UNIVERSITY OF CALIFORNIA, SAN DIEGO

Central, peripheral, and contextual regulation of food intake

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Neurosciences

by

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2012

The Dissertation of Sarah Lynne Parylak is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2012

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ACKNOWLEDGEMENTS

This work is eternally indebted to all the neuroscientists that have made my graduate school experience a memorable one. You know who you are.

Chapter 2, in full, is a reprint of the material as it appears in Parylak SL, Cottone P, Sabino V, Rice KC, Zorrilla EP. 2012. Effects of CB1 and CRF1 receptor antagonists on binge-like eating in rats with limited access to a sweet fat diet: lack of withdrawal-like responses. Physiol Behav 107:231-242. The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is currently in preparation for submission for publication as the manuscript Parylak SL, Zorrilla EP. Acute binge-related cues increase, but chronic binge-like intake of a palatable diet decreases, intracranial self-stimulation thresholds in rats. The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, is a reprint of material as it has been submitted for publication as Parylak SL, McElroy JF, Chorvat R, Zorrilla EP. (submitted). Joint blockade of peripheral and central CB1-mediated signals is required to suppress binge-like, but not ad libitum, palatable food intake in rats. The dissertation author was the primary investigator and author of this paper.

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PUBLICATIONS

Parylak SL, Zorrilla EP. (in preparation). Acute binge-related cues increase, but chronic binge-like intake of a palatable diet decreases, intracranial self-stimulation thresholds in rats.

Parylak SL, McElroy JF, Chorvat R, Zorrilla EP. (submitted). Joint blockade of peripheral and central CB1-mediated signals is required to suppress binge-like, but not ad libitum, palatable food intake in rats.

Parylak SL, Cottone P, Sabino V, Rice KC, Zorrilla EP. 2012. Effects of CB1 and CRF1 receptor antagonists on binge-like eating in rats with limited access to a sweet fat diet: lack of withdrawal-like responses. Physiol Behav 107:231-242.

Parylak SL, Koob GF, Zorrilla EP. 2011. The dark side of food addiction. Physiol Behav. 104:149-56.

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ABSTRACT OF THE DISSERTATION

Central, peripheral, and contextual regulation of food intake

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Doctor of Philosophy in Neurosciences

University of California, San Diego, 2012

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Near-unlimited access to energy-dense foods high in fat and sugar is widely viewed as a major contributing factor to the obesity epidemic of modern industrialized nations. Excessive consumption of these highly palatable foods, however, may have consequences on physiological and psychological function that extend beyond gains in body weight. Like drugs of abuse, palatable foods acquire enhanced incentive salience with repeated exposure, reflecting increased motivation to obtain and consume them. Whether palatable foods, similar to drugs of abuse, can also produce a withdrawal-like syndrome upon cessation of access remains unclear.

This dissertation describes a model of rapid, excessive 'binge-like' intake of a highly palatable sweet fat diet in rats, investigates whether evidence of a withdrawal-like syndrome or impaired reward function is apparent during the abstinent period between binges, and explores the contribution cannabinoid type 1(CB1) receptor mediated signals in regulating binge-like behavior . Results indicated that limited daily access to the sweet fat diet induced robust binge-like behavior in the absence of negative emotional symptoms of anxiety-like behavior or impaired reward function upon withdrawal. However, rats with a history of binge-like palatable food intake did show evidence of enhanced reactivity to binge-related cues. A binge "prime" consisting of a minimal dose of the sweet fat diet and contextual cues associated with the binge increased locomotor indices of activity and elevated intracranial self-stimulation thresholds. This suggests that the primary factors motivating continued binge-like intake are the hedonic value of the food and the appetitive drive associated with binge cues rather than an attempt to compensate for a negative state of withdrawal. Using a novel CB1 receptor inverse agonist, the hypothesis that central and peripheral cannabinoid receptors contribute independently to regulation of binge-like food intake was also tested. Peripheral CB1 blockade reduced ad libitum intake of both less palatable chow and the palatable diet, but joint blockade of central and peripheral receptors was required to suppress binge-like intake. This suggests that central cannabinoid circuitry is preferentially recruited when incentive to consume a palatable food is enhanced by limited access.

Chapter 1: Introduction

The obesity epidemic

Obesity is an enormous public health problem. Not only does it increase an individual's risk of cardiovascular disease, type-2 diabetes, cancer, osteoarthritis, and major depression [1], but the financial burden it places on society is immense, with obesity-related healthcare costs estimated to reach $210 billion as of 2012 [2]. Over the past 30 years, the prevalence of obesity has skyrocketed. Between 1960 and 1980 the percentage of obese American adults (BMI>30) held relatively stable at approximately 10% [3], but by 2010 over 35% qualified as obese [4]. Rising obesity rates have cut across boundaries of race, gender, and even age: among children and adolescents, approximately 17% already meet the criteria for obesity [5].

On the surface, the causes of obesity appear straightforward. Even a small imbalance in calories consumed and energy expended will, if maintained over time, lead to the progressive accumulation of body weight. Yet beneath this superficial analysis lies a host of biological, environmental, and sociocultural factors that all encourage excess weight gain. Genetic variations promoting intake of energy-dense foods and maintenance of weight during times of food scarcity would have provided a selective advantage throughout most of human history. However, in a modern environment that provides near-constant access to palatable foods high in fat and sugar, these variations are likely to encourage maladaptive amounts of food intake.

Further, cultural changes that have advanced sedentary occupations have diminished the amount of physical exertion required by individuals during normal daily activities.

Similarly, the seemingly obvious prescription for the obesity problem -- a recommendation for the obese to eat less and exercise more – downplays the magnitude of the challenge. Most people who attempt to lose weight will fail. Of overweight or obese individuals trying to lose weight, only 15-20% are successful in achieving and maintaining moderate weight loss of at least 10% of their starting body weight for a period of one full year [6, 7]. In the coming decades, one of the major challenges facing public health policy will be to enable individuals to understand and to combat the forces that drive them to overeat. Successful intervention strategies will require a better understanding of the biological factors that make intake of highly palatable foods so irresistible for many.

Binge eating: an example of "food addiction?"

Recent work has begun to examine obesity not merely as a failure to properly regulate energy intake and energy expenditure, but to view the consumption of palatable foods using the same conceptual framework as the consumption of drugs of abuse. One problematic type of food consumption in particular – binge eating disorder – bears striking similarity to patterns of intake normally seen in drug addiction.

Drug addiction is a chronic, relapsing disorder characterized by three distinct phases [8]. Although initial experimentation with drugs of abuse produces a

hedonically rewarding state of intoxication (the binge/intoxication phase), these effects inevitably subside and are replaced by the negative symptoms of withdrawal (the withdrawal/negative affect phase). Withdrawal can include both somatic signs, such as the tremors associated with withdrawal from alcohol or the severe pain associated with withdrawal from opiates, as well as psychiatric symptoms of heightened anxiety, depression, or anhedonia [8-10]. Following a period of abstinence, drug users may then enter a period of renewed preoccupation with or anticipation of further drug taking (the preoccupation/anticipation phase). As drug users progress from experimentation to addiction, it has been suggested that the primary factor motivating additional drug use changes. Instead of focusing on achieving a pleasurable state or "high," addicted individuals may be compelled to continue drug use predominantly to avoid the negative consequences of withdrawal [11].

Binge eating disorder (BED), as described in the DSM-IV, is characterized by repeated bouts of excessive eating ("binges") in which a person rapidly consumes large quantities of food beyond the amount a typical person would be expected to eat under similar circumstances [12]. These binges occur at least twice weekly for a period of six months or more and are accompanied by feelings of distress or loss of control. This behavior looks qualitatively similar to the behavior of an addict: individuals are unable to control their consumption of a rewarding substance, are abstinent for a period of time, then ultimately relapse and repeat the behavior. Further, binge eating, like drug dependence, persists despite mounting negative repercussions

on health and social functioning. Severe obesity (BMI>40) is more common among individuals with BED [13], and obese individuals with BED perceive greater impairment of their weight on quality of life, including measures of work performance, self-esteem, and distress in social situations, than similarly obese individuals without BED [14]. Such similarities in the features of drug use and binge eating have spurred efforts to categorize these addictive-like eating patterns as a form of "food addiction" [15].

With estimates of the lifetime prevalence of BED at ~2.8% [13], binge eating alone cannot account for the rise in obesity. However, the same mechanisms that go awry in BED may also contribute to less severe but more common forms of binge-like eating, such as in dieters who successfully maintain a negative energy balance on weekdays but "cheat" and indulge in greater intake on weekends [16]. Examining the extreme case may aid in identifying environmental and biological risk factors relevant to a much broader population.

It remains unclear whether the factors triggering binge eating, as in the case of addictive drug use, also involve a transition from pleasure-seeking to withdrawal-avoidance. The initial development of binge eating behavior likely depends on the availability of foods with high hedonic value. Foods consumed during a binge tend to be highly palatable items rich in calories from carbohydrates, fats, or both, such as cookies, ice cream, chips or other salty snacks, pastas, and fried meats [17]. Binge episodes are also more likely to occur subsequent to meals or snacks that contain such highly craved foods [18]. However, the pleasurable aspects of food consumption may

rapidly be replaced by a negative emotional state. Indeed, the diagnostic criteria for BED include feelings of disgust, guilt, or depression after overeating [12]. Rates of mood, anxiety, impulse control, and substance abuse disorders are also extremely high in the BED population. Over 75% of individuals with BED meet the criteria for at least one of these core DSM-IV disorders [13]. The prevalence of severe mood impairment is particularly concerning, with a third of teenage binge eaters reporting suicidal ideation [19]. It is possible that high comorbidity reflects commonalities underlying vulnerability to both BED and other psychiatric illnesses. An alternative explanation, however, is that BED itself may exacerbate symptoms of anxiety, depressed mood, or impaired impulse control. Repeated bouts of excessive palatable food consumption may generate a negative affective state during the abstinent period between binges, culminating in renewed binge eating as an individual seeks relief from binge "withdrawal." One of the major aims of this dissertation was to determine the role of positive (pleasure-seeking) vs. negative (reward-avoidance) reinforcement in driving binge-like behavior using a novel rodent model of binge eating. These ideas are discussed more thoroughly in Chapters 2 and 3.

Modeling binge eating behavior in rodents

Studying binge eating in rodents offers clear advantages in the investigation of underlying biological mechanisms by providing greater control over diet content and availability and by allowing for techniques too invasive to be used in humans. Nevertheless, this approach also has several important limitations. First, rodent models are unable to encompass the full range of social and cultural factors involved

in human food intake, which are as diverse as family and peer preferences, perceived health benefits or costs, and advertising [20, 21]. Second, measuring a rodent's subjective experience of distress or loss of control during a binge is not realistic given our current ethological understanding. Finally, an ideal animal model of binge eating would possess construct, predictive, and face validity. The unclear etiology of BED in humans, however, hinders an accurate assessment of construct validity. The scarcity of effective pharmacological treatment options for BED similarly hinders the assessment of predictive validity: the antidepressant fluoxetine appears to reduce the frequency of binge episodes [22] and is the only currently available FDA-approved treatment. Due to these considerations, the model utilized in this dissertation has focused on achieving face validity. Details of the model are described in Chapter 2. The quintessential feature of a binge -- rapid, excessive consumption of a palatable food – is also the primary feature of the following model and the metric against which potential pharmacological treatments and environmental manipulations are measured.

(Neuro)anatomy of a binge

Any attempt to link binge eating behavior to its biological underpinnings must contend with the highly distributed nature of networks involved in energy balance. Early study of central nervous system (CNS) regulation of food intake focused narrowly on the hypothalamus, within which selective lesions profoundly affected consummatory behavior in rodents. Lesions of the ventromedial nucleus increased food intake, whereas lesions of the lateral hypothalamus suppressed feeding and body weight, leading to the conceptualization of the ventromedial nucleus as an "inhibitory"

or satiety center and the lateral hypothalamus as an “excitatory” or feeding center [23]. Over time, these theories have been supplanted by more nuanced models acknowledging important contributions from other regions. Food intake, like addictive drugs such as cocaine and amphetamine, engages reward circuitry in the midbrain and basal forebrain. Food rewards stimulate firing of dopaminergic neurons in the ventral tegmental area of primates [24] and raise extracellular dopamine levels in the nucleus accumbens of rodents [25]. Food cues further recruit frontal cortical circuits involved in executive control, decision making and goal-directed behavior. Neurons within the orbitofrontal cortex show increased activity during the delay between cue presentation and delivery of a food reward in primates, and a subset of these cells respond preferentially to more highly preferred foods, suggesting that these neurons aid in encoding the anticipated value of a reward [26]. Numerous signals from the periphery are also integrated by CNS structures, with gustatory information from the facial and glossopharyngeal nerves [27] and post-ingestive gastrointestinal feedback via the vagus nerve converging on the brainstem in the nucleus of the solitary tract [28]. Leptin, which is produced by adipose tissue and circulates in proportion to fat stores [29], provides a gauge of longer term energy reserves and targets receptors not only in the hypothalamus [30], but also in dopaminergic neurons of the ventral tegmental area [31]. Control of food intake thus engages not a single, concentrated system but rather a broad network of neural structures positioned to process the hedonic value of a food, anticipation in response to food-related cues, and overall metabolic need. Different types of food intake – of more vs. less palatable

foods, under ad libitum or binge-like conditions, or during eating bouts triggered in response to salient food cues – may in turn rely preferentially on different components of this network.

One particular neurotransmitter system with a similarly broad distribution to hypothalamic, brainstem, midbrain, cortical, and peripheral structures – the endocannabinoid system [32-35] – is a particularly attractive target in the search for molecular regulators of binge-like eating. Ligands for the cannabinoid type 1 (CB1) receptor are known to modify food intake, but the critical sites of action for the anorectic effects of CB1 antagonists and inverse agonists [36-39] remain disputed. Early work assumed that anorectic effects resulted from interactions with brain CB1 receptors [40, 41]. More recent efforts, however, have identified putatively peripherally-selective antagonists and inverse agonists that maintain anorectic efficacy without occupying CB1 receptors in the brain [42-44]. Subtle differences in the context of food intake, as alluded to previously, may underlie these seemingly contradictory results. The specific role of this system in regulating binge-like food intake is explored in detail in Chapter 4.

Specific Aims

In summary, the overarching goal of the work described in this dissertation was to identify neurobiological and behavioral factors contributing to and consequences of binge-like food intake. Within this broader context, studies sought to achieve three specific aims:

1.) To induce binge-like eating in rodents using a paradigm that incorporates known triggers of human binges and to examine the effects of binge-like eating on metabolic outcomes.

2.) To examine the consequences of binge-like eating on behavior during the abstinent period between binge events:

    a. To establish whether binge-like intake causes negative emotional symptoms of withdrawal, and whether these symptoms in turn contribute to maintaining a binge-like eating pattern.

    b. To establish whether binge-like intake results in reward system dysregulation or anticipatory behavior when binge-related cues are present.

3.) To determine the role of both peripheral and central cannabinoid receptors in regulating binge-like food intake.

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Chapter 2: Effects of CB1 and CRF1 receptor antagonists on binge-like eating in rats with limited access to a sweet fat diet: lack of withdrawal-like responses

Abstract:

Positive reinforcement (e.g., appetitive, rewarding properties) has often been hypothesized to maintain excessive intake of palatable foods. Recently, rats receiving intermittent access to high sucrose diets showed binge-like intake with withdrawal-like signs upon cessation of access, suggesting negative reinforcement mechanisms contribute as well. Whether intermittent access to high fat diets also produces withdrawal-like syndromes is controversial. The present study therefore tested the hypothesis that binge-like eating and withdrawal-like anxiety would arise in a novel model of binge eating based on daily 10-min access to a sweet fat diet (35% fat kcal, 31% sucrose kcal). Within 2-3 weeks, female Wistar rats developed binge-like intake comparable to levels seen previously for high sucrose diets (~40% of daily caloric intake within 10 min) plus excess weight gain and adiposity, but absent increased anxiety-like behavior during elevated plus-maze or defensive withdrawal tests after diet withdrawal. Binge-like intake was unaffected by pretreatment with the corticotropin-releasing factor type 1 (CRF_1) receptor antagonist R121919, and corticosterone responses to restraint stress did not differ between sweet-fat binge rats and chow-fed controls. In contrast, pretreatment with the cannabinoid type 1 (CB1) receptor antagonist SR147778 dose-dependently reduced binge-like intake, albeit less effectively than in ad lib chow or high fat controls. A priming dose of the sweet fat diet did not precipitate increased anxiety-like behavior, but rather increased plus maze

locomotor activity. The results suggest that CB_1-dependent positive reinforcement rather than CRF_1-dependent negative reinforcement mechanisms predominantly maintain excessive intake in this limited access model of sweet-fat diet binges.

Introduction

Binge eating in humans is defined as repeated bouts of rapid, uncontrollable and excessive food intake. Individuals differ in which specific foods trigger binge eating; common foods include high carbohydrate items, such as breads and pastas, or high fat items, such as chips and fried meats [1]. Typical rodent models of binge eating thus provide palatable foods high in sugar or fat during limited access periods with intervening phases of food deprivation or access to a less preferred food [2-6]. Such schedules lead to hyperphagia when palatable food is available, mimicking the intermittent but intense character of human binges. For example, rats provided with only 10-min daily access to a chocolate-flavored high sucrose/low-fat diet ultimately consumed >40% of their total daily caloric intake within those 10 min [7]. This binge-like behavior was accompanied by increased weight gain, metabolic feed efficiency, and adiposity. As part of the current study, we sought to determine whether binge-like food intake and increased obesity risk similarly developed in a novel putative model of binge eating based on daily 10-min access to a sweet, high fat diet.

It remains unclear whether motivational mechanisms that drive binge eating are shared among palatable foods, or whether certain factors differentially contribute to bingeing on sugar vs. fat. Anecdotal reports and the popular lay literature promote

a concept of "sugar addiction" or "sugar craving" [8-10] that has begun to receive empirical support in animal models. For example, intermittent access to palatable, high sugar diets can produce withdrawal-like behavioral effects upon cessation of access. Hoebel, Avena and colleagues found that rats provided with daily 12-hr access to sugar solutions showed increased anxiety-like behavior in the elevated plus-maze (10% sucrose) [2] and opiate-like somatic withdrawal signs (25% glucose) after 1-2 days of food deprivation, effects that could be precipitated at earlier time points by the opioid antagonist naloxone [11]. We similarly found that rats 'cycled' between continuous access to a chocolate-flavored, nutritionally complete high-sucrose diet (50%kcal from sucrose, 2 days) vs. chow (5 days) each week developed increased anxiety-like behavior when the palatable food was withdrawn, binge eating upon renewed access to the food, and reduced intake of and reinforcing efficacy of otherwise acceptable chow. Activation of brain stress systems via corticotropin-releasing factor (CRF) partly mediated these effects of 'diet cycling'; pretreatment with the CRF_1 antagonist, R121919 normalized anxiety-like behavior, reduced overeating upon renewed access, and partly restored chow intake at doses that did not alter behavior of controls [12, 13]. Rats withdrawn (24 hr) from daily 10-min access to the same high-sucrose diet also showed increased anxiety-like behavior [7]; moreover, greater anxiety-like behavior correlated directly with larger binges, further supporting the hypothesis that relief from anxiety-like effects of withdrawal may promote binge-like intake of high sucrose diets in negative reinforcement fashion.

In contrast to the anxiogenic-like effects reported for withdrawal from high sugar diets, the ability of abstinence from high-fat or sweet-fat diets to induce a negative state in rodents is more controversial. One group reported increased open field locomotor activity following a switch from *ad libitum* high fat diet access to standard chow in mice, along with an increase in CRF mRNA in the central amygdala [14]; however, anxiety-like changes in plus-maze behavior were not observed. Furthermore, whereas withdrawal (24 hr) from *ad libitum* access to a sweet fat diet did increase plus-maze anxiety-like behavior in rats, anxiogenic-like behavior did not result following intermittent access [15]. Moreover, neither spontaneous nor naloxone-precipitated opiate-like somatic withdrawal signs were seen in rats withdrawn from the sweet fat diet [15].

In the present study, we sought to test the hypothesis that limited access to a sweet fat diet produces motivationally-relevant withdrawal-like signs, akin to our previously studied high-sucrose diet [7, 12], evident as either increased anxiety-like behavior or greater sensitivity to the effects of a CRF_1 antagonist on food intake. Because exposure to a craved food can precipitate binge behavior in humans with bulimia nervosa [16] and animal models [17], we also tested the hypothesis that a priming dose of the sweet fat diet might precipitate anxiety-like behavior if further binge food was not available. Finally, because high fat diets engage neurotransmitter systems other than endogenous opioids, such as the endocannabinoid system [18-20], we tested the hypothesis that administration of a cannabinoid type 1 (CB1) receptor

antagonist might differentially precipitate anxiety-like behavior or reduce food intake in binge -fed sweet-fat diet animals.

Whereas unexpected withdrawal of a palatable diet can intrinsically increase anxiety-like behavior, access to palatable diets reportedly attenuates acute neuroendocrine responses to external stressors. For example, rats with *ad libitum* access to sucrose or lard in addition to standard chow showed reduced ACTH and CORT responses to restraint stress [21-23]. Even brief twice-daily access to a sucrose or saccharin solution reduced ACTH and CORT restraint stress responses [24, 25]. The ability of palatable diets to dampen the hypothalamic-pituitary-adrenal (HPA) axis stress response has been proposed to motivate both rodents and humans to consume "comfort food" under periods of stress [26]. In the present study, we therefore tested the hypothesis that a history of binge-like intake of a sweet fat diet alters basal or restraint-induced CORT levels. We also tested whether recovery to basal levels following restraint was facilitated if rats thereafter were allowed to binge.

Materials and methods

Animals

Adult Wistar rats (n = 90, Charles River, Raleigh, NC) were group-housed in wire-topped plastic cages (19 x 10.5 x 8 inches) in a temperature- (22°C) and humidity- (60%) controlled vivarium with a 12:12hr reverse light cycle with lights off at 8:00am or 10:00am. Because bulimia and binge eating disorders are more prevalent in women than men [27], female rats were chosen as subjects. All procedures adhered

to the guidelines for animal use provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication number 85-23, revised 1996) and were approved by the Institutional Care and Use Committee of The Scripps Research Institute.

Binge Protocol 1 - Diets and development of 10-min binge-like behavior

Animals weighed 186-251 (M = 219) g at the start of experiments. Except as described below, all animals had access to water and standard rodent chow (Harlan Teklad LM-485, 3.1 kcal/g, 17% kcal from fat, 58% kcal from carbohydrates, 25% kcal from protein, Harlan, Indianapolis, IN) *ad libitum*. To induce binge-like eating (Fig 2.1), animals were transported daily to a testing room at dark cycle onset and placed individually into cages with wire mesh floors to permit monitoring of food spillage. Animals were then food-deprived for 2 hr, after which food was provided for a 10-min "binge" session in the testing room. The 10-min binge food consisted of either the standard rodent chow already described (Chow group) or a nutritionally-complete high-fat diet (Binge group: Bio-serv F06190, 4.1 kcal/g, 35% kcal from fat, 46% kcal from carbohydrates, 18% kcal from protein, Bio-serv, Frenchtown, NJ, ~85% of fat as saturated fat, 67% of carbohydrate as sucrose) that rats strongly prefer to chow (>99% preference ratio when provided daily concurrent access for 1-hr,Fekete and Zorrilla, unpublished observations). Diets were never concurrently presented to the same animals in the present study. Immediately after the 10-min binge, animals were removed from the testing room and returned to their home cages. Animals from a given home cage were assigned to the same diet groups, so home cage chow intake

was estimated by dividing total intake by the number of rats. Degrees of freedom from subsequent statistical analyses were lowered accordingly. One Binge animal that developed a malocclusion was dropped from all analyses, and its cagemate was not included in any outcome measure that involved home cage intake.

Body composition

Total fat mass and lean mass were determined after 6 weeks of the Chow or Binge diet access schedules using a whole body nuclear magnetic resonance machine (Echo-MRI-900, Echo Medical Systems, Houston, TX).

Drugs

The CRF_1 antagonist R121919 [28] (generously provided by Kenner Rice, NIH) was solubilized first in 1M HCl (10% of final volume) then diluted in 2-hydroxypropyl-beta-cyclodextrin (Sigma-Aldrich, 20% wt/vol final concentration) and titrated to pH 4.5 with 1M NaOH. The effects of R121919 treatment on intake during the 10-min binge session were determined by administering doses of 0, 5, 10, and 20 mg/kg s.c. 60 min prior to the 10-min binge in a within-subject Latin square design (n=7-8/diet group). We have previously observed anxiolytic-like effects of R121919 on the elevated plus maze test and increases in progressive ratio responding for a less-preferred chow reinforcer using this same 60 min pretreatment interval at a dose of 20 mg/kg [12]. Progressive ratio effects were evident within the first 5 min of food access.

The CB1 antagonist surinabant [29] (SR147778, 5-(4-Bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide), an analog of rimonabant, was provided by Sanofi-Aventis (Paris, France) and suspended in an 18:1:1 mixture of 0.9% saline:ethanol:cremophor prior to injection. The effects of SR147778 treatment on intake during the 10-min binge session were determined by administering doses of 0, 0.3, 1, 3, and 10 mg/kg i.p. 30 min prior to the 10-min binge in a within-subject Latin square design (*n*=8/diet group). Effects of SR147778 treatment on plus-maze behavior were determined by administering a dose of 1 mg/kg or vehicle 30 min prior to the plus-maze test.

The effects of SR147778 treatment on 10-min intake of the sweet fat diet were also examined in a separate set (*n*=8) of rats with no history of binge-like intake to help determine whether any differences across diet groups were in fact due to diet history (Binge vs. Chow) as opposed to the diet presented during the binge session itself (sweet fat vs. chow). Rats previously maintained on standard chow were allowed *ad libitum* access to the sweet fat diet in the home cage for 24 hr prior to the start of drug treatments. As in Binge Protocol 1, rats were then transported daily to a separate testing room, food deprived for 2 hr, then provided with 10 min access to the same sweet fat diet. At the conclusion of this 10 min session, rats were returned to their home cages to continue consuming the sweet fat diet *ad libitum*. SR147778 was administered at doses of 0, 0.3, 1, 3, and 10 mg/kg i.p. 30 min prior to the 10-min session in a within-subject Latin square design.

Estrous cycle synchronization

To control for estrous cycle-related variability, estrous cycles were synchronized by administering (s.c.) two doses of 2 □g each of the potent gonadotropin releasing hormone (GnRH) agonist [D-Trp^{6}, Pro^{9}-NEt]-GnRH [30], generously provided by Dr. Jean Rivier (The Salk Institute, La Jolla, CA) to simulate the GnRH surge at proestrus. Rats received all drug treatments as well as the restraint stress and elevated plus-maze tests during diestrus.

Elevated plus-maze

Previously, we observed that rats receiving the present schedule of binge-like access, but to a high-sucrose diet, showed increased anxiety-like behavior on the elevated plus-maze when the diet was not available [7]. Therefore, we sought to determine whether increased anxiety-like behavior also developed in relation to removal of the sweet high-fat diet. Elevated plus-maze tests were conducted on a black Plexiglas maze [31] that consisted of four arms with dimensions 50 cm long x 10 cm wide, two of which were enclosed by 40 cm high walls (closed arms), and two of which had 0.5 cm ledges along the sides (open arms). Each animal received a 5-min session on the maze following a minimum of 90 min of habituation in an adjacent room. Plus-maze testing was performed in a dimly-illuminated room (~3 lux on open arms) during the first half of the dark cycle. Sessions were recorded for subsequent treatment-naïve scoring of time spent on the open vs. closed arms and the number of entries into each arm.

Spontaneous and precipitated withdrawal from binge-like sweet fat diet intake

To determine whether rats with a history of binge-like sweet fat diet intake showed increased spontaneous anxiety-like behavior, rats (*n*=48) were tested on the elevated plus maze during the 6th week of exposure to the Chow or Binge diet access schedules, approximately 24 hr after the previous 10-min binge session. To determine whether anxiogenic-like effects could be precipitated in Binge animals by a CB1 antagonist, a class of compounds known to both reduce food intake [32] and to increase anxiety-like behavior [33] and which can precipitate withdrawal-like signs in cannabinoid-dependent animals [34], rats received either SR147778 (1.0 mg/kg, i.p.) or vehicle 30 min prior to being placed on the maze. The dose of SR147778 was sufficient to reduce food intake in both Binge and Chow rats (Fig 2.4). No food deprivation period or 10-min binge session were given on plus-maze test days.

Plus-maze behavior following pellet priming

To determine whether a priming dose of the rats' respective binge foods would acutely alter anxiety-like behavior, a separate cohort of animals (*n*=16) was tested on the elevated plus maze at the end of the 10th week of exposure to the Chow or Binge diet access schedules. Rats were food deprived at dark onset and placed in the binge session cages, but they received only a small priming dose (three 45 mg high-fat diet pellets [only 0.6% of an average binge during the preceding week] or the equivalent weight of chow) of their 10-min binge session diet, without additional access to food. After the priming dose was consumed, rats were immediately placed on the elevated plus-maze in an adjacent room.

Plasma corticosterone release after restraint stress

To determine the effects of a history of binge-like intake of a sweet fat diet on the function of the hypothalamic-adrenal-pituitary axis, tail blood was collected from rats (*n*=16) at 3 time points surrounding the 10-min binge session: 1) baseline conditions – prior to the 2-hr food deprivation period, 2) stressed -- immediately following 60 min of restraint stress in plastic semicylindrical restraint chambers that prevented the rat from freely moving or turning (stress onset immediately following the baseline bleed, ~2hrs prior to the binge session), and 3) post-binge recovery – 30 min following the conclusion of the binge session (~100 min after the termination of the stressor). Tail blood was also collected on unstressed binge days to permit a within-subject comparison of plasma corticosterone (CORT) levels at each time point. All tail blood samples were collected during weeks 13-15 of Binge or Chow diet access. Samples were collected over chilled tubes containing 10 □L of 0.1 M EDTA and centrifuged at 4°C for 10 min at 1341 *g*. The plasma fraction was stored at -80°C until analysis. Corticosterone levels were quantified by radioimmunoassay with a commercially available kit per the manufacturer's instructions (MP Biomedicals, Solon, OH).

Binge Protocol 2 – Individual housing with chow pre-presentation

The 10-min binge protocol described in Binge Protocol 1 was modified slightly from that used in our previous report of binge-like behavior associated with 10-min access to a high sucrose diet [7]. To address the alternative interpretation that

procedural changes, rather than changes in diet composition, may have led to the different outcomes on anxiety-like behavior between studies, we performed an additional experiment that matched the previous protocol [7] precisely.

Binge-like intake was developed in a separate set of adult female Wistar rats (n=16) as described in Binge Protocol 1 with the following alterations. First, animals were singly rather than group-housed upon arrival and weighed 210-237 (*M* = 224) g at the start of experiments. Second, a pre-presentation of chow was included immediately following the 2-hr food deprivation period, wherein both Chow and Binge rats were provided a feeder containing standard chow (Feeder 1) for 10 min. At the conclusion of 10 min, rats then received a second 10-min access period (Feeder 2) to either chow (Chow group, n=8) or the sweet-fat diet (Binge group, n=8) before being returned to their home cages. Third, no estrous cycle synchronization was performed. Fourth, the elevated plus maze test was conducted on day 16 as described previously, but in the absence of any drug or priming treatment.

To test the acute role of CRF_1 in control of food intake under these conditions, the effects of R121919 treatment on 10-min intake and subsequent home cage chow intake were determined by administering doses of 0 or 20 mg/kg s.c. 60 min prior to the first 10-min session in counterbalanced order in a within-subject design.

Defensive withdrawal

To determine whether anxiogenic-like effects could be detected after a longer duration of withdrawal from the sweet fat diet, rats from Binge Protocol 2 also

underwent a defensive withdrawal task on day 26, approximately 48 hr after their final 10-min binge session. The testing arena was constructed of black foamed polyvinylchloride with dimensions of approximately 79 x 79 cm with 50 cm high walls. A darkened withdrawal chamber (a 2-L Pyrex beaker shielded from outside light by wrapping in brown packing tape) was placed inside the arena with the opening ~22 cm from one of the corners. Rats were habituated to an anteroom outside of the testing room for a minimum of 60 min. After habituation, rats were placed individually into the withdrawal chamber, oriented facing the back, and allowed to freely explore the arena for 10 min under bright room light (~250 lux on open field). Sessions were videorecorded for subsequent scoring of latency to emerge from the withdrawal chamber, withdrawals into the chamber, total time spent in the chamber, and time spent in the chamber per withdrawal by two reliable, treatment-naïve raters (intraclass correlation coefficients >0.99 for all outcome measures [35]).

Statistical Analysis

Intake during the 10-min binge and the intervening home cage periods was analyzed with two-way repeated measures analyses of variance (ANOVA) with Diet Access group (Chow or Binge) as a between-subjects factor and Day as a within-subjects factor. Pairwise comparisons between Chow and Binge groups on specific days (Fig. 2.2) were obtained with Student's *t*-test. Ten-min intake following R121919 or SR147778 pretreatment was analyzed by two-way repeated measures ANOVA with Diet Access group as a between-subjects factor and Dose as a within-subjects factor. Ten-min intake in the *ad libitum* sweet fat-fed group was analyzed by

one-way repeated measures ANOVA with Dose as a within-subjects factor. Linear contrast ANOVAs were used to identify monophasic dose-responsive effects, as defined by a log-linear dose-response function [36]. Stability of individual differences in 10-min binge session intake across the 6-week study period was assessed by calculating two-way, random effect intraclass correlations of consistency agreement [35].Total cumulative intake, body weight gain, and feed efficiency during the 6-week study period, as well as final fat mass and lean mass, were analyzed with Student's *t*-test.

For the spontaneous and CB1-antagonist precipitated testing in the elevated plus maze, percentage of time spent on or entries into the open arms (inverse measures of anxiety-like behavior) and total arm entries were analyzed by two-way ANOVA with Diet Access group and Drug Treatment (vehicle or SR147778) as between-subjects factors. For the food-primed elevated plus-maze test and untreated plus-maze test, differences between Chow and Binge groups on the same dependent measures were compared with Student's *t*-test. Two animals in the binge group were removed from the analysis of the food-primed plus-maze test – one for failing to consume any of the priming pellets and one that was determined to be an outlier for total entries and latency to consume the food prime by deviating more than five standard deviations from the jackknifed group mean.

For defensive withdrawal testing, latency to emerge from the withdrawal chamber, number of withdrawals into the chamber, total time spent in the withdrawal

chamber, and time spent in the chamber per withdrawal were analyzed with Student's *t*-test.

Plasma corticosterone levels following restraint stress were analyzed by three-way repeated measures ANOVA with Diet Access group as a between-subjects factor and Stress Condition (stressed or unstressed) and Timepoint (baseline, post-stress, post-binge) as within-subjects factors. Intake during the 10-min binge following 60-min restraint was analyzed by two-way ANOVA with diet access group as a between-subjects factor and stress condition as a within-subjects factor. Higher order effects were interpreted by additional ANOVAs run separately for the interacting factors.

Results

Binge Protocol 1

Development of binge-like eating behavior and reduced home cage chow intake

Rats in the Binge group rapidly escalated their intake of the palatable high-fat diet over consecutive 10-min binge sessions, with intake plateauing after 2-3 weeks (Fig 2.2a). By the third week of exposure, Binge rats ate 5-fold more calories than the Chow rats during the 10-min session (M ± S.E.M 26.9 ±1.3 vs. 5.2±0.3 kcal, $t(61)=16.446$, $p<0.001$, Student's *t*-test), obtaining 38.0±1.3% of their total daily caloric intake during just 10 min compared to 7.8 ±0.4% in the Chow group. High-fat diet intake did not further escalate between weeks 3-6 (Week effect for Binge group only $F(5,150)=43.37$, $p<0.001$; Week 1 < all subsequent weeks $p<0.001$, Week 2 < all subsequent weeks $p<0.01$, Weeks 3-6 p's not significant). Chow animals, in contrast,

ate at a low, consistent rate during the 10-min session throughout the study (Diet effect: $F(1,61)=299.18$, $p<0.001$; Diet X Day effect: $F(38, 2318)=19.86$, $p<0.001$). The magnitude of intake during binge sessions showed individual differences across the entire 6-week observation period in both groups (Chow group *ICC*[2,35] consistency = 0.92, Binge group *ICC*[2,35], consistency=.98), but the stable individual differences were notably greater from day-to-day within the Binge group (Binge group, *ICC*[2,1] = .61 vs. Chow group *ICC*[2,1] = 0.25) .

When returned to their home cages, Binge rats reduced their chow intake during the interval between daily 10-min binges. Chow hypophagia became more pronounced across time (Diet effect: $F(1,21)=101.08$, $p<0.001$; Diet X Day effect: $F(36,756)=3.38$, $p=0.001$.Overall, over 6 weeks, total energy intake of Binge rats was significantly, but only slightly (5.4%), greater than that of Chow rats (Fig 2.2c, 2723.4±54.6 vs. 2584±33.4 kcal, $t(60)=2.21$, $p<0.05$). Self-selected overall macronutrient intake in Binge rats consisted of23.6±0.2% kcal from fat, 53.8±0.1% from carbohydrate, and 22.5±0.1% kcal from protein vs. the respective 17, 58 and 25% proportions of Chow rats.

<u>Excess weight gain and adiposity in rats with a history of binge-like, high-fat diet intake</u>

Binge rats gained more weight across 6 weeks than did Chow controls (Table 2.1, $t(61)=2.57$, $p<0.05$). This weight gain reflected a selective increase in fat mass ($t(61)=2.91$, $p<0.01$), rather than lean mass ($t(61)=0.71$, ns). Further, excess weight

gain was not completely accounted for by the excess caloric intake, because feed efficiency also was significantly (12.7%) greater in Binge rats (23.9±0.8 vs. 21.2±1.0 mg weight gained per kcal consumed, $t(60)=2.16$, $p<0.05$).

Pretreatment with a CB1, but not CRF_1, antagonist reduces binge-like intake

To determine whether CRF_1 receptors help maintain binge-like intake of the sweet-fat diet, rats were pretreated with the CRF_1 antagonist R121919. R121919 at doses up to 20 mg/kg had no effect on 10-min intake of either chow or the palatable high-fat diet (Fig 2.3a,b; no Dose effect $F(3,39)=0.75$; no Dose X Diet effect $F(3,39)=0.86$; p's>0.4). These negative results contrast with our previous finding that 20 mg/kg of R121919 reduced intake of a palatable, high-sucrose diet upon renewed access in diet-cycled rats [12].

In contrast, pretreatment with the CB1 antagonist SR147778 dose-dependently reduced 10-min binge session intake in both Chow and Binge groups (Fig 2.3c,d; Dose effect $F(4,56)=25.298$, $p<0.001$; linear contrast effect of Dose $F(1,14)=128.69$, $p<0.001$). SR147778 had greater anorectic potency in the Chow rats than Binge rats (Dose X Diet effect $F(4,56)=5.387$; $p<0.01$; minimum effective dose 0.3mg/kg in Chow vs. 1.0mg/kg in Binge) and also produced greater maximal suppression of intake in Chow (8.2%±3.7 of vehicle intake) than Binge rats (41.9%±6.7 of vehicle intake)

In rats provided *ad libitum* sweet fat diet access, SR147778 also dose-dependently reduced 10-min intake of the sweet fat diet (Fig 2.3e; Dose effect

F(4,28)=9.94, p<0.001; linear contrast effect of Dose F(1,7)=29.5, p<0.01). Maximal suppression of intake at the highest dose of 10mg/kg (6.3±6.0% of vehicle intake) resembled that seen in Chow rather than Binge rats (Fig 2.3f, t(14)=3.98, p<0.01 ad lib sweet-fat vs. Binge;).

Elevated plus maze behavior is altered by a priming dose of sweet fat diet, but not binge-like diet history alone

To determine whether anxiety-like behavior increased in Binge rats when the sweet fat diet was not available, rats were tested on the elevated plus-maze ~24 hrs after a 10-min binge. Withdrawal (24 hr) from the high-fat diet did not enhance anxiety-like behavior in the Binge animals, as measured by the % of arm time spent on (Fig 2.4a, $F(1,43)=0.40$, ns) or entries into (Fig 2.4b, $F(1,43)=0.49$,ns) the open arms of the maze. Pretreatment with an effective anorectic dose of SR147778 (1.0 mg/kg i.p.) in an attempt to "precipitate" withdrawal also had no effect on the % of arm time spent on or entries into the open arms (Fig 2.4a-b, $F(1,43)=0.65$ and 0.19, respectively, not significant). Anxiety-like behavior did not correlate with 10-min binge intake in Chow or Binge rats under either vehicle or SR147778-treated conditions (all r's<0.5, ns). Locomotor activity, as indicated by total arm entries, also did not differ between Chow and Binge groups or as a function of SR147778 treatment (Fig 2.4c, $F(1,43)=0.30$ and 0.49, respectively, ns).

A priming 'dose' of the rats' respective binge diet immediately before the plus-maze test also did not elicit differences in anxiety-like behavior between the diet

groups, as measured by the % time spent on or entries into the open arms (Fig 2.4a-b, $t(12)=0.60$ and 0.61, respectively, ns). However, pellet priming did increase overall locomotor activity, as indicated by more total arm entries in the Binge relative to the Chow group (Fig 2.4c, $t(12)=3.44$, $p<0.01$). Priming pellets were consumed more rapidly by the Binge group than the Chow group (18.8±1.7s vs. 55.4±12.3s, $t(12)=2.54$, $p<0.05$).

HPA-axis reactivity to restraint stress and binge-like sweet fat diet intake

Restraint stress (60 min) significantly increased CORT levels by ~2-3-fold in both Binge and Chow rats, with recovery of CORT to basal levels by the post-binge timepoint (Table 2.2; Stress X Time point: $F(2,26)=5.02$, $p<0.05$). Binge and Chow rats did not differ from one another, at the baseline, stressed, or recovery time points under either unstressed or restraint conditions (no effect of Diet: $F(1,13)=0.001$, ns, no interactions involving Diet, all p's>0.6). Average basal CORT levels across stressed and unstressed days also did not differ between diet groups (205±39ng/ml for Chow vs. 247±26 for Binge rats, ns). Although restraint was sufficient to increase CORT levels markedly, it did not alter intake during the 10-min period in either Diet group (no effect of stress: $F(1,13)=1.16$, ns).

Binge Protocol 2

Single housing conditions and chow pre-presentation does not alter binge-like intake

To determine whether changes in housing conditions or feeder presentation relative to our previous report [7] significantly impacted the development of binge-like

behavior or weight gain, singly housed rats were assigned to Chow or Binge groups and provided with two consecutive 10 min feeding sessions: the first feeder contained standard chow regardless of diet group, whereas the second feeder contained either chow (Chow group) or the sweet fat diet (Binge group). As observed previously with a high–sucrose diet [7], binge rats suppressed their intake from the 1st feeder relative to Chow rats (F,1,14)=17.01, p<0.01; Fig 2.5a), but rapidly escalated their intake of the sweet fat diet from the 2nd feeder at levels similar to those seen in group-housed rats of Protocol 1 with only a single feeder presentation (Fig 2.5b). This escalation, as before, was not observed in Chow rats (diet effect: F(1,14)=116.4, p<0.001; Diet X Day effect: F(22,308)=9.50; p<0.001).

When returned to their home cages, Binge rats again reduced their chow intake during the interval between daily 10-min binges (Fig 2.5c). As with Protocol 1, chow hypophagia again became more pronounced across time (Diet effect: F(1,14)=22.2, p<0.001; Diet X Day effect: F(21,294=2.80, p<0.001). Total energy intake over the 24-day study period was also significantly greater in Binge rats than in Chow rats (Fig 2.5d, 1897.5±41.7 vs. 1668.0±49.2 kcal, t(14)=3.56, p<0.01); this overall caloric difference was greater than that seen in group-housed rats of Protocol 1 despite the shorter study period (13.8±2.5% vs. 5.4±2.1% t(18.3)=2.55, p<0.05, Welsh's t-test). Binge rats also gained more weight over the study period than Chow controls (41.9±2.8 vs. 28.5±2.0g; t(14)=3.91, p<0.01) and had greater feed efficiency (22.0±1.2 vs 17.2±1.2 mg weight gained per kcal consumed; t(14)=2.88, p<0.05).

Lack of effects of CRF_1 antagonist treatment on 10-min binge-like intake

The procedural differences of Protocol 2 (isolation housing, chow pre-presentation) did not alter the lack of effect of R121919 administration on binge-like intake of the sweet fat diet. R121919 at 20 mg/kg did not affect 10-min intake from the 1st feeder (Fig 2.6a, no Drug effect F(1,14)=0.17, p=ns; no Drug X Diet effect F(1,14=0.01, p=ns) or from the 2nd feeder in either Chow or Binge groups (Fig 2.6b, no Drug effect F(1,14)=0.08, p=ns, no Drug X Diet effect F(1,14)=1.01, p=ns). Further, R121919 treatment did not significantly affect home cage intake in either diet group (Fig 2.6c, no Drug effect F(1.14)=2.18, p=ns; no Drug X Diet effect F(1,14)=0.92, p=ns).

Lack of anxiogenic-like behavior during withdrawal from 10-min binge-like intake

Similarly, the procedural differences of Protocol 2 did not lead to increased anxiety-like behavior in rats withdrawn from daily 10-min binge access. In the elevated plus maze (Fig. 2.6c), a history of binge-like intake did not alter % time spent on (t(14)=0.36, p=ns) the open arms of the maze, % entries into the open arms (t14)=0.32, p=ns), or total arm entries (t(14)=0.79, p=ns) 24 hr following the previous binge. Further, neither % time spent on the open arms nor % entries in the open arms correlated with 10-min intake from either feeder in Binge rats (all *r*'s<0.6, ns). There was a trend towards a correlation between reduced open arm time and increased intake from the 2nd feeder in Chow rats only, which did not reach significance (r=.627, p=0.10).

To determine whether an anxiety-like state might become apparent at a later timepoint in sweet-fat withdrawn rats, we conducted a defensive withdrawal test at 48 hr, rather than 24 hr, after the final 10-min binge session. In opposition to the hypothesis of increased anxiety-like behavior during withdrawal, rats with a history of binge-like sweet fat diet intake emerged from the withdrawal chamber more rapidly than did chow rats (Fig 2.6d, $t(14)=2.52$, $p<0.05$), an anxiolytic-like effect. Non-significant trends for anxiolytic-like effects also were seen for Binge rats withdrawing less frequently into the withdrawal chamber ($t(14)=1.86$, $p=.09$) and spending less total time in the withdrawal chamber ($t(14)=1.75$, $p=0.10$). No group differences were seen in the time spent in the chamber per withdrawal ($t(14)=.67$, p=ns).

Discussion

The present study finds that daily, but highly limited (10 min), access to a sweet fat diet leads to binge-like intake, excess weight gain, and increased adiposity, without signs of a stress-like state upon withdrawal of the palatable diet. At 24 hr after their most recent binge, rats with a history of binge-like sweet fat diet intake did not show increased anxiety-like behavior on the elevated plus-maze under spontaneous, binge-food primed or CB1-antagonist treated conditions. Binge history rats also did not show altered basal or restraint-induced circulating CORT levels, indicating normal adrenocortical responses to stress. Further, binge-like intake was unaffected by pretreatment with a CRF_1 receptor antagonist, which reduces excess intake of rats withdrawn from a high sucrose diet [12], ethanol [37-41], opiates [42], nicotine [43], or cocaine [44] in models of addiction. Instead, binge history rats

showed locomotor activation in response to a sweet-fat food prime and reduced their binge-like intake in response to a selective CB1 antagonist, albeit less potently than did chow controls. Anticipatory negative contrast did develop when rats were provided with a feeder of less-preferred chow immediately prior to the 10-min sweet-fat binge, similar to the reduced acceptance of chow reported for a high sucrose diet [7]. This anticipatory negative contrast did not, however, appear to require concurrent withdrawal-based anxiety-like behavior. Indeed, with a longer duration of withdrawal (48 hr), sweet-fat binge history actually showed anxiolytic-like effects on a defensive withdrawal task. The collective results from this novel binge eating model contrast with reports of increased anxiety-like behavior and opiate-like withdrawal signs upon withdrawal of diets high in sugar [2, 7, 11, 12] and add to a growing literature finding mild or nonexistent withdrawal-like effects for high fat or sweet fat diets [15, 45].

Both limited access sweet-fat diet protocols utilized in this study produced similar degrees of binge-like intake and chow hypophagia as we previously observed in female rats receiving the same schedule of access to a high sucrose diet [7]. In the protocol matched precisely to our previous report, adverse metabolic effects of increased weight gain and feed efficiency were also comparable to animals with binge-like sucrose diet access. Yet, here, unlike with the high sucrose diet, increased anxiety-like behavior was not seen. Exactly which features of the high fat vs. high sugar diets yield different withdrawal-like outcomes remain to be determined. While differences in withdrawal-like signs might relate to general physiochemical properties of sugars vs. fats, they may also result from correlated, but not immutable, properties

of the macronutrients. For example, although both diets were highly preferred to chow, perhaps the high sucrose diets studied yielded a more intense hedonic value than did the high fat diets. Consistent with this possibility, non-deprived rats showed greater acceptance of or preference for non-nutritive sweet tastants vs. non-nutritive 'fatty' tastants in previous studies [46, 47], suggesting greater sweet vs. fat oral reward. Moreover, intra-gastric self-infusions of fats were less effective than iso-caloric self-infusion of sugars in producing conditioned solution intake [48] or flavor preferences [49-51] in rats, suggesting greater post-oral reward of sugar than fat under those test conditions. However, studies in C57BL/6J mice [52, 53] have found comparable post-oral rewarding effects of fats vs. sweets, indicating that any macronutrient-associated differences in hedonic intensity are not absolute. One of the few findings of withdrawal-like responses following high-fat diet withdrawal involved altered open field behavior in mice withdrawn from access to a high fat diet that the mice strongly preferred to a high sugar (35% sucrose) diet [14]. In this case, the less-preferred high sugar diet did not elicit withdrawal-like behavioral outcomes, consistent with the above interpretation that greater initial hedonic effects lead to more severe negative emotional effects upon withdrawal.

Another potentially key difference between fats and simple sugars concerns differences in their kinetics of digestion, absorption and clearance. Dietary simple sugars like glucose, sucrose and maltose, after rapid intestinal digestion and absorption, yield rapid spikes in blood glucose. The pronounced elevations strongly drive insulin secretion leading to similarly rapid glucose clearance. In contrast,

dietary fat must be digested and absorbed in a much lengthier process that requires emulsification, hydrolysis to fatty acids, micelle aggregation, intestinal conversion to triglycerides for incorporation into chylomicrons and, ultimately, transport via the lymphatic system to blood circulation. Reflecting this protracted digestion and absorption process, post-oral rewarding effects of dietary fat in the mouse are later in onset and more gradual and sustained in magnitude than those of simple sugars [52]. By analogy, perhaps, a given unit dose of cocaine is less reinforcing when infused over a delayed or longer interval [54-58] or via a less rapidly absorbed route [59]. Furthermore, several substances of abuse, such as benzodiazepines, have rebound anxiety withdrawal potential if they have shorter elimination half-lives [60]. Thus, the rapid digestion, absorption, and clearance of simple sugars may be more conducive to post-oral addiction-like processes than the protracted absorption of fat. Addition of fat to diets high in simple sugars slows gastric emptying and both delays and inhibits absorption of the dietary sugar [61-65]. This may explain why sugar-containing sweet fat diets do not reliably elicit withdrawal-like effects, despite having sugar contents similar to or even greater than sugar solutions or low-fat but sugar-rich foods [15] and supporting binges of comparable magnitude.

A defining feature of binges is their discrete, intermittent nature with bouts of intense intake separated by periods of abstinence or dietary restriction. The lack of increased anxiety-like behavior at the time that rats would otherwise initiate their next binge (24 hr post-binge) suggests that under the present conditions, withdrawal "anxiety" did not acutely motivate binge intake of the high-fat diet in negative

reinforcement fashion. Additional support for this interpretation comes from the inability of an anxiolytic-like dose [66, 67] of a CRF_1 antagonist (R121919) to attenuate binge-like intake and the normal basal and restraint-induced HPA-axis responses to stress of Binge rats. Furthermore, Binge rats did not increase their intake of the sweet fat diet when challenged by restraint stress, unlike previous reports of stress triggering enhanced "comfort feeding" of palatable foods [4, 23, 26]. Although the aforementioned differences in fat vs. sugar digestion and absorption might suggest that a withdrawal-like state would occur later in sweet-fat bingeing animals, anxiety-like phenotypes on a defensive withdrawal task were still not observed in binge history rats even at 48 hr after the last binge session. The major implication of sustained binge-like intake (~40% of daily energy consumed within 10 min across months of access) without signs of increased anxiety-like behavior or stress reactivity upon withdrawal is that the primary factor driving binge intake of the sweet fat diet was not the relief from a negative emotional state, a key component of relapse in drug addiction, alcoholism, and high sugar diet consumption [11, 68]. Instead, the binge intake may reflect ongoing appetitive, rewarding effects of the highly palatable food and/or learned,habitual responses. Our observation that a history of binge-like intake decreased latency to emerge from a protected withdrawal chamber may be a reflection of these ongoing appetitive effects.

A key trigger of binge eating is the initial sensory cues associated with the diet itself. In animal models, rats trained to lever press for palatable food pellets and then extinguished for this response will reinstate food-seeking (non-reinforced) behavior if

primed with a single palatable food pellet [69, 70]. A palatable food prime also can potentiate palatable food context-induced bingeing on chow [71]. In the present study, a taste of the binge food, which also can precipitate binges in humans [16], increased overall activity levels of Binge rats in the plus-maze without altering their anxiety-like behavior. The locomotor activation may reflect arousal, anticipatory behavior, or foraging entrained to scheduled palatable food access [72-74] or a response analogous to frustrative non-reward [75, 76] in Binge rats, differences that were not present in the absence of the food prime. The results further support the hypothesis that binge foods acquire conditioned incentive motivating properties.

A second, not mutually exclusive interpretation of sustained binge-like behavior without accompanying anxiety is that the daily binges become compulsive learned behaviors or habits that are resistant to environmental factors and independent of energy need. Consistent with this possibility, rodents will endure adverse conditions such as a brightly lit arena [14] or environmental cues previously paired with foot shock [77] in order to obtain a palatable diet, suggesting the development of compulsive intake patterns despite mounting negative consequences. Data from the current study that support the hypothesis that the binge-like behavior was resistant to change include the marked day-to-day stability of individual differences in binge magnitude, the stability of the binge despite 1-hr restraint or blood sampling, and the diminished potency and efficacy of SR147778, a CB1 antagonist, to attenuate binge-like intake of the high-fat diet, as compared to chow. The latter finding is noteworthy because previous reports have suggested that CB1 antagonists preferentially target the

intake of more palatable foods [32, 78, 79] by reducing their acute hedonic value [80]. Accordingly, CB1 antagonists more effectively reduce *ad libitum* intake of palatable high-fat, than chow diets [[78], but see also [81]]. Indeed, we observed greater efficacy of a CB1 antagonist in reducing 10-min sweet fat diet intake when this diet was provided *ad libitum* in the home cage rather than solely during the 10-min binge. The relative resistance of the binge-like intake specifically to CB1 antagonist treatment suggests that acute food reward may be less important for maintaining binge-like eating, once established. However, the minimum effective dose that reduced 10-min intake was similar in Binge and *ad libitum* sweet fat rats. An alternative possibility is thus that perhaps the more intense reward of the binge and its associated palatable diet requires a greater dose of CB1 antagonist to surmount endocannabinoid action.

Conclusions

Binge-like intake of a sweet fat diet under limited access conditions persists for weeks or months in the absence of a negative anxiety-like state upon sweet fat diet removal, suggesting that unique biological mechanisms may contribute preferentially to excessive intake of sugars vs. fats. The resilience of binge-like eating behavior to modification by either restraint stress or CB1 antagonists further suggests that learned, habitual, or cued intake of palatable foods may be of particular importance in sustaining the binge-like intake. Because the typical Western diet is comparable in fat proportions (32-34%, [82]) to the present sweet fat diet (35%), clinical interventions to

reduce binge intake of fat-containing “Western” foods may more effectively target aspects of cued intake or positive reinforcement, rather than negative reinforcement.

Disclosure Statement

EPZ is an inventor on a patent filed for CRF1 antagonists (USPTO Application #: #2010/0249138).

Acknowledgements

The authors thank Michael Arends for editorial assistance with the manuscript and Jean Kim and Daniel Le for technical assistance. Financial support for this work was provided by the Pearson Center for Alcoholism and Addiction Research, the Harold L Dorris Neurological Research Institute, and grants DK070118, DK076896, DA023680, DA026690, and 1T32NS061847 from the NIH. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This is manuscript number 21675 from The Scripps Research Institute.

Chapter 2, in full, is a reprint of the material as it appears in Parylak SL, Cottone P, Sabino V, Rice KC, Zorrilla EP. 2012. Effects of CB1 and CRF1 receptor antagonists on binge-like eating in rats with limited access to a sweet fat diet: lack of withdrawal-like responses. Physiol Behav 107:231-242. The dissertation author was the primary investigator and author of this paper.

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Group	Lights on	Lights off		
Chow	Chow *ad libitum*	2hr deprivation	10min Chow	Chow *ad libitum*
Binge	Chow *ad libitum*	2hr deprivation	10min Sweet Fat	Chow *ad libitum*

Figure 2.1: Limited access protocol to promote binge-like intake of a high-fat diet. Female Wistar rats were allowed to consume standard chow (Harlan Teklad LM-485, 3.1 kcal/g, 17% of kcal from fat) ad libitum in their home cages throughout the light cycle. At dark onset, all rats were transported to individual test cages and food deprived for 2 hr. Following the deprivation, animals received 10-min access either to the same standard chow (Chow group) or a highly palatable high-fat diet (Bio-serv F06190, 4.1kcal/g, 35% kcal from fat, Binge group). All rats were returned to their home cages following the 10-min Binge. Diets were never presented concurrently to the same animal.

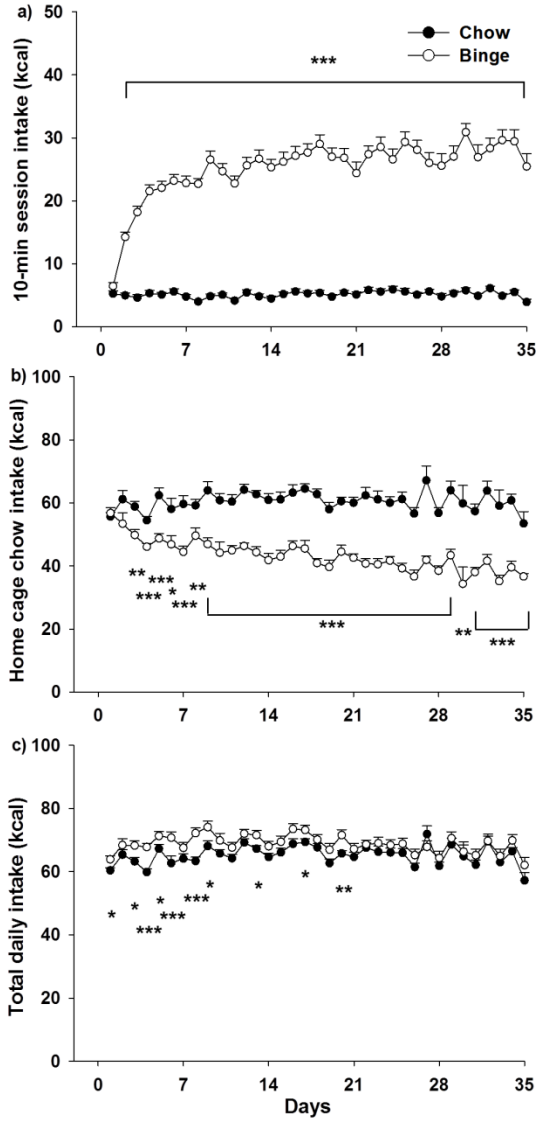

Figure 2.2: Binge-like intake of a high-fat diet developed rapidly with daily exposure to a mere 10-min diet access following a 2-hr food deprivation period. Data are expressed as *M* ± SEM in panels *a*) 10-min intake (kcal) of either standard chow (Chow) or a highly palatable sweet fat diet (Binge). Binge rats escalate 10-min intake across the first 3 weeks of exposure and plateau at a level of ~40% total daily kcal intake during the 10-min binge. b) *Ad libitum* home cage intake of standard chow during the intervening time between binge sessions was decreased in Binge rats. c) Total daily combined intake of both standard chow and the highly palatable diet. Binge rats consumed slightly more kcal cumulatively across the 6-wk study period than Chow rats. *Differs from Chow $p<0.05$, **$p<0.01$, ***$p<0.001$

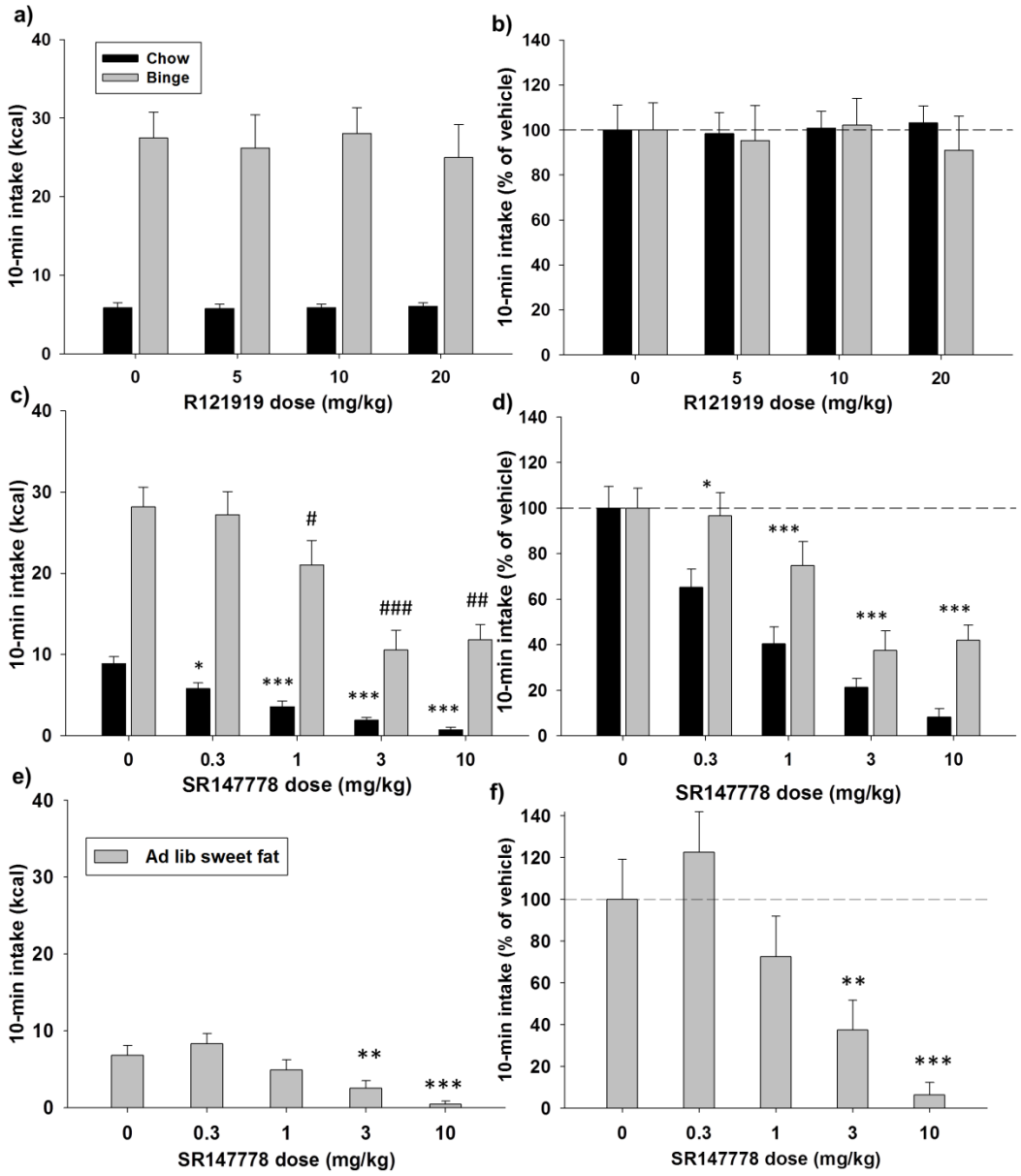

Figure 2.3: Binge-like intake of a sweet fat diet was blocked by a CB1 receptor antagonist, but not by a CRF1 receptor antagonist. a,b) Pretreatment with the CRF1 antagonist R121919 did not impact intake of either sweet fat diet or standard chow during a daily 10-min binge as measured by raw kcal consumed (a) or % of vehicle intake (b). c,d) Pretreatment with the CB1 antagonist SR147778, in contrast, dramatically reduced 10-min intake of sweet fat diet and standard chow (raw kcal, c; % vehicle intake, d). e) Pretreatment with SR147778 also reduced 10-min intake of the sweet fat diet in rats with ad libitum access to the same sweet fat diet in their home cages. f) Maximal suppression at the highest dose mirrored the efficacy observed in Chow, rather than Binge rats. *Differs from vehicle $p<0.05$, **$p<0.01$, ***$p<0.01$; # differs from binge/veh $p<0.05$

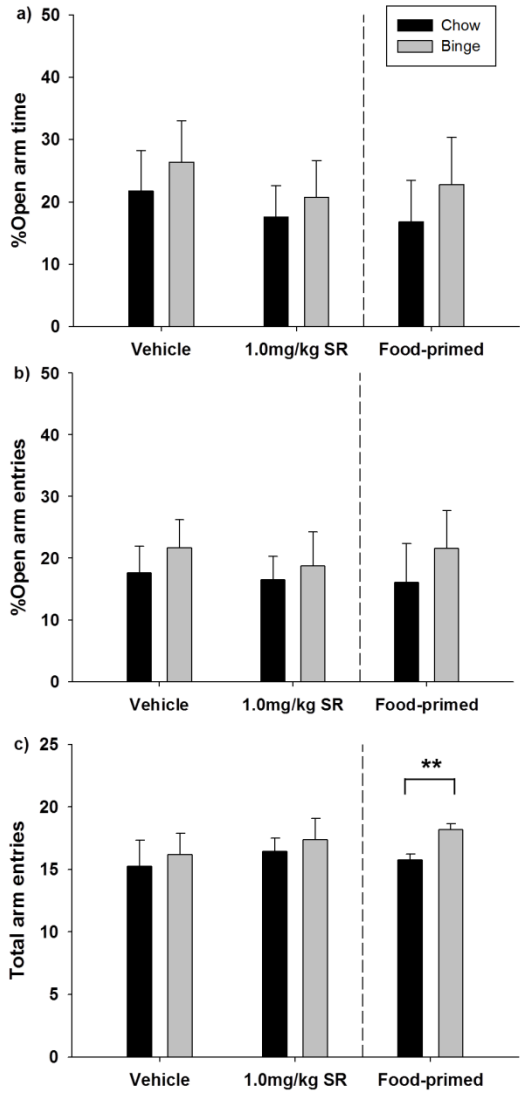

Figure 2.4: No evidence of increased anxiety-like behavior on the elevated plus-maze 24 hr following last high-fat diet access in rats with binge-like intake history. Separate groups of rats were treated (i.p.) with vehicle, or the CB1 antagonist SR147778, or given a food prime prior to being placed on the maze. For food priming, rats received 3 pellets of sweet fat diet (Binge group) or an equivalent weight of standard chow (Chow group). Data are expressed as M ± SEM in panels. a,b) Neither percentage of time spent on the open arms nor percentage of entries into the open arms differed between Chow and Binge rats under vehicle conditions or following treatment with an anorectic dose of SR147778 in an attempt to "precipitate" withdrawal from the high-fat diet. Food priming also did not alter open arm time or entries. c) Overall locomotor activity, as measured by total arm entries was enhanced in Binge relative to Chow rats following a food prime only. **Differs from Chow $p<0.01$

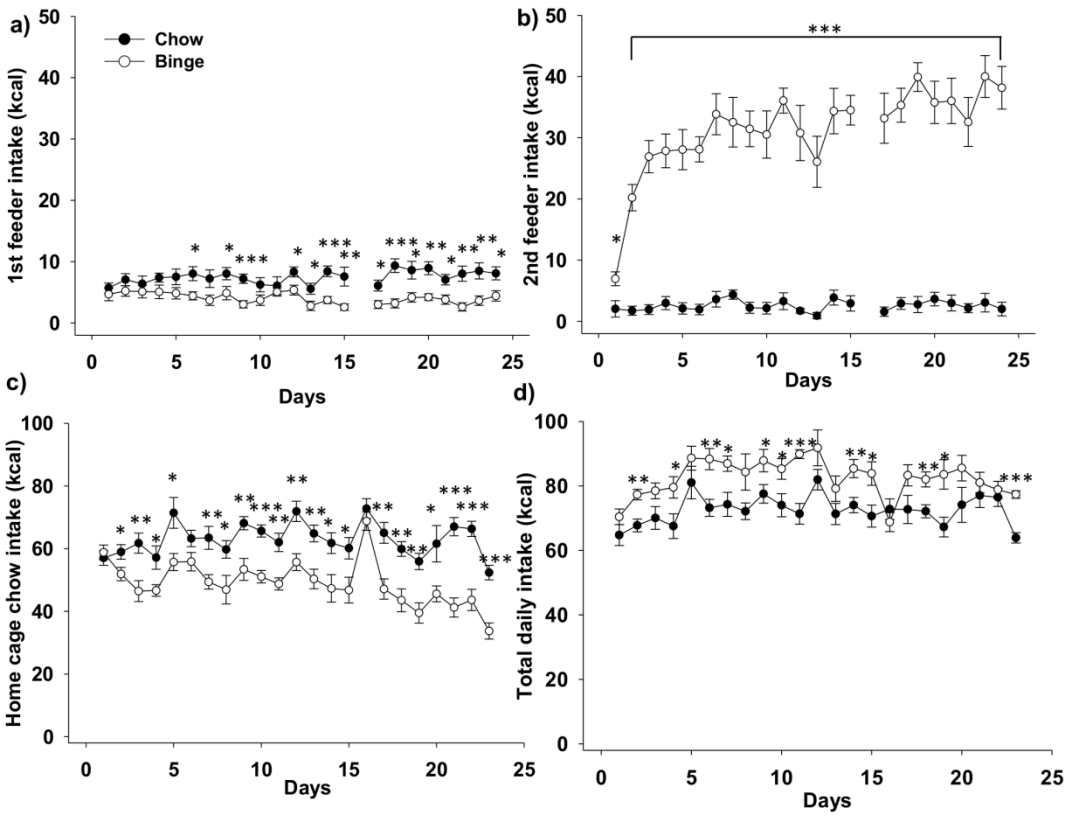

Figure 2.5: Binge-like intake of a high-fat diet and concurrent chow hypophagia in singly housed rats with chow feeder pre-presentation. Two consecutive 10-min feeding sessions were performed, with all rats receiving standard chow from the 1st feeder. Binge rats received the sweet fat diet in the 2nd feeder, whereas Chow rats again received standard chow. Data are expressed as M ± SEM in panels a) 10-min intake (kcal) of standard chow from the 1st feeder is reduced in Binge rats in anticipation of receiving the sweet fat diet from the 2nd feeder. b) Binge rats escalate 10-min intake of the sweet fat diet from the 2nd feeder at levels comparable to those seen in group-housed animals with only a single feeder presentation. c) Ad libitum home cage intake of standard chow during the intervening time between binge sessions was decreased in Binge rats. d) Total daily combined intake of both standard chow and the highly palatable diet. Binge rats consumed more kcal cumulatively across the study period than Chow rats. *Differs from Chow $p<0.05$, **$p<0.01$, ***$p<0.001$

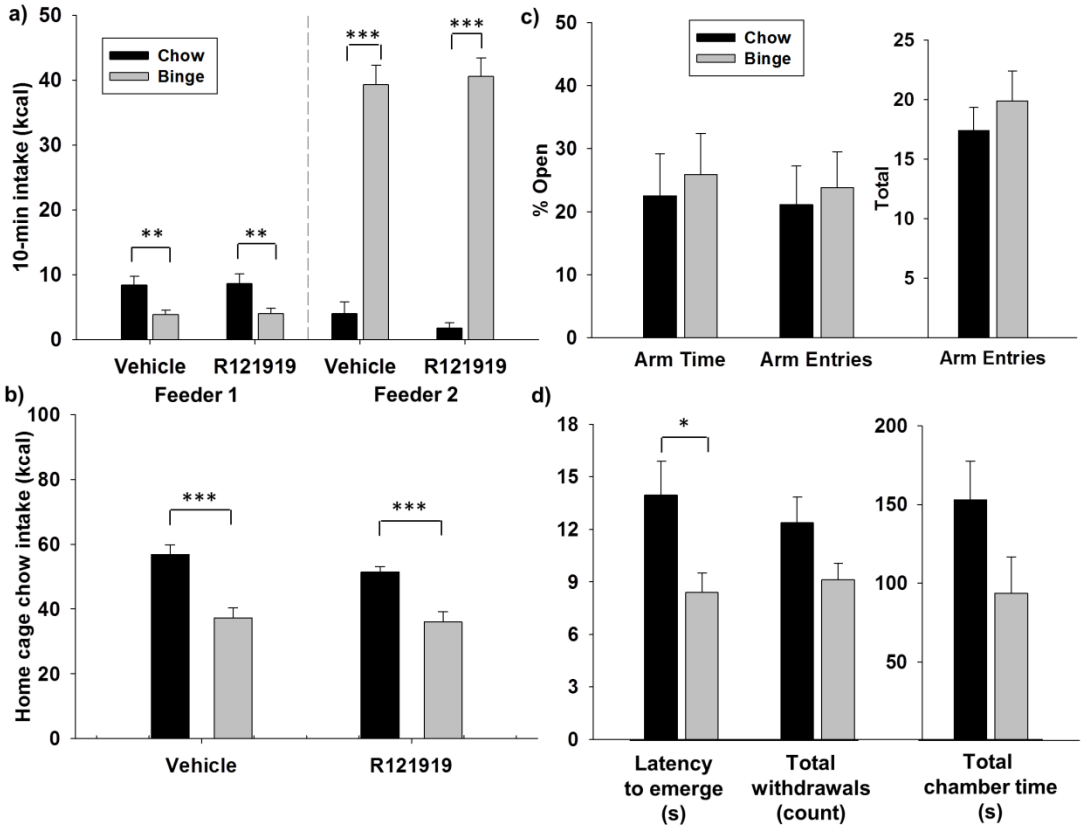

Figure 2.6: No evidence of CRF-driven binge-like intake or increased anxiety-like behavior in singly housed rats with chow feeder pre-presentation. Data are expressed as M ± SEM in panels. a) Pretreatment with the CRF1 antagonist R121919 did not impact 10-min intake of either standard chow or sweet fat diet. b) R121919 treatment also did not alter home cage chow intake in either Chow or Binge rats. c) On the elevated plus maze, percentage of time spent on the open arms, percentage of entries into the open arms, and overall locomotor activity as measured by total arm entries did not differ between Chow and Binge rats at 24 hr after the most recent binge. d) During a defensive withdrawal task, Binge rats emerged more rapidly from a darkened withdrawal chamber than Chow rats when tested 48 hr after the most recent binge. Total time inside the withdrawal chamber and total number of withdrawals did not differ significantly between diet groups. *Differs from Chow $p<0.05$, **$p<0.01$, ***$p<0.001$.

Table 2.1: Increased weight gain and adiposity in female rats with a history of binge-like sweet fat diet intake.

	Chow (n=32)	**Binge (n=31)**
Body weight gain (g)	59.3 ± 2.9	69.2 ± 2.5*
Feed efficiency (mg weight gain/kcal consumed)	21.2 ± 1.0	23.9 ± 1.8*
Fat mass (g)	39.8 ± 1.6	47.2 ± 2.0**
Lean mass (g)	197.0 ± 3.2	199.9 ± 2.6
*Differs from Chow $p<0.05$, **$p<0.01$ Data represent *M* ± SEM All measurements represent values after 40-41 days of binge-like access		

Table 2.2: Plasma corticosterone levels (ng/ml) and 10-min food intake in Binge and Chow rats under unstressed and restraint stressed conditions.

	Chow (n=7)		Binge (n=8)	
	Unstressed	**Stressed**	**Unstressed**	**Stressed**
Baseline (0min)[a]	233 ± 64	177 ± 41	214 ± 31	279 ± 44
Post-stress (60min)[b]	249 ± 43	717 ± 334	229 ± 35	647 ± 220
Post-binge (160min)[c]	241 ± 68	142 ± 20	217 ± 31	185 ± 27
10min intake (kcal)	6.69 ± 1.13	5.13 ± 1.28	23.87 ± 3.63	21.56 ± 3.06

[a]Baseline samples collected immediately prior to the daily 2hr food deprivation period
[b]Post-stress samples collected immediately following 60min of restraint stress or no stress
[c]Post-binge samples collected approximately 30min following the conclusion of the 10min binge period, or approximately 160min after the baseline measurement
Data represent *M* ± SEM

Chapter 3: Acute binge-related cues increase, but chronic binge-like intake of a palatable diet decreases, intracranial self-stimulation thresholds in rats

Abstract

Obesity is associated with reduced striatal dopamine function in both humans and animal models. Overconsumption of palatable foods may impact reward function indirectly by promoting weight gain, but repeated exposure to hedonically valuable foods may also alter reward function directly. Using intracranial self-stimulation to index reward function, we examined whether limited 'binge-like' access to a palatable diet or binge-related cues would alter reward thresholds independently of obesity. Female Wistar rats were first trained to administer lateral hypothalamic stimulation using a discrete-trial rate-independent procedure. Changes in reward thresholds were tracked throughout seven weeks of daily 2-hr food deprivation followed by 10-min access to a palatable sweet fat diet (Binge group) or standard chow (Chow group), with chow available at all other times. Binge rats rapidly escalated their sweet fat diet intake, obtaining ~45% of their total daily calories during the 10-min session by Week 3, but were not significantly heavier than Chow rats by Week 7. In contrast to previous reports of increased thresholds with extended palatable food access, thresholds were reduced in Binge rats relative to Chow controls. Binge-related cues (feeder presence) and priming with a minimal dose (<1% of an average binge) of the 10-min diet, however, increased thresholds selectively in Binge rats. An anorectic dose of the CB1 inverse agonist SR147778 also increased thresholds independent of diet history. These results indicate that binge-like palatable food intake does not

chronically impair reward function, but that binge-related cues may acutely impair reward function in the absence of opportunity to binge.

Introduction

Altered brain reward function has been linked to obesity and excessive intake of palatable food. For example, similar to drug addicts [1, 2], obese humans have reduced D2 receptor availability in the striatum vs. lean controls; [3, 4], with reduced D2 receptor availability correlating with greater body mass index (BMI) among the obese [4]. Accordingly, excessive intake of palatable foods by obese individuals has been hypothesized to compensate for deficient mesolimbic dopamine reward function [5, 6]. Consistent with this "hypofunction" hypothesis, tasting palatable food elicits less activation of the striatum as measured by BOLD signal in obese vs. lean individuals [7], and especially in carriers of the Taq1A polymorphism of the DRD2 gene, which can reduce striatal D2 expression by 40% [8].

Animal studies suggest that the altered reward system function seen in obese individuals may be consequences, rather than or in addition to antecedents, of chronic palatable food intake. Intermittent access to a 25% glucose solution alternated with food restriction (12 hr each/day), reduced D2 receptor binding in the dorsal striatum [9], similar to reviewed findings in obese humans. In rats, intermittent access to 10% sucrose with intervening 12-food deprivation reduced nucleus accumbens (NAc) extracellular dopamine levels following a 36-hr fast [10], and a*d libitum* access to a palatable cafeteria diet lowered both basal extracellular and evoked dopamine release

in the NAc [11]. Consumption of standard chow no longer stimulated NAc dopamine release after 14 weeks of cafeteria diet exposure, whereas the cafeteria diet continued to be effective, suggesting a devaluation of the previously acceptable chow. Perhaps accordingly, intermittent access to a very highly preferred chocolate-flavored, high sucrose diet reduced the reinforcing efficacy and intake of an otherwise palatable, sweetened chow [12].

Further evidence for palatable-food induced changes in reward function comes from intracranial self-stimulation (ICSS) experiments. In this model, rats respond to obtain current delivery from an electrode implanted within a component of brain reward circuitry, most commonly the medial forebrain bundle at the level of the lateral hypothalamus or the ventral tegmental area [13]. The threshold current amplitude that sustains responding is operationalized as an index of brain reward function. Drugs of abuse, including cocaine [14], morphine [15], and ethanol [16] acutely reduce current thresholds, indicating facilitation of reward function. In contrast, extended access to the same drugs of abuse [17-19] increases thresholds upon withdrawal, suggesting impaired brain reward function or hypohedonia in dependent animals [20]. Chronic (40 days), extended access (18-23 hr/day), but not limited access (1 hr/day), to a palatable cafeteria diet also increased ICSS thresholds in rats relative to chow-fed controls [21]. Diet-induced increases in ICSS threshold were accelerated by knockdown of striatal D2 receptor expression and persisted across 2 weeks of diet abstinence with attendant weight loss. Yet, because *ad libitum* cafeteria-diet fed rats

became profoundly obese [21], it remains unclear whether intake of palatable food increased ICSS thresholds independent of diet-induced obesity.

Palatable foods, like drugs of abuse [20], acquire enhanced incentive salience with repeated exposure, reflecting increased motivation to obtain and consume them [22, 23] and exemplified in conditioned responses to food-related cues. For example, in contrast to their blunted response to the taste of palatable food, obese subjects show greater insula activation in anticipation of palatable food receipt [24] and greater insula and striatal activation in response to pictures of high calorie foods [25], as compared to lean subjects. A taste of a craved food can also precipitate binge eating in women with bulimia nervosa [26]. Further, a single 45 mg palatable food pellet can reinstate food-directed lever pressing in rats with a history of palatable food self-administration [27-29], and a palatable food prime (2 g) can also trigger binge-like intake of standard chow in rats with a cyclic history of food restriction alternating and refeeding on palatable foods [30]. Contextual cues of palatable food availability increase standard chow consumption even if no palatable food prime is provided [31]. Yet, while food cues can clearly influence subsequent consummatory behavior, it remains unknown whether palatable-food cues elicit changes in brain reward function as measured by ICSS thresholds.

A possible reconciliation of these data is that the motivation to consume palatable foods may be maintained or enhanced even as the reward experienced in consuming them diminishes [23, 32, 33]. Accordingly, the current study tested the hypotheses in female rats that 1) chronic 10-min daily ‘binge’ access to a sweet fat

diet would increase ICSS "reward" thresholds tonically, and 2) exposure to binge-related cues would increase thresholds acutely. In contrast to previous studies that confounded effects of palatable food intake *per se* with the resulting diet-induced obesity, the present "binge" model permits the comparison of animals with similar body weights but vastly different palatable food consumption.

Finally, we tested the hypothesis that administration of an inverse agonist of the cannabinoid type 1 (CB1) receptor would differentially alter reward thresholds in binge-fed rats vs. chow-fed controls, CB1 receptors subserve the binge-like intake of palatable food [34], and chronic intake of palatable, high-fat food produces neuroadapative changes in CB1 reward-related circuitry [35-37]. By analogy, opioid receptor antagonists differentially increase ICSS thresholds in rodents with a history of chronic opiate self-administration [38].

Methods and Materials

Animals

Adult Wistar rats were group-housed upon arrival in wire-topped plastic cages in a temperature- (22°C) and humidity- (60%) controlled vivarium with a 12:12hr reverse light cycle (lights off at 10:00am). Per our previous studies with the binge model [34] and the greater prevalence of binge eating disorders in women [39], females were used as subjects. While in their home cages, rats were provided with standard rodent chow and water *ad libitum*. Additional diet manipulations are described below. Procedures adhered to the National Institutes of Health Guide for

the Care and Use of Laboratory Animals (NIH Publication number 85-23, revised 1996) and were approved by the Institutional Care and Use Committee of The Scripps Research Institute (PHS assurance number A3194-01).

Surgery

Under isoflurane (2-5%) anesthesia, rats (*n*=44, 196-272 g, *M* = 228 g) were stereotaxically implanted with bipolar stainless steel electrodes (Plastics One) targeting the medial forebrain bundle in thelateral hypothalamus (AP -0.5, ML±1.7 from bregma, DV -9.5 mm from skull, with the incisor bar raised 5.0 mm above interaural zero [40]). Rats were allowed to recover ≥7 days before testing.

Intracranial self-stimulation training

Rats acquired ICSS behavior via a discrete trial, rate-independent procedure [17, 41]. Sessions were conducted in sound-attenuated operant chambers (MED Associates) with a wheel manipulandum on one wall that elicited electrical stimulus pulses. Rats were connected to the apparatus via swivel commutators with bipolar leads that allowed free movement throughout the chamber. A quarter turn of the wheel delivered a current of 500 ms duration and 100 Hz frequency on a fixed-ratio 1(FR1) schedule. The stimulus current amplitude was tittered individually to support responding during training (*M*=146 μA, range=100-220 μA). Intertrial interval (ITI) "timeout" delays were gradually incremented from 1-15 s across training sessions once subjects obtained >50% of rewards at a given delay. Responses during the "timeout" reset the ITI. Subjects that completed training in this manner attained

discrete-trial performance (typically >90% of rewards obtained at the 15 s delay). Thereafter, sessions were switched to a series of four descending and ascending current columns with a step size of 5μA and mean ITI of 15 s. At trial onset, rats received a single noncontingent intracranial current delivery and had 7.5 s to respond for a second stimulus of the same amplitude. Responses during the ITI did not elicit current delivery, and those occurring after the first 2 sec of the "timeout" period reset the ITI. Within each column of descending currents, two successive amplitude steps in which the rat failed to respond for least 2 of 3 contingent stimuli triggered a reversal to a series in the ascending direction. Within columns of ascending current amplitudes, successful responses for at least 2 of the 3 contingent stimuli triggered a reversal to a descending series. Thresholds for each column were defined as the mean amplitude (in μA) of the two steps prior to reversal. Session thresholds were defined as the average of all 4 column reversals. Response latencies were also recorded to distinguish reward-modulating effects from performance effects [42].

Rats underwent at least 2 weeks of column reversal training before calculating a baseline threshold value across the subsequent 5 consecutive sessions. Thresholds at baseline stabilized at 98.7±2.0% across the entire subject pool compared to the previous week, consistent with stable performance.

Induction of binge-like palatable food intake

After attainment of stable ICSS thresholds, binge-like intake of palatable food was induced by a previously described procedure [34]. Rats were transported daily

(M-F) to a test room at dark cycle onset and placed individually into cages with wire-mesh floors to permit measurement of food spillage. Rats were then food-deprived for 2 hr, after which food was provided for a 10-min "binge" session. The 10-min binge food consisted of either standard rodent chow (Chow group: Harlan Teklad LM-485, 3.1 kcal/g, 17% kcal from fat, 58% kcal from carbohydrates, 25% kcal from protein, Harlan, Indianapolis, IN) or a nutritionally-complete sweet fat diet (Binge group: Bio-serv F06190, 4.1 kcal/g, 35% [kcal]fat, 46% carbohydrate, 18% protein, Frenchtown, NJ, ~85% of fat as saturated fat, 67% of carbohydrate as sucrose). Rats strongly prefer the sweet-fat diet to chow (>99% preference ratio when provided daily concurrent access for 1 hr, Fekete and Zorrilla, unpublished observations). Diets were never concurrently available in the present study. After the 10-min binge, rats were removed from the test room and returned to their home cages with standard chow available *ad libitum*. Rats from a given home cage were assigned to the same diet groups to permit measurement of home cage chow intake. Degrees of freedom for analyses of home cage intake were reduced accordingly.

To determine the effects of binge-like palatable diet intake on ICSS "reward" behavior, current thresholds were tracked daily throughout the binge protocol. Mean weekly thresholds were calculated and expressed as a percent of an individual's baseline threshold amplitude. Rats that did not complete the seven week study period due to technical problems (e.g. cap loss, failure to acquire or maintain ICSS responding) were eliminated from all analyses, and n=25 rats completed the study. To determine whether the timing of the ICSS session relative to the 10-min binge

impacted ICSS thresholds (e.g. to account for differences in recent access to or a withdrawal-like state from the palatable food), *n*=9 rats completed their ICSS sessions during the light cycle immediately prior to the daily 2-hr food deprivation period, while the remaining *n*=16 rats completed ICSS sessions within 6 hrs of the 10-min binge.

Estrous cycle synchronization

To control for estrous-cycle related variation, estrous cycles were synchronized by administering (s.c.) two doses of 2 μg each of the potent gonadotropin releasing hormone (GnRH) agonist [D-Trp6, Pro9-NEt]-GnRH [43], generously provided by Dr. Jean Rivier (The Salk Institute, La Jolla, CA), to simulate the GnRH surge at proestrus. Rats received all binge cue and SR147778 treatments during diestrus.

Effects of binge-related cues on ICSS thresholds

To determine the effects of binge-related cues on ICSS thresholds, rats (*n*=13) were food deprived for 2 hr but did not receive a 10-min binge session. Instead, they received an ICSS session at the typical binge session time, and the feeders used for the 10-min binge were placed in one corner of the ICSS chamber. Feeders only contained a "priming" dose of three 45 mg sweet fat diet pellets (<1% of an average binge), however, or the equivalent weight of chow. Thus, the compound binge "prime" included the scheduled time of day, the preceding 2-hr food deprivation and anticipatory holding in the test cage, and the feeder and food prime presentation in the ICSS apparatus. Priming was performed on two separate days during weeks 8 and 9

of binge access, with approximately half of the rats in each diet group completing the priming session on each day. Thresholds after the food prime were compared to the average of those from unprimed ICSS days during the remainder of the week.

Effects of a CB1 receptor antagonist on ICSS thresholds

The CB1 receptor inverse agonist surinabant [29] (SR147778, 5-(4-Bromophenyl)-1- (2,4-dichlorophenyl)- 4-ethyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide), an analog of rimonabant, was generously provided by Sanofi-Aventis (Paris, France) and suspended in an 18:1:1 mixture of 0.9% saline:ethanol:cremophor. To determine whether doses of SR147778 known to differentially suppress binge-like sweet fat diet intake vs. chow intake[34] would also alter ICSS "reward" behavior differentially in Binge vs. Chow rats (n=14 total), SR147778 (3 mg/kg) or vehicle were administered i.p. 20 min prior to the start of the daily ICSS session in counterbalanced order during Weeks 6 and 7 of binge access. Rats then received an additional treatment of 1 mg/kg SR147778 in counterbalanced order after the priming experiment was completed, during weeks 11 and 12 of binge access. Note that treatment days were excluded from the weekly averages used to calculate the effects of binge-like food intake alone on reward thresholds, and carryover effects were not observed on ICSS behavior on the subsequent non-treatment day.

Statistical Analysis

Weekly averages were calculated for 10-min binge intake, 22-hr home cage chow intake between binge sessions, and ICSS "reward" thresholds (as % of baseline)

and analyzed by separate two-way repeated measures ANOVAs with Diet (Chow vs. Binge) as a between-subjects factor and Week as a within-subjects factor. Potential effects of the time of ICSS session (pre- or post-binge) were analyzed by three-way repeated measures ANOVA with Diet and Time as between-subjects factors and Week as a within-subjects factor. Group differences during individual weeks were examined by post hoc tests as described for two-way ANOVA using the Bonferroni method [44] [see http://www.graphpad.com/quickcalcs/posttest1.cfm]. Diet group differences in cumulative energy intake, body weight, and weight gain across seven weeks of binge access were analyzed by Student's *t*-test.

The effects of binge-related "priming" cues on ICSS "reward" thresholds and response latency were analyzed by two-way repeated measures ANOVA with Diet as a between-subjects factor and Priming condition (Primed or Unprimed) as a within-subjects factor. One-sample *t*-tests were performed for each diet group to determine if the percent change in threshold relative to unprimed days differed significantly from 100%.

Effects of SR147778 treatment on ICSS "reward" thresholds and response latency were analyzed by two-way repeated measures ANOVA with Diet as a between-subjects factor and SR treatment as a within-subjects factor. Doses (1 or 3 mg/kg vs. vehicle) were analyzed separately to account for cap loss (n=2 rats) and potential drift in thresholds across the intervening 4-week period, during which the priming test had been performed.

Pearson correlation coefficients were used to analyze potential relationships between ICSS behavior and measures of food intake, body weight, and weight gain.

For ease of visualization, change in ICSS "reward" thresholds and response latency after both the priming experiment and the SR147778 treatment are expressed as percentage of the relevant control condition (unprimed days or vehicle-treated days, respectively).

Results

Escalation of binge-like sweet fat diet intake and concurrent chow hypophagia

Binge rats rapidly escalated their 10-min intake of the sweet fat diet (Figure 3.1a) such that mean 10-min "binge" intake accounted for ~45% of their total daily caloric intake by the third week of access and then stabilized at this plateau (Diet X Week interaction $F(6,138)=18.923$, $p<0.001$; Week effect within Binge group: $F(6,66)=18.701$, $p<0.001$), consistent with previous results [34]. Rats in the Chow group, in contrast, ate at a low but consistent rate (~6-7 kcal) throughout 7 weeks of binge sessions (no Week effect within Chow group: $F(6,72)=1.131$, p=ns). When returned to their home cages, Binge rats underconsumed the standard chow relative to Chow rats (Figure 3.1b, Diet effect: $F(1,10)=36.464$, $p<0.001$), and this relative hypophagia became more pronounced across time (Diet X Week effect: $F(6,60)=2.978$, $p<0.05$) as binge-like intake escalated. The net effect was that cumulative energy intake across seven weeks of binge access did not differ significantly between diet groups (Figure 3.1c, $t(23)=1.357$, p=ns). Final body

weights were also not significantly greater in Binge rats than Chow rats (307±7.1 vs. 298±5.2 g, $t(23)=1.006$, p=ns), although Binge rats did reliably gain slightly more weight than Chow rats across the study period (Figure 3.1c, $t(23)=2.619$, $p<0.05$).

Divergence of ICSS thresholds after exposure to Binge vs. Chow diet schedules

Baseline reward thresholds did not differ between diet groups ($t(23)=-0.184$, p=ns; Chow 68.2±6.7 μA, Binge 66.8±3.1 μA). ICSS reward" thresholds diverged after the onset of diet schedules (Figure 3.1d), with Binge rats trending lower relative to baseline over the course of binge-like sweet fat diet exposure and Chow controls trending upward (Diet X Week interaction: $F(6,138)=4.956$, $p<0.001$; Diet effect $F(1,23)=3.801$, $p=0.06$). Pairwise comparisons showed that Binge rats had significantly lower ICSS reward thresholds (% baseline) than Chow rats during Weeks 5, 6, and 7 of binge access. By Week 7, thresholds were significantly greater than baseline (100%) within Chow rats ($t(12)=2.653$, $p<0.05$) and lower than baseline within Binge ($t(11)=2.349$, $p<0.05$) rats.

The effects of binge-like sweet fat diet access on ICSS “reward” thresholds were independent of those on body weight, because no reliable correlation was observed between changes in ICSS thresholds on the one hand and either final body weight or weight gain on the other during the 7-week period in either diet group (r’s<0.2, p’s>0.6). Changes in ICSS thresholds were also unrelated to the magnitude of binge-like intake during the 10-min session in either diet group (r’s<0.3, p’s>0.4). Baseline ICSS amplitudes also were unrelated to baseline body weight (r’s <0.4,

p's>0.3) in both diet groups. Interestingly, however, baseline ICSS amplitudes were inversely correlated with 10-min intake during the 1st week of binge-like access in Binge rats only (Figure 3.2, r=-0.577, $p<0.05$).

The timing of the ICSS session relative to the daily 10-min binge also did not impact ICSS threshold changes; because rats that completed their ICSS session immediately before the 2-hr deprivation did not show a different pattern of threshold changes across the study period than those that completed it 1-6 hrs after the binge (Time: $F(1,21)=0.188$, Time X Diet: $F(1,21)=2.754$, Time X Diet X Week: $F(6,126)=0.747$, all p's$\geq$0.11). Further, thresholds of rats tested pre-binge on Mondays (after ~70 hr of binge withdrawal), did not differ from those tested post-binge (Time: $F(1,21)=0.021$, Time X Diet: $F(1,21)=1.852$, p's>0.18).

Effects of binge priming cues on ICSS behavior

Following priming exposure to binge-related cues, ICSS thresholds were increased in Binge rats (Figure 3.3a). Although Priming appeared to increase thresholds in both groups ($F(1,11)=17.855$, $p<0.001$)), there was a trend towards a Diet X Priming interaction ($F(1,11)=4.499$, $p=0.057$). Planned comparisons revealed that the percent increase relative to unprimed days was significant only in the Binge group (Binge group: $t(6)=3.931$, $p<0.01$; Chow group: $t(5)=2.052$, $p=0.095$). Latency to respond during successful trials also was increased during Primed sessions (Figure 3.3b, Priming effect: $F(1,11)=13.990$, $p<0.01$), but this was independent of diet history (Diet: $F(1,11)=0.922$; Diet X Priming effect $F(1,11)=0.010$, p's>0.35). Moreover,

increases in response latency and increases in ICSS "reward" threshold were not correlated (r=0.10, p>0.7), indicating that diet-related differences in thresholds were independent of performance deficits.

Effects of CB1 inverse agonism on ICSS behavior

When pretreated systemically with the CB1 inverse agonist SR147778, ICSS "reward" thresholds increased at the 3 mg/kg dose (Figure 3.3c, F(1,12)=20.093, p<0.01), but not at the 1 mg/kg, dose (F(1,10)=3.177, p=0.106), regardless of diet history (no Diet X SR147778 interaction at 1 mg/kg: F(1,10)=0.272; or 3 mg/kg: F(1,12)=1.294, *p's*>0.25). Eliminating the two animals with lost caps from the 3 mg/kg analysis did not abrogate significance (F(1,10)=12.578, p<0.01). SR147778 treatment did not significantly alter the latency to respond during successful trials at either dose (Figure 3.3d, at 1 mg/kg: F(1,10)=0.094; at 3 mg/kg: F(1,12)=2.159, *p's*=ns) regardless of diet history (no Diet X SR147778 interaction at 1 mg/kg: F(1,10)=1.974; at 3 mg/kg: F(1,12)=0.578, *p's*=ns).

Discussion

In this study, we have demonstrated that daily 10-min binge-like sweet fat diet intake alters reward thresholds both chronically – after several weeks of binge access-- and acutely -- in response to food-related cues -- in female rats. In contrast to our initial hypothesis, binge-like sweet fat diet intake alone did not increase reward thresholds, but rather reduced them, in comparison to chow-fed controls. This indicates that limited access to a palatable food, despite promoting rapid, excessive

binge-like intake, does not impair reward function, but under the conditions of the current study may instead facilitate it. Binge-like eating is thus unlikely to be maintained as a compensatory response to overcome a state of chronic reward deficit. Further, baseline ICSS thresholds were inversely correlated with 10-min sweet fat diet intake during the 1st week of binge access, suggesting that rats with greater reward sensitivity escalate binge-like intake more quickly. When 'primed' with a taste of the sweet fat diet (<1% of an average binge) and tested in the presence of binge-related cues, however, this pattern was reversed. Relative to unprimed days, priming increased reward thresholds in Binge rats but not Chow rats. This suggests that the lack of opportunity to binge in the presence of cues that have previously predicted availability of the sweet fat diet may negatively impact reward circuitry.

Cue-elicited threshold increases may reflect a state of frustrative non-reward, in which the presentation of an appetitive cue without subsequent opportunity for reward consumption is hypothesized to induce an aversive state [45]. Support for this perspective comes from human studies using the acoustic startle response, which is typically potentiated in response to negative or aversive stimuli and attenuated by positive stimuli [46, 47]. In food-deprived individuals, exposure to favorite foods attenuates the startle response if participants are informed that they will be allowed to eat these foods later in the experiment [48]. In contrast, if no opportunity to eat is anticipated, food imagery potentiates the startle response [49]. This pattern is similar to that observed in smokers, who when presented with an appetitive cue (a lit cigarette) reported greater negative mood on trials in which they were informed there

would be no opportunity to smoke compared to trials in which smoking was permitted [50]. Binge eaters may be particularly sensitive to food cues. A history of binge eating, independent of food deprivation, enhances acoustic startle responses after viewing images of foods relative to other positive stimuli [49]. Startle responses were elevated most dramatically in binge-eating subjects who reported greater food cravings prior to the test session, further supporting a link between food cravings and a subsequent negative state. Women with BED also have enhanced long latency event-related potentials in response to pictures of high calorie, but not low calorie foods, suggesting that food cues command greater sustained attention from those with BED [51].

Independent of cues, the ability of a 10-min daily sweet fat binge to reduce reward thresholds stands in contrast to a previous report in which limited (1-hr) access to a cafeteria diet failed to significantly alter thresholds [21]. Any trend in the previous report indeed appeared to shift in the opposite direction, towards increased thresholds. Diets used in both studies were energy dense, palatable, and included significant calories from fat and simple sugars. However, the cafeteria diet allowed free choice between foods of varying tastes and macronutrient content (e.g. bacon, cheesecake, chocolate), whereas the sweet fat diet in the current study was presented as the only palatable option. It is possible that the introduction of choice between several palatable foods could have produce disparate effects on reward circuitry. A recent study of the effects of alternating 5 days of standard chow access with 2 days of a single chocolate-flavored high sucrose diet, however, also found no change in

reward thresholds in response to the palatable diet [52]. Subtle differences in diet composition may be critical, as high fat or sweet fat diets appear to produce fewer deleterious effects on anxiety- or withdrawal-like symptoms than sugar-based diets [34, 53].

One additional feature that differentiates the current study from both prior limited access studies is the use of female instead of male rats. Although current rates of obesity do not differ in men and women (35-36% in both sexes as of 2010 estimates [54]), lifetime prevalence of bulimia nervosa and binge eating disorder is higher in women than in men (1.5% and 3.5%, respectively, for women and 0.5% and 2.0% in men [39]; although some authors have challenged the clinical underrepresentation of men [55, 56]). Effects of binge-like food intake and food cues on reward function in females, if different from effects in males, will thus be critical for developing effective interventions for uncontrollable food intake in humans. Notably, in a comparison of men and women with BED, women reported eating more in response to negative emotional states of anxiety, anger/frustration, and depression than men [57]. Including females in reward function studies may introduce additional estrous-cycle related variability, but this additional variability was controlled for during the priming tests by synchronizing cycles with a GnRH agonist and testing all animals during diestrus. During binge training, animals were permitted to cycle naturally, but food intake and threshold levels were averaged across weekly intervals. Daily variations in food intake or ICSS thresholds due to estrus cycle phase were thus unlikely to differentially impact diet groups.

It should be noted that reward thresholds can be altered by food deprivation and satiety. Food deprivation or reductions in body weight can increase response rates, particularly at currents near the threshold value [58, 59]. In contrast, overfeeding via intragastric delivery of a liquid diet reduces response rates [60]. With sufficient overfeeding and obesity, lateral hypothalamic stimulation may even become aversive, seen as increased responses to terminate stimulation in acutely overfed or obese rats [61]. Differences in satiety level between Binge and Chow rats are unlikely to explain the current results, however. Most importantly, timing of the ICSS session relative to the daily binge did not appear to alter thresholds on its own or in combination with diet history. If satiation were a factor, Binge rats would have been expected to differ when satiated post-binge (1-6 hrs and within the dark cycle) vs. after comparatively low levels of chow intake during the light cycle pre-binge (immediately prior to the food deprivation period at dark onset). Further, changes in reward thresholds after binge-like sweet fat diet access were independent of obesity, as body weights did not differ between Binge and Chow groups.

Our results also demonstrated that the CB1 receptor inverse agonist surinabant increased reward thresholds acutely independent of diet history. Previous studies examining the effects of cannabinoid drugs on ICSS responding have been equivocal. Some authors report an increase in reward thresholds or reduced response rates following CB1 agonists [62, 63], inhibitors of endocannabinoid hydrolysis [64] and reuptake [65] inverse agonists; other studies have found no effect of CB1 agonists on self-stimulation [66]. CB1 inverse agonists have also been observed to increase

thresholds [66-68], but have also repeatedly failed to increase thresholds [62-65, 68]. In the current study, the higher 3 mg/kg dose of SR147778 significantly increased thresholds in both diet groups, while the lower dose of 1 mg/kg had no significant effect. Both of these doses are sufficient to reduce 10-min intake in both Chow and Binge rats, but anorectic effects within this dose range are more pronounced in Chow rats, reflecting a possible resistance of binge-like sweet fat diet intake to modulation by CB1 inverse agonists [34]. In combination with the current study, these results demonstrate that CB1 inverse agonists are capable of reducing food intake at doses that do not impair reward function. Because cannabinoid drugs interact with reward circuitry to modulate responses to palatable foods [69-72], one might have expected threshold increases and intake-suppressing actions to be linked. If these two actions are indeed dissociable, then this further suggests that distinct neuronal populations mediate these effects. A growing body of work has implicated peripheral CB1 receptors in mediating anorexia associated with CB1 receptor blockade [73-76]; receptors in the periphery might thus impact consummatory behaviors without impacting reward function.

In summary, limited access to a sweet fat diet that promotes pronounced binge-like intake also results in a reduction in reward thresholds relative to chow-fed controls. These effects are reversed in the presence of binge-related cues, with sweet-fat bingeing rats but not chow-fed controls showing elevated thresholds. A CB1 receptor inverse agonist previously shown to reduce binge-like intake also increases thresholds, but the minimum dose required to increase thresholds exceeded the

minimum dose required to reduce intake. Binge-like food intake is thus likely to be maintained not as an attempt to overcome a chronic state of reward deficit, but in response to increased incentive salience of food-related cues.

Acknowledgements

Chapter 3, in full, is currently in preparation for submission for publication as the manuscript Parylak SL, Zorrilla EP. Acute binge-related cues increase, but chronic binge-like intake of a palatable diet decreases, intracranial self-stimulation thresholds in rats. The dissertation author was the primary investigator and author of this paper.

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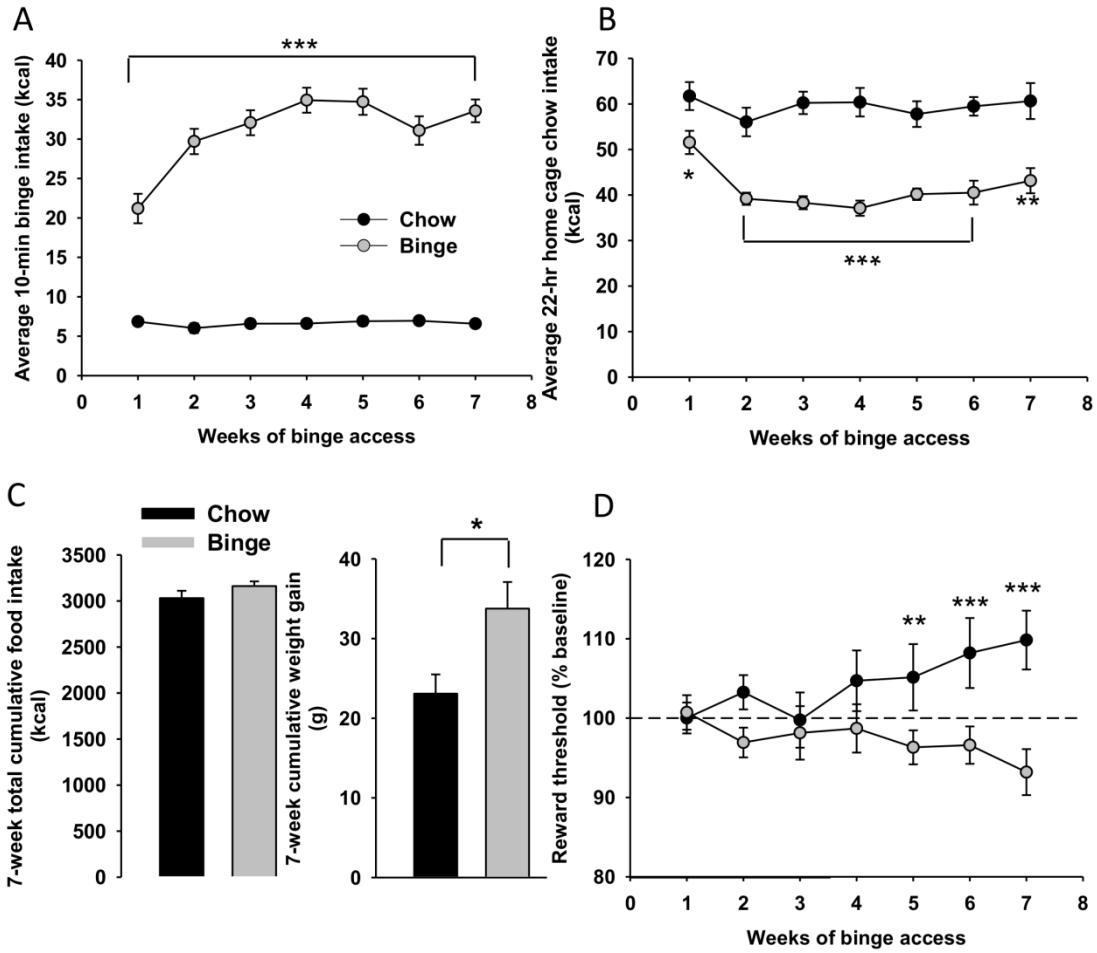

Figure 3.1: ICSS thresholds are reduced in rats with 10-min daily 'binge-like' access to a sweet fat diet. A) Binge-like intake developed rapidly in rats provided daily 10-min access to a palatable, sweet fat diet (Binge group) following 2-hr of food deprivation and plateaus at ~45% of total daily caloric intake. Rats refeeding on standard chow during the 10-min session (Chow group) ate at a dramatically reduced and stable level across weeks. B) When allowed *ad libitum* standard chow access between binge sessions, Binge rats reduced their consumption of standard chow relative to Chow rats. C) Binge rats gained more weight over seven weeks of binge access despite no difference in total caloric intake across the study period. D) Compared to pre-binge baseline levels, reward thresholds decreased in Binge relative to Chow rats. Values in panels represent means ± S.E.M. *differs from Chow $p<0.05$, **$p<0.01$, ***$p<0.001$.

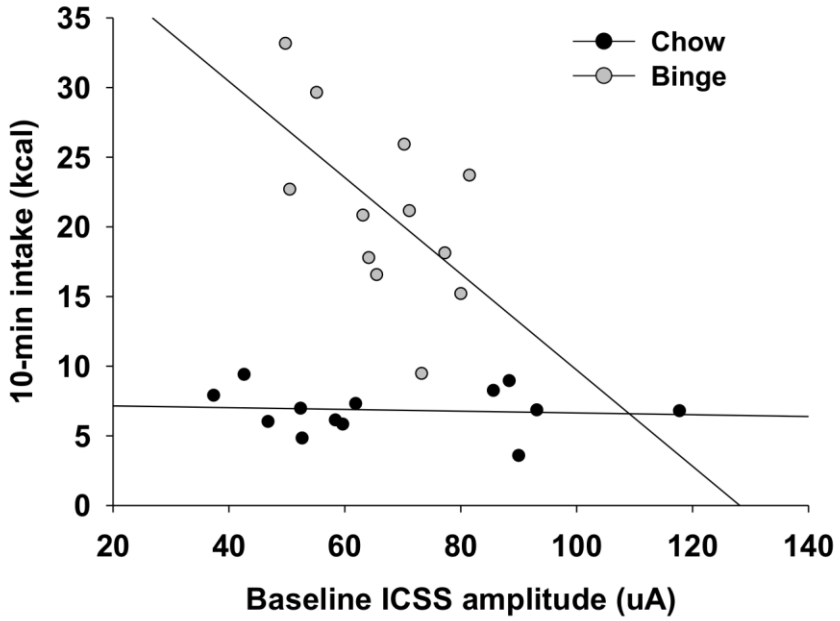

Figure 3.2: Baseline ICSS thresholds are inversely correlated with 10-min intake during the first week of access to the sweet fat diet in Binge rats ($r=-0.577$, $p<0.05$). No correlation was observed between 10-min intake and ICSS baseline amplitude in Chow rats.

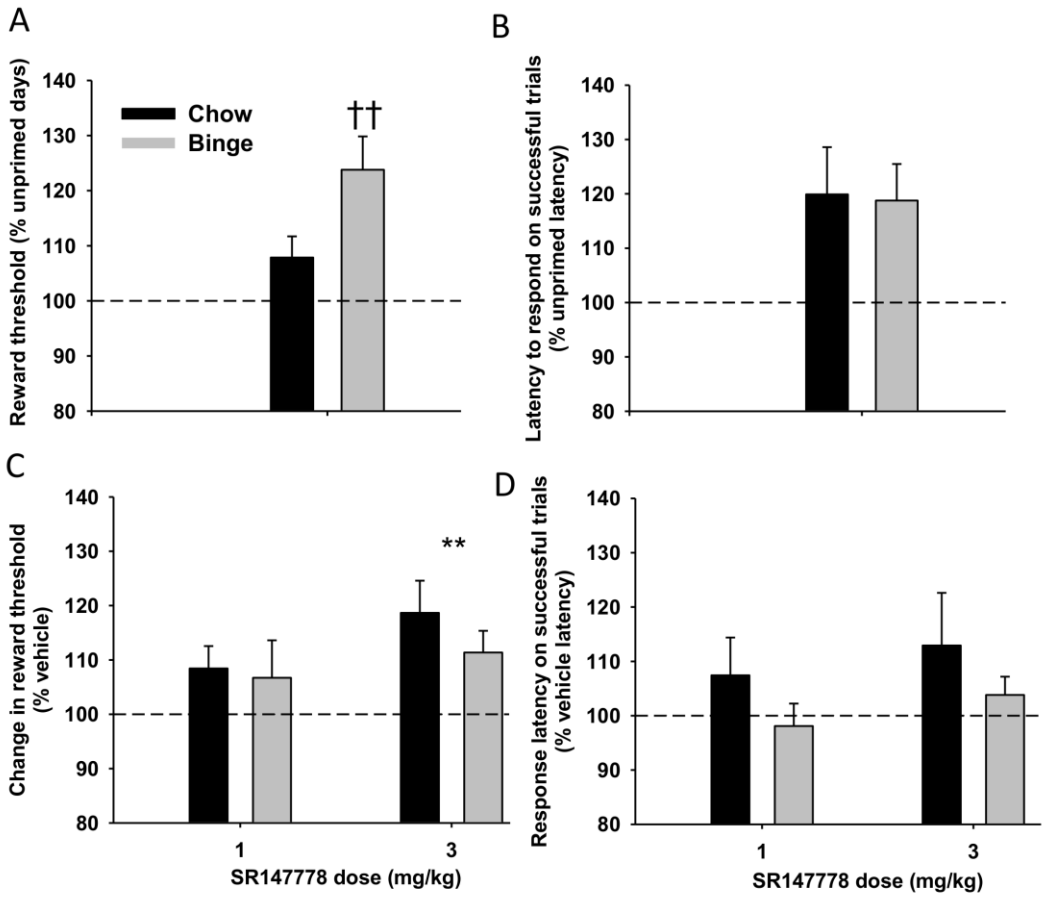

Figure 3.3: Effects of binge 'priming' and CB1 inverse agonist administration on reward thresholds. A) When rats were "primed" with a minimal dose of their 10-min binge food (three 45 mg sweet fat diet pellets or the equivalent weight of chow, <1% of an average binge) and provided with an otherwise empty feeder throughout the ICSS session, reward thresholds increased preferentially in Binge rats. B) Latency to respond on successful trials increased in both diet groups but was uncorrelated with threshold increases (see Results section). C) A dose of the CB1 inverse agonist SR147778 with known anorectic effects increased thresholds in both diet groups. D) Latency to respond on successful trials was unaffected by SR147778 treatment. Values in panels represent means ± S.E.M. ††differs from unprimed days $p<0.01$; **differs from vehicle-treated days $p<0.01$.

Chapter 4: Joint blockade of peripheral and central CB1-mediated signals is required to suppress binge-like, but not ad libitum, palatable food intake in rats

Abstract

Cannabinoid type 1 (CB1) receptor antagonists and inverse agonists have anorectic and weight-reducing properties, but centrally-mediated psychiatric side effects prompted the clinical withdrawal of rimonabant, a brain-penetrant CB1 ligand. CB1 antagonist anorexia was initially assumed to be centrally mediated, but recently-developed "peripherally-restricted" CB1 antagonists also reduce food intake in rodents. Whether such effects are entirely independent of central involvement and CB1 specific is uncertain, however. The influence of diet properties or access conditions on recruitment of peripheral vs. central CB1 signals also remains unclear. Here, we tested the hypothesis that peripheral and central CB1-mediated signals independently control food intake under both *ad libitum* and limited access conditions. JD5006, a novel CB1 inverse agonist, was shown to be peripherally selective and was administered alone or in combination with centrally-infused hemopressin, a CB1 inverse agonist, to female rodents. JD5006 alone reduced intake of both chow and a palatable, sweet fat diet in *ad libitum*-fed rats and mice, effects that were absent in CB1 knockout mice. Combining peripheral JD5006 and central hemopressin infusion had additive effects, reducing chow intake acutely (1-2 hr). However, when motivation to consume the sweet fat diet was enhanced by providing it exclusively during a daily 10-min 'binge,' JD5006 was insufficient to suppress intake. Reducing 'binge' intake required joint antagonism of peripheral and central CB1 receptors. The

results indicate that peripheral and central CB1 signals interact synergistically to modulate binge-like palatable food intake and suggest that central endocannabinoid circuitry is engaged differentially when a food's incentive value is high.

Introduction

Cannabinoids are powerful modulators of appetite. In rats, agonists of the cannabinoid type 1 (CB1) receptor, such as the endogenous ligand anandamide [1] and delta-9-tetrahydrocannabinol (THC), the principal psychoactive component of marijuana (*Cannabis sativa*) [2], stimulate feeding when administered systemically at low doses (high doses, which elicit sedative and cataleptic effects, become anorectic [e.g. THC [3]]). In humans, smoking marijuana also increases food intake [4, 5], as can systemic administration of low doses of THC or the synthetic cannabinoid dronabinol [6-10]. Inversely, CB1 receptor antagonists, such as AM4113, and inverse agonists, such as SR141716A (rimonabant), SR147778, and AM251, suppress food intake and reduce body weight in rodents [11-15], non-human primates [16], and humans [17]. CB1 systems were pursued as a therapeutic target for human obesity [18], in part because CB1 antagonists robustly suppress intake of palatable, energy-dense foods [19]. Similarly, mice with genetic knockout of the CB1 receptor are leaner than wildtype littermates not only when fed standard chow, but especially when fed a highly palatable, high-fat diet *ad libitum* [20] -- a situation putatively relevant to the etiology of human obesity.

Until recently, the appetite-modulating effects of CB1 ligands were often attributed solely to brain CB1 receptors [21-23]. Indeed, the expression of the CB1

receptor in hypothalamic, brainstem, and striatal regions [24-26], positions it to impact feeding behavior via the integration of circuits that subserve energy homeostasis and hedonic behavior. CB1 agonists elicit hyperphagia when administered locally to numerous brain nuclei, including the ventromedial hypothalamus [27], paraventricular hypothalamus [28], parabrachial nucleus [29], and the nucleus accumbens [30]. However, while central pretreatment with CB1 antagonists or inverse agonists consistently prevents the hyperphagia induced by centrally applied CB1 agonists, intrinsic anorectic effects of CB1 antagonists are less reliably seen. Whereas some studies found that intracerebroventricular (i.c.v.) infusion of CB1 antagonists reduced chow intake [31-33], others found no such anorectic effects [34, 35]. Such inconsistent findings contrast with the reliable reduction of intake seen following systemic administration of the same brain-penetrant CB1 ligands [11, 14, 15].

Due to the unacceptable psychiatric liability of central CB1 receptor blockade in the clinic as exemplified by rimonabant [17, 36, 37], attention has shifted to the functional significance of peripheral CB1 receptors. CB1 receptors are also found in adipose tissue, the gastrointestinal tract, liver, pancreas, and skeletal muscle [38]. Accordingly, "peripherally-selective" CB1 antagonists have been sought, including small molecules that are expelled by P-glycoprotein from the CNS or that were designed not to permeate the blood-brain barrier readily [39]. Compounds with these properties have been reported to still suppress food intake and reduce body weight acutely in chow-fed rats [40] and mice [41, 42] and in high-fat diet induced obese mice [39, 42] as well as following repeated daily administration (1-3 weeks) in obese

Zucker rats [40], *ob/ob* mice [41], and high-fat diet induced obese mice [39, 43]. The degree of CNS involvement of these novel antagonists is uncertain, however. Some of these putatively selective and "peripherally-restricted" CB1 antagonists have non-CB1 effects [URB447 [41]; LH-21 [44]] or maintain greater, pharmacologically-significant brain penetration than originally believed [LH-21 [44]; AM6545 [45]]. Because of the uncertainty of the presence of these compounds in the brain, as determined by more conclusive methods, the functional significance of central vs. peripheral CB1 receptors in mediating the anorectic and weight-reducing effects of CB1 antagonists remains unclear.

Further, most studies of intake reduction following allegedly peripherally-restricted CB1 antagonists have provided *ad libitum* access to chow or a single palatable diet. Such conditions do not resemble the intake of humans with dieting or restrained eating styles, which often involves compulsive binge eating of palatable food after caloric restriction or abstinence from preferred foods. In rodents, alternating periods of palatable food access with periods of food deprivation or access to a less-preferred food models 'binge-like' eating during the limited time when the palatable food is available [46-51]. To our knowledge, only one study has examined the effects of a putatively peripheral CB1 receptor antagonist on binge-like food intake [52]; AM6545, a neutral CB1 antagonist, reduced intake of both high-fat and high-carbohydrate diets in rats receiving limited (30 min/day) access to the palatable diets three times per week. AM6545 is not fully peripherally-restricted however, because it reaches the brain at 15-20% of plasma levels after intraperitoneal (i.p.) dosing [45].

In light of these issues, the present study sought to achieve four objectives. First, we tested the hypothesis that systemic administration of JD5006, a novel, truly peripherally-restricted CB1 inverse agonist, would suppress food intake and body weight in rodents receiving *ad libitum* access to either chow or a highly palatable sweet fat diet. Second, we tested whether JD5006 would also suppress intake in rodents receiving binge-like access to the sweet fat diet. Third, we tested whether joint administration of systemic JD5006 and central hemopressin (a peptide inverse agonist at the CB1 receptor [53]), to block peripheral and central CB1 receptors simultaneously, would produce greater anorectic and weight-reducing effects than blocking either peripheral or central receptors alone. Finally, we tested the hypothesis that the anorectic effects of JD5006 were, in fact, CB1-dependent.

Materials and methods

Animals

Adult Wistar rats (Charles River, Raleigh, NC) were group-housed upon arrival in wire-topped plastic cages in a temperature- (22°C) and humidity- (60%) controlled vivarium with a 12:12hr reverse light cycle with lights off at 1000. Adult CB1 knockout mice (CB1-/-; $Cnr1^{tm1Zim}$/ $Cnr1^{tm1Zim}$) [54] and wildtype littermates (CB1 +/+) on a C57/Bl6J background were singly-housed in plastic cages under a 12:12hr reverse light cycle with lights off at 1400. Mice were offspring of heterozygous pairings in our colony and were descendants of breeding pairs generously provided by Dr. Carl Lupica (NIDA).

Because the studies involved a model of binge eating, which is more prevalent in women [55], female rats and mice were chosen as subjects. Except as described below, animals had access to water and standard rodent chow (Harlan Teklad LM-485, 3.1 kcal/g, 17% kcal fat, 58% kcal carbohydrates, 25% kcal protein, Harlan, Indianapolis, IN) *ad libitum*.

Ethics Statement

All procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication number 85-23, revised 1996) and were approved by the Institutional Care and Use Committee of The Scripps Research Institute (PHS assurance number A3194-01). Efforts were made to minimize pain and distress from surgical procedures via the use of isoflurane anesthesia and postoperative analgesic treatment.

Drugs

The active enantiomeric form of JD5006 was generously provided by Jenrin Discovery (Wilmington, DE). Synthesis was carried out as previously described [56] (precursors) and as in US Patents 8,088,809, 7,666,889, 7,482,470 - Scheme 3 for 3-(4-Chlorophenyl)-*N*'-[(4-chlorophenyl)sulfonyl]-*N*-methylenecarboxamide-4-phenyl-4,5-dihydro-*1H*-pyrazole-1-carboxamidine (JD-5006). This compound functions as an inverse agonist at the CB1 receptor with high affinity (racemic 5006, CB1 receptor affinity, IC_{50} 18 nM, inverse agonist IC_{50} 46 nM (cAMP)) and selectivity (>400x vs. CB2) [57]. Doses of up to 30 mg/kg do not occupy brain CB1 receptors in mice [57].

For all *in vivo* experiments, JD5006 was suspended in a vehicle of 18:1:1 saline:cremophor:ethanol. Because JD5006 was found to be unsuitable for central administration due to its low aqueous solubility, hemopressin (PVNFKFLSH), a peptide inverse agonist at the CB1 receptor [53], was obtained from Cayman Chemical and dissolved in saline for i.c.v. infusions. Drug solutions were prepared fresh on test days.

Estrous cycle synchronization

To control for estrous-cycle related variation, estrous cycles were synchronized by administering (s.c.) two doses of 2 μg each of the potent gonadotropin releasing hormone (GnRH) agonist [D-Trp6, Pro9-NEt]-GnRH [58], generously provided by Dr. Jean Rivier (The Salk Institute, La Jolla, CA), to simulate the GnRH surge at proestrus. Rats then received all anti-CB1 treatments during diestrus.

Surgery

Anesthetized rats (2-5% isoflurane) for experiments 2 and 3 (n=35, body weight range 190-224 g) were secured in a stereotaxic frame and implanted with unilateral 22-gauge guide cannulas targeted just dorsal to the lateral ventricle (AP -0.6, ML ± 2.0 from bregma, DV -3.3 mm from skull, with the incisor bar raised 5.0 mm above interaural zero; [59]). Rats were allowed to recover from surgery for a minimum of 10 days before testing. Drug infusions were delivered through 28-gauge injectors that extended 1.2 mm past the guide cannula (final injection DV of -4.5mm). Cannula placements were validated functionally by a hyperdipsic response (>10 ml

water intake within 30 min) to angiotensin II (1 μg in 0.5x PBS) during the dark cycle. Rats that failed the angiotensin II test (*n*=1 in Experiment 2, 6 in Experiment 3) or whose cannulas became lost or occluded during the study (*n*=3 in Experiment 2, 3 in Experiment 3) were eliminated from analyses.

Experiment 1: Anorectic effects of JD5006 in rats with a history of binge-like palatable diet intake

Diets and induction of 10-min binge-like behavior

To induce binge-like eating, rats (*n*=16) were transported daily (M-F) to a testing room at dark cycle onset and placed individually into test cages with wire-mesh floors to permit monitoring of food spillage. Rats were then food-deprived for 2 hr, after which food was provided for a 10-min "binge" session. The 10-min binge food consisted of either the standard rodent chow already described (Chow group) or a nutritionally-complete sweet fat diet (Binge group: Bio-serv F06190, 4.1 kcal/g, 35% [kcal]fat, 46% carbohydrate, 18% protein, Frenchtown, NJ, ~85% of fat as saturated fat, 67% of carbohydrate as sucrose). Rats strongly prefer the sweet-fat diet to chow (>99% preference ratio when provided daily concurrent access for 1 hr, Fekete and Zorrilla, unpublished observations). Diets were never concurrently presented in the present study. Immediately after the 10-min binge, animals were removed from the testing room and returned to their home cages with standard chow available *ad libitum*. Animals from a given home cage were assigned to the same diet groups to permit monitoring of home cage chow intake within diet groups.

Confirmation of peripheral restriction of JD5006

To confirm peripheral restriction of JD5006 to plasma vs. brain at 30 min and 5 hr post-administration, rats were treated with 10 mg/kg JD5006 i.p. and transcardially perfused 30 min or 5 hr later. Rats were deeply anesthetized with sodium pentobarbital and briefly perfused with phosphate-buffered saline for 5 min to reduce blood in the cerebrovasculature. Brains were removed and homogenized in ice-cold phosphate-buffered saline at a volume of 1 ml/g tissue. Blood was collected via cardiac puncture over 0.5 M pH 8.0 EDTA and centrifuged at 4°C for 10 min at ~1300 *g* to isolate plasma. Whole brain homogenate and plasma were stored at -80°C and sent to Apredica, Inc (Watertown, MA) for quantitation of JD5006 by LC-MS/MS (*n*=3/time point).

Effect of JD5006 on binge-like eating

To determine whether a peripherally-restricted CB1 inverse agonist would suppress binge-like intake of the sweet fat diet, rats were pretreated with JD5006 30 min prior to the 10-min binge session at doses of 0, 0.3, 1, 3, and 10 mg/kg in a within-subject Latin square design. Injections were spaced a minimum of 3 days apart. Intake during the binge session and body weight change overnight were analyzed by repeated measures ANOVA with diet history (Chow or Binge) as a between-subjects factor and dose as a within-subjects factor. To assist with visual presentation of the data and because baseline intake differed ~5-fold between Binge vs. Chow groups under vehicle conditions, 10-min intake after JD5006 treatment is

expressed as the percentage of intake relative to the group mean following vehicle treatment. Linear contrast ANOVAs were used to identify monophasic dose-responsive effects, as defined by a log-linear dose-response function [60]. Drug treatments began after 16 weeks of binge-like access.

Based on the results of the dose-response experiment, two additional, longer pretreatment times were then studied. Rats received vehicle or JD5006 (i.p., 10 mg/kg) either 60 min or 5 hr prior to the 10-min binge session in counterbalanced order. Following the 60 min pretreatment, rats remained singly-housed for 4 hr after the 10-min binge session to test for delayed anorectic effects of JD5006 on home cage chow intake. Intake during the binge session, overnight body weight change, and home cage intake were analyzed by two-way repeated measures ANOVA with diet history (Chow or Binge) as a between-subjects factor and JD5006 treatment (vehicle, 60 min, or 5 hr) as a within-subjects factor.

Experiment 2: Effects of joint peripheral and central CB1 receptor inverse agonism on chow intake

To determine the role of peripheral and central cannabinoid signaling in the control of chow intake, we compared the effects of inverse agonism of peripheral and central CB1 receptors singularly or jointly. Rats with i.c.v. cannulas (n=8) were transported to a testing room at dark onset, placed individually into plastic cages with wire-mesh floors to monitor food spillage, and food deprived for 2 hr. At 30 min before the end of the food deprivation period, rats were treated in a 2 (Central CB1

inverse agonist) X 2 (Peripheral CB1 inverse agonist) design, receiving i.c.v. hemopressin (30 nmol) or vehicle (5 ul saline) immediately followed by i.p. JD5006 (10 mg/kg) or vehicle (2 ml/kg). Each of the 4 conditions was given in a Latin square design, spaced by a minimum of 3 days. Intake of chow was measured 1, 2, and 6 hr after refeeding. Rats were then returned to their housing room overnight. Body weight and overnight chow intake were measured the following morning at dark onset (~22 hr post-drug treatments). To confirm that hemopressin effects were not due to leakage of the peptide from the central nervous system into the periphery, the same dose of hemopressin (30 nmol) or vehicle (2 ml/kg saline) was administered i.p. to the same rats in counterbalanced order, and chow intake was recorded at the same time points. Hemopressin was administered centrally rather than JD5006, because of the latter's inadequate solubility for central infusion in a tolerable vehicle.

Chow intake and body weight change after the combination treatment were analyzed by 2-way repeated measures ANOVA with JD5006 treatment and hemopressin treatment as within-subjects factors. Group differences in the presence of interactions were examined by post hoc tests as described for two-way ANOVA using the Bonferroni method [61] [see http://www.graphpad.com/quickcalcs/posttest1.cfm]. Chow intake and body weight change for the peripheral hemopressin control were analyzed by paired *t*-tests.

Experiment 3: Effects of joint peripheral and central CB1 receptor inverse agonism on binge-like palatable diet intake

To determine the role of peripheral and central cannabinoid signaling in the control of binge-like food intake, we compared the effects of inverse agonism of peripheral and central CB1 receptors singularly or jointly. Female Wistar rats with i.c.v. cannulas (*n*=14) were assigned to Chow or Binge groups and received daily (M-F) 10-min binge sessions for 3 weeks after which drug treatments began. Rats were treated in a 2 (Central CB1 inverse agonist) X 2 (Peripheral CB1 inverse agonist) design, as per Experiment 2, 60 min before the 10-min binge session. Five animals required an extra infusion due to technical problems during their initial treatment. To confirm that effects seen after joint i.c.v. and i.p. treatment were not merely attributable to a greater total dose of inverse agonist, rats, in counterbalanced order, also received dual i.p. injections of JD5006+hemopressin or of vehicle 60 min before the 10-min binge.

Intake and overnight body weight change after the combination treatment were analyzed by 3-way repeated measures ANOVA with JD5006 and hemopressin as separate within-subjects factors and diet history as a between-subjects factor. Intake and overnight body weight change for the dual peripheral treatment were analyzed by two-way repeated measures ANOVA with diet history as a between-subjects factor and drug treatment as a within-subjects factor.

Experiment 4: Effects of JD5006 on *ad libitum* food intake in rats and CB1-/- vs. wildtype mice

To determine whether the reduced effectiveness of JD5006 against binge-like intake of the high-fat diet, as compared to chow, resulted from the diet *per se* (High-fat vs. Chow) or the limited, binge-like diet availability (10 min/day vs. 22+ hr/day), a separate group of adult female Wistar rats (*n*=8) were first acclimated to *ad libitum* sweet fat diet access for a period of 3 days. JD5006 (10mg/kg, i.p.) or vehicle was administered at dark onset in counterbalanced order 4 days apart. Intake of the sweet fat diet was measured at 1, 2, 6, and 24 hr post-injection. Intake and body weight changes were analyzed by paired *t*-tests.

To determine the CB1 receptor dependency of JD5006 suppression of *ad libitum* chow and high-fat diet intake, adult female CB1-/- mice and wildtype littermates were singly housed and fed *ad libitum* chow. Mice were treated with JD5006 (30 mg/kg, i.p) or vehicle at dark onset in counter-balanced order. Food intake was measured at 1, 2, 6, and 24 hr post-injection. The same mice were then acclimated to *ad libitum* access to only the sweet fat diet for 2 weeks after which they received 30 mg/kg JD5006 or vehicle injections in counter-balanced order. Sweet fat diet intake was measured at 1, 2, 6 and 24 hr post injection. Treatments were spaced by 7 days.

Results

Experiment 1 – Delayed anorectic effects of JD5006

Consistent with previous results [62], Binge rats rapidly escalated their intake of the sweet fat diet during the 10-min binge period; within 3 weeks, “binge” intake

plateaued at ~35 kcal and accounted for 45% of daily caloric intake (Figure 4.1a). In contrast, Chow rats maintained a stable, significantly lower rate of intake (~4-6 kcal). When returned to their home cages, Binge rats decreased their consumption of the standard chow by approximately 40% vs. Chow rats (Figure 4.1b). Binge overeating and home cage chow hypophagia persisted at these levels across 19 further weeks of diet exposure (Table 4.1).

JD5006 treatment did not alter 10-min binge intake in Chow or Binge rats (Figure 4.1c) (Dose: $F(4,56)=0.943$, ns; Dose X Diet interaction $F(4,56)=1.796$, ns), In contrast, JD5006 dose-dependently induced significant overnight weight loss (Figure 4.1d, Dose: $F(4,56)=5.646$, $p<0.01$; linear contrast Dose $F(1,14)=7.779$, $p<0.05$), independent of diet condition (Dose X Diet: $F(4,56)=0.650$, ns).

Pharmacokinetic studies confirmed excellent plasma exposure of JD5006 30-min and 5-hr post-administration with negligible blood-brain barrier penetration (<2% of measured plasma values) after treatment with 10 mg/kg i.p (Figure 4.2a), confirming peripheral restriction of the CB1 inverse agonist across the pretreatment intervals used in the present studies.

Because JD5006 reduced body weight while being ineffective against 10-min binge intake, we sought to address the possibility that JD5006 had failed to reduce binge intake because an insufficient pretreatment interval had been used. Following each of 2 longer pretreatment intervals (60 min and 5 hr), JD5006 still failed to reduce binge-like intake of the sweet fat diet (Figure 4.2b). In contrast, JD5006 significantly

suppressed 10-minute chow intake in the Chow group when a 5-hr pretreatment interval was used (Diet X JD5006: $F(2,28)=4.086$, $p<0.05$; JD5006 in Chow only: $F(2,14)=12.760$, $p<0.01$ vs. JD5006 in Binge only: $F(2,14)=0.598$, ns; 5 hr pretreatment differs from both vehicle and 60-min pretreatment $p<0.01$)

To determine whether JD5006 perhaps reduced body weight by suppressing food intake outside the 10-min binge period, home cage chow intake was measured 1, 2, 4 and 22 hr after the binge (or ~2, 3, 5, and 23 hr after the 60-min pretreatment). JD5006 treatment reduced home cage chow intake with delayed onset; cumulative intake was reduced at 4 hr (Figure 4.2c, $F(1,14)=8.439$, $p<0.05$) and 22 hr (Figure 4.2d, $F(1,14)=14.742$, $p<0.01$), but not at 1 or 2 hr (Fs$(1,14)<2.58$, ns). Anorectic effects on home cage chow intake were independent of diet condition (Diet X JD5006: (Fs$(1,14)<2.17$, ns). JD5006 also reduced overnight (22-hr) home cage chow intake following the 5-hr pretreatment interval (Figure 4.2d) ($F(2,10)=13.783$, $p<0.01$), again irrespective of binge condition (JD5006 X Diet: $F(2,10)=0.495$, ns). JD5006 induced overnight weight loss after both 60-min (JD5006: ($F(1,14)=21.282$, $p<0.001$) and 5-hr pretreatment intervals (JD5006: $F(1,14)=9.897$, $p<0.01$, data not shown).

Experiment 2 - Effects of joint peripheral and central CB1 receptor inverse agonism on chow intake

Treatment with JD5006 to block peripheral CB1 receptors and with hemopressin to block central CB1 receptors had additive anorectic effects over 1-2 hrs of refeeding (Figure 4.3a) (2hr: JD5006 $F(1,7)=6.835$, $p<0.05$; hemopressin:

$F(1,7)=7.487$, $p<0.05$; JD5006 X hemopressin interaction $F(1,7)=0.074$, ns) (1 hr: JD5006: $F(1,7)=6.178$, $p<0.05$; hemopressin $F(1,7)=5.509$, $p=0.051$; JD5006 X hemopressin $F(1,7)=0.018$, ns). Post hoc comparisons revealed that only joint treatment with JD5006 and hemopressin summated to differ significantly from vehicle treatment ($p<0.05$ at 1 hr, $p<0.01$ at 2 hr).

At later time points, only peripheral CB1 inverse agonism via JD5006, but not central CB1 inverse agonism via hemopressin, maintained a cumulative anorectic effect. This pattern was apparent by 6 hr post-refeeding (Figure 4.3b) (JD5006 $F(1,7)=21.593$, $p<.01$; hemopressin $F(1,7)=0.226$, ns; JD5006 X hemopressin $F(1,7)=1.510$, ns) and was maintained overnight (24 hr: JD5006 $F(1,7)=24.005$, $p<.01$; hemopressin $F(1,7)=0.296$, ns; JD5006 X hemopressin $F(1,7)=0.653$, ns). Similarly, body weights were reduced overnight by JD5006, but not hemopressin, treatment (Figure 4.3c) (JD5006 $F(1,7)=7.836$, $p<0.05$; hemopressin $F(1,7)=0.597$, p=ns; JD5006 X hemopressin $F(1,7)=0.053$, ns).

Unlike i.c.v. administration, systemic administration of the same dose of hemopressin (Table 4.2) did not reduce intake at 1 hr ($t(6)=-0.566$ ns), 2 hr ($t(6)=0.385$, ns), 6 hr ($t(6)=0.109$, ns), or 24 hr ($t(6)=-0.557$) or reduce body weight overnight ($t(6)=-0.515$, ns), consistent with a central site of action for i.c.v. hemopressin.

<u>Experiment 3 - Effects of joint peripheral and central CB1 receptor inverse agonism on binge-like palatable diet intake</u>

Binge-like intake during the 10-min limited access session was reduced significantly by joint treatment with JD5006 and hemopressin but not by either drug treatment alone (Table 4.3 and Figure 4.3d). To facilitate visualization of effects within each diet condition and because baseline intake differed ~4-fold between Binge vs. Chow groups under vehicle conditions (Table 4.3), Figure 4.3 presents 10-min intake after drug treatments as a percentage of intake of vehicle-treated rats. Consistent with a synergistic effect of inverse agonism of peripheral and central CB1 receptors, analysis of raw kcal intake (Table 4.3) indicated an interaction between the two treatments (JD5006 X hemopressin interaction: $F(1,12)=5.253$, $p<0.05$) in addition to a main effect of hemopressin ($F(1,12)=8.083$, $p<0.05$) and a trend for JD5006 ($F(1,12)=4.11$, $p=0.07$). Only the combined hemopressin and JD5006 treatment reduced intake significantly below vehicle levels under both raw (Table 3, $p<0.01$) and vehicle-normalized analysis (Figure 4.3, $p<0.05$). The three-way interaction of JD5006 X Hemopressin X Diet was not significant ($F(1,12)=1.959$, ns). The anorectic effects of joint JD5006+hemopressin treatment did not result from a greater total dose of inverse agonist, because joint peripheral administration of JD5006 and hemopressin did not reduce 10-min binge intake (Table 4.4, $F(1,14)=0.957$, ns) when analyzed as percentage of intake of vehicle-treated rats. A slight reduction in raw kcal intake was observed in the Binge group only (Table 4.4, Drug X Diet interaction $F(1,14)=8.597$, $p<0.05$, Binge group differs from vehicle $p<0.01$) with no corresponding suppression in the Chow group. The overall mean kcal reduction in intake was ~3-fold greater following combined central + peripheral route of

administration condition than after the dual peripheral administration condition (-5.1±1.8 kcal vs. -1.3± 0.7 kcal; $F(1,12)=6.261$, $p<0.05$), further supporting the synergistic action of joint central and peripheral blockade.

As was seen with chow-fed rats, peripheral CB1 inverse agonist treatment reduced overnight body weight of binge-fed rats (Figure 4.3d, JD5006 $F(1,12)=38.128$, $p<0.001$), in the absence of any additional weight-reducing effect of hemopressin ($F(1,12)=0.202$, ns).

Experiment 4 - Effects of JD5006 on *ad libitum* food intake in rats and CB1-/- vs. wildtype mice

Unlike its lack of efficacy against binge-like intake when administered alone, JD5006 significantly reduced intake of the palatable sweet fat diet in *ad libitum* fed rats, with a similar delay as was seen for chow intake in Experiment 1. As shown in Figure 4.4, JD5006 reduced cumulative 6-hr ($t(7)=5.158$, $p<0.01$), but not 1-hr ($t(7)=0.574$, ns) or 2-hr sweet fat diet intake ($t(7)=1.197$, ns) (Figure 4.4a). JD5006 also induced significant overnight weight loss in sweet-fat diet-fed rats ($t(7)=4.493$, $p<0.01$, Veh 3.0±0.6g, JD5006 -2.1±1.2g) .

Consistent with the hypothesized CB1-mechanism of action, JD5006 significantly reduced 2-hr *ad libitum* chow intake in wildtype mice ($t(14)=2.480$, $p<0.05$), but not CB1(-/-) knockout mice ($t(13)=1.024$, p=ns) (Figure 4.4b) (JD5006 X Genotype interaction: $F(1,27)=6.273$, $p<0.05$). A genotype effect also was seen

whereby CB1 KO mice ate less than their wildtype counterparts at the 2 hr time point ($F(1,27)=9.737$, $p<0.01$).

JD5006 also reduced *ad libitum* intake of the sweet fat diet selectively in wildtype, but not CB1 KO, mice at the 2 hr and 6 hr time points (Figure 4.4c) (JD5006 X Genotype: $F(1,24)=5.034$, $p<0.05$ at 2hr; $F(1,24)=14.346$, $p<0.01$ at 6hr; JD5006 vs. Veh differences in wildtype mice at 2hr, $t(14)=3.830$, $p<0.01$, and 6 hr, $t(14)=5.959$, $p<0.001$, but not in CB1 knockout mice $ts(10)<1.080$, ns).

Discussion

The present study shows that selective inverse agonism of peripheral CB1 receptors alone via the small molecule JD5006 is sufficient to reduce intake of rodents fed chow or a highly palatable sweet fat diet *ad libitum*. Furthermore, blockade of central CB1 receptors via i.c.v. administration of the CB1 inverse agonist hemopressin, also was sufficient to block chow intake, but synergistically interacted with peripheral JD5006. In particular, joint inverse agonism of both central and peripheral CB1 receptors most effectively reduced binge-like intake of the palatable sweet fat diet. Inverse agonism of peripheral CB1 receptors with JD5006 promoted overnight weight loss in all diet conditions, with no incremental effect of blocking central CB1 receptors by hemopressin. The results support the hypotheses that peripheral and central CB1 receptors each modulate food intake and that inverse agonism of peripheral CB1 receptors with the novel ligand JD5006 is sufficient to reduce body weight as well as *ad libitum* intake of highly palatable, sweet-fat food.

The results also indicate, in contrast, that signals from both central and peripheral CB1 receptors may need to be blocked in order to suppress intake effectively when the motivation to consume a palatable food is enhanced by limited access conditions, as may occur with intermittent dieting or binge eating of "forbidden" foods.

The novel small molecule JD5006 was confirmed to be peripherally-restricted in rats, reaching the brain at negligible levels (<2% of plasma) previously shown to be insufficient to occupy CB1 receptors in mice [57], and produced delayed-onset anorexia under *ad libitum* access conditions. JD5006 reduced feeding via a CB1-mediated mechanism, as shown by its lack of effect in CB1(-/-) mice. JD5006 treatment was at least as, if not more, effective at reducing intake of the palatable sweet-fat diet (e.g., 57% reduction in 2-hr intake in wildtype mice) than of the standard chow diet (33%). This finding was unexpected given that most previous studies have ascribed endocannabinoid-system modulation of responses to and intake of palatable food to central CB1 receptors [22, 63-66]. For example, systemic administration of brain-penetrant CB1 antagonists reduced hedonic reactions to sucrose at low doses and produced conditioned gaping and taste aversions at higher doses, actions not shared by the peripherally restricted antagonist AM6545 [45, 67]. Also consistent with a role for brain CB1 receptors, injections of CB1 agonists into the nucleus accumbens increased hedonic 'liking' reactions to a palatable sucrose solution [68] and elicited hyperphagia [30, 69]. Similarly, local microinfusion of 2-AG [29] or of the fatty acid amide hydrolase inhibitor, arachidonoyl serotonin [70], into the parabrachial nucleus increased intake of highly palatable diets, but not chow, via a

CB1 antagonist-reversible mechanism. Further, CNS-specific knockdown (~70%) of CB1 receptor expression attenuated the anorectic effects of the brain-penetrant antagonist rimonabant on high-fat diet intake [64], whereas surgical abrogation of neural routes for feedback to the hindbrain (via subdiaphragmatic vagotomy, vagal deafferentation (SDA), and celiac-superior mesenteric ganglionectomy) did not prevent rimonabant suppression of a highly palatable liquid diet (Ensure: [63]). Here, in apparent contrast, blockade of peripheral CB1 receptors alone was sufficient to reduce *ad libitum* intake of a palatable, high-energy diet and also necessary, but insufficient, to reduce binge-like intake.

Upon closer inspection, the present results are not inconsistent with previous findings, because they show interacting, but distinct, central and peripheral CB1 modulation of appetite. Accordingly, in previous studies, rimonabant still significantly reduced high-fat diet intake in mice with CNS knockdown of CB1 receptors, just not to the same degree as in wildtype mice, potentially consistent with dissociable central vs. peripheral components of CB1 anorexia [64]. The ability to occupy brain CB1 receptors is also uncorrelated with anorectic potency, as a dose of the CB1 inverse agonist SLV319 that occupied only 11% of brain CB1, but not a dose of rimonabant that occupied 48% of brain CB1, reduced intake of a high fat diet in rats [71]. In contrast, surgically ablating neural routes for peripheral CB1 feedback to the CNS was not sufficient to blunt rimonabant anorexia of a palatable Ensure diet, seemingly implicating central CB1 receptors [63]. The present study may reconcile these apparently contradictory findings by demonstrating independent contributions of

central and peripheral CB1 signaling to the control of palatable, high-energy food intake. Concurrent blockade of *both* was needed to reduce intake synergistically and maximally under the most highly motivating conditions: binge-like access.

It remains uncertain what information peripheral CB1 activation is encoding that led to the greater effectiveness of JD5006 to reduce intake of sweet fat diet vs. chow. Dietary fat (and not intake of sucrose or protein solutions), elicits the release of the endocannabinoids 2-AG and anandamide in the proximal portion of the small intestine via descending signals from the vagus nerve in rats [72]. This endocannabinoid release occurs in sham-fed rats where only the orosensory properties of fat are present, and has been proposed to represent a positive feedback mechanism that promotes further intake when fat is detected. Accordingly, local infusion of rimonabant into the duodenum or i.p. injection of the peripherally-restricted CB1 antagonist/CB2 agonist URB447 suppressed sham-feeding of fat [72]. In the current study, the sweet fat diet provided a more concentrated source of dietary fat than the chow diet (1.46 fat kcal/g sweet fat vs. 0.53 fat kcal/g chow; 35% total kcal from fat vs. 17% kcal from fat, respectively), and the disparity in fat content may have produced greater intestinal endocannabinoid release in response to sweet fat consumption. In humans, plasma 2-AG levels are also greater following a meal of highly palatable food than following a meal of matched macronutrient content but rated as less pleasant and producing a lesser urge to eat [73]. Thus, peripheral CB1 receptor inverse agonism may have produced a greater anorectic effect on *ad libitum*

intake of sweet fat than chow diet intake by blocking a greater stimulatory influence of sweet fat diet on intestinal endocannabinoid release.

The mechanisms via which the anorectic effects of JD5006 were more evident 4-6 hr post-treatment are unclear. Because plasma levels of JD5006 were similar at 30 min vs. 5 hr, delayed anorectic effects are unlikely to result from a failure of JD5006 to reach peripheral sites of action by 30 min, though we cannot rule out pharmacokinetics of drug distribution or accumulation in specific tissues. An alternative hypothesis is that peripheral CB1 activation is interacting with post-oral/ingestive signals that accumulate secondary to food intake during the post-treatment interval. Consider, for example, that JD5006 did not reduce 10-min chow intake following a 2-hr pretreatment interval that consisted of a fast, whereas it did reduce 10-min chow intake following a 5-hr pretreatment interval when rats were allowed to feed during the first 3 hr. Another hypothesis is that blockade of peripheral CB1 receptors may initiate a cascade of transcriptional and organizational changes that requires hours to complete, as has been suggested for the adipocyte hormone leptin [74] and the type 2 urocortins [75, 76], each of which also produce delayed-onset anorexia.

Under limited access conditions, peripheral CB1 receptor antagonism by JD5006 was ineffective in reducing binge-like intake even though the exact same palatable sweet fat diet was provided. Because binge-like intake was most attenuated only when both central and peripheral CB1 receptors were blocked, this suggests that additional, central endocannabinoid circuitry is engaged when the incentive value of

the sweet fat diet is increased, for example, here, by limiting its availability. The ability of systemically delivered CB1 agonists to reduce the latency of presatiated rats to consume chow in an open field suggests that cannabinoids can enhance appetitive components of food seeking, and not merely hedonic responses [77]. Accordingly, systemic treatment with CB1 antagonists increased the latency to initiate lever pressing for a sucrose reward [78] and reduced the willingness to work for a chocolate-flavored chow [79] or liquid diet [80] under a progressive ratio reinforcement schedule. It remains to be seen which neural circuits subserve the incentive properties of CB1 activation and how they synergistically interact with peripheral CB1 feedback, because the relevant motivational constructs have been understudied. Overall, however, our results support the hypothesis that distinct central and peripheral endocannabinoid systems differentially subserve appetitive, hedonic, and energy homeostatic aspects of food consumption [21, 81].

It is also important to note that both JD5006 and hemopressin [53] act as inverse agonists at the CB1 receptor. The inverse agonist activity of compounds such as rimonabant has been proposed to underlie some of their aversive effects, such as nausea and emesis [82]. We cannot rule out that there may be a requirement for inverse agonism centrally in order to reduce binge-like palatable food intake. Clarifying this issue would require development of neutral antagonists with solubility parameters that allow for reliable, site-specific infusion into the brain.

The synergistic requirement for joint blockade of peripheral and central CB1 receptors to suppress binge-like intake is consistent with a neuroanatomical integrator

of central and peripheral CB1 signals. One hypothesized candidate for such an integration site is the parabrachial nucleus of the brain stem. As alluded to earlier, the parabrachial nucleus expresses CB1 receptors, and infusion of 2-AG or the FAAH inhibitor arachidonoyl serotonin directly into this nucleus stimulates intake of high-fat high-sucrose chow, pure fat, or pure sucrose, but not a standard chow diet [29]. Major inputs to the parabrachial nucleus arrive via the nucleus of the solitary tract [83], which in turn receives input from cranial nerves carrying gustatory information, visceral feedback from vagal afferents [84], and blood-borne endocrine feedback at circumventricular organs [85]. CB1 receptor expression in the nucleus of the solitary tract is responsive to changes in energy stores: CB1 receptor mRNA increases in response to 6 weeks of *ad libitum* sweet fat access [86]. This increase relative to chow-fed controls was abolished when body weights were used as a covariate, suggesting that obesity, rather than an acute effect of the sweet fat diet, caused the increase. Within vagal afferent fibers themselves at the level of the nodose ganglion, CB1 receptor mRNA expression undergoes robust changes in response to short-term dietary changes. Although CB1 mRNA is expressed at very low levels in sated rats, fasting for 6-48 hrs dramatically increases CB1 expression [87, 88], and refeeding for 2.5 hrs abolishes this increase [88]. This integrative network of brainstem sites, responsive to both gustatory information and metabolic need, presents a promising target for continued investigation.

Acknowledgments

The authors thank Natalie Gill, Gustavo Chavez, Jean Kim, Daniel Le, and Ryan Chen for technical assistance, Dr. Carl Lupica and Dr. Jean Rivier for providing research materials, and Mary Gichuhi for administrative assistance. This is manuscript #21881 from The Scripps Research Institute.

Chapter 4, in full, is a reprint of material as it has been submitted for publication as Parylak SL, McElroy JF, Chorvat R, Zorrilla EP. (submitted). Joint blockade of peripheral and central CB1-mediated signals is required to suppress binge-like, but not ad libitum, palatable food intake in rats. The dissertation author was the primary investigator and author of this paper.

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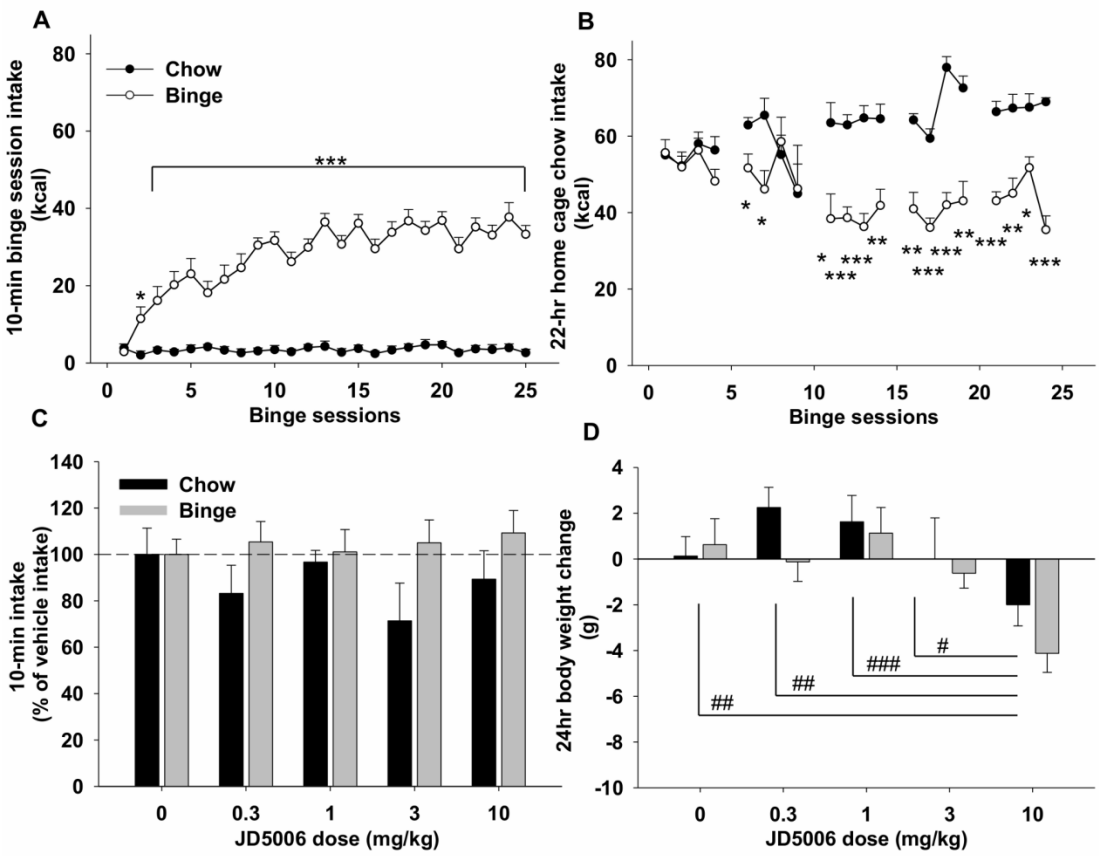

Figure 4.1: The peripherally-selective CB1 inverse agonist JD5006 reduces body weight but not binge-like palatable diet intake. Data are expressed as *M* ± SEM in all figure panels. a) Binge-like intake of a sweet fat diet developed rapidly with daily exposure to a mere 10 min of sweet fat diet access (Binge group) following a 2-hr food deprivation period. Chow controls received standard chow during the 10 min. b) Intake of standard chow in the 22 hr between successive 10-min binge periods is reduced in Binge compared to Chow rats. c) 10-min binge intake of the sweet fat diet was unaffected by pretreatment with the peripherally-restricted CB1 receptor inverse agonist JD5006, in contrast to our previous finding that 10-min binge intake was strongly attenuated by pretreatment with a centrally-penetrant CB1 receptor inverse agonist. d) JD5006 treatment resulted in overnight body weight loss despite the lack of effect on 10-min binge-like intake. *Differs from Chow group $p<0.05$, **$p<0.01$, ***$p<0.001$; #differs from vehicle $p<0.05$, ##$p<0.01$, ###$p<0.001$

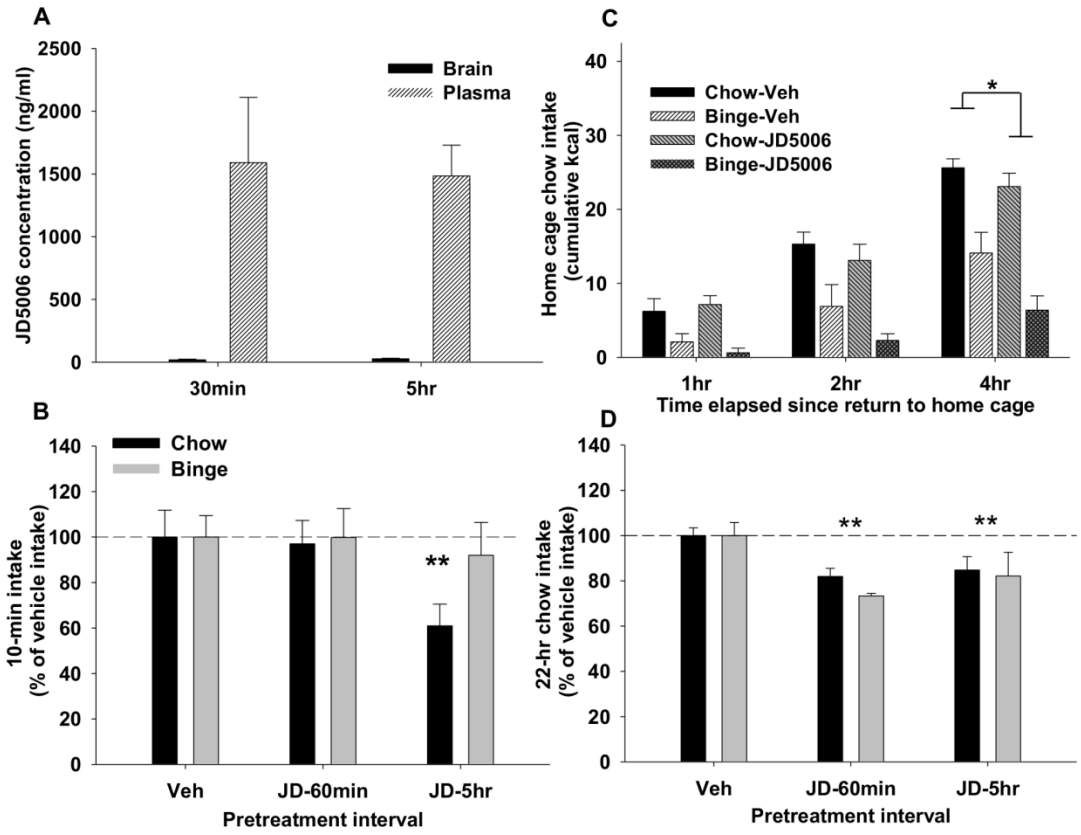

Figure 4.2: JD5006 reduces standard chow intake and remains peripherally restricted when these anorectic effects become apparent. a) Levels of JD5006 in whole brain homogenate remain under 2% of detected plasma levels at 30 min and 5 hr after i.p. administration of 10 mg/kg. b) JD5006 treatment reduced home cage intake of standard chow in rats with a history of binge-like sweet fat diet intake, but only at a delay. c) JD5006 reduced 10 min intake of standard chow, but only at a delay of 5 hr and not 1 hr post injection. d) JD5006 reduced 22 hr intake of standard chow at comparable levels at both pretreatment intervals. *Differs from vehicle treatment $p<0.05$, **$p<0.01$

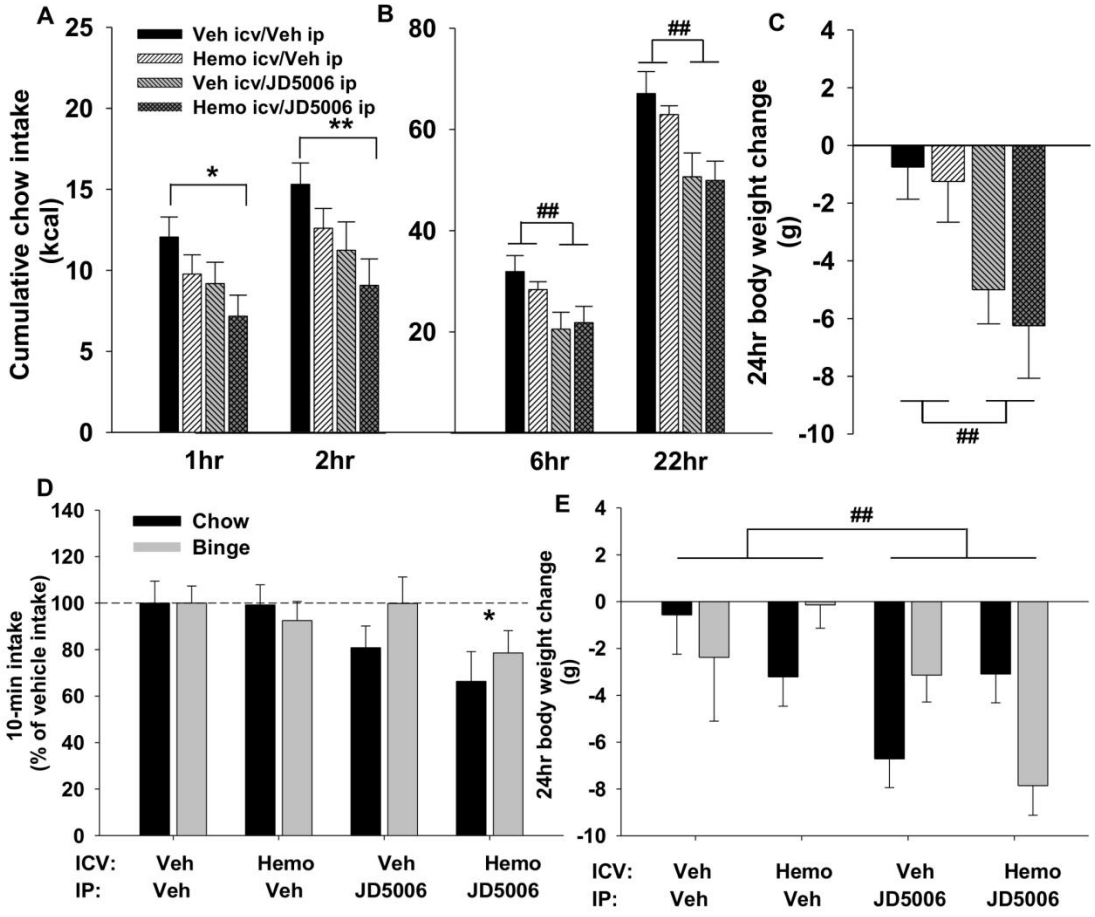

Figure 4.3: Joint peripheral and central CB1 inverse agonism is required to attenuate binge-like sweet fat diet intake. Rats were food deprived for 2 hr and allowed to refeed on standard chow (a-c) or given 10-min access to chow or a sweet-fat diet (d-e). At 30-60 min prior to refeeding, rats received an i.p. injection of a peripherally-restricted CB1 receptor inverse agonist (JD5006, 10 mg/kg) alone, an i.c.v. infusion of a peptide CB1 inverse agonist (hemopressin, 30 nmol) alone, or both drugs in combination. a) Peripheral and central CB1 receptor inverse agonists had additive anorectic effects at 1 hr and 2 hr after refeeding. b) Peripheral CB1 receptor inverse agonist alone was sufficient to reduce chow intake at 6 hr and 22 hr. c) Peripheral CB1 receptor inverse agonist alone also reduced body weight overnight. d) Joint treatment with JD5006 and hemopressin was required to significantly reduce intake during the 10-min binge session. e) JD5006 treatment produced weight loss overnight without any additional effects from hemopressin co-treatment. *Differs from vehicle/vehicle $p<0.05$, **$p<0.01$; ##JD differs from vehicle $p<0.01$.

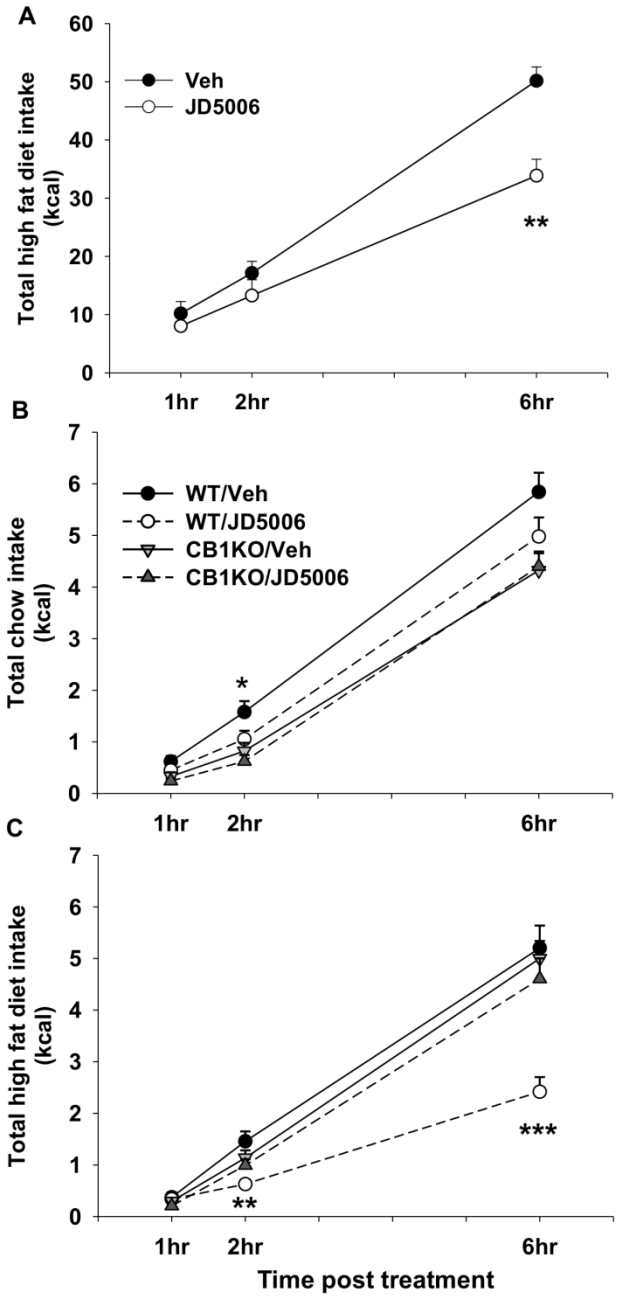

Figure 4.4: JD5006 reduces *ad libitum* sweet-fat diet intake, and these effects are CB1 dependent. a) JD5006 reduces *ad libitum* intake of a sweet fat diet in rats with delayed onset. b,c) JD5006 reduces ad libitum intake of both chow and sweet fat diets in mice but has more pronounced effects on sweet fat diet intake. Sweet-fat intake reduction was specific to wildtype CB1 (+/+) mice and absent in CB1 (-/-) littermates. *WT/Veh differs from WT/JD $p<0.05$, ** $p<0.01$, ***$p<0.001$

Table 4.1: Stable binge-like high-fat diet intake with concurrent home cage chow hypophagia

		Weeks of binge access (average across weeks, kcal)				
Intake measure	**Group**	**4-7**	**8-11**	**12-15**	**16-19**	**20-22**
10-min 'binge'	Chow	3.7±0.5	3.8±0.8	5.7±0.7	6.4±0.6	6.0±0.6
	Binge	34.1±2.0***	36.2±2.2***	37.5±2.9***	36.5±3.3***	35.2±3.7***
22-hr home cage chow	Chow	66.7±1.0	62.4±2.7	66.9±3.5	64.7±2.7	61.6±2.6
	Binge	41.1±2.5***	36.1±2.2***	41.7±2.4**	42.1±1.3***	44.6±1.8**

**Differs from Chow p<0.01, ***p<0.001
Data represent M ± SEM
10-min intake means and errors reflect n=8 rats per diet group
Home cage intake means and errors reflect on n=4 cages of pair-housed rats per diet group.

Table 4.2: A dose of hemopressin that is active centrally does not suppress intake peripherally

	Cumulative chow intake (kcal)				Weight gain (g)
Treatment (i.p.)	**1 hr**	**2 hr**	**6 hr**	**22 hr**	**24 hr**
Vehicle (saline)	11.5±1.1	16.2±1.0	34.9±2.6	68.0±1.5	1.0+0.5
Hemopressin (30 nmol)	12.3±1.0	15.8±1.3	34.7±1.2	70.2±3.3	1.6±0.8

Data represent M ± SEM
n=7/treatment

Table 4.3: Combined JD5006 and hemopressin treatment reduces binge-like intake

Group	**Route**	**10 min binge session intake (kcal)**			
	ICV:	**Vehicle**	**Hemopressin**	**Vehicle**	**Hemopressin**
	IP:	**Vehicle**	**Vehicle**	**JD5006**	**JD5006**
Chow (n=7)		8.25±0.78	8.19±0.71	6.67±0.77	5.47±1.06**
Binge (n=7)		34.08±2.50	31.51±2.80	34.01±3.89	26.76±3.26**

Data represent M ± SEM
JD5006 = 10 mg/kg i.p., hemopressin = 30 nmol i.c.v.
**differs from Vehicle/Vehicle p<0.01

Table 4.4: Dual peripheral administration of hemopressin and JD5006 does not substantially reduce 10-min intake

	10-min binge session intake			
	% of vehicle intake		**Raw kcal**	
Group	**Vehicle + vehicle**	**JD5006 + hemopressin**	**Vehicle +vehicle**	**JD5006 + hemopressin**
Chow (n=8)	100.0±12.5	100.7±13.8	5.95±0.74	5.99±0.82
Binge (n=8)	100.0±11.4	88.2±12.3	30.65±3.50	27.04±3.78**

Data represent M ± SEM
All treatments administered i.p.; JD5006 = 10 mg/kg, hemopressin = 30 nmol
**differs from Vehicle/Vehicle p<0.01

Chapter 5: Conclusions

The studies described in this dissertation sought to achieve three aims: 1) to induce binge-like eating in rodents using putative triggers of human binges, 2) to examine the extent to which binge-like intake produces a withdrawal syndrome or altered reactivity to cues when the preferred food is unavailable, and 3) to determine the role of central and peripheral CB1 receptor mediated signals in regulating binge-like intake. Major findings corresponding to these aims and their implications for future work are discussed below.

In combination with mild food deprivation, limiting access to a highly-preferred sweet fat diet to a mere 10 min per day elicited rapid, intense binge-like consumption in rats during the limited access period. Rats in the Binge diet condition obtained ~45% of their total daily caloric intake during the 10 min of sweet fat access. Binge-like intake consistently produced greater weight gain and increased weight gain per kcal consumed in Binge rats relative to chow-fed controls. As reported in Chapter 2, excess weight gain stemmed from increases in fat mass rather than the addition of lean muscle mass. The magnitude of excess weight gain in Binge rats was sensitive to modulation by environmental enrichment, with individually-housed animals gaining weight more rapidly and consuming a greater overall caloric excess than their socially-housed counterparts. Although standard chow consumption was consistently reduced in Binge rats relative to Chow controls, this reduction in intake was insufficient to compensate for the daily binge. These data support the inclusion of binge-like intake as a risk factor for the development of obesity in humans and further suggest that

attempts to minimize caloric intake by restricting or "forbidding" access to particular foods, as is common in those attempting to lose weight, may ultimately stimulate excessive intake if access to those foods is renewed.

To examine consequences of binge-like intake beyond the propensity towards increased weight gain, Chapters 2 and 3 sought to establish a connection between food intake and the conceptual framework traditionally applied to intake of addictive drugs. Would repeated, intermittent binge-like eating produce withdrawal-like symptoms akin to the anxiety or anhedonia of drug withdrawal? In contrast to the initial hypothesis, Binge rats did not show evidence of increased anxiety-like behavior, an altered neuroendocrine response to stress, or impaired reward function during the period between binge sessions, up to 24-48 hrs after the most recent binge. On some measures, such as the defensive withdrawal test and intracranial self-stimulation thresholds, a history of binge-like intake even appeared to produce anxiolytic effects and facilitation of reward function. These data strongly argue against the hypothesis that binge-like eating behavior is maintained by negative reinforcement mechanisms to provide relief from a negative affective state achieved during the abstinent period between binges. Instead, the hedonic value of the sweet fat diet may continue to serve as the driving force motivating binge-like intake even after binge-like patterns have been stably established for weeks or months.

Notably, the results also suggest that foods rich in both sugars and fats may differ from foods consisting predominantly of sugars alone in the extent to which they produce a withdrawal-like syndrome. Several studies have demonstrated that limited

access to sugar-based diets elicits opiate-like withdrawal signs and increases in anxiety-like and depressive-like behavior after cessation of access [1-5]. The failure of the current sweet fat diet to do the same, despite not differing in 1-hr preference tests from a high-sucrose chocolate diet that did produce anxiety-like effects [2], indicates that macronutrient content, rather than hedonic value alone, contributes to withdrawal potential. Indeed, the sweet rat diet sustained robust binge-like behavior comparable in magnitude with previous reports of high-sucrose diet binges [2], but pretreatment with a CRF1 receptor antagonist known to exert anxiolytic effects [1] had no impact on the magnitude of subsequent binge-like intake.

Whereas binge-like intake of a sweet fat diet had no measurable withdrawal-like component, it did alter reactivity to binge-related cues in the absence of access to the preferred food. Chapters 2 and 3 investigated the effects of a compound binge "prime" including a minimal dose of the sweet fat diet (<1% of an average binge) and contextual cues associated with sweet fat diet access, including the 2-hr food deprivation period and feeder typically presented with the binge diet, on subsequent behavior. Priming led to an increase in subsequent locomotor activity on the elevated plus maze with no concurrent increase in anxiety-like behavior. This increase is perhaps reflective of anticipatory behavior and appetitive drive activated by binge-related cues. However, the inability to satiate this now-activated appetitive drive may induce a negative state, perhaps akin to frustrative nonreward. In support of this view, priming also led to an acute and selective increase in intracranial self-stimulation "reward" thresholds in Binge, but not Chow rats. Binge-related cues, in addition to

the acute rewarding properties of the sweet fat diet, may be critical for sustaining binge-like eating.

In search of molecular mechanisms with the capacity to regulate binge-like eating, Chapters 2 and 4 described the sensitivity of intake behaviors to modulation by drugs targeting the endocannabinoid system. Although systemic administration of a CB1 inverse agonist reduced 10-min intake of both the sweet fat diet and chow, both potency and efficacy appeared to be reduced in the Binge group. If animals were instead provided with the sweet fat diet in their home cage as well as during the binge, efficacy to reduce 10-min intake increased to a level comparable to that seen in Chow controls. Thus, binge-like intake specifically, rather than intake of the palatable diet, produced resistance to CB1-mediated anorectic effects. These results were surprising in light of previous reports in which CB1 inverse agonists appeared to selectively reduce intake of more palatable food options [6, 7].

The comparison of centrally- and peripherally-acting CB1 inverse agonists in Chapter 4 provided a possible explanation for the resistance of binge-like intake to modification. Blocking peripheral CB1 receptors alone was sufficient to reduce intake of standard chow or the sweet fat diet in rodents with *ad libitum* access. Blocking either peripheral or central CB1 receptors was also sufficient to reduce chow intake after brief (2-hr) food deprivation, and these effects appeared to sum additively. In contrast, selective administration of a CB1 inverse agonist either peripherally or centrally had no effect on binge-like intake of the sweet fat diet. Only via joint blockade of both peripheral and central CB1 receptors was a substantial reduction in

binge magnitude achieved. These observations provide support for the hypothesis that distinct components of the endocannabinoid system regulate different types of food intake. Peripheral CB1 receptors, expressed in areas such as adipose tissue and the gastrointestinal tract [8], may be ideally positioned to modify feedback relating to metabolic need. Brain CB1 receptors, expressed throughout the cortex, hypothalamus, and striatum [9, 10], may be well-suited to modify reward-driven components of food intake. In the case of binge-like intake, in which motivation to consume the palatable diet is enhanced by limited access, engagement of brain reward circuitry may override peripheral signals that would reduce intake under less powerfully motivating conditions.

Several avenues exist for future work to explore the connection between the endocannabinoid system and binge-intake in greater detail. In particular, the current studies do not address which specific components of binge-like intake are responsive to CB1 inverse agonists. Reductions in binge magnitude might result from a change in perceived palatability or hedonic value of the sweet fat diet, reduced salience of the binge diet or binge-related cues, or alterations of post-ingestive feedback. Each of these possibilities has some support in existing literature. Indeed, CB1 agonists enhance hedonic 'liking' reactions to palatable sucrose solutions [11], increase progressive ratio breakpoints for food 'seeking' under operant conditions [12], and stimulate fat intake via feedback from the gastrointestinal tract [13]. The potential of CB1 receptor inverse agonists to shift food preferences in rats with a binge history, block the cue-induced changes in anticipatory behavior or reward thresholds, or alter

circulating signals such as leptin and insulin would all be fertile ground for future study.

Further, this dissertation focused on factors contributing to the maintenance of binge-like behavior once stable patterns of intake had already been established. However, individual differences in binge magnitude were both significant and persistent. It is unclear whether pre-existing differences in the endocannabinoid system might pre-dispose some individuals to faster acquisition of binge-like behavior, a higher plateau of intake, or greater weight gain. Identification of such risk factors will be invaluable for identifying vulnerable individuals and developing targeted therapies to combat binge eating in humans.

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