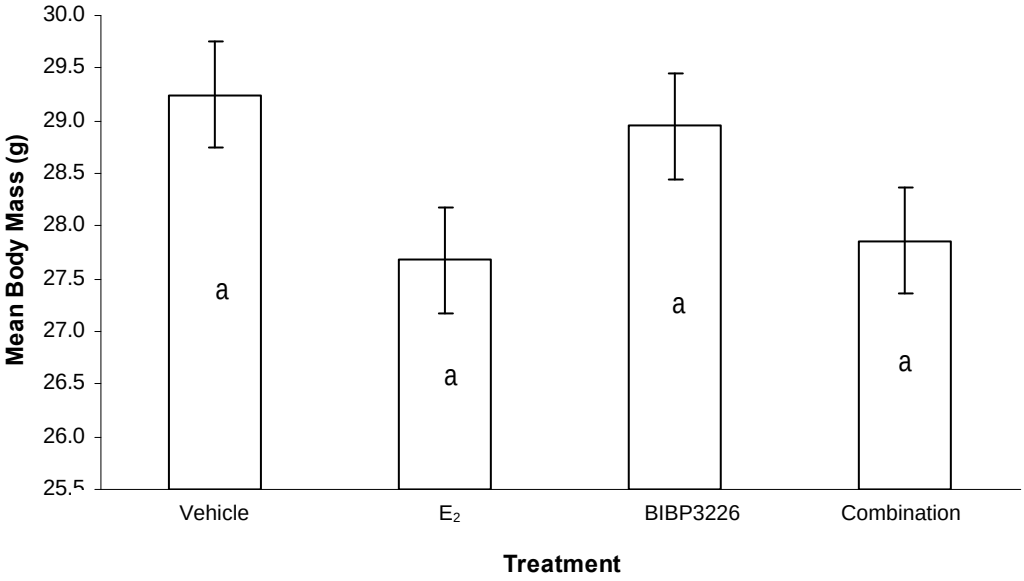

FIGURE 12: Mean body mass for each treatment group.

TABLE 16: Average body mass by treatment group.
[a,b] Means with different superscript letters are significantly different.

Treatment	n	Average Body Mass
Vehicle	14	29.25 (±0.33) [a]
E_2	15	27.68 (±0.24) [a]
BIBP3226	15	28.95 (±0.25) [a]
Combination	15	27.86 (±0.22) [a]

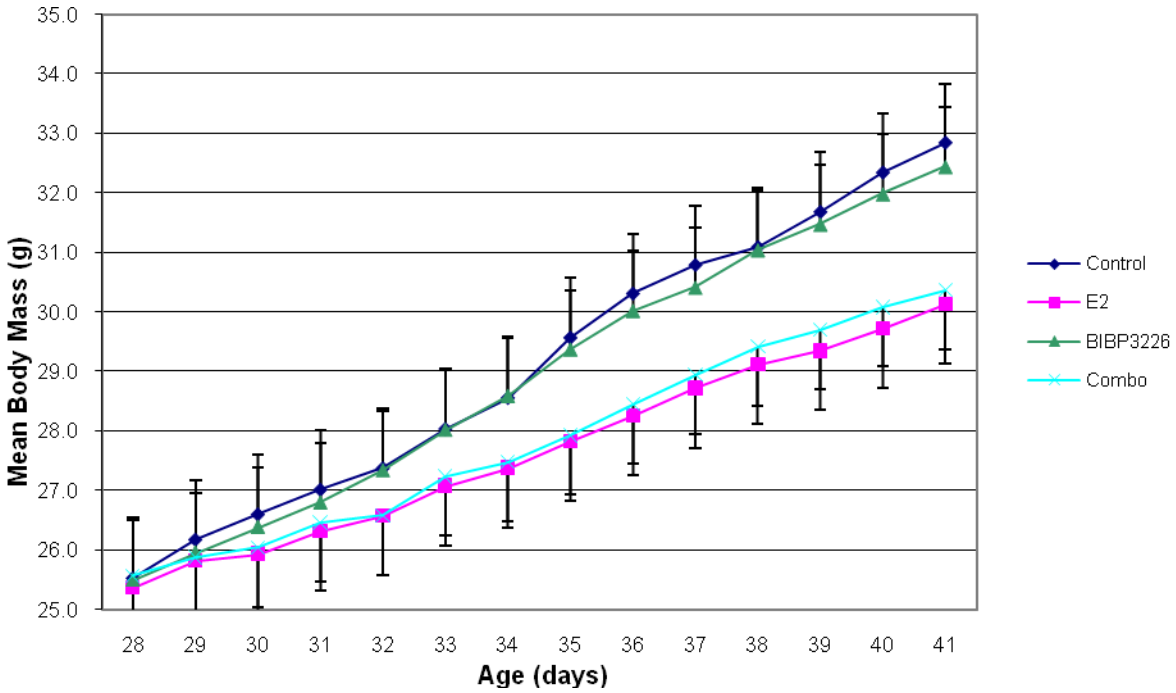

FIGURE 13: Body mass by age for each treatment group.

TABLE 17: Average body mass for weeks 1 & 2 by treatment group.
[a,b] Means with different superscript letters are significantly different.

Treatment	n	Week 1 Average Body Mass	Week 2 Average Body Mass
Vehicle	14	27.26 (±0.37)[a]	31.23 (±0.40)[b]
E_2	15	26.36 (±0.30)[a]	29.02 (±0.46)[b]
BIBP3226	15	26.94 (±0.29)[a]	30.96 (±0.30)[b]
Combination	15	26.46 (±0.28)[a]	29.27 (±0.28)[b]

End of Week Body Mass Measurements for Week 1 and Week 2

Time had a significant effect on end-of-week body mass between week 1 and week 2 (*p*-value <0.001, Tables 18, 21); as before, body mass increased at the end of week 2. Though a statistically significant difference did not exist between treatment groups, mice in the vehicle and BIBP3226 combination treatment groups weighed more than those in the E_2 and combination treatment groups. A strong within-subject effect was evident for end of week body mass between week 1 and week 2 indicating body mass shifted significantly with a *p*-value < 0.001 (Tables 18, 19): overall end of week body mass for week 1 was 28.06 g versus 31.42 g for week 2. Treatment alone did not have a significant effect on end of week or mean body mass (Tables 20, 21). Time and treatment did have a statistically significant interaction effect on end of week body mass between week 1 and week 2 where body mass shifted significantly for all groups with a *p*-value <0.001 (Table 18 & 22).

TABLE 18: F and *p* values for end of week body mass.

Effect	**F-value**	***p*-value**
Time	$F_{(1,55)}$= 925.854	<0.001 (see Figure 10, Table 17)
Treatment	$F_{(3,55)}$=1.474	0.232 (see Figure 11, Table 18)
Time x Treatment	$F_{(3,55)}$=8.322	<0.001 (see Figure 12, Table 20)

TABLE 19: End of week body mass for week 1 and week 2.
[a,b] Means with different superscript letters are significantly different.

Week	n	End of Week Body Mass
1	59	28.06 (±0.42)[a]
2	59	31.42 (±0.48) [b]

TABLE 20: Total end of week body mass by treatment group.
[a] Means with different superscript letters are significantly different.

Treatment	n	End of Week Body Mass
Vehicle	14	29.77 (±0.91) [a]
E_2	15	28.76 (±0.62) [a]
BIBP3226	15	30.51 (±0.65) [a]
Combination	15	28.92 (±0.56) [a]

TABLE 21: Mean body mass for *ob/ob* treatment groups at day 41.
[a] Means with different superscript letters are significantly different.

Treatment	n	Mean Body Mass
Vehicle	14	29.25 (±0.33) [a]
E_2	15	27.68 (±0.24) [a]
BIBP3226	15	28.95 (±0.25) [a]
Combination	15	27.86 (±0.22) [a]

TABLE 22: End of week body mass for weeks 1 & 2 by treatment group.
[a,b] Means with different superscript letters are significantly different.

Treatment	n	End of Week 1 Body Mass	End of Week 2 Body Mass
Vehicle	14	28.84 (±1.08)[a]	32.83 (±1.34) [b]
E_2	15	27.38 (±0.79) [a]	30.13 (±0.97) [b]
BIBP3226	15	28.59 (±0.77) [a]	32.44 (±0.83) [b]
Combination	15	27.48 (±0.72) [a]	30.37 (±0.71) [b]

Uterine Mass Analysis and Reproductive Behavior

One-way ANOVA indicated a statistically significant treatment effect on uterine mass. Subsequently, a pair-wise comparison with Duncan's Multiple Range test resulted in the distinction of three groups of mice. In the first group, the average uterine masses of mice which received vehicle injections or BIBP3226 injections were homogenous, low and statistically equivalent: 24.5 mg for the vehicle group and 24.2 mg for the BIBP3226 group (Table 23, Figure 14). In the second group, the average uterine mass of mice which received E_2 only was 53.2 mg and significantly greater than those in the first group (Table 23). Finally, a third group of mice, those which received the combination treatment had the highest uterine mass at 92.1 mg (Figure 14). Following overnight cohabitation on day 14 with a stud male, a vaginal plug was evident in each female mouse that had received E_2 or the combination treatment, but none of the females in the vehicle or the BIBP3226-only group mated (Table 24).

TABLE 23: Mean (± SEM) adjusted uterine mass (mg of uterine mass/ 25g body mass) at age 44 days (n=number of mice per treatment group).
[a,b,c] Means with different superscript letters are significantly different.

Treatment	n	Uterine Mass; Mean(±SEM) mg/25g body mass
Vehicle	14	24.5 (±0.9) [a]
E_2	15	53.2 (±3.9) [b]
BIBP3226	15	24.2 (±2.0) [a]
Combination	15	92.1 (±5.3) [c]
Treatment Effect, $F_{(3,54)}= 84.48$, $p<0.001$		

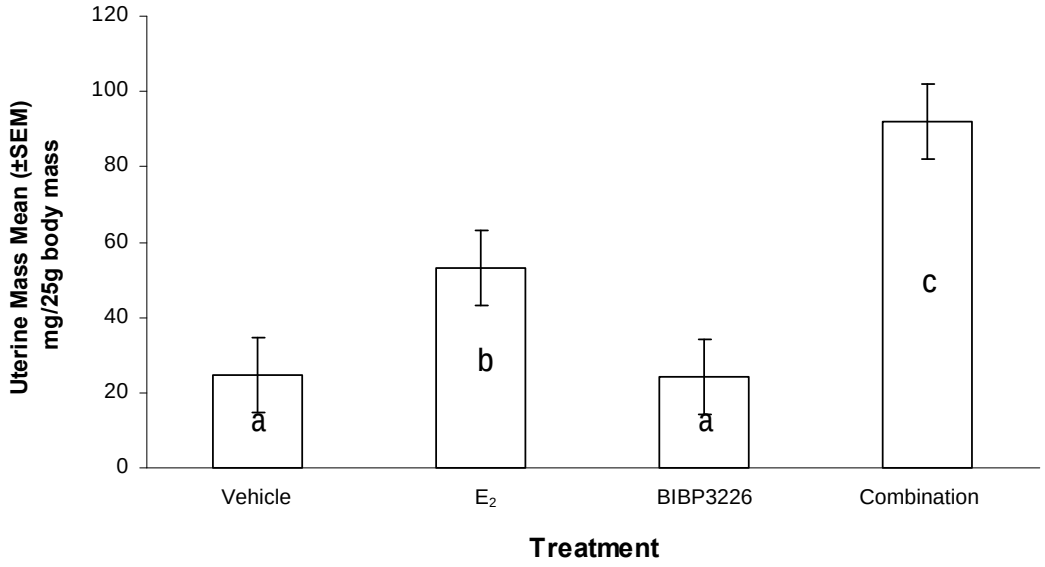

FIGURE 14: The effects of drug treatment on uterine mass in leptin-deficient female mice. Different letters indicate statistically significant differences.

TABLE 24: Presence or absence of vaginal plug in female mice following 12 hours with stud male (n=number of mice per treatment group).

Treatment	n	Percent of females with vaginal plug
Vehicle	14	0%
E_2	15	100%
BIBP3226	15	0%
Combination (17-β Estradiol & BIBP3226)	15	100%

CHAPTER IV

DISCUSSION

This project evaluates the effects of peripheral NPY Y1 receptor blockade and exogenous estradiol activity in prepubertal leptin-deficient female mice while assessing appetite regulation, body mass and reproductive development and behavior.

The premise for using the BIBP3226 compound, an NPY Y1 receptor antagonist, to impair peripheral NPY Y1 receptor activity is the proposed antagonistic relationship between the leptin and the NPY signaling pathways. Leptin is a protein that signals satiety in hypothalamic networks as part of a physiological mechanism to maintain an optimal body mass. Conversely, NPY is a signal that activates appetite via its interactions with various receptors throughout the central, and possible the peripheral, nervous system. Regions of the ARC in the hypothalamus are heavily innervated with both leptin and a variety of NPY receptors. Clarifying the specific roles of the NPY sub-receptors and their influence on appetite, body mass and reproductive development and behavior would be a significant breakthrough in decoding the complex signaling network that maintains energy homeostasis.

Ovarian sex steroid hormones, especially E_2, play a crucial role in normal reproductive development. The estrogens have anorexigenic consequences, much like leptin, that influence energy homeostasis. Though estrogen receptors are broadly distributed, E_2's direct mode of action to decrease body mass is thought to result from its

interactions with the leptin pathway. In addition, E_2 might cause an increase in energy expenditure with or without its proposed interactions on the leptin pathway and appetite. Utilizing a model with a specific disruption in the leptin signaling pathway, and consequent changes in NPY and other pathways, allowed me to examine the role of such pathways in regulating appetite, body mass and reproductive behavior. Likewise, this *ob/ob* mouse model allowed me to assess the influence of Y1 activity in the presence and absence of active estrogen.

Neuropeptide Y

NPY mediates its effects by binding its receptors; however, the search for the central and peripheral locations of each of the six NPY receptor subtypes is ongoing. By targeting a specific receptor, i.e., NPY Y1 receptor, I examined the receptors' role in metabolism and reproduction. Theory holds that the absence of leptin causes a chronic elevation of NPY and hyperphagia, and diminished energy expenditure, both of which contribute to an obese phenotype.

Based on the results reported here, the NPY Y1 receptors in the periphery are unlikely candidates in mediating the orexigenic effects of NPY on appetite and body mass. The NPY Y1R antagonist, BIBP3226, failed to reduce hyperphagia and body mass. It is possible that the central Y1 receptors were not antagonized by the peripheral administration of BIBP3226. Future studies could examine the effects of central administration (intracerebroventricular injections) of BIBP3226 in the *ob/ob* model.

Though *ob/ob* mice administered the Y1 receptor antagonist, BIBP3226, weighed slightly less than mice administered vehicle, the differences were not statistically significant. Summarizing BIBP3226's effects on food intake and body mass, there were no significant effects of BIBP3226 when delivered alone. As noted earlier, CNS administration of BIBP3226 might produce different results.

Research suggesting that Y1 receptor activity prevents reproductive organ development as well as reproductive behavior was supported by the results of this experiment as previously hypothesized. *Ob/ob* mice treated only with BIBP3226 did not exhibit uterine hypertrophy nor did they exhibit reproductive behavior characterized by a vaginal plug following 12 hour behavioral test with stud males. Endogenous E_2 signaling is directly controlled by the hypothalamic pituitary gonadal axis, and in the absence of E_2 signaling, BIBP3226 had no direct effects on uterine development. However, in the presence of E_2, as in the combination treatment group, BIBP3226 caused significant uterine hypertrophy than did E_2 treatment alone. This finding was consistent with my hypothesis that the combination treatment would have additive effects on reproductive development.

17-β Estradiol

Whether by itself or in combination with the NPY-Y1R antagonist, E_2 exerted its expected stimulatory effects on uterine hypertrophy and on sexual receptivity. Moreover, E_2 reduced food intake and body mass in the same pattern.

Following 14 days of treatment, all mice treated with E_2, whether E_2 only or the combination group, exhibited significantly greater uterine mass than those in the vehicle or BIBP3226 treatment groups. As expected, and consistent with my hypothesis, E_2 did exert uterotrophic effects in the *ob/ob* mouse model. This finding suggests that disturbances in hypothalamic peptide levels, specifically leptin and NPY, lead to a reduction in endogenous E_2 release, resulting in the reproductive impairments characteristic of the *ob/ob* mice. Though both the E_2 and the combination treatment groups unanimously exhibited lordosis, as evidenced by vaginal plugs, the presence of exogenous E_2 appears to have a greater effect on reproductive behavior than did BIBP3226. This finding is supported in that 100% of mice in the E_2 and combination treatment groups exhibited copulatory behavior, but the BIBP3226 treatment alone was insufficient to stimulate lordosis when female mice were partnered with their stud male in the 12 hour behavioral test.

Exogenous E_2 reduced food intake and body mass over the 14 day treatment period in this study: the leptin-deficient mice administered E_2 exhibited significantly lower food consumption than did mice in the vehicle and BIBP3226 treatment groups. This finding supports my hypothesis that E_2 exerts anorexigenic effects on food intake in leptin-deficient mice.

The pubertal transition in mice occurs during a phase of somatic growth, so it's no surprise that mice in all four treatment groups gained weight, as expected, over the duration of the experiment. However, E_2 did not significantly reduce body mass compared to the vehicle, BIBP3226 and combination treatment groups over the 14 day

treatment. In future studies, extending the treatment period or increasing the dosage might result in a significant E_2 effects on body mass.

Combination Treatment

NPY-influenced aspects of reproductive development are linked to the presence of sex steroids, as my findings suggest an uterotrophic role for NPY Y1 receptor blockade only when E_2 was added. Uterine growth was evident in all mice who received either the E_2 or the combination treatment, though the uterine development was significantly more pronounced in mice which received the combination treatment. This finding supported my hypothesis that peripheral Y1 receptor blockade can contribute to reproductive organ development. Likewise, all (100%) mice who received the combination treatment exhibited copulatory behavior as evidenced by a seminal plug. This "ceiling effect" prohibits discernment of a separate role for Y1 receptor blockade. However, in the absence the E_2, the Y1 receptor antagonist had no significant effect on reproductive development and behavior. The presence of E_2 appears necessary to induce the peripheral effects of BIBP3226 on Y1 receptors to stimulate reproductive organ development and behavior, where BIBP3226 alone was insufficient to stimulate reproductive development and elicit reproductive behavior. Research by Hill et al. supports a role for E_2 in reducing Y1 receptor mRNA, where female rodents administered estrogen *in vivo* exhibited a decrease in Y1R mRNA [48]. This finding, in combination with research indicating that E_2 increases excitatory inputs to POMC neurons, the precursor to α-MSH, may explain, in part, the additive effects of the combination

treatment on uterine mass results [34]. Moreover, the combined treatment effects of E_2 and BIBP3226 supports my hypothesis that an interaction between E_2 and the Y1 receptor subtype exists and that simultaneous activation of the E_2 path and inactivation of the Y1 receptor path mediates reproductive organ development, as evidenced by the combination treatment group having a significantly higher average uterine weight than the E_2 only treated group.

Mice in the combination treatment group exhibited a lower average food intake than did the E_2 only treated mice, but this difference was not significantly different. The results of this experiment do support my hypothesis that E_2 has anorexigenic effects on food intake leading to reduced body mass, though the Y1 receptor antagonist did not elevate the effects of E_2 as was expected to occur in the combination treatment group. This finding may be explained by a different NPY receptor subtype, other than the Y1, in mediating the orexigenic actions of NPY.

Body mass of the combination treatment mice was less than those of mice which received either vehicle or BIBP3226-only. However, the combination treatment did not have greater effects in reducing body mass than E_2 alone. This finding suggests that a continued combination treatment may result in a significantly lower body mass during an extended treatment interval than did vehicle and BIBP3226 treatments. Moreover, an extended treatment period may support a sex steroid dependent mechanism for NPY on adipocytes as well as reproductive organs. The presence of active estrogen may be required to limit the anti-lipolytic actions of NPY, as results indicate that the combination treatment group had a reduced body mass compared to the BIBP3226 treatment only.

Future studies could examine the effects of central administration of BIBP3226 in combination with E_2 to reduce body mass in the *ob/ob* mice.

CHAPTER V

CONCLUSION

In conclusion, exogenous E_2 does appear to have a dual function in both energy homeostasis, with anorexigenic actions similar to that of leptin, and reproductive development, where reproductive growth is enhanced by the presence of a NPY Y1 receptor antagonist. Peripherally administered E_2 and NPY Y1R antagonist, BIBP3226, stimulate reproductive (uterine) development in *ob/ob* mice. Consequently, E_2 or combination treatments promote copulatory behavior in this leptin-deficient model. Exogenous E_2 does lead to reductions in appetite and body mass, but BIBP3226 treatments alone do not successfully minimize caloric intake or body mass in *ob/ob* mice. From these experimental findings, I conclude that the Y1 receptor activity does not mediate its effects on energy homeostasis, but rather reproductive development and behavior, and these reproductive changes are E_2 dependent. In addition, exogenous E_2 does play a vital role in appetite and body mass regulation and reproductive ability whether administered alone or in combination with NPY Y1R antagonist.

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