EFFECTS OF OVARIAN HORMONES ON BINGE-TYPE EATING IN RATS

A Dissertation in

Nutrition

by

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ABSTRACT

Binge eating is more common in females than in males. Ovarian hormones, estradiol and progesterone, can modulate food intake and have shown possible effects on binge eating in both humans and animals. In addition, binge eating shares behavioral and neurobiological similarities with substance abuse. Although estradiol generally inhibits food intake, it has been reported to increase the sensitivity of females to the rewarding effects of substances of abuse and promote the development of drug addiction in females. Those who binge may exhibit features of both homeostatic eating during non-binge times and hedonic addictive-like eating during binge times. Therefore, a series of studies was conducted to investigate the effects of ovarian hormones on ingestive behavior and neurological correlates of eating in rats maintained on a binge-eating protocol.

Study one investigated the effects of the ovarian hormones estradiol and progesterone on food intake and body weight in binge rats. Three groups of hormone-treated ovariectomized (OVX) rats and three groups of oil vehicle rats were used. Within each hormone treatment condition, rats were divided into three feeding schedules: chow, low-restriction access to shortening (daily), and high-restriction access to shortening (binge). All of the rats were non-food-deprived throughout. The main finding was that binge size during the 1-h binge access period was tonically, but not cyclically, reduced in hormone-treated binge rats. In contrast, daily total food intake was both tonically and cyclically reduced in hormone-treated binge rats. The results indicate that the normal cyclic inhibitory influence of ovarian hormones on eating, but not their normal tonic inhibitory influence, is disrupted by conditions leading to binge-type eating.

Study two investigated the individual roles of estradiol and progesterone on food
intake and body weight in binge rats. Two groups of estradiol-treated rats, two groups of estradiol and progesterone co-treated rats, two groups of progesterone-treated rats, and one group of oil vehicle rats were used. Within each hormone treatment condition, rats were divided into two feeding schedules: chow, and high-restriction (binge). Oil vehicle rats only had chow access. The main findings were that binge size was not different among the three hormone-treated groups (E, EP, and P) and was not cyclically reduced in any of the groups. In contrast, daily total food intake was both tonically and cyclically reduced in E and EP rats but not in P and OIL rats. The results indicate that administration of E alone has the same effect as co-administration of E and P on feeding and body weight in binge rats. Progesterone alone had no effect on feeding and body weight in binge rats.

Study three investigated the effects of estradiol and progesterone on the inhibitory effect of the GABA-B agonist baclofen on binge fat intake. The first experiment of this study included the same six groups that were used in study one. Five doses of baclofen (0, 0.6, 1.0, 1.8, 3.2 mg/kg) were administrated to each rat. The main finding was that baclofen reduced fat intake percentage change at 3.2 mg/kg in EP-treated binge rats and at 1.8 and 3.2 mg/kg in Oil-treated binge rats, indicating that baclofen was less effective in the EP-treated binge rats. The second experiment included the same six groups that were used in study two (no oil group). Four doses of baclofen (0, 1.0, 1.8, 3.2 mg/kg) were administrated to each rat. The main findings were that baclofen reduced fat intake at 1.0, 1.8 and 3.2 mg/kg in EP binge rats, but only at 3.2 mg/kg in P binge rats and was without effect in E binge rats. These results indicate that baclofen was even less effective
in the rats treated with E-alone or P-alone. Taken together, the inhibitory effect of baclofen on binge fat intake was influenced by hormones in this way: OIL ≥ EP > P > E.

Study four investigated the neural correlates of eating behavior in binge rats and the influences of estradiol and meal size by using c-Fos expression, a marker of stimulus-induced neural activation. The first experiment of this study included two groups of estradiol-treated rats (E2), another two groups of estradiol-treated rats (E4), and two groups of oil vehicle rats (O). Within each hormone treatment condition, rats were divided into chow, and high-restriction (binge) groups. After 6-weeks of binge training, rats had 50-min access to shortening and were perfused 30 min after the end of access. E2 and E4 rats were perfused on the day 2 and day 4 of hormone treatment cycles respectively. Brain tissues were collected and processed for c-Fos-like immunoreactivity (c-Fos-LI). Several findings are reported. First, there was more activation in the anterior cingulate cortex (Cg 1) and ventral tegmental area (VTA) in BO than in CO and less activation in VTA, basolateral amygdala, arcuate and lateral hypothalamus in BE2 than CE2. These results indicate that a history of repeated binge behavior promotes the activation of reward-related neural circuitry and this activation appears to be subject to the modulation of estradiol. The second experiment included three groups of estradiol-treated rats and three groups of oil vehicle rats. All rats were binge rats in this experiment. Within each hormone treatment condition, rats were given 50-min access to 0g, 1g, or 3g shortening on the last day and then were perfused. Brain tissues were processed for c-Fos-LI. The main findings include that there were main effects of meal size in CeA, VTA and midNTS but only meal size-dependant effects in the VTA of oil-treated binge rats. There were no hormone treatment effects on c-Fos response in any brain area. The
relatively small amount of food provided on the last day, as well as the perfusion of rats on day 4 of the treatment cycle may account for the negative effects.

The results of the present studies indicate that ovarian hormones, especially estradiol, may contribute to increased meal size during a binge and may reduce the therapeutic effect of baclofen. These effects may be due to effects of estradiol on reward-related dopaminergic mesocorticolimibic circuitry. The results of the present research provide a preliminary understanding of the effects of estradiol on eating behavior and the neurobiology of binge eating in females, and may help explain the contribution of estradiol to binge vulnerability in females.
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CHAPTER 1

LITERATURE REVIEW
1. Understanding binge eating

Bingeing-related eating disorders (BRED) including Binge Eating Disorder (BED) and Bulimia Nervosa (BN) are increasing health problems in western countries (1-3). Lifetime prevalence estimates of BRED are about 5% among women and about 2.5% among men according to the National Comorbidity Survey Replication (NCS-R), a nationally representative psychiatric epidemiological survey of the US household population that estimates the prevalence and correlates of mental disorders (4). As shown in Tables 1 and 2, the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) defined binge eating disorder as recurrent episodes of binge eating (at least twice a week for 6 months); and bulimia nervosa as recurrent episodes of binge eating (at least twice a week for 3 months) and recurrent use of inappropriate compensatory behaviors to prevent weight gain. An episode of binge eating for BED and BN is characterized by “eating in a discrete period of time (usually less than 2 hr) an amount of food that is larger than most individuals would eat under similar circumstances” (1), which is not necessarily driven by hunger or metabolic need (5). Individuals engaging in binge eating often feel loss of control and experience psychological distress (1,6).

Like other eating disorders, BED and BN are more common in females than in males. American women are 1.5 times more likely to develop BED and 3 times more likely to develop BN than are men (7,8). In Norway, the female-male ratios are 1.7:1 for lifetime prevalence of BED and 3:1 for BN in adolescents (9). Furthermore, people who do not meet the criteria for bingeing-related eating disorders (bulimia nervosa, binge eating disorder, binge/purge subtype of anorexia nervosa) also binge eat. For instance, one study reported a binge eating prevalence of 24% in a randomly sampled population
of women, whereas the prevalence of bulimia nervosa was only 1.5% (10). Various etiological mechanisms for the behavior of binge eating have been proposed and studied. Among these are psychological, environmental and biological mechanisms. The work included in this dissertation is limited to some of the possible biological mechanisms.

Table 1 DSM-IV Diagnostic Criteria for Binge Eating Disorder (307.50)

(A) Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the followings:

1. Eating, in a discrete period of time (e.g., within any 2h period), an amount of food that is definitely larger than most people would eat in a similar period of time under similar circumstances
2. The sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating)

(B) Binge-eating episodes are associated with three (or more) of the following:

1. Eating much more rapidly than normal
2. Eating until feeling uncomfortably full
3. Eating large amounts of food when not feeling physically hungry
4. Eating alone because of being embarrassed by how much one is eating
5. Feeling disgusted with oneself, depressed, or very guilty after overeating

(C) Marked distress regarding binge eating is present

(D) The binge eating occurs, on average, at least 2 days a week for 6 months

(E) The binge eating is not associated with the regular use of inappropriate compensatory behavior (e.g. purging, fasting, excessive exercise, etc.) and does not occur exclusively during the course of anorexia nervosa or bulimia nervosa

Table 2 DSM-IV Diagnostic Criteria for Bulimia Nervosa (307.50)

(A) Recurrent episodes of binge eating characterized by both:

1. Eating, in a discrete period of time (e.g., within any 2-hour period), an amount of food that is definitely larger than most people would eat during a similar period of time and under similar circumstances
2. A sense of lack of control over eating during the episode, defined by a feeling that one cannot stop eating or control what or how much one is eating

(B) Recurrent inappropriate compensatory behavior to prevent weight gain

1. Self-induced vomiting
2. Misuse of laxatives, diuretics, enemas, or other medications
3. Fasting
4. Excessive exercise

(C) The binge eating and inappropriate compensatory behavior both occur, on average, at least twice a week for 3 months.

(D) Self evaluation is unduly influenced by body shape and weight.

(E) The disturbance does not occur exclusively during episodes of anorexia nervosa.
2. Understanding the neurobiology of food intake regulation, substance abuse and binge eating

2.1 The neurobiology of food intake regulation

The central nervous system control of food intake is complex and consists of both short-term and long-term signaling pathways (11-13).

Short-term meal-related hormonal gut-brain signaling was proposed by Gibbs and Smith in the 1970s. These signals were proposed to control meal initiation and termination in response to acute energy needs and are responsive to the volume and energy content of food, as well as eating duration (14). During a meal, food digested in the gut generates “satiety signals” such as cholecystokinin (CCK) that are transmitted via vagal afferent fibers to the nucleus of the solitary tract (NTS) in the hindbrain resulting in meal termination. A separate “lipostatic model” regulatory system originally proposed by Kennedy addresses the long-term regulation of energy homeostasis, i.e. body weight and maintenance of energy stores in the form of body fat (15). Energy homeostasis is achieved when there is a balance between catabolic and anabolic influences. During periods of positive energy balance, adiposity signals such as leptin and insulin are secreted in proportion to body fat mass and then act on central effector pathways in the hypothalamus to suppress food intake and increase energy expenditure. Central effector pathways include those that stimulate food intake and promote weight gain (anabolic
pathways), such as the neuropeptide Y (NPY) system, and those that reduce food intake and promote weight loss (catabolic pathways), such as the hypothalamic melanocortin system (16-20). The arcuate nucleus (ARC) plays a critical role in the integration of adiposity signals due to a high density of NPY neurons and Pro-opiomelanocortin (POMC) neurons, the precursor molecule for hypothalamic melanocortins. From ARC, NPY and POMC neurons project to other hypothalamic regions such as the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), and perifornical area (PFA), where neurons synthesize several food-intake regulatory neuropeptides and send projections to the hindbrain (19,21-23) (24). In the PVN, neurons express thyrotropin-releasing hormone (TRH), corticotrophin-releasing hormone (CRH), and oxytocin, all of which decrease food intake (25-27). In the LHA and PFA, neurons express orexins and melanin-concentrating hormone (MCH), which increase food intake (23,28-30). In short, a complex integrative system exists for the control of energy homeostasis.

In addition to homeostatic eating driven by energy deficits, food intake can also be regulated by “hedonic eating” or “addictive-like eating” normally driven by high palatability of foods (31,32). Considerable evidence in both animals and humans indicates that the hedonic pathways share commons with reward-related pathways in substance of abuse (33,34). Much of work has focused on the mesolimbic dopamine pathway since both substance of abuse and natural food reward induce the release of dopamine into the nucleus accumbens (35). Other involved brain regions within this pathway include frontal cortices, amygdala, ventral striatum, anterior cingulate cortex, as in the similar pathway that will be described next (36).
2.2 The neurobiology of substance abuse

It has long been recognized that one of the reward-related neuronal circuits in substance abuse is the mesocorticolimbic dopamine pathway. In response to motivationally relevant events, the ventral tegmental area (VTA) stimulates the release of dopamine to projection terminals in brain regions such as the nucleus accumbens (Acb), basolateral amygdala (BLA), and prefrontal cortex (PFC), and. Dopamine within these regions is thought to facilitate cellular changes that establish learned associations between the drug and the context in which it is delivered (37,38). A wide variety of abused substances (e.g. cocaine, amphetamine, ethanol, and opiates) increase the release of dopamine from tegmental projection areas, especially in Acb (39-41). It is also believed that the behavioral responses such as reinforcing and locomotor effects of several substances of abuse are related to the increased dopamine release in Acb (42,43).

The VTA is part of the midbrain and is the location of dopaminergic cell bodies that project to mesocorticolimbic target regions, such as Acb, BLA, and PFC. The Acb has two distinct subregions: shell and core. Both shell and core are interconnected with other regions within the mesocorticolimbic system and are critical to either establishing or mediating the expression of the learned associations between motivated behavior and environmental perceptions (44-48). Similar to nucleus accumbens, the amygdala is critical in establishing learned associations between motivationally relevant behaviors (49). The glutamatergic projections from the basolateral amygdala to the prefrontal cortex and accumbens are required for the learned associations (48). The anterior cingulate cortex, a component of the PFC, is associated with executive function, which is composed of neural processes involved in detecting conflict or errors, preparing and
anticipating upcoming responses, performance monitoring, and implementation of control (50-53).

DA is associated with many types of motivated behavior (54). Although substances of abuse all result in increased dopamine in Acb, different drugs may act in different ways and activate different substrates within the mesocorticolimbic system.

In addition to mesocorticolimbic dopamine, orexin neurons in the LHA have also been suggested to play a role in reward processing and drug abuse (55). There was increased activation of LH orexin neurons, as indicated by c-Fos staining, in rats conditioned with morphine, cocaine or food relative to non-conditioned rats. In addition, direct microinjections of orexin into the VTA stimulated neuronal firing and elicited morphine preference in animals in which preference previously had been extinguished (56). These actions of orexin were suggested to be due to increased glutamate inputs and plasticity in the VTA (55,57). Thus, orexin projection from the LH to the VTA provides a critical connection between the homeostatic and hedonic signaling regions of the brain and has been proposed to play an important role in drug addiction.

2.3 The neurobiology of binge eating

Considerable evidence exists indicating similarities between binge eating and substance abuse. Many studies have documented the co-morbidity of eating disorders and substance abuse, especially in women (58). For example, the percentages of bulimics who report alcohol or substance abuse and/or dependence range from 2.9% (59) to 48.6% (60). The percentages of bulimics who report a past history of substance abuse and/or dependence range from 0% (61) to 55% (62). Conversely, the percentages of drug abusers who report a current or past history of bulimia or bulimic behaviors range from
8% (63) to 40.7% (64). In a community-based sample, severe bingeing was consistently associated with alcohol abuse in young adult women (65).

Animal studies also suggest similarities between the neurobiology of binge eating and neurobiology of substance abuse (66,67). In rats, binge eating of sugar produced behavioral and neurochemical changes that are similar to those observed with some drugs of abuse (68). The behavioral changes included: 1) opiate-like withdrawal signs, such as aggressive behavior, dysphoria, depression and specific somatic and physiological signs when withdrawal is precipitated with an opioid antagonist (e.g., naloxone) or when food and sugar are removed (67,69,70), 2) enhanced sugar intake following abstinence (71,72), and 3) locomotor and consummatory cross-sensitization between sugar and amphetamine (73,74). The neurochemical changes include release of accumbens dopamine (DA) upon bingeing each day, which is coupled with a delay in Ach release (75). During “withdrawal”, a reversal of accumbens DA-Ach balance has been reported, i.e. DA is low and Ach is high (67,69), a neural state that has been observed during withdrawal from drugs of abuse.

In humans, alterations within the dopamine system have been identified in individuals with BED and BN. Specifically, differences in dopamine receptor and dopamine transporter gene expression have been found in binge eaters compared to non-binge eaters (76). In addition, human brain imaging studies have shown activation of areas within reward neuronal circuitry in response to food cues in binge eaters. In a fMRI case study, increased activation in the anterior cingulate cortex was reported during response anticipation and response conflict processing in women with BN (77). In another study, in response to food stimuli, especially binge food stimuli, more activation
was seen in the prefrontal cortex of obese binge eaters than obese non-binge eaters (78). In contrast, after recovering from a bulimic-type eating disorder, women had significantly lower activation in the right anterior cingulate cortex and the left cuneus compared to control women (79). Food pictures also induced greater arousal, ACC activation and insula activation in bulimic patients than in healthy controls (80). Chocolate is one palatable food that often is craved by binge eaters. One study found more activation in the medial orbitofrontal cortex and ventral striatum of chocolate cravers than non-cravers in response to the image of dark chocolate. (81). Given the above, the orbitofrontal cortex and anterior cingulate cortex seem to be consistently activated in disordered eating patients or cravers.

In addition, binge eaters generally prefer foods that are high in fat and sugar (82). It has been reported that cues associated with palatable foods and drugs can induce similar regional activation within mesocorticolimbic circuitry. Drug- and food-related cues can also result in similar conditioned gene expression within the shared areas (83). Taken together, binge eating may involve neuronal reward circuitry that is similar to that which is involved in substance abuse.

3 The involvement of GABA-B receptors in substance abuse and food intake

3.1 GABA and GABA-B receptor

GABA is the dominant inhibitory neurotransmitter in the central nervous system and acts on two types of receptors: GABA-A and GABA-B receptors. GABA-A receptors are ligand-gated ion channels, whereas GABA-B receptors are G-protein-coupled receptors. GABA-B receptors are heterodimers that are expressed both pre- and
post-synaptically throughout the central nervous system. Presynaptically, GABA-B receptors are localized on bodies and/or dendrites of GABAergic neurons (autoreceptors) as well as non-GABAergic neurons (heteroreceptors). Postsynaptically, GABA-B receptors are localized on non-GABAergic neurons (84,85). Baclofen, which was synthesized by Heinrich Keberle in the 1960s, is an agonist selective for mammalian GABA-B receptors.

3.2 The use of baclofen in the treatment of substance abuse and possible mechanism

Recently, baclofen has shown promise for the treatment of substance abuse (86,87). Preclinical studies indicate that baclofen reduces the self-administration of many drugs of abuse including cocaine, methamphetamine, nicotine, heroine, morphine, and ethanol. For example, baclofen suppresses cocaine self-administration under a variety of experimental conditions (88-91), reduces ethanol intake (92,93), and reduces self-administration of heroin (94). Therefore, stimulation of GABA-B receptors negatively affects the intake of cocaine, alcohol, and heroin. Clinically, baclofen showed promise in the treatment of cocaine dependence and alcohol abuse or dependence (95,96). Baclofen also altered the sensory properties of smoked cigarettes, i.e. increased rating of ‘harsh’ and decreased ratings of ‘like cigarette’s effects’ (97).

One hypothesis for the effect of baclofen in substance abuse involves a modulation of dopamine transmission. It has been suggested that stimulation of inhibitory GABA-B receptors could dampen dopamine neuronal firing in the VTA, thus reducing dopamine release in the Acb (86,98-100). Drugs of abuse generally increase extracellular dopamine in Acb (101-105) while GABAergic drugs reduce the stimulation of dopamine in Acb induced by drugs of abuse (methamphetamine, heroin, and ethanol) (94,106,107).
3.3 The use of baclofen in the treatment of binge eating and possible mechanisms

Baclofen can also reduce binge eating. In a limited fat access binge eating rat model, intraperitoneal (IP) injection of baclofen reduced binge eating of fat (108-110), fat emulsions (111), and sugar and high-fat mixtures (112), but had no effect on or stimulated regular chow intake (108-110) or binge intake of sugar-rich diet (112) in male rats. In addition, in an open-label trial, baclofen showed promising results in women with BED or BN: 71% of patients reduced binge eating frequency by greater than 50% (113). However, how baclofen acts and whether baclofen reduces binge eating via the same GABAB-dopamine neural system remains unclear.

3.4 The effect of baclofen on non-binge type food intake and possible mechanisms

Baclofen generally has no effect on (109,114) or stimulates the consumption of standard diets in a variety of animal species including rat, mice, chickens, and pigs (115-118). This effect can be mediated via both GABA-A receptors and GABA-B receptors. The candidate neuronal sites for the stimulatory effect of GABA were suggested to be brain areas that control feeding including the paraventricular (PVN), ventromedial (VMH), and arcuate hypothalamic nuclei, where GABA is found in high concentrations (119-123).

4 Understanding the modulation of food and drug intake by ovarian hormones

4.1 Modulation of food intake by ovarian hormones and possible neural sites

Estradiol and progesterone are two ovarian hormones within the hypothalamus – pituitary – gonadal (HPG) axis. Both hormones but mainly estradiol can influence food intake (124). Across the menstrual cycle, food intake in women varies with a decrease in
the peri-ovulatory phase, when plasma estradiol concentration peaks; conversely, food intake generally increases in the luteal phase, when plasma progesterone levels are high (125-127). Adult female rats and mice also eat different amounts of food across their estrous cycle, which is usually 4 d in length. Rats and mice eat the least near the time of ovulation, during what is called the estrous phase, which occurs just after estradiol peaks, and eat the most during diestrus, when estradiol levels are low.

This cyclic food intake pattern is thought to be due to inhibitory effects of estradiol on eating (128). The decrease in eating during the peri-ovulatory phase of the ovarian cycle is referred to as the cyclic inhibitory effect of estradiol on food intake (129). In addition, ovariectomy (OVX) dramatically increases food intake and body weight in rats and mice, and administration of estradiol brings food intake and body weight back to a normal physiological level (124,130,131). There is some evidence for a similar effect in women (132). Thus, in addition to its cyclic effects, estradiol also has tonic inhibitory effects on food intake (124,129,130). In rats, pharmacological progesterone treatment can attenuate the intake-reducing effects of estradiol, but no physiological action of progesterone on eating has yet been shown (124,130,131).

The modulation of non-binge type eating by estradiol was suggested to be due to the increased satiating potency of negative-feedback controls of meal size that are elicited by gastrointestinal actions of ingested food (133-135). Specifically, the potential neural sites of estradiol’s action include the nucleus of the solitary tract in the hindbrain (NTS), the first central target of vagal afferent fibers mediating the satiating action of gut peptides such as CCK. In addition, estradiol is thought to also act in the PVN of hypothalamus (136,137). More recently, estradiol was found to negatively regulate
GABA-B receptors in the hypothalamus of female rats (138) and to rapidly attenuate the potency of baclofen to activate G-protein-gated inwardly rectifying K+ (GIRK) channels in hypothalamic POMC and dopamine neurons in female guinea pigs (139). It is likely that estradiol reduces inhibition of GABA on hypothalamic POMC neurons and thus facilitates the ability of POMC to reduce food intake.

In addition to its effects on non-binge eating, estradiol also appears to modulate binge-type eating. The frequency of binge-type eating has been reported to change during the menstrual cycle. In women with bulimia nervosa, binge frequency increased during the luteal phase and menses, when estradiol levels are low (140-142). Specifically, a significant negative association between estradiol and binge frequency as well as a significant positive association between progesterone and binge frequency were reported in women with BN (143). In none of theses studies, however, have alterations in binge size with the menstrual cycle been reported. Neither have the tonic and cyclic effects of ovarian hormones on binge behavior been investigated. Finally, bingeing may disrupt menstrual cyclicity and ovarian hormone function (144-146); in three studies, a range from 37-64% of women with BN experienced oligomenorrhea (147-149).

4.2 Modulation of drug seeking/taking by ovarian hormones on and possible neural sites

Although estradiol reduces food intake and binge frequency, it has very different effects on drug-seeking and taking behavior. In animal studies, estradiol enhances drug-seeking and taking behavior during acquisition, withdrawal, relapse and treatment (150). For example, intact females and E-treated OVX rats tend to acquire cocaine or heroin self-administration faster and take more drug once the behavior is established (150,151). Cocaine-stimulated locomotor activity is highest during proestrus and estrus phases, as
well as when estradiol is administrated to intact rats (152,153). Estradiol treatment given to OVX rats enhances responding on a progressive ratio schedule (154). In contrast to estradiol, progesterone treatment given concurrently with estradiol counteracts the effect of estradiol on acquisition of cocaine self-administration (154); however, progesterone alone does not affect cocaine self-administration (155).

One proposed hypothesis for the effect of estradiol on drug seeking and taking is a direct enhancing effect of estradiol on dopamine release in the Acb (156). Another possible idea is that estradiol mediates Acb and striatal dopamine release indirectly by its inhibitory effect on intrinsic GABAergic neurons (157). Estradiol was shown to decrease GABA-B-stimulated G-protein activation in the VTA of female rats (158). GABA-B receptors are located mainly, but not exclusively, on dopamine projection neurons in the VTA. Therefore, desensitization of GABA-B receptors by estradiol may curtail the inhibitory effect of baclofen on dopamine neuronal firing, resulting in greater dopamine concentrations in terminal regions such as the Acb and PFC. Little information about the association between progesterone and GABA-B function is known; but one study reported that progesterone increased the density of baclofen binding to GABA-B receptors in the neocortex of female rats (159).

5 Proposed neural mechanisms of eating behaviors in female binge eaters

Those who engage in binge eating may exhibit features of eating during non-binge times and “addictive-like eating” during bingeing. Therefore, I propose that the central neural control of non-binge eating and the central neural control of “addictive-like
“eating” both exist in binge eaters and both neural circuits are modulated by ovarian hormones especially estradiol.

Specifically, it is proposed here that the regulation of chow intake during non-binge times is modulated by ovarian hormones especially estradiol acting via feeding-regulatory circuitry involving the hypothalamus and hindbrain. Therefore, estradiol will reduce chow intake via increasing the satiating potency of negative-feedback controls of meal size in the NTS and PVN. Estradiol can also reduce chow intake by facilitating the anorexigenic ability of POMC neurons by attenuating the inhibition of GABA on POMC neurons in the Arc.

It is additionally proposed that the regulation of the intake of highly palatable fatty food during binge times is modulated by estradiol via mesocorticolimbic hedonic circuitry. Periodic bingeing may induce more dopamine release from the VTA into projection areas such as the PFC, Acb, and BLA. These areas together with the VTA are part of an integrated neuronal circuit that involves dopaminergic, glutamatergic and GABAergic interconnections (37,48,160). Estradiol will increase dopamine release by attenuating the inhibition of GABA on dopaminergic cell bodies in the VTA and therefore stimulate binge size during binge times.

6 Aims and hypothesis

Aim 1 To determine if food intake is affected by ovarian hormones – estradiol (E) and progesterone (P) in binge rats.

Study 1 To examine the effect of E and P on binge size and body weight in OVX rats.

Hypothesis:
1) binge size and daily food intake will be reduced in hormone-treated binge rats compared to vehicle-treated binge rats due to tonic inhibitory effects of estradiol on eating

2) binge size and daily food intake will be reduced on the day of the hormone treatment cycle modeling estrus (day 4) due to cyclic inhibitory effects of estradiol

3) day-to-day intake patterns of daily food intake will be different in hormone-treated and vehicle-treated binge rats due to the cyclic influence of ovarian hormones.

Study 2 To examine the effects of estradiol and progesterone independently and together on binge eating.

Hypothesis:

1) binge size will be reduced in E and EP rats compared to P rats due to tonic inhibitory effect of estradiol. Binge size would not be reduced on the day of treatment cycle modeling estrus (day 4) in E and EP rats due to the lack of cyclic inhibitory effect of estradiol on binge size in binge rats

2) daily energy intake will be reduced in E and EP rats compared to P rats and vehicle-treated rats due to tonic inhibitory effect of estradiol. Daily energy intake would be reduced on day 4 in E and EP rats but not in P rats due to cyclic inhibitory effect of estradiol on daily food intake

**Aim 2** To determine the effect of ovarian hormones on GABA-B-induced reductions of binge fat intake.
Study 3 To determine the effect of estradiol and progesterone on GABA-B receptor activity (peripherally) in OVX rats under binge-type condition.

Hypothesis:

1) combined administration of estradiol and progesterone will attenuate the inhibitory effects of baclofen on fat intake due to the attenuation of estradiol on GABA-B signaling

2) administration of estradiol alone will further attenuate the intake reducing effects of baclofen due to the removal of counteracting effects of progesterone

3) administration of progesterone alone would enhance the intake reducing effects of baclofen

Aim 3 To determine estradiol’s modulation of neuronal activation in response to feeding in female binge rats.

Study 4 To determine the neural correlates of binge eating in rats and the influence of estradiol. To determine whether the neuronal activation is meal size dependant

Hypothesis:

1) binge eating will activate the hedonic reward-related mesocorticolimbic pathway such as VTA, ACC, BLA, Acb evidenced by c-Fos expression

2) estradiol will enhance the activation of this pathway in binge rats

3) estradiol will also activate homeostatic food intake regulatory brain regions such as NTS and Arc of hypothalamus

4) Larger amount of shortening intake is associated with more c-Fos expression in several brain areas including NTS in binge rats
5) E treatment increases food-induced c-Fos expression in those hind brain areas

6) Amount of shortening intake is not associated or negatively associated with c-Fos expression in several other brain areas including ACC, Acb, VTA
References


CHAPTER 2

STUDY ONE

OVARIAN HORMONES INHIBIT FAT INTAKE UNDER BINGE-TYPE CONDITIONS IN OVARIECTOMIZED RATS

Introduction

Bingeing-related eating disorders including binge eating disorder (BED) and bulimia nervosa (BN) have become important health issues in western countries (1-4). Like other eating disorders, BED and BN are more common in females than in males. American women are 1.5 times more likely than men to develop BED and 3 times more likely to develop BN (4-6). In Norway, the female-male ratio is 1.7:1 for lifetime prevalence of BED and 3:1 for lifetime prevalence of BN in adolescents (7). Furthermore, people who do not meet the criteria for bingeing-related eating disorders (bulimia nervosa, binge eating disorder, binge/purge subtype of anorexia nervosa) also binge eat. For instance, one study reported a binge eating prevalence of 24% in a randomly sampled population of women, whereas the prevalence of bulimia nervosa was only 1.5% (8).

Although biological sex differences ultimately arise from the different genotypes of males (XY) and females (XX), after early development most sex differences are mediated through hypothalamic-pituitary-gonadal (HPG) axis function, especially the actions of gonadal steroid hormones – androgens, estrogens and progesterones (9,10). Several effects of gonadal steroid hormones on food intake have been well documented in both humans and animals (11-13). Food intake in women varies with the phase of the menstrual cycle, with a decrease in the peri-ovulatory phase, when plasma estradiol concentration peaks; conversely, food intake generally increases in the luteal phase, when plasma progesterone levels are high (14-16). Adult female rats and mice also eat different amounts of food across the estrous cycle, which is usually 4 d in length. Rats and mice eat least near the time of ovulation, during what is called the estrus phase, which occurs just after estradiol peaks, and eat most during diestrus, when estradiol levels are lower.
This cyclic food intake pattern is thought to be due to inhibitory effects of estradiol on eating (17). In rats, pharmacological progesterone treatment can reduce the intake-reducing effects of estradiol, but so far no physiological action of progesterone on eating has been shown (11-13). The decrease in eating during the peri-ovulatory phase of the ovarian cycle is referred to as the cyclic inhibitory effect of estradiol on eating (18). In addition, ovariectomy (OVX) dramatically increases food intake and body weight in rats and mice, and administration of estradiol brings food intake and body weight back to a normal physiological level (11-13). There is some evidence for a similar effect in women (14). Thus, in addition to its cyclic effects, estradiol also has tonic inhibitory effects on eating (11,12,18).

The frequency of binge–type eating has been reported to change during the menstrual cycle. In women with bulimia nervosa, binge frequency increased during the luteal phase and menses (19-21). In one study of women with BN, a significant negative association between estradiol and binge frequency as well as a significant positive association between progesterone and binge frequency were reported (22). In a community sample of women, changes in a modified Emotional Eating subscale of the Dutch Eating Behavior Questionnaire (DEBQ) also were associated with cyclic hormone fluctuations. Specifically, higher scores (consistent with binge eating) were obtained when estradiol was low and progesterone was high (23). In none of these studies, however, have alterations in binge size with the menstrual cycle been reported. Finally, bingeing may disrupt menstrual cyclicity and ovarian hormone function (24-26); in three studies, 37-64% of women with BN experienced oligomenorrhea (27-29). How binge behavior and HPG function might interact has not been established, and the necessary
mechanistic studies are difficult in human subjects. Animal models, therefore, are needed.

Several animal models have been developed to study binge eating (30). In the present study a limited-access binge-eating model is used. In this model, rats are given access to a source of dietary fat for one or two hours per day three times a week, with nutritionally complete rat chow and water always freely available. Fat intakes during the fat-access period are much higher under this 3-day limited-access condition than when rats are offered fat for similar periods every day (31-35). The model has face validity in that it reproduces a key criterion for human binge eating: the consumption of more food during a brief period than is normally consumed under similar circumstances. In addition, body weight typically does not differ between binge rats and chow controls. This is also similar to the maintenance of normal body weight by most patients with bulimia nervosa (1) as well as recent data showing that most people who binge are not obese (4). Therefore, rats with 3-day limited access are referred to as bingeing rats, and the three weekly fat access days are called binge days. Although the rats consume large amounts of energy on binge days relative to controls, they eat less chow on the non-binge days. Due to this “overeat/undereat” or “sawtooth” intake pattern, body weight typically does not differ between binge rats and chow controls. Thus, factors involved in binge behavior can be studied without obesity-related confounds that might influence food intake.

Although both intact female and intact male rats exhibit binge behavior with this protocol, the energy consumed during the limited-access period by females is much smaller than that consumed by males (32,33,35). In addition, the day-to-day intake patterns are different; that is, the overeat/undereat pattern is not as regular in females (33).
A possible reason for the smaller binge size in intact females compared to males may be the tonic inhibitory effect of estradiol on eating. The irregular day-to-day intake pattern, on the other hand, may be due to estradiol’s cyclic inhibitory effect during the estrus phase, which falls randomly on binge and non-binge days.

The rationale for the present study rests on reports, first, that higher levels of estradiol have been associated with decreased binge frequency in women with BN (22) and, second, that estradiol can elicit both tonic and cyclic inhibitory effects on eating under non-binge conditions in women and in female animals (17,36). Tonic and cyclic effects of ovarian hormones on binge behavior, however, have not been investigated. Therefore, we sought to investigate the tonic and cyclic effects of ovarian hormones on binge eating behavior in the limited fat access animal model. Specifically, we hypothesized that in this model: 1) binge size and daily food intake would be reduced in hormone-treated binge OVX rats compared to vehicle-treated binge OVX rats due to tonic inhibitory effects of estradiol on eating, 2) binge size and daily food intake would be reduced on the day of the hormone treatment cycle modeling estrus (day 4) due to cyclic inhibitory effects of estradiol, and 3) day-to-day intake patterns of daily food intake would be different in hormone-treated and vehicle-treated binge rats due to the cyclic influence of ovarian hormones.

Materials and methods

1. Subjects

Seventy-eight female Sprague-Dawley rats (Harlan, Indianapolis, IN; 60 days of age and initially weighing 184-218 g) were individually housed in stainless-steel cages
and pelleted chow (Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN; macronutrient content (g/kg diet, kcal/kg diet, percent of calories): protein (234, 936, 28%), fat (45, 405, 12%), carbohydrate (490, 1960, 60%); total, 3.3 kcal/g). The vivarium was maintained at 22 ± 2°C with a 12/12h light-dark cycle (lights off at 1900 h). All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

2. OVX and cyclic hormone treatment

After a one-week period of adaptation to the vivarium, rats were given overnight access to a bowl of solid fat [Crisco® All-Vegetable Shortening (partially hydrogenated vegetable oil), J.M. Smucker Co., Orrville, OH; 9.17 kcal/g] clipped to the front of cage, in addition to their continuously available chow and water. This was done to prevent neophobia during the rest of the study. Three days later, the rats were anaesthetized (1 ml/kg body weight, intraperitoneally: IP) with a mixture of 70 mg/kg Ketamine (Phoenix Science Inc., St. Joseph, MO) and 2 mg/kg Xylazine (Phoenix Science Inc., St. Joseph, MO), with 0.2 ml/kg supplements given as needed, and a bilateral OVX was performed using a dorsal approach.

After 4-5 days of postoperative recovery, when body weights had returned to their pre-surgical levels, rats were matched for body weight and overnight fat intake and divided into two groups. One group (OVX+EP, n = 39) was subcutaneously (SC) injected with 17-β-estradiol-benzoate (Sigma, 2 µg/100µl sesame oil) in the middle of the light phase every fourth day and with progesterone (Sigma, 500 µg/100µl sesame oil) 1 day later; the other group (OVX+OIL, n = 39) was injected with the sesame oil vehicle on the
same days. Injection days were followed by 2 non-injection days. The hormone treatment regimen is shown in Table 1. These hormone injection regimens produce near-physiological levels of estradiol (17) and progesterone (37), and maintain normal body weight, food intake, spontaneous meal patterns, and sexual receptivity (lordosis) in OVX rats (17). Note that the day of estradiol injection is labeled day 2 of the treatment cycle and the progesterone injection day is labeled day 3. This is done so that the last day of the cycle models the last day of the typical 4-d estrus cycle of intact rats; that is, Day 1: Diestrus 1, Day 2: Diestrus 2, Day 3: Proestrus, Day 4: Estrus.

Table 1 Cyclic ovarian hormone treatment regimen

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP</td>
<td>—</td>
<td>E</td>
<td>P</td>
<td>—</td>
</tr>
<tr>
<td>OIL</td>
<td>—</td>
<td>Oil</td>
<td>Oil</td>
<td>—</td>
</tr>
</tbody>
</table>

Cyclic ovarian hormone treatment regimen used throughout the study. All rats were ovariectomized. The 24-h test days begin and end at the tick marks, i.e. at 1600 h, 3 h prior to lights off. Hormones were injected at 1300 h (the middle of the light phase) on Day 2 and Day 3 of the cycle, so that Day 4 of the treatment cycle models the estrus phase of the ovarian cycle in intact rats. E = 2 \( \mu \)g \( \beta \)-estradiol 3-benzoate/100\( \mu \)l sesame oil/rat; P = 500 \( \mu \)g progesterone/100\( \mu \)l sesame oil/rat.

3. Feeding protocols

After two cycles of hormone treatment or one cycle of vehicle treatment, rats within each group were matched for current body weight and assigned to one of three
subgroups (n = 13/group), which were then maintained on one of the three fat-access schedules shown in Table 2: **Chow only (C)**, which had no additional fat; **Low-restriction access (L)**, which had access to a bowl of additional fat for 1 h/day every day, 2 h prior to lights off; and **High-restriction access (H)**, which had access to a bowl of additional fat for 1 h/day on Monday, Wednesday and Friday, 2 h prior to lights off. All rats had continuous access to chow and water throughout the study. These schedules were based on previous work from our laboratory (32,33,35). The H group is also referred to as the bingeing group. Chow intake was measured every 24-h prior to the fat access period. One-h fat and chow intakes were measured at the end of the 1-h fat access period. Food intake and weekly body weight were monitored as an indicator of the efficacy of the OVX and hormone-treatment protocols.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hormone Treatment</th>
<th>Fat Access</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP-C</td>
<td>Estradiol + Progesterone</td>
<td>None</td>
</tr>
<tr>
<td>EP-L</td>
<td>Estradiol + Progesterone</td>
<td>Low-restriction</td>
</tr>
<tr>
<td>EP-H</td>
<td>Estradiol + Progesterone</td>
<td>High-restriction</td>
</tr>
<tr>
<td>OIL-C</td>
<td>OIL</td>
<td>None</td>
</tr>
<tr>
<td>OIL-L</td>
<td>OIL</td>
<td>Low-restriction</td>
</tr>
<tr>
<td>OIL-H</td>
<td>OIL</td>
<td>High-restriction</td>
</tr>
</tbody>
</table>

4. **Body composition**

Body composition analysis was undertaken to investigate the source of the body weight differences observed at the end of the study. After 12 weeks on their respective diet protocols, rats were sacrificed via CO₂ asphyxiation. First, the gonadal, abdominal (mesenteric + non-gonadal), and retroperitoneal fat pads were removed, weighed and returned to the carcass for analysis. Then, body water, fat, ash, and protein were analyzed.
using methods previously described (32).

5. Data analysis

Data were analyzed using SAS 9.1 for Windows (SAS Institute, Cary, NC). The outcomes analyzed were 1-h energy intake (kcal), 24-h energy intake (kcal), weekly energy intake (kcal), body weight (g), fat pad weight (g), and body composition (dry mass in g, and water, mineral, fat, and protein in g and percent). All data are presented as means ± SEM. One-h energy intake and 24-h energy intake on binge days and non-binge days were analyzed by 3-way ANOVA (fat access schedule × hormone treatment × cycle day), with cycle day as a repeated factor. Cycle day 1 data was calculated by averaging all of the day 1 food intake data for each 4-day hormone treatment cycle on binge days (Mon, Wed, Fri) or non-binge days (Tues, Thurs, Sat, Sun) across the 6-week study. Similarly, food intake data on days 2, 3, or 4 of the cycle were averaged across all 6 weeks. Within each treatment group and under each fat access schedule, 1-h energy intake and 24-h energy intake on each cycle day were analyzed by 1-way ANOVA, with day as a repeated factor. Following the 1-way ANOVA, Tukey’s HSD post hoc tests were used to determine significant differences among cycle days. Within each hormone treatment group, average weekly 24-h energy intake was analyzed by 2-way ANOVA (fat access schedule × week), with week as a repeated factor. Body weights (before OVX, 5 d postovariectomy and 45 d postovariectomy), total and individual fat pad weights (gonadal, abdominal and retroperitoneal), and body composition data were analyzed by 2-way ANOVA (fat access schedule × hormone treatment). Planned comparisons among groups were examined using a Least Square differences (LS means) table, with the Bonferroni correction applied to insure an experiment-wide $\alpha < 0.05$. Tukey tests (for repeated
measures) and ANOVA outcomes were considered significant when \( \alpha < 0.05 \).

**Results**

1. 1-h energy intake

   Energy intake in the 1-h fat access period was significantly affected by fat access schedule, ovarian hormone treatment, and day of treatment cycle (Fig 1). There were main effects of the fat access schedule [main effect of fat access \( F(2,72)=148.22, p<0.0001 \)]. In both the OIL and EP groups, the 1-h energy intake of the H rats was significantly greater than that of the L rats, which in turn was significantly greater than that of the C rats (\( p<0.0001 \); Fig 1). In other words, bingeing, as operationally defined by H intake greater than L intake, occurred in both OIL and EP groups.

   There were also main effects of ovarian hormone treatment due to greater energy intake overall by the OIL rats relative to EP rats [main effect of hormone treatment \( F(1,72)=34.62, p<0.0001 \)]. In other words, the EP treatment tonically reduced 1-h energy intake (EP < OIL) in both H and L rats (\( p<0.0001 \); Fig 1).

   Hormone treatment interacted with fat access schedule [\( F(2,72)=8.99, p<0.0005 \)], apparently because intake was reduced only in the groups with access to dietary fat (H and L), but not in the group with access to only chow (C). This was likely due to a floor effect in the chow groups, as 1-h chow intake was quite low. Hormone treatment also interacted with cycle day [\( F(3,216)=5.25, p<0.05 \)] due to lower 1-h energy intake on Day 4 relative to Day 2 in the EP-L rats only [main effect of day \( F(3,216)=4.38, p<0.01 \)]. That is, the cyclic effect of EP treatment [1-h food intake in EP-treated rats was less on cycle day 4 than on cycle day 2, (17)] occurred only in the L rats (1-way repeated
ANOVA F (3,36)= 16.92, p<0.0001), not in the H rats (1-way repeated ANOVA F (3,36)=1.07, NS). There was no 1-h energy intake difference between day 2 and day 4 in any OIL group (Fig 1).

**FIGURE ONE**

Fig 1 Effect of cyclic hormone treatment and fat access schedule on 1-h energy intake. D=Day. # indicates significant differences among the C, L, and H groups within the OIL and EP rats (P<0.0001). * indicates significant differences between OIL and EP groups on the H and L feeding schedules (P<0.0001). † indicates intake on day 4 significantly different from intake on day 2 in EP-L group (P<0.05).

2. **Daily energy intake**

Twenty-four hour energy intake was significantly affected by fat access schedule, hormone treatment, and day of cycle on binge days as well as on non-binge days (Fig 2). There were main effects of the fat access schedule [binge days: main effect of fat access F(2,72)=85.04, p<0.0001; non-binge days: main effect of fat access F(2,72)=11.43, p<0.0001]. These were due to the fact that in both OIL and EP groups, H rats ate
significantly more than did L rats and C rats on binge days and significantly less on non-binge days (p<0.0001), i.e. the H rats exhibited an overeat/undereat, sawtooth pattern of consumption.

The main effect of ovarian hormone treatment revealed tonic inhibitory effects of EP on daily energy intake in all EP-treated groups on binge days (main effect of hormone F(1,72)=124.24, p<0.0001) and EP-L and EP-C groups on non-binge days (main effect of hormone F(1,72)=73.85, p<0.0001; Fig 2). In other words, the 24-h energy intakes were significantly lower in EP rats than OIL rats in the L and C groups on both binge and non-binge days, and in the H group on binge days (EP < OIL; p<0.0001). The lack of tonic inhibitory effect of EP in H groups on non-binge days resulted in an interaction between hormone treatment and fat access on non-binge days [F(2,72)=11.43, p<0.0001].

There also were cyclic effects of EP on 24-h energy intakes (Day 4 < Day 2). On binge days there were interactions between cycle day and fat access [F(6,216)=2.5, p<0.05] as well as between cycle day and hormone treatment [F(3,216)=15.95, p<0.0001]. On non-binge days, a similar profile emerged, i.e. there were interactions between cycle day and fat access [F(6,216)=4.62, p<0.005], as well as cycle day and hormone treatment [F(3,216)=10.74, p<0.0001]. These results were due to significant daily energy intake differences between day 2 and day 4 (day 4 < day 2) in all EP-treated groups on both binge and non-binge days, while there was no significant daily energy intake difference between day 2 and day 4 in any of the OIL groups. On binge days 1-way ANOVA F (3,36) and p values for the EP-L, EP-H and EP-C groups, respectively, were 23.32, P<0.0001, 6.58, p<0.005, and 19.97, p<0.0001, and on non-binge days, were 14.26, P<0.0001, 9.88, p<0.0001, and 31.93, p<0.0001.
Fig 2 Effect of cyclic hormone treatment and fat access schedule on 24-h energy intake on binge days (Mon, Weds, Fri) and non-binge days (Tues, Thurs, Sat, Sun). D=Day. # indicates H rats ate significantly more than C and L rats on binge days (P<0.0001) and significantly less on non-binge days (P<0.0001). * indicates significant differences between OIL and EP groups on the C, L and H feeding schedules on binge days, and in C and L groups on non-binge days (P<0.0001). † indicates intake on day 4 significantly different from intake on day 2 within the EP-C, EP-L and EP-H groups (P<0.05).
3. Weekly energy intake

Weekly average energy intakes were compared between chow control and fat-restricted rats in order to assess the compensatory abilities of the different groups (Table 3). In the OIL groups, the L rats consumed more energy than was consumed by the C rats in the first three weeks and then ate statistically the same amount of energy as did C controls thereafter. The H rats, on the other hand, consumed statistically the same amount as did the chow rats during all six weeks (interaction of week × fat access F(10,180)=6.71, p<0.0001; main effect of fat access F(2,36)=3.27, p<0.05).

Table 3  Weekly cumulative energy intake across the study

<table>
<thead>
<tr>
<th></th>
<th>Wk 1</th>
<th>Wk 2</th>
<th>Wk 3</th>
<th>Wk 4</th>
<th>Wk 5</th>
<th>Wk 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OIL GROUPS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>521.3±6.8a</td>
<td>510.2±8.9a</td>
<td>497.6±8.0a</td>
<td>481.7±9.5</td>
<td>470.3±9.3</td>
<td>452.5±7.8</td>
</tr>
<tr>
<td>L</td>
<td>590.1±10.8b</td>
<td>562.9±14.5b</td>
<td>535.8±12.9b</td>
<td>490.4±17.0</td>
<td>451.9±10.8</td>
<td>453.6±10.2</td>
</tr>
<tr>
<td>H</td>
<td>536.5±6.2a</td>
<td>517.4±7.5a</td>
<td>493.1±9.4a</td>
<td>475.7±9.6</td>
<td>454.2±9.0</td>
<td>449.9±10.3</td>
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<td><strong>EP GROUPS</strong></td>
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<td></td>
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</tr>
<tr>
<td>C</td>
<td>403.8±4.7a</td>
<td>391.6±4.2a</td>
<td>394.7±5.6a</td>
<td>379.3±5.5</td>
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<tr>
<td>L</td>
<td>454.8±8.2b</td>
<td>433.6±6.5b</td>
<td>422.7±9.4b</td>
<td>393.2±9.8</td>
<td>386.5±5.1</td>
<td>402.7±8.2b</td>
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<tr>
<td>H</td>
<td>437.7±6.8b</td>
<td>423.5±4.7b</td>
<td>411.9±10.1a</td>
<td>400.7±7.1</td>
<td>396.6±6.7</td>
<td>401.6±6.3b</td>
</tr>
</tbody>
</table>

Different lower case letters indicate different weekly energy intake within OIL or EP groups in each week (p<0.05).

In the EP-L rats, results were similar to those described above for the OIL-L rats. That is, the EP-L rats consumed more energy than did the EP-C rats in the first three weeks and then ate statistically the same amount of energy as did C controls for the next two weeks. By week 6, however, L intakes again exceeded C intakes. Results differed in the EP-H rats, in that average weekly intakes of the EP-H group exceeded those of the C group in the first two weeks and in week six, but were statistically similar to the chow.
4. Body weight

Body weight before OVX and at day 5 post-OVX did not differ significantly among groups. Body weight at the 6th week of the study, however, was significantly affected by hormone treatment, with the OIL rats (324 ± 2 g) weighing significantly more than the EP rats (248 ± 2 g) [Fig 3; interaction effect of (hormone treatment × fat access) F(2,72)=3.68, p<0.05; main effect of hormone treatment: F(1,72)=818.66, p<0.0001]. The interaction effect was due to significantly greater body weights in the EP-L and EP-H relative to EP-C (p<0.0167). Such differences did not emerge in the OIL rats. OIL rats gained 106 ± 4 g between day 5 and day 45 post-OVX, whereas EP rats gained only 29 ± 2 g during the same period. There was no significant difference in final body weight among the OIL groups.

Due to an intervening drug study, during which the EP and OIL treatments were maintained, there was an ~40-day interval between the end of the 6-wk feeding study and the day of sacrifice for the carcass analysis. Rats were sacrificed about 10 days after the end of the drug study, which allowed sufficient time for the drug to clear. The overall pattern of group differences was not affected by the drug study. Specifically, there was no significant difference in sacrifice body weight among the OIL groups. However, among the EP groups, only the sacrificed body weight of EP-L was statistically greater than that of EP-C. EP-H was ~10 g greater than EP-C; however, this was no longer statistically significant.
Fig 3 Body weight at day 5 of postovariectomy and weekly across the 6-week study. # indicates final body weight of L and H rats greater than C rats in EP groups (P<0.05). * indicates significant difference between OIL and EP groups that had the same feeding schedule (P<0.001).

5. Body composition

As analyses of the masses of the retroperitoneal, abdominal, and ovarian fat pads yielded similar results as analysis of the sum of the three pads’ weights, only the latter are presented (Table 4). The summed masses of the OIL groups' fat pads were significantly greater than those of the EP groups (Table 4). In addition, fat pad mass was greater in OIL-L rats than in OIL-H rats, which in turn were greater than OIL-C rats (Table 4; p<0.0167). Fat pad mass was not significantly affected by fat access in the EP
rats. Carcass analysis was done to determine if total body fat changed in proportion to the retroperitoneal, abdominal, and ovarian fat pads and to determine if there were also differences in fat deposition in regions other than the fat pads measured (e.g. subcutaneous fat). This analysis revealed that total body fat content, body protein, body water, body mineral, and wet carcass weight were all increased in OIL rats compared to EP rats (Table 4; P<0.0167), with the relative changes quite similar to those observed in

<table>
<thead>
<tr>
<th>Sacrifice body weight</th>
<th>C</th>
<th>H (Binge)</th>
<th>L</th>
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<td>326.4±4.21</td>
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<td>12.8±1.3ab, 1</td>
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</table>

Different lower case letters indicate significant differences within OIL or EP groups. Different numbers indicate significant differences between OIL and EP groups which had the same fat access schedule (p<0.0167).

† Fat pads are the combined mass of gonadal, abdominal and retroperitoneal fat pads.
the three fat pads measured. Body mineral content, body water content and wet carcass
were not different among the different diet protocols in the OIL rats, although in EP rats,
body water and wet carcass were greatest in the EP-L group (p<0.0167). Protein mass
was not affected by fat access in either OIL or EP rats.

Discussion

The major finding of this study is that binge size was tonically, but not cyclically,
reduced in the EP-H rats, i.e., rats in which highly restricted scheduled access to fat led to
significantly less energy than did the OIL rats during the 1-h fat access period, indicating
a tonic reduction of binge size in the EP rats. However, 1-h energy intake did not vary
cyclically across the 4-day hormone treatment cycle in the EP-H rats, but did vary
cyclically in the EP-L rats. One-h energy intake also did not vary in the EP-C rats, but we
attribute this to the very low 1-h intake in this group. Finally, 24-h energy intakes varied
cyclically in all three groups. Together these data indicate that, whereas the normal tonic
inhibitory effect of ovarian hormones on eating persists in this binge eating model, the
normal cyclic inhibitory effect on eating is disrupted during binge-type eating episodes.

The tonic and cyclic decreases in eating produced by cyclic hormone treatment in
OVX EP-L rats extends reports of such effects under several other conditions
(11,12,17,18,38) and indicates that these effects occur even with limited access to an
optional source of dietary fat. Cyclic estradiol, however, failed to produce cyclic
inhibition of eating during the 1-h fat access period in EP-H rats. The fact that cyclic
effects on 1-h fat intake were evident in the L rats indicates that the lack of effect in the

55
binge rats is not simply due to the availability of fat in addition to chow. Rather, it appears to be related to the consumption of large amounts in brief periods of time relative to controls, e.g. bingeing. Estradiol’s cyclic inhibitory effect on eating under non-binge conditions is expressed as reduced meal size, with no reduction in meal frequency (17,38). Thus, the present results suggest that the cyclic inhibitory effects of estradiol are compromised by binge-type consumption of large fatty meals.

We know of no comparable human data. Although binge frequency and subjective correlates of eating have been reported to change across the menstrual cycle in women with bulimia nervosa (22,23), alterations in binge size across the menstrual cycle have not. However, there are some reports indicating that the ability to limit meal size is compromised in women with bulimia nervosa. For instance, patients with bulimia nervosa eat significantly more of both single and multiple-item meals than do control women when instructed to binge eat (39); patients with bulimia nervosa also need to eat more than do controls to produce equivalent self-reported fullness during a meal (40). Such results suggest that the normal physiological inhibitory controls of eating, including the effects of estradiol, may be weakened in women who binge frequently. These effects may be related to a reduced satiation effect of CCK, because both blunted postprandial cholecystokinin release and delayed gastric emptying were seen in women with bulimia nervosa (11,12,41,42). In contrast, in one report using a 24-h naturalistic laboratory feeding situation, the majority of meals were of normal size in bulimic women; binges represented the minority of meals but were calorically rich, primarily due to increased consumption of fatty foods (43).
Because pharmacological doses of progesterone (1 mg or more) can reverse estradiol’s inhibitory effect on eating in rats (44), it is possible that the loss of estradiol’s cyclic effects during the binge was due to an enhanced effect of progesterone at this time. This does not seem likely, however, because there was still a cyclic decrease in 24-h intake in the same rats and because our progesterone dose (0.5 mg) was smaller than those reported to antagonize estradiol’s eating-inhibitory effect.

Alternatively, the lack of cyclic hormonal effects during the binge may have been due to other influences of estradiol that might affect binge behavior. The inhibitory action of estradiol on eating is generally thought to relate to “homeostatic” rather than “hedonic” controls of food intake (45-47). In addition, however, estradiol can have stimulatory effects on other behaviors, including drug intake (48,49) and drug-induced locomotor activity (50), that are considered hedonic rather than homeostatic. Binge eating is also likely to be more related to non-homeostatic, hedonic processes (45,46). This suggests that it is possible that the hedonic, stimulatory effects of estradiol predominated during consumption of the binge food in the EP-H rats, while the homeostatic, inhibitory effects of estradiol predominated during consumption of the chow. The net result would be an elevated binge size in the estrus phase of the ovarian cycle in the EP-H rats, eliminating the typical cyclic food intake pattern during the bingeing period, while still leaving cyclic effects on chow and overall daily intake intact.

Although the cyclic eating-inhibitory effect of estradiol on binge intake was eliminated in the binge rats, the tonic eating-inhibitory effect was still present, both in 1-h and in 24-h energy intake. In general, the average 24-h energy intake of the EP rats was lower than that of the OIL rats on both binge days and non-binge days. The one exception
to this occurred in the EP-H group; 24-h intake of EP-H was not significantly lower than OIL-H on non-binge days (Fig 2). This appeared to be due to the failure of the EP-H groups to undereat on non-binge days during the first few weeks of the study (24-h intake data not shown), thus increasing the 6-week mean data presented. During the latter weeks of the study, the tonic inhibitory effect of EP was indeed more clear.

Previous studies in male rats indicate that H rats overeat on binge days when fat is present and undereat on non-binge days, resulting in a net energy intake comparable to C rats (35). This occurred here in the OIL-H group but not in the EP-H group. In OIL groups, weekly average energy intake did not differ between H and C control rats in any of the 6 weeks. In contrast, weekly average energy intakes were greater in the EP-H rats relative to the EP-C controls in the first two weeks and in week six. However, in a previous study from our laboratory (33) intact bingeing female rats tended to undereat more than they overate until the fifth week. Whether this difference represents an activational effect of ovarian hormones on energy homeostasis warrants further investigation.

The effects we report here are likely to be of physiological relevance because we used near physiological amounts and patterns of estradiol and progesterone. For example, in previous work with OVX Long Evans rats, cyclic 2 μg estradiol benzoate administered on day 3 of a 4-day cycle produced estradiol concentrations comparable to those of intact cycling rats, with low and high levels all close to minimum and maximum intact values (10-30 and 180-300 pmol/L, respectively). Furthermore, normalized body weight and food intake patterns have been reported when this estradiol replacement regimen was used (17). Others have shown that in OVX Sprague-Dawley rats, progesterone doses
larger than those used here (~1000 µg/rat) injected 20 h following estradiol, produced peak plasma progesterone levels that were near to peak levels assayed during the proestrus phase in intact females (37,51-53) Therefore, the 500 µg/rat progesterone dose used in this study was lower than that which would be considered physiological. Although relatively low, this dose of progesterone has been reported to produce normal sexual receptivity in E-treated OVX rats (17). The EP rats in the present study gained 29 ± 2 g during the initial 6-weeks of the study, which is comparable to the 35 g body weight gain reported previously in intact female Sprague-Dawley rats maintained for 6 weeks on the same feeding schedule (33). Thus, the 4-day cycle of estradiol/progesterone administration used herein would not be considered pharmacological.

We included progesterone in the injection protocol in the present study because progesterone seems to be important for ligand binding to GABA-B receptors in the neocortex of female rats (37), and because the GABA-B agonist baclofen has been shown to reduce binge-type eating in rats (54) and to produce promising results in an open-label trial in humans (55). In addition, estradiol was found to negatively regulate GABA-B receptors in the pituitary and hypothalamus of female rats (56) and to rapidly attenuate the potency of baclofen in hypothalamic POMC neurons in female guinea pigs (57). Thus, desensitization of GABA-B receptors by estradiol could theoretically interfere with inhibitory/compensatory controls under binge-type conditions. Whether such alterations can explain the present results remains to be determined. We included progesterone also because high progesterone levels were associated with increased binge frequency independent of estradiol in women with BN (22). More recently, progesterone was independently positively associated with higher emotional eating scores in a community
sample (23). Therefore, it would be of interest to determine the individual roles of estradiol and progesterone under binge-type conditions.

The delayed compensatory behavior, however, is not likely due to alterations in the ability of the EP-H rats to learn how to adapt to the feeding protocol, because increases in estradiol or progesterone in serum, cortex, and hippocampus have been reported to enhance cognitive performance in both intact and hormone-primed OVX rats (58).

One aspect of human binge eating that group analyses using this model do not capture is that of individual differences in susceptibility. It may be possible, however, to pursue this issue in the context of this model, as individual variability in the amount of shortening consumed has been seen in this study as well as in previous studies using male rats. Within the EP-H (binge) rats in the current study, for instance, shortening intakes ranged from 13.4 to 73.3 kcal, with the top 24% of the rats (3 rats) consuming 60.5 kcal, and the bottom 24% consuming 16.5 kcal. Thus, as in humans (8), individual vulnerability to the effects of exposure to binge-inducing stimuli appears to exist under the conditions used in the present study.

As previously reported (11,13,59), OIL rats weighed significantly more than EP rats. Body weight and wet mass did not differ among the OIL rats; however, dry mass of the OIL-L group was significantly greater than that of the OIL-C controls (Table 4). The elevated dry mass of the OIL-L rats was primarily due to a higher fat mass; body water, mineral, and protein mass did not significantly differ among the OIL rats. This effect in the OIL rats is similar to a previous study in intact females (33), in which fat mass accounted for the somewhat higher carcass mass of the L rats relative to C controls. This
similarity suggests that repeatedly consuming large amounts of fat in brief periods of
time may have disrupted estrus cycling in the previous study.

In summary, this study reports that administration of estradiol and progesterone
no longer exerts cyclic inhibitory effects on the size of brief bouts of fat intake under
binge-type eating conditions. Clearly, further work is warranted to determine the
mechanisms involved in this effect and to determine if the phenomenon is of relevance to
binge size in human binge-type eating.

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Research Program (WISER) funded by the Pennsylvania Space Grant Consortium.
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CHAPTER 3

STUDY TWO

INDIVIDUAL EFFECTS OF ESTRADIOL AND PROGESTERONE
ON FOOD INTAKE IN OVARECTOMIZED BINGE RATS
Introduction

Ovarian hormones (e.g. estradiol and progesterone but mainly estradiol) affect food intake in both humans and animals (1-3). Estradiol has both tonic and cyclic inhibitory effects on eating (1,4,5). Tonic inhibition is evidenced by the increases in food intake and body weight that occur with ovariectomy (OVX) in animals, and (in many cases) with menopause in humans; administration of estradiol to OVX animals normalizes food intake and body weight (1-3,5). Cyclic inhibition is evidenced by changes in food intake across the estrous cycle in rats and across the menstrual cycle in women. Female rats and mice eat less during the estrus phase, which occurs just after estradiol peaks, and eat more during diestrus, when estradiol levels are lower (1,2). In women, food intake generally decreases in the peri-ovulatory phase, when estradiol is highest, and increases in the luteal phase, when estradiol is low (6-8). Physiological levels of progesterone, on the other hand, do not affect eating in rats; however, pharmacological progesterone treatment can reverse the inhibitory effect of estradiol on eating (3,6). In addition, in women with normal menstrual cycles, food intake increases in the luteal phase, when progesterone levels are high (6-8).

Binge eating also is modulated by ovarian hormones. In humans, the frequency of binge eating varies with the menstrual cycle, with higher binge frequency occurring during the luteal phase and menses, when estradiol levels are low and progesterone levels are high (9-11). A negative association between estradiol level and binge frequency, and positive association between progesterone level and binge frequency, has been reported in women with bulimia nervosa (BN) (12). In addition, higher emotional eating scores (consistent with binge eating) on an eating behavior questionnaire were obtained when
estradiol was low and progesterone was high in a community sample (13). Although binge frequency is negatively associated with estradiol in humans, associations between estradiol and binge size have not been reported.

In current animal models of binge-type eating, opportunities to binge are experimentally controlled; therefore, binge frequency cannot be used as an outcome measure. However, effects of ovarian hormones on binge size can be examined. In a previous report of ours, binge size was tonically, but not cyclically, reduced in OVX rats treated with both estradiol and progesterone (14). In the same rats, total daily food intake was reduced both tonically and cyclically, suggesting that the modulatory effects of estradiol on meal size differ depending upon the conditions under which food is consumed. Although effects were thought to be due primarily to estradiol-mediated events, the relative contributions of estradiol and progesterone could not be ascertained, since both hormones were administered. In women with BN physiological progesterone levels have been associated with increased binge frequency independent of estradiol (12). Furthermore, progesterone was independently and positively associated with higher emotional eating scores in a community study (13). Therefore, it is possible that progesterone contributed to the loss of cyclic inhibitory effects of estradiol on binge size in our previous report (14).

This study was designed to determine the individual roles of estradiol (E) and progesterone (P) as well as their combined effects (EP) on food intake in bingeing rats. We hypothesized: (1) that binge size would be reduced tonically in E and EP rats relative to P rats, but would not show cyclic variation in any of the groups, and (2) that daily energy intake also would be reduced tonically in E and EP rats relative to P and OIL rats.
However, in contrast to binge intake, we predicted that daily energy intake would show cyclic variation in E and EP, but not in P and OIL (14,15).

**Materials and Methods**

1. **Subjects**

   Female Sprague-Dawley rats (Harlan, Indianapolis, IN; 60 days of age) were individually housed in stainless-steel cages with ad libitum access to water and pelleted chow (Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN; macronutrient content (kcal/kg diet, percent of calories): protein (936, 28.05%), fat (405, 12.14%), carbohydrate (1960, 59.81%); 3.3 kcal/g). The vivarium was maintained at 22 ± 2°C with a 12/12h light-dark cycle (lights off at 1900 h). All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

2. **Ovariectomy (OVX)**

   After one-week adaptation to the vivarium, rats were given overnight access to fat (Crisco® shortening [hydrogenated vegetable oil], J.M. Smucker Co., Orrville, OH; 9.17 kcal/g) in a bowl clipped to the front of the home cage. This was done to prevent neophobia during the rest of the study. Three days later, the rats were anaesthetized (1 ml/kg body weight, IP) with a mixture of 70 mg/kg ketamine (Phoenix Science Inc., St. Joseph, MO) and 2 mg/kg xylazine (Phoenix Science Inc., St. Joseph, MO), with 0.2 ml/kg supplements given as needed, and bilaterally OVX using a dorsal approach.

3. **Experimental design**

   Sixty-two OVX rats were used. After 5 days of postoperative recovery from OVX surgery, rats were matched for body weight and overnight fat intake and divided into
seven groups. Two groups (n=9/group) were maintained on the same 4-day hormone treatment cycle as EP groups in our previous study; that is, subcutaneous injection with 17-β-estradiol-benzoate (Sigma, 2 µg/100µl sesame oil) at the end of day 2 followed by progesterone (Sigma, 500 µg/100µl sesame oil) 1 day later (14); two groups (n=9/group) had only E injections on day 2 and another two groups (n=9/group) had only P injections on day 3; the seventh group (n=8) had oil vehicle injections on day 2 and day 3. This hormone treatment regimen models the typical 4-d estrous cycle of intact rats; that is Day 1: Diestrus 1, Day 2: Diestrus 2, Day 3: Proestrus, Day 4: Estrus (14). The doses of E and P injected here produce near-physiological levels of estradiol (15) and progesterone (16). Cyclic estradiol maintains sexual receptivity (lordosis) in OVX rats (15), and normal body weight and food intake in OVX rats fed chow or allowed to binge on fat (14,15). The hormone treatment was continued throughout the experiment.

### Table 1 Summary of Experimental Groups

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<th>Group</th>
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<th>Fat Access</th>
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After four hormone treatment cycles, rats within each hormone treatment group were assigned to one of two fat-access protocols: Chow only (chow available ad libitum with no optional fat access) and our standard high-restriction “binge” protocol (chow available ad libitum with optional fat provided for 1h/day on Mon, Weds and Fri, 2h prior
to lights off). The experimental groups are summarized in Table 1. Chow intake was measured every 24h prior to and at the end of the 1-h fat access period on Mon, Weds, and Fri in all groups. Fat intake was measured at the end of the 1-h fat access period on Mon, Weds and Fri in the H groups. Body weight was measured before OVX, daily during the 6 days postovariectomy and once a week thereafter.

4. Data analysis

Data were analyzed using SAS 9.1 for Windows (SAS institute, Cary, NC). The outcomes analyzed were 1-h energy intake (kcal), 24-h energy intake (kcal), and body weight (g). All data are presented as means ± SEM. One-h energy intake includes 1-h fat intake and 1-h chow intake. Twenty four-h energy intake includes 1-h fat intake and 24-h chow intake. One-h energy intake and 24-h energy intake were analyzed by 3-way ANOVA for the 6 hormone-treated groups (fat access schedule × hormone treatment × cycle day), in order to determine main effects of access schedule and hormone treatment. In order to compare results among all groups, including the OC group, data also were analyzed using a 2-way ANOVA (group × cycle day). Within each treatment group and under each fat access schedule, 1-h energy intake and 24-h energy intake on each cycle day were analyzed by 1-way repeated ANOVA with day as a repeated factor. Cycle day 1 data was calculated by averaging all of the day 1 food intake data for each 4-day hormone treatment cycle across the 4-week study. Similarly, food intake data on day 2, 3, or 4 of the cycle were averaged across all 4 weeks. Tukey’s post hoc tests following the 1-way ANOVAs were used to determine significant differences between intakes on days 2 and 4. Body weights (before OVX, 5 d postovariectomy and 45 d postovariectomy) were analyzed by 2-way ANOVA for the 6 hormone-treated groups (fat access schedule ×
hormone treatment), in order to determine main effects of the hormone treatments, as well as by 1-way ANOVA in order to determine differences among all 7 groups, including OC. Differences were considered significant when $\alpha < 0.05$.

Results

1. 1-h energy intake

![1h energy intake by cyclic hormone treatment](image)

Figure 1 Effect of cyclic hormone treatment and fat access schedule on 1-h energy intake. D=Day, E=Estradiol, P=Progesterone, EP=Estradiol/Progesterone. 1-h energy intake did not differ among three hormone-treated groups. It also was not reduced in estrus phase compared to diestrus phase in any hormone-treated binge group.

Energy intake in the 1-h fat access period was significantly affected by the fat access schedule with binge rats consuming significantly more than did chow rats (3-way ANOVA: main effect of fat access schedule $F(1,48)=306.22$, $p<0.0001$; 2-way ANOVA: main effect of group $F(6,55)=65.46$, $p<0.0001$). There were no significant differences in 1-h energy intake among the different hormone-treated groups within any fat access schedule. There was also no difference between day 4 and day 2 of treatment in all groups; that is 1-h energy intake was not reduced in the estrus phase (day 4) compared to
diestrus (day 2).

2. Daily energy intake

Daily energy intake was significantly affected by hormone treatment (3-way ANOVA: main effect of hormone treatment F(2,48)=72.53, p<0.0001; 2-way ANOVA: main effect of group F(6,55)=25.58, p<0.0001). The main effect of hormone treatment was due to a reduction in daily energy intake in E and EP rats relative to P and OIL rats. That is, estradiol had a tonic inhibitory effect on daily 24-h food intake. There were also effects of cycle day on 24-h energy intake in E and EP rats. That is, daily energy intake was reduced in the estrus phase (day 4) compared to diestrus (day 2) in E and EP-treated rats (one-way repeated ANOVA: EC F(3,24)=16.08, p<0.0001; EH F(3,24)=12.11, p<0.0001; EPC F(3,24)=16.58, p<0.0001; EPH F(3,24)=13.61; p<0.0001) but not in P and OIL rats.

![Daily energy intake by cyclic hormone treatment](image)

Figure 2 Effect of cyclic hormone treatment on daily energy intake. D=Day, E=Estradiol, P=Progesterone, EP=Estradiol/Progesterone. * indicates OC > EC=EPC; PC > EC=EPC. # indicates intake on day 4 significantly different from intake on day 2.
3. Body Weight

Body weight before OVX and at day 5 post-OVX did not significantly differ among groups. At the end of the study (4th week), however, body weight was significantly affected by hormone treatment (2-way ANOVA: main effect of hormone treatment $F(2, 48)=237.36$, $p<0.0001$; 1-way ANOVA (group as the independent measure) $F(6, 55)=50.30$, $p<0.0001$) with P and OIL rats weighing significantly more (PH: 307±3.6 g; PC: 311±3.7 g; OC: 309±11.2 g) than did E and EP rats (EH: 244±3.4 g; EC: 243±4.7 g; EPH: 242±3.1 g; EPC: 238±2.7 g).

![Body weight graph](image)

Figure 3 Body weight at day 5 postovariectomy and weekly across this 4-week study. D=Day, W=Week. * indicates significant difference between P or OIL and E or EP groups, i.e. OC=PC=PH > EH=EC=EP=EPH=EPC.

Discussion

Results from the current study demonstrated different roles of estradiol and
progesterone in food intake control and body weight regulation in binge rats. The main findings include: (1) binge size was not cyclically reduced on day 4 of the hormone treatment cycle, a phase that models estrus, in any of the hormone-treated rats (E, EP, and P). Furthermore, binge size did not differ among E, P or EP-treated rats; (2) 24-h energy intake was tonically reduced by E and EP treatment and cyclically reduced on day 4 of the treatment cycle in E and EP rats but not in P and OIL rats; (3) Both E and EP treated rats maintained lower body weights than did P or Oil treated rats. Together, the results indicate that administration of E alone has the same effect as co-administration of E and P on feeding and body weight in binge rats. Progesterone alone had no effect on feeding and body weight.

The first finding of the current study extends findings from our previous report. That is, 1-h energy intake was not cyclically reduced in EP-treated binge rats (14). Others have shown that the cyclic inhibitory effect of estradiol is accomplished by reduced meal size, without a fully compensatory increase in meal frequency under non-binge conditions (15,17,18). Under binge-type conditions, however, it appears that opposite results occur. Specifically, in humans, estradiol level and binge frequency are negatively associated across the menstrual cycle (12), i.e. binge frequency is reduced when estradiol levels are elevated. Furthermore, the present results indicate that binge size remains large across the estrus cycle. Thus, it appears that the influence of estradiol on binge eating patterns is different from its influence on non-binge patterns. While the mechanisms that account for this are not yet known, the present results indicate that the cyclic inhibitory effects of estradiol on meal size are compromised by binge-type consumption of large fatty meals independent of progesterone modulation.
In contrast to our previous report, 1-h energy intake was not tonically reduced in the present study. That is, 1-h intakes did not differ among E, EP, and P binge rats. We defined the tonic inhibitory effect of estradiol as reduced intake in EP rats compared to vehicle-treated OVX rats in our previous report (14). In that study, EP rats consumed significantly less energy (38.9±4.2 kcal) during the 1-h fat access period than was consumed by OIL rats (63.8±4.5 kcal). The binge size in EP binge rats was comparable to binge size in female intact rats (35–40 kcal) (19) and the binge size in OIL rats was comparable to binge size in male rats under similar limited access conditions (~60 kcal) (20,21). In the present study, binge size was larger in the E and EP rats than was previously reported: 52.7±6.0 kcal in EP rats, 61.9±5.2 kcal in E binge rats, and 64.2±5.8 kcal in P rats, approaching the physiological capacity of the stomach for rats of this size (22). Although estradiol has been reported to stimulate consumption of a highly palatable food in female rats (23), the majority of evidence indicates that estradiol reduces food intake (18,24,25). The present data indicate that the tonic inhibitory effects of estradiol on meal size can be attenuated by binge-type consumption of fatty food. The slightly smaller, but not significantly different, binge size in EP rats relative to E rats is consistent with previous reports, in which combined restoration of estradiol and progesterone produced a more pronounced decrease in food intake and meal size than did estradiol treatment alone in OVX rats (26). Results differ, however, from those reported in another study, in which progesterone increased food intake, thus opposing the intake inhibitory effect of estradiol (27). The second study looked at the pattern of two-day food intake for consecutive 30 days rather than the meal size. Our results suggest that any opposing
effect of progesterone is weak, at best, when meal size is large, such as occurs during a binge.

The second finding that E alone or co-administrated with P reduced daily food intake relative to vehicle-treated rats is consistent with previous work under either similar binge-type conditions (14) or other feeding conditions in OVX rats (15,18,26). Administration of progesterone alone had no effect on food intake either tonically or cyclically in the absence of estrogen in both chow and binge rats. This is consistent with previous reports that progesterone alone had no effect on food intake, meal size or meal frequency in OVX rats (18). Together, the present results indicate that estradiol, but not progesterone, regulates the consumption of non-binge foods in female rats even under binge-type conditions.

The third finding that administration of either E or EP prevented OVX-induced body weight gain in both binge and non-binge rats confirms the effect of estradiol on body weight reported before (14,15,28). The body weight gains (~30g) within 4 weeks in E or EP rats were comparable to weight gains in binge intact female rats and EP-treated binge rats in previous studies (14,19), indicating the efficacy of the hormone treatment. Our results indicate that normal levels of estradiol with or without progesterone can maintain body weight at a normal level in rats under binge-type conditions. Similar to the effect on food intake, there was no individual effect of progesterone on body weight in OVX rats. This is also consistent with a previous report that progesterone alone had no effect on body weight (18). The present results indicate that OVX-induced body weight gain is primarily due to loss of estradiol even under binge-type conditions.

In summary, administration of estradiol alone or co-administrated with progesterone
resulted in both tonic and cyclic inhibition of daily food intake, neither tonic nor cyclic inhibition of binge size, and maintenance of body weight at normal levels in binge OVX rats. In contrast, administration of progesterone alone had no effect on either food intake or body weight in binge OVX rats. This indicates that estradiol is the primary ovarian hormone responsible for food intake and body weight regulation under binge-type conditions in rats. Furthermore, the present results indicate that the inhibitory effects of estradiol on meal size are compromised, independent of progesterone modulation, when large fatty meals are consumed.

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References


CHAPTER 4

STUDY THREE

ESTRADIOL AND PROGESTERONE ATTENUATE EFFECTS OF BACLOFEN ON BINGE EATING OF FAT IN FEMALE RATS
Introduction

Similar neurobiological mechanisms may contribute to substance abuse and disordered eating, and these mechanisms may involve γ-aminobutyric acid B (GABA-B) receptors. GABA is the dominant inhibitory neurotransmitter in the central nervous system and acts on GABA-A and GABA-B receptors (1). GABA-A receptors are ligand-gated ion channels, whereas GABA-B receptors are G-protein-coupled receptors. GABA-B receptors are heterodimers that are expressed both pre- and post-synaptically throughout the central nervous system. Stimulation of GABA-B receptors negatively affects the intake of cocaine, alcohol, and heroin. Specifically, peripheral or intrategmental administration of the GABA-B agonist baclofen suppresses cocaine self-administration under a variety of experimental conditions (2-5), reduces ethanol intake at certain doses (6,7), and reduces self-administration of heroin (8) in animals. Clinically, baclofen has shown promise in the treatment of cocaine dependence and alcohol abuse or dependence (9,10). Similar to its effect on drug self-administration, intraperitoneal (IP) injection of baclofen reduces binge-type consumption of optional fatty foods. Specifically, baclofen reduced intake of solid fat (11), solid fat emulsions (12), and solid sugar and high-fat mixtures (13), while having no effect on or stimulating chow intake (11) in male rats. In an open-label clinical trial, baclofen showed promising results in women with Binge Eating Disorder (BED) or Bulimia Nervosa (BN): 71% of patients reduced binge eating frequency by greater than 50% (14).

The effect of baclofen on substance abuse has been observed to vary with sex, indicating the influence of ovarian steroids. For example, female rats pretreated with baclofen took longer to acquire cocaine self-administration than did male rats and fewer
females met acquisition criterion (15). This would suggest that female hormones might enhance the inhibitory effects of GABA-B activation and attenuate reward signaling. Indeed, baclofen binding to brain synaptic membranes in neocortex, hippocampus, and hypothalamus varies with the rat estrous cycle (16); progesterone, in particular, has been reported to increase the density of baclofen binding to GABA-B receptors in the neocortex of female rats (17). Thus, the enhanced inhibitory effects of baclofen on the acquisition of cocaine self-administration in females may have been due to actions of progesterone on GABA-B receptor binding.

Estradiol, on the other hand, appears to have effects that counter those reported for progesterone. Estradiol decreased GABA-B-stimulated G-protein activation in the VTA of female rats, suggesting that GABA-B binding sites are reduced or uncoupled to their intracellular Gαi/o proteins by estrogen (18). While GABA-B receptors are present on different cell types within the VTA, density is highest on DA cell bodies (19,20). Therefore, desensitization of GABA-B receptors by estradiol may attenuate the inhibitory effect of baclofen on DA neuronal firing and enhance reward signaling.

Similar effects have been reported in other brain regions. Estradiol negatively regulated the expression of GABA-B receptors in the hypothalamus of female rats both at the mRNA and protein levels (21). Estradiol also rapidly reduced the potency of baclofen to activate G protein-gated inwardly rectifying K+ (GIRK) channels in hypothalamic neurons (22). The hypothalamus is critical to the regulation of energy homeostasis and portions of it are also involved in reward processing. Therefore, it is possible that by reducing GABA-B-mediated inhibitory activity in the VTA, hypothalamus and other brain regions involved in food reward; estradiol may attenuate GABAergic inhibition of
reward processing. Indeed, differential activation of reward-related circuitry has been reported in males and females, with activation being stronger in females during reward delivery (23). This could explain why females are more likely to develop eating disorders that involve binge-type consumption of highly palatable fatty foods, i.e. binge eating disorder (BED) and bulimia nervosa (BN), than are males (24,25). Estradiol, therefore, may have dual effects on ingestive behavior, i.e in addition to its well known ability to enhance satiating signals relevant to homeostatic control of food intake (26,27), estradiol may also enhance reward signals relevant to food hedonics. Indeed, recent work from our group has shown that, while chow intake is both tonically and cyclically reduced by hormone replacement in female ovariectomized rats, binge intake of fat no longer displays cyclic regulation (28).

The present research sought to determine the influence of progesterone and estradiol on the ability of baclofen to reduce fat intake in female rats under binge type conditions. We predicted that estradiol and progesterone would have differential effects on the ability of baclofen to reduce fat intake. Specifically, we hypothesized that: 1) combined administration of estradiol and progesterone (4-day treatment, (28)) would attenuate the inhibitory effects of baclofen on fat intake due to the attenuation of GABA-B signaling by estradiol; that is, we anticipated that effects of estradiol on GABA-B actions would predominate over those of progesterone; 2) administration of estradiol alone would further attenuate the intake reducing effects of baclofen due to the removal of counteracting effects of progesterone; 3) administration of progesterone alone would enhance the intake reducing effects of baclofen.
Materials and methods

1. Animals

Female Sprague-Dawley rats (Harlan, Indianapolis, IN; 60 days of age) were individually housed in stainless-steel cages with ad libitum access to water and pelleted chow (Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN; macronutrient content (kcal/kg diet, percent of calories): protein (936, 28.05%), fat (405, 12.14%), carbohydrate (1960, 59.81%); 3.3 kcal/g). The vivarium was maintained at 22 ± 2°C with a 12/12h light-dark cycle (lights off at 1900 h). All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

2. Ovariectomy (OVX)

After one-week adaptation to the vivarium, non-food-deprived rats were given overnight access to a bowl of fat (Crisco® shortening [hydrogenated vegetable oil], J.M. Smucker Co., Orrville, OH; 9.17 kcal/g) clipped to the front of the cage. This was done to prevent neophobia during the rest of the study. Three days later, the rats were anaesthetized (1 ml/kg body weight, IP) with a mixture of 70 mg/kg Ketamine (Phoenix Science Inc., St. Joseph, MO) and 2 mg/kg Xylazine (Phoenix Science Inc., St. Joseph, MO), with 0.2 ml/kg supplements given as needed, and bilaterally OVX using a dorsal approach.

3. Experimental Design

Experiment 1

Experiment 1 was conducted to test the hypothesis that combined administration of estradiol and progesterone would attenuate the inhibition of baclofen on fat intake relative to oil vehicle in ovariectomized rats.
Seventy-eight OVX rats were used in experiment 1. After 4-5 days of postoperative recovery from OVX surgery, rats were matched for body weight and overnight fat intake and divided into six groups of 13 rats each. Three groups (OVX+EP) were subcutaneously (SC) injected with 17-β-estradiol-benzoate (E; Sigma, 2 µg/100µl sesame oil) on day 2 and progesterone (P; Sigma, 500 µg/100µl sesame oil) on day 3 every four days as described in our previous report (28); the other three groups (OVX+OIL) were injected with the OIL vehicle on the same days. The injections were maintained throughout experiment 1. This regimen and the dose of ovarian hormones maintain normal body weight, food intake, and mimics the typical 4-d estrus cycle in intact rats (29), that is, Day 1: Diestrus 1, Day 2: Diestrus 2, Day 3: Proestrus, Day 4: Estrus.

After two cycles of hormone treatment, rats within each of the three hormone treatment groups were assigned to one of three fat-access schedules: Chow only (C): No fat was provided to this group at any time during the study; Low-restriction access to fat (L): Fat was provided for 1h/day every day, 2h prior to lights off; High-restriction access to fat (H): Fat provided for 1h/day only on Monday, Wednesday and Friday, 2h prior to light off. These schedules were based on previous work from our laboratory (28). The high-restriction group is also referred to the bingeing group, operationally defined as 1h food intake in H group being greater than that of the L group. The experimental groups are summarized in Table 1. Chow intake was measured during the 23 h prior to the 1-h fat access period as well as during the 1-hr fat access period (chow was available to the rats for 24-h per day). Fat intake was measured during the 1 h-fat access period daily in the L group or on Mon, Weds and Fri in the H group. Body weight was measured before OVX,
daily during the 6 days postovariectomy and then once a week thereafter.

Table 1 Summary of Experimental Groups*

<table>
<thead>
<tr>
<th>Group</th>
<th>Hormone Treatment</th>
<th>Fat Access</th>
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<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
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<tr>
<td>Experiment 1 (n=13/group)</td>
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<tr>
<td>EP-C</td>
<td>Estradiol</td>
<td>Progesterone</td>
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<td>EP-L</td>
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<td>Progesterone</td>
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<td>EP-H</td>
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<td>Oil-H</td>
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<td>Experiment 2 (n=9/group)</td>
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<tr>
<td>EP-C</td>
<td>Estradiol</td>
<td>Progesterone</td>
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<tr>
<td>EP-H</td>
<td>Estradiol</td>
<td>Progesterone</td>
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<td>E-H</td>
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<td>P-C</td>
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<td>P-H</td>
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* Hormone injections were given at the middle time of the light phase on Day 2 and Day 3 of the cycle, so that Day 4 of the treatment cycle corresponds to estrus phase of ovarian cycle in intact rats. E = 2 µg β-estradiol 3-benzoate/100 µl sesame oil; P = 500 µg progesterone/100 µl sesame oil; O = sesame oil. Control (C) groups had no access to fat; low-restriction (L) groups were offered fat for 1 h/day each day, 2h prior to light off. High-restriction (H) groups were offered fat for 1 h/day on Mondays, Wednesdays and Fridays, 2h prior to light off.

Experiment 2

Experiment two was conducted to test the hypotheses that administration of estradiol alone would further attenuate, while administration of progesterone alone would enhance, the inhibition of baclofen on fat intake relative to co-administration of both hormones.

Fifty-four OVX rats were used in experiment 2. After 5 days of postoperative recovery from OVX surgery, rats were matched for body weight and overnight fat intake and divided into six groups of 9 rats each. Two groups were maintained on the same 4-
day hormone treatment cycle as EP groups in experiment 1; two groups had only E injections on day 2 and another two groups had only P injections on day 3. The hormone treatment was maintained throughout the experiment 2.

After four cycles of hormone treatment, rats within each hormone treatment group were assigned to one of two fat-access schedules: Chow only (no fat access) and High-restriction. The experimental groups summarized in Table 1. Chow intake, fat intake and body weight were measured as described in experiment 1.

4. Drug Test

After 6 weeks on the fat access protocols in experiment 1 and 4 weeks in experiment 2, effects of the GABA-B agonist baclofen (Tocris, Ellisville, MO) were assessed. Baclofen was dissolved in 0.9 % saline and administered intraperitoneally at a volume of 1 ml/kg. Four doses of baclofen (0.6, 1.0, 1.8, and 3.2 mg/kg) as well as the saline vehicle were used in experiment 1. Three doses of baclofen (1.0, 1.8 and 3.2 mg/kg) and saline were used in experiment 2. Baclofen was administered 30 min prior to fat access on Mondays and Fridays. Intervals between two consecutive baclofen administrations were at least two days. Doses were assigned to each rat according to a uniformed Latin Square, with each rat receiving all doses in a given experiment.

5. Data Analysis

Data were analyzed using SAS 9.1 for Windows (SAS institute, Cary, NC). All data are presented as means ± SEM. Body weights and 1-h energy intake in the last two weeks before baclofen tests was analyzed by 2-way ANOVA (fat access × hormone treatment). One-h fat intake and 1-h chow intake under each fat access schedule were analyzed by 2-way ANOVA (hormone treatment × drug dose) for two hormone-treated
groups in experiment one and for three hormone-treated groups in experiment two in order to determine main effects of drug doses. Differences in 1-h fat intake between different baclofen doses and saline were also expressed as percentage of saline. Within each hormone treatment group, one-way repeated ANOVAs were used to examine the effect of baclofen dose on 1-h fat intake, 1-h chow intake, and percentage of saline with doses as the repeated factor for all groups in baclofen tests. Tukey’s post-hoc tests were used to analyze significant differences among individual means. Differences were considered significant when $\alpha <0.05$.

Results

Experiment 1

Energy intake and body weight

Rats were maintained on the assigned feeding protocols for 6 weeks prior to baclofen testing. During the last two weeks, mean energy intake during the 1-h fat access period was significantly different among the groups, with greater energy intakes in the H groups than in the L groups, which, in turn, were significantly greater than in the C groups (interaction effect, $F(2,72)=8.37$, $p<0.001$; main effect of schedule $F(2,72)=147.17$, $p<0.0001$). Thus, high-restriction rats were considered binge rats, based upon the operational definition of a binge as H intakes being greater than L intakes. There were also main effects of ovarian hormone treatment due to greater energy intake by the OIL rats relative to EP rats (main effect of hormone treatment $F(1,72)=32.58$, $p<0.0001$).

Body weight at the conclusion of the 6-week period was different, with the OIL rats (324±1.9 g) weighing significantly more than did the EP rats (248±1.9 g) (interaction
effect $F(2,72)=3.68$, $p<0.05$; main effect of hormone treatment: $F(1,72)=818.66$, $p<0.0001$). The interaction effect was due to significantly greater body weights in the EP-L and EP-H rats relative to EP-C ($p<0.0167$).

Fat intake and Baclofen

As shown in figure 1, under the high-restriction (H) condition, higher doses of baclofen significantly reduced 1-h fat intake in both OIL and EP groups (main effect of drug dose $F(4, 96) = 26.35$, $p<0.0001$). Specifically, baclofen reduced 1-h fat intake at 1.8 and 3.2 mg/kg in the OIL group ($F (4,48) = 22.97$, $P<0.0001$) and 1.8 and 3.2 mg/kg in the EP group ($F (4,48)= 5.54$, $P<0.001$). One-h fat intake percentage indicated 1-h fat intake had significant reduction at 1.8 and 3.2 mg/kg compared to saline in the OIL group ($F (4,48) = 19.59$, $P<0.0001$), but only at 3.2 mg/kg in the EP group ($F (4,48) = 5.38$, $P<0.005$). Under the low-restriction (L) condition, there was main effect of drug on 1-h fat intake in both OIL and EP groups ($F(4, 96) = 14.23$, $p<0.0001$); however, baclofen did not significantly reduce fat intake at any dose in the OIL or EP group according to Tukey’s post hoc test.

Chow intake and baclofen

As shown in figure 2, baclofen either stimulated or had no effect on chow intake in all groups during the 1-h fat access period (main effect of drug dose $F (4, 96) = 5.56$, $p<0.0005$). Specifically, baclofen increased chow intake at 1.8, 3.2 mg/kg in the OIL High restriction (H) group ($F (4,48) = 6.00$, $P<0.0005$), at 1.0, 1.8 mg/kg in the OIL Low restriction (L) group ($F (4,48)= 4.12$, $P<0.01$), and had no effect in the OIL Chow (C) group. Baclofen had no effect on chow intake in the EPH group, increased chow intake at 1.8 mg/kg in EPL group ($F (4,48)=7.26$, $P<0.0001$), and at 3.2 mg/kg in EPC group ($F$...
Figure 1 Effect of baclofen on fat intake in OIL rats and EP rats under conditions of high-restriction access to fat and low restriction to fat. * # indicate different from saline administration in OIL rats and EP rats respectively. Percentage numbers indicate the 1-h fat intake percentage at different doses compared to saline in Oil and EP rats respectively.
Figure 2 Effect of baclofen on chow intake in OIL rats and EP rats under conditions of high-restriction access to fat and low restriction to fat. * # indicate different from saline administration in OIL rats and EP rats respectively.
Experiment 2

Energy intake and body weight

Rats were maintained on their assigned feeding protocols for 4 weeks prior to baclofen testing. During the last two weeks, 1-h energy intake was significantly affected by schedule, with greater energy intake in the H group than in the C group (main effect of schedule $F(1, 48) = 290.84, P<0.001$).

At the end of the 4-week period, body weight significantly differed among the 6 groups, with the P groups weighing significantly more than the E and EP groups ($F(6,54)= 105.89, P<0.0001$).

Figure 3  Effect of baclofen on fat intake in EP, E and P rats under condition of high-restriction access to fat. * # indicate different from saline administration in EP rats and P rats respectively. Percentage numbers indicate the 1-h fat intake percentage at different doses compared to saline in EP, E, and P rats respectively.

Fat intake and Baclofen

As shown in figure 3, higher doses of baclofen significantly reduced 1-h fat intake
(main effect of drug dose $F (3, 95) = 18.95$, $P<0.0001$). Specifically, baclofen reduced 1-h fat intake at 1.0, 1.8, and 3.2 mg/kg in the EP group ($F (3, 24) = 20.56$, $P<0.0001$), at 3.2 mg/kg in the P group ($F (3, 24) = 4.49$, $P<0.05$) and had no inhibitory effect on fat intake in the E group ($F (3,24) = 2.15$, $P = 0.12$). One-h fat intake percentage indicated 1-h fat intake had significant percentage reduction at 1.0, 1.8, and 3.2 mg/kg compared to saline in the EPH group ($F (3, 24) = 26.25$, $P<0.0001$), but had no reduction at any dose in the EH and PH groups.

Figure 4 Effect of baclofen on chow intake in EP, E and P rats under conditions of high-restriction access to fat and no access to fat.
Chow intake and Baclofen

As shown in figure 4, baclofen had no effect on chow intake in all groups during the 1-h fat access period.

Discussion

Several new findings are reported. In study one, baclofen reduced fat intake at 1.8 and 3.2 mg/kg in both OIL and EP rats under binge-type condition (high-restriction fat access schedule). One-h fat intake percentage indicated baclofen caused significant reduction at 1.8 and 3.2 mg/kg in OIL rats but at 3.2 mg/kg in EP rats, i.e. the potency of baclofen was reduced in the EP rats, supporting our hypothesis that hormone treatment attenuates the inhibitory effects of baclofen on fat intake. Study two was designed to determine which hormone provides the predominant attenuating influence on the potency of baclofen. In study two, baclofen reduced fat intake at 1.0, 1.8 and 3.2 mg/kg in EP rats, but only at 3.2 mg/kg in P rats and was without effect in E rats. This result indicates that baclofen was even less potent in the rats receiving estradiol or progesterone alone than in rats in which the hormones were co-administered. The loss of effect in rats administered estradiol supports our hypothesis that estradiol alone attenuates the intake reducing effects of baclofen to a greater extent that does co-administration of estradiol and progesterone. However, the reduced potency in the progesterone rats was unexpected.

Taken together, the inhibitory effect of baclofen on fat intake was influenced by ovarian hormones in the following manner (from greatest effect to least effect): oil vehicle ≥ administration of both estradiol and progeterone > progesterone alone > estradiol alone.

The finding that baclofen reduced fat intake under limited access conditions while
increasing or having no effect on chow intake is consistent with previous reports from this lab (11-13,30). Administration of estradiol once every four days followed by progesterone to binge rats seems to attenuate the reducing effect of baclofen on fat intake compared to OIL rats based on fat intake percentage change data in study one. Therefore, baclofen was less potent in the EP rats, i.e. there was a shift to the right in the baclofen dose-effect function. In addition, baclofen was also less effective in the EP rats, i.e., the percentage of reduction by baclofen was smaller in EP rats than in Oil rats (EP: 43% of reduction at 3.2 mg/kg; OIL: 73% of reduction at 3.2 mg/kg). When administered separately in study two, estradiol further attenuated the effect of baclofen compared to EP rats; that is, baclofen was even less potent in E rats. Indeed, baclofen showed no inhibition of fat intake in E rats. Furthermore, baclofen was also less effective in the E rats compared to EP rats, i.e., the percentage of reduction by baclofen was smaller in E rats than in EP rats (E: 37% of reduction at 3.2 mg/kg; EP: 79% of reduction at 3.2 mg/kg). The attenuation of the fat intake-reducing effect of baclofen in EP-treated high-restriction rats in study one, and in E high-restriction rats in study two, may be due to modulation of GABA-B receptors by estradiol. Estradiol has been reported to down regulate GABA-B receptors in the central nervous system. Estradiol decreased GABA-B stimulated G-protein activation in the VTA of female rats (18). Since activation of the GABA-B receptor suppresses both basal and drug-induced DA firing in the VTA (8,19,31) and reduces DA levels in the nucleus accumbens (32), it is possible that desensitization of GABA-B receptors by estradiol curtailed the inhibitory effect of baclofen on DA neuronal firing. This, in turn, could increase feeding-induced DA release in terminal regions such as the nucleus accumbens and prefrontal cortex, thus altering food reward
In addition, estradiol has been reported to markedly reduce GABA-B receptor expression in hypothalamic neurons in adult female rats (21) and to rapidly attenuate the potency of baclofen to activate G protein-gated inwardly rectifying K+ (GIRK) channels in dopamine neurons in the arcuate nucleus of female guinea pigs (22,36). This suggests that a similar mechanism may exist in other brain areas as well. Indeed, besides its role in tuberoinfundibular pathway to regulate the secretion of prolactin, dopamine neurons in the arcuate nucleus appears to also be involved in the mediation of reward. For example, blockade of dopamine-2 receptors in the arcuate decreased cocaine-induced beta-endorphin secretion in the nucleus accumbens (37).

Baclofen has also been reported to increase POMC expression in the arcuate nucleus in diet-induced obese rats, a mechanism proposed to explain its intake reducing effects (38). Since estradiol attenuates GABA-B function, the ability of baclofen to increase POMC expression (and reduce fat intake) would be attenuated by estradiol, a scenario consistent with the present results. It is not known, however, if baclofen reduces food intake via actions at POMC in normal weight rats that binge on fat.

Progesterone treatment given concurrently with estradiol counteracts the effect of estradiol on many behaviors such as food intake (39,40) and acquisition of cocaine self-administration (41). This might explain why estradiol alone attenuated intake reductions induced by baclofen to a greater extent than co-administration of estradiol and progesterone. There is little information about the effects of progesterone on GABA-B function. Our data indicate that progesterone attenuated the effects of baclofen in binge rats compared to binge rats given both estradiol and progesterone, albeit to a lesser extent.
than did estradiol alone. Progesterone administration has been reported to increase baclofen binding in the neocortex of female rats (17), which could indicate increased cortical inhibitory tone. This may attenuate baclofen’s ability to reduce fat intake, by reducing cortical glutamate neuronal signalling. Cortical glutamatergic projections to sites such as the nucleus accumbens may be involved in modulating food intake, since blockade of glutamate receptors in the accumbens robustly stimulates feeding (42,43). If progesterone enhances baclofen binding to GABA-B receptors on cortical glutamate cell bodies, this could reduce glutamatergic innervation to the accumbens and attenuate the fat intake-reducing effects of baclofen.

The reduced efficacy of baclofen in the hormone treated groups was not due to inactivity of the hormones. Near physiological amounts and patterns of estradiol and progesterone injections were used (28) and estradiol treatment resulted in the well-characterized reductions in 24-h food intake (data not shown) and body weight that has been described by others (28,29).

In this study, baclofen injections were administrated every 3 or 4 days at a 2-day or 3-day intervals. Clinically, baclofen is taken several times a day due to its short half-life. While tolerance to its effects could be of concern, this has not been shown in an open label trial in binge eaters (14), or in a more recent randomized cross-over study (Corwin et al., Appetite, abstract submitter). In rats, repeated daily administration of baclofen (2 mg/kg, i.p.) for 27 days had the same effects on 1-h food intake each day indicating tolerance did not develop to the short-term effect of baclofen (44).

Our findings indicate that female hormones may influence the actions of GABA-B receptors on binge-type consumption of fat. This is not only of biological interest but
also of therapeutic interest because: 1) different therapeutic strategies in the treatment of binge eating may need to be applied in female and male subjects; 2) female hormones may contribute to the higher vulnerability to binge eating in women, since naturally cycling estradiol may attenuate inhibitory effects of GABA in the CNS and facilitate the initiation and maintenance of binge eating.
References


CHAPTER 5

STUDY 4

EFFECTS OF ESTRADIOL AND MEAL SIZE ON FEEDING-INDUCED BRAIN C-FOS EXPRESSION IN OVARIECTOMIZED BINGE RATS
Introduction

Bingeing-related eating disorders (BRED) including Binge Eating Disorder (BED) and Bulimia Nervosa (BN) are increasing health problems in western countries (1-3). Lifetime prevalence of BRED in the United States is about 5% among women and about 2.5% among men according to a recent national survey (4). Altered reward/hedonic-related neuronal measures within the mesocorticolimbic dopamine system have been identified in individuals with BED and BN.

For example, differences in dopamine-4 receptor and dopamine transporter (DAT1) gene expression have been found in binge eaters compared to non-binge eaters (5,6). In addition, human brain imaging studies have indicated altered brain activation in areas within reward-related neuronal circuitry in binge eaters. Critical neural areas include the ventral tegmental area (VTA), where dopaminergic neurons are located, and nucleus accumbens (Acb), ventral striatum (VS), amygdala, and prefrontal cortex (PFC), which are all terminal regions of dopamine projections (7,8). In a fMRI case study, increased activation in the anterior cingulate cortex (ACC), a subregion of the PFC, was seen during response anticipation and response conflict processing in women with BN (9). In another study, in response to food stimuli, especially binge food stimuli, more activation was seen in the prefrontal cortex (PFC) of obese binge eaters than obese non-binge eaters (10). Food pictures also induced greater arousal and ACC activation in bulimic patients than in healthy controls (11). Binge eaters generally prefer foods that are high fat and sugar, likely due to the reward value of these foods (12). One study found that in response to an image of dark chocolate, the medial orbitofrontal cortex (mOFC) and ventral striatum (VS) were more highly activated in chocolate cravers than in non-
cravers (13). Altered reward-related neuronal circuitry by binge eating has also been supported by animal work (14,15). Rats trained to binge on sugar had higher dopamine (DA) levels in the nucleus accumbens when bingeing each day and decreased accumbens DA during “withdrawal” (15-17). A long history of sugar bingeing also resulted in increased dopamine D1 receptor binding in the nucleus accumbens shell and core in rats (14,17). Thus, both human and animal research indicates that regions of the brain involved in the processing of food reward/hedonics are differentially activated in bingeing and control subjects.

In addition to hedonic influences on food intake, food intake and energy balance are also regulated by homeostatic mechanisms (18-22). Food intake driven by acute energy needs is regulated by a variety of signaling molecules. CCK, a molecule signaling satiety, is released after a meal from the GI tract and acts upon vagal afferent fibers that terminate in the nucleus of the solitary tract (NTS) in the hindbrain. Long-term energy homeostasis and body weight are regulated, in part, by a “lipostatic” regulatory system, in which adiposity signals such as leptin and insulin are secreted in proportion to body fat mass. These molecules act via the arcuate nucleus of the hypothalamus on central effector pathways in the hypothalamus to suppress food intake and increase energy expenditure. Involved brain regions include the arcuate nucleus (ARC), paraventricular nuclei (PVN), dorsomedial hypothalamus (DMH), lateral hypothalamic area (LHA) and perifornical area (PA). (23-25).

Not all binge eaters are obese, despite the fact that they take in large amounts of energy during short time periods during a binge. This suggests that the control of energy homeostasis may still function during non-binge times. In other words, binge eaters may
exhibit features of both homeostatic eating during non-binge times and hedonic eating during bingeing. One aim of the present investigation was to determine which brain regions are differentially activated under various eating conditions in our rat model of binge eating. Regions of interest included areas involved in hedonic eating and motivational processing (VTA, Acb, ACC, PFC, LH, BLA, CeA) and areas involved in homeostatic food intake control (NTS, LH, Arc).

In addition, both food intake and the central neuronal systems controlling food intake are modulated by estradiol (26-32). Furthermore, both binge frequency and binge size have been reported to be modulated by estradiol (33-35). Differential activation of reward-related circuitry has been reported in males and females, with activation being stronger in females during reward delivery (36). To our knowledge, the effect of estradiol on neural activation in those who binge has not been assessed. Therefore, the second aim of this work was to investigate the influence of estradiol on neuronal activation in binge rats and controls.

During a binge, meal size is increased dramatically, suggesting that neuronal activity may differ from that of non-binge eating episodes. Neuronal activation in response to meal size has not been studied under binge-type conditions, to our knowledge. However, studies of non-bingeing rats have demonstrated that activation of brain regions such as the NTS or PVN is quantitatively related to the amount of food consumed (37,38). Moreover, estradiol can increase this activation in some areas (38). In contrast, the activation of food reward regions might not be related to the amount of food consumed. Therefore, a third aim of this work was to investigate whether the activation of neural
regions involved in homeostatic and hedonic eating is amount dependent under binge-type conditions.

C-Fos expression was used to investigate the neural activity in rats in this study. We hypothesized that: 1) more c-Fos expression would be seen in binge rats in brain areas such as VTA, Acb, ACC, and PFC within reward-related mesocorticolimbic regions. 2) Because stronger activation of reward-related circuitry is usually seen in females (36), we also predicted that estradiol would enhance c-Fos expression in these areas. 3) Others have reported that estradiol enhances neuronal activation induced by CCK and food (38-40), therefore, we further predicted that estradiol would enhance c-Fos expression in regions mediating satiety such as the NTS. 4) Finally, because others have reported that satiety effects are amount dependant, but rewarding effects of food are not, we predicted that the consumption of a large amount of shortening would be associated with more c-Fos expression in brain areas associated with satiety but not in brain areas associated with reward (37,38).

Materials and methods

1. Animals

Female Sprague-Dawley rats (Harlan, Indianapolis, IN; 60 days of age) were individually housed in stainless-steel cages with ad libitum access to water and pelleted chow (Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN; macronutrient content (kcal/kg diet, percent of calories): protein (936, 28.05%), fat (405, 12.14%), carbohydrate (1960, 59.81%); 3.3 kcal/g). The vivarium was maintained at 22 ± 2°C with a 12/12h light-dark cycle. All procedures were approved by the Pennsylvania State University
2. **Ovariectomy (OVX)***

After five-day adaptation to the vivarium, rats were given overnight access to a fat (Crisco® shortening [hydrogenated vegetable oil], J.M. Smucker Co., Orrville, OH; 9.17 kcal/g) in a bowl clipped to the front of cage. This was done to prevent neophobia during the rest of the study. One-week later, the rats were anaesthetized (1 ml/kg body weight, IP) with a mixture of 70 mg/kg Ketamine (Phoenix Science Inc., St. Joseph, MO) and 2 mg/kg Xylazine (Phoenix Science Inc., St. Joseph, MO), with 0.2 ml/kg supplements given as needed, and bilaterally OVX using a dorsal approach.

3. **Experimental design***

**Experiment one***

Experiment one was conducted to determine if c-Fos was differentially expressed under various eating conditions in our rat model of binge eating and to determine the influence of estradiol on that expression. In order to test if there are cyclic inhibitory effects of estradiol on brain activation (35), two different E groups were included, i.e. one group of rats was sacrificed on day 2 of the injection cycle (E2) and another on day 4 of the cycle (E4).

Seventy-two ovariectomized rats were used in experiment one. After 4-5 days of postoperative recovery from OVX surgery, rats were matched for body weight and overnight fat intake and divided into six groups of 12 rats each. As shown in Table 1, three groups of rats were 3X rats, which had continuous access to chow and water throughout the study and access to a dietary fat on only three occasions, i.e., overnight access before the study, 1-h access in week 1, and 1-h access on the day of sacrifice.
Three other groups of rats were “binge” rats, which had continuous access to chow and water, overnight access to fat before the study and highly limited access to fat, i.e., 1-h access every other day for 6 weeks until the sacrificed day. The 1-h fat access started at 1230pm, 2h prior to lights off and ended 1h later in all groups. One-h fat intake was measured at the end of the 1-h fat access period. Weekly body weight was monitored as an indicator of the efficacy of the OVX and hormone-treatment protocol.

Table 1 Summary of experimental groups*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hormone treatment</th>
<th>Fat Access (shortening)</th>
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</thead>
<tbody>
<tr>
<td>Experiment One</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3X-O</td>
<td>Oil</td>
<td>1h in Week1</td>
</tr>
<tr>
<td>3X -E2</td>
<td>E</td>
<td>1h in Week1</td>
</tr>
<tr>
<td>3X –E4</td>
<td>E</td>
<td>1h in Week1</td>
</tr>
<tr>
<td>Binge-O</td>
<td>Oil</td>
<td>1h every other day in Week1-6</td>
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<tr>
<td>Binge-E2</td>
<td>E</td>
<td>1h every other day in Week1-6</td>
</tr>
<tr>
<td>Binge-E4</td>
<td>E</td>
<td>1h every other day in Week1-6</td>
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<tr>
<td>Experiment Two</td>
<td></td>
<td></td>
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<tr>
<td>OO</td>
<td>Oil</td>
<td>1h every other day in Week1-6</td>
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<tr>
<td>OL</td>
<td>Oil</td>
<td>1h every other day in Week1-6</td>
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<tr>
<td>OH</td>
<td>Oil</td>
<td>1h every other day in Week1-6</td>
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<tr>
<td>EO</td>
<td>E</td>
<td>1h every other day in Week1-6</td>
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<td>EL</td>
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<td>1h every other day in Week1-6</td>
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<tr>
<td>EH</td>
<td>E</td>
<td>1h every other day in Week1-6</td>
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</tbody>
</table>

*All rats have continues access to chow and water throughout the study

Within each feeding schedule, two groups were injected every fourth day with estradiol benzoate (2µg/100µl sesame oil) on day 2 of injection cycle as described in our previous report (35). The third group was injected with oil vehicle on the same days. At the end of week 6, two estradiol treatment groups within each feeding schedule were matched for 24 h food intake and 1h shortening intake and divided into E2 and E4 groups. Rats in E2 group were sacrificed on day 2 of the 4-day estradiol injection cycle. Rats in
E4 group were sacrificed on day 4 of the injection cycle. Since rats were given shortening every other day and given hormone injections on day 2 every fourth day. The days 2 and days 4 actually all fell on binge days; that is, the day rats got 1h access to shortening. On one of the binge days in the seventh week, chow was removed from the cages for all rats ~ 4 hours before the shortening was given. All rats were then given 50 minutes access to 3 grams of shortening 1-2.5 hours prior to light off and anaesthetized at the end of the 50-minute access period. Three grams of shortening was selected based upon the average 1h shortening intake previously reported in hormone-treated binge rats (35). The same amount of shortening was provided to all of the rats in order to prevent brain activation differences caused by different amount of food. Brain tissues were collected and processed for c-Fos-like immunoreactivity (FLI).

Experiment two

Experiment two was conducted to determine whether c-Fos would be differentially expressed when different amounts of fat were consumed. In addition, this study determined the effect of estradiol on amount-dependent c-Fos expression.

Fifty-two ovariectomized rats were used in experiment two. After 4-5 days of postoperative recovery from OVX surgery, rats were matched for body weight and overnight fat intake and divided into six groups (8 or 9 rats per group). As shown in Table 1, three groups of rats were E rats, which received estradiol benzoate injections every fourth day on day 2 of injection cycle as in experiment one. Another three groups were Oil rats, which received oil vehicle on the same injection days. All rats had continuous access to chow and water throughout the study plus highly limited access to a dietary fat, i.e., 1-h access every other day for 6 weeks. Therefore, all rats were
considered binge rats in this study. The 1-h fat access started at 1330pm, 2h prior to light off and ended 1h later in all groups. One-h fat intake was measured at the end of the 1-h fat access period. Weekly body weight was monitored as an indicator of the efficacy of the OVX and hormone-treatment protocol.

On one of the binge days in the seventh week, chow was removed from the cages for all rats ~ 4 hours before the shortening was given. Rats within each hormone treatment group were then given 50 minutes access to 0, 1, or 3 grams of shortening respectively 1-2.5 hours prior to light off and anaesthetized at the end of the 50-minute access period. Brain tissues were collected and processed for c-Fos-like immunoreactivity (FLI).

4. Perfusion and Tissue Collection

Thirty minutes after the end of 50-minute feeding test, rats were anesthetized (1 ml/kg, IP) with the same mixture of 70 mg/kg Ketamine and 2 mg/kg Xylazine as used in OVX and killed by transcardiac perfusion fixation. Perfusates consisted of ~150 ml of heparinized 0.15 M saline followed by ~150 ml of 4% paraformaldehyde in 0.01 M phosphate buffer (PB). The brains were dissected and postfixed overnight in paraformaldehyde at 4 C. Brains then were immersed in 10% sucrose solution at 4 C overnight and in 20% sucrose solution for 24 h at 4 C. Brains were stored in 30% sucrose solution at 4 C before sectioning. Whole brains were blocked between forebrain and hindbrain and sectioned at 50 µm on a cryostat. In experiment one, brain sections were collected every two out of five sections for whole brains. In experiment two, brain sections were collected for a serial 12 slices for each interested structure (see details below). Tissue sections were stored in 0.1 M phosphate buffer saline (PBS) with azide.
5. Immunohistochemistry

In experiment one, sections that are representative for areas anterior cingulate cortex (Cg1), nucleus accumbens (Acb), arcuate nucleus (Arc), lateral hypothalamus (LH), basal lateral amygdala (BLA), and ventral tegmental area (VTA) in forebrain and nucleus of solitary tract (NTS) in hindbrain were processed for c-Fos-like immunoreactivity. Each structure has three representative sections. Based on templates contained in the Paxinos and Watson’s atlas (41), Cg1 and Acb are included in three consecutive templates from the place (2.70 mm anterior to bregma) to place (1.70 mm anterior to bregma). Arc, LH and BLA are included in three consecutive templates from place (2.80 mm posterior to bregma) to place (3.30 mm posterior to bregma). VTA is included in three consecutive templates from the place (4.80 mm posterior to bregma) to place (5.30 mm posterior to bregma). NTS is included in three consecutive templates from the place (13.68 mm posterior to bregma) to place (14.08 mm posterior to bregma).

In experiment two, sections that are representative for areas anterior cingular cortex (Cg1, Cg3), prefrontal cortex (IL), nucleus accumbens (Acb), arcuate nucleus (Arc), lateral hypothalamus (LH), basal lateral amygdala (BLA), central nucleus of amygdala (CeA), ventral tegmental area (VTA) in forebrain and midNTS and caudalNTS in hindbrain were processed for c-Fos-like immunoreactivity. Different from in experiment one, two consecutive sections were used for each structure this time. Some new areas including IL and CeA are included in experiment two. This was done because: (1) IL has been shown to be a critical area for eating disorders in human brain image studies (10,42). (2) Both CCK and feeding increased c-Fos-positive cells in CeA, which suggests its role in satiation signaling and meal size regulation (38,39). Based on the
templates contained in the Paxinos and Watson’s atlas, Cg1, Cg3, IL and Acb are included in one template plate (2.20 mm posterior to bregma). Arc, LH, BLA and CeA are included in one template plate (2.56 mm posterior to bregma). VTA is included in one template plate (4.80 mm posterior to bregma). MidNTS is included in one template plate (13.80 mm posterior to bregma). CaudalNTS is included in one template plate (14.30 mm posterior to bregma).

Before immunocytochemical procedure, brain sections were washed with TPBS, blocked with 50% ethanol for 30 min, and washed with TPBS again. Then incubated for 24 h at room temperature with c-Fos primary antibody (Arnel Rabbit a c-Fos, 1:500,000 dilution; lot # D00029671, Calbiochem). Tissue sections were then washed with TPBS and incubated in secondary antibody (Biotin-SP-conjugated AffiniPure Donkey anti-Rabbit IgG, 1:500 dilution) for at least 18 h. Next, brain sections were treated with ExtraAvidin solution (1:1300 dilution; Sigma) for at least 3 h. Diaminobezidine (DAB, Sigma) intensified with nickel sulfate was used to stain Fos-like products black. Sections were then mounted on microscope slides and cover-slipped. Each assay has positive and negative controls to verify staining.

6. Quantification of c-Fos-like immunoreactivity

Quantitative analysis of c-Fos immunoreactivity was done using Image-Pro Plus 5.1 software (Media Cybernetics, Bethesda, MD). The darkness of background was balanced each time for each section. In each section, the borders of nuclear structures were outlined based on the templates in the Paxinos and Watson’s atlas and only c-Fos-positive cells within these borders were counted. The c-Fos-positive cells were counted
for each structure by the imaging program by setting minimum optical density levels or by the researcher if visible dark, nuclear staining not counted.

7. Statistical Analysis

Data were analyzed using SAS 9.1 for Windows (SAS institute, Cary, NC). Body weight and food intake data are presented as means ± SEM. C-Fos data was log transformed for normalization of data distribution. Body weights (before OVX, 5 d postvariecyctomy and 57 d postovaricetomy) and quantification of c-fos activation were analyzed by 2-way ANOVA (fat access ×hormone treatment in experiment 1, amount of fat ×hormone treatment in experiment 2). One-h fat intake in the last two weeks in experiment 2 was also analyzed by 2-way ANOVA (amount of fat ×hormone treatment). One-h fat intake in binge rats and 50-min fat intake on sacrifice days were analyzed by 1-way ANOVA followed by Tukey’s HSD post hoc tests to determine significant differences among individual means. Differences were considered significant when $\alpha <0.05$.

Results

Experiment one

1. Body Weight

Body weight before OVX and at day 5 post-OVX did not differ significantly among groups. At the end of this study (week 6); however, body weight was significantly affected by hormone treatment with OIL rats (CO and BO) significantly heavier than E rats (CE2, CE4, BE2, and BE4) ($F(2, 66) = 125.33, p<0.0001$).

2. Food Intake
At the end of this study, the average 1h fat intake in the last two weeks in binge rats was significantly affected by hormone treatment with OIL rats consuming more than E rats consumed (1-way ANOVA, F (2, 33) = 9.67, p=0.0005).

Fifty-min fat intake on the perfusion day did not significantly differ among groups. The average 50-min fat intakes on perfusion day were 2.8±0.2 grams in CO group, 2.6±0.2 grams in CE2 group, 2.3 ± 0.2 grams in CE4 group, 2.9 ± 0.1 grams in BO group, 2.6 ± 0.1 grams in BE2 group, 2.5 ± 0.2 grams in BE4 group. In all, 70% of the rats finished 3 grams of shortening and the lowest intake of shortening for any individual rat was 1.5 grams.

![Body Weight Graph](image)

Figure 1 Body weight at day 5, day 15 of postovariectomy and weekly across the 6-weeks of Study 1. * indicates significant difference between OIL and E2 or E4 groups that had the same feeding schedule (P<0.001).

3. c-Fos expression
Figure 2 Effects of estradiol and fat access schedule on c-Fos counts in different brain areas in Study 1. C-Fos counts were log transformed to normalize the distribution. * indicates significant differences between binge and 3X rats that had the same hormone treatment (p<0.0167). Different letters indicates significant difference among oil, E2 and E4 within either 3X or binge groups (P<0.0167).
C-Fos expression in Cg1 area was significantly affected by both hormone treatment and fat access schedule (main effect of hormone treatment $F(2, 64) = 9.50, p<0.0002$; main effect of schedule $F(1, 64) = 5.32, p = 0.0243$). In addition, C-Fos expression in the Acb was affected by hormone treatment (main effect of hormone treatment $F(2, 64) = 4.46, p = 0.0153$) and in the BLA by fat access schedule (main effect of schedule $F(1, 64) = 8.19, p<0.0057$).

With hormone treatment, LSmeans table indicated there was less activation in Arc, LH, BLA, and VTA in BE2 than in CE2 ($p<0.0167$). Without hormone treatment, there was more activation in Cg1 and VTA ($p<0.0167$) and more but not statistically significant activation in Acb ($p=0.0504$) in BO than in CO. In addition, more activation was found in Cg1, Acb and VTA in CE2 than in CO ($p<0.0167$).

**Experiment two**

1. **Body Weight**

   Body weight before OVX and at day 5 post-OVX did not differ significantly among groups. At the end of this study (week 6), body weight was significantly affected by hormone treatment with OIL rats being significantly heavier than E rats ($F(1, 46) = 270.35, p<0.0001$).
Figure 3 Body weight at day 5, day 11 of postovariectomy and weekly across the 6-weeks of Study 2. * indicates significant difference between OIL and E groups that had the same feeding schedule (P<0.001).

2. Food Intake

At the end of this study, the average 1h fat intake in the last two weeks was significantly affected by hormone treatment with OIL rats ate more than E rats (2-way ANOVA, main effect of hormone treatment F (1, 46) = 18.39, p<0.0001).

Fifty-min fat intake on the perfusion day was also significantly affected by hormone treatment with OIL rats consuming more than did E rats (main effect of hormone F (1, 44) = 5.07, p<0.05). This was mainly caused by higher fat intake in OH group than EH group. Fifty-min fat intake was also significantly affected by amount of fat provided (main effect of amount of fat F (2, 44) = 536.79, p< 0.0001). The average 1h fat intakes on perfusion day were 0 grams in both EO and OO groups, 1.15 ± 0.04 grams in EL group and 1.13 ± 0.04 grams in OL groups, 2.43 ± 0.13 grams in EH group and
2.89 ± 0.13 grams in OH group. Hormone interacted with amount of fat provided (F (2, 44) = 5.72, P = 0.0062), likely due to slightly higher fat intake in EL group than in OL group, but higher fat intake in OH group than in EH group.

3. C-Fos expression

C-Fos expression was significantly affected by amount of fat provided, with higher amounts of fat inducing more c-Fos expression in CeA (main effect of amount of fat F(2, 43) = 4.78, p = 0.0133), VTA (main effect of amount of fat F(2, 43) = 4.17, p = 0.0222) and midNTS (main effect of amount of fat F(2, 43) = 5.59, p = 0.0069). C-Fos expression was not affected by hormone treatment in any brain area. LSmeans table indicated that 1 and 3 grams of fat induced more c-Fos activation in VTA in oil binge rats relative to 0 grams of fat.

Discussion

Activation of brain regions involved in the control of energy homeostasis and food reward was examined in binge and non-binge OVX rats treated with estradiol. Several key findings are reported. In study one: 1) c-Fos expression was greater in the Cg1 and VTA and to some extent in the Acb in OVX binge rats than in 3X controls, in the absence of estradiol replacement; 2) when treated with estradiol (E2), c-Fos expression was lower in the VTA, as well as in the Arc, LH, and BLA in binge rats than in 3X controls; (3) among the 3X rats, c-Fos expression was greater in Cg1, VTA and Acb in E2 rats than in Oil rats. However, among the binge rats, no hormone treatment differences were detected among E and Oil groups in these areas. In study two, all of the rats were “binge” rats. Consistent with study 1, there were no differences in c-Fos expression between E and oil treatment groups. However, main effects revealed that
larger meal size stimulated more c-Fos expression in the CeA, midNTS, and VTA. Within the VTA of the Oil treated rats, both the 1g and the 3g meals stimulated more c-Fos expression than did an empty bowl.

Figure 4 Effects of different amount of fat (0g, 1g, 3g) on c-Fos counts in different brain areas in Study 2. C-Fos counts were log transformed to normalize the distribution. Different letters indicate significant differences among different meal size in oil rats (p<0.0167). $ indicates the main effect of amount of fat in different brain areas.
C-Fos expression was higher in the Cg1 and VTA, and tended to be higher in the Acb, in OVX binge rats that were not treated with estradiol. This indicates that repeated binge access to a fatty food activates the mesocorticolimbic dopamine system, a system which is thought to be critical to the mediation of food reward. Our results are consistent with previous reports that Acb dopamine was elevated in rats that binge on sucrose relative to non-binge controls (43). The present results are also consistent with findings from human brain imaging studies, in which regions of the prefrontal cortex were more highly activated in response to food cues in binge eaters relative to non-binge controls (10,11). When a motivational event such as seeking a palatable food becomes familiar by repeated exposure, event-induced dopamine release is likely to be constant but moderate (44-46). This prolonged moderate DA release in prefrontal cortex will activate the dopamine-1 like receptor, which enhances the activity of glutamatergic neurons in PFC (47,48). One of major projection areas of glutamatergic neurons in PFC is nucleus accumbens. Therefore, the non-significant but greater c-Fos expression in Acb might be due to the stimulation of Acb neurons by glutamate.

In estradiol-treated rats (E2), c-Fos expression was lower in the VTA, as well as the Arc, LH, and BLA in binge rats than in 3X controls. The decreased c-Fos expression in VTA and BLA is consistent with a report in which low dopamine neuronal activity was associated with high estradiol levels during proestrus, and in which estradiol enhanced the inhibitory effects of cocaine on VTA DA neurons (49). Similar to cocaine administration, repeated binge consumption of shortening may increase dopamine concentration in VTA terminal regions. Increased dopamine concentrations, in turn, inhibit the firing of DA neurons though a feedback mechanism via the presynaptic D2
autoreceptor (50). Autoinhibition by DA has been shown to be enhanced by estradiol (49), an effect that would be consistent with the reduced activation of VTA neurons seen in the present E-treated binge rats, relative to the 3X controls, as well as relative to binge rats treated with oil.

Reduced activation of the Arc and LH may be related to the effect of estradiol on GABA-B receptors in the hypothalamus. Within a short time period after administration, estradiol rapidly attenuates GABA-B receptor-mediated autoinhibition of GABAergic neurons and therefore increases GABA release in the hypothalamus (51). Reduced autoinhibition of GABAergic neurons by estradiol would increase the release of GABA and therefore enhance the inhibition of inhibitory neurotransmitters on other neurons. This may explain the reduced neural activation in Arc and LH in E2 binge rats. Because this attenuating effect of estradiol on GABA-B activation is an acute event and only lasts for 24 hours, it did not occur in the E4 rats.

In 3X rats, c-Fos expression was higher in the VTA, Cg1, and Acb, in E2 rats than in Oil rats. However, there were no hormone treatment differences between the E2 and Oil binge rats in these areas. The binge groups were repeatedly exposed to shortening whereas the 3X groups were only exposed to shortening three times. Thus, the shortening provided on the sacrifice day to the 3X groups can be considered acute exposure to a palatable food. The results suggest that the neuronal response to acute and repeated exposure to a palatable food are differentially regulated by estradiol. If this regulation involves dopaminergic mechanisms, as suggested by the present results, then the effects of repeated and acute exposures would be expected to differ.
A previous study reported increased NTS activation in binge rats compared to non-binge controls (52). We did not see this in Study 1. One explanation for the different results may be that our rats were sated, whereas rats in the previous study were food-restricted prior to sacrifice. Perhaps that when rats are already sated, the satiety signaling in the hindbrain is not stimulated as much as when they are hungry. In addition, the amount of food consumed in the previous study was slightly larger than that of the present study. Stimulation of satiety signals is thought to be stomach distension-related but not energy content-related (37,38). The 3 grams of fat may not have been large enough to produce stomach distension and provide robust activation of NTS neurons. Therefore, binge access did not induce activation of the NTS that was different from that of the 3X rats in our study. The concern of smaller amount of shortening provided on sacrificed day may also explain the non-difference in NTS in study two, although there was a main effect of meal size in midNTS.

There were no hormone treatment effects on c-Fos expression in any brain area in study two. This might be due to the fact that the rats were sacrificed on day 4 of the hormone treatment cycle rather than on day 2. We chose this cycle day because day 4 of the treatment cycle mimics the estrus phase of the estrus cycle in rats, during which the regulatory effects of estradiol on food intake are maximally demonstrated. However, the results obtained in study one suggest that neuronal activity in response to intake under binge-type conditions might be modulated by rapid effects of estradiol. Our E2 rats in study one were sacrificed ~ 5 hours after E administration, and effects of hormone treatment were more consistently seen in rats sacrificed on that day rather than day 4. The effects of hormone treatment on body weight and 1-h energy intake are consistent
with previous reports (35) and indicate that the hormone was active. The present results, therefore, suggest that the regulation of neuronal activity by estradiol under binge-type conditions is an activational effect rather than an organizational effect.

In study two, fat consumption stimulated more c-Fos expression in the VTA than did empty jars in oil binge rats. This indicates that the actual consumption of fat will stimulate more neuronal activity in this region, than will simple exposure to cues associated with fat ingestion. The VTA has not been suggested to relate to satiety signals; therefore our results are not likely related to a satiety effect. Instead, the stimulatory effect of fat consumption was surprising because dopamine is thought to be critical to “wanting”, but not to “liking”, during goal-directed behavior (53,54). The rats in Study 2 all had repeated binge access to shortening. After rats got familiar with this schedule by repeated exposure, one would expect that empty jars would stimulate “wanting” and more neuronal activation in the VTA. Why actual fat consumption stimulated more activity than the cues in this study warrants further investigation.

The present results provide a greater understanding of the neuronal activation in binge eaters. The effect of estradiol on brain activation in binge will also help explain the contribution of estradiol to binge vulnerability in females.
References


CHAPTER 6

SUMMARY AND CONCLUSION
The overall goal was to (1) examine the effects of ovarian hormones on bingeing and food intake and body weight regulation in a diet-related binge eating rat model, and (2) investigate the neural systems that are associated with the effects of ovarian hormones on bingeing.

**Aim 1** To determine if food intake is affected by ovarian hormones – estradiol (E) and progesterone (P) in binge rats.

Study one revealed that administration of ovarian hormones estradiol and progesterone no longer exerts cyclic inhibitory effects on the size of brief bouts of fat intake under binge-type eating conditions. Specifically, energy intake during 1-h binge time was tonically, but not cyclically, reduced in the hormone-treated high-restriction (binge) rats. In addition, the tonic and cyclic inhibitory effects of estradiol were still present in total daily food intake in the same batch of rats.

Study two revealed that administration of estradiol alone or co-administration of estradiol with progesterone resulted in both tonic and cyclic inhibitions of daily food intake, a loss of cyclic inhibition of binge size, and maintenance of body weight at normal levels in binge OVX rats. In contrast, administration of progesterone alone had no effect on either food intake or body weight in binge OVX rats.

The results of these two studies indicate that estradiol is the primary ovarian hormone responsible for non-binge food intake and body weight regulation even under binge-type conditions in rats and that part of the normal inhibitory influence of estradiol on eating is disrupted during a binge.
**Aim 2** To determine the effect of ovarian hormones on GABA-B-induced reductions of binge fat intake.

Study three revealed that both estradiol and progesterone attenuate the inhibitory effect of GABA-B agonist baclofen on binge fat intake. Specifically, in experiment one, a higher dose of baclofen was required to reduce fat intake in EP-treated rats relative to Oil-treated rats. In experiment two, baclofen reduced binge fat intake at an even higher dose in P-treated rats than in EP-treated rats and baclofen had no inhibitory effect in E-treated rats. Overall, the inhibitory effect of baclofen on binge fat intake was influenced by hormones in this way: OIL ≥ EP > P > E.

The results indicate that both estradiol and progesterone influence the actions of GABA-B receptors on binge-type consumption of fat. Estradiol has greater influence on GABA-B receptor action than progesterone in binge rats. Therefore, different therapeutic strategies in the treatment of binge eating may need to be applied in women vs men or in cycling vs non-cycling women. In addition, this may partially explain why females are more vulnerable to binge eating; that is, naturally cycling estradiol may attenuate inhibitory effects of GABA in the CNS and facilitate the initiation and maintenance of binge eating.

**Aim 3** To determine estradiol’s modulation of neuronal activation, as assessed by c-Fos immunocytochemistry, in response to feeding in female binge rats.

Study four revealed that there was more neural activation in anterior cingular cortex (Cg 1) and ventral tegmental area (VTA) in oil-treated binge rats than in chow rats and less activation in VTA, lateral hypothalamus, basolateral amygdala and arcuate in E-
treated binge rats than E-treated chow rats. In addition, more activation was found in Cg1, nucleus accumbens and VTA in E-treated chow rats than in oil-treated chow rats but no difference in these areas between E-treated binge rats and oil-treated binge rats. These results suggest that repeated binge access to shortening engages a reward-related neural circuit in rats. Estradiol may promote the sensitivity of this neural circuit to binge access by increasing the autoinhibition of dopaminergic neurons in VTA.

These four studies support several conclusions. First, although estrogens, of which estradiol is the most important, are the primary ovarian hormones responsible for food intake and body weight regulation under binge-type conditions as under normal feeding conditions in rats, binge eating disrupts the normal cyclic inhibitory influence of estrogens on eating. Second, an effect of estrogens to attenuate GABA-B receptor-mediated inhibitory effects of GABA in the CNS may contribute to these effects. Third, the data provide further evidence that GABA-B neuronal function may facilitate the development of binge eating and reduce the therapeutic effect of medication on binge eating. Finally, the data also support the idea that binge eating induced by repeated exposure to a palatable food stimulates a reward-related neural circuit in rats and that estrogens may promote the sensitivity of this neural circuit under binge conditions.

These conclusions suggest that estrogens can have different actions in the CNS of rats maintained under binge-type conditions. These are shown in Figure 1. I proposed that non-binge eating is mediated by the “feeding-regulatory pathway” centered in the hypothalamus, whereas binge-type eating is mediated by more rostral
“mesocorticolimbic pathways”. Both non-binge and binge-type eating occur in binge eaters. In addition, both neural circuits can be modulated by ovarian hormones, especially estrogens. During non-binge feeding, estrogens regulate food intake predominately via feeding-regulatory circuitry. The literature suggests that estrogens may act in three sites to produce this regulation: 1) enhancing satiety signaling in the NTS, or 2) in the PVN, or 3) facilitating the anorexigenic effect of POMC neurons by attenuating the inhibition of GABA on POMC neurons in the arcurate. In contrast, I propose that estrogens have different effects during a binge, via a fourth site of action within mesocorticolimibic circuits. Within this circuitry, estrogens promote dopamine release by attenuating the inhibition of GABA on dopaminergic neurons in the VTA, thus stimulating binge-type eating. This model, and the studies upon which it is based, should be useful in the design of future analyses of these actions of estrogens on eating.

Results of studies in chapters 2 and 3 support the hypothesis that both neural control systems are present in the rats under binge conditions and these two systems are modulated by estrogens. Food intake over a longer time (daily food intake) and body weight was modulated by estradiol, as evidenced by tonic and cyclic inhibitory effects of estradiol on daily food intake and maintenance of body weight at control levels in estradiol-treated binge rats. This is consistent with actions of estradiol on feeding-regulatory circuitry. In contrast, the lack of a cyclic effect of estradiol on binge intake is consistent with modulatory effects of estradiol on a different neural circuit (possible mesocorticolimibic dopamine pathway).
The diminished cyclic inhibitory effects of estradiol might involve GABA-B receptors. Estradiol reduced the inhibitory effect of the GABA-B agonist baclofen on binge fat intake as described in chapter 4. Thus, estradiol may attenuate the inhibitory effects of GABA on hedonic feeding-stimulatory neurons and thereby increase binge size (1-3). However, the neural sites of modulation of estradiol on binge eating are still not clear.
The neural sites of modulation of estradiol on non-binge eating have been suggested to be located in the PVN of the hypothalamus and in the NTS of the hindbrain (4-7). Therefore, I proposed that estradiol would reduce chow intake by increasing the satiating potency of negative-feedback controls of meal size in the NTS and PVN. However, there was no hormone effect on c-Fos expression in the NTS in either binge or chow rats in study four (described in chapter 5). This is not consistent with a previous report that estradiol increased feeding-induced c-Fos expression in the NTS (5). The lack of effect in study four may have been due to the lack of food-deprivation and to the relatively small amount of shortening that was used to stimulate c-Fos expression; that is, the amount provided may not have been enough to produce stomach distension and stimulate satiety signals in the NTS. Alternatively, satiety signaling in response to a fatty food may be compromised when limited and intermittent access to that food has been provided. Therefore, the conditions used for c-Fos expression in the present study may not have reflected effects of estradiol as reported for non-binge food consumption.

I also proposed that the regulation of fat intake during a binge is modulated by estradiol via mesocorticolimibic circuitry. First, does this research provide any evidence that mesocorticolimibic circuitry is involved in the regulation of eating during a binge? Results described in chapter 5 support this hypothesis since more c-Fos expression was found in oil-treated binge rats relative to oil-treated 3X rats in the VTA, Cg1, and Acb, brain areas that are part of mesocorticolimibic pathways. Brain regions within the mesocorticolimibic pathway involved in binge eating in animals or in binge eating patients has also been reported by others (8-13). It is possible that restricted access to shortening induces more dopamine release from VTA into projection areas, including the
PFC and Acb. This neuronal circuit may help rats establish learned associations with the limited access to shortening and repeatedly induce dopamine release (14-16). However, how these areas interconnect with each other and through what neurotransmitters to establish the association is not known.

Second, what has the present research demonstrated about the kind of modulation estrogens may have on mesocorticolimibic activation in binge rats? I proposed that estrogens may increase dopamine release by attenuating the inhibition of GABA on dopaminergic neurons in VTA. By these actions, estrogens would enhance feed-forward signaling, and increase binge eating during a binge. However, if this were the case, then one would expect more c-Fos expression in the VTA of estradiol-treated binge rats, due to greater activation of dopaminergic neurons in that brain region. In contrast, there was less c-Fos expression in the VTA of estradiol-treated binge rats compared to non-binge rats. While this does not support my hypothesis, the result is consistent with some previous findings. Instead of increasing dopamine release in terminal areas of dopaminergic neurons, estradiol reduces dopamine transport and reduces dopamine release in the nucleus accumbens in rats (17,18). Basal firing rate of dopamine neurons in the VTA is lowest during proestrus when the estradiol level is highest. In addition, estradiol increased the inhibitory effect of cocaine on VTA dopamine neurons (19). This might suggest that similar to cocaine administration, repeated binge consumption of shortening will increase dopamine concentration. Increased dopamine concentration, in turn, inhibits the firing of DA neurons though a feedback mechanism via the presynaptic D2 autoreceptor, as discussed in that study. This
autoinhibition was enhanced by estradiol. Estrogens may have similar effects during a binge, thus contributing to binge vulnerability in women.

In conclusion, results from studies one through four are consistent with most of the proposed mechanisms but still leave some questions unanswered. In particular, why and how estradiol has both cyclic and tonic inhibitory effects on chow and fat intake during non-binge eating episodes but does not have cyclic inhibitory effects on fat intake during a binge warrants investigation. In addition, brain sites that are involved in the modulating effects of estradiol, as well as the exact mechanisms, need to be investigated.

**Implications and limitations**

This series of studies provide a preliminary understanding of the effects of ovarian hormones on binge eating in females. The findings help explain the contribution of estrogens to binge vulnerability and ultimately may contribute to the development of improved treatments for binge eating in women. In addition, studies one and two showed that the cyclic inhibitory effect of estradiol on 1h fat intake was lost in estradiol-treated binge rats. That is, estradiol did not reduce binge size on day 4 of the hormone treatment cycle. In humans, estradiol level and binge frequency are negatively associated across the menstrual cycle (20), i.e. binge frequency is reduced when estradiol levels are elevated. This suggests that estradiol has opposing modulatory effects on binge size and binge frequency. In fact, under non-binge conditions estradiol reduces meal size and increases in meal frequency. However, overall estrogens demonstrates a cyclic inhibitory effect on food intake due to a not fully compensated increase in meal frequency (21-23). Thus, it would be of scientific interest to investigate the effects of estrogens on binge size in
women with binge eating disorders. Moreover, study three reported that both estradiol and progesterone attenuated the “therapeutic” inhibitory effect of baclofen on binge fat intake in ovariectomized binge rats. This suggests that different therapeutic strategies in the treatment of binge eating, such as different doses of medication, may need to be applied in female and male patients.

There are some limitations to the present series of studies. First, an oil-treated ovariectomized binge rat control group in study two would have helped clarify the tonic inhibitory effects of ovarian hormones on binge size. Second, a larger meal size (not necessarily energy amount) may have been helpful in study four in order to have increase the stimulation hindbrain neurons mediating satiation and make possible detection of more effects. Third, the specific neural cell type expressing c-Fos in study four was not examined.

**Future directions**

Several future studies could be derived from the results of current series of studies. These are discussed below in relation to several potentially relevant aspects of brain function.

**Activational and organizational effects of estradiol**

Sex differences can be driven by gonadal steroid hormones via two mechanisms: organizational and activational effects of hormones. Organizational effects of hormones are long-lasting permanent effects on the developing systems and thereafter influence the behaviors. Activational effects influence behaviors temporarily and lose their influence after the hormones are gone. Sex differences have been reported in binge prevalence (24-
26), binge frequency (20,27-30), binge size (12,31), and other clinical characteristics of binge eaters (32-34). Many of the sex differences occur in adulthood after brain development has taken place. Thus, one could speculate that steroid hormones have activational effects that influence binge eating in adults. The results of the current studies are consistent with activational effects of estradiol on food intake, body weight, and neural responses to dietary fat in bingeing rats. Studies involving neonatal estradiol administration in female rats and male rats could be used to test the organizational effects of ovarian hormones on binge eating. In fact, neonatal exposure to gonadal hormones in rats can influence the development of central nervous system and potentially influence the food intake and body weight. For example, neonatal estradiol administration in female rats has been shown to decrease the central concentration of progesterone, increase GABA-A receptor expression, and produce less sensitivity to anxiolytic drugs (35).

**Estrogen receptors**

There are two predominant types of classical nuclear estrogen receptors: ER-α and ER-β. Binding of estradiol to these ERs initiate gene transcription (36). In addition, recent evidence indicates that ER-α and ER-β, as well as novel ER types, are also found in the cell membrane and can mediate both genomic and non-genomic effects (37-39). These are known as membrain ER (mER).

The inhibitory effects of estradiol on non-binge-type food intake is thought be mediated through ER-α (40,41), although ER-β may also contribute (42). Which receptor subtype mediates estradiol’s effects on binge eating is not clear. Stimulation of ER-β, but not ER-α, has been reported to enhance cocaine-seeking behavior in rats (43). In addition,
stimulating ER-β has been shown to increase D2 receptor binding in the nucleus accumbens core (44). Estradiol, therefore, might have inhibitory effects on food intake through ER-α actions and stimulatory effects on drug-seeking through ER-β or other non-ER-α actions. In addition, a novel mER that elicits rapid effects has been identified in the hypothalamus (45,46). A ligand (STX) that is selectively bound to this mER can regulate excitability and gene transcription in CNS neurones and influence food intake and body weight gain in ovariectomized females (47). Moreover, evidence that estradiol rapidly enhances striatal DA release and the motivation to take cocaine is consistent with the rapid response of mERs and supports the idea that the mERs may be involved in the effects of estradiol on drug seeking behavior (1). The results of the current studies (especially study four) suggest that the effect of estradiol on binge eating and on the neural response to binge consumption of fat are rapid effects. Membrane-associated ERs, therefore, may be involved in the mediation of estradiol on binge eating. The development of specific ER agonists or antagonists could be used to help determine the key estrogen receptors that are involved in binge eating.

**Serotonin system**

Considerable evidence indicates comorbidity between eating disorders, including BED and BN, and anxiety and depressive disorders, as well as obsessive-compulsive disorder (48-50). Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that has an enormous influence over many brain functions, including those involved in appetite, mood, and depression. Consistent with the comorbidity of eating disorders and depressive disorders, disturbances of 5-HT system function have been observed in many patients
with eating disorders. Thus, 5-HT has been suggested to be involved in the pathology of eating disorders (51-55).

Indeed, many medications used in the treatment of psychiatric disorders have shown promising effects in the treatment of eating disorders. For example, selective serotonin reuptake inhibitors (SSRIs), anti-depressant drugs that block the reuptake of serotonin back into the cell and therefore increase serotonin levels, are well tolerated and reduce binge episodes in patients with eating disorders such as BED and BN (56).

Ovarian hormones influence SSRI function and therefore are thought to influence 5-HT levels. One study reported that both estradiol and progesterone can inhibit the ability of an SSRI to slow the clearance of 5-HT (57). Estradiol has also been reported to influence 5-HT receptor activity and 5-HT binding in different areas of the brain, and thus has direct or indirect effects on mood and mental states (58,59). Physiologically this would suggest that serotonin may contribute to the development of binge eating and that serotonergic actions can be modulated by ovarian hormones. Pharmacologically, this is consistent with the findings in study three, in which both estradiol and progesterone attenuated the potency and efficacy of the GABA-B agonist baclofen on binge fat intake. Therefore, future studies should be conducted to test the involvement of serotonin in binge eating in the current binge eating rat model.

**GABA-B antagonist**

As a GABA-B receptor agonist baclofen has reduced binge fat intake in both ovarian hormone-treated and oil-treated binge rats. In addition, estradiol and progesterone attenuated the inhibitory effect of baclofen on binge fat intake. These results indicate that centrally acting GABA might be involved in binge-type consumption of fat. If this is the
case, then blockade of GABA-B receptors via the administration of a GABA-B antagonist would be predicted to have the opposite effect on binge fat intake; that is, GABA-B blockade should stimulate binge consumption of fat. In order to further investigate whether GABA-B receptors influence binge eating, GABA-B antagonists such as saclofen or CGP35348 could be tested in binge rats. The ability of the antagonist to block baclofen-induced effects or effects of the antagonist alone could be assessed. In fact, central administration of saclofen in either nucleus accumben shell or the VTA area can block the baclofen-induced feeding behavior in rats (60-62). In another study, both peripheral and central administration of CGP35348 significantly reduced cumulative non-binge food consumption in hungry rats (63). The same laboratory also reported that the administration of baclofen stimulated non-binge food consumption (64,65). Therefore, it is suggested that endogenous GABA acting at central GABA-B receptors plays a physiological role in the regulation of feeding behavior. However, the effect of GABA-B antagonists on binge intake has not been studied to my best knowledge.

**Dopamine system**

Although I emphasized the effect of ovarian hormones on GABA via inhibitory GABA-B receptor, other aspects of the dopamine-related mesocorticolimbic system are likely under the modulation of ovarian hormones and therefore may help explain the higher vulnerability to binge eating in females. For example, previous studies have shown that estrogen stimulates a significant increase in dopamine type-2 (D2) receptor binding via ER-β in rat striatum and nucleus accumbens (44), but decreases D2 receptor binding in VTA (66). After repeated exposure to cocaine, the effect of estradiol was reversed. That is, estradiol decreased D2 receptor binding in Cg 2 and increased D2 receptor
binding in VTA (66). If the release of dopamine is regulated by a feedback mechanism via the presynaptic D2 autoreceptor in VTA, then decreased D2 receptor binding in VTA by estradiol should activate more dopaminergic neurons and lead to more dopamine release. This was suggested to be a mechanism by which estrogen potentiates behavioral sensitization to cocaine in the female rat. Estradiol may have the same effect on D2 receptors in female rats under binge type conditions. Therefore, future studies should be carried out to test this hypothesis.

**Phenotypes of activated cells**

Study four reported more c-Fos expression in Cg 1 and VTA and a tendency in Acb in oil-treated binge rats relative to non-binge oil rats indicating that binge eating activated regions within the mesocorticolimbic pathway in the absence of estradiol. In addition, there was less activation in the VTA and BLA in binge E2 rats than non-binge E2 rats indicating that feeding-induced neuronal activation was attenuated, rather than enhanced, in the presence of estradiol. More c-Fos expression means more brain cells were activated in those neural sites. It does not alone indicate what type of neurons was activated. For example, although the majority of neurons in the VTA are dopaminergic, a population of GABAergic neurons and a small population of glutamatergic neurons also exist in the VTA (67-69). Therefore, future studies need to identify the phenotypes of activated cells in key areas including VTA, Acb, BLA, hypothalamus, and NTS. Some possible techniques include double labeling immunohistochemistry or microarrays measuring genome-wide gene expression profiles for specific neuronal cell types (70-72).
References


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APPENDIX A

OVARIECTOMY PROCEDURE

STUDY ONE THROUGH FOUR
Set-up

Surgery area
  Surgery logbook
  good lighting
  heating pad
  disposable underpads
  surgery packs
  glass bead sterilizer
  suture material (Coated vicryl violet braided 4/0 with cutting needle)

Anesthesia
  ACE Cocktail
  Controlled substances logbook
  30 gauge needles
  1ml syringes
  scale with cage/box

Animal prep
  eye lubricant
  electric clippers
  iodine
  70% ethanol
  cotton swabs/gauze
  tape to stabilize body

Surgeon prep
  lab coat
  hair bonnet
  masks
  sterile gloves

Surgery Pack
  surgery instruments
  drapes
  gauze
  wound clip applicator
  wound clips

Surgical instruments
  needle holder
  hemostats
  forceps
  scissors

Recovery
  recovery cage with heat and no loose bedding
  analgesic
  Surgery logbook
  wound clip remover
Anesthesia

**Ket/Xyl Cocktail** is used for anesthesia with a 1ml syringe and 30 gauge needle. It is given **IP** at the dose of **1 ml per kg** of animal weight. So, if the rat weighs 200g then the anesthesia dose would be 0.20ml. This dose and type of anesthesia has lasting effects of approximately 30-45 minutes. It is safe to offer booster injections if the surgeon feels necessary, the dose determined by individual cases. Monitor the withdrawal reflex response to the toe/pad-pinç and corneal reflexes to determine anesthesia effectiveness. Depth, pattern and rate of respirations may also be monitored as an index of anesthesia depth. Anesthesia amounts must be logged in the “Surgery Logbook” and the “Controlled substances Logbook”.

**Animal Prep**

After the animal is fully anesthetized, begin the prep work. Prep work is ideally done away from the area in which the surgery is to take place.

1. Put **eye lubricant** in the eyes to prevent the eyes from drying out due to the lack of a blink reflex while under the affects of the anesthesia.
2. **Remove the hair** from the surgical site. There are several methods of doing this, but the method of choice is electric clippers. It is NOT necessary to remove fine hairs with a razor.
3. **Position the animal** at the surgery area. The animal should be positioned on its ventral surface with its tail towards the surgeon. Tape may be used to secure the animal in position.
4. **Clean the surgery site with antiseptics.** Use an iodine(betadine) solution with either cotton swabs or gauze squares and clean from the middle of the surgical area working
your way to the outer edges, thereby “pushing” the contaminants out. Next, use a 70% ethanol solution, following the same cleaning pattern as a rinse. Alternate iodine and ethanol solutions for a total of 3 cycles.

**Surgeon Prep**

1. Put on labcoat, hair bonnet and face mask.
2. Wash hands.
3. Open outside cover of surgery pack, paying particular attention as to not touch inside wrap, leaving it sterile.
4. Open suture material aseptically and drop onto open surgical pack.
5. Open sterile glove pack and aseptically put on gloves.
6. Open surgery pack and find the drapes. Cut a corner hole in one of the drapes and position it over the surgical site of the animal.

**Dorsal Approach**

A midline dorsal incision (1.0-2.0 cm) is made through the skin about halfway between the hump of the back and the tail base. An incision will then be made into the underlying abdominal musculature on both the left and right side of the rat to enter the abdomen approximately 1/3 to ½ down either side of the body. The ovary will be pulled out through the incision. If need be, the mesenteric fat may be cleared away. The uterine horn and blood vessels will be clamped and a 4-knot ligature will be placed around the blood vessels and uterine horn just below the clamp. The ovary will be cut off and the clamp released. If no bleeding is observed, the uterine horn will be replaced into the abdominal cavity. Absorbable suture material (4-0 vicryl or a generic equivalent) will be used to close the muscle layer. Individual 4-knot ligatures are preferred rather then a
running stitch when closing the muscle layer. The muscle incision and ovary removal is then repeated on the other side. Surgical staples will be used to close the skin.

Recovery

1. Following surgery, animal should be gently cleaned up and placed in a heated recovery cage. The temperature of the recovery cage should not exceed \(40-42^\circ\text{C}\). Refer to the “Recovery Cage temperature Experiment” for suggestions on preferred recovery cage set-ups. The cage should NOT have any loose bedding material. Disposable underpads work well in recovery cages. The heating pad should be insulated and the recovery cage should provide an area that the animal can move off away from the heat if they need to.

2. Monitor animal closely until it can stand up, not be easily pushed over and locomotion is present. Animal may then be returned to its homecage.

3. During the recovery period of 7-10 days, the animal should be monitored frequently and notations should be made in the “Surgery logbook”. Particular attention should be made to defecations, weight change, behavior and incisions. The would clips should be removed after 7-10 days.
APPENDIX B

PERFUSION PROCEDURE FOR RAT BRAINS

STUDY FOUR
Perfusion Procedure

1. Set up perfusion apparatus in fumigation hood.
2. Fill right (red) jar with 4% paraformaldehyde solution and fill left (blue) jar with 0.15M saline solution with heparin.
3. Clear perfusion apparatus first with paraformaldehyde solution and then with saline solution.
4. Have instruments ready: perfusion needle, 1 small sharp scissors, 1 large pointy, 1 large ribcage ronguers, small spatula and jars with paraformaldehyde solution (labeled for brains).
5. Deeply anaesthetize animal with large dose of ACE Cocktail (1ml/kg)
6. When animal is sedated, place on wire rack over fluid collection tubs in hood. Incise into abdominal cavity directly beneath zyphoid process. Cut through abdominal muscles laterally on both sides. With other large scissors, cut anterior along sides through the rib cage. Snip through the diaphragm to expose the heart. Detach heart from muscle by clearing away membranes. With small scissors snip through carotid on animals’ right (right ventricle) and there should be a strong blood flow out. Quickly insert perfusion needle into left ventricle near apex with a firm jab. Begin perfusion with saline solution. Continue to profuse until the fluid exiting the carotid runs clear - approximately 250-300ml. Close saline tube and begin perfusion with paraformaldehyde solution. Watch for muscle activation and stiffness in the muscles of the neck. When the animal appears well fixed (approximately 250-300ml of formalin) the perfusion is complete.

Notes/hints:
1. Don’t use very sharp surgical scissors for cutting ribs.

2. Use a BLUNT 16g needle.

3. Make sure that saline is flushed last (through perfusion tubes) so it goes into rat first.

4. Use scissors instead of ronguers to cut rib cage.

5. Don’t use rat-toothed forceps or towel clamps.

6. If doing multiple animals, after perfusing with paraformaldehyde, and before taking brain out, move on to next animal.

7. Use 1ml TB syringes for anesthesia.

8. The anesthesia is given IM in hind leg muscle.

9. Use a permanent marker on tail to ID rats after removing from cage.

10. If you perfuse into right ventricle, then it goes into lungs and this is BAD!

11. Put rat bodies into freezer in basement, into CBL barrel. Label bags with date, sex, advisor and number of animals.

**Taking the Brain following Perfusion**

1. Using a utility blade, incise into the scalp, first towards the nose then down the center to the neck. Spread the skin down around the neck.

2. Remove the muscles around the base of the neck, the spinal cord and along the temples using large pointy scissors and the utility blade. Be careful not to damage the underlying tissue.

3. When the muscles are cleared away, crush C1 or C2 with the ronguers.

4. Carefully begin to clear away the bone from the spinal cord and the candela base of the skull. Snip off small pieces to avoid damaging the brain stem beneath.
5. Once the occipital bones are cleared away, begin to cut down the mid-line of the skull. Proceed up to the olfactory bulbs and then peel away the temporal plates laterally. Clean away the dura around the cerebellum.

6. Using a rotating action, pry away the bone around the cerebellum and brain stem, being careful to avoid damaging the tissue with bone fragments or dura.

7. Using the small spatula, cut through the spinal cord and olfactory bulbs to free the tissue. Lift the brain and cut through the optic and trigeminal nerves. Cut through the remaining nerves on the brain stem to free the brain. Carefully lift the brain, checking that no attachments remain to tear the tissue and drop it into the jar of paraformaldehyde solution.

8. Allow the brain to postfix in the 4% paraformaldehyde solution overnight. Transfer the brain to a solution of 10% sucrose (weight by volume in .1M PBS). Leave overnight in the refrigerator, allowing the brain to soak up the sucrose and sink. Then transfer brain into a 20% sucrose solution and leave overnight in the refrigerator. Then transfer brain into a 30% sucrose solution and leave overnight in the refrigerator. Cut brains as soon as possible after soaking in 30% sucrose overnight.
APPENDIX C

MOUNTING AND CUTTING THE BRAIN

STUDY FOUR
1. Collect:
   
   a. jars, slides or well plates for sections (labeled)
   b. 0.1M PBS with azide
   c. magnifying glass
   d. permanent marker
   e. glass rods
   f. TPBS
   g. OCT Compound
   h. utility blades
   i. 0.01% gelatin
   j. disposable pipets
   k. slide holder

2. Put tissue holder (chuck) into cryostat to freeze.

3. Set new blade on cutting platform, lock in place and **shift with blade guard**. Set section thickness to 50um.

4. Block Brain. Cut vertically through the brain at level of Rostral tip of cerebellum perpendicular to plane of hard working surface. Block through spinal cord.

5. Place slides to be used to freeze brain in cryostat to freeze. Place a drop of 0.01 % gelatin (9mls dd-water and 1ml .1% gelatin stock from refrigerator) on slide. Set brain on slide vertically. Set on freezing platform in cryostat. Coat the brain with 0.01% Gelatin. Allow gelatin to freeze. Continue to build up gelatin and freeze. When tissue is completely frozen, trim excess gelatin away from the base of the brain thus releasing it from the slide. Place a drop of OCT onto a frozen chuck and quickly attach the tissue that was frozen with gelatin. Use additional OCT to edge the base of the tissue to the chuck.

6. Set chuck with tissue onto chuck mount. Position tissue and align to cutting surface. Take sample sections onto slide or visualize sections to examine for asymmetry or
use magnifying glass and look at tissue in holder directly. If reasonable symmetrical
- begin to cut sections.
APPENDIX D

C-FOS IMMUNOHISTOCHEMISTRY

STUDY FOUR
Notes:
  a. Shake plates at slow speed on lab rotator.
  b. It is useful to use colored paper under the plates to facilitate visualizing sections through plates.
  c. Fill plates with enough solution to cover sections.
  d. Move sections from one plate to the next using a paintbrush. Pick up section in a clump, not on one end, to avoid tearing.
  e. It is recommended to keep notes on each plate, noting the start and stop times and anything different or unusual.

**Day 1**

1. Wash sections in 3 x 5 min with TPBS. (> than 5 min is okay)
2. Treat sections with 1ml (or just enough to cover sections) of 50% ETOH for 30 min (exact 30 min!).
3. Pull out 10% NHS from refrigerator. Wash sections in 3 x 5 min with TPBS.
4. Block section with 1ml of 10% NHS for at least 30 min (can go longer). 10% normal horse serum (NHS) is made diluting 100% NHS with ddH\textsubscript{2}O to 10%. The shelf life of the NHS is about 2 weeks. If it becomes cloudy, discard and make fresh. NHS goes foamy, so pour gently, like a beer.
5. Add 1\textsuperscript{°} Ab directly to 10% NHS in vials. 1\textsuperscript{°} Ab is Arnel Rabbit a C-Fos at a dilution of 1:50,000 (For 2mls of 10% NHS add 4ul of R a Fos 1:100. This will give you a final dilution of 1:50,000). As long as the solution is made correctly, then how much you put on sections is irrelevant as long as the sections are covered. Leave the 1\textsuperscript{°} Ab on overnight (24hr), with lid on and shaker on.
Notes:

a. You can do step 4 and just add 4ul primary if you use exactly 2ml NHS but you may also do it in 2 separate steps.

b. 1° Ab is in 5ul aliquots in -80 freezer.

c. example volumes:

1:100 1° Ab soln = 500ul 10% NHS + 5ul stock 1° Ab  
vortex

1:50,000 1° Ab soln = 60ul 1:100 soln + 30ml 10% NHS

This will give you enough for one plate at 1ml/well.

**Day 2**

6. After 24 hours, wash sections in 3 x 5min with TPBS.

7. Make up the 2° Ab solution (Donkey a Rabbit biotin 1:500) Use 2mls of 1% NHS (fridge) per well plus 1ml(extra ml/batch). So for 8 wells, use 17 mls 1% NHS and add 34ul of Donkey a Rabbit biotin neat stock. This will give you a final dilution of 1:500. Put 2mls of this 1:500 solution in each well. As long as the solution is made correctly, then how much you put on the sections is irrelevant as long as sections are covered. Leave 2° Ab on overnight as close to 24 hours as possible but minimally 18 hr. 2° Ab is in 50ul aliquots in -80 freezer.

Note: To make enough for one 24-well plate

a. 2°Ab/plate(24wells) = 25ml 1% NHS + 50ul 2°Ab

b. Add small amount of 1% NHS from 25ml aliquot to the 50ul of 2°Ab in the centrifuge tube using a Pasteur pipette.

c. Thoroughly vortex.
d. Empty centrifuge tube with 2°AB into 25ml 1% NHS using Pasteur pipette.

Don’t forget lid! Repeatedly swish solution in and out of Pasteur pipette to get all of the 2°Ab into the 25ml 1% NHS solution.

**Day 3**

8. First thing in the morning, wash section 3 x 5min with TPBS.

9. Make up the XAv solution (in fridge) at 1:1300. For 1 plate (24 wells), mix 25ml TPBS and 19ul XAv to get 1:1300 solution. Vortex XAv before using. Maintain XAv in the refrigerator as much as possible to avoid warm/cool cycles. Leave the XAv on for at least 3 hours.

10. After 3 hours, wash sections 3 x 10min with TPBS.

11. Run NiDAB reaction using the protocol that follows.
Nickle-DAB reaction

**WARNING** - DAB (3,3’-diaminobenzidine) is a **potential carcinogen**!

- Wear gloves while handling DAB or anything with DAB in it.
- In general, use disposable items for DAB reactions or items labeled for DAB use only.

**Stock Solutions:**

DAB tablets - 10mg tabs (Sigma Cat# D-5905)

1% Nickel ammonium sulfate (Ammonium nickel sulfate)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>nickel ammonium sulfate</td>
<td>0.5g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>50mls</td>
</tr>
</tbody>
</table>

0.4M sodium phosphate buffer, pH 7.4

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>0.2g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>50mls</td>
</tr>
</tbody>
</table>

0.4% NH₄Cl, store at 4°C

20% D-glucose containing 0.05% sodium azide, store at 4°C

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>10g</td>
</tr>
<tr>
<td>Na Azide</td>
<td>0.025g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>50mls</td>
</tr>
</tbody>
</table>

Glucose oxidase (Sigma Cat# G-6891), store at 4°C
**Preparation of Pre-incubation solution**

**Notes:**

a. Keep solutions on lab rotator at slow speed as you are working with them.

b. Only use DAB labeled glassware.

<table>
<thead>
<tr>
<th></th>
<th>20ml</th>
<th>40ml</th>
<th>60ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Final Volume</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In a graduated cylinder put</td>
<td>(1 plate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4M PBS, pH7.4 (room temp)</td>
<td>5ml</td>
<td>10ml</td>
<td>15ml</td>
</tr>
<tr>
<td>0.4% NH₄Cl (fridge)</td>
<td>200ul</td>
<td>400ul</td>
<td>600ul</td>
</tr>
<tr>
<td>20% glucose (fridge)</td>
<td>200ul</td>
<td>400ul</td>
<td>600ul</td>
</tr>
<tr>
<td><strong>Distilled water</strong></td>
<td>13.8ml</td>
<td>27.6ml</td>
<td>41.4ml</td>
</tr>
<tr>
<td>Total volume is now</td>
<td>19.2ml</td>
<td>38.4ml</td>
<td>57.6ml</td>
</tr>
</tbody>
</table>

**Put in 10mg DAB tablet(s) (freezer)**

1 tab       2 tabs       3 tabs

Allow DAB tablet(s) to dissolve.

After DAB tablets dissolve add:

1% Nickel ammonium sulfate   0.8ml  1.6ml  2.4ml

Filter solution through a disposable filter (use a syringe filter, no smaller than .45u, .8u better)
Nickle-DAB Protocol

1. Add 1ml per vial of freshly prepared pre-incubation solution.
2. Agitate on shaker for 10min (can go longer).
3. Just before the 10min pre-incubation is over, make the reaction mix:
   Measure out \([(1\text{ml} \times \text{the number of wells}) + 1\text{ml}]\) of pre-incubation solution [eg, 7mls if there are 6 wells; 10mls if there are 9 wells]. For each 1ml of pre-incubation solution measured out add 2ul of glucose oxidase [eg, 14ul if there are 7mls; 20ul if there are 10mls].
   Note: For 1 plate you will need 26mls pre-incubation solution + 52ul glucose oxidase.
4. Add 1ml per well of freshly prepared reaction mix (total volume in each well will now be 2ml with a final concentration of glucose oxidase of 1ul/ml).
5. React wells for **EXACTLY 4 MIN**.
6. Rinse sections at least 3 times with TPBS.
7. After 3rd TPBS rinse, sections can stay in 4th rinse for up to a few days in the fridge until able to put onto slides.
8. Mount sections on slides and allow to completely dry.
11. Clear slides by putting them in each of the following solutions 1 time for 5min each:
   a. 95% ETOH
   b. 100% ETOH
   c. 100% ETOH
   d. Histoclear
   e. Histoclear
12. Blot excess Histoclear off the back and bottom of the slide, but do not dry the slide.
13. Put 3-5 drops of DPX on each slide and coverslip.
EDUCATION

2005-2010 Ph.D. Major: Nutritional Sciences
The Pennsylvania State University, University Park, PA
2001-2004 M.S. Major: Food Science
Tianjin University of Science & Technology, Tianjin, China
1996-2000 B.S. Major: Food Science and Engineering
Hebei University of Science & Technology, Shijiazhuang, China

PROFESSIONAL EXPERIENCE

Jan. 2005 – Dec. 2009 Title: Research Assistant
Department of Nutritional Sciences, Penn State University, University Park, PA
Investigated the effects of ovarian hormones on binge eating behavior and the underneath neurobiological mechanism in rats

Feb. 2009- Aug. 2009 Title: Dietetic Intern
Tulane Dietetic Internship Program, Tulane University, New Orleans, LA
Completed dietetic practices in several clinical, administrative, and community settings

Sep. 2001 – Feb. 2004 Title: Research Assistant
Department of Bioengineering and Food Science, Tianjin University of Science and Technology (TUST), China
Improved methods of D-ribose overproduction by optimizing fermentation parameters and by Metabolic Flux Analysis

PUBLICATIONS

Zhiping Yu, N. Geary, R.L. Corwin. Inhibition of Fat Intake by Ovarian Hormones under Binge-type Conditions in Ovariectomized Rats, Physiology & Behavior, 95 (3): 501-507, 2008

TEACHING EXPERIENCE

2008 Teaching Assistant for NUTR 380 (Leadership Principles in Nutrition Services)
2008 Teaching Assistant for NUTR 453 (Diet in Disease)
2007 Teaching Assistant for NUTR 100 (Contemporary Nutrition Concerns)

MEMBERSHIP IN PROFESSIONAL SOCIETIES

American Society for Nutrition
American Dietetic Association
Society for the Study of Ingestive Behavior
Society for Neuroscience

HONORS/AWARDS

2008 Grace M. Henderson Scholarship in the College of Health and Human Development at Penn State
2008 Third place winner of 2008 Graduate Exhibition Award at Penn State
2007 Honor mention of Penn State Institute for Diabetes and Obesity Wine and Cheese Posters Competition
2006 Woot-Tsuen Wu Lueng Scholarship in Nutrition at Penn State