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Nicole M. Avena *Editor*

Animal Models of Eating Disorders

Second Edition

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Animal Models of Eating Disorders

Second Edition

Edited by

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Foreword

The features of the most common eating disorders are simple to describe and, in practice, not difficult to recognize. Severe calorie restriction and, often, increased physical activity are the hallmark features of anorexia nervosa and are unchanged since the syndrome was first clearly described. Clinicians have also come to understand the syndromes of bulimia nervosa and binge eating disorder, both of which are characterized by the recurrent occurrence of binge eating. These patterns of eating behaviors have been examined objectively in laboratory studies. However, it is often difficult to understand the etiology and biochemistry of how these disturbing behaviors arise, and, once they have become established, why they are often so persistent and difficult to reverse.

In many areas of medicine, it has been possible to examine critical features of illnesses by using laboratory animal models. For example, mechanisms underlying the development of cardiovascular illnesses, cancers, and pulmonary diseases have been well studied in laboratory animals, and such investigations have led to major advances in understanding pathological processes and to the development of treatments. The development of animal models to study psychiatric disorders has been more difficult to employ, as these disorders concomitantly involve cognitive and emotional disturbances that are difficult to measure in animals. However, in recent decades, however, significant progress has been made in probing the neural circuitry of psychiatric disturbances.

The current volume focuses on the use of animal models to better understand facets of eating disorders. Part I focuses on binge eating, the salient feature of both bulimia nervosa and binge eating disorder. The chapters in this part usefully describe a range of methods by which animals can be induced to engage in behavior that resembles the binge eating of individuals with eating disorders. Methods include simply making palatable foods available in the environment, restricting access to such foods, increasing the level of stress, and requiring operant behavior to obtain access to palatable foods.

Part I also explores a long-standing and controversial area in human eating disorders, namely, the significance of the striking parallels between eating disorders and addictions. With impressive frequency and conviction, individuals with binge eating describe their struggles with food in remarkably similar terms to those used by individuals who struggle with drugs of abuse. Chapters in this part describe complementary approaches to examining this issue, including changes in behavior and in dopamine signaling associated with binge eating of sugar, the relationship between saccharin preference and vulnerability to drug abuse, and the persistence of food-seeking despite aversive consequences.

Part II is comprised of chapters describing a range of innovative approaches which may provide insights into the striking behavioral syndrome of anorexia nervosa. Several of the chapters address the circumstances and controls of increased physical activity which, under certain experimental conditions, become so marked that weight loss is life-threatening. Other chapters probe the contributions of genetic factors and neurotransmitters on reduced food intake and the effect of weight loss on the functioning of the reward system. Part II of this volume also focuses on the critical issue of weight loss. There is a chapter in this part that focuses on understanding the effects of weight loss after gastric bypass surgery.

In summary, this volume brings together a range of valuable perspectives on how aspects of animal eating behavior can be manipulated to resemble key features of human eating

disorders, and thereby provide provocative insights into the factors that facilitate the development and persistence of disturbed eating in humans. This valuable research tool will enable others to apply animal models of eating disorders to their research and to better understand the biological significance disordered eating behavior.

Mark S. Gold

Preface

This volume of the series *Neuromethods* provides an in-depth review of preclinical laboratory animal models used in the study of eating disorders. The prevalence of eating disorders in the USA and other developed countries continues to pose a problem, and clinicians continue to struggle treating these disorders of complex etiologies. Many researchers turn to the use of animal models to assist in their investigation and characterization of the behaviors and neurochemical alterations associated with them. As such, animal models have become integral to understanding the biological basis of eating disorders. This volume consists of chapters contributed by experts in the field who are well-versed in the development and implementation of these models.

The study of eating disorders is a burgeoning field. In recent years there have been many new discoveries and theories on their biological bases. The growth of the field has led to a vast array of empirical articles on the study of eating disorders, and the development of new models that can be used to study these disorders continues to stimulate new research. This book serves as a collection of detailed techniques that scientists can follow. Since eating disorders are complex and likely due to a combination of environmental, genetic, and social causes, the following chapters have been designed to highlight different contributing factors. Collectively, these chapters give a comprehensive and representative overview of both recently developed and classic methodologies used in the study of eating disorders.

Gratitude is extended to all of the contributing authors for their hard work and excellent chapters. It is an honor to work with such incredible scientists and scholars. I would like to acknowledge the assistance of Anne Lewandowski, who helped with the formatting and organization of the book. I would also like to thank Wolfgang Walz (Series Editor), Patrick Marton (Executive Editor), and Anna Rakovsky (Assistant Editor) and the rest of the team at Springer for their guidance and interest in this important topic.

New York, NY, USA *Nicole M. Avena*

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Chapter 1

Highly Processed Food and Binge Eating

Ashley N. Gearhardt

Abstract

Binge eating is an extreme form of loss of control over the consumption of food. The foods consumed in a binge are typically highly processed (HP) foods with unnaturally high levels of refined carbohydrates and/or fat (e.g., chocolate, pizza, cookies). Animal models play a key role in identifying the underlying mechanisms implicated in binge eating. Dysfunction in systems associated with reward and craving, impulsivity, stress, and negative affect has been identified as key contributors to binge eating in animal models, which converges with human research. In animal models, intermittent patterns of access appear particularly likely to trigger binge eating. This also mirrors the intermittent pattern of binge-restrict consumption in humans. Based on existing animal and human research, interventions that target reward-related dysfunction, inhibitory control difficulties, and the management of stress and negative affect are of key importance. Further, understanding how properties of HP foods (or ingredients in these foods like sugar) impact reward, inhibitory control, and stress systems in a manner that drives forward binge eating is a particularly important area of future study. Animal models will continue to play an important role in highlighting novel intervention targets and inform our understanding of binge eating in humans.

Key words Binge eating, Bulimia, Processed food, Reward, Impulsivity, Stress, Intermittency

1 Introduction

The modern food environment has changed drastically in the last 50 years. Highly processed (HP) foods with unnaturally high levels of refined carbohydrates and/or fat (e.g., chocolate, pizza, cookies) have become increasingly cheap, accessible, and marketed. As the food environment has shifted, disorders associated with excess consumption of HP foods have rapidly increased, including obesity, diabetes, and heart disease [1]. Excess consumption of HP foods is very common, and attempts to cut down on HP foods typically fail [2, 3]. Binge eating is an extreme form of loss of control over the consumption of HP foods. Objective binge eating episodes (OBEs) are marked by consumption of an objectively large amount of food in a discrete period of time that is also accompanied by a subjective sense of losing control [4]. The presence of frequent OBEs is a core indicator of both bulimia nervosa (BN, where the OBEs are

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accompanied by purging behavior) and binge eating disorder (BED, where the OBEs are *not* accompanied by purging behavior) [4]. In these disorders, OBEs occur despite negative emotional (e.g., shame, guilt) and physical (e.g., excessive weight gain) consequences and continue despite a strong desire to stop [4]. While effective treatments for BED and BN have been developed (particularly cognitive behavioral therapy), only about half of patients respond to these treatments [5, 6]. Thus, there is a significant need to further understand the causes of binge eating to improve treatment effectiveness.

Animal models play a key role in identifying the underlying mechanism implicated in binge eating. The human diet is extremely complex, and the ability in humans to tightly control eating behavior (especially from birth) to understand the causes of binge eating is functionally impossible and unethical. However, animal models provide tightly controlled, rigorous experimental designs, which provide insight into the potential causes of binge eating in humans. In this introduction, I will briefly review core mechanisms implicated in binge eating in humans that parallel findings from animal models: reward and craving, intermittency, impulsivity, stress, and negative affect.

2 Reward and Craving

Reward plays a key role in binge eating tendencies. The desire to experience pleasure, not necessarily to address homeostatic deficits, appears to be important in binge eating [7]. The foods that are most likely to be consumed in a binge are HP foods [8]. Emerging research suggests that HP foods are more effective at activating reward-related neural systems and triggering dopaminergic release than healthier, minimally processed options (e.g., fruits, vegetables) [9, 10]. Thus, the ability of HP foods to trigger higher levels of reward may increase the likelihood of excessive binge consumption. Binge eating (but not body mass index, BMI) is associated with increased striatal dopamine release to food [11]. Individuals who binge eat generally have higher levels of craving for HP foods, and these cravings are frequent triggers for a binge episode [12–14]. The cues that signal the availability of HP foods can become powerful triggers of reward-related systems, and individuals with binge eating exhibit attentional biases for these cues and greater reward-related neural response to food cues [15, 16]. Intermittent access to a rewarding substance can enhance its rewarding properties and sensitize the dopamine system to be more reactive to cues [17]. There is a clear pattern of intermittent consumption in binge eating, where individuals binge on large quantities of HP food and then restrict to counteract the excess caloric intake [4]. This intermittent pattern of HP food consumption may further enhance the

reward responses to HP food and related cues, thus driving forward a compulsive pattern of binge intake.

3 Impulsivity

There are also important individual differences that can increase the risk for binge eating. Elevated levels of impulsivity are associated with both BED and BN [18, 19]. Individuals who binge eat have been found to have difficulty maintaining attention, inhibiting behavioral responses, and delaying gratification of rewards [20]. This is consistent with neuroimaging studies that have found individuals with BED and BN to have greater activation in executive control regions during tasks that require cognitive control, suggesting additional effort may be needed to exert top-down control processes [21, 22]. Thus, an increased tendency toward impulsivity and difficulty with inhibitory control may increase the likelihood that individuals with struggle to inhibit a strong reward drive for HP foods and may be more likely to discount future negative consequences.

4 Stress and Negative Affect

A greater propensity to experience stress and difficulties managing negative affect (e.g., sadness, anxiety, boredom) also appears to be a factor in binge eating. Hormones associated with stress (like cortisol) appear to prime reward-related neural systems in the brain to be more reactive, which may increase the salience of HP foods [23]. The experience of stress also inhibits the effectiveness of executive functioning, which may further diminish the ability to inhibit the desire for HP foods [23]. Negative affect has been identified as a common precursor for binge-eating episodes, and the motivation to use food to cope with negative affect is associated with binge eating [14, 24]. Difficulties regulating negative affect are associated with binge eating, which may further increase the reliance on food to cope [25]. Finally, individuals who have high negative urgency (i.e., more prone to act impulsively in the context of negative affect) are also at elevated risk for binge eating [26].

5 Summary

In sum, the mechanisms implicated in animal models of binge eating converge with those in human studies of binge eating. Given that binge eating is associated with high levels of emotional distress and negative health outcomes [27], it is important to improve current treatment outcomes by developing a better

understanding of the mechanisms that contribute to binge eating. Based on existing animal and human research, interventions that target reward-related dysfunction, inhibitory control difficulties, and the management of stress and negative affect are of key importance. Further, understanding how properties of HP foods (or ingredients in these foods like sugar) impact reward, inhibitory control, and stress systems in a manner that drives forward binge eating is a particularly important area of future study. Animal models will continue to play an important role in highlighting novel intervention targets and inform our understanding of binge eating in humans.

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Chapter 2

The Binge Eating-Prone/Binge Eating-Resistant Animal Model: A Valuable Tool for Examining Neurobiological Underpinnings of Binge Eating

Natasha Fowler, Nicollette Russell, Cheryl L. Sisk, Alexander W. Johnson, and Kelly L. Klump

Abstract

Binge eating is a common form of disordered eating, affecting approximately 8% of Americans and characterizing several eating disorders, including binge-eating disorder, bulimia nervosa, and anorexia nervosa binge-purge subtype. While the etiology of binge eating remains largely unknown, there is increasing evidence to support a role for neurobiological underpinnings of the behavior. Animal models are a valuable tool for examining neurobiology. The binge eating-prone (BEP)/binge eating-resistant (BER) rodent model of binge eating is a tool that can help expand our understanding of the neurobiological risk factors that contribute to binge eating etiology. Following repeated, intermittent exposure to palatable food (PF), rats that are BEP versus BER can be identified based on their pattern of under- or overconsumption of PF. BEP/BER phenotypes emerge upon first presentation to a PF (and thus are not learned patterns of behavior) and share striking similarities with the continuum of binge eating that is present in humans. In this chapter, we outline the protocol for the BEP/BER model and describe subsequent iterations of the model that reveal additional parallels between BEP rats and human binge eating, including sex differences in BEP phenotypes, binge eating in the absence of hunger and with intact satiety signals, preference for and hyperphagia of PF when stressed, motivation to obtain and consume PF despite aversive consequences, binge eating development that is independent of obesity, and emergence of BEP phenotype during puberty. These behavioral profiles render the BEP/BER model a useful tool to uncover key neurobiological substrates of binge eating that can assist in the development of more targeted treatments and prevention methods for individuals suffering with binge eating.

Key words Binge eating, Eating disorder, Bulimia nervosa, Binge eating disorder, Obesity, Rat, Sex differences, Puberty

1 Introduction

Binge eating affects approximately 8% of Americans [1, 2] and is a common feature of many eating disorders, including bulimia nervosa (BN), binge-eating disorder (BED), and anorexia nervosa binge-purge subtype (AN-BP) [3]. Binge eating is defined as

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repeated overconsumption of food (e.g., >1000 calories) accompanied by a sense of loss of control (i.e., once an individual starts a binge eating episode, they feel like they cannot stop or control what or how much they consume) [3]. Binge eating is often accompanied by significant psychological distress, including reduced quality of life, a general decrease in psychological functioning, and anxiety and depressive symptoms [4]. Human studies have pointed to a role for psychosocial factors in binge eating development, with increasing evidence supporting a role for biological underpinnings [5–8]. Nevertheless, the neurobiology of binge eating remains largely unknown.

Animal models of binge eating are one means to expand our understanding of neurobiological factors that contribute to the etiology of binge eating. Unlike human studies, animal studies provide us with the unique opportunity to manipulate specific variables (e.g., stress exposure, hormone level, neuronal activity, brain circuitry, genes) and control for extraneous factors (e.g., sociocultural factors) that are unable to be controlled in human studies. Thus, animal models of binge eating are a great tool for advancing our understanding of binge eating development, maintenance, and prognosis.

In this chapter, we focus on the binge eating-prone (BEP) versus binge eating-resistant (BER) rodent model of binge eating. The BEP/BER rodent model was developed following the observation that rats of the same age and sex display innate differences in consumption of intermittently presented, highly palatable food (PF), despite eating relatively similar amounts of standard lab chow (i.e., nutritive food). A key observation in this model is that approximately 10–30% of rats *consistently* eat the highest and ~10–30% of rats *consistently* eat the lowest amount of PF [9–13]. This stable pattern of PF consumption is consistent with the chronic and stable pattern of binge eating that is present in humans. Importantly, binge eating is not expressed in the BEPs until they come into contact with the PF, i.e., BEPs and BERs are indistinguishable from one another in terms of chow intake. Additionally, BEPs do not learn to overeat PF; this behavior is innate and can be observed following the first PF exposure.

The BEP/BER model has numerous parallels with binge eating in humans that validate its use as a preclinical tool. BEP rats exhibit stable patterns of binge eating during a discrete period of time period (i.e., 4 h), and they (1) binge eat on PF only, not on chow; (2) endure high levels of pain (via foot shock) to consume PF even when sated (perhaps indicative of loss of control over eating in human binge eating – *see* [14]); and (3) tend to overeat, not undereat, when stressed. These consumption patterns parallel very closely the binge eating behavior that is present in women with BN and BED. Additionally, binge eating phenotypes do not emerge until mid- to late puberty onset, which mirrors findings

from humans showing that binge eating pathology is rare before puberty and increases substantially in late to postpuberty [15, 16]. Importantly, BEP and BER rats do not differ in body weight or their propensity to develop obesity. This is much like the human condition, where the genetic contribution to binge eating is independent of weight status, and individuals who engage in binge eating display a wide range of body weights [16–18]. Indeed, four subgroups of rats can be identified in this model (i.e., BEP rats with obesity, BEP rats without obesity, obesity without BEP, and control rats) that are similar to different subgroupings of binge-related disorders in humans (i.e., BN/BED with obesity, BN/BED without obesity, obesity without BN/BED, controls) [9]. These subgroups may be useful in genetic and neurobiological studies that aim to distinguish propensity for each of these conditions.

In this chapter, we first describe the BEP/BER rodent model of binge eating to increase its dissemination. Next, we discuss troubleshooting problems with the base model before moving on to describing new advances in the model that can be implemented to yield specific behavioral responses that parallel features of BED, bulimia nervosa, and AN-BP. These advances have helped to identify neurobiological risk factors for binge eating. Lastly, we end with suggestions for future studies using this model to continue expanding our knowledge of the neurobiological underpinnings of binge eating.

2 Materials and Methods

2.1 Animals

The age and sex of the rats used depends on the research question. Most typically, young adult female Sprague-Dawley rats (Envigo, MI) are used to model the higher female prevalence and age of onset of binge eating disorders [1, 19]. Young adult male rats have also been used in this paradigm [11] as have prepubertal and adolescent rats [20, 21]. In order to obtain sufficient sample sizes, the BEP/BER model requires a group size that is at least three times the number of desired rats in the BEP and BER groups. For example, the minimum number of rats needed to obtain $n = 10$ BEPs and $n = 10$ BERs is $n = (10 \times 3)$ or 30 rats. Past studies confirm the reliability of the $n \times 3$ formula because approximately one-third of female rats will be classified as BEPs and one-third will be classified by BER criteria [9, 12, 14, 21, 22]. However, more recent studies have shown percentages closer to 10–30% of rats meeting BEP/BER criteria [10, 11, 13, 23], so group sizes may need to be increased. Ultimately, the number of BEP/BER rats required depends on the complexity of the study design (e.g., hormonal manipulations versus control conditions, stress versus nonstress). More complex study designs may require 20 or more rats per group, especially if interactions (e.g., sex × experimental manipulation) are examined.

Rats have traditionally been single-housed in clear Plexiglas cages (45 × 23 × 21 cm^3) with a wire cage lid prior to testing and during the testing sessions. But there is no reason why animals cannot be pair-housed prior to testing. If rats are pair-housed during testing, they should be transferred to their own individual cage during the testing sessions. Animals should be acclimated before the start of the study to the conditions of the holding room. Temperature should be maintained at 21 ± 2 °C, and the animals should be kept on a 12:12-h dark/light cycle. The onset of the dark phase should coincide with the start of the feeding tests. For example, if feeding tests wish to be conducted at 1200 h, then lights should go off at 1200 h. This allows the feeding tests to capture the initial 1 h of intake following onset of the dark period, when rats typically begin their daily food consumption. Any changes to the light-dark schedule require re-acclimation, just as with any other study.

2.2 Diet

The animals are maintained on ad libitum water and chow (Rodent diet 8640; Harlan Teklad Global Diets, Madison, WI; 3.3 kcal/gm) for the duration of the studies. A PF must be introduced to establish BEP and BER groups. The PF that has been used most consistently is Betty Crocker™ Vanilla Rich and Creamy Frosting (4 kcal/gm). The PF is introduced into the home cage of the rat in a petri dish attached to a wire hook that is hung over the side of the cage. Frosting has worked well in binge eating models [11] and includes the high-fat and high-sugar contents that are typically consumed in binge eating episodes in humans [24, 25]. However, other PFs have successfully been used in feeding tests as well (e.g., Oreos – see more on this below; [9, 12, 21]). One important component of the study is that PF is always provided alongside the standard lab chow. Without simultaneous presentation of chow with the PF, it is not possible to determine whether increased food intake in BEPs is specific to the hedonic properties of PF (a critical parallel feature of human binge eating; [3]) or whether it generalizes to any food, palatable or not.

2.3 Identifying BEP and BER Rats

The rats are identified as BEP/BER following completion of several feeding tests. To avoid effects of neophobia, the rats are exposed to a few grams of PF in their home cage prior to the first feeding test. The rats are then subjected to intermittent PF exposure by alternating feeding test days and nonfeeding test days, typically on a MWF schedule, as feeding tests must be separated by at least one day of nonfeeding test days. A range for the number of feeding tests have been used, but six are sufficient to identify the BEP/BER phenotypes [11].

For adult rats, each feeding test consists of preparing a premeasured amount of PF (e.g., two Oreo cookies, ~29 g; 25–30 g Betty Crocker Vanilla Frosting in a petri dish) and chow pellets

(e.g., 70 g) inside of or hanging from the side of the home cage of the rat just prior to lights out [11]. For prepubertal rats, a lower amount of PF (e.g., ~15 g of Betty Crocker Vanilla Frosting in a petri dish) and chow (~60 g) are initially given, and amounts increase with advancing age until they reach adult PF and chow measures [20, 21]. Prior to PF exposure, the amount of chow accessible from the wire lid should be recorded. Intake of PF and chow is measured at 4 h following PF exposure. It is important to note that because all feeding tests are conducted in the rat's dark phase, consumption measures are obtained under dim red light. Experimenters should not expose rats to bright light at these times to avoid causing shifts in their circadian rhythm, which could affect subsequent feeding tests. When measuring PF and chow consumption, the cage should be checked to account for any spillage of PF or chow. The critical window that allows for measurable differences in PF and chow intake between groups in models of binge eating occurs at the 4-h mark [21, 22, 26–29]. Other studies have measured chow at 1 and 2 h, in addition to 4 h [9], which can help determine when binge eating begins in rats. However, the 4-h intake measurement is the key measurement. For chow, another key window for measurement occurs at 24 h to ensure that total chow intake does not differ between BEPs and BERs [21]. Body weights are not necessary for identification of BEP/BER status; however, recording body weights on a daily basis is critical to examine and control/account for any group differences or changes in body weight over the study period. Body weights should be measured when 24-h chow intake is measured. On nonfeeding test days, there is no presentation of the PF, and chow and body weights are measured at the 2-h time point only. It is important to measure chow and body weight at the same time every day.

2.4 Phenotype Determination: Classifying BEP and BER Rats

Upon completion of the feeding tests, rats will be classified as BEP, BER, or binge eating neutral (BEN). Daily PF tertiles are calculated using the 4-h PF consumption values across the full sample of all rats. This means that for each animal, on each feeding test day, the amount of PF consumed will fall into one of three groups: top, middle, or bottom PF intake group. It is more important to attend to how consistently a rat falls into a particular tertile than the absolute kcal value of PF that they consume. A range of cutoffs based on the number of times that a rat falls in the top (for BEP) or bottom (for BER) tertile have been used to determine binge eating phenotypes.

In the most lenient phenotyping criteria, the Boggiano method of BEP/BER phenotyping, rats are categorized as BEP or BER depending on whether they ate more or less than the median PF intake score for each feeding test day [9]. In the Boggiano method, BEP and BER classifications are determined based on how consistently the rat's PF intake is above or below the median score for

each feeding test day. The 20 most consistently high-PF-intake rats are assigned BEP, and the 20 most consistently low-PF-intake rats are assigned BER. This method yields the highest amount of BEPs and BERs; however, the number of studies using this method of phenotyping is small.

The most commonly used phenotyping method classifies rats whose PF consumption falls in the top tertile on ≥50% of feeding test days and *never* in the bottom tertile will be classified as BEP. Rats whose PF intake falls in the bottom tertile on ≥50% of feeding test days and *never* scores in the top tertile will be classified as BER. All other rats will be classified as BEN. For more stringent classifications, a ≥67% or 83% of feeding test days criterion has been used [11]. Unlike the Boggiano phenotyping method, it is important to remember that in this alternate phenotyping method, BEPs should never fall into the bottom tertile on any feeding test days, and BERs should never fall into the top tertile on any feeding test days. While the Boggiano method does produce larger numbers of BEPs and BERs (because it allows BEPs and BERs to fall into the top or bottom tertile), results are similar between studies that have used both methods.

When phenotyping groups that are expected to have baseline differences in PF consumption (e.g., males versus females, ovariectomized (OVX) versus intact), the tertiles can be examined either within the groups (e.g., within males only or females only) or in an aggregated group (e.g., groups of males and females combined) [11]. It is common for studies to phenotype the animals using both methods to ensure that results are robust.

In order to capture the full spectrum of binge eating behavior and avoid discarding a significant portion of rats from analyses (i.e., BEN rats), a continuous phenotyping approach can be used instead of the categorical BEP versus BER groups. With the continuous phenotyping approach, a continuous "binge eating proneness" variable is calculated doing a count variable of the number of times a rat fell in the highest tertile of PF consumption across all feeding test days (e.g., if a rat fell in the highest tertile on four of the six feeding tests days, their score on the binge eating proneness count variable will be 4) [21]. A similar score can be calculated for a binge eating-resistant count variable.

2.5 Time Required

The feeding tests require at least 2 weeks if the experimenter is interested in using six feeding tests (conducted on MWF) to define BEP and BER status.

2.6 Data Preparation for Analysis

Raw PF and chow intake (in grams) (e.g., [11]) have been used in analyses, as have kcals (e.g., [9]). It is important to note that when comparing groups that are expected to differ in body weight (e.g., males versus females, OVX versus intact), it is typical to standardize the PF and chow data by body weight using the following formula:

intake (g)/body weight $(g)^{2/3}$ [30]. This eliminates any confounds from natural variations in body weight that could unduly influence tertile calculations and BEP/BER phenotyping.

To compare differences in PF and chow intake and body weight between BEPs and BERs, a Student's *t*-test or ANOVA can be used. If there is a need to compare more than two groups, an ANOVA with post hoc tests can be used. Other complex designs have been used, for example, repeated measures and mixed linear models (MLMs), especially when measuring changes over time and/or development (e.g., pubertal development) [21]. It is always useful to include effect sizes since group sample sizes for BEP and BER rats can be small and may lead to difficulty detecting clinically and behaviorally significant medium and even large effect sizes [12].

3 Modifications to the Base Model

3.1 Using Other PF to Identify BEP/BER Rats

Most studies have used either Betty Crocker Vanilla Frosting (General Mills, MN) [12, 81] or Oreo cookies [9, 14, 26] as the PF. Rats who were first identified as BEP/BER with Oreos exhibited the same binge-like patterns on other PFs, e.g., Oreo-flavored pellets (Research Diets, NJ), high-fat pellets (Research Diets, Diet #D12266B), Crisco® (Proctor & Gamble, OH) [9], Froot Loops® (Kellogg, MI), and M&Ms [14]. Therefore, it is possible to identify BEPs and BERs with non-sugar fatty (e.g., Crisco) and nonfat sugary (e.g., Froot Loops) PFs and not just mixed macronutrient PFs like Oreos and frosting. Nevertheless, it is recommended to first test any new PFs to ensure that it can produce stable patterns of binge eating behavior. One PF that has been tested and failed to yield a significant difference between groups is candy corn (Brach's Confections, TN), despite being preferred over chow [9]. This demonstrates that not all PFs may differentiate BEPs from BERs, as it is possible that some PFs are less pleasurable than others. Salty snack foods have not yet been tested as a PF, but they are predicted to dissociate BEPs from BERs given that rats find them rewarding [31].

3.2 Using Other Rat Strains or Species

While the majority of studies have used Sprague-Dawley rats, other rat strains have been examined as well. Hildebrandt and colleagues compared adult female Sprague-Dawley and Wistar rats with adult male Sprague-Dawley rats on binge eating and proportions of BEP/BER phenotypes [32]. As expected, given sex differences in binge eating in humans [33], the female Sprague-Dawley rats ate significantly more PF and were more likely to be classified as BEP compared to their male counterparts. The Wistar female rats, however, exhibited similar PF consumption as the male Sprague-Dawley rats and were more likely to be classified as BER rather

than BEP. This suggests that female Sprague-Dawley rats may be particularly vulnerable to binge eating versus other rat strains. Male Wistar rats have also been used in this model [34]. Mice have not been used in this model, but they do show clear preferences for PF, including food used in the BEP/BER model here with rats [35, 36]. Therefore, it may be possible to use mice if their patterns of PF intake are determined to be stable.

4 Enhancements of the Base Model

4.1 Examining Sex Differences

Binge eating is notoriously a sexually dimorphic behavior, with female-to-male ratios up to 8:1 [3, 33]. In the first study to examine sex differences in binge eating patterns, Klump and colleagues reported that while males are capable of presenting with the BEP phenotype, rates of BEP phenotypes were two to six times higher for female versus male rats, despite both sexes having equal access to the PF [11]. Because females exhibit a stronger preference for PF versus males in this and several other studies [37–39], phenotyping the females and males together may have skewed BEP classifications to the females. To test this, the authors also conducted within-sex phenotypes (i.e., within males only; within females only) and found that sex differences in PF consumption remained even when phenotyping male and female rats within their respective sex separately. Subsequent studies have confirmed these sex differences [32]. Importantly, Klump and colleagues found that the nature of the BEP phenotype in males is similar to that of the females, in that males also binge only on PF and never on chow, and binge eating occurs within a discrete time period (e.g., 4 h) [11].

4.2 Effect of Gonadal Hormones on BEP Phenotypes

The substantial sex differences in binge eating suggested that ovarian hormones may play an important role in binge eating development. Klump and colleagues [12] examined this possibility directly by examining the effects of ovariectomy (OVX, i.e., ovaries removed) on rates of binge eating adult female rats. Results revealed that OVX in adulthood increased PF intake in all rats, as would be expected, given the effects of OVX on food intake [40]. Importantly, however, OVXed BEP rats continued to consume significantly more PF than OVXed BERs [12]. Because BEP/BER status remained stable across ovarian hormone manipulations in adulthood, the authors concluded that other processes may be important for determining BEP/BER group status. Their results suggest that differences in PF consumption between BEPs and BERs cannot be explained by differential sensitivity to the activational effects of estrogen on PF or food consumption in adulthood.

Even though BEP phenotypes are not responsive to OVX in adulthood, that does not mean that they are not responsive at other time points. Given that rates of binge eating increase significantly from pre- to postpuberty [16] and the organizational effects of gonadal hormones on the development of other sex-specific behaviors that present during puberty (e.g., anxiety, sexual receptivity; [41]), puberty may be a critical sensitive period for BEP development. To test this hypothesis, Klump and colleagues followed two samples of female rats from prepuberty to adulthood and found that, much like in humans, the BEP phenotype did not emerge until puberty [12]. Specifically, BEP/BER groups did not differ in their PF intake in prepuberty – they only began to emerge in mid- to late puberty (P39–P58), suggesting that puberty (and potentially ovarian hormones) is a critical developmental process for binge eating onset in female rats. Importantly, and consistent with previous studies [9, 12], BEP/BER rats did not differ in chow intake, suggesting a specific effect of puberty on PF intake rather than food intake in general.

A recent study by the same group replicated and extended these findings [81] by following a sample of intact and prepubertally OVXed rats from prepuberty to adulthood and observing BEP phenotype status in adult female rats. Once again, these investigators found that BEP phenotypes emerged during mid- to late puberty, but importantly they also found a significant effect of prepubertal OVX on adult BEP phenotypes. Rats that underwent OVX in prepuberty displayed significantly increased rates (~2–8 ×) of BEP phenotypes in adulthood as compared to intact rats [81]. These data, together with the findings from the adult OVX study [12], suggest a specific effect of pubertal hormones on adult risk for binge eating. These results also suggest potential mechanistic differences in ovarian hormone influence on binge eating across development [12]. Specifically, it appears that during adulthood, estrogen exerts activational effects on binge eating, i.e., binge eating levels acutely change following removal of estrogen in adulthood, but BEP phenotypes remain stable. In contrast, pubertal estrogen appears to exert long-lasting and organizational effects on binge eating development, i.e., prepubertal estrogen removal induces significantly increased rates of BEP phenotypes in adulthood compared to intact pubertal rats. Overall, results from this study suggest that pubertal estrogen may serve as a protective factor against binge eating in adulthood.

Because males exhibit lower rates of binge eating [3, 33] and testosterone is the predominant male gonadal hormone, testosterone may also have protective organizational effects against binge eating development. To test this hypothesis, Culbert and colleagues examined whether perinatal testosterone exposure decreases risk for BEP status in male and female rats in adulthood [20]. As expected, fewer males were classified as BEP than control females.

Interestingly, females that were perinatally (P0, P1, and P5) treated with testosterone propionate (100 μg of testosterone propionate dissolved in 0.1 ml sesame oil; Sigma-Aldrich, St Louis, MO, USA) showed similarly low rates of BEP classifications as male rats. Testosterone-treated female and male rats did not differ on BEP status at any point from prepuberty to adulthood. These results suggest that testosterone, like estrogen, may also exert protective effects against binge eating in males and females, perhaps by organizing the brain perinatally so that risk for binge eating is not activated (or at least mitigated) during puberty.

5 Examination of Neurobiological Contributors to Binge Eating

5.1 Conducting Tests of Reward/Motivation

Studies have hypothesized alterations in reward processing as a contributing factor to binge eating development [42]. To test this hypothesis using the BEP/BER model, Sinclair and colleagues compared neural activation (assessed via Fos expression) to PF in brain regions associated with reward (e.g., nucleus accumbens, medial prefrontal cortex [mPFC]) in female BEPs and BERs [43]. They found that BEP female rats exhibited significantly higher activity to PF in brain reward regions compared to BERs, even after adjusting for differences in PF intake. Differences in neural response to PF were more robust in the mPFC versus the nucleus accumbens, confirming alterations in reward processing in BEP vs BER. Based on these findings, the authors concluded that BEP status is associated with increased reward response to PF.

Because binge eating predominantly affects females compared to males (up to 8:1 female/male; [3, 33]), subsequent studies by this same group hypothesized that the reward response to PF differs between females and males. To examine this hypothesis, Sinclair and colleagues used the conditioned place preference paradigm[1] to assess sex differences in behavioral aspects of reward response to PF in BEP and BER rats [44]. In this paradigm, rewarding efficacy of the PF was determined by shifts in preference for the rats preferred chamber (typically a dark chamber) to their non-preferred chamber (a light chamber) that was paired with a rewarding stimulus (e.g., PF).

Results revealed that female rats displayed a more robust shift in preference for the non-preferred chamber paired with PF versus males [44]. Based on these results, the authors concluded that increased reward response to PF in females versus males may increase motivation for PF during binges.

Subsequent studies reported that female BEPs exhibit an increased reward response to PF and increased neural activity in

[1] For more details about the conditioned place preference paradigm, *see* [44].

brain reward pathways (e.g., nucleus accumbens core and shell, mPFC areas) compared to female BERs following PF exposure [45]. While this study did not compare brain reward activity between females and males, it does further support altered reward processing to PF as a contributor to binge eating development.

5.2 Effect of Early Versus Chronic Stage Binge Eating

Despite studies suggesting associations between binge eating and hyper-reward responsivity [46], other studies have demonstrated hypo-reward responsivity in binge eating [46]. Similar mixed results have been observed in the addiction literature where acute drug use elicits a hyper-rewarding response; however, after chronic drug use, the rewarding response becomes blunted, and individuals become hyporesponsive to the drug [47, 48]. This change from hyper- to hypo-reward is thought to contribute to increased drug intake following prolonged use to elicit the same rewarding effects as before [49]. Because of this and the similarities observed between addiction and binge eating [42], Hildebrandt and colleagues hypothesized that a similar pattern of reward responsivity may be occurring in BEP rats following chronic exposure to PF. To test this hypothesis, the BEP/BER model was used to categorize adult female rats into BEP versus BER before randomly splitting them into early or chronic stage binge eating groups [45]. Rats in the early binge eating group were exposed to 6 feeding test days, and rats in the chronic binge eating group were exposed to 24 feeding test days.

Results showed that brain reward activity was highest (i.e., increased Fos expression in the nucleus accumbens core and shell) in the early stage BEPs versus early stage BERs and chronic stage BEPs [45]. However, brain reward activity decreased (i.e., decreased Fos expression in the nucleus accumbens core and shell) in the chronic stage BEPs [45]. Chronic stage BEPs and BERs showed similar levels of brain reward activity, confirming that the initial hyper-rewarding response to PF in BEPs becomes blunted with chronic PF exposure and intake [45]. It is important to note that chronic stage binge eating does *not* alter BEP/BER status; instead, it changes the reward response to PF only in the BEP rats. Because of this, the authors concluded that changes in reward response to PF in BEPs may serve as a risk factor early on and then become a maintenance factor for binge eating behavior.

5.3 Effect of Stress on BEP Versus BER Rats

Stress is a strong risk factor for binge eating in humans [49–51] and rats [9]. Studies have hypothesized that the hedonic properties of PF may help ameliorate the stress response and promote binge-like eating following stress exposure [52–54]. But the mechanisms underlying stress-induced binge eating remain poorly understood. To examine potential neurobiological mechanisms of stress-induced binge eating, Calvez and Timofeeva [55] exposed adult

female rats to five sessions of 1-h access to sucrose and then subjected them to three unpredictable foot shock stress sessions.[2]

As expected, stress increased food consumption in BEPs, but not BERs. Of note, only PF consumption increased in the BEPs, and chow consumption remained unchanged post-stress. Additionally, BERs exhibited a decrease in chow consumption post-stress with no change in PF consumption. To better understand why stress increases PF consumption in BEPs, the authors also examined sucrose microlicking structure (measure of hedonic evaluation and motivational properties of PF; [56]) and PF intake in a light-dark box paradigm (measure of compulsivity) in this same study. They found that stress increases the hedonic properties of PF in BEPs, but not BERs, and that the increased hedonic value of PF was associated with increased compulsive-like intake of PF in BEPs, even in aversive environments (i.e., higher sucrose consumption in the light versus dark chamber of a light-dark box).

Inherent sensitivity to stress in BEP rats may help explain the above effects. Subsequent studies from Calvez and colleagues using a similar stress paradigm as described above have explored this hypothesis. Their results show that unlike BERs, who exhibit the expected increase in corticosterone levels to stress, BEPs exhibit a blunted corticosterone response to stress, indicative of a hypoactive stress response [55, 57]. Additionally, BEPs also exhibit decreased corticotropin-releasing factor (CRF) receptor expression in brain regions responsible for terminating the stress response (e.g., paraventricular nucleus of the hypothalamus) and increased CRF receptor expression in brain regions involved in potentiating the stress response (e.g., bed nucleus of the stria terminalis) compared to BERs [57]. These results suggest increased susceptibility to stress in BEPs versus BERs that may potentiate the reinforcing properties of PF to increase binge-like eating in BEPs versus BERs.

5.4 Tests of Impulsivity and Decision-Making in BEP Versus BER Rats

Individuals who engage in binge eating are known to display deficits in higher-order cognitive functioning, including increased impulsivity, impaired decision-making, and inflexible or compulsive behavioral responding [42, 58]. To date, only two studies have used the BEP/BER model to examine differences in higher-order cognitive functioning between BEP and BER rats. The first study used male rats to examine differences in impulsivity using a delay discounting paradigm in which rats chose between two levers, one that rewards one food pellet with little to no delay or another that rewards five food pellets after an increasingly large delay [34]. Of note, this study used the Boggiano phenotyping method to classify their rats as BEP versus BER. The second study compared female rats on their ability to act in a goal-directed manner based on

[2] For more details on the foot shock paradigm, *see* [54].

reductions in outcome value of two different food reinforcers using an instrumental reward devaluation paradigm [13].

As expected, BEPs exhibited increased impulsivity and a need for immediate gratification (i.e., preference for high reward lever when delays to food pellet are short, but shift to low reward lever when delays increase; [34]). The increased impulsivity in BEPs can be observed as early as following a 10-sec delay from lever press to reward delivery. Additionally, BEPs exhibited an insensitivity to act in a goal-oriented manner and thus decrease lever responding for a devalued, sated food reinforcer, demonstrative of an inability for BEPs to flexibly adjust their choices based on outcome value [13]. One potential interpretation of these latter findings is that BEP rats may be more readily predisposed to the establishment of habitual behavior [59], which would be consistent with their persistently high rates of PF intake [13]. Collectively, these results suggest that preexisting impairments in impulse control and goal-oriented decision-making may be present in BEPs that mirror the impairments in higher-order cognitive functioning that we see in individuals who binge eat [42].

5.5 Effect of Pharmacological Manipulation

BEPs demonstrate significant impairments in inhibitory control and decision-making [13, 34]. To examine potential mechanisms underlying these impairments, a recent publication by Sinclair and colleagues examined the role of the mPFC (critical for executive functioning and cognitive control; [61]) in PF consumption patterns in BEPs versus BERs [23]. Neural activity in the mPFC was measured via PF-induced Fos expression in excitatory and inhibitory mPFC neurons. Following quantification of neural activity, the mPFC was pharmacologically inactivated using muscimol (a GABA-A receptor agonist) to determine effects on PF intake.

Results revealed that while most Fos-expressing neurons in the mPFC of BEPs and BERs were excitatory, fewer excitatory mPFC neurons were active in BEP rats following PF exposure [23]. Additionally, pharmacological inactivation of the mPFC significantly increased PF intake in both BEPs and BERs. However, PF increases were more robust in BEPs versus BERs [23]. These data suggested that reduced excitatory tone in the mPFC of BEP rats following PF exposure may contribute to a decreased ability to inhibit binge-like eating of PF.

6 Conclusions

Binge eating is a core symptom of most eating disorders and is associated with significant comorbidity and morbidity. While psychosocial factors (e.g., peer group, societal standards of body image) contribute to the development of binge eating [61–63], there is a growing focus on neurobiological risk factors. The BEP/

BER model offers a simple, quick, and reliable model with which to study potential neurobiological mechanisms and better understand human binge eating behavior. While exposure to PF is difficult, if not impossible, to control in humans, the overwhelming salience of PF in human binge eating behavior (e.g., its consideration as "forbidden" and role in craving induction; [64]) underscores the importance of understanding the behavioral and neurobiological response to PF to better understand why some individuals develop binge eating and others do not.

In addition to describing the BEP/BER model, this chapter discussed studies that have used the model to dissect hormonal and neurobiological mechanisms related to reward processing, higher-order cognitive functioning, and stress in BEPs versus BERs. Evidence so far suggests that BEPs are more sensitive to the rewarding properties of PF versus BERs [43, 44] and that the initial hyper-reward response to PF decreases over time [45]. Evidence also indicates that BEPs are more susceptible to stress [55, 57], show poorer impulse control [23, 34], and exhibit inflexible decision-making as compared to BERs [13]. However, studies of BEP/BER rats are in the early stage, and additional work is needed to increase our understanding of how these (and other) hormonal and neurobiological mechanisms may contribute to binge eating.

Given the profound sex differences in binge eating [3, 16] and the established role of gonadal hormones in binge eating development [12, 20, 65, 66, 81], future studies should further examine the organizational versus activational effects of gonadal hormones on binge eating development. Current findings from prepubertal and adult OVX studies suggest that the organizational effects of estradiol during the pubertal period, and not the activational effects of gonadal hormones in adulthood [81], are important for binge eating development. However, because these studies did not readminister gonadal hormones following OVX, it is unknown when in development gonadal hormones are necessary in order for their protective effects to be expressed (i.e., are gonadal hormones necessary prior to puberty only, in adulthood only, or both?).

To better understand how increased activity in brain reward regions (e.g., nucleus accumbens) and increased reward responsivity to PF in BEPs contribute to binge eating, it would be helpful to examine potential differences in neuronal connectivity between the brain reward system and other brain regions that regulate binge eating behavior. Studies have repeatedly reported strong interconnectivity between the nucleus accumbens shell and core with the infralimbic and prelimbic cortical subdivisions of the mPFC [67–69]. In fact, findings from the addiction literature support a role for infralimbic cortex to nucleus accumbens shell projections in suppressing cocaine-seeking behavior, while projections from the prelimbic cortex to the nucleus accumbens core play a role in initiating cocaine-seeking [67–69]. These pathways have also been

implicated in impulse control of drugs of abuse [70, 71] and are suggested to contribute to the habitual pattern of drug taking that develops following prolonged drug use [71]. Addiction and binge eating share many similarities (e.g., altered reward processing, impaired impulse control; [72, 73]), and some have argued for shared underlying circuitry between the two behaviors [73]. Future studies should examine the role of the nucleus accumbens to mPFC pathways as they relate to BEP phenotypes.

Neurotransmitters such as dopamine, serotonin, and brain-derived neurotrophic factor (BDNF) play a crucial role in reward, impulsivity, and decision-making, and they all exert actions via the mPFC-nucleus accumbens pathway [74, 75]. Studies have also posited a role for alterations in these neurotransmitters in the risk for binge eating [74, 75]. However, the specific role that each neurotransmitter contributes to the above behaviors and neural pathways remains to be fully elucidated. Therefore, future studies should examine how alterations in these neurotransmitters influence the above systems and behaviors to contribute to binge eating risk and development. Results have the potential to profoundly increase our understanding of mechanisms underlying alterations in reward processing (e.g., shifts from hyper- to hyporesponsivity to PF reward) and impairments in higher-order cognitive functioning (e.g., increased impulsivity, inflexible decision-making) observed in individuals and animals who binge eat.

Lastly, stress is known to alter reward processing and increase preference for palatable food [76, 77], as well as decrease inhibitory control over binge eating [79]. However, the exact mechanisms through which stress does so remain poorly understood. Potential mechanisms of interest include alterations in glucocorticoid receptor (GR) expression in brain regions related to reward and higher-order cognitive control. The GR is ubiquitously expressed throughout the brain and body to help mediate the stress response [79]. Within the brain, GRs are expressed on a variety of neurons, including inhibitory (i.e., GABA), excitatory (i.e., glutamate), and dopaminergic neurons [80], all of which have been implicated in reward, higher-order cognitive functioning, and binge eating behavior [73]. Differences in GR expression patterns between BEPs and BERs may suggest innate differences in stress susceptibility between BEPs and BERs that perpetuate the binge eating phenotypes. Examining and identifying differential expression of GRs may also provide insight into differences in neuronal connectivity and communication between brain regions related to binge eating that can be used to inform future pharmacological studies and aid in the development of targeted treatments for binge eating.

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Chapter 3

Food Seeking in Spite of Harmful Consequences

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Abstract

In industrialized nations, overeating is a significant problem leading to overweight, obesity, and a host of related disorders; the increase in these disorders has prompted a significant amount of research aimed at understanding their etiology. Eating disorders are multifactorial conditions involving genetic, metabolic, environmental, and behavioral factors. Considering that compulsive eating in the face of adverse consequences characterizes some eating disorders, similar to the way in which compulsive drug intake characterizes drug addiction, it might be considered an addiction in its own right. Moreover, numerous review articles have drawn a connection between the neural circuits activated in the seeking/intake of palatable food and drugs of abuse. Based on this observation, "food addiction" has emerged as an area of intense scientific research, and accumulating evidence suggests it is possible to model some aspects of food addiction in animals. The development of well-characterized animal models would advance our understanding of the etiologic neural factors involved in eating disorders, such as compulsive overeating, and it would permit to propose targeted pharmacological therapies.

Key words Food and eating addiction, Compulsion, Animal model

1 Introduction

1.1 Food and Eating Addiction

Feeding behaviors are mostly innate and flexible behaviors allowing individual's survival depending on the strict interaction between physiological needs and environmental resource availability. These behaviors are driven by, in addition to the biochemical (i.e., physiological) balance, cognitive/emotional factors, which are not always feasible to separate.

Clinical and preclinical studies show these fundamental behaviors can turn into maladaptive behaviors, threatening individual's environmental fitness, physical health, and psychosocial functioning. Peculiar symptoms for feeding and eating disorders (FEDs) are reported in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) [1] where it is evidenced how these pathological behaviors – in particular those characterized by overconsumption (e.g., binge eating disorder, bulimia nervosa) – could resemble

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those observed in patients with substance use disorders (SUDs), with whom comorbidity is reported. Shared behavioral manifestations include lack of control, impulsivity, tolerance, withdrawal, distress/dysfunction, overconsumption, and craving patterns, suggesting alterations in the motivational processes and response to specific stimuli in the absence of "physiological" need.

To note, the interpretation (and planning) of clinical and preclinical studies in this field has so far resulted in different theoretical frameworks, differently emphasizing a central role for specific food stimuli (food addiction) or behavioral response to them (eating addiction) in addiction-like eating pathological phenotypes. The still ongoing debate on these dichotomous, but not totally mutually exclusive, points of view [2–7] does not represent a mere terminological issue but has critical implications to the understanding of the underlying processes and mechanisms as well as means for targeted prevention and treatment strategies for eating disorders [8].

Studies in humans and laboratory animals show that, in addition to energy balance, eating is regulated by factors unrelated to metabolic control, and several studies suggest a link between stress, access to highly palatable food, and eating disorders [9–12]. Eating in response to negative emotional states suggests the possibility that individuals overeat to self-medicate, for example, with "comfort foods." However, clinical data suggest that some individuals may develop addiction-like behaviors when consuming palatable foods [13, 14]. It has been proposed that overeating of palatable food may produce long-term neuroadaptations in the reward and stress networks of the brain [15, 16], similar to those produced by long-term drug abuse [14].

Drug addiction is a chronic, relapsing disorder characterized by an inability to stop or limit drug intake, extremely high motivation to take the drug (with activities focused on its procurement and consumption), and continued use of the drug despite harmful consequences [17, 18]. These behaviors have been reproduced in animal models of drug addiction [17, 18], and some of them have been reported in animal models in response to the consumption of highly palatable foods. Moreover, common neurobiological adaptations have been suggested to be involved in both drug- and food-related disorders [9, 14, 19, 20]. Therefore, a critical question is the legitimacy of the term "food addiction."

Several authors support the validity of the "food addiction" definition evidencing how only some particular foods (i.e., processed palatable foods high in carbohydrate and/or sugar, fat, salt) [21] can have an addictive potential in some circumstances [21–25], supporting the hypothesis that the overconsumption of "refined" foods can be described as an addiction conforming to the Diagnostic and Statistical Manual of Mental Disorders [1]. On the other hand, because food is crucial for survival and a clear

"addictive" agent has not been identified, unlike from other pharmacological addictive stimuli (e.g., drugs, ethanol), pathological behaviors in the eating domain have been proposed to be non-substance-related behavioral addictions (i.e., "eating addiction"), similar to gambling disorder (within the "Addictive Disorders" section of the DSM-V manual) [26–28].

These two approaches have so far resulted in different instruments to evaluate the addiction-like eating/food addiction in humans. Among these, the Addiction-Like Eating Behavior Scale (AEBS; [27]) has been recently developed and preliminary validated to quantify the behavioral features of a potential "eating addiction" phenotype, while the Yale Food Addiction Scale (YFAS) has been proposed to measure the food addiction based on the diagnostic criteria for SUDs, according to the DSM-V [29, 30]. Investigation of neurobiological substrates sustaining eating-related pathological behaviors has focused on brain circuits involved in the motivated behaviors. In particular, despite the differences in addictive potential between food and drugs [31–35], animal and human studies highlighted several alterations within the catecholaminergic cortico-accumbal reward circuitry, evolutionarily developed to mediate the response to natural (and later artificial) stimuli.

A substantial body of evidence suggests the possibility of producing animal models of "food addiction," and several studies have used palatable diets to induce overeating, obesity, binge eating, withdrawal, and food relapse in animal models [11, 15, 36–48]. In addition, one study suggests that sugar-bingeing rats show cross-sensitization with some drugs of abuse [49].

1.2 Neuronal Circuits Underlying Food Addiction

In addition to the behavioral manifestations described above, several studies also support the idea that overconsumption of certain foods has parallels with drug addiction [8, 14, 50–52].

In fact, over the years, increasing evidence has indicated how high-calorie/palatable foods can affect the reward circuitry of susceptible individuals in a similar way to the drugs of abuse [8, 14, 19, 20, 50–54].

Several areas of the brain, as well as neurotransmitter systems, are involved in the reinforcement effects of both food and drugs [9, 55–61]. It appears that under certain circumstances the potent rewarding capacity of palatable food can lead to behavioral and neurochemical changes resembling those produced by drug abuse [14, 40, 42, 44, 55, 60, 62].

Like addictive drugs, certain palatable foods can activate brain circuitry involved in reward, motivation, and decision-making [50–52, 63, 64]. In particular, it has been proposed that adaptation in the reward, motivational, memory, and control circuits occurring with repeated exposure to palatable food is similar to that observed with repeated drug exposure [14, 65]. In vulnerable individuals,

consumption of high quantities of palatable food (or drugs) can affect the balance between motivational, reward, learning, and control circuits, increasing the reinforcing value of food (or drugs) and weakening the control circuits [65, 66].

The most clearly established mechanism common to food and drug intake is the activation of the dopamine (DA)-containing link in brain reward circuitry [9, 51, 65, 66]. The major sites of these neuroadaptations are thought to be the mesolimbic and nigrostriatal DA circuits. Repeated stimulation of DA reward pathways is believed to trigger neurobiological adaptations in different neural circuits that may make seeking behavior "compulsive" [8] and lead to a loss of control over food and drug intake [65, 66]. In addition, the degree of DA release seems to correlate with subjective reward from both drug and food use in humans [63, 66]. Repeated DA stimulation induced by exposure to addictive drugs produces plastic changes in the brain resulting in compulsive drug intake. Similarly, repeated exposure to palatable foods can induce compulsive food consumption through the same mechanisms [14, 40, 60], and neuroimaging studies in obese subjects have revealed alterations in DA receptor expression that are similar to those found in drug-addicted subjects [9, 50–52, 60, 66]. Thus, overconsumption of palatable foods seems to downregulate dopaminergic reward circuitry through the same mechanisms observed in drug addiction, namely, reduced striatal DA D2 receptor availability and blunted DA release [51, 52, 65, 66]. It has been hypothesized that the switch from voluntary drug use to habitual and progressively compulsive drug use represents a transition at the neural level from prefrontal cortical to striatal control over drug-seeking and drug-taking behaviors; there is also a progression from ventral to more dorsal domains of the striatum, which is mediated at least in part by its stratified dopaminergic innervations [67, 68]. This progressive shift from use to compulsion seems to be related to a change in the balance of behavioral control processes from the prefrontal cortex to the striatum [68]. Striatal D2 receptor availability in obese subjects correlates with glucose metabolism in some frontal cortical areas, such as the dorsolateral prefrontal cortex (PFC), which is involved in inhibitory control [66]. It has been suggested that reduced dopaminergic modulation from the striatum may lead to impaired inhibitory control over food intake and an increased risk of overeating [16, 65, 66]. The same direct correlation between striatal D2 availability and glucose metabolism in the dorsolateral cortex has been reported in alcoholics [66]. In addition, the lack of availability of D2 has been proposed as a risk factor for developing compulsive behaviors related to general addiction [60], and reduced striatal D2 availability has been reported in obesity and addiction [50]. Several areas of the PFC are implicated in the motivation to eat [69], and a substantial body of evidence points to a critical role of PFC in motivated animal and human behavior

related to food or drugs [9, 44, 50, 58, 62, 70–72]. Thus, some prefrontal regions could reflect a neurobiological substrate common to the drive to eat or take drugs [50–52]. Abnormalities in these regions could enhance either drug-oriented or food-oriented behavior, depending on the established habits of the subject [9].

Abundant data suggest prefrontal cortical dysfunction in drug and food addicts and are increasingly supported by experimental studies in both animals and humans [20, 65, 68, 73]. Dysfunctional regions of the PFC involved with emotional processing [46] and inhibitory control [74] are particularly important to understanding addiction, as their disruption is linked to compulsive behaviors and poor impulse control [64]. Besides the dopaminergic system, many different neurotransmitter systems are involved in the rewarding effects of drugs of abuse and food [50]. Among these, the opioid pathway plays an important role in normal and pathological consumption of palatable, high-calorie foods [51, 72].

Opioids are thought to process the hedonic responses to food, which in turn reinforces the assignment of incentive salience to cues associated with palatable reward [75, 76]. Opioid receptors are located in several brain regions related to reward processing and the regulation of energy homeostasis. Among the different opioid receptor subtypes, mu-opioid receptors (MORs) are strongly implicated in reward [51]. Accumbal MORs promote the approach to palatable food only in the absence of a homeostatic need for calories, thus suggesting that these receptors contribute to a neural mechanism that drives intake of calorie-dense food specifically in the state of satiety [72]. Moreover, the opioid system is also a regulator of incentive motivation for rewards and reward-associated cues [77, 79], key factors in action-outcome versus stimulus-driven habitual overeating [64, 80]. The opioid system is especially related to the hedonic impact arising from drugs of abuse and palatable food intake [51, 72].

Many classes of addictive drugs induce euphoria acting on opioid system [51, 72]. Human positron emission tomography (PET) studies [81–83] show that alcohol and cocaine dependence are associated with higher brain MOR availability in the reward circuitry, while opiate use leads to downregulation of MOR [84]. Similar to opiate addictions, decreased MOR availability has been found in obesity, BED, and bulimia nervosa in some brain regions of reward processing [85, 86], and diminished MOR availability has been hypothesized to promote overeating to compensate for a blunted MOR response [86].

Overall, numerous evidences indicate that both dopaminergic and opioid pathways play an important role in both substance abuse and consumption of palatable foods [50, 51, 72]. Opioid and DA receptors are found in multiple brain reward regions, and MOR and DA receptor availability in regions like striatum and VTA are highly

correlated in humans [87]. The crosstalk between these two neurotransmitter systems has also been shown [87]. Dampening of DA signaling and downregulation of the MOR have been reported in both food and drug addiction, thus supporting a key role for dopaminergic and opioid systems in these pathological phenotypes [50, 68].

The norepinephrine (NE) system is involved in cognitive and mood disorders, and altered NE transmission is involved in overeating and emotional eating (EE) seen in obesity. Collectively, studies suggest that NE transmission is impaired in substance use disorder, obese, and EE individuals. Variations in NET availability have been suggested to contribute to motivated behavior alteration, a key feature underlying substance use disorder and obesity [50]. Moreover, NE transmission in medial prefrontal cortex (mPFC) has been shown to be involved in the behavioral and central effects of drugs of abuse [88–92] and critical for food-related motivational behavior [58, 59], and we have previously reported that prefrontal NE transmission also plays a major role in aberrant motivation related to the seeking of palatable foods [93]. However, additional studies are needed to fully define the role of NE and NET in addictive behavior to drugs and food.

Acetylcholine (ACh) is released by local interneurons of NAc, critically influencing activity of the cortico-accumbal motivational system. Based on the observation that increased activity of the cholinergic interneurons in the NAc reduces palatable food consumption, accumbal ACh has been suggested to play a modulatory effect on feeding behavior. The effects of drugs of abuse on cholinergic interneurons in the accumbens reflect their effects on feeding behavior: drugs increasing food intake decrease (or produce no change on) ACh release in the NAc, while drugs reducing food intake induce increased accumbal ACh release. Anticholinergic drugs can be abused thanks to their ability to increase DA activity in the striatum, and DA normally exerts an inhibitory action on striatal ACh interneurons in rats. In addition, ACh is increased in the NAc during drug withdrawal, and enhanced accumbal ACh interneuron functioning prevents addictive behaviors for cocaine and morphine [52, 94]. Collectively, these data support an antagonistic association between DA and ACh in the NAc and striatum. Findings from many studies support the observation of a significant, although not identical, neural overlap in response to high-calorie/palatable food and drugs of abuse in food and drug addiction conditions. Further studies in both animal models and humans will advance our understanding of these neural mechanisms allowing developing focused therapeutic interventions.

1.3 Epigenetic of Food Addiction: The Link Between Genetics and Environment

Epigenetic is the capability of environment to "switch on/off" specific genes (by DNA methylation, chromatin modification, and noncoding RNA silencing), silencing or enhancing the gene expression without changes in DNA sequence [95]. These mechanisms that allow rapid adaptation to environment and are fundamental for evolution can also be "hijacked" toward addictive behaviors [96]. The importance of the link between different addictive behaviors and epigenetic processes is well established. This interaction has a bilateral influence: on the one hand, epigenetic modifications (e.g., due to early experiences) are associated with increased vulnerability of drug addiction [97]; on the other hand, drug exposure can induce epigenetic modifications that reinforce addictive behavior, making individuals more vulnerable to relapse [98]. However, although many studies have investigated the role of epigenetic modifications in drug addiction, less is known about the relation between epigenetic mechanisms and food addiction.

Evidence suggests that stressful experiences and altered diet can induce epigenetic changes that increase vulnerability to pathologies like obesity, binge eating disorder, and bulimia nervosa. Longitudinal studies on offspring of women that lived natural disaster and famine during the first trimester of gestation reported increased DNA methylation and higher probability to develop obesity [99, 100]. Moreover, early traumas (like childhood physical and emotional abuse), able to induce epigenetic modifications, have been linked with bulimia nervosa and binge eating disorder [101]. To investigate the connection between epigenetic modifications and eating disorders, some authors focused on genes known to be related with stress reaction, reward processing, and eating behavior. Due to their involvement in both reward circuit and eating behavior, dopaminergic genes received particular attention. Studies analyzing methylation of the dopamine transporter (DAT) and D2 receptor (DRD2) genes in patients with disorders like anorexia and bulimia nervosa have showed altered expression of DAT mRNA and downregulation of DRD2 due to hypermethylation of the genes [102, 103]. Moreover, based on the role of glucocorticoid receptors (GR) in stress reactivity, Steiger et al. [104] tested the hypothesis that stressful experiences increase the risk of bulimia nervosa through epigenetic modification of the GR genes. Results show a significant hypermethylation of the GR gene promoter [104].

Thaler et al. [105] reported significant hypermethylation of the BDNF promoter in patients with bulimia nervosa compared to control group. This alteration was particularly evident when bulimia nervosa co-occurred with childhood abuse or borderline disorder [105].

However, studies investigating epigenetic modifications in eating disorders have mainly focused on DNA methylation, neglecting other epigenetic mechanisms (like histone modification

and noncoding RNA). Moreover, small size sample and high heterogeneity of human tissues are limiting factors in this promising field of research [106]. The data available until now are not sufficient, and future researches are needed to understand possible epigenetic mechanisms involved in eating disorders and in food-related addiction behaviors.

1.4 Eating Disorders, Palatable Foods, and Stress

Eating disorders are multifactorial conditions caused by environmental and genetic factors and the complex interactions among them [9, 107–109]. Although some human and preclinical studies have begun investigating the genetic basis of susceptibility to pathological addiction-like eating [106, 110], suggesting genetic similarities between substance use disorders and maladaptive eating patterns, further studies are necessary to define the genetic basis of these disorders and identify tailored pharmacological intervention for their treatment. On the other side, several studies have focused on environmental conditions able to "increase" the likelihood to develop these pathological phenotypes highlighting, among others, a critical role for stressful condition exposure and dieting style characterized by prolonged availability of specific palatable food.

1.4.1 Dieting Style and Availability of High-Calorie/Palatable Food

Of the environmental factors that influence eating disorders such as binge eating disorder and bulimia nervosa, the availability of palatable foods is the most obvious [9]. It has been demonstrated that different foods induce different levels of compulsive behavior [9, 36]. In particular, processed foods with high concentrations of sugar or other refined sweeteners, carbohydrates, and fat have been suggested to have an addictive potential and rewarding properties in both humans and animals [21, 23, 25, 36] representing the first-choice food in these eating disorders. An evolutionary explanation for the preference for these particular foods reflects their higher reward value and high nutrient content [111].

Despite difference in addictive potential between drugs and foods, several studies have reported how the exposure to palatable food affects the structure and functionality of the same motivational brain circuits evolved to respond to rewarding and aversive (i.e., stressful) stimuli, hijacking learning/conditioning processes and finally resulting in increased likelihood for seeking and consumption of these foods [112]. This could explain why many people lose control of their ability to regulate intake of such palatable foods [36]. Of all palatable foods, chocolate has been shown to have particularly rewarding properties in animals [58, 59, 62, 113], and it is the food most commonly associated with reports of food craving in humans. Thus, chocolate craving and addiction have been proposed in humans [114].

Voluntary or induced cycles of consume/deprivation from "forbidden" palatable foods represent a common finding in

patients suffering from eating disorders [115–118]. Accordingly, the most reliable way to induce maladaptive eating in animal models consists in repeated and alternated access to standard and palatable foods for variable periods of time. To note, intermittent food access appears to be necessary to induce binge-like behavior in rodent models, while continued food availability devaluates rewarding value, suggesting that compulsive eating behaviors derive from the uncertainty of availability [119]. In addition, it has been evidenced how intermittent (but not continuous) access to palatable food results in profound alterations in different structures of the corticolimbic circuit as well as in systems involved in stress response, analogously to what has been reported in studies of drug addiction [40, 120–122].

However, the validity of these protocols in mimicking human condition where availability of palatable food is constant has been questioned [5]. In fact, prolonged food availability does not necessarily result in prolonged intake of such "addictive foods" in humans. To better investigate this discrepancy, a prolonged ad libitum alternation of different palatable foods could be employed in animal models. Moreover, species-specific differences must also be taken into account.

1.4.2 *Stress*

Stress represents another critical factor in the development and manifestation of eating disorders. It affects the development, course, outcome, and relapse after periods of remission of both drug and food addiction [123–128]. In research on eating disorders, stress is considered a factor able to disturb the regulation of both qualitative and quantitative aspects of food intake; assessment of stressful conditions that increase vulnerability to the development of eating disorders is one of the major goals of preclinical eating disorder research. Acute or chronic stress has been shown to influence food intake (as well as the propensity to take drugs) [9], while chronic stress has been shown to increase the consumption of certain palatable foods, commonly referred to as "comfort food" [12, 127, 128], and to precipitate binge eating [10, 48]. Finally, several studies have reported a synergistic relationship between stress and food restriction in promoting eating disorders, such as binge eating, in humans and animal models [16, 37, 38, 107, 129].

Periods of food restriction, or dieting, are a very common finding among individuals with eating disorders [48], and theorists posit that diet exposure increases overall risk for onset and maintenance of eating disorders, such as binge eating disorder and bulimia nervosa [115, 130, 131]. Indeed, recurrent periods of caloric restriction are consistently the strongest predictors of overeating in response to stress [132], whereas hunger alone appears not to be enough to induce such binge eating phenomena [115, 132].

Caloric restriction is used in several animal models because, in addition to ecological validity and strong adaptive and motivational value, it also mimics typical human dietary conditions. Moreover, caloric restriction represents an extremely stressful experience for rodents [133], and many studies have shown that this manipulation alters levels of hormones, such as glucocorticoids, adrenocorticotropic hormone, and corticotrophin-releasing factor, which are involved in the physiological response to stressful conditions [134–137]. Finally, previous exposure to caloric restriction produces changes in stress neurocircuitry that increase stress sensitivity and the tendency to overeat high-fat foods [138].

Food restriction in rodents is a stressful condition that alters the sensitization of brain reward systems [139, 140], and greater sensitization of the reward systems can lead to excessive intake of highly palatable foods [107]. In fact, repeated stimulation of reward pathways through highly palatable food consumption may lead to neural adaptations that make consumption more compulsive [9]. Moreover, stress is an important trigger of binge eating behavior, and a prior history of caloric restriction is the most important factor influencing the neural adaptation of rats that develop binge eating behavior [37]. It has been proposed that food deprivation that reduces extracellular DA levels in the nucleus accumbens (NAc), such as incontrollable stressors, induces anhedonia-like behavior and that refeeding reverses this pattern by increasing DA levels in the NAc [15]. Translating these observations to humans, the increase in DA induced by refeeding would increase the reinforcement of palatable food for individuals under caloric restriction more than for individuals in a normal, non-deprived state [15].

2 Animal Models of Food Seeking/Intake in Spite of Harmful Consequences

Although animal models cannot explain or reproduce all the complex internal and external factors that influence eating behavior in humans, they do make it possible to distinguish the relative roles of genetic and environmental variables, thus allowing tighter variable control and the ability to investigate behavioral, physiological, and molecular mechanisms [16]. Animal models can be used to investigate molecular, cellular, and neuronal processes underlying normal or pathological behavioral patterns. Thus, the use of animal models can advance our understanding of many different factors involved in the development and expression of eating disorders. In recent decades, the use of animal models in preclinical research has contributed significantly to the study of the etiology of some human psychiatric disorders and has provided a useful tool for the development of appropriate therapeutic interventions. Inbred strains of mice provide one of the most common and useful animal models to

investigate gene/environment interactions involved in psychiatric disorders.

As previously highlighted, accumulating evidence suggests the possibility to model food addiction in animals. A hallmark feature of drug addiction is compulsive drug use in the face of adverse consequences [17, 18, 38, 68]; similar compulsion despite negative consequences is evident in some eating disorders, such as binge eating disorder and bulimia nervosa [19]. Consuming large quantities of palatable foods can indicate increased motivation for food. However, consuming large quantities of palatable foods despite harmful consequences of this behavior, i.e., tolerating punishment to obtain it, represents strong evidence of a pathological motivation for food [141]. Although there is little evidence of continued food seeking/intake despite its possible harmful consequences (an index of compulsion) in rats [45, 141] and mice [93, 142], animal models that have reproduced this behavior indicate that adaptive food seeking/intake can be transformed into maladaptive behaviors under specific experimental manipulations.

2.1 Food Seeking/Intake Despite Possible Harmful Consequences in Rats

In order to evaluate the compulsive nature of eating palatable food, preclinical models measure the motivation to seek and consume palatable food despite potentially harmful consequences. Negative consequences are usually modeled by pairing an unconditioned stimulus (US, i.e., foot shock) with a conditioned stimulus (CS, i.e., light); subsequently, a test session assesses the effects of exposure to CS on palatable food seeking and consumption in spite of the signaled incoming punishment or measures the voluntary tolerance of punishment in order to obtain the palatable food. Otherwise, negative conditions can be modeled by directly pairing the rewarding stimulus with an unconditioned aversive one (e.g., light, taste).

The major models of compulsive eating of palatable foods to study obesity and binge eating disorders [45, 141] have been developed by exposing rats to environmental conditions characterized by limited periods of availability of high palatable food, a condition shown to be able to induce pathological overeating [143, 144]. Rossetti and colleagues [145] used a well-characterized paradigm to induce binge-like eating exposing adolescent female rats to two different dieting protocols for seven cycles (7 days each): standard chow (C/C group, control) or alternating phases of standard (C) and high-fat chocolate-flavored food (5 days chow and 2 days with palatable food (P); C/P group) [47, 143, 146, 147]. The results of these experiments show how, compared to standard chow, alternating dieting produces binge-like eating with specific pattern of overeating during the P phases and under-eating during the C phases as previously reported in other studies with similar manipulation [46, 47, 143, 146].

In addition, this condition is able to induce delayed (but not "immediate") compulsive binge-like eating selectively for palatable food (but not for a novel different food, i.e., sweet pellet), as demonstrated by persisting lever press despite the harmful consequences (foot shock).

The emergence of compulsive behavior has been interpreted as deriving from negative reinforcement mechanisms arising from negative states accompanying withdrawal [47].

Dore and colleagues [148] tested compulsive behavior in male rats using a shortened version of the abovementioned dieting protocol consisting in eight cycles where standard chow (2 days) was alternated to highly palatable food (chocolate-flavored, high-sucrose; 1 day).

Alternating exposure between chow and palatable food (chow/palatable, C/P versus chow/chow, C/C groups) produced profound alterations in feeding pattern, with progressive escalation in consumption of the palatable food over time, resembling the behavioral tolerance observed in addictive behaviors [149, 150].

In addition, the withdrawal period from this intermittent and extended access to the palatable diet increased the motivation to compulsively seek and consume the sugary diet while facing aversive conditions (the enlightened aversive compartment in a light/dark conflict box), as reported in another study [152]. Paralleling results from drugs of abuse studies [153–156], the authors highlighted how these pathological behaviors were selectively abolished by systemic administration of cannabinoid type 1 receptor (CB1) inverse agonist, suggesting an important role for the cannabinoid system in modulating the hedonic value of food and offering a possible pharmacological target for binge eating disorder.

Velázquez-Sánchez [157] modeled binge-like phenotype in male rats by limited palatable food self-administration procedure and investigated its extended effects on conditioned seeking response and compulsion. After the acquisition of a stable self-administration response with standard food, animals were divided in two groups depending on the food they would receive during the future self-administration sessions (1 h-FR1) in the successive 12 days: palatable (chocolate-flavored, high-sucrose pellet) [46, 47] or chow (standard chow).

Results confirmed a rapid increase of preferred food consumption [157] as well as increased palatable food seeking as measured by conditioned place preference and second-order schedule of reinforcement (FI5(FR10:S)) tasks.

In addition, the authors proved the compulsive aspects of binge eating phenotype by testing rats for cue-induced conditioned suppression (with palatable and chow food) that resulted blunted in animals that had been exposed to prolonged and intermittently palatable food.

The same procedure of limited access to the same "naturally" preferred palatable foods was used in male rats by Smith and

colleagues [158] to evaluate the effects of systemic and local administration of an NMDA antagonist able to decrease the reinforcement effects of drugs of abuse and alcohol [159–162] on pathological feeding behavior.

The authors highlighted that systemic administration of antagonist selectively reduced seeking behavior in the second-order schedule of reinforcement (FI5(FR10:S)), binge-like intake, and compulsive behavior (dark/light conflict test) toward a preferred food in palatable rats (see above/below), without affecting standard food consumption in chow rats with a history of food restriction (30% for 10 days).

This research also highlights a critical role for the shell portion (but not core) of NAc, critically involved in drug addiction and in binge-like eating behavior. Despite obesity is currently not included within the FED in DSM-V due to its etiological heterogeneity, it is clinically associated with eating disorders (e.g., binge eating disorder). Johnson and Kenny [45] evaluated compulsive eating in obese male rats. In the first phase of their study, animals were assigned to one of three experimental conditions with different levels of exposure to palatable, energy-dense foods similar to those readily available for human consumption: chow only, restricted access, and extended access. Under all three conditions, rats had ad libitum access to standard laboratory chow. Chow-only rats did not have access to a cafeteria-style diet containing highly palatable foods, while restricted- and extended-access groups had 1 h per day and 18–23 h per day of access to the cafeteria-style diet, respectively. This procedure was maintained for 40 consecutive days, until a statistically significant body weight increase in rats with extended access was observed. In a second phase of the study, all of the animals were permitted 30 min of access per day to the cafeteria-style diet for 5–7 days in an operant chamber. Within each of the three groups, half of the rats were exposed to light-foot shock pairing (punished group), whereas the other half were exposed to the cue light in the absence of foot shock (unpunished group).

On the test day, the effect of exposure to the cue light on the consumption of palatable food was examined. Results demonstrate that extended access to palatable, energy-dense food induced compulsive-like behavior in obese rats, as measured by the consumption of palatable food despite the application of a negative conditioned stimulus (light). Moreover, results show that striatal DA D2 receptors were downregulated in obese rats, a phenomenon also reported in drug-addicted humans, thus confirming addiction-like neuroadaptive responses in compulsive eating. In addition, lentivirus-mediated D2 knockdown in the striatum rapidly accelerated the development of binge-like eating as well as the onset of compulsive-like food seeking in rats with extended access to palatable high-fat food.

A recent study [152] investigated the compulsive behavior in young adult female rats prone or resistant to stress-induced (shock) binge-like behaviors (BEP and BER, respectively).

Intermittent and random access to 10% sucrose solution (five sessions, 1 h each) in combination with stress exposure (four 3-sec foot shocks repeated for 3 days) was followed by evaluation of sucrose bingeing (1-h session) in undisturbed or stress-induced (foot shock) condition. The results indicate that sucrose consumption increases with the repeated access to sucrose independently from stress exposure in BEP rats, which show a high consumption also in the absence of physical hunger.

This behavioral pattern is paralleled by a high motivational drive to sucrose intake in non-stressful condition and an increased hedonic value for sucrose after stress exposure, as measured by qualitative analysis of the behavioral pattern during intake pattern.

Interestingly, the combination of the prolonged access to food and stress produced increased the proneness of BER rats to seek and consume sucrose despite an ecological aversive condition (assessed by dark/light test). In this work, the authors extended previous results [163] obtained using the same model evidencing an alteration of the HPA axis in binge-prone animals in response to stressful condition, coherently with other results from clinical and preclinical studies on binge eating phenotypes.

In another study, Oswald and colleagues [141] investigated whether binge eating-prone (BEP) rats also demonstrated compulsive palatable food eating. The heightened (aberrant) motivation for palatable food was measured as the voluntary tolerance of punishment to obtain a particular palatable food. Generally, rats of the same age and sex consume very similar amounts of standard rat chow; however, the amount consumed can vary when provided the opportunity to choose between highly palatable foods and standard chow. BEP rats were selected on the basis of stable differences in consumption of palatable food in a discrete, 1–4-h period of time. BEP rats were those that consumed 40% more palatable food than rats that consistently consumed the least amount of these foods, known as binge eating-resistant (BER) rats. This model and its results are described in greater detail in Chapter 1 of previous edition of this volume.

Moshe et al. [164] recently proposed a similar model to test compulsive behavior in binge-prone female adult rats by using a delayed internal aversive unconditioned stimulus (i.e., pain). After evaluating for natural proneness to binge eating behavior (palatable hypercaloric liquid intake in three test sessions, 5 days), rats were tested (after glucose exposure) for palatable food intake (Oreo cookies). Rats from the BEP and BER groups were then exposed to a lactose conditioning protocol (LCP, three tests for 5 days) where palatable food consumption was preceded by lactose (or glucose as control) administration. Finally, BEP and BER rats

were tested in the last phase for delayed memory of LCP conditioning (in the absence of lactose).

Results evidenced a stable profile of overeating for BEP rats with both liquid and solid palatable rewards (*see* also [165]). Interestingly, these results also suggest that this phenotype is accompanied by high motivation to obtain palatable food even during the experience of discomfort (caused by lactose ingestion), evidencing a devaluation of the expected future consequences, thus mirroring alterations in decision-making processes typical of addiction-like behaviors.

2.2 Food Seeking/Intake Despite Possible Harmful Consequences in Mice

Despite intrinsic differences between mice and rats, to date just few studies have examined the compulsive features of addiction-like behaviors related to food and eating in mice.

Inbred mouse strains could be useful to understand the individual differences in behavioral, pharmacological, physiological, and biochemical phenotypes underlying susceptibility to addiction-like eating disorders. They may offer a starting point for more extensive genetic studies aimed at identifying genes involved in these pathological phenotypes [166–168], using a model previously reported to induce binge-like behaviors in mice [169] and two inbred strains characterized by differences in susceptibility to drugs of abuse [15, 43, 44, 47, 59, 142]. C57(BL/6 J) and DBA (2/J) inbred mouse strains were exposed to intermittent exposure (six times for 20 days) to sweetened palatable food counterbalanced with standard food (same for macronutrient but differing for sweetness) in a conditioned place preference apparatus. Food intake was used to evaluate binge eating phenotype, and after this protocol, mice were tested for seeking (CPP) and compulsive behavior (assessed by dark/light test conflict). The results evidenced that regardless of genotype, prolonged intermittent exposure led to increased consumption of palatable food; however, a dramatic escalation in palatable food intake and compulsive-like eating selectively occurred in the DBA strain.

The major sites of food-induced neuroadaptations are thought to be the DA mesolimbic and nigrostriatal circuits, and overconsumption of palatable food is accompanied by dopaminergic reward circuitry downregulation through the same mechanisms observed in drug addiction [14, 65]. However, several areas of PFC are implicated in influencing the motivation to feed [69], and several lines of evidence point to a critical role of PFC in motivated behavior related to food or drug consumption in animals and humans [9, 44, 58, 62, 70, 71, 93], thus suggesting that some prefrontal regions could contain a common neurobiological substrate [9].

Using a paradigm of conditioned suppression, we have previously investigated compulsive eating behavior by modeling food seeking in spite of harmful consequences in mice [93].

Moreover, we assessed, in C57 mice, the hypothesis that prefrontal NE transmission plays a major role in maladaptive food-related behavior, assessing the effects of selective norepinephrine inactivation in mPFC on compulsive eating behavior [93]. To test this hypothesis, we assessed the effects of selective norepinephrine inactivation in mPFC on conditioned suppression test in stressed (calorie-restricted) mice. While control (nonfood-deprived) animals showed a profound conditioned suppression of chocolate seeking during the presentation of conditioned stimulus, previously food-restricted animals showed food seeking/intake despite the possible harmful consequences. Moreover, food seeking in spite of harmful consequences was prevented by selective NE inactivation, thus suggesting that prefrontal cortical NE is critical for maladaptive food-related behavior. These findings indicate that adaptive food seeking/intake can be transformed into maladaptive behavior and point to a "top-down" influence on eating disturbances, as well as new targets for treatment of aberrant eating behaviors [58, 59] paralleling results indicating a critical role for prefrontal NE transmission in mediating the behavioral and central effects of drugs of abuse [88, 92].

In a later study, we compared compulsion-like eating, in the form of conditioned suppression of palatable food seeking in C57 and DBA inbred mouse strains, previously exposed to chocolate (pre-exposure) and chronic mild stressful experience (food restriction) in order to determine the influence of gene-environment interplay on this behavioral phenotype [142].

3 Materials and Procedures

3.1 Animals

Male mice of the inbred C57BL/6J (C57) and DBA2/J strains (Charles River, Como, Italy), which are commonly used in neurobehavioral phenotyping, 8–9 weeks old at the time of the experiments, were housed and maintained in a 12-h/12-h light/dark cycle (light on 07.00–19.00 h). Each experimental group was composed by eight animals.

3.2 Drugs

Chloral hydrate, 6-hydroxydopamine (6-OHDA), and GBR 12909 (GBR) were used for the experiments investigating the role of prefrontal NE in compulsive eating in C57 mice. Chloral hydrate (350–450 mg/kg) and GBR (15 mg/kg) were dissolved in saline (0.9% NaCl) and injected intraperitoneally (i.p.) in a volume of 10 mL/kg. The light-sensitive 6-OHDA neurotoxin was freshly prepared every day, dissolved in saline containing Na-metabisulfite (0.1 M) and stored at 4 °C.

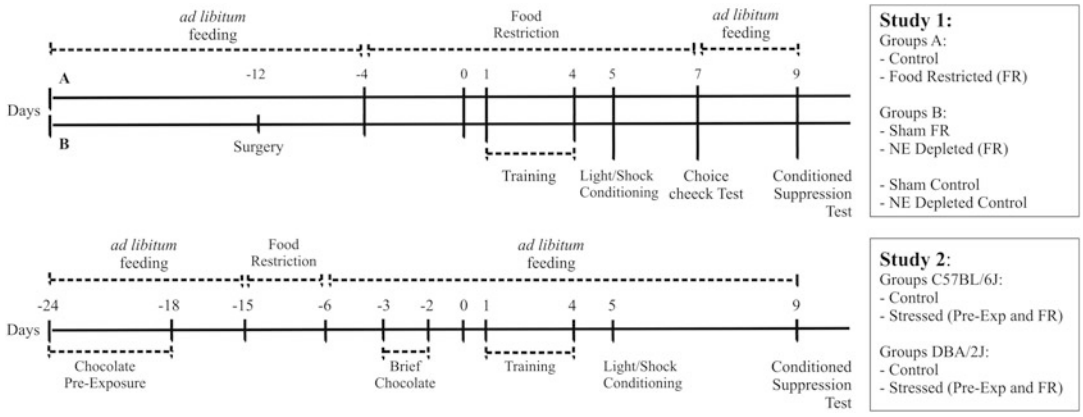

Fig. 1 Schematic timeline of the experimental procedure. *See* Table 1 for detail

3.3 Intermittent Pre-exposure to Chocolate

The experimental timeline is shown in Fig. 1. In study 2, stressed mice from the two strains (C57 and DBA) were intermittently pre-exposed to chocolate for 7 days (from day −24 to day −18) before the conditioned suppression procedure began. Mice were daily "randomly" isolated daily for 4 h; milk chocolate and standard food were delivered ad libitum. Two days after the end of this schedule (day −15, *See* Fig. 1), mice in the stressed group were subjected to caloric restriction (food restriction, FR) (*See* Table 1).

3.4 Food Restriction

We used food restriction as stressful experience in both studies investigating food seeking in spite of harmful consequences [133]. In the food restriction condition, food was delivered once daily (07.00 p.m.) in a quantity adjusted to induce a loss of 15% of the original body weight. In the ad libitum condition, food was given once daily (07.00 p.m.) in a quantity adjusted to exceed daily consumption [38].

In study 1 (investigating the role of prefrontal NE in compulsive eating), FR procedure began 5 days before the conditioned suppression procedure started and mice returned to ad libitum feeding 2 days before testing in order to rule out any effects of dietary deficiencies on the conditioned suppression test day (*See* Fig. 1, Table 1, study 1).

In study 2 (gene-environment interplay), FR schedule lasted for 10 days (from day −15 to day −6), until 6 days before the conditioned suppression procedure began (day 1, *see* Fig. 1; Table 1, study 2). Six days before the training phase started, the animals were returned to ad libitum feeding in order to rule out any possible effects of dietary deficiency on the conditioned suppression test day.

Table 1
Detailed experimental design for Study 1/2

Study 1:
Groups A: Two groups of mice, control and food restricted (FR), were used.
Days − 4 to 7: FR mice were placed on a moderate food restriction schedule 5 days before the test began; this schedule was maintained until 48 h before the conditioned suppression test.
Days 1 to 4: Control (not Food-Restricted) and FR mice were subjected to training phase.
Day 5: Animals were exposed to light/shock conditioning.
Day 6: Mice were left undisturbed in their home cage.
Day 7: Animals were subjected to the choice check test, and then FR animals were returned to ad libitum feeding.
Day 8: Mice were left undisturbed in their home cage.
Day 9: Animals were subjected to conditioned suppression test.
Groups B: Two groups of FR mice, sham (sham FR) and norepinephrine (NE) depleted (NE depleted FR), were used.
Days − 4 to 7: Both groups were placed on a moderate food restriction schedule; this schedule was maintained until 48 h before the conditioned suppression test. Moreover, other two groups of animals were used to evaluate the effects of prefrontal NE depletion in control (nonfood-deprived) animals: Sham control and NE depleted control. Before the training phase started, mice were randomly assigned to one of the two groups (sham, NE depleted) and subjected to surgery. Both control-deprived and FR groups were subjected to surgery, and after 7 days they were used for behavioral test. From day −4 to 7, FR mice (sham, NE depleted) were subjected to food restriction procedure.
Days 1 to 4: Both Control and FR groups were subjected to the training phase.
Day 5: Animals were exposed to light/shock conditioning.
Day 6: Mice were left undisturbed in their home cage.
Day 7: Animals were subjected to choice check test, and then FR groups were returned to ad libitum feeding.
Day 8: Mice were left undisturbed in their home cage.
Day 9: Animals were subjected to the conditioned suppression test.
Study 2:
Two groups of mice, control and stressed (pre-exposed and FR), were used for both strains (C57Bl/6J and DBA/2 J).
Days − 24 to − 18: Stressed animals were pre-exposed to chocolate. This schedule was maintained for 7 days after that mice were left undisturbed in their home cage with ad libitum feeding.
Days − 15 to − 6: Stressed animals were placed on a moderate food restriction schedule. This schedule was maintained for 10 days after that mice were left undisturbed in their home cage with ad libitum feeding.
Days − 3 to − 2: Both control and stressed groups were briefly pre-exposed to chocolate.
Days − 1 to 0: Mice were left undisturbed in their home cage.

(continued)

Table 1
(continued)

Days 1 to 4: Both control and stressed groups were subjected to the training phase.
Day 5: Animals were exposed to light/shock conditioning.
Days 6 to 8: Mice were left undisturbed in their home cage.
Day 9: Animals were subjected to the conditioned suppression test.

3.5 Brief Pre-exposure to Chocolate

In study investigating gene-environment interplay in compulsive eating (study 2), two groups of C57 and DBA mice were exposed to chocolate using the same protocol 2 days before the conditioned suppression procedure started (for 2 days) to prevent unspecific novelty response to chocolate in the groups that were not subjected to the pre-exposure condition described above (i.e., control groups) (*See* Fig. 1; Table 1, study 2).

3.6 Selective NE Depletion in Medial Prefrontal Cortex

Surgery procedure in study 2 was carried out under anesthesia as previously described [58, 90, 91]. Animals were injected with GBR (15 mg/kg) 30 min before the 6-OHDA microinjection in order to protect dopaminergic neurons. Bilateral injection of 6-OHDA (1.5 μg/0.1 μl/2 min for each side) was made into the medial prefrontal cortex (coordinates: +2.52 AP; ± 0.6 L; −2.0 V with respect to bregma [90]) through a stainless steel cannula (0.15 mm outer diameter, UNIMED, Switzerland) connected to a 1 μl syringe by a polyethylene tube and driven by a CMA/100 pump (NE depleted group). The cannula was left in place for an additional 2 min after the end of the infusion. Sham animals were subjected to the same treatment but received intracerebral vehicle after GBR administration. Animals were used for behavioral experiments 7 days after surgery. NE and DA tissue levels in the medial prefrontal cortex were assessed by high-performance liquid chromatography, with electrochemical detection analysis, as previously described [90, 91], to evaluate the extent of depletion.

3.7 Apparatus for the Conditioned Suppression Test

In both studies reported [93, 142], the apparatus used for the conditioned suppression test was a modified version of the place conditioning apparatus (*See* Fig. 2); it consisted of two gray Plexiglas chambers (15 × 15 × 20 cm) and a central alley (15 × 5 × 20 cm). Two sliding doors (4 × 20 cm) connected the alley to the chambers. A Plexiglas cup (3.8 cm diameter) was placed in each chamber: in one, the cup contained 1 g of milk chocolate (Kraft); in the other, the cup was empty.

Acquisition of the conditioned stimulus CS (light)-shock association was established in a different apparatus (*See* Fig. 3) comprised of one, 15 × 15 × 20 cm Plexiglas chamber with a black and white striped pattern on two walls (to make it very different from

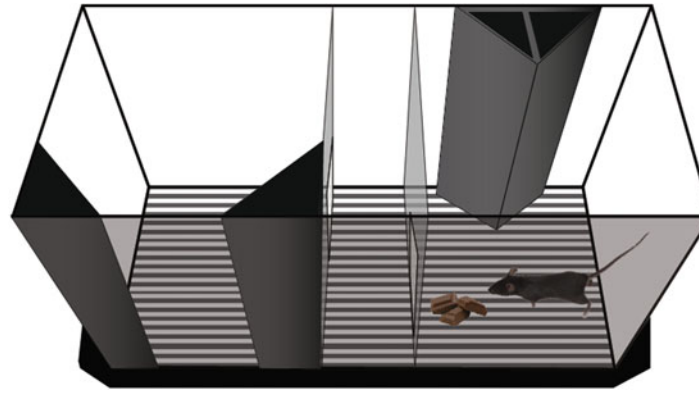

Fig. 2 Schematic apparatus used for training phase. The apparatus used for the conditioned suppression test was a modified version of a place conditioning apparatus [90, 91, 133]; it consisted of two gray Plexiglas chambers (15 × 15 × 20 cm), a central alley (15 × 5 × 20 cm), and black spatial patterns, with a stainless steel grid floor. Two sliding doors (4 × 20 cm) connected the alley to the chambers. In each chamber, two triangular parallelepipeds (5 × 5 × 20 cm) made of black Plexiglas and arranged in different patterns (always covering the same surface of the chamber) were placed to make it easier for the animals to distinguish the two chambers. A Plexiglas cup (3.8 cm diameter) was placed in each chamber: in one, the cup contained 1 g of milk chocolate (Kraft); in the other, the cup was empty. From days 1 to 4 (training phase), mice were placed individually in the alley; the sliding doors were opened to allow them to enter freely in both chambers and to explore the entire apparatus for 30 min. The time spent (s ± SEM) in each of the two chambers (i.e., the one with the cup containing chocolate and the one with the empty cup) and in the center was recorded throughout

the conditioned suppression apparatus) and with a stainless steel grid floor through which the shocks were delivered. The light was produced by a halogen lamp (10 W, Lexman), located under the grid floor, that was turned on for five, 20-s periods every 100 s; in each period, after the light had been on for 19 s, a 1-s, 0.15 mA scrambled foot shock was delivered. This session of light-shock association lasted 10 min and was followed by a 10-min rest period and then by another identical 10-min light-shock association session; overall, the mice received ten light-foot shock pairings in a 30-min session.

All experiments were carried out in experimental sound-attenuated rooms indirectly lit by a standard lamp (60 W). For all behavioral tests, data were collected and analyzed by the "EthoVision" (Noldus, The Netherlands), a fully automated video-tracking system [170]. The acquired digital signal was then processed by the software to extract "time spent" (in seconds) in the chambers, which was used as raw data for preference/aversion scores in each sector of the apparatus for each subject.

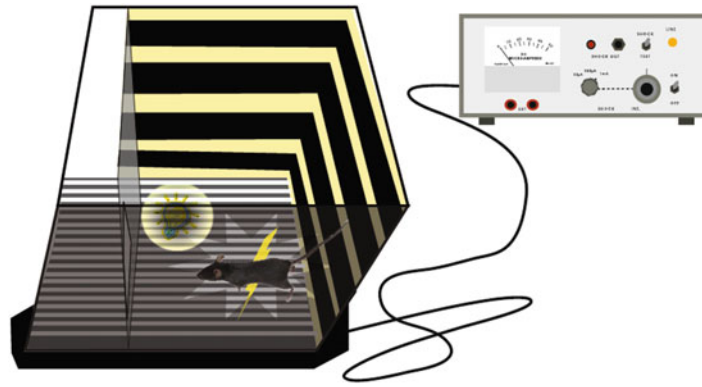

Fig. 3 Schematic apparatus used for light-shock pairing. Acquisition of the conditioned stimulus CS (light)-shock association was established in a different apparatus comprised of a 15 × 15 × 20 cm Plexiglas chamber with a black and white striped pattern on two walls (to make it very different from the conditioned suppression apparatus) and with a stainless steel grid floor through which the shocks were delivered. The light was produced by a halogen lamp (10 W, Lexman), located under the grid floor, which was turned on for five, 20-s periods every 100 s. In each period, after the light had been on for 19 s, a 0.15 mA scrambled foot shock was delivered for 1 s. This session of light-shock association lasted 10 min and was followed by a 10-min rest period and then by another identical 10-min light-shock association session; overall, the mice received ten light-foot shock pairings in a 30-min session

3.8 Chocolate Consumption and Body Weight

Chocolate consumption was assessed during the various phases of the conditioned suppression procedure (pre-exposure, training, test) by weighing leftover chocolate at the end of each session. Mice were weighed throughout the experiment, and analyses were carried out on critical experimental phases: the first day of the experiment (before the experimental procedure began), the training phase days, and the conditioned suppression test day.

3.9 Conditioned Suppression Procedures

For both studies, behavioral training took place from day 1 to day 4 (training phase), when mice were individually placed in the alley; the sliding doors were opened to allow them to enter both chambers freely and to explore the entire apparatus for 30 min (*See* Fig. 4). The time spent in each of the two chambers (i.e., the one with the cup containing chocolate and the one with the empty cup) and in the center was recorded throughout. The choice of the chamber containing chocolate was assessed by the time spent in it. On day 5, animals were exposed to light-foot shock pairings; acquisition of the conditioned stimulus (CS, i.e., light)-shock association was established in an apparatus with a visual-spatial pattern that was different from the one used for conditioned suppression (*See* Fig. 4). The light was produced by a halogen lamp located under the grid floor that was turned on for five, 20-s periods every 100 s; in each period, after the light had been on for 19 s, a 1-s, 0.15 mA scrambled foot shock was delivered. Overall, the mice

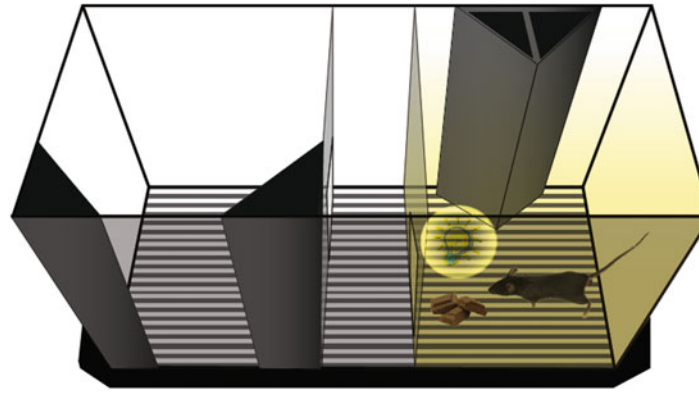

Fig. 4 Schematic apparatus used for conditioned suppression test. Conditioned suppression of chocolate seeking was assessed in a test session (day 9 of experimental procedure) that lasted 20 min in which the mice had access to chocolate in one of the two chambers in which chocolate had been placed during the training phase. In the chamber containing chocolate, CS (light) was presented according to the paradigm used for the light-foot shock association (except for the 10-min rest period, which was discontinued). Light was produced by a halogen lamp located under the grid floor and was turned on for 20-s periods every 100 s. This session lasted 20 min; overall, the mice received ten, 20-s periods in a 20-min session. The testing session began with the first 20-s period of light. The time spent in each of the two chambers, one containing chocolate and the other empty but "safe" (the chamber in which no conditioned threatening stimulus was present), was recorded throughout the session

received ten light-foot shock pairings in a 30-min session comprising a session of light-shock association lasting 10 min and a 10-min rest period followed by an identical 10-min light-shock association session.

On day 6, the mice were left undisturbed in their home cage. On day 7, the animals were subjected to the same procedure as in the training phase to evaluate whether the previous light-foot shock pairings would affect, in a nonspecific way, the choice of the chamber containing chocolate (Choice Check Test, only for study 1). The mice were then returned to ad libitum feeding to rule out any effect of dietary deficiencies on the conditioned suppression test day. On day 8, the mice were left undisturbed in their home cage. Finally, on day 9, the conditioned suppression of chocolate seeking was assessed in a test session that lasted 20 min during which the mice had access to chocolate in one of the two chambers as during the training phase (*See* Fig. 4). In the chamber containing chocolate, a CS (light) was presented according to the paradigm used for the light-foot shock association (except for the 10-min rest period, which was discontinued). The light was produced by a lamp located under the grid floor that was turned on for 20-s periods every 100 s. This session lasted 20 min; overall, the mice received ten 20-s periods in a 20-min session. The testing session began with the

first 20-s period of light. The time spent in each of the two chambers – the one containing chocolate and the other empty but safe one (the chamber in which no conditioned threatening stimulus was present) – was recorded throughout the session.

3.10 Dopaminergic and Noradrenergic Receptor Expression in Naive C57 and DBA Mice

In study 2 [142], baseline D1R and D2R receptor expression in the mpFC, NAc, and CP and alpha 1 noradrenergic receptors (α1R) in the mpFC was measured in animals subjected neither to environmental conditions (pre-exposure to chocolate, FR) nor to the conditioned suppression procedure (naive groups) in order to test the hypothesis that low striatal D2 receptor availability is a genetic risk factor for food compulsion-like behavior.

3.11 Dopaminergic and Noradrenergic Receptor Expression in Control and Stressed DBA Mice

In study 2 [142], α1R, D1R, and D2R receptor expression in three brain regions (mpFC [α1R, D1R, D2R], NAc [D1R, D2R], and CP [D1R, D2R]) was measured by Western blot in control and treated groups used in the conditioned suppression experiment.

4 Notes

4.1 Food Restriction and Different Stressful Conditions

We investigated whether the ability of a foot shock-paired conditioned stimulus to suppress chocolate-seeking behavior was reversed by a combination of previous food restriction experience and its combination with intermittent exposure to palatable food. However, the role of single variables should be assessed, and other stressful conditions could be employed to investigate whether our results may be generalized to different environmental conditions and acute or chronic stressful events.

Compulsive drug seeking has been shown only to emerge following an extended history of drug-taking [17, 18, 45]. In agreement with these data, we suggested that extended access to chocolate is able to transform adaptive food seeking/intake behavior into compulsive eating, depending on genotype [142]. Finally, our experimental procedure could be used to test relapse to compulsive-like behavior at different times from the end of the conditioned suppression test, proposing a withdrawal model for "abused" food.

4.2 Selective NE Depletion and Pharmacological Treatments

To test the hypothesis that prefrontal NE transmission plays a major role in maladaptive food-related behavior, we assessed the effects of selective norepinephrine inactivation in medical prefrontal cortex on a conditioned suppression test in stressed (calorie-restricted)

mice. However, our behavioral procedure could be used to investigate effects of many pharmacological treatments and to test the selective role of different neurotransmitters in specific brain areas. Moreover, genetically modified mice (mutant, transgenic, knockout) may be employed.

4.3 Chocolate Consumption and Body Weight

Chocolate consumption and body weight have to be assessed during the different steps of conditioned suppression experiments. Significant chocolate intake increase, together with increased chocolate seeking, during test phase can be considered like compulsion-like behavior.

During the training phase and test, a Plexiglas cup (3.8 cm diameter) was placed in each chamber: in one, the cup contained 1 g of milk chocolate (Kraft); in the other, the cup was empty. However, since animals tend to move cups, these have to be fixed on the floor to prevent mice to moving cups.

4.4 Criticisms

Our data indicate that excessive chocolate seeking observed in food-deprived mice was not determined by general motivation to eat, akin to hunger, but rather by a more specific motivational state, akin to craving. However, since in study 1 food-deprived mice were exposed to chocolate in the test apparatus while being food deprived (see experimental procedures), chocolate might be more rewarding in the food-deprived than in the control mice, thus making the food-deprived mice more motivated to consume chocolate during the final test. Moreover, conditioned suppression in previously food-restricted animals may involve an incentive learning process that allows the animal to assign an appropriate value to a reward that is modulated by its motivational states. This learning process is engaged when animals contact and experience the reward in the relevant state. Thus, exposure to chocolate during training, that is, when animals are still in food restriction, may have increased the perceived salience of chocolate due to the motivational state induced by feeding regimen that would lead to an increased value of the reinforce at the moment of test, that is, when animals are yet in free feeding for 2 days. We are currently evaluating this point testing animals many days after the end of food restriction and using different stressful conditions rather than food restriction. Further experiments will be carried out in order to assess this point.

4.5 Choice Check Test

An important point is to rule out any unspecific effects of food restriction or different manipulations that could affect either associative or mnemonic processes, on light-shock association. For this aim, a conditioned avoidance test or different associative tests can be used. In our study 1, the conditioned avoidance test was conducted like the conditioned suppression test, but there was no chocolate in either of two chambers.

4.6 Shock Sensitivity

Finally, it is needed to rule out differences in shock sensitivity between different strains or between different experimental groups subjected to environmental treatments. To rule out alterations in sensitivity depending on the genetic background, mice from the two inbred strains were tested for foot shock sensitivity. Individual mice were placed in the testing apparatus for a 1–2-min acclimation period; no background noise was presented during the testing period. Then, they received six series of six shocks (1 s), ranging from 15 to 150 μ A, delivered at 20-s intervals through the grid floor. The series of shocks were delivered in alternating ascending and descending order; the first series was in ascending order. Shock threshold was defined as the lowest shock intensity (μA) at which an animal's hindfoot left the grid floor. For each mouse, the mean value of shock thresholds recorded in each series was calculated.

5 Conclusion

Compulsive eating despite negative consequences is evident in some people who suffer from obesity and some eating disorders such as bulimia nervosa or binge eating disorder and is similar to the phenomenon observed with compulsive seeking/intake of drugs among addicted individuals. Because increasingly compulsive use of drugs in the face of well-known detrimental consequences is a critical behavioral feature of drug addiction, it has been suggested that compulsive overeating, particularly of refined foods, can be described as a food addiction.

Peculiar symptoms for feeding and eating disorders (FED) are reported in the last edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-V) where it is evidenced how pathological behaviors characterized by overconsumption could resemble those observed in patients with substance use disorders (SUDs). Shared behavioral manifestations include lack of control, impulsivity, tolerance, withdrawal, distress/dysfunction, overconsumption, and craving patterns.

Different theoretical frameworks, emphasizing a central role for specific food stimuli (food addiction) or behavioral response to them (eating addiction), have been proposed. These two approaches have so far resulted in different instruments, such as the AEBS and the YFAS, to evaluate the addiction-like eating/food addiction in humans.

Although they are needed, studies of gene/environment interactions in human eating disorders are very rare; to date, only a few animal studies have investigated the specific role of environmental and genetic factors (and their interaction) on the development and expression of continued food seeking/intake despite possible harmful consequences (i.e., an index of compulsion) in rats [13, 45, 141] and mice [93, 142]. We developed a new model of

compulsive eating in mice in order to test the hypothesis that environmental factors affect eating behavior depending on genetic background. Using C57BL/6 and DBA/2 mice, two very well-characterized inbred strains, we have found that extended access to chocolate can transform adaptive food-seeking and intake behavior into compulsive eating in C57BL/6 mice, which were previously shown to be more sensitive to drug effects than DBA/2 mice [133]. This study confirms that compulsive eating emerges following extended access to a highly palatable diet [45], similar to how compulsive drug seeking emerges following an extended history of drug-taking [17, 18] but only in genetically susceptible subjects [142].

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Chapter 4

Assessment of Binge-Like Eating Behavior in Mice Utilizing a Weekly Intermittent Access Paradigm

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Abstract

In humans, binge eating (BE) is central to the harmful effects of bulimia and binge eating disorder (BED). An estimated 30% of the obese population in the United States meets the diagnostic criteria for BED. Thus, BED is likely a major contributor to the current obesity epidemic. We developed a novel model to examine binge-like eating behavior in rodents that utilizes a schedule of 24-h weekly access to a highly palatable, nutritionally complete energy-dense diet (HED). This method for inducing BE has advantages over previous methods in that it does not require the use of exogenous stressors, caloric restriction, or entrained food anticipatory activity to induce the binge episode. Herein, we report that the BE response induced by this intermittent feeding paradigm can be maintained for at least 9 months in C57BL/6 mice. However, answers to a fundamental question remain. Can BE increase the risk of metabolic syndrome above and beyond the risk associated with obesity alone? Recent evidence in humans and rodents suggests that this may be the case. Given the high prevalence of BED in obesity, it is to be expected that there will be metabolic consequences of BE in this model and potentially in other BE models. However, the exact nature and if it is similar to that observed in frank obesity remains to be determined. We report on what is known about the metabolic consequences of long-term exposure to BE in mice with 24-h weekly access to an HED. While the changes we observed are subtle, over time they could have a significant impact on overall metabolism. Alterations in opioid receptor signaling pathways after repeated bingeing are discussed and may be one mechanism that links binge-like eating behavior with peripheral metabolism. Mice have particular advantages as a preclinical model mainly due to the sophisticated genetic techniques that are available in this species. Extensive characterization of the physiological, behavioral, and molecular changes associated with intermittent access to palatable diets will provide opportunities to identify and test novel therapeutic approaches to reduce BE and to understand its clinical translatability.

Key words Binge eating disorder (BED), Genetically engineered mice (GEMs), Intermittent access schedule, C57BL/6, Metabolic syndrome, Insulin resistance, Palatable energy-dense diets (HED), Energy expenditure, Adipose tissue, Kappa opioid receptor (KOR), Time domain nuclear magnetic resonance (TD-NMR), Nor-binaltorphimine (nor-BNI), Selective serotonin reuptake inhibitors (SSRIs)

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1 Introduction

Binge eating disorder (BED) is now recognized as a distinct disorder in the Diagnostic and Statistical Manual of Mental Disorders [1, 2]. BED is characterized by repeated brief periods of overeating in the absence of hunger and in the absence of compensatory behaviors such as purging. The estimated prevalence of BED is 3–5%, with a greater number of females being affected compared to males (3:2 females/males) [3–6]. It is clear that BED is a significant contributor to the current obesity epidemic in this country. At least 30% of the obese individuals in the United States meet the diagnostic criteria for BED, with a majority of these patients being morbidly obese. Binge eating (BE) may increase the rate of obesity by altering metabolism in such a way that causes more fat to be stored in the organism above and beyond what can be accounted for by caloric intake alone. Such obesogenic switches may hinder the ability to lose weight even when caloric intake is significantly reduced by dieting and could increase the risk for the development of metabolic syndrome, which is characterized by dyslipidemia, hyperglycemia, hyperinsulinemia, and decreased insulin sensitivity [7].

An outstanding question is whether BE increases the risk of metabolic syndrome above and beyond the risk associated with frank obesity. Recent studies have suggested that BE can increase the severity of metabolic syndrome and exacerbate complications associated with type 2 diabetes in humans [8–15]. Furthermore, the rate of BED has been reported higher (5.6–8%) in patients with type 2 diabetes compared to the overall prevalence of BED [8, 9, 11]. However, the mechanisms driving these changes in BED remain unknown. BE often occurs outside of normal scheduled meal times. Disruption in normal feeding patterns can be expected to alter circadian-like rhythms of hormonal release, including insulin from the pancreas and gut hormones such as ghrelin and the incretin glucagon-like peptide-1. These changes, along with changes in the central nervous system (CNS) that are driven by repeated BE on high-fat and high-sugar foods, could collectively alter glucose homeostasis, whole-body energy expenditure, and peripheral lipid metabolism [13]. Therefore, it is likely that BED can impact the progression of metabolic disease, and thus it is important to understand how these changes differ from that observed in frank obesity.

In order to identify the mechanisms driving BED and its metabolic consequences, we need reliable preclinical models with predictive validity. Many of the early rodent models for eating disorders utilized a history of food restriction, stress, and/or limited access to palatable diets that have varied in macronutrient composition. However, models involving exogenous stressors

have been challenging to reproduce consistently across different laboratories [16]. Therefore, stress-independent intermittent access models have been favored to induce binge-like eating in rodents. Several different paradigms had been previously utilized that have varied binge-food exposure times from 1 h daily to 2 h three times per week. However, shorter access intervals can lead to entrained food intake as has been observed in mice and rats [17–22]. For example, daily access for 1–2 h leads to entrained food intake behavior where the animal is observed to take in all of its daily calories during the 1–2-h access period even though chow is available the rest of the day and there is no change in overall 24-h caloric intake compared to controls [21].

The development of a clinically translatable model of BE behavior specifically in mice has been highly desirable to take advantage of the vast genetic toolbox available that can be utilized to understand the pathways and molecular mechanisms involved in BE. However, developing a mouse model of BE has proven to be difficult since minor perturbations can significantly inhibit food intake in this species [23]. This prompted us to develop a rapid and relatively simple model of BE in mice that does not require food deprivation, the application of exogenous stressors, or entrained food anticipatory activity [24]. This model utilizes weekly 24-h free-choice access to a nutritionally complete high-fat and high-sugar diet (HED) that is highly relevant since humans typically binge on foods that are a complex mixture of fats, sugars, and proteins. We have previously shown that mice will significantly reduce chow intake after access to the HED is removed. Moreover, it can take at least 5 days for mice to recover back to pre-binge chow intake. Thus, weekly access reduces the chance of entraining HED intake. Moreover, our weekly intermittent access schedule can be used to determine how more infrequent and "casual" BE behavior could progress to BED.

Over the years, this model has gained considerable acceptance, and genetic mouse models have been used to isolate the neural mechanisms that regulate BE behavior [25–28]. However, complex interactions between genetic manipulations, binge behavior, and metabolic adaptations could confound interpretations. Therefore, it is critical to consider these factors to understand its translatability. Many sophisticated techniques to probe the neural circuitry involved in BE behavior, including the use of optogenetics, are now available [29]. Herein, we describe a mouse model of BE behavior that uses a schedule of once weekly 24-h access to an HED in mice. We discuss key factors that can influence the model such as stress, sex, and genetic background. Evidence is also provided that several subtle metabolic changes, accompanied by changes in the expression of opioid receptors in the CNS, occur

with prolonged exposure to binge-like eating behavior. Furthermore, the potential impact of these observed changes on the development of metabolic syndrome is discussed.

2 Materials

2.1 Equipment and Setup

Before initiating any studies described herein, an animal-use protocol that is in accordance with the NIH Guide for Care and Use of Laboratory Animals must be approved by an Institutional Animal Care and Use Committee (IACUC). The studies shown here were approved by the Mayo Clinic in Arizona IACUC and were in accordance with the NIH Guide for Care and Use of Laboratory Animals. Mice should be acclimated to being individually housed for at least 1 week in a standard shoebox cage (approximately 435 cm^2). Nestlets or a similar nesting material should always be available for environmental enrichment and to reduce "shredding" of the food pellets. Mice are typically maintained in a temperature-controlled room (approximately 22–23 °C) with a 12:12-h light/dark cycle. HED should be offered during early light phase, a time period when mice are typically satiated and exhibit reduced locomotor activity. Mice should be reared on a standard low-fat chow diet with a fat content of $\leq$13% total calories from fat. Drinking water should be made available to the mice at all times throughout the experiment. A major consideration is whether or not there is an automatic watering source to supply the mice with water or if a water bottle is utilized. If there is an automatic watering source, then the standard metal cage tops will suffice. Otherwise, an additional divider should be vertically inserted into the food holder to separate the chow and HED. Alternatively, food hoppers that hang into the cage need to be used. Removable and autoclavable metal dividers may be custom generated by either the investigators, the manufacturers of the caging system, or a local machine shop. The location of the HED should be randomized so that one-half of the mice receive HED on one side of the divided food area or in the food hopper, while the others receive it on the opposite side. Lastly, care should be taken to reduce stress associated with transport and handling. A balance (0.01 g minimal sensitivity) for measuring food intake and body weight should be nearby to minimize the distance cages must be transported from their normal holding racks.

3 Methods

3.1 Weekly 24-h, Free-Choice Access Schedule

The procedure used to initiate and maintain BE behavior with once weekly access is outlined in Fig. 1. Adult mice should be randomized by body weight into one of three experimental groups. The chow-only and continuous HED access groups serve as control

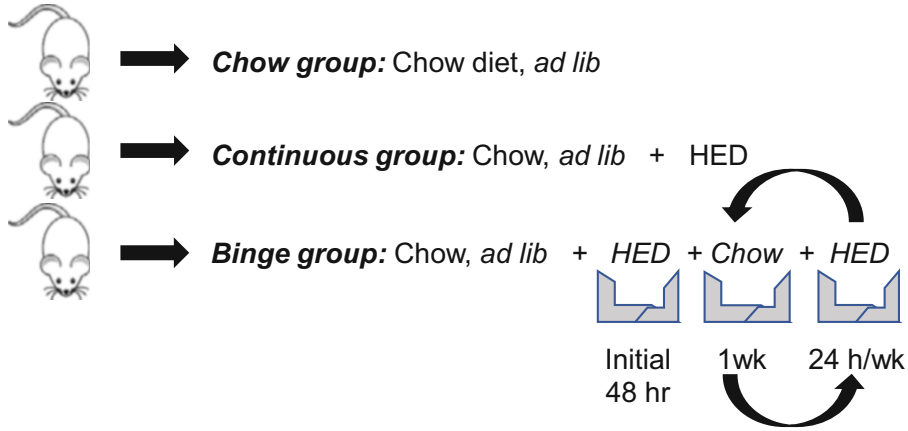

Fig. 1 Schematic depiction of experimental design to induce BE in mice. A 24-h weekly intermittent access induces BE in mice. The binge group receives an initial 48-h access to the HED and then 5 days of ad libitum chow only before access to the HED for 24 h. In successive binge cycles, mice in the binge group have free-choice access to standard chow for 6 days and HED once weekly for 24 h. A chow control group has ad libitum access to a standard chow diet, and a continuous access control group has ad libitum access to both the standard chow and HED

groups. Chow-fed mice receive continuous access to a standard low-fat chow diet (e.g., TD2014, 13% fat, 67% carbohydrate, 2.9 kcal/g, Teklad, Madison, WI). Mice in the continuous access group receive continuous free-choice access to both the standard low-fat chow diet and HED (e.g., Teklad 95,217, 39.8% fat, 16% sucrose, 4.3 kcal/g). Mice in the intermittent binge group receive an initial 48-h free-choice access to both chow and the HED to prevent neophobia to the diet. After 48 h, the HED is removed for 5 days, during which time only the standard chow is available. This provides sufficient time for chow intake to return to levels measured prior to HED access. The HED is then presented back to the rodent approximately 4 h into the light cycle, and intakes of both the chow and HED are monitored at 2.5 h and 24 h after HED presentation. The 2.5-h time point represents the most rapid rate of consumption and is defined as the BE period. The 24-h time point is used to monitor any compensatory changes in caloric intake that may occur following the BE episode. Following the 24 h of free-choice access to both chow and HED, the HED is removed from mice in the binge group completing binge cycle #1. For subsequent binge cycles, animals have 6 days of access to chow only (again to allow daily caloric intake values to return to "normal" levels), followed by another 24 h of free-choice access to both diets. It should be pointed out that animals in the binge group are never deprived of chow. A significantly reduced amount of chow intake is commonly observed following the removal of HED. However, the amount of chow intake is completely determined by the mice and not the investigator.

3.2 Data Analysis

Intakes should be normalized to body weight since mice that continually have access to HED gain significantly more weight throughout the study. Daily food intakes across two groups (e.g., chow versus the binge group) should be done with repeated measures ANOVA followed by post hoc analysis.

3.3 Experimental Variables

Other than measuring chow intake, daily manipulations are typically not required with this weekly 24-h free-choice access method. However, care should be taken in measuring food intake. The investigator should consider limiting the number of food pellets offered to each mouse to five to six full-sized pellets of each diet during the days that food intake is being monitored. This limits spillage and also facilitates weighing. Small and visibly eaten pellets should be discarded. Otherwise, the smaller pellets might fall through the grates of the lid and result in an overestimation of food consumption. Both chow and HED should be "refreshed" consistently across all groups to avoid the issues of palatability and novelty between fresh new pellets and stale older pellets. Refrigerated diets should be held at room temperature for 1 or 2 days prior to use to avoid issues with novelty associated with evaporation of moisture from the diet during warming. The composition of standard chow diet that is being offered should be reevaluated if significant HED consumption is not observed. In addition, 24–48-h initial exposure to the HED should occur 1 week prior to initiating limited access to HED.

Reduction in chow intake after removal of HED is inevitable. When utilizing the weekly 24-h access model in different genetic backgrounds, food intake should be monitored daily until their chow intake returns to pre-HED levels before each subsequent HED BE episode is initiated. The initial overconsumption of the HED during the light phase is not compensated for by decreasing nocturnal feeding; thus, 24-h caloric intake is elevated [24]. Interestingly, almost all the extra caloric intake in the binge group comes from consumption during the first 2.5 h after HED access. In addition, all of the calories consumed during the 24-h access period are from the HED.

In studies that include pharmacological intervention and behavioral testing, the timing of compound administration is important. At least one binge cycle should be completed before testing pharmacological agents. One can also test the drug before, during, or after the binge (i.e., exposure to the HED). Therefore, variations in the timing of compound administration may influence results depending on the mechanism of reducing HED intake. For all experiments, mice should be block randomized into treatment groups based on 2.5-h intake of the HED in the prior binge cycle to account for inherent preferences that might exist for the HED among a given cohort of mice. It is necessary to determine the question being addressed, e.g., preventing development of BE,

reducing intake by blocking initiation of the BE episode, or limiting HED intake during the test session. As an example, if we are trying to prevent development of BE, what would happen following withdrawal of the drug treatment? Under these conditions, one might expect that there is a rapid rebound response, or alternatively there may be a slow gradual rise in the intake of the HED.

4 Notes

4.1 BE Induced with Weekly Intermittent Access to HED Is Highly Durable in Mice

With this weekly access paradigm, we have been able to obtain reliable binge-like eating during the first binge cycle in both C57BL/6 and 129SvEv mice. When HED is present, 100% of the total calories consumed by mice in the binge group are from HED intake. Interestingly, as long as the HED is present, mice do not compensate by reducing their nocturnal intake. Thus, 24-h food intakes remain significantly elevated after access to the HED in the binge group. Typical food intakes within 2.5 h after receiving the HED average between ~0.8 and 1.2 g for 129SvEv and C57BL/6 mice, respectively [24]. During this time period, mice consume approximately 33% of their typical daily caloric intake (measured relative to the day prior to presenting the HED). A significant compensatory reduction in chow consumption in the 24 h following HED access in the binge group can be expected, and it takes several days for chow intake to return to pre-binge levels. These data have been recapitulated in several other laboratories [25–28]. We found that the BE induced with these methods is highly consistent when measured weekly for at least 16 weeks in C57BL/6 N mice and 37 weeks in C57BL/6 J mice (*see* Fig. 2). Both males and females display equally robust BE behavior during these time periods. Interestingly, total 24-h caloric intake may increase slightly over time. Thus, this model to induce BE is reproducible, and the durability of the behavior allows one to perform long-term studies with confidence that the binge-like behavior remains intact. We also found that BE is dose-dependently decreased by fluoxetine [24]. A subsequent study by Xu et al. [27] demonstrated that this effect is driven largely by the activation of serotonin 2C receptors on dopamine (DA) neurons. Genetic deletion of these receptors (5-HT_{2C} null mice) globally abolished the ability of fluoxetine to reduce BE, whereby re-expression of these receptors specifically in DA neurons significantly inhibited 2.5-h HED intake in mice in the intermittent access group. These data support the clinical relevance of this mouse model of BE, as fluoxetine has been shown to reduce binge frequency in human subjects with BED [30].

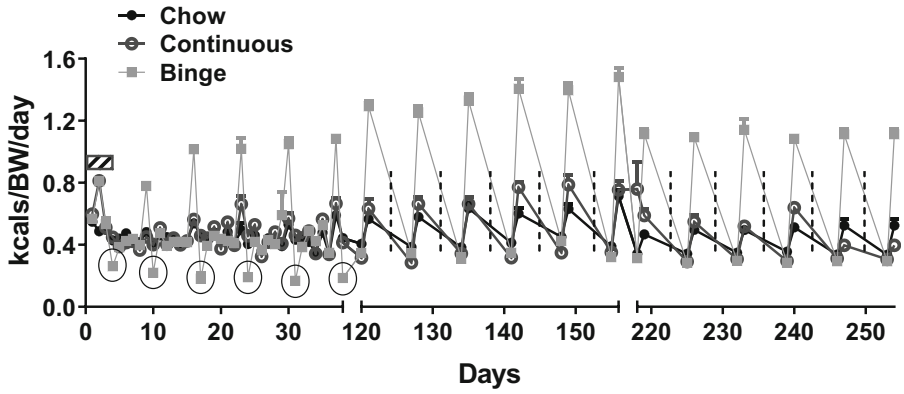

Fig. 2 BE behavior is consistent in mice over 37 weeks. (Left) Daily caloric intake (kcals/body weight/day) is shown for the first 6 weeks on the left side of the graph (days 1–38) in male and female (not shown) C57BL/6 J mice. The horizontal rectangle (hatched bar) indicates initial 48-h exposure to the HED. Chow intake is significantly reduced in the binge group in the 24 h after the HED is removed (highlighted with ovals). (Middle) Shown in the middle are weeks 18–23, with daily caloric intake for 2 consecutive days shown for each week corresponding to the day prior to the binge and the binge day for each week. Note that metabolic testing had occurred in this cohort of mice during this time period. This could have influenced caloric intake. (Right) The right side of the graph shows weeks 32–27. Stable binge eating was able to be obtained for at least 37 consecutive weeks. Dotted lines indicate break in food intake measurements until following week ($N = 9$–10 mice per group)

4.2 Long-Term Exposure to BE Leads to Changes in Energy Expenditure and Glucose and Lipid Metabolism in Mice

Our model of bingeing reflects a common ingestive pattern in modern societies where episodes of intermittent bingeing are followed by periods of self-induced caloric restriction. It is in essence a hedonic feeding paradigm in that food intake is not driven by hunger but rather the high palatability of the food and the unpredictability of its availability. Interestingly, a recent clinical study reported that females ended up consuming more of a comfort food (in this case, macaroni and cheese) when it was served only once a week compared to those that became habituated to it and consumed it every day [31]. Repeated "casual" BE (i.e., once weekly) might exaggerate obesity and the risk of diabetes by reducing energy expenditure and insulin sensitivity, as well as increasing adiposity, independent of total caloric intake or gross changes in overall body weight.

We initially observed that 6 weeks of binge-like eating did not grossly alter body weight or percent fat mass compared to chow-fed controls [24]. Furthermore, no changes were observed in circulating leptin or corticosterone levels at that time. We have now maintained male and female mice that have been bingeing well over 6 weeks to determine if longer exposure to BE would lead to observable metabolic changes. Intermittent bingeing on energy-dense foods negatively affected metabolism as early as 10–12 weeks after initiating binge cycles. Specifically, we found that male mice that had 12 weeks of intermittent access to the HED displayed [1]

increases in adiposity (*see* Fig. 3a), [2] elevated fasting blood glucose values (*see* Fig. 3b), and [3] decreased oxygen consumption compared to chow-fed controls (*see* Fig. 3c, d) even in the absence of significant changes in body weight (*see* Fig. 3e). In female mice, no differences in adiposity were observed, but a reduction in oxygen consumption was observed similar to males. Such metabolic alterations, even though subtle, suggest that both the pattern of ingestion and composition of the diet might interact to affect body weight, insulin sensitivity, and lipid metabolism. Collectively, these changes could lead to the worsening of metabolic syndrome in BED. One potential mechanistic sequela could be that BE leads to an impairment in the ability of adipose tissue to store fat resulting in ectopic lipid accumulation in insulin-sensitive tissues such as skeletal muscle and liver and followed by insulin resistance. Such impairments could be enhanced further by disruptions in the circadian release of hormones.

Accumulating evidence suggests that the gut microbiome plays an important role in the extraction of energy from the diet and its storage as fat [32–35]. One intriguing possibility is that BE could alter the gut microbiome and metabolism in such a way that energy extraction from the diet is enhanced leading to an increase in fat deposition and thus promoting obesity. Such a switch toward an obesogenic gut microbiome may hinder the ability to lose weight even when caloric intake is significantly reduced by dieting and thus would further contribute to metabolic syndrome. Changes in diet would be expected to alter the microbiome and lead to changes in absorption. Indeed, we have found alterations in intestinal absorption in bingeing mice using direct calorimetry methods to analyze fecal caloric content (*see* Fig. 3f, g). Although intriguing, changes in gut microbiome will need to be measured directly in these mice.

4.3 Practical Considerations in the Analysis of Metabolic Parameters

Given the clinical relevance of metabolic disease to BED, it is suggested here that investigators working with preclinical models of BE perform some basic analysis of metabolism when establishing new models or when expanding on studies involving existing BE models (e.g., addition of exogenous stressors plus weekly intermittent access). Body weight is a minimum measurement that should be taken at least weekly. If changes in body weight are observed, further studies should investigate body composition. It is appreciated that most investigators outside of fields directly studying metabolism may find it a daunting task to measure metabolic changes in vivo. However, there are several steps an investigator can take to perform some basic measurements. One can consider collaborations with local investigators. In addition, investigators can take advantage of internal or external fee-for-service arrangements including the NIDDK metabolic cores [36]. While it would be logistically more challenging to ship mice as the stress of transport could significantly impact both the behavior and metabolic

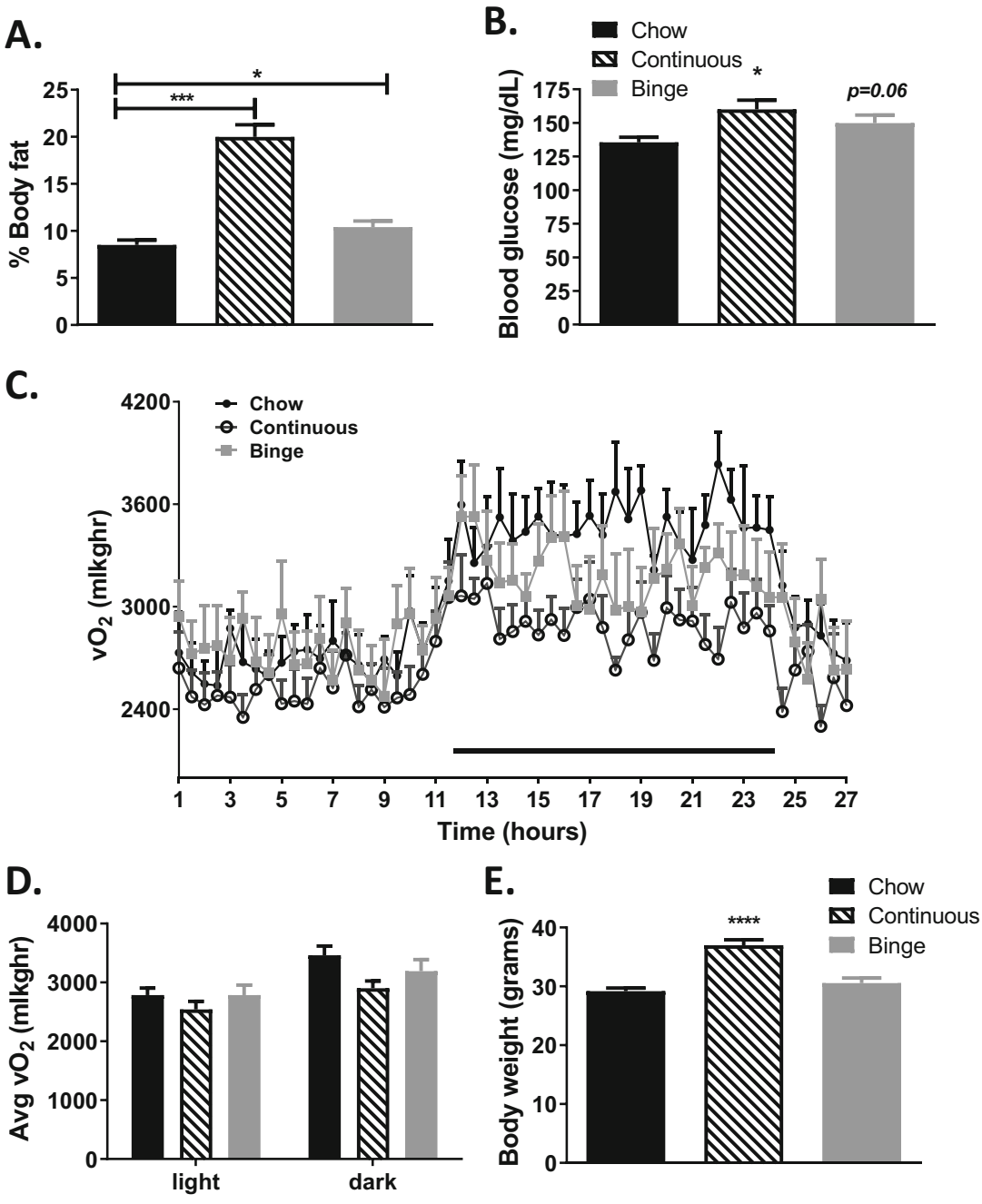

Fig. 3 Repeated weekly intermittent access to an HED induces subtle changes in metabolic physiology in male C57BL/6 mice. (**a**) Body composition analysis with TD-NMR shows that the relative content of body fat was significantly increased in the binge group. Mice are C57BL/6 J mice after 21 weeks of either continuous or 24-h weekly access to HED (ANOVA, Tukey's post hoc test, *** $= p < 0.0001$, * $p = 0.03$, unpaired t-test). (**b**) Fasting blood glucose levels trended to be elevated in male C57BL/6 N mice after 10 weeks of 24-h weekly access to HED ($p = 0.06$, unpaired t-test compared to chow). All measurements were taken 1 week after the last binge indicated, and thus caloric intakes had returned to chow control levels at the time of measurement. (**c**) vO_2 plotted over 24 h in C57BL/6 N mice after 12 weeks of 24-h weekly access to HED. Mice in the continuous group had reduced vO_2 levels during the dark phase (indicated by black bar). Mice in the binge

physiology, it is a more feasible option to send tissue and serum to obtain measurements of hormones such as leptin and ghrelin or lipids. Time domain nuclear magnetic resonance (TD-NMR) is a rapid and reliable way to measure whole-body composition of fat and lean mass in non-anesthetized mice. The purchase of such an instrument could be cost prohibitive for a single lab. However, one could weigh individual fat pads. Typically, three major fat pads (reproductive, inguinal, and retroperitoneal) are dissected out and weighed [37]. The individual fat pad weights are added together and expressed as a percentage of body weight and can be used as a surrogate for overall fat mass. Typically, a standard glucometer that can be purchased at a pharmacy is used to measure blood glucose levels from a single drop of blood from the tail vein. Multiple sampling can be performed during glucose and insulin tolerance tests using this method [38]. Total blood volume taken should not exceed IACUC guidelines.

There is a complicated interaction between caloric restriction, stress, and access schedule of energy-dense diets that must be

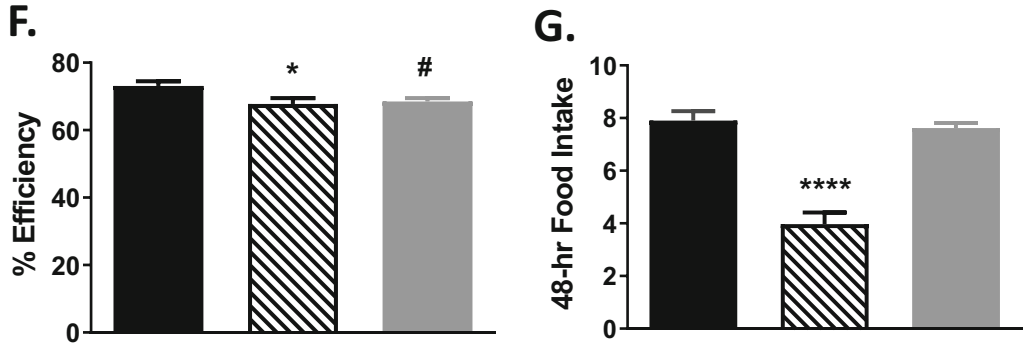

Fig. 3 (continued)

Fig. 3 (continued) group exhibited an intermediate phenotype even though locomotor activity was significantly elevated in this group during the dark phase compared to both chow and continuous groups (not shown). (**d**) Shown is the average 24-h vO_2 consumption from C in the light and dark cycles. (**e**) Body weights from mice in C show no significant differences between chow and binge groups. (**f**) Shown is nutrient assimilation as determined by direct calorimetry methods to measure total fecal caloric content. (**g**) Food intake during the 48-h fecal collection period. Fecal caloric content is normalized to the amount of food intake to obtain % efficiency in (**f**). All groups were fed a pulverized chow diet at the time of fecal collection. Percent efficiency decreased similarly in both mice with a history of BE for 12 weeks and those that previously had continuous access to an HED (i.e., diet-induced obese mice). These data support the hypothesis that when obese mice are switched to a "diet" of lower calorie food, they actually are less efficient at extracting calories from that food source which in turn might be working against net weight loss. BE might have a similar "obesogenic" effect (ANOVA, Tukey's post hoc test, ****$p < 0.0001$, *$p = 0.05$, #$p = 0.09$). Panels a–e, $N = 8$–10 mice per group. Panels f and g, $N = 6$ mice per group

4.4 Factors Impacting BE Behavior and Potential Effects on Metabolism

4.4.1 Stress

considered when evaluating BE in mice. Both stress and food deprivation can quickly lead to physiological changes which, by themselves, likely alter food intake. Even short periods of food restriction can reduce circulating leptin levels, increase hepatic gluconeogenesis, reduce energy expenditure, cause hyperlocomotion, and increase circulating corticosterone levels [39–42]. Furthermore, there are significant limitations with stress models since stress is variable and its physiological consequences can be dependent on animal husbandry conditions, vendor sub-strains, and other environmental variables that are difficult to control across institutions. Lastly, while baseline levels of circulating corticosterone in bingeing mice appear to be similar to chow controls [24], these observations were done at single time points shortly after a binge episode. However, the release of this stress hormone is regulated in a circadian manner, and it is currently not known whether chronic BE disrupts the normal temporal patterns of corticosterone release or how responses to acute stressors may be altered. At least three reported attempts have been made to induce BE with exogenous stressors and have involved using a combination of stress and food restriction [23, 43, 44]. These models had severe limitations in reproducibility, and one model employing exposure to an unpredictable daily stressor ultimately led to a reduction in overall food intake.

It is well known that BED in humans is associated with a history of early life stress. It is also known that both dieting and stress can precipitate BE episodes. The 24-h weekly intermittent access model described here allows one to study the mechanisms driving the initiation and maintenance of BE behavior without the confounds of caloric restriction or exogenous stress. Thus, this model is well suited to test how glucocorticoids may be involved in the initiation and maintenance of BE and can influence its metabolic consequences. Indeed, we and others have utilized this model to demonstrate that exposure to both prenatal and postnatal stress maintains BE behavior and exacerbates metabolic syndrome in mice [45, 46].

4.4.2 Sex

The prevalence of BED in females is reported to be higher than in males. While social factors may reduce the likelihood that males will report BE as problematic, it is also possible that female binge eaters suffer more negative consequences from bingeing and thus are more likely to seek treatment. Furthermore, it is not known how sex-specific differences in stress responses could differentially influence BE in males and females. Removal of a high-fat diet after 12 weeks increased BE during subsequent periods of its intermittent access in female but not male mice [47]. Ovariectomized mice exhibit normal BE behavior consuming ~1.2 g of HED during the first 2.5 h of access. 17β-estradiol treatment in these mice significantly reduced HED intake [25]. Thus, while it is clear that sex hormones can influence BE, it is unknown how this impacts the

observed increase in BED in females. Interestingly, Schroeder et al. [26] found that females that had been exposed to stress prenatally exhibited increased BE. Thus, there are complex interactions between sex hormones, environmental factors, and bingeing that impact metabolism, but they have yet to be determined.

4.4.3 Genetic Background

Strain-dependent effects must be taken into account when investigating models of BE in mice. We found that both C57BL/6 and 129SvEv mice will binge using the methods described herein [24]. However, it should be noted that 129SvEv mice have an overall lower caloric intake compared to C57BL/6 mice. Other inbred strains may behave differently in these paradigms, and the response of each strain needs to be empirically determined. Background strain can also influence macronutrient preference and should be considered when selecting an appropriate mouse strain to study [48–52]. For example, the obesity-prone C57BL/6 strain will consume more fat when presented with a choice of protein-, carbohydrate-, and fat-rich diets compared to the obesity-resistant C3H/Hej [49] and 129/J [52] inbred strains. In addition, known differences in metabolism among the strains must be taken into account. For example, C57BL/6 J mice have a naturally occurring deletion in the gene encoding nicotinamide nucleotide transhydrogenase leading to glucose intolerance [53]. Thus, it is likely that there will be strain-dependent effects of BE behavior and on the observed metabolic outcomes.

Investigators should be cautious when working with genetically engineered mice (GEMs) until caloric intake studies are performed and baseline results compared to appropriate wild-type control mice. Wild-type littermates are the best controls to account for age and rearing conditions. When investigating GEMs, it is important to discern the effects of the genetic mutation on preference for specific macronutrients. For example, ghrelin receptor knockout (KO) mice consume less fat regardless of whether it is presented continuously or intermittently [54]. If a reduction of BE occurs in the GEM, one should test whether these mice have a preference for one or more of the three macronutrients. Other factors to consider are overall reductions in 24-h chow intake, energy expenditure, regulation of gastrointestinal and neuroendocrine mechanisms, and general locomotor activity levels in the individual GEM.

4.4.4 Species

This 24-h weekly access paradigm has been observed to elicit BE behavior in both mice and rats. However, we have observed that while all mice binge, not all rats do. We and others have noted significant variation in HED intakes in rats using this paradigm and that they can be grouped further as having binge-prone and binge-resistant phenotypes [55–57]. The advantage of mice is the homogeneity in their response, small size, and the use of sophisticated

genetics including knockout and transgenic models and the ability to temporally and spatially restrict gene expression. However, one could argue that the heterogeneity observed in rats is more similar to humans and that they would be a better model to study the mechanisms driving both the behavior and its impact on metabolic outcomes. This remains to be determined.

4.4.5 Other Factors

Several additional factors should be considered including housing conditions (group vs. single housed), macronutrient content of chow diet which often differs across institutions, and source of fat in the HED (i.e., lard vs. vegetable fat). Group housing has the advantage of reducing cage number and reducing stress associated with social isolation. However, it is nearly impossible to measure individual food intake when group housing conditions are used. Lastly, the age of mice studied should be considered as changes in gonadal hormones over time can influence both behavior and metabolism [58].

4.5 Long-Term Binge Behavior Causes Changes in the CNS that Can Drive Metabolic Outcomes: Opioid Receptors May Be Key Modulators of Both BE Behavior and Metabolism

The consumption of an HED high in fat and sucrose increases extracellular DA levels in the nucleus accumbens (NAcc) [17, 20, 59, 60]. It is expected that pathways that control hedonic feeding behavior overlap with those involved in substance abuse. Indeed, a significant comorbidity with substance abuse exists in patients with BED [61, 62]. Mu (MOR), kappa (KOR), and delta (DOR) opioid receptors and their endogenous ligands β-endorphin, dynorphin, and enkephalin, respectively, are abundant throughout brain regions known to be responsible for reward. Within the NAcc, it is now generally accepted that these opioid receptors control hedonic feeding behavior including the (over) consumption of highly palatable foods [63]. We and others have shown that activation of opioid receptors contributes to the development of obesity specifically during prolonged consumption of high-fat calorically dense diets [64–69]. Furthermore, these receptors appear to play distinct roles, and KO mice lacking each of these genes are protected from many of the negative metabolic consequences of prolonged high-fat diet exposure.

Since binge foods are often enriched in fat and sugar, it is likely that activation of opioids can drive at least part of the food intake during BE. Indeed, there are several lines of evidence that suggest opioid receptor pathways do regulate BE behavior in both rodents and humans. Importantly, the increases in NAcc DA release in response to consuming a nutritionally complete, high-fat diet can be blocked by administration of the pan-opioid antagonist LY255582 in both mice and rats [20, 70]. Interestingly, MOR and KOR have opposing actions on DA levels in the brain. Whereas MOR agonism generally causes euphoria associated with increased DA levels, KOR agonists cause dysphoria associated with decreased DA levels. This has added significantly to the complexity of

understanding how opioid receptors control BE. Moreover, it is unknown how these receptors (which are also expressed in several metabolically relevant peripheral tissues including brown adipose tissue and pancreas) may influence metabolism in the context of BED.

Genetic studies in mice have not yet given definitive answers as to how opioid receptors may modulate BE. We previously reported that while MOR and DOR KO mice exhibited no differences in BE, KOR KO mice had increased 2.5-h HED intake compared to wild-type control mice when utilizing the 24-h weekly intermittent access model described here [70]. A gain-of-function variant of the MOR allele (A118G) was found to be present more frequently in patients with BED [71]. However, this allele when expressed in mice did not alter BE behavior in an intermittent access model (30 min of access to sweetened fat diet consisting of hydrogenated vegetable shortening +10% sucrose 3 days a week) [72]. Since the A118G single-nucleotide polymorphism was expressed in the context of the mouse MOR sequence, it is possible that there are species-specific differences in how this variant affects MOR function. Furthermore, only female mice were tested. Thus, species- and/or sex-specific effects of this variant may account for the observed differences.

In preclinical models, increases in KOR signaling positively regulate both depressive-like and drug-seeking behaviors [73]. This has led us to the hypothesis that repeated BE leads to an upregulation in KOR signaling and that blocking KOR may reduce BE in mice. Indeed, we found increases in mRNA expression of KOR and the gene encoding its ligand prodynorphin in the dorsal striatum (but not in the hypothalamus) after 15 weeks of bingeing (*see* Fig. 4) . While these changes were not statistically significant, they could reflect more robust alterations at the level of protein expression and receptor signaling. Furthermore, we found that a 48-h pretreatment with the long-acting KOR antagonist nor-binaltorphimine [74] could reduce BE when administered alone and may enhance the ability of fluoxetine to reduce BE (*see* Fig. 5) . These data collectively support that opioid receptors may be upregulated after chronic bingeing and that pan-opioid or KOR-specific antagonists may be useful alone or in combination with selective serotonin reuptake inhibitors (SSRIs) to reduce binge eating. Furthermore, alterations in opioid receptors both in CNS and the periphery represent at least one potential mechanistic link between the maintenance of BE behavior and the metabolic changes observed with chronic BE.

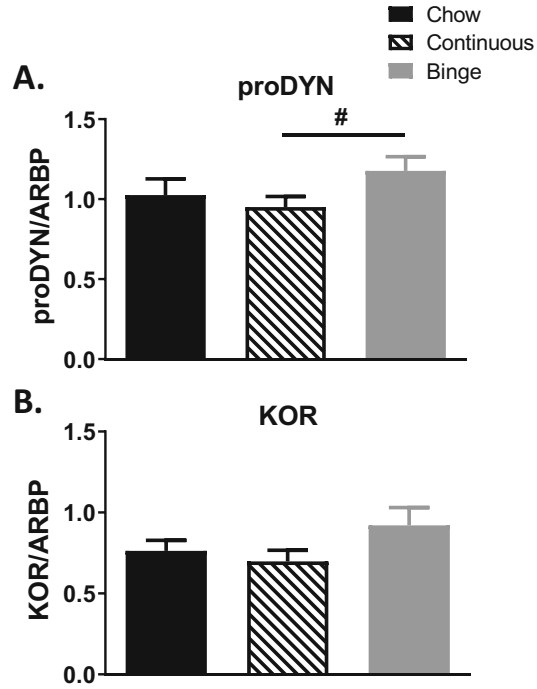

Fig. 4 qPCR analysis of prodynorphin (proDYN) and KOR in the dorsal striatum of C57BL/6 N mice with 15 weeks of prior binge exposure. (**a**) Prodynorphin (proDYN) and (**b**) KOR mRNA levels were elevated (n.s.) in the dorsal striatum of male mice with a history of binge-like eating behavior. There were no differences in expression of these same genes in the hypothalamus (not shown) suggesting that there are region-specific changes in transcription after repeated BE. Data were normalized to the ARBP housekeeping gene. $N = 5$ per group. (#$p = 0.08$, unpaired t-test)

5 Conclusion

A 24-h weekly intermittent access to an HED allows one to obtain reproducible BE behavior that can be maintained consistently for several months. Chronic BE behavior is likely to lead to alterations in metabolism that are independent of changes in total caloric intake since mice with intermittent access reduce their food intake for several days following the HED access period. There are several adaptations in both the CNS and periphery with BE that occur which could significantly alter metabolic physiology and likely include the complex regulation by opioid receptor signaling pathways. Important considerations include strain effects on macronutrient preferences and baseline metabolism and sex-specific differences. The changes that occur both in the brain and on whole-body metabolism could significantly affect therapeutic outcomes. Thus, it is important for BED therapies to address the long-

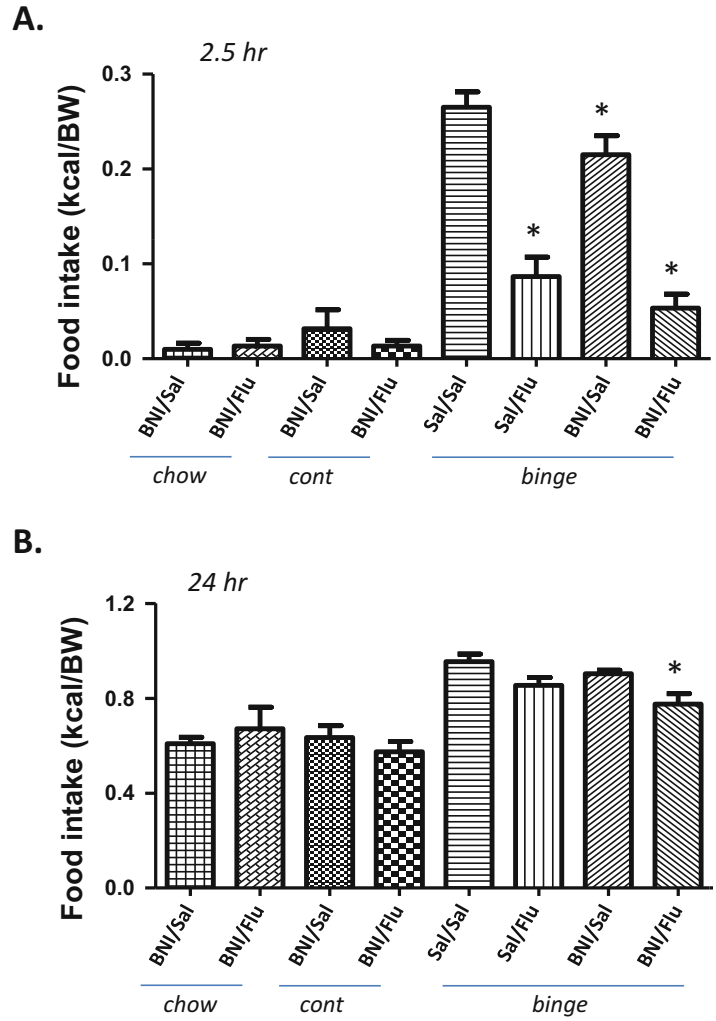

Fig. 5 Treatment with the long-acting KOR antagonist nor-binaltorphimine (nor-BNI) can reduce BE behavior. (**a**) Shown are 2.5-h food intakes after reinstatement of the HED on binge cycle #1 in female mice. All mice in the binge group had an initial 48-h exposure to the HED the week prior to testing. Food intake was significantly reduced at this time point after 20 mg/kg IP fluoxetine (1-h pretreatment, Sal/Flu) or 10 mg/kg IP nor-BNI (48-h pretreatment, BNI/Sal) treatment. The combination treatment with fluoxetine and nor-BNI also significantly reduced food intake 2.5 h after HED reinstatement. (**b**) Only the combination of norBNI and fluoxetine resulted in significant reductions in 24-h food intake suggesting an interaction between KOR and serotonin signaling pathways in this binge model. Data were analyzed with one-way ANOVA and Dunnett's post-test. *$p < 0.05$ vs. binge Sal/Sal. $N = 6$/treatment

term changes that occur after bingeing in both the CNS and in peripheral organs. Since a history of early life stress is associated with BED, and knowing that dieting and stress are also precipitating factors inducing BE in humans, future studies should focus on

the metabolic outcomes in mice with a history of stress and BE. It is predicted that the interaction between stress and BE may result in more robust metabolic phenotypes that may be distinct from what is observed in frank obesity. The above should guide future research with this BE model and others, with careful consideration to translational caveats. Consideration of metabolic consequences of new and existing models of BE is imperative as this field is arguably in its infancy and we need to better understand the translatability of individual preclinical BED models. Collaborations among BED and metabolic researchers will be key in advancing our understanding of BED and how this disease may be contributing to the rapid rise in metabolic syndrome worldwide. Furthermore, it may provide opportunities for novel therapeutic interventions in BED patients.

Acknowledgments

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Chapter 5

Predicting and Classifying Rats Prone to Overeating Fat

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Abstract

The availability and overconsumption of palatable foods rich in fat are contributors to the worldwide epidemic of obesity. With environmental factors and genetic predisposition both playing important roles in this serious health problem, it is crucial to recognize and properly treat individuals who have a greater propensity to eat excessive amounts of fatty foods. Animal studies have been instrumental in this regard, allowing researchers to identify and examine early factors that are associated with and can predict future fat consumption and obesity. The methods for classifying and subgrouping these animals focus on early behavioral and physiological measures. Herein, we describe three of these measures, namely, fat intake over 5 days, novelty-induced locomotor activity, and fat-induced triglyceride levels, which have been successfully used to identify adult outbred rodents that are prone (i.e., have higher propensity) to overeating fat. The early identification of animals prone to overeating fat, in conjunction with tests to characterize their specific phenotype, can yield valuable information regarding the underlying behavioral, physiological, and neurochemical pathways involved in driving excessive fat intake, ultimately leading to an obese state.

Key words Fat consumption, Obesity, Behavioral prediction, Locomotor activity, Triglycerides

1 Introduction

The consumption of palatable foods, particularly those rich in fat, has increased dramatically during the last several decades [1] and is one contributing factor to the worldwide obesity epidemic [1–3]. Such unhealthy eating patterns may be a result of environmental factors, such as greater availability of high-fat junk food, and also polygenic factors that predispose individuals to overconsume or gain weight on fatty foods [4–7]. Clinical studies have suggested that food preferences and feeding patterns, symptoms of hyperactivity, and dyslipidemia at a young age are associated with and can identify individuals that are prone to overeating and gaining weight later in life [8–11]. It is especially important to identify these "predisposed" individuals prior to or during very early stages of overeating. This early identification can help to initiate proper intervention prior to the onset of chronic metabolic disturbances,

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such as obesity and heart disease, and also to understand the disruptions in behavior or even neurochemistry that may be driving such maladaptive processes.

Studies with rodent models have been particularly informative for understanding the inherent forces driving unusually high consummatory behavior. Outbred rat strains such as Sprague-Dawley (SD) have been especially relevant for understanding mechanisms of human feeding behavior. Similar to humans, SD rats exhibit genetic heterogeneity and show a wide spectrum of behavioral and physiological outcomes, allowing them to be subgrouped based on their inherent differences in these measures. Using outbred SD rats as a model, studies have identified distinctive characteristics in animals prone to consuming high amounts of fatty foods and in some cases gaining excessive weight on these foods. Predictive indicators of such behavior include (1) consumption patterns during initial exposure to a high-fat diet [12–15], (2) novelty-induced locomotor activity prior to diet exposure [16], and (3) circulating triglyceride levels (TGs) following consumption of a fat-rich meal [17]. From an evolutionary perspective, animals showing these three inherent traits – preference for calorically dense foods (early consumption patterns), enhanced novelty-seeking (locomotor activity), and increased storage of energy (TG response to fat) – are more likely to survive in the wild when food is scarce. However, when fat-rich foods are abundantly available, these behavioral and metabolic traits are maladaptive and can lead to chronic pathological states, such as obesity. The goal of this chapter is to describe, in detail, the methods and procedures for classifying animals, based on the predictor measures of early consummatory patterns, locomotor activity, and fat-induced TGs, as being prone (having an increased propensity) to overeating fat.

2 Materials

2.1 Animals

To examine patterns of fat consumption, researchers have used a variety of rodent strains, including those that are outbred or selectively bred, as well as mouse lines that are genetically altered or inbred [12, 13, 18–22]. For translational purposes, however, we consider that the outbred animal for several reasons may be the most appropriate model of human fat overconsumption, as illustrated in our studies of feeding behavior in SD rats [12, 13, 16]. These animals, typically purchased from Charles River or Taconic Laboratories, are easy to handle and are genetically heterogeneous, thus more accurately representing the individual genetic variability demonstrated by humans. Most importantly, some of these animals naturally overconsume a high-fat diet relative to a standard diet even at initial exposure, and as a group they exhibit

considerable variability in their intake, which allows them to be rapidly and easily classified as higher or lower volume fat consumers [12, 13, 16].

2.2 Diet Recipe

To examine natural patterns of fat consumption, different types of high-fat diets have been employed by various laboratories, including those rich in animal fat, such as lard [13], or vegetable fat, such as Crisco All-Vegetable Shortening® [23], as well as a palatable junk food diet, such as one including Oreos® [24]. From our experience using a lard-based high-fat diet, we find that the optimal amount of fat for accurate prediction of future fat intake is 30–60% [13]. The majority of our feeding prediction studies have used a diet composed of 50% fat, containing a total of 5.2 kcal/g of diet (*see* Table 1). This diet has three macronutrient constituents, 50% fat composed of 75% lard (Armour Star, Peoria, IL) and 25% vegetable oil (Crisco, Orrville, OH); 25% carbohydrate from 30% dextrin (ICN Pharmaceuticals, Costa Mesa, CA), 30% cornstarch (ICN Pharmaceuticals, Costa Mesa, CA), and 40% sucrose (Domino Foods Inc., Yonkers, NY); and 25% protein from casein (Bio-Serv,

Table 1
Composition of a 50% high-fat diet

Fat	
Lard	230
Vegetable oil	50
Carbohydrate	
Dextrin	97
Cornstarch	98
Sucrose	130
Protein	
Casein	325
Vitamin mix	30
Mineral mix	40
Total weight (g)	**1000**
Energy density (kcals/g)	5.15
% Macronutrient kcals	
Fat	50
Carbohydrate	25
Protein	25

Reprinted with permission from Ref. [16]
Ingredients expressed by weight (g)

Frenchtown, NJ) and 0.03% L-cysteine hydrochloride (ICN Pharmaceuticals, Costa Mesa, CA). This solid diet is supplemented with minerals (USP XIV Salt Mixture Briggs; ICN Pharmaceuticals, Costa Mesa, CA) and vitamins (Vitamin Diet Fortification Mixture; ICN Pharmaceuticals, Costa Mesa, CA) to make it nutritionally complete.

2.3 Open Field Activity Chambers

For activity measures, animals are tested in an activity chamber. Historically, the apparatus used for these measurements in rats was a 182.9 cm by 182.9 cm box constructed from plywood painted black with the bottom composed of 16 equally sized squares, and scoring was performed manually. However, activity measurements can now be made using more advanced systems, involving infrared beams or video tracking. The computerized open field activity chamber that is commonly used consists of a Plexiglas box (43.2 × 43.2 cm) with a white floor, opaque walls, and infrared photocells which detect both vertical and horizontal movements (e.g., MED Associates, St. Albans, VT, USA). Video tracking (e.g., EthoVision from Noldus, Leesburg, VA, USA) can also be used, which monitors and analyzes behavior, movement, and activity in any arena. These latter two approaches provide more precise analyses of locomotor behavior and also track other activity-related behaviors. With the use of parameters such as vertical counts and time, distance traveled, average velocity, and stereotyped behavior, these systems can more accurately detect behaviors related to exploration and affect.

2.4 Lipid Determinations

For determination of circulating lipid levels in serum samples, an enzyme kit from Sigma-Aldrich (St. Louis, MO) is commonly used. The Serum Triglyceride Determination Kit utilizes free glycerol reagent, triglyceride reagent, and a glycerol standard. The assay involves enzymatic hydrolysis of serum triglycerides to glycerol and fatty acids. The glycerol undergoes several enzymatic reactions, resulting in a quantifiable color change that is directly proportional to the triglyceride concentration of the sample. The color change is detected using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, USA) set at a wavelength of 540 nm.

3 Methods

3.1 Predicting Fat Intake Based on Initial Consumption Patterns

Several important earlier studies examining patterns of fat consumption demonstrated that initial fat preference and weight gain on a high-fat diet had a strong relationship with long-term consumption of fat, body weight gain, and body fat accrual [25–27]. Building on these phenomena, subsequent publications from our lab and others have successfully utilized measures of early preference for fat and initial weight gain on a high-fat diet to

identify animals with a greater propensity to overconsume a high-fat diet over the long term and to gain more weight and accumulate heavier fat deposits [13, 14, 21, 28]. These studies, performed in SD rats, have demonstrated that animals preferring fat over the other macronutrients, carbohydrates or proteins, are more likely to eat greater amounts and gain more weight on a high-fat diet. Likewise, animals that gain the most weight over the first 1 or 2 weeks of high-fat diet exposure have an increased propensity to develop an obese-like state when allowed to consume this diet for several months.

In more recent articles, a modified version of the fat preference or weight gain model has been used with rats to more easily and accurately classify them based solely on their initial consumption of (rather than preference for or weight gain from) a fat-rich diet [12, 16]. Using this protocol, animals can be subgrouped into high-fat consumers (HFC) and controls based on their initial intake of a high-fat diet consisting of 50% fat. Specifically, after a 1-week acclimation period to laboratory conditions, chow intake is monitored for 3 days, and then the high-fat diet is provided as a 15 kcal high-fat meal over 3 consecutive days in addition to the daily chow diet, allowing all subjects to learn to consume the entire high-fat meal at least once by the end of the third day of exposure. After this acclimation period, the chow is then removed, and the animals are allowed to consume the high-fat diet ad libitum for 5 days, with fat intake and body weight measured daily. Animals are then rank ordered based on their fat intake, with the top third forming the HFC group, which consumes about 35% more daily calories with the high-fat diet than with chow, and the bottom third forming the control group, which tends to consume equal calories from both diets. This separation of groups is consistent with a cluster analysis of intake under similar conditions in mice, which shows that individual subjects naturally exhibit a near tertile distribution [20].

In order to obtain accurate measures of feeding behavior, stressful environmental factors should be kept to a minimum, and measurements should be consistent from day to day, with the animals maintained on a 12-h light-dark cycle. For at least 1 week prior to diet exposure, they should be acclimated to standard housing conditions and handled daily. They should also be acclimated to the diets themselves prior to ad libitum feeding in order to avoid effects of neophobia. To prevent the diets from spoiling or becoming unpalatable, they should be prepared fresh at least once a week and kept refrigerated until served, and the animals should be given fresh diet each day. Measurements of food intake should be recorded daily at the same time each day, preferably immediately after the end of the dark cycle.

Using the tertile-split classification procedure, HFC rats have been shown to be more prone over the long term to consuming excessive fat, during both chronic high-fat diet access and at re-exposure after a 2-week withdrawal from the diet [12]. When

maintained on the high-fat diet chronically, HFC rats ultimately gain more weight and develop larger fat pads [12]. This model is also validated by other studies in rats and mice showing similar measures of energy intake and weight gain to be accurate indicators of long-term patterns of fat consumption and mild obesity [13, 15, 21, 28–31].

3.2 Predicting Fat Intake Based on Activity Measures

Locomotor activity has been closely associated with food seeking and can be particularly high immediately prior to scheduled meals of palatable food, a phenomenon referred to as "food anticipatory activity" [32, 33]. Several studies have related high activity levels to the consumption of palatable foods and other reinforcing substances [16, 34–36], suggesting that locomotor activity may serve as a good indicator of future patterns of fat intake.

Locomotor activity is generally tested in specialized open field activity chambers (described above), in which either an observer blind to the study (if scoring manually) or a computerized program or video tracking system records behaviors related to horizontal and vertical motion. Testing should be carried out during the animals' waking time (for rodents, this is the dark cycle), when baseline activity is highest [37]. Depending on whether animals have been acclimated to these chambers or are experiencing them for the first time, the behavioral analysis can be used to determine, respectively, general locomotor activity or novelty-induced locomotor activity. Several published articles have suggested that novelty-induced locomotor activity is a particularly reliable predictor of subsequent intake of reinforcing substances, including fat [16, 38–40], and a high locomotor response to novelty has been found to be a significant predictor of vulnerability to addiction [41, 42]. An important factor to consider for novelty-induced locomotor activity is the duration of the test. Optimally, activity measures should be examined for the first 5–30 min of exposure to the arena, a time range when the novelty of the environment appears to have the greatest impact [37, 43, 44]. The specific measurement frequently employed to represent locomotor activity is either line crossings when scored manually or ambulatory distance when measured by automated programs. For novelty-induced locomotor activity, an additional measure of number of rearings (manual) or vertical counts or time (automated) can be recorded.

Using the measure of novelty-induced line crossings to represent novelty-induced locomotor activity, SD rats have successfully been characterized as HFC and controls [16]. With this protocol, each rat prior to any fat exposure and in the middle of the dark cycle is placed in the center of the open field, and the number of lines crossed is recorded for a minimum of 5 min, with the placing of both front paws and torso into a new square counted as a line crossing. The apparatus between the tests is thoroughly cleaned

with disinfectant and allowed to dry. After activity testing, animals are given the high-fat diet for classification into HFC or controls as described above. These initial measurements of line crossings are found to be greater in HFC rats that are prone to overconsuming fat over the long term, suggesting that this measure of novelty-induced locomotor activity may itself be a strong predictor of future fat consumption [16]. Novelty-induced hyperactivity is also found to be strongly and positively correlated with consumption of ethanol and cocaine, suggesting that this behavioral trait may be important for identifying animals prone to overconsuming an array of reinforcing substances [16, 38, 45, 46].

3.3 Predicting Fat Intake Based on Meal-Induced TG Levels

Circulating lipid levels such as serum TGs, which are elevated by the consumption of a high-fat diet [17, 47, 48], are a good indicator of metabolic activity and efficiency. In the body, these lipid molecules are either stored in adipose tissue for future use or are broken down for immediate energy in the form of fatty acids that can produce a feeling of satiety [49]. When they accumulate in serum, however, they may signify inefficient metabolism and a disruption in energy homeostasis [50], much like high levels of glucose in serum following a glucose tolerance test can indicate the presence of diabetes [51]. Based on this information, it is likely that animals showing elevated levels of TGs after a fatty meal find this diet to be less satiating and therefore go on to overconsume the diet. This principle has been supported in animal studies showing that, compared to a low-fat meal, a meal high in fat content that markedly elevates TG levels also leads to hyperphagia in a subsequent test meal [52]. In light of this idea that exaggerated fat-induced TG levels may be an indicator of metabolic inefficiency and reduced feeding satiety, these lipid levels may serve as a valid predictor of future fat overconsumption as well as obesity.

According to published work, measurements of fat meal-induced TGs can be used to predict increased caloric intake and dietary obesity in rats [17]. In order to use TGs to identify these prone animals, SD rats are first acclimated to standard housing conditions and trained to consume a high-fat meal in a manner similar to the high-fat acclimation for ad libitum consumption. The meal is 15 kcal of a 50% lard-based high-fat diet (*see* Table 1), given for 30 min each day over 3–4 days until all subjects have eaten the entire high-fat meal at least once. This should occur at dark onset since animals are more likely to consume this meal during their early waking hours. After this training, animals are tested for their TG response to fat. Chow is removed prior to dark onset in order to prevent nonspecific food intake, which could interfere with TG measures, and also to motivate animals to consume their entire test meal prior to blood sampling. Animals are then exposed to a 15 kcal meal of high-fat diet (once daily over 3 nonconsecutive days), and tail vein blood is collected 1 h after each exposure, for

measurement of serum TG levels using the Serum Triglyceride Determination Kit described above. Based on their average fat meal-induced TG levels, animals can then be rank ordered, with the top third representing the predicted fat overconsumers and the bottom third being their lower volume fat-eating (control) counterparts.

Using this approach, a high-TG response to fat can reliably identify animals with a greater propensity to consume excess amounts of fat during chronic access and subsequently exhibit certain metabolic disturbances. This model differs from the 5-day fat intake and activity models described above, as it classifies animals based on their TG response to test meals of a high-fat diet, which is a metabolic rather than behavioral parameter. While the animals characterized as high-TG responders consume similar amounts of daily high-fat diet (117 ± 2.6 kcal) as the HFC animals described above (124 ± 3.5 kcal), it is not clear if they in fact represent the same subgroup of animals. However, with TG levels also increased in the HFC rats identified based on their 5-day fat intake, it is likely that animals predicted based on their initial consummatory patterns and TG response to fat are indeed the same subgroup of prone rats. Other support for the TG prediction model comes from studies demonstrating that elevated fasting TG levels predict future weight gain [53], lipid-lowering drugs reduce food intake in obesity-prone rats [54], and fat-induced TG levels predict level of ethanol drinking [38, 46].

3.4 Experimental Variables

When using 5-day fat consumption to identify HFC rodents, there are two main variables to monitor, namely, diet texture and day-to-day consumption. Since this prediction is based on the animals' consumption of this diet over a short 5-day period, it is important to keep identical the ingredients and texture of the diet for each animal. While lower-fat diets may have a more powdered texture, a 50% high-fat diet should have a completely smooth consistency. This smooth consistency allows the diet, for the 3-day acclimation, to be made into a small 15 kcal ball that is placed directly into the cage and, for the period(s) of ad libitum feeding, to be kept in a metal bowl or glass jar. This minimizes spillage which, when unaccounted for, can lead to incorrect rank ordering of animals and thus unreliable results. It is important to keep the fat concentration near 50%, since animals find this amount highly palatable and therefore overconsume it in the short 5-day time period. Whereas a higher-fat diet of 60%, shown previously to predict long-term fat consumption and weight gain, may also be used [13, 28, 30], more recent studies have found a 45–50% fat diet, which is closer to the human diet, to yield reliable and replicable results [12, 16, 35]. Aside from the diet texture, it is important to carefully examine and correlate animals' fat consumption each day. Animals should only be subgrouped once their day-to-day consumption is stable, as indicated

by a positive daily correlation of >0.60 as in previous publications [12, 16]. Lack of a stable consumption pattern could signify a problem either with the animal's health or with the diet consistency and therefore should be further examined prior to classifying animals as prone to overeating.

When using novelty-induced locomotor activity to classify animals as fat overconsumers, measurements of activity should be made in a uniform and standardized manner, and careful attention should be paid to environmental stressors. Standardizing the measurements is especially important for when recordings are made manually, as some behaviors may be difficult to capture and track with the human eye. For example, line crossing should be counted only if an animal has both paws and torso in a new square, and rearing behavior should be counted only if the animal is fully on its hind paws for a minimum of 2 s. If the observer has any doubts about these measures, a videotape can be made of the test session, and analysis of the behavior can be performed later, based on the recording. Due to their ability to reduce or completely eliminate experimental bias, automated systems (computerized program or video tracking) are now strongly preferred. Also, since anxiety can mask increases in novelty-seeking behavior, it is important that animals are well handled and, when tested in the activity apparatus, are placed into it gently.

When using circulating TG levels as a predictor of fat consumption, variables involving high-fat meal consumption must be well controlled. It is important that the animals consume the entire 15 kcal meal within the first 30 min of exposure and that the tail vein blood is collected for TG measurements within 1 h of fat access. To ensure that this timing is consistent for each animal, there are three steps that should be taken. First, animals must be trained to expect the meal at the same time each day and therefore wait to consume this palatable fatty food. Second, animals should be food deprived for some time (1–4 h) before being given access to the high-fat meal, to increase motivation to consume the meal quickly. Third, prior to collection of tail vein blood, each cage should be thoroughly checked for any remaining high-fat diet, as animals that do not consume their daily meal should not be included in the analysis. If these three steps are correctly followed, elevations in fat-induced TG levels can accurately and reliably identify animals prone to fat overeating.

4 Notes

4.1 Anticipated Results

Results from a typical experiment using two of the discussed predictors of fat consumption, initial consumption patterns and meal-induced TG levels, are shown in Tables 2 and 3. From the first table (*see* Table 2), it is clear that animals characterized as HFC show a

Table 2
Caloric intake and body weights of high-fat consumers (HFC) compared to controls during different periods of chow and high-fat diet access

	Control	HFC
Intake (kcal/day)		
Chow (before high-fat diet)	91 ± 6	89 ± 4
High-fat diet (5 d)	95 ± 4	121 ± 10*#
High-fat diet (2 wk)	94 ± 5	118 ± 10*
Body weight (g)		
Chow (before high-fat diet)	302 ± 10	300 ± 8
High-fat diet (5 d)	371 ± 9	392 ± 12#
High-fat diet (2 wk)	427 ± 15	475 ± 19*#

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Data are mean ± S.E.M. *$p < 0.05$ vs. control; #$p < 0.05$ vs. chow

Table 3
Measures of meal size, daily intake, body weight, and adiposity hormones in subgroups differentiated by their HF-induced TG levels

	Low-TG responders	High-TG responders
Chow test meal		
Meal size (kcal)	5.7 ± 0.5	12.4 ± 1.8*
Daily intake (kcal)	78 ± 2.2	75 ± 1.3
Body weight (g)	444 ± 7.7	453 ± 5.4
4 weeks on HF diet		
Body weight (g)	505 ± 7.0	545 ± 12*
Daily intake (kcal)	101 ± 1.8	117 ± 2.6*
Feed efficiency (kcal/g)	0.21 ± 0.01	0.23 ± 0.01
Fat pad weights (g)	31 ± 2.1	38 ± 3.5*
Leptin (ng/ml)	8.0 ± 0.4	11 ± 1.1*
Insulin (ng/ml)	5.3 ± 0.2	6.1 ± 0.5

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*$p < 0.05$ for comparisons between high-TG and low-TG responders

significant increase in fat consumption within the first 5 days of ad libitum high-fat diet access, an effect that persists for an additional 2-week period and is still evident (data not shown) after re-exposure to the diet following a 2-week gap without it. This increase in fat consumption is accompanied by greater body weight

in the HFC rats, also shown in a previous publication using a similar prediction model [13, 28].

The data presented in Table 3 describe the typical characteristics of animal subgroups predicted by their fat-induced TG levels. These data demonstrate that animals that show a higher TG response to a high-fat meal go on to consume more chow in a subsequent test meal and, after 4 weeks on a high-fat diet, have greater body weight, consume more calories each day, have larger fat deposits, and show disturbances in circulating adiposity signals. These two data sets provide important information regarding the use of early consumption patterns and fat-induced TG levels to predict which animals are prone to overeating fat and beginning to develop more chronic metabolic disturbances.

4.2 Troubleshooting

In order to obtain reliable and reproducible results using the three predictors of fat intake described above (initial consumption, novelty-induced locomotor activity, and serum TG levels), it is important to consider the following points:

4.2.1. For the early consumption predictor model, animals should be properly adapted to the high-fat diet, with a minimum of three daily exposures. This will prevent the occurrence of neophobia when the diet is introduced ad libitum, for the test period. While rare in occurrence, animals that do not adapt well to the high-fat diet, as measured by a lack of interest in this diet upon its presentation into the home cage and by a failure to consume the entire 15 kcal meal at least once during the training days, should be omitted from the study.

4.2.2. For the early consumption model, it is also critical that measurements of daily fat intake during the 5-day prediction period are taken at the same time each day. In order not to disturb the animals from eating their daily meals, these measurements should be performed during the light cycle, when animals are more likely to be resting. Since animals may vary in their individual patterns of feeding behavior, taking a measurement either during a time of high consumption or low consumption may provide unreliable results and therefore reduce the reliability of this model.

4.2.3. For the novelty-induced locomotor activity predictor, a standardized measurement system for recording activity is essential. This is especially important if an automated system is not available, and data are recorded manually. The observer must have a uniform way to measure locomotor activity. It is best that a single individual be assigned to take measurements, as animals may change their behavior when

exposed to different researchers. Again, due to their ability to reduce or completely eliminate experimental bias, the use of an automated system (computerized program or video tracking) is strongly encouraged.

4.2.4. Since rodents are naturally anxious, the room conditions during testing should be well controlled. For example, during novelty-induced locomotor activity testing, it is important that the testing room is set at a temperature between 23 and 25 °C. The room should also be noise- and light-controlled. This can be achieved either by having a constant low-level background noise or complete silence in the room and by having on a continuous red light in the room. Once the animal is placed in the chamber, the observer should stand at an angle where the animal cannot see them. If using an automated program, the researcher if possible should leave the room immediately after placing the animal into the activity test chamber.

4.2.5. For the fat-induced TG model, it is important to collect the tail vein blood for TG measurements at exactly the same time on the test days. Circadian fluctuations in TGs may contribute to variability in results and incorrect subgroupings. It is additionally important not to wait too long after the meal to collect the blood samples, since serum TG levels may be cleared from plasma or broken down with prolonged time periods. To capture the immediate TG response to fat, blood should be collected within 1 h of high-fat meal exposure.

4.2.6. For the fat-induced TG model, animals should be removed from the analysis if they fail to completely consume the 15 kcal meal on test days and/or if sufficient blood cannot be obtained for analysis. Since the measure of TG levels is meant to reflect the efficiency of metabolizing the same-sized fatty meal, it is critical that the animals be faced with the same fat challenge. Additionally, blood plasma must be obtained from tail vein blood on each test day in a sufficient volume to test samples in triplicate in the Serum Triglyceride Determination Kit, to ensure reproducibility.

5 Conclusion

In summary, the procedures described above for using initial consumption patterns, locomotor activity, and circulating lipid levels have successfully and reliably been used to classify animals prone to overeating a palatable high-fat diet. These models are well supported by clinical literature in which early patterns of food consumption, hyperactivity, and dyslipidemia have been associated

with a greater probability of consuming excess amounts of fat or developing obesity later in life [9–11, 55]. In animal studies, these predictive measures have been instrumental in allowing researchers to probe the neurochemical mechanisms underlying excessive fat intake and also obesity. For example, by thoroughly examining animals identified as prone to overconsuming fat, several studies have shown that these animals also exhibit elevated levels of orexigenic hypothalamic peptides, suggesting that disturbances in these central systems may contribute to an overeating phenotype [12, 13, 16, 17]. With these unique patterns in behavior, physiology, and neurochemistry occurring either prior to exposure or during very early periods of consumption, it is important to understand how similar inherent traits could lead to human overindulgence in palatable fatty foods and thus increase the propensity to become obese.

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Chapter 6

Psychosocial Stress and Dietary Environment Promote Emotional Feeding in Female Rhesus Monkeys

Vasiliki Michopoulos, Kelly Ethun, and Mark E. Wilson

Abstract

One proposed contributor to the recent surge in obesity prevalence is the increased availability of highly palatable foods coupled with the drive to consume these foods under stressful conditions. Studies of humans suggest that stress exposure promotes increased caloric intake and a preference for energy dense foods, and this may be particularly true for women, as they more often show higher rates of obesity and report a higher incidence of emotional feeding relative to men. Socially housed female rhesus macaques provide a unique, ethologically relevant model for studying the effects of psychosocial stress on appetite within varying dietary environments. Macaque groups, regardless of size, are organized by a matrilineal dominance hierarchy that functions to maintain group stability. Lower ranking animals receive more aggression from higher ranking group mates and terminate these interactions by emitting submissive behavior. Subordinates have less control over their environment, and continual harassment from dominant animals results in dysregulation of the limbic hypothalamic pituitary adrenal (LHPA) axis. Metabolic and anthropometric phenotypes differ between dominant and subordinate monkeys when maintained on a standard low-fat, high-fiber laboratory diet, as dominant females are more often heavier with greater fat and bone mass. Recent studies, using validated automated feeders, suggest that under conditions of a low-caloric-density diet (LCD), subordinate monkeys consume similar calories but are more active during the daytime relative to dominant monkeys. However, once a highly palatable, high-caloric-density diet (HCD) is added to the LCD environment, subordinate females become significantly hyperphagic and exhibit significant increases in fat mass within a 2-week period. More recent long-term studies of the effects of psychosocial stress and dietary environment show that access to an HCD impacts reward pathways similar to what has been shown in obese humans. Future studies are warranted to explore the chronic effects of psychosocial stress on appetite within a rich dietary environment analogous to that of humans.

Key words Psychosocial stress, Social subordination, Diet choice, Monkeys, Emotional feeding

1 Introduction

As of 2009, approximately 73 million adults in the United States were obese, representing 28% of the total population and an increase of 7% over obesity prevalence in 2001 [1]. Additionally, 34% of adults in the United States were classified as overweight [2]. While obesity can be explained in biological terms as the

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consequence of prolonged positive energy imbalance (i.e., energy intake exceeding energy expenditure), a number of complex environmental and social factors affect both sides of this equation. One proposed contributor to the increase in weight gain is the availability of highly palatable foods rich in calories from fat and sugar coupled with the drive to consume these foods under stressful conditions [3–5]. Emotional feeding often results from exposure to stressors [6], and attempts to lose weight often fail [7] because feeding behaviors become disinhibited and people overeat in response to emotional states [8]. Furthermore, psychopathologies whose etiologies stem from chronic stress exposure are highly comorbid with eating disorders, as well as stress-induced obesity [9, 10].

Any perceived situational or environmental threat elicits a physiological stress response from the limbic-hypothalamic-pituitary-adrenal (LHPA) axis. Under normal conditions involving an acute (short-duration) stressor, corticotropin-releasing hormone (CRH) is released from the hypothalamus triggering the subsequent release of adrenocorticotropic hormone (ACTH) from the pituitary, which enters systemic circulation to induce the release of cortisol from the adrenal glands. As a glucocorticoid, cortisol mobilizes energy stores allowing an individual to respond appropriately to the stressor. Following cessation of the perceived stressor, the LHPA axis is capable of turning itself off via a negative feedback mechanism that attenuates the release of cortisol and facilitates a return to allostasis [11]. These acute perturbations in physiology occur with every insult, and the constant hassles and repetitive struggles associated with everyday life can result in chronic stress exposure and a subsequent dysregulation of the LHPA axis [12]. Exposure to such chronic stress can lead to an array of highly comorbid adverse health outcomes [13, 14].

Because the health [15] and economic burdens [16] imposed by obesity are enormous, effective programs to prevent or alleviate its impact on society are a high priority. However, studies of humans assessing how exposure to chronic stress interacts with the dietary environment to influence eating behavior employ a limited degree of experimental control. To effectively study the causative nature of this relationship, an appropriate animal model of chronic stress exposure is necessary. While infection, injury, and other physical stressors can directly activate the LHPA axis, it is the psychogenic component of chronic exposure to social and environmental stressors that is critical to the prolonged activity of the LHPA axis [17–20]. Rodent models of chronic stress have shown that feeding resulting from chronic exposure to stressors is a probable contributor to excess food intake [21–24]. However, these models elicit hormonal and behavioral responses that are unique to the particular type of stress employed in a laboratory setting, and in many cases, animals adapt to stressors as evidenced by extinction of

hormonal and behavioral responses [25–30]. Thus, it is important to focus on stressors that are likely to be shared by human populations, namely, prolonged psychogenic, uncontrollable, unpredictable stress [31–33], when investigating the biobehavioral effects of stress as they relate to the development of human disorders.

Another important consideration for the study of stress-induced disruptions in feeding behavior that is often ignored in animal models is gender. In humans, individuals suffering from eating disorders [34, 35], including emotional feeding and affective disorders [36–40], are most often women. Given this clear sex difference in humans surrounding the adverse effects of chronic stress exposure on feeding and affect, there is a dearth of animal models of chronic stress in females as most models reported in the literature use males. However, socially housed female macaques provide a translational model to study the effects of psychosocial stress on a range of health outcomes in women, including emotional feeding.

2 Materials and Procedures

2.1 Chronic Psychosocial Stress Exposure in Female Rhesus Monkeys

The organization of socially housed rhesus monkeys (*Macaca mulatta*) is a matrilineal linear dominance hierarchy that functions to maintain group stability, regardless of group size [41]. Any individual animal's rank within the social hierarchy is enforced both by contact aggression and by the threat of aggression or harassment from higher ranking animals [41–44]. Lower ranking animals terminate these agonistic encounters by emitting submissive behaviors. Thus, lower social rank is defined by an animal emitting an unequivocal submissive act toward another group mate [41–44]. In social groups comprised of five adult females, a model previously used to study the effects of subordination on physiology and behavior, females ranked 1 and 2 are typically categorized as dominant, while those of ranks 3, 4, and 5 are considered subordinate [45–52]. Figure 1a shows characteristic differences in agonistic behavior in females living in five-member groups and shows the categorization of social status.

Subordinate status also results in reduced control over an individual's social and physical environment [53]. This exposure to daily stressors leads to long-lasting physiological alterations in the functioning of the LHPA axis that are similar to those associated with stress-induced disorders in humans. Subordinate macaques have enlarged adrenal glands [54], show diminished glucocorticoid negative feedback as assessed by a dexamethasone suppression test (*see* Fig. 1b), and exhibit diminished cortisol reactivity in response to ACTH administration (*see* Fig. 1c) [55]. Thus, the psychogenic, uncontrollable, unpredictable nature of the imposition of social rank in macaque social groups leads to a dysregulation of the

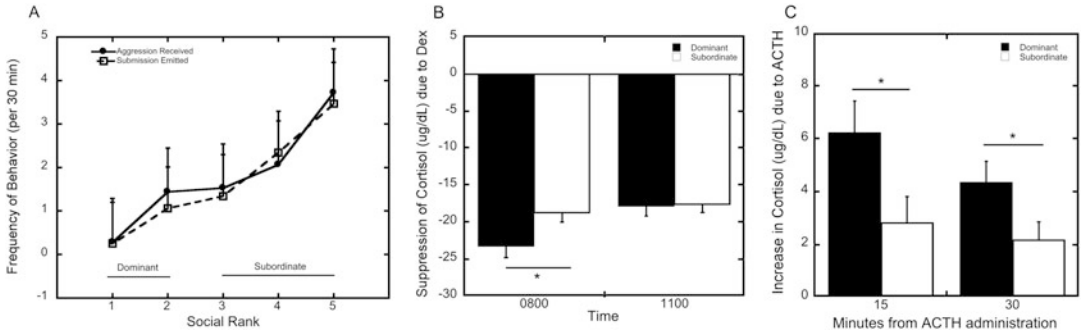

Fig. 1 (**a**) Mean ± SEM rates of agonistic behavior for animals categorized as dominant (ranked 1 and 2) and subordinate (ranks 3 through 5). Dominant females receive less aggressive behavior (closed square) than those categorized as subordinate, while subordinate animals emit more submissive behaviors (open square) than dominant animals. (**b**) Mean ± SEM change of serum cortisol at 0800 and 1100 h following dexamethasone administration at 1750 h the evening before for dominant and subordinate females. Asterisk denotes significantly greater decrease in cortisol in dominant compared to subordinate females. (**c**) Mean ± SEM change in serum cortisol 15 and 30 min following ACTH administration for dominant and subordinate females. Asterisks denote significant status differences in increases of cortisol levels. (Reproduced with permission from Ref. [55])

LHPA axis, providing a unique model to study how chronic psychosocial stress and dysregulation of the LHPA axis affect behavior and physiology as it relates to human health [46, 48, 56–63].

2.2 Social Subordination as a Model of Stress-Induced Alterations in Feeding Behavior in Women

Implementation of an automated feeding system that monitors continuous food intake in socially housed monkeys at the Yerkes National Primate Research Center has provided the opportunity to quantify food intake in socially housed monkeys [64] and assess how varying dietary conditions influence food intake and energy balance differentially in dominant and subordinate monkeys.

2.2.1 Availability of a Standard Laboratory Diet

When fed a standard laboratory monkey diet, low in sugar and fat and high in fiber [58, 65], subordinate females weigh significantly less than dominant animals (*see* Fig. 2a) . This decreased body weight is associated with reduced fat, but not lean mass (*see* Fig. 2a). Additionally, subordinate females have lower levels of circulating leptin and insulin and higher levels of adiponectin compared to dominant females [65]. The reduced body weight in subordinate females corroborates findings of diminished body weight in rodents exposed to repeated restraint stress [66, 67], chronic variable stress [68], or exposure to a predator [69]. These results in rodents are consistent with other data from rodents showing that exposure to chronic stressors [70, 71] or CRH administration [72–75] attenuates intake of these low-calorie, laboratory diets.

The lower body weight and fat mass in subordinate animals could be due to lower intake of the LCD, greater energy

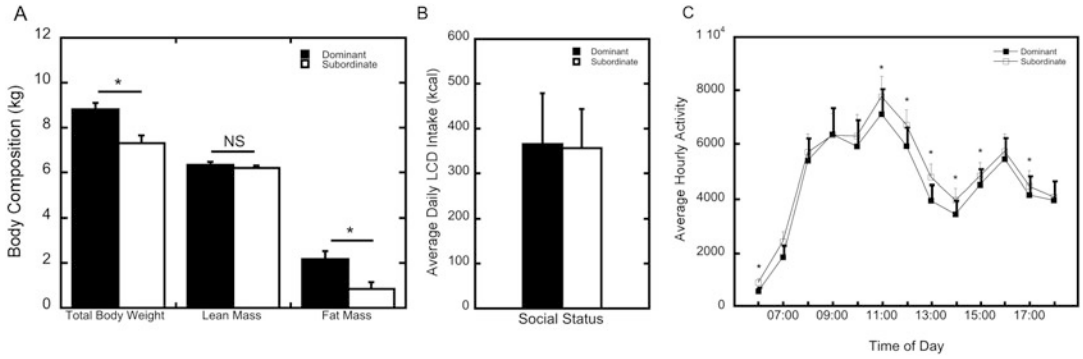

Fig. 2 (**a**) Body weight and body composition (kg) in dominant (dark bar) and subordinate (open bar) females. Asterisks denote increased body weight due to increased fat mass in dominant compared to subordinate females. (**b**) Average daily caloric intake (kcal) of a standard low-calorie diet (LCD) in dominant (dark bar) and subordinate (open bar) animals. (**c**) Average daily daytime activity in dominant (dark square) and subordinate (open square) females. Asterisks denote increased activity in subordinate animals compared to dominant females

expenditure, or both. Assessment of LCD intake over the course of 2 weeks in socially housed animals using the automated feeders yields no status differences in LCD intake. Subordinate females do not consume fewer calories of an LCD than dominant females in this dietary environment (*see* Fig. 2b), suggesting that the status difference in body weight is due to greater energy expenditure in subordinate females compared to their dominant counterparts. Indeed, assessment of activity bouts with Actical accelerometers, as a surrogate measure of energy expenditure [76], in these group-housed animals, shows that subordinate animals are more active during daytime hours, suggesting that subordinates expend more overall energy over the course of a day than do dominant animals (*see* Fig. 2c). Increased activity in subordinate animals could indeed be a consequence of the constant threat of aggression and harassment and the need to avoid and withdraw from higher ranking females. Nonetheless, these data indicate that lower body weights in subordinate females are associated with higher levels of activity.

The imposition of social subordination following new group formation [46] reduces body weight, and these lower body weights are sustained over time [65]. It is important to emphasize that these observations occur in the presence of a standard LCD. The more relevant question as it pertains to the increase in emotional eating and obesity in humans is: What happens to food intake and preference when the dietary environment is expanded to include highly palatable, high-calorie foods? Do socially subordinate females show a preference for a high-fat and high-sugar diet? And do they increase overall calorie intake in a rich dietary environment when a choice of food is available, analogous to the dietary environment in human societies?

2.2.2 Short-Term Availability of a Diet Choice Including Highly Palatable Food

Availability of an HCD in combination with an LCD for just 2 weeks has a profound impact on caloric intake, most notably for subordinate females [77]. Although both dominant and subordinate females prefer to consume the HCD when given a choice, overall caloric intake is markedly different in dominant and subordinate animals during this diet condition. While dominant females consume similar amounts of total calories during both the choice condition and LCD-only conditions, subordinate females significantly increase total caloric intake when HCD is offered concurrently with an LCD option. This augmented intake of calories upon HCD availability in subordinate animals is greater than both subordinate baseline intake during the LCD-only condition and dominant female intake under the choice condition (*see* Fig. 3). These data in monkeys corroborate findings from a number of rodent models [78–80] and data from humans [3, 5, 6, 81] indicating that exposure to stress facilitates augmented calorie intake and preference for a high-calorie diet.

Despite having only 2 weeks of access to this rich dietary environment, the increase in caloric intake during a choice diet condition is associated with an increase in body weight in subordinate females [52]. Because the data indicate that appetite in dominant animals is unaffected by dietary environment, it is plausible that the efficacy of satiety signals could be diminished and orexigenic signals augmented in subordinates [52] resulting in increased caloric intake among subordinate females during the choice diet condition. Taken together, these data indicate that the chronic psychosocial stress experienced by subordinate female monkeys increases total caloric intake only when these females are exposed to a rich dietary environment for a short period of time.

2.2.3 Long-Term Availability of a Diet Choice Including Highly Palatable Food

More recent studies have assessed the long-term effects of a rich dietary environment on food intake in dominant and subordinate female rhesus monkeys. Females that direct less aggression toward group mates (a characteristic of lower social status) ingest more total calories over a 4-month period when an LCD and HCD were both available ("choice dietary environment") [82]. Importantly, this relationship is not present in females with only access to an LCD for 4 months [82], supporting our previous observations. The impact of the dietary environment on appetite in socially housed rhesus monkeys is also evident after a 12-month availability to a rich dietary environment, as females with access to a choice between an LCD and HCD eat more overall calories and gain more weight and body fat than females with only access to an LCD [83]. In females with access to this rich dietary environment, higher cortisol concentrations, characteristic of low social status, predict greater total caloric intake and greater HCD intake [83]. Overall, these latest findings are directly relevant to the increase in emotional feeding and obesity in humans that occurs when the dietary environment includes highly palatable, high-calorie foods [21, 23].

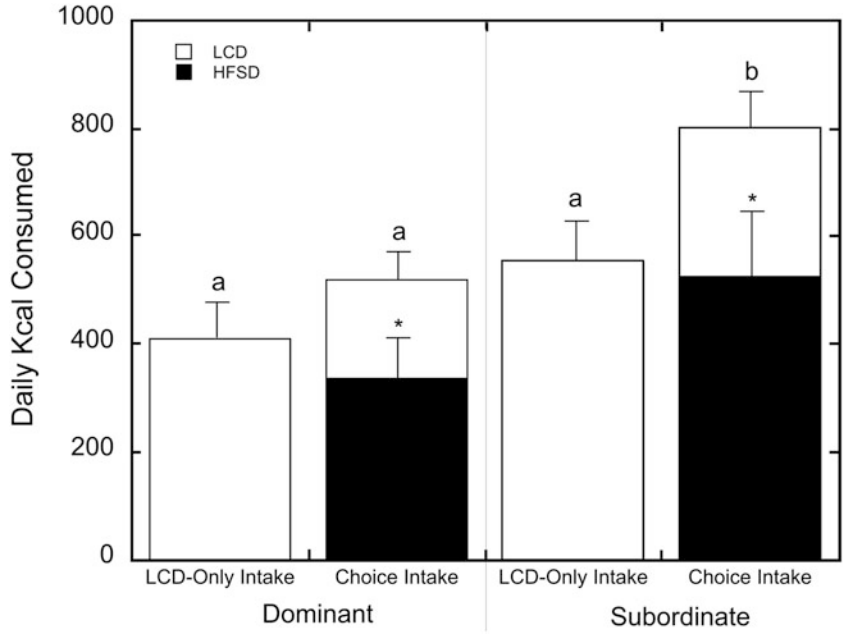

Fig. 3 Average daily caloric intake (kcal) in dominant and subordinate females during a no choice and choice diet condition. Open bars represent intake of the low-calorie diet (LCD), and closed bars indicate caloric intake of the high-fat and high-sugar diet (HFSD) diet. Asterisks denote overall preference of the HFSD over the LCD during the choice condition. Letters denote differences in overall caloric intake during each diet condition. Subordinate animals with a choice to consume an HFSD increase their total caloric intake to levels higher than those seen in subordinates on an LCD diet only and to dominant females, regardless of diet condition. (Reproduced with permission from Ref. [77])

2.3 Long-Lasting Effects of HCD Availability on Subsequent LCD Food Intake

One question that is also of interest as it pertains to emotional eating and obesity in humans is why attempts to lose weight often fail [7]. To assess how a previous short-term (2-week) exposure to diets high in fat and sugar might affect food intake in a "healthy" dietary environment, similar to what humans strive for when "dieting," caloric intake of an LCD upon the removal the HCD was quantified for 2 weeks [77]. As illustrated in Fig. 4a, dominant monkeys continued to eat a similar number of calories regardless of diet history. In contrast, subordinate females continued to eat significantly more calories compared with dominant females during this post-HCD phase (*see* Fig. 4b). While caloric intake was lower than observed during the choice phase when HCD was available, it was significantly higher than the previous LCD phase before any exposure to an HCD (*see* Fig. 4b).

Although these data suggest that a background of chronic psychosocial stress can interact with diet history (and access to an HCD) to increase caloric intake even in a healthier dietary environment [77], more recent data indicates that the duration of the dietary intervention may impact food intake and body weight after animals have been cycled from a choice dietary environment

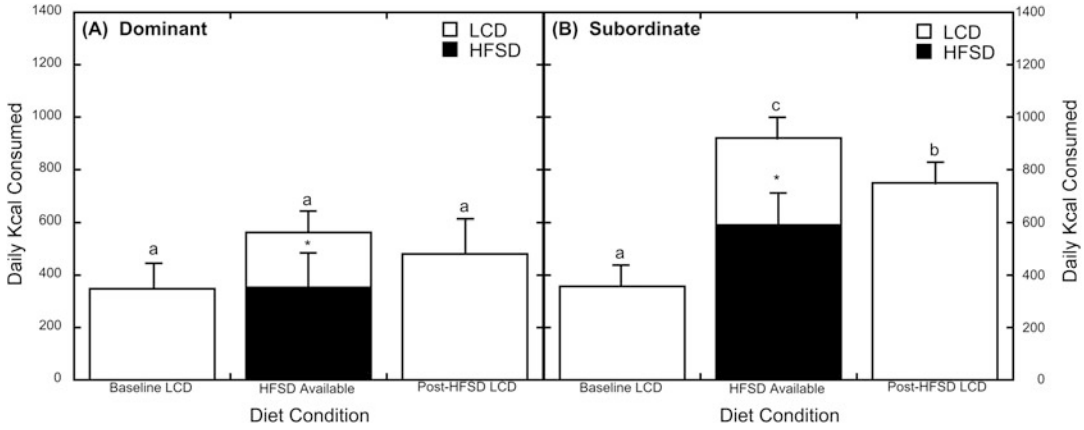

Fig. 4 Average daily caloric intake in dominant (**a**) and subordinate (**b**) females. Open bars represent intake of the low-calorie diet (LCD), and closed bars indicate caloric intake of the high-fat and high-sugar diet (HFSD). Asterisks denote overall preference of the HFSD over the LCD during the choice condition. Letters denote differences in intake during each diet condition. Subordinate animals consume more overall calories of the LCD diet following exposure to an HFSD than they did prior to HFSD exposure (**b**), whereas prior HFSD exposure does not affect caloric intake of an LCD in dominant animals following removal of HFSD. (Reproduced with permission from Ref. [77])

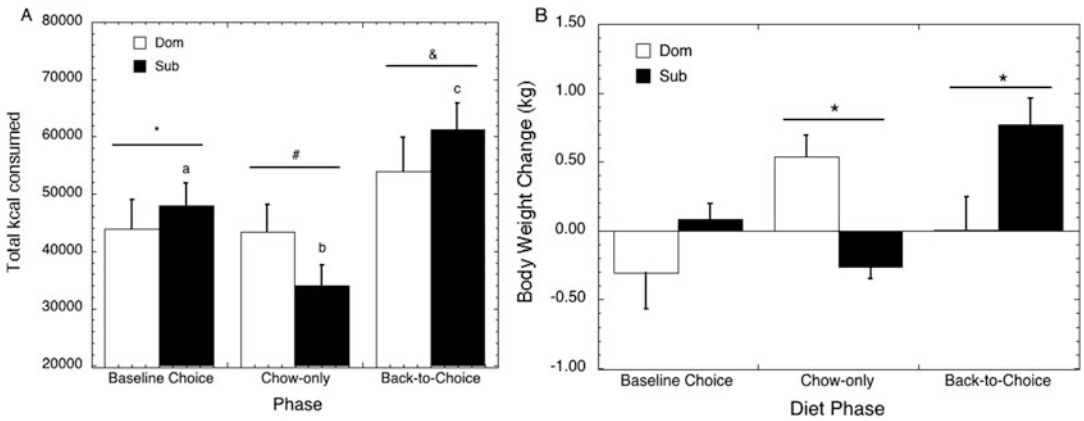

Fig. 5 (**a**) Mean ± SEM cumulative total calories consumed in each of the three dietary phases for dominant (Dom) and subordinate (Sub) females. Symbols denote the main effect of dietary condition, as food intake was significantly different between the three dietary conditions. Letters denote significant differences in intake within subordinate females between phases. (**b**) Mean ± SEM body weight change (kg) during each of the three dietary conditions for dominant (Dom) and subordinate (Sub) females. Asterisks (*) denote significant differences in body weight change between dominant and subordinate females during the chow-only phase and the back-to-choice phase. (Reproduced with permission from Ref. [84])

to an LCD-only condition [84]. Indeed, subordinate females maintained in a choice dietary environment for 14 months who are diet-cycled to an LCD-only environment for 15 weeks show a significant reduction in caloric intake (*see* Fig. 5a) and body weight (*see* Fig. 5b). Dominant females, on the other hand, do not show

changes in food intake and body weight during the same dietary manipulation (*see* Fig. 5). Following the switch from a choice to an LCD-only condition, all animals were then cycled back to a dietary environment wherein an LCD and HCD were once again available [84] to assess the effects of social status and reintroduction to a choice dietary environment on food intake and body weight to model the consequences of "dieting" in people. While this diet cycling did not impact food intake and body weight in dominant females (*see* Fig. 5), subordinate animals increased their caloric intake to amounts significantly higher than observed in the initial diet choice condition prior to the initiation of the diet interventions (*see* Fig. 5a). Consequently, these subordinate animals had a significant increase in body weight (*see* Fig. 5b). Taken together, these recent findings suggest that psychosocial stressor exposure may contribute to the "yo-yo dieting" phenomenon described in humans who start and stop dieting and show parallel decreases and increases in their body weight. Future studies leveraging this translational model can address the biological mechanism by which a history of psychosocial stress and HCD intake may reprogram appetite regulation and metabolism to make it difficult to avoid an HCD-type diet and lose weight.

The lasting effects of HCD exposure on appetite regulation in subordinate female macaques could be due to acute and long-lasting changes in specific satiety signals. While intake of an HCD is also associated with an increase in insulin and glucose levels in all animals regardless of social status, only subordinate females continue to consume more overall calories once the HCD is removed [77]. The finding that leptin levels are increased only in subordinate females following HCD exposure during the subsequent period of increased LCD-only intake [77] further supports the notion that satiety signaling might be altered in subordinate females [77]. Studies in rodents have shown that glucocorticoids can lead to both weight gain and increased feeding by inducing leptin insensitivity [85, 86]. Additionally, excess glucocorticoids counteract the activity of insulin and can facilitate the development of insulin insensitivity, resulting in increased lipogenesis, central adiposity, and leptin levels [87]. Furthermore, diet cycling, or a switch from an HCD dietary environment to an LCD-only environment, is a stressor in and of itself, as making this dietary change upregulates hypothalamic gene expression of stress-related signals in rodents [88]. A consequence of these physiological changes includes augmented food intake and increased body weight as described in individuals suffering from excess cortisol levels due to Cushing's disease [89]. However, longer HCD exposure can lead to insulin resistance and reduce satiety signaling in subordinate females [90] that could possibly contribute to excess dietary consumption. Taken together, these data suggest that the exposure to an HCD alters sensitivity to

signals that are critical for the maintenance of energy homeostasis and could explain the high failure rate of weight loss attempts among human populations.

3 Notes

3.1 Individual Variability in Food Intake

While social status and stress background account for a significant proportion of variance in total caloric intake when an HCD is available, there is nonetheless variability in feeding behavior within each social status category in this dietary environment. Animals classified as dominant show some variability in HCD intake during its availability (*see* Fig. 6), whereas females categorized as subordinate show a greater amount of variance in HCD intake. This variability among members of each social status category indicates that other variables interact with social status to contribute to this feeding phenotype. Genetic factors confer individual differences and contribute to increased individual vulnerability to stress-induced disorders [91]. An example of this is the polymorphic region in the promoter of the serotonin reuptake transporter, whose short allele variant has been linked to increased susceptibility to depression [91]. Likewise, a polymorphism in the dopamine D2 receptor gene is linked to increased incidence of obesity [92]. Thus, polymorphisms in genes regulating feeding behavior and stress axis reactivity could interact with the environment and account for the variability observed in emotional feeding within subordinate monkeys [93, 94]. Furthermore, epigenetic changes in gene

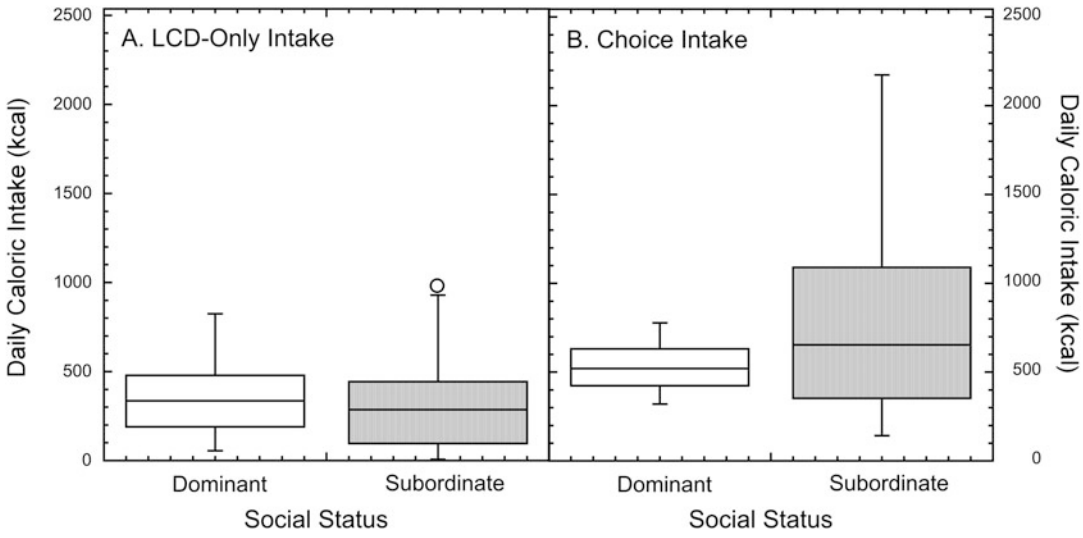

Fig. 6 Box plots depicting variability of overall caloric intake and emotional feeding within animals of each social status categorization during (**a**) the LCD-only with no previous history of HFSD and (**b**) a diet choice condition where HFSD was available

expression due to life experiences and the environment likely facilitate individual variability in emotional feeding and other stress-induced phenotypes [95]. Importantly, social subordination in female rhesus monkeys provides us with a unique model to differentiate how these factors contribute to the feeding phenotypes observed in both dominant and subordinate animals.

3.2 What Sustains Emotional Feeding?

The mechanisms responsible for the increased motivation to consume calories in the presence of highly palatable diet in individuals experiencing chronic stress remain uncertain. A possible explanation for altered feeding behavior is altered sensitivity to appetite and satiety signals under conditions of chronic stress. An example of this is the increased sensitivity to ghrelin observed in subordinate females, and not dominant females, that is linked to increased caloric intake and LHPA dysregulation in subordinate animals [96]. Leptin signaling might also be disrupted by social subordination as HCD intake in subordinate females increases circulating leptin levels while still maintaining increased caloric intake [77]. Thus, changes in sensitivity to appetite and satiety signals can occur in concert with one another and depend on the dietary environment. Future studies are necessary to delineate how psychosocial stress exposure and activity of the LHPA axis influence the regulation orexigenic and satiety signals in complex dietary environments to dysregulate feeding behavior.

Another possible mechanism is that comfort food ingestion diminishes activation of the stress response, as has been shown in rodents [24, 80, 97, 98] and in high-stress premenopausal women [99]. However, because glucocorticoids are a critical component in initiating emotional feeding [100–102], a reduction in stress hormone responsivity by comfort foods is likely not a sustaining factor that maintains this phenotype. In contrast, the availability of an HCD in female macaques increases the diurnal rhythm of cortisol [52], and HCD consumption augments cortisol responsivity to an acute social separation stressor, regardless of social status [52]. These data in subordinate female monkeys not only support data from rodents with access to an HCD [103–107] but also are consistent with clinical data linking enhanced LHPA activity to measures of central adiposity [108–110]. This increased LHPA activity linked to ingestion on HCD in subordinate monkeys supports the notion that emotional feeding is a behavior that results in the reinforcement and continued motivation to engage in emotional feeding via an increase in glucocorticoids and LHPA activation.

Because ingestion of HCDs is linked to the activity of the LHPA axis and LHPA axis activity can modulate behavior [58, 96], it could be possible that consumption of these highly palatable diets might affect mood and socioemotional behavior. While some studies in rodents and in rhesus monkeys have shown

that availability of an HCD reduces aggression and anxiety-like behavior [52, 98, 107, 111, 112], other data from monkeys show that HCD availability has no effect on socioemotional behavior [77]. Additionally, while the lack of behavioral effects upon HCD availability could indicate that HCD intake does not affect behaviors, it is more plausible that the increased LHPA activation associated with HCD availability is actually having an adverse or neutral effect on socioemotional behaviors in this particular social context. Indeed, if comfort food ingestion actually increases stress hormone responsivity, consumption of these diets may actually be anxiogenic. Further studies are necessary to determine how HCD availability and ingestion affect social and emotional behaviors in this model and other social contexts. Additionally, determining how diet affects behavioral responses to acute threatening situations is important to better understand why individuals engage in emotional eating.

A final hypothesis for sustained emotional feeding involves the reward pathways. Chronic psychosocial stress exposure in humans increases individual vulnerability for addictive phenotypes [113], including psychostimulant abuse [114], by reducing dopamine D2 receptors in mesolimbic regions and producing a hypodopaminergic condition in cortico-limbic regions of the brain that are important for reward processing [115–117]. Intake of calorically dense diets reduces dopamine D2 receptors (D2R) in these reward regions of the brain in obese humans [118, 119] and in some animals [118]. Additionally, studies in rats and in humans indicate that HCD availability activates reward circuitry in forebrain structures [120–123]. Because social subordination in macaques also results in a reduction in D2R binding potential [62, 124], it is possible that emotional eating may act as a self-prescribed treatment for increasing the activation of an already dysfunctional reward system [77].

The notion that emotional eating is maintained by altered reward function is supported by findings from the more recent study of caloric intake across 12 months in socially housed female rhesus monkeys provided access to either an LCD-only condition or a dietary choice between an LCD and HCD (described in Subheading 2.2.3) [82, 83]. These studies quantified food intake, a number of physiological markers, and D2R binding potential (BP) and functional connectivity (FC) using PET and resting-state MRI (magnetic resonance imaging) within reward neurocircuitry, including subregions of the striatum (e.g., nucleus accumbens or NAcc) and the prefrontal cortex [82, 83]. After 4 months on the choice diet but not in the LCD-only condition, an association emerged between more total caloric intake and lower D2R BP in the orbitofrontal cortex (OFC) [82], a brain region involved in goal-directed behavior and the inhibitory control of behavior [125]. Importantly, after 12 months, the effects of the adverse

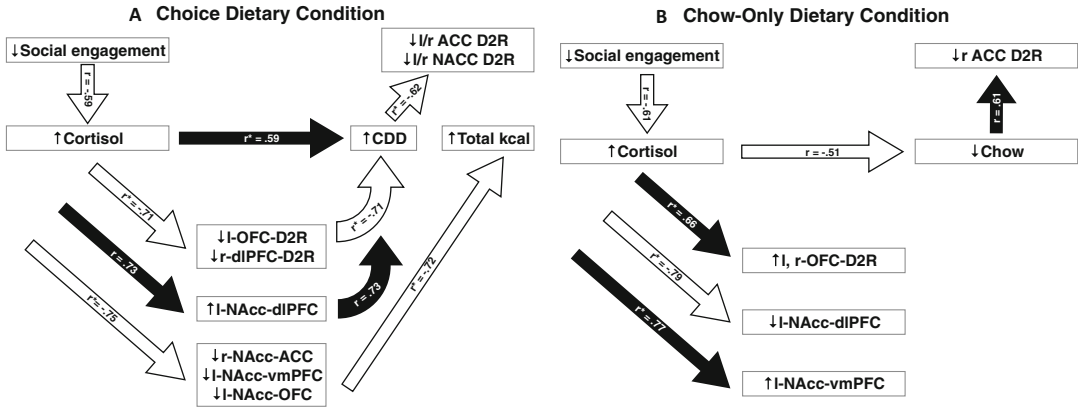

Fig. 7 Bivariate correlations between social behavior, measures of cortisol release, D2R BP, and FC for females maintained in the (**a**) dietary choice condition and the (**b**) chow-only condition for 12 months. The designation of r* reflects a composite correlation coefficient. All correlations are significant ($p < 0.05$). (Reproduced with permission from Ref. [83])

social experience of being a subordinate female were evident on brain reward systems and appetite, but these outcomes varied significantly by dietary condition [83]. Regardless of diet condition, the social phenotype of subordination was associated with higher basal cortisol (*see* Fig. 7). In the dietary choice condition, these higher concentrations of basal cortisol predicted more HCD intake as well as decreased D2R BP in the OFC and the dorsolateral prefrontal cortex (dlPFC) (*see* Fig. 7a), a brain region critical for incentive-based behavioral responses including those in response to food [126]. Additionally, greater concentrations of cortisol of females in the dietary choice condition predicted reduced FC between the NAcc and the ventromedial PFC but increased FC between the NAcc and the dlPFC that, in turn, predicted more calorie intake (*see* Fig. 7a). These relationships between the subordination social phenotype, glucocorticoids, food intake, and reward neurobiology were significantly different in females maintained for 12 months in an LCD-only dietary environment, with higher cortisol predicting reduced intake of LCD, increased D2R BP in the OFC, increased NAcc-vmPFC FC, and reduced NAcc-dlPFC (*see* Fig. 7b) [83]. Taken together, these data indicate that the consequences of higher concentrations of cortisol associated with adverse social experience differ depending on the availability of an HCD, with the changes in D2R BP and reward neurocircuitry observed in females consuming more HCD possibly compromising reward pathways and impairing the cognitive control of food intake.

4 Conclusion

The ongoing studies of food intake in socially housed female rhesus monkeys indicate that social subordination provides an important model to study stress-induced emotional eating and its impact on obesity in females. The use of this translational model in nonhuman primates may help fill gaps in knowledge surrounding stress-induced alterations in feeding behavior and the interaction with dietary environments for women. Critical questions regarding emotional eating in females that remain unanswered include, but are not limited to: Are differences in D2R binding and functional connectivity within corticostriatal circuits sustained in a diet cycling condition? Do satiety signals become ineffective at curbing emotional feeding? Are these both mechanisms for why dieting fails so often in humans? How does sustained intake of HCDs affect LHPA activity and feeding efficiency superimposed on a background of social chronic stress? Does social history account for individual variability in stress-induced changes in feeding and socioemotional behavior and physiology? Does the alleviation of social stress normalize corticostriatal circuits to reduce intake of calorically dense diets?

The animal model of stress-induced emotional eating described in this chapter will allow investigators to answer these questions using tools such as genetics, epigenetics, and neuroimaging simultaneously. These results will be critical for understanding the etiology of emotional eating and factors that might increase individual vulnerability to stress-induced eating and obesity, thus providing the basis for treatments that may benefit millions of individuals worldwide (*see* Fig. 8).

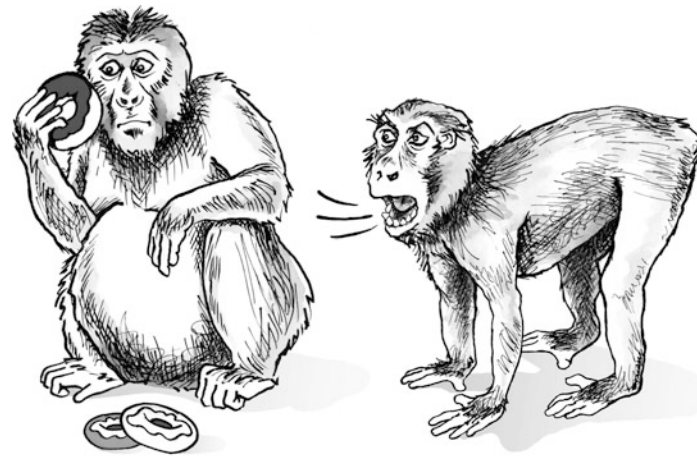

Fig. 8 Illustration of social stress induced emotional feeding by a subordinate female monkey

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Chapter 7

Artificial Sweeteners in Animal Models of Binge Eating

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Abstract

Rising rates of obesity in most industrialized countries are a major cause of serious medical conditions, including diabetes, heart disease, and mental health disorders. Binge eating, characterized by excessive consumption of highly palatable food within a short period of time, contributes significantly to these problems, even in individuals who are not diagnosed with binge eating disorder (BED). Over the last half century, the use of nonnutritive substitutes has been promoted as a means to reduce fat and sugar consumption, potentially minimizing obesity-related illnesses and associated health conditions. Paradoxically, increased intake of artificial sweeteners is associated with weight gain, which may be linked to alterations in metabolic processes. Artificial sweeteners also increase food intake in both humans and rodents, raising intriguing possibilities that these substances are altering biological processes that underlie feeding behavior (e.g., homeostatic control). We explore this idea by summarizing the clinical and preclinical literature on behavioral and biological mechanisms of artificial sweeteners. As a starting point, we review evidence that nonnutritive sweeteners are rewarding in rodent models and then provide a comparison of neural systems mediating the rewarding properties of natural and artificial sweeteners. We then summarize data pointing to sexual dimorphism in behavioral and biological responses to sucrose, with preliminary evidence suggesting that responses to artificial sweeteners may follow a similar pattern. Finally, we provide an overview of the relationship between binge eating and substance use disorders, noting findings from animal studies that artificial sweeteners could contribute to this comorbidity.

Key words Feeding, Reward, Dopamine, Opioid, Sucrose, Saccharin

1 Introduction

Binge eating disorder (BED), the most common of all eating disorders [1], is characterized by consumption of large amounts of food within a discrete period of time in the absence of compensatory behaviors [2]. According to World Health Organization estimates, the lifetime prevalence of BED is 1.9%, with a median onset age of 20 years old [3]. Not surprisingly, BED is more common in women than in men, although these ratios are distributed more evenly than in other eating disorders [3]. Binge eating is highly comorbid with a number of medical conditions, other psychiatric disorders [4], and a reduced quality of life [5]. Of the medical

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conditions associated with BED, obesity is one of the most prevalent, likely due to the lack of purging or exercise following binge intake [6]. Adult patients with BED have significantly higher obesity rates than individuals with no eating disorder [7], and even those who are not obese are distressed by their bingeing behavior.

Many patients with obesity and BED attempt to limit caloric intake and counter weight gain by adopting hypocaloric diets, specifically restricting highly palatable foods [8]. This is generally ineffective, as individuals who are food restricted tend to binge on foods that are high in sugar and/or fat, ingesting significantly more calories than nonrestricted individuals [9]. The pattern is exacerbated in modern society with the prevalence and availability of highly palatable food. Consumption of these foods activates brain reward circuits [10], increasing the probability that behaviors leading to their intake will be repeated. These factors undoubtedly contribute to growing rates of obesity [11], which have tripled since 1975 (WHO). Obesity dramatically increases the risk of medical conditions such as type 2 diabetes, hypertension, and dyslipidemia, although these metabolic syndrome disorders may develop in BED patients, even in the absence of obesity [12].

Artificial sweeteners are an appealing alternative to restrictive diets in that they allow individuals to consume highly palatable food with minimal calories. If bingeing in humans is a reaction, at least in part, to restriction of forbidden "pleasure" foods, consumption of these commodities should reduce binge eating. Weight gain should also be minimized when individuals opt for artificial over natural or processed sugars simply due to a reduction in calorie intake. It is therefore surprising that the two have grown in parallel: higher rates of obesity and higher use of artificial sweeteners.

1.1 Animal Models of Binge Eating

Despite the prevalence and associated problems, the etiology of BED is not clearly understood [13]. Animal models provide a means to unravel the causal mechanisms of this disorder as rodents, like humans, exhibit binge eating when they are provided with intermittent access to highly palatable food or when they undergo periods of food restriction and stress [14]. The intermittent access protocol (12 h food deprivation followed by 12 h access to sucrose or glucose and food) simulates behavioral aspects of BED, including escalation of intake and withdrawal-like symptoms [15, 16]. This pattern reflects eating patterns of BED as patients often display excessive food intake during the evening after self-imposed restriction during the day [17]. Importantly, the rat intermittent access model produces compulsive responding for palatable food, mimicking the loss of control over food intake that characterizes patients with BED [18]. Caloric restriction, itself, is not a necessary prerequisite for bingeing in that fat bingeing occurs when access to this commodity is limited, but regular chow is freely available [19].

Stress is also a potent trigger of binge eating, particularly when it is combined with restriction of palatable food [20, 21]. For example, manipulations, such as tail pinch, induce hyperphagia for palatable food [22] and increase consumption of standard chow [23]. Females may be more sensitive to stress-induced feeding, showing more rapid increase of palatable food intake than males [24]. Developmental factors, including level of maternal care and exposure to stressors during adolescence, also increase vulnerability to binge intake [25].

Few studies have investigated the impact of artificial sweeteners on binge eating: in most animal studies, access to these substances is a control condition, used to separate intake that is driven by caloric versus hedonic properties of food [26–29]. Nonetheless, inspection of data from these control groups reveals unique patterns of intake associated with access to artificial sweeteners. For example, intermittent access to saccharin produces binge-like intake in mice [26, 29], an effect that appears to be absent in rats [18, 28]. In addition to species differences, the concentration of saccharin may be a critical factor in the elicitation of bingeing behavior, as this was much higher in the rat experiments. Moreover, a 0.4% saccharin solution induced bingeing in rats that matched the intake of rats given access to an isohedonic sucrose solution (4%) [30]. Both groups exhibited more rapid escalation of intake than control groups given unlimited access to either solution. Importantly, either intermittent or unlimited access to an isocaloric solution, maltodextrin, that provides calories with no sweet taste did not induce binge intake.

2 Artificial Sweeteners

2.1 History of Artificial Sweeteners

Figure 1 provides a brief overview of the history of natural and artificial sweetener use in Western countries. Artificial sweeteners were introduced to the public on a large scale to deal with sugar rationing during WWII. The use of these compounds expanded in the latter part of the twentieth century to address growing rates of obesity, which are often attributed to the overconsumption of added sugars. Health directives generally recommend a maximum sugar consumption of 10% of total calorie intake per day [31, 32], whereas most adults in industrialized countries consume between 15% and 21% of their daily calories in sugar. The proportion of sugar intake in children is even higher (16–26%) [33]. Fructose may be a primary culprit in sugar overconsumption, particularly with the increased use of high-fructose corn syrup as a sweetener beginning in the 1970s [34]. Fructose is particularly detrimental to secondary medical conditions, such as diabetes, because it increases insulin resistance, oxidative stress, and inflammatory responses [35]. Table 1 compares the relative sweetness of common non-nutritive sweeteners.

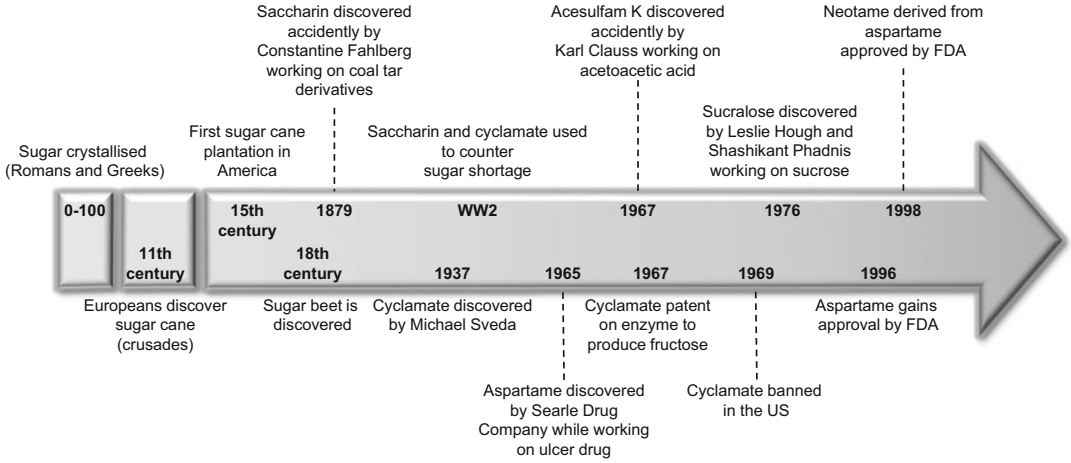

Fig. 1 Historical timeline of natural and artificial sweetener availability

2.2 Paradoxical Effects of Artificial Sweeteners

2.2.1 Artificial Sweeteners and Weight Gain

Despite the apparent appeal, widespread use of artificial sweeteners has not been associated with reduced obesity. Indeed, a number of studies indicate that these substances are linked to both weight gain and metabolic disorders [36, 37]. For example, a prospective study of male and female participants who normally consumed artificially sweetened beverages revealed a positive correlation between the quantity of beverages consumed and weight gain over time, although the effects were not sweetener specific [38]. The findings were confirmed in a separate study, which also showed that weight gain was independent of the macronutrient content of meals. More specifically, both male and female participants using artificial sweeteners consumed the same amount and type of food as a control group (not consuming artificial sweeteners) but still gained weight [39]. Although intriguing, these findings require further investigation, particularly as serving sizes in each study were not reported.

More in line with conventional thinking, other studies report stable or decreased weight in individuals who consume artificial sweeteners [40]. This could reflect the fact that people who use artificial sweeteners are often on a weight loss diet, thereby attempting to reduce the number of calories they consume. Indeed, weight loss was observed in an intervention study in which male and female adults followed a 12-week diet, with half of the participants drinking beverages with nonnutritive sweeteners daily (701 mL) and the other half consuming the same amount of water. Moreover, other measures such as systolic pressure, waist circumference, and blood triglycerides improved in the group consuming artificial sweeteners compared to their water-consuming counterparts [41]. At least in these conditions, nonnutritive sweeteners appear to be beneficial in reducing problems associated with overeating.

Table 1
Relative sweetening strength of nonnutritive sweeteners

Nonnutritive sweetener	Sweetening strength compared to sugar
Acesulfame potassium	200×
Aspartame	200×
Cyclamate	40×
Neotame	8000×
Saccharin	300×
Sucralose	600×

Note: In animal studies, the concentration of a sugar solution is usually 4–20%

The impact of artificial sweeteners on weight gain is also controversial in children, with some studies reporting a positive correlation between diet soda consumption and body mass index [42], although the effect may be limited to males [43]. The findings should be viewed with caution as children who drink a lot of sodas also tend to eat more junk food and be less active [44]. Indeed, a controlled randomized trial (over 600 healthy children followed for 18 months) found a significant reduction of weight gain and body fat mass in a group that was instructed to consume sugar-free beverages each day, compared to a group that consumed sugar-sweetened beverages [44]. In addition, in contrast to adults, children modify their food intake following consumption of artificially sweetened beverages [45] with the timing of the sweetener preload (lunch or snack) differentially impacting the later consumption of calories. Regardless of the mechanism, children's intake of noncaloric beverages requires further attention because consumption of these drinks often increases over time [46], and the impact of artificial sweeteners on developmental processes is unknown.

The controversy surrounding artificial sweeteners and weight gain is difficult to untangle in humans as eating is influenced by a combination of physiological, social, and subjective factors. For instance, participants consuming artificial sweeteners ingest fewer calories when they are not aware that sucrose has been replaced with a low-calorie substitute [47], but increase their calorie intake when they are informed of the substitution [48]. Of note, research studies are often funded by agencies with a potential conflict of interest (e.g., food industry) that could impact the interpretation of scientific or epidemiological studies [49]. Animal studies, which minimize extraneous factors that may impact eating in humans, can help to elucidate the relationship between artificial sweeteners and weight gain. At least some of this work supports a positive correlation between the two: rats given intermittent access to

saccharin consumed more calories and gained more weight than a group given intermittent access to glucose [50]. The findings were confirmed in studies using saccharin or aspartame versus sucrose [51, 52]. Although further work needs to be done, preliminary evidence from animal studies supports the idea that consumption of artificial sweeteners is associated with weight gain.

2.2.2 Artificial Sweeteners and Metabolic Changes

The effect of artificial sweeteners on weight gain may be related to reduced basal metabolism as postprandial thermogenesis is lower in rats given access to a low-calorie sweetener compared to sucrose [50]. Moreover, in a mouse model of diet-induced obesity, animals given access to the nonnutritive sweetener, aspartame, exhibited reduced oxygen consumption during the dark phase and increased visceral fat due to hyperinsulinemia [53]. Similarly, saccharin-consuming rats show a decrease in glucagon like peptide 1, an anorexigenic hormone which could explain increased food intake and increased blood glucose following a glucose tolerance test [54]. In contrast to aspartame, saccharin does not appear to affect insulin levels, although this may depend on overall diet consumption. For example, differences in energy intake, weight gain, and adiposity following saccharin versus sucrose consumption are only observed when rats have access to a high-fat diet [55].

If artificial sweeteners do impact weight gain, they may do so by altering metabolic processes in the periphery [56]. Like natural sugars, these substances activate sweet taste receptors which are located, primarily, on the tongue but are also expressed in the bladder, pancreas, and gut [57]. This provides a mechanism by which artificial sweeteners can alter metabolism. As an example, male rats provided access to saccharin exhibited reduced thermal responses compared to a sucrose group, suggesting that lower energy expenditure and storage of nutrients were responsible for the increased weight gain in these animals [50]. Interestingly, the effect of artificial sweeteners on metabolic effects may depend on the molecular structure of the substance. Both saccharin and acesulfame K (500-fold sweeter than sucrose) stimulate glucose-induced insulin secretion and promote glucose uptake [58], as well as induce adipogenesis and repress adipocyte lipolysis [57]. In contrast, aspartame, which is only 200-fold sweeter than sucrose, produces the opposite effect, reducing adipogenesis by downregulating the expression of adipogenic markers. The latter effects were produced in vitro so must be replicated using in vivo measures [59]. These inconsistent effects of artificial sweeteners on metabolism may explain, at least in part, the contradictory findings regarding artificial sweeteners and weight gain.

2.2.3 Artificial Sweeteners and Eating

Any association between increased use of artificial sweeteners and weight gain is somewhat paradoxical in that individuals who substitute non- or low-nutritive sweeteners for sugary foods should be consuming fewer calories. The literature on this question in humans is controversial. Some studies show no effect of low-calorie sweeteners on appetite [60, 61], whereas others suggest that artificial sweeteners, specifically aspartame [62] and saccharin [63], reduce food intake. Again, this could be a specific effect of the sweeteners under study (aspartame and saccharin), which are less commonly used in food products today than are sucralose or acesulfame K [40].

As with weight gain and metabolic effects, animal studies point to a positive relationship between consumption of artificial sweeteners and increased food intake. For example, rats given access to saccharin during the first 2 h of the dark cycle exhibit a 10–15% increase in food intake, compared to rats given access to water. Sucrose produces the same effect suggesting that sweet tastes stimulate appetite, regardless of the calorie content [64]. In line with this idea, saccharin preload increases chow intake in rats [65], and even fruit flies exhibit increased food intake following access to an artificial sweetener [66].

The effect of artificial sweeteners may be exacerbated in binge eating models with food intake being higher in animals given limited access to saccharin, compared to those given limited access to sucrose [26]. This difference could not be explained, entirely, by the caloric content of sucrose (i.e., animals consuming calories in sucrose would be expected to eat less food) because animals ingesting saccharin consumed more total calories per day than animals given either intermittent or continuous access to sucrose. Intriguingly, rats are able to monitor food intake in anticipation of sucrose access, but do not show the same effect when provided access to saccharin. More specifically, animals consumed less chow when a sweet taste cue predicted access to sucrose-flavored yogurt, suggesting that they were regulating calorie intake over the entire session and therefore did not gain weight [50]. In contrast, a cue predicting access to saccharin-flavored yogurt increased food intake and led to greater weight gain, suggesting that artificial sweeteners were altering the homeostatic control of energy intake. In sum, artificial sweeteners may impair the ability to predict caloric intake by uncoupling signals related to sweet taste and calorie content [50, 51].

3 Behavioral and Biological Effects of Artificial Sweeteners

3.1 Rewarding Effects

Animal studies support the idea that artificial sweeteners produce rewarding effects in that saccharin is self-administered by rats [67] and elicits binge-like intake in mice given limited daily access [26]

or 2-day intermittent access [68] to a saccharin solution. Interestingly, increasing a no-access period to saccharin reinforces the bingeing behavior [30]. Saccharin also elicits "craving" responses in rats, mimicking those produced by either sucrose or cocaine [67]. In these experiments, animals learn to self-administer cocaine, sucrose, or saccharin and then undergo 1 or 30 days of forced abstinence. When presented with a cue predicting access to the reinforcer, rats in the prolonged abstinent groups showed significantly higher seeking responses for all three commodities, reflecting a common phenomenon of "craving incubation." Low-calorie sweeteners, therefore, produce a similar pattern of reinstatement to those of natural reinforcers in an animal model of relapse. This suggests that artificial sweeteners may be poor substitutes for sugars as they could increase the risk of relapse in eating disorder patients.

On the other hand, the effects of artificial and natural sweeteners on reward-related behaviors in rats do not always overlap. For example, extended intermittent access to sucrose blocks the reinforcing effect of sucrose in the conditioned place preference paradigm [28] and induces compulsive responding for sucrose in the conditioned suppression paradigm [18]. These effects were absent in groups given extended intermittent access to saccharin, although it should be noted that saccharin did not elicit binge behavior in these experiments. Intriguingly, intermittent access to saccharin appears to increase sensitivity to develop a morphine conditioned place preference paradigm in male rats [28], suggesting that it may modulate the rewarding value of other reinforcers. Moreover, rats prefer saccharin over cocaine in a two-choice operant paradigm, an effect that is independent of prior drug experience [69].

3.2 Neural Mechanisms

Artificial sweeteners provide little or no nutritive value, suggesting that consumption of these substances is driven, primarily, by their hedonic properties: these may be mediated by the same neural systems that underlie the rewarding properties of palatable food. In studying this assumption, Frank [70] reported that higher concentrations of both sucrose (up to 32%) and sucralose (concentrations matched to sucrose for sweetness) increased activation in the primary gustatory cortex of humans (frontal operculum and anterior insular), although sucrose activated additional regions implicated in feeding (e.g., the midbrain, substantia nigra, and ventral striatum) [70]. The study also revealed differential functional connectivity associated with ingestion of the two substances, specifically recruitment of reward pathways for sucrose, but not sucralose.

Similarly, sucrose and low-calorie sweeteners both regulate the hypothalamic neuropeptide, orexin, although the two effects are not completely overlapping. For example, the selective orexin 1 receptor antagonist, SB-334867, blunts saccharin and sucrose drinking in mice [71, 72], and sucrose and saccharin bingeing decrease orexin mRNA in the lateral hypothalamus [73]. The latter

is associated with reduced phosphorylated cyclic AMP response binding protein (pCREB) in orexin neurons, at least in female rats [74]. This reduction was observed in melanin-concentrating hormone (MCH) neurons but only following sucrose consumption, pointing to a potential dissociation of mechanisms mediating consumption of natural and artificial sweeteners. The fact that orexin processes are reduced in bingeing animals appears to contradict evidence that this neuropeptide reduces satiety and increases appetite [75]. The relationship between orexin levels and binge eating, however, likely involves a complex interaction with brain reward systems that could impact non-homeostatic eating. More specifically, orexin enhances dopamine levels in brain reward systems, so reduced orexin activity could reflect a hypodopaminergic state. Animals may compensate for this deficit by increased consumption of sucrose or saccharin in an attempt to maintain homeostatic levels of dopamine.

At the same time, there are subtle differences in the impact of dopamine manipulations on the intake of natural versus artificial sweeteners. Administration of either a D1 or a D2 receptor antagonist (SCH23339 or raclopride) dose-dependently reduces sucrose, but not saccharin, intake [76] in a two-bottle choice paradigm. In addition, cues associated with either sucrose or saccharin presentation evoke dopamine release in the nucleus accumbens (NAc) core, but the effect produced by sucrose-paired cues is much larger [77]. In line with this evidence, sucrose induces a larger dopamine release in the ventral striatum compared to saccharin [69]. A similar pattern of findings emerges regarding the relationship between feeding-related peptides and artificial versus natural sweeteners. Saccharin, like sucrose, increases mRNA levels of neuropeptide Y (NPY), orexin, and agouti-related peptide (AgRP) [78, 79], changes that may underlie sweetener-induced increases in energy intake and weight gain. However, there is a distinct pattern of time-dependent changes following ingestion of the two commodities. More specifically, saccharin consumption leads to an immediate increase in NPY and orexin expression, whereas sucrose produces an immediate decrease in NPY and AgRP that is followed by increased expression of these peptides within 10 min post ingestion. Moreover, NPY infusions into the ventral tegmental area, NAc, or lateral hypothalamus consistently increase sucrose consumption and/or the motivation to obtain sucrose [80]; in contrast, effects of these manipulations on saccharin intake are inconsistent [78, 81].

A separation of neural systems mediating the rewarding effects of sucrose and nonnutritive substitutes could be explained within the context of incentive sensitization. According to this theory [82], the “wanting” component of reward depends on mesolimbic dopamine systems, whereas “liking” is mediated by opioidergic mechanisms. As noted previously, dopamine may have dissociable

roles controlling responses to natural and artificial sweeteners, particularly in terms of cues predicting the presentation of one commodity or the other. There is some degree of overlap in the mediation of "liking" natural and artificial sweeteners in that mice with deletion of the ion channel TRPM5 and TRPM4 taste receptors consume less sucrose and saccharin than wild-type mice [83], and mu-opioid receptor knockout mice show reductions in both sucrose and saccharin bingeing [26]. Moreover, general opioid antagonism dose-dependently decreases palatable food bingeing [84], as well as saccharin preference and consumption [85]. On the other hand, mu-opioid receptor knockout animals exhibit decreased licking of sucralose, compared to sucrose [86], and the mu-opioid receptor agonist, DAMGO, selectively increases saccharin drinking in rats [87, 88]. Thus, although the neural substrates mediating hedonic responses to artificial and natural sweeteners share common elements, these are not completely overlapping.

4 Artificial Sweeteners and Sex Differences

As with other basic biological processes, behavioral responses to palatable food often vary across sexes. Sex differences are attributed to a combination of chromosomal and hormonal differences between males and females, combined with gender constructs related to societal expectations [89]. These help to explain sex differences in behavioral processes and the disproportional representation of one sex or the other in disorders or diseases. As an example, women transition more rapidly to compulsive drug use in addiction than males [90], and female rats exhibit enhanced escalation of heroin intake compared to male rats [91]. An increased rate of developing maladaptive drug use in females could reflect sex differences in dorsal striatal activity as this system controls the transition to compulsive drug use in addiction [92]. The effect may also be related to sexual dimorphism in brain reward systems, as these have been observed for dopamine D1 and D2 receptors in the frontal cortex and striatum of juvenile rats [93]. These findings fit with clinical evidence of differential activation of brain reward circuitry during craving in male and female patients with cocaine dependence [94].

Similar to drug reinforcers, sex differences emerge in male/female responses to palatable food. For example, women are more likely than men to exceed recommended limits on sugar intake [95] and to perceive sweet taste more intensely than men [96]. Female rats also show a higher preference than males of their species for both glucose and saccharin, an effect that is maintained even at the highest concentrations of saccharin [97]. Both males and females in this study preferred saccharin over glucose in conditions of short-term access; when access was extended, only males reverted to a

higher preference for the natural sugar. These findings are reflected at the biological level with a higher proportion of female rats showing increased neuronal firing in the parabrachial pons in response to sucrose [98]. A similar profile of sex differences was observed in female and male rats provided with a saccharin solution, suggesting that the effect is driven by taste, not nutrient content [99]. Gonadal sex hormones likely contribute to these effects in that dopamine release in the NAc shell is significantly increased when female rats are self-administering sucrose during the estrous phase of their cycle [100]. Finally, in choice paradigms, female rats are more likely than their male counterparts to select sucrose over cocaine [101], suggesting that sweet solutions exert a more powerful control over behavior in this sex. Taken together, these data suggest that intake of palatable food is driven more strongly by hedonic properties, regardless of metabolic state, in females than males. It should not be surprising, therefore, that there is a higher proportion of female, compared to male, rats in binge-prone versus binge-resistant groups [102]. Sexual dimorphism in binge eating appears to extend to nonnutritive sweeteners as binge intake of saccharin is associated with increased chow intake in female, but not male, mice [26]. Although the data are preliminary, sex differences in reward processing related to palatable food may extend to artificial sweeteners.

5 Artificial Sweeteners and Substance Use Disorders

Binge intake of highly palatable food overlaps with many of the behavioral and biological features of drug abuse [15]. This suggests that BED and substance use disorder (SUD) may share a common etiology, although this contention remains controversial [103, 104].

Regardless of the interpretation, commonalities in maladaptive feeding and drug intake help to explain the high comorbidity between eating disorders and SUDs [6, 105]. Although it is difficult to untangle the causal relationship between the two, animal studies suggest that maladaptive eating precedes drug use in that rats that binge on fat later exhibit increased intake and motivation to consume alcohol [106]. Moreover, epidemiological studies in humans reveal that binge eating is associated, prospectively, with alcohol-related problems [107]. That is, individuals who met the criteria for eating disorders were more likely to report negative consequences of alcohol use even if they did not drink more than non-eating disordered counterparts. Interestingly, the relationship between binge eating and drinking in a student population was stronger in males [108], which could explain the higher comorbidity of SUD with BED in men than women [109].

In humans who binge eat, intake of sweet foods is a significant predictor of the frequency of binge episodes [9]. Responses to sweet tastes, therefore, may play an important role in the emergence and acceleration of food or drug intake. This could explain why sucrose bingeing in rats alters subsequent responses to drugs, manifested as increased locomotor sensitization to psychostimulants, such as cocaine or amphetamine [110, 111]. There is some evidence that nonnutritive sweeteners produce similar effects. Rats bred for high saccharin intake show more rapid acquisition of cocaine self-administration, slower rates of extinction, and increased reinstatement to cocaine seeking [101]. The relationship could be bidirectional as rats bred for high intake of alcohol show increased saccharin consumption [112].

6 Conclusions

Binge eating is a common element of many eating disorders and one of the primary factors in growing rates of obesity. Both binge eating and obesity are driven by overconsumption of highly palatable food that is high in sugar and/or fat. This intake reflects hedonic, rather than metabolic, processing suggesting that sweet-tasting food that contains minimal calories should help to reduce both binge eating and obesity. Neither clinical nor preclinical studies confirm this idea, although the data across experiments is often contradictory. Further work is required to unravel the relationship between behavioral and biological mechanisms mediating the rewarding effects of natural versus artificial sweeteners. Animal models are an important tool in this endeavor, particularly in terms of understanding sex differences in response to palatable food.

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Chapter 8

Intermittent Extended Access Rodent Models of Compulsive Eating

Eric P. Zorrilla, Alison D. Kreisler, and Samantha R. Bagsic

Abstract

Compulsive eating is prevalent in binge-type eating disorders, present in some obese individuals, and often conceptualized in relation to the recently operationalized construct of food addiction. Compulsive eating putatively involves escalated intake of highly preferred foods, finickiness toward otherwise acceptable alternatives, increased effort and time spent to obtain preferred foods, eating behavior despite incorrect or adverse outcomes, and eating of palatable food in order to soothe abstinence- and stress-induced negative emotional states. We review theoretical and empirical bases for an opponent-process affective dysregulation model of compulsive eating, adapted from the addiction field, whereby intermittent, extended access to palatable food progressively dampens reward circuitry and potentiates activation of stress circuitry. We then detail corresponding protocols for two rodent models of intermittent, extended access to palatable food, describe methods for assessing compulsive-like outcomes, and discuss possible adaptations that can be used to understand better the prevention, biology, and treatment of compulsive eating.

Key words Rat, Animal model, Compulsive eating, Intermittent extended access, Palatability, Food reward, Diet cycling, Food addiction, Bulimia nervosa, Binge eating disorder, Obesity

1 Introduction

1.1 Compulsive Eating and Food Addiction

A cultural epidemic, "compulsive eating" is the most frequently described "compulsive" behavior in the modern English language, constituting ~2% of the written use of "compulsive" [1]. Defined as eating behavior that results from or is related to an irresistible urge and that persists despite negative or incorrect outcomes, compulsive eating is often experienced as unwanted and uncontrollable [1]. Compulsive eating is a construct captured in the Yale Food Addiction Scale (YFAS). Adapted from the *Diagnostic and Statistical Manual of Mental Disorders, 4th Edition*, (DSM-IV) criteria for substance dependence [2, 3] and revised per DSM-V criteria for substance use disorders (YFAS 2.0) [4–6], YFAS items measure (1) food reward tolerance, (2) escalation of intake, (3) increased

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effort/time to obtain food, (4) loss of control over intake, (5) eating despite (risk of) adverse consequences, and (6) negative emotional symptoms upon abstinence, with food eaten in order to relieve affective "withdrawal" symptoms (*see* also [7]). Compulsive eating is represented in YFAS items that assess "loss of control," "escalated intake," "increased time/effort to obtain food," and "eating despite negative consequences" [1, 8–10]. Per YFAS criteria, compulsive eating is more prevalent in women than in men and in younger populations [5, 11, 12] and common in binge-type eating disorders, such as bulimia nervosa (~84–100%) and binge eating disorder (BED: ~47–57%) [2, 3, 5, 13, 14], which affect ~8–10% of Western women [15–17]. Compulsive eating also is seen in approximately half of bariatric surgery patients [2, 3, 5, 13, 14] and a subset (~15–33%) of overweight/obese individuals. Most men and women are overweight [18], implying that tens of millions of Americans present compulsive eating. Normal-weight individuals without diagnosed eating disorders also meet YFAS criteria, emphasizing the extent of this public, mental health problem.

1.2 Predictive Validity

Compulsive eating, as reflected by YFAS scores, is related to greater binge eating, night eating, caloric and fat intake, and body mass index (BMI) in both obese and nonobese populations [14, 19–26]. YFAS scores also predict negative emotional symptoms (e.g., depression, anxiety, and post-traumatic stress symptoms), emotional reactivity, insomnia, and the use of food to self-soothe [20, 27–40]. Consistent with the hypothesis that there are shared, competing substrates for food and substance use disorders [10, 41, 42], adolescents with food addiction symptoms were more likely to have used alcohol, cannabis, and cigarettes [43]. Conversely, food addiction was more prevalent in men with heroin use disorder [44]. Food addiction also predicts poor ponderal and metabolic outcomes. These include visceral adiposity (a marker of cardiometabolic risk) even in women with a normal BMI [45] and a higher prevalence of type 2 diabetes [46, 47]. Inferior weight intervention results are often seen among those with food addiction [48], such as greater attrition from weight management programs [49], less weight loss [36, 50], and more "transfer addictions" after bariatric surgery [50].

1.3 Animal Model of Compulsive Eating: Intermittent, Extended Diet Access

Limiting the duration of access to preferred food can increase intake. Accordingly, we and others have used animal models of highly restricted access to palatable food with durations as brief as 10 min/day in order to study resulting changes in binge-like intake and emotional function [1, 10, 51–63]. These "limited access" models can help us to understand the biology, prevention, and treatment of highly restrained, pathologic binge eating behavior in humans [7, 58, 59, 64]. On the other hand, the intermittent,

extended access animal models of compulsive eating described in this chapter stem from the hypothesis that cycles of extended duration overeating vs. dietary restriction may be more akin to chronic, relapsing cycles of extended drug use vs. abstinence that are seen in addiction [8, 10, 51–53, 65–67]. The underlying premise is that extended access to palatable food has addiction-like effects because it (1) often involves dietary cycles of contrasting high vs. low reward and (2) promotes persistent counterregulatory opponent processes to restore emotional homeostasis that downregulate brain reward function and enhance stress system responsivity.

1.4 Diet Cycling of Reward

Intermittent access reflects dietary patterns that are common in humans who develop disordered eating. Energy-dense foods initially promote overeating and weight gain [68–70]. However, many people diet thereafter [71, 72] because of cultural norms for thinness or fitness, health worries, and unrealistic body ideals [73–77]. A survey review found that approximately half of one million respondents had attempted to lose weight during the past year. Even higher rates of dieting are seen in overweight individuals, women [78], and young adults. Many individuals diet despite being at a "healthy" BMI [79, 80]. When dieting, many people abstain from certain "illicit" foods based on their "richness," macronutrient composition (e.g., sugary, starchy, or fatty), or caloric content [78, 81–84] and limit themselves to typically less tasty, but nominally healthier, choices. As we and others have discussed [8, 51–53, 65–67, 85, 86], this dietary pattern of reward contrast can increase the reinforcing value of "illicit" foods and devalue alternatives. Dieting can thereby lead to cyclic patterns of reward and intake with overeating (high reward) vs. abstinence from (low reward) palatable food. Diet cycling is a putative causal risk factor for compulsive eating [8, 53, 64, 67], binge eating disorders [87–89], and weight gain [90–92].

1.5 Opponent-Process Affective Dysregulation

In addition to diet cycling, extended intake of highly palatable food also is hypothesized to drive addiction-like changes via the counterregulatory, opponent-process downregulation of brain reward circuitry and recruitment of brain stress circuitry [1, 10, 93–101]. This premise follows from findings in animal models of addiction that more extended durations of access to drugs of abuse promote addiction-like changes in drug use, seeking, reinforcement, and withdrawal [102–115]. For example, extended access periods (e.g., 6–23 hr/day) promoted escalated intake of several psychostimulants and opiates, whereas briefer access periods did not (e.g., 1–3 hr/day). Extended access periods also increased the reinforcing efficacy of drugs; compulsive drug seeking despite punishment, risk, or non-reinforcement; negative emotional withdrawal symptoms; and recruitment of brain stress circuitry.

Repeated cycles of extended consumption of palatable food, similar to drugs of abuse, are hypothesized to elicit opponent processes that occur earlier, have a greater magnitude, and are more persistent. This putatively leads to a deficit emotional state whereby increasingly palatable food is needed to sustain intake; also, greater amounts of palatable food must be eaten to maintain or approach euthymia. If palatable food is not eaten, then negative emotional signs emerge, such as irritability, anxiety, dysphoria, and subjective feelings of need. Thus, as individuals progress to compulsive intake, the prime motive for eating palatable food is proposed to shift from reward to relief (i.e., self-medicating the hypohedonia and negative emotional states that emerge when preferred foods are not eaten) [1, 93].

1.6 Human Neurobiological Evidence of Dampening of Reward Circuitry

Consistent with this opponent-process, affective dysregulation model, humans with conditions in which compulsive eating is overrepresented exhibit fewer dopamine D2 receptors in the striatum, which subserves reward processing, and lower basal or food-stimulated striatal dopamine release [116–124]. Food-deprived obese volunteers had smaller dorsal striatal extracellular dopamine responses to food [125] compared with normal-weight subjects [126]. This has been proposed to exemplify food reward tolerance [1] and may explain why adolescents who frequently eat ice cream exhibit lower activation of the putamen and caudate in response to an ice-cream milkshake, defined by lower functional magnetic resonance imaging blood-oxygen-level-dependent signals, compared with less frequent ice-cream eaters [127].

Human findings also support the related hypothesis that dampened reward system function [42, 128–132] in the mesolimbic dopamine pathway [42, 133–138] promotes further overeating of palatable food in humans. For example, obese individuals exhibit lower striatal dopamine D2 receptor levels compared with nonobese controls in direct relation to higher BMIs [131, 139]. Lower caudate activation responses to a palatable milkshake also are seen in obese vs. lean individuals [140] and prospectively predict increases in BMI over a 6-month period in women [141]. Conversely, many psychotropics that show efficacy to treat disorders with compulsive eating may do so because they enhance mesolimbic dopamine signaling. These medications include lisdexamfetamine dimesylate, a prodrug of *d*-amphetamine used to treat BED [142–148]; tesofensine, a triple monoamine reuptake inhibitor used to treat obesity [149–152]; and Contrave, a sustained release formulation of bupropion (dopamine/norepinephrine reuptake inhibitor)-naltrexone with reported efficacy against obesity [153] and obesity with comorbid binge eating [154, 155].

Differences in opioid system function, which has been linked to reward, also are seen in conditions in which compulsive eating is present. For example, the opioid receptor antagonist naltrexone

elicited greater cortisol stress responses and aversive nausea in obese women with food addiction symptoms and reward-driven eating than in obese women low in these measures [156].

1.7 Dampening of Reward Circuitry in Extended Access Models

Results from animal models support the hypothesis that allostatic decreases in reward function occur in rodents with extended access to palatable food. Intracranial self-stimulation thresholds in the lateral hypothalamus increased in rats following extended (23 hr/day), but not limited (1 hr/day), access to a palatable high-fat/high-sugar cafeteria diet [57]). Elevated self-stimulation thresholds, an index of impaired brain reward function, develop with obesity and persist despite forced 2-week abstinence from a cafeteria diet. Extended access to a cafeteria diet also differentially reduced striatal dopamine D2 receptor levels in rats. Lentiviral-mediated knockdown of D2 receptor expression accelerated diet-induced elevations of reward thresholds, suggesting a causal role in impaired brain reward function [57]. Chronic exposure to palatable food also decreased basal [157, 158] and amphetamine-induced dopamine release in the nucleus accumbens [157, 159] and decreased dopamine transporter expression and function [160]. Consistent with the hypothesized food reward tolerance, standard chow no longer increased dopamine efflux in chronic cafeteria-diet-fed rats, whereas it did so in chow-fed rats [157]. Nonetheless, the cafeteria diet still elicited dopamine release, implying that continued intake of the cafeteria diet is needed to prevent a dopamine deficit. Conversely, the therapeutically effective monoamine transporter inhibitors lisdexamfetamine and tesofensine both increased striatal dopamine efflux and opposed chronic palatable-food-induced decreases in mesolimbic dopamine signaling [161–165].

As in humans, there also is evidence of altered opioidergic system following extended access to palatable tastes with reduced NAc preproenkephalin mRNA levels [166] and increased sensitivity to naloxone-precipitated withdrawal in rats with a history of intermittent access to sugar solutions [167].

In our models of intermittent, extended access, binge-like escalation of intake and operant self-administration are seen upon renewed access to the palatable food [8, 53, 54, 66, 67, 168]. Rats with weekend access to a palatable diet exhibited progressively greater cumulative weekend intake across cycles of access and two-fold greater intake within the first 3 hr of renewed intake compared with chow-fed rats [8, 66]. Rats with intermittent (Monday-Wednesday-Friday [MWF]), extended access (24 hr/day) developed even more pronounced overeating with binge-like home cage intake or operant self-administration during the first 30 min of renewed access that was comparable in magnitude to that of rats that received intermittent, highly restricted (30 min/day) access. Under the MWF extended access schedule, rats showed almost twofold greater daily intake on access days vs. rats that received

chow or the palatable diet ad libitum [53, 67, 168]. The results demonstrate a key role for intermittency, and not only restrictedness, in escalating intake of palatable food [53, 67, 168]. Additionally, the results are consistent with the hypothesis that rats with intermittent, extended access undergo a shift in food reward set point or develop food reward tolerance.

Also consistent with this hypothesis, in our animal models, other previously acceptable rewards become less effective at supporting intake or operant responding. Rats that received cycles of weekend access to a palatable, chocolate-flavored, sucrose-rich diet showed lower progressive ratio (PR) break points than chow-fed controls when responding for a less preferred, but otherwise palatable, corn syrup-sweetened chow [8, 66]. Likewise, weekend or MWF extended access to highly preferred diets led to the underconsumption of otherwise acceptable chow even when it was the only food available, leading to voluntary weight loss [8, 51, 57, 65, 66, 169–171]. The undereating of chow increased with longer durations of access to palatable food [53] and became progressively larger in magnitude and more persistent with repeated diet cycles [53]. This undereating may result from extended diet exposure, because rats that received chronic ad libitum access to a highly preferred diet continued to undereat chow for at least 2 weeks after it was the only diet available, despite having returned to normal body weight and adiposity [53]. This "finickiness" may be as problematic as the initial overeating behavior because it drives diet choices toward more palatable and ostensibly less healthy options in a vicious circle.

1.8 Human Evidence of Stress Circuitry Activation

Several results support the hypothesis that abstinence from food reward activates negative emotional, stress-like circuitry. Dieting, for example, prospectively predicts higher self-reported "stress" [172] and depressive symptoms [173] in both overweight and non-overweight individuals [174–177]. On a briefer time scale, individuals who habitually skip breakfast have higher distress, depressive symptoms, and suicidal ideation [178–184]. The portmanteau "hangry," now recognized in the Oxford English Dictionary, refers to increased irritability during abstinence from food. As one colorful study showed, women with lower glucose levels more often stabbed pins into a voodoo doll that represented their spouse and delivered more extreme aversive blasts of noise to their spouse [185]. As another example, volunteers who were switched to a presumptively less palatable diet after eating a high-fat diet for one month reported more subsequent anger, anxiety, and hostility vs. volunteers assigned to keep eating the high-fat diet [186]. Many randomized, controlled studies find that changes in diet lead to changes in mood [187–189]. These effects often are attributed to physiochemical properties of the diet (e.g., fat, carbohydrate, specific fatty acids, cholesterol, electrolytes). Less attention

has been given to the role of preference, liking, or other subjectively evaluated properties of the diets [190, 191], as proposed in the models that are described below.

Several neuroimaging studies also support the hypothesis that stress circuitry is differentially activated in disorders with compulsive eating (*see* also [41]). Food addiction symptoms and obesity are each linked to heightened amygdala reactivity to pictures of a milkshake [192, 193]. Women who have obesity similarly exhibited greater amygdala responses to pictures of palatable high-calorie foods (e.g., cheesecake) vs. less preferred, low-calorie foods (e.g., steamed vegetables) [194]. Drug and alcohol cues also elicit greater amygdala responses in volunteers with substance use disorders [195–199]. We have proposed that this cue-induced amygdala activation reflects aversive circuitry, engaged by frustrative reward omission (e.g., not receiving the milkshake) or conditioned opponent processes [1]. Consistent with a relationship of altered amygdala function to compulsive eating, healthy controls exhibited smaller amygdala responses to sucrose when sated (vs. fasted), whereas women recovered from bulimia nervosa did not. As a result, they showed greater amygdala responses to sucrose compared with controls when sated [200], as do obese children [201].

Pharmacological evidence of stress circuitry activation in volunteers with a history of intermittent palatable food intake came from a double-blind, placebo-controlled trial in individuals with restrained eating. Pexacerfont, an antagonist of the stress-related corticotropin-releasing factor type 1 (CRF_1) receptor, showed promise to reduce food craving and laboratory-stress-induced eating [202]. The study was halted by the National Institutes of Health Institutional Review Board because of human subject protections that were unrelated to efficacy or adverse events. At the time that the study was stopped, pexacerfont descriptively reduced laboratory-stress-induced eating ($r = 0.30$) and craving for sweet foods ($r = 0.28$–0.49). In bogus taste tests whose genuine purpose was to allow observation of total food intake, pexacerfont reduced palatable food intake across all imagery scripts ($r = 0.34$). Finally, nightly food addiction symptoms, measured by the YFAS, were lower in subjects who received pexacerfont ($r = 0.39$). Both Bayes factor and counternull analyses indicated a positive potential of pexacerfont [202], providing a rationale to study the neurobiological role of CRF_1 system activation in compulsive eating.

1.9 Recruitment of Stress Circuitry in Extended Access Models

Signs of a negative emotional state also were seen in animals that were withdrawn from extended access to palatable food. An early finding was increased "nippiness" during withdrawal from sucrose solutions [203]. We similarly observed an increase in irritability-like behavior with more aggressive behavior directed toward a bottle brush (e.g., mounting, biting, following/chasing) in rats that were withdrawn for 24 hr onto chow diet from our MWF extended access model [168].

Hoebel, Avena, and colleagues found that rats with daily 12-hr access to high-sugar solutions plus chow alternated with 12-hr food deprivation exhibited somatic and anxiogenic-like signs of opioid withdrawal when challenged with the opioid receptor antagonist naloxone [167] or after a 24–36 hr fast [64]. This anxiogenic-like phenotype was accompanied by a "harm-avoidance profile" [204] of higher NAc extracellular acetylcholine levels vs. lower dopamine levels [167], similar to morphine withdrawal [205, 206].

We similarly found that weekend-diet-cycled rats showed an increase in anxiety-like behavior in the elevated plus maze and defensive withdrawal tests when tested during the chow phase of their diet cycle ([8, 66]; but *see* [170]). Using the same model, Cottone, Sabino, Iemolo, and colleagues also observed an increase in forced swim immobility, a depressive-related behavior in withdrawn diet-cycled rats [207]. Boutrel and colleagues found lower locomotor activity in a novel open field, a measure of greater emotionality [170].

When withdrawn from palatable food, rats in the weekend access model showed increased expression of CRF in the central nucleus of the amygdala (CeA) [8]. Similarly, CeA CRF systems are activated during withdrawal from alcohol [208–211], opiates [212–215], cocaine [216], cannabinoids [217], and nicotine [110, 218] in animal models. Both the undereating of chow and the motivational deficits in responding for otherwise acceptable food seen in our weekend access model were mitigated by pretreatment with a CRF_1 receptor antagonist [8]. This may be analogous to the ability of a CRF antagonist to reverse the decrease in reward function seen during nicotine or alcohol withdrawal [219, 220]. Pretreatment with the selective CRF_1 antagonist R121919 also blocked anxiogenic-like behavior during food withdrawal at doses that did not alter behavior in chow-fed controls [8]. Analogously, CRF_1 receptor antagonists also reduce aversive- and anxiety-like states during withdrawal from alcohol [211, 221, 222], opiates [223, 224], cocaine [225, 226], and nicotine [110]. Finally, CRF_1 receptor antagonist pretreatment also blunted the degree to which weekend-cycled animals overate the preferred diet upon renewed access at doses that did not alter intake of chow-fed controls or of rats fed with sucrose-rich diet but without a history of weekend diet cycling [8]. Similarly, CRF_1 antagonists reduce the intake of alcohol [209, 227–232], cocaine [233], opiates [102], and nicotine [110] in animal models of dependence while having less effect on self-administration in nondependent animals.

When access to the palatable diet was restored, the plus-maze behavior, forced swim immobility, and levels of CeA CRF in weekend-diet-cycled animals normalized to levels of chow-fed controls, consistent with the hypothesis that activation of the amygdala CRF system and associated negative emotional behavior resemble

an acute withdrawal-like state [8, 66, 207]. Similar to findings during alcohol withdrawal [210], weekend-diet-cycled rats are more sensitive to the ability of CRF_1 receptor antagonists to modulate CeA-aminobutyric acid (GABA)ergic transmission. Specifically, R121919 reduced evoked inhibitory postsynaptic potentials to a greater degree in diet-cycled rats. Finally, Cottone, Sabino, Iemolo, and colleagues also found that intra-CeA infusion of R121919 reduced the anxiogenic-like behavior and palatable diet overeating of withdrawn weekend-diet-cycled rats [169]. The latter results resemble the ameliorating effects of intra-CeA CRF antagonist administration on withdrawal-associated negative emotional symptoms and self-administration in models of alcohol and substance use disorders [234]. In summary, weekend-diet-cycled rats exhibit abstinence-associated activation of central extended amygdala CRF systems, similar findings in many animal models of addiction. The activation is hypothesized to contribute to compulsive eating in humans, as suggested by the halted NIH pexacerfont clinical study.

Neuroadaptations in the brain endocannabinoid (eCB) system also are seen in the weekend access model. Amygdala eCB-cannabinoid-1 receptor (CB1) signaling has been conceptualized as an anti-stress buffer that is activated during stress in compensatory fashion [235, 236], the deficiency of which may increase vulnerability to negative emotional symptoms [237]. Consistent with a stress-like response, withdrawal from cyclic weekend access to palatable food increased (compensatory) levels of the eCB 2-arachidonoylglycerol and its CB1 receptor in the CeA [238]. Systemic or intra-CeA infusion of the CB1 receptor inverse agonist rimonabant more potently precipitated anxiogenic-like behavior and anorexia in rats that were withdrawn from cyclic palatable food compared with chow-fed controls [238, 239]. These findings may explain why rimonabant and taranabant, another CB1 receptor inverse agonist, produced adverse psychiatric side effects in obese patients, including anxiety, depression, and irritability, that led to their withdrawal from the clinic and drug development [240].

1.10 Motivational Measures of Compulsivity

The above findings of altered food reward function, voluntary intake cycling, and abstinence-associated negative emotional behavior with activation of brain stress circuitry support the hypothesis that rats with intermittent, extended access to palatable food may develop compulsive eating. To test this hypothesis further, we incorporated operant measures of performance into the MWF access model to determine whether rats continue to respond to palatable food despite incorrect [241] or adverse [242] outcomes. Consistent with the former, the rats exhibited disproportionately increased responses during "timeout" periods when responding was not reinforced and qualitatively greater PR break points compared with rats that received chow or the palatable diet

ad libitum [67, 168]. Consistent with the latter, the majority of the rats developed punishment-resistant responding in which they continued to self-administer food excessively despite receiving contingent footshock with food delivery [168]. We also found that optogenetic inhibition of an excitatory projection from the anterior insula to the ventral striatum modulated both PR and intermittently punished responding in rats that developed highly compulsive eating behavior, but not in controls, thus implicating altered insular-striatal function in highly compulsive eating [168].

2 Materials

2.1 General Notes

Our models of intermittent, extended access to palatable food were developed from Wistar rats. Studies have been conducted in both male and female rats, ranging in ages from 6 to 9 weeks at the onset of the study. A variant of the weekend access model in C57BL/6J mice has been published [243], and we have recapitulated key features of the MWF extended access model in a variant using C57BL/6J mice as well (Spierling, Hui, Fang, Williams, Huang, Pucci, Murphy, and Zorrilla, in preparation). To our knowledge, the models have not been studied in other genetic backgrounds or species.

Rodents should be housed in a climate-controlled vivarium (~21–22 °C, 60% humidity) with a 12-hr light/dark cycle. To facilitate experimental procedures near the onset of the dark cycle, we typically house subjects under a reversed-light cycle (e.g., lights off at 9 a.m.). Experimental procedures (including handling, housing, and husbandry) must adhere to relevant government and institutional regulations and should be conducted in accordance with NIH's *Guide for the Care and Use of Laboratory Animals* and approved Institutional Animal Care and Use Committee (IACUC) protocols.

Rodents should be divided into experimental vs. control groups (at least $n = 6$–10/group) through either random assignment or matched for relevant measures. We typically assign rats to treatment groups that are matched for baseline body weight and daily chow intake using *z*-scores. When relevant, one can use body composition (e.g., via EchoMRI) and baseline operant performance as additional matching variables. If one seeks to compare subgroups of rats that do vs. do not develop highly compulsive behavior following intermittent, extended access, then the sample size should be doubled for the intermittent access group. Potential control groups are discussed in Subheading 3.

2.2 Caging and Animal Preparation

Animals should be housed in a manner that allows for the accurate measurement of individual food intake throughout the experiment. This can involve individual housing or, alternatively, pair housing in a cage, separated by clear plastic dividers perforated with holes. The

latter "buddy barrier" approach can reduce effects of isolation stress while permitting individual measurement of intake [244]. Because deprivation is not experimentally imposed in the model, rodents can be housed in wire-topped, plastic shoebox-style cages using noncaloric contact bedding (e.g., no corncob- or rice hull-based bedding).

Upon arrival, rodents should be allowed at least 5 days to acclimate to the environment and the chow diet of their new vivarium, during which they are handled daily. To facilitate rapid, less invasive measurement, food is provided to each rat in a J-feeder (e.g., GPF20, Ancare, Bellmore, NY) or similar food hopper, rather than on the wire top or in the bedding. Food and water are provided ad libitum for the duration of the studies. Water can be provided via steel-ball valve sipper bottles or automatic watering systems.

If desired, acute intake measurements also can be collected with the rodents housed over a wire mesh or wire grid, such as in operant test cages or with a wire pedestal insert placed in shoebox cages. This can reduce potentially confounding effects of pica or hoarding and facilitate measurement of spillage. Wire-bottom cages are not recommended for chronic housing, however, due to potential stress-potentiating effects [245, 246].

One important note is that if the studies will involve an operant component, then the home cage "chow" diet for all rats, including controls, must be the same diet that they receive for operant studies. This is necessary because commonly used chow operant pellets (e.g., TestDiet 5TUM) are highly preferred to standard vivarium chow. We found that this also was true even when we precision-pelleted standard vivarium chow, perhaps reflecting the binders used, texture, or portion size of precision pellets. An undesired cyclic binge-like/rejection-like pattern of intake develops in controls if they do not receive the identical diet between the operant and home cage settings.

Both home cage intake and, where relevant, operant performance should be confirmed as being stable across three consecutive days (<15% variation from mean) before assigning subjects to diet schedules. This interval will be longer than 5 days (e.g., ~2–3 weeks) for subjects undergoing operant training.

2.3 Palatable Diet

Cyclic overeating vs. rejection behavior is elicited using a highly preferred, nutritionally complete diet. The diet used in most of our studies is a chocolate-flavored, high-sucrose (50% kcal), AIN-76A-based purified, open-source diet (originally made as chocolate-flavored Formula PJPPP by Research Diets and P.J. Noyes and now by TestDiet as formula 5TUL). The diet is nutritionally complete and broadly similar in macronutrient proportions and energy density (66.7% [kcal] carbohydrate, 12.7% fat, 20.6% protein, metabolizable energy 3.44 kcal/g; retrieved from TestDiet,

10/1/2019, ducm04_026412.pdf) to many commonly used vivarium diets. We chose such a diet in part to increase focus on the effects of diet preferredness, as opposed to malnourishment, macronutrient composition, or caloric density. The preferred diet was selected in two-diet choice studies from a range of purportedly palatable rodent diets and flavorings that were commercially available in 2004–2005 and are highly and universally preferred in every rodent we have tested vs. both standard vivarium chow (e.g., corn-based Harlan Teklad/Envigo LM-485 7012: 65% [kcal] carbohydrate, 13% fat, 21% protein, metabolizable energy 3.41 kcal/g, 90–92% preference ratio over 24 hr) [53, 66] and traditional operant chow pellets (e.g., No/Yes/Formula A/I/5TUM; 80–91% preference ratio) [53, 65]. The diet is precision-pelleted (45 mg for rats, 20 mg for mice) even for non-operant studies to increase its preferredness and facilitate binge-like intake [247].

In variants of the procedures, we also observed increasingly cyclic intake patterns by alternating standard vivarium chow vs. an even less preferred vivarium corn-based chow (Teklad LM-485 vs. Purina 5012; LabDiet) [66] and by alternating operant chow pellets vs. standard extruded vivarium chow. Because a given diet can serve as either the "accepted" or "rejected" diet depending on the alternative diet available, relative preferredness per se, rather than any specific diet ingredient, is proposed at least in part to drive the cyclic intake behavior.

Preweighed food (using a scale of 0.1 g and 0.01 g precision for rats and mice, respectively) is provided in approximately twofold excess of the upper range of intake that is observed in the subjects, post-weighed using the same scale, and replenished daily. Food spillage should be separated from bedding and feces, the former assisted by sifting through a mesh grate, and included in measurements. Water bottles should be changed twice weekly. Cages and bedding should be once to twice weekly for individually housed subjects and pair-divided subjects.

3 Procedures

3.1 "Weekend" Model

The main experimental group each week will receive 2 days of continuous access to the preferred palatable diet (e.g., TestDiet 5TUL) and 5 days of access to the less preferred chow diet (e.g., Envigo LM-485 or, if operant studies will occur, TestDiet 5TUM). Although the procedure is referred to as the "weekend" model, the rat's 2-day "binge weekend" can be scheduled to occur during the traditional work week. This facilitates the measurement of changes in intake and behavior that occur when access to palatable food is restored vs. withdrawn. An alternate form of the "weekend" model has been developed in which 2 days of palatable food access are alternated with only 1 day of chow [239]. Preweighed food is

provided at the onset of the dark cycle and measured daily or not less often than at the beginning and ending of each diet phase. Intake can be expressed as either grams or calories. Changes in intake occur after the first diet cycle and progressively grow across weeks [8, 66].

3.2 "Monday-Wednesday-Friday" Model

The main experimental group will receive 24-hr/day access to the preferred palatable diet only on Monday, Wednesday, and Friday of each week (three nonconsecutive days 1–2 days apart). On all other days, they will only receive ad libitum access to the less preferred, control chow diet. Preweighed food is provided within the 30 min preceding the onset of the dark cycle and measured daily. Intake can be expressed as either grams or calories.

3.3 Model Comparison

Changes in binge-like intake, daily overeating, and chow hypophagia are greater in the MWF model than in the weekend model. The MWF model exposes subjects to more palatable food each week (both in time and quantity) than the weekend model and also offers three of each type of dietary switch each week in which to perform causal or observational analyses, rather than only one as in the weekend model. The more sustained palatable food exposure of the weekend model may promote more lasting adaptations in food reward circuitry, because the progressive rejection of less preferred food appears to persist across more days in the weekend model. For both models, the 7-day length of diet cycles reduces the likelihood that the estrous cycle (4–5 days) will account for observed effects and resembles designs that were used in previous diet-cycling studies [248]. In both models, the daily time commitment will vary depending on the number of subjects studied and, if operant studies are performed, on the number of self-administration apparatus available. In our laboratory, this is typically up to 1 hr/day for free-feeding and up to 2 hr/day for studies with operant components.

3.4 Comparison Groups

Potential control groups for both models, depending on the experimental question, can include the following:

1. *Ad libitum chow.* This group allows comparison to the normal behavior and biology of animals without a history of palatable diet access.
2. *Ad libitum palatable diet.* This group allows one to determine whether observed effects are actually due to the intermittent nature of access as opposed to simply the palatable diet per se. We typically do not also provide concurrent chow access to this group because then animals also would differ from the experimental group by having dietary choice/variety at any moment in time. Depending on the experimental question, this group could have ad libitum access for the entire duration of the study, or alternatively, they could have continuous ad

libitum chow access for same number of chow days experienced by the experimental group and then followed by continuous ad libitum palatable diet access for the total number of palatable diet access days received by the experimental group.

3. *Pair-restricted chow group*. Because the diet-cycling groups engage in self-restriction and substantially undereat the less preferred diet to the point of negative energy balance and weight loss, it can be useful to determine the degree to which ponderal, behavioral, and biochemical changes that are seen in the experimental group directly result from this undernutrition. To determine this, a pair-restricted chow group can be run in which rats are pair-restricted during the nonaccess phase, limited to a ration that is equal to the mean intake of the experimental group rats on their corresponding nonaccess day. The ration is provided in two portions during the dark phase approximately 6–8 hr apart. Chow is provided ad libitum to the pair-restricted group during the "access phase" of the experimental group.
4. *Brief palatable diet ("intermittent-short")*. To determine which effects are due to the *extended* nature of palatable food access as opposed simply to diet intermittency, one also can include a comparison group that receives much briefer access to the palatable diet on each occasion (e.g., 30–60 min). Such highly restricted access also elicits binge-like intake but seems less able to produce signs of food reward tolerance, such as persistent undereating of less preferred food to the point of voluntary weight loss and weight cycling [53, 54, 168]. Note that the intermittent-extended experimental group also conversely can serve as a useful comparison for the intermittent-short group in order to identify effects that uniquely result from highly restricted access.

3.5 Operant Responding Analysis Options

At the outset of all studies, rodents are trained to self-administer standard chow pellets (5TUM) in previously described operant chambers [67] with two levers (active lever, inactive lever). Water is available ad libitum via a steel-ball valve sipper tube. After completing a ratio requirement at the active lever, one precision pellet will be delivered 0.5 sec later, followed by a 3.25-sec post-reinforcement timeout interval, during which responses have no scheduled consequences. Responses during this non-reinforced period are deemed "timeout" responses [249] and increase even more than reinforced responses in rats that receive intermittent access to palatable food. Responses at the inactive lever have no scheduled consequences. No food restriction, explicit cues, or lights are utilized in training or test sessions; house lights remain off for the entire session.

Prior to diet assignment, all rats are trained over a 2–3-week period reinforced by 5TUM chow, during which they also receive ad libitum 5TUM chow in their home cages. Training begins with a single 24-hr fixed-ratio 1 (FR1) session that is run together with the cagemate, which, in our laboratory, accelerates initial performance. This pair training only occurs on the first day. To accustom the rats to briefer opportunities to self-administer, they then receive individual 6-hr and then 2-hr FR1 individual sessions until a criterion of $\geq$75% discrimination between the active vs. inactive lever is achieved (typically 1–2 sessions of each duration). These are followed by individual 30-min FR1 sessions until each rat attains a criterion of 10 pellets/session (typically 3–5 sessions). Finally, the rats receive one progressive ratio (PR) session in which the response requirement increases exponentially with successive reinforcers per the following reinforcement schedule progression [65]: response ratio requirement = $[4(e^{\#\text{ of reinforcer}*0.075}) - 3.8]$, rounded to the nearest integer. The PR session ends when a rat does not acquire another reinforcer in 14 min, with a maximum duration of 2 hr. The "break point" is defined as the last response requirement completed and is qualitatively increased in MWF rats as opposed to ad libitum-fed controls. Following training, the rats are assigned to experimental vs. control diet conditions as described in Subheading 2.

Once diet cycling begins, all rats in the MWF model (including ad libitum-fed controls) receive two weekly FR1 sessions and one weekly PR session, all given at dark onset. All rats receive these sessions at the onset of the dark cycle on days that experimental rats receive access to the preferred diet. Access to the preferred diet on these days begins at the start of the operant session, and each rat will respond for its group's respective diet for 30 min on FR days or until the break point is reached on PR days (typically 30–60 min and no longer than 2 hr). After completing the FR or PR session, the remaining duration of preferred diet access for the day then occurs in the home cage. Asymptotic, escalated FR and PR performance are typically reached within 4–6 weeks for MWF rats, after which causal or observational studies can occur.

If one wishes to assess punishment-resistant responding, then three "preparatory" intermittent shock punishment (FR3, 0.1 mA, 0.5-sec footshock) and continuous food reinforcement (FR1) operant sessions (i.e., food reinforcement every lever press with concurrent shock punishment every third lever press) are performed before the test days of interest. As a desirable punishment schedule, a random-ratio schedule of 30% probability of footshock per trial can instead be used in order to avoid artifacts that can occur with predictable, FR punishment schedules. Each of these preparatory punished sessions is separated from the other by a standard 30-min non-punished FR1 session to promote the recovery of responding. Thereafter, the subjects can be compared for punishment-resistant self-administration in untreated control vs. causally manipulated

(e.g., drug treatment, optogenetics, chemogenetics) conditions, with a non-punished session inserted between each punished session. Note that punishment-resistant rats may also reduce their responding during the first 1–2 preparatory punished sessions. What defines them as being punishment-resistant is that they thereafter resume self-administering palatable food in subsequent sessions despite punishment. Following each operant session, the rats are returned to their home cage where their assigned diet and water are available ad libitum.

If one instead wishes to assess the magnitude of invigorated extinction bursts and subsequent resistance to extinction as measures of compulsivity, then five 30-min extinction sessions can be performed on consecutive days during which responses at the active lever no longer elicit food delivery. A subject's extinction burst is defined as the number of responses that occur within the 5 min after their first response and is analyzed as the delta from that subject's average number of responses during the analogous first 5 min of 2–3 reinforced self-administration sessions. Compulsive-like responding is interpreted as a larger extinction burst over previously reinforced responding and as resistance of the extinction burst to decay across the five extinction sessions [250].

Operant sessions can similarly be performed using weekend-model rats, but they are limited to only one operant session per week that can occur only on the first of the two "weekend" days because intake and operant performance between the two "weekend" days substantially differ.

4 Notes

4.1 Individual Differences

These models generate robust, stable individual differences in the magnitude of initial (e.g., 30 min) binge-like eating upon renewed diet access, total daily overeating, and rejection of otherwise acceptable chow diet [53, 66, 168], evidenced by extremely high intraclass correlations. The biological basis of these robust differences in consummatory responses to diet cycling has yet to be determined, but they are related to fat gain [66] and may be translationally relevant.

The models also lead to the stable divergence of instrumental performance in both male and female rats. For example, PR performance begins to separate during the second week of access [67], with a subset of diet-cycled rats dramatically escalating their PR performance at that time and other diet-cycled rats not escalating their performance. These stable differences in PR escalation are important because reinforcement efficacy-based measures in humans may better predict ponderal outcomes than intake alone [251–255]. PR performance also has been used to indicate compulsive-like responding for substances of abuse

[241, 256]. Consistent with the latter notion, PR performance in diet-cycled rats correlates strongly with their punishment-resistant responding ($r = 0.78$) [168].

To define subgroups using PR performance, we have defined "high-PR" rats as those that develop responding >2 standard deviations above the mean of all ad libitum-fed rats [67, 168], corresponding to ~315 active lever presses in a recent study [168]. Similarly, we have defined "punishment-resistant" responders as those that persisted with self-administration at levels >2 standard deviations above the mean of controls [168]. As reference, in a recent study [168], the high-PR and punishment-resistant groups constituted 54% and 63% of subjects, respectively, with almost uniform concordance between subgroups.

Attesting to the validity of these indices of compulsive-like behavior, we have found that "high-PR" diet-cycled rats exhibited greater (1) daily overeating of the preferred diet, (2) rejection of chow diet on nonaccess days, and (3) binge-like self-administration upon renewed access compared with "low-PR" diet-cycled rats. High-PR female rats also exhibited higher respiratory exchange ratios in indirect calorimetry analyses [168], a predictor of weight gain in humans [257] that indicates sparing of fat as a fuel substrate. Finally, high-PR rats showed a unique endocrine profile of jointly heightened glucagon-like peptide-1 and pancreatic polypeptide with lower ghrelin compared with all other groups [168].

4.2 Sex Differences

Although the models robustly generate cyclic intake in both sexes, several sex differences may be relevant to study design and expected outcomes. More females than males meet the criteria for high-PR responding, and high-PR females develop their associated differences in overeating and chow rejection earlier than do high-PR males [67]. Only high-PR female rats and not high-PR male rats developed the fat-sparing phenotype of elevated respiratory exchange ratios [67]. This aligns with the finding that MWF diet-cycled females gain more body weight and fat than chow-fed controls, whereas diet-cycled MWF males do not [67]. Finally, if the results are to be compared between sexes, then it is recommended that measures related to intake are normalized to body weight scaled to the 2/3 power [258].

4.3 Sweet-Fat Variation for Operant Studies

As an alternative diet, if one wishes to study a sweet-fat diet in operant studies, we designed a custom, open-source high-fat diet that uses palm oil as the fat source, making it possible to precision-pellet at room temperature (Bio-Serv F06190, 4.1 kcal/g, 35% kcal from fat, 46% kcal from carbohydrates, 18% kcal from protein, Bio-Serv, Frenchtown, NJ). This diet involves ~85% of fat as saturated fat and 67% of carbohydrate as sucrose and is strongly preferred by rats over chow in 1-hr, two-choice taste tests (>99% preference ratio) [52]. On an average basis, it is comparably

preferred to the traditional chocolate-flavored, high-sucrose diet (~50% mean preference ratio). However, some individual rats stably prefer the sweet-only diet, while others prefer the sweet-fat diet. As mentioned earlier, because a given diet can serve as the "accepted" or "rejected" diet depending on available alternatives, there may be flexibility in the types of foods or solutions that can be used to induce diet cycling.

4.4 Diet Choice

The models can also be varied to provide chow to subjects at all times and instead only to cycle the presence vs. absence of the palatable diet [53, 239]. Under these conditions, the subjects have diet choice and variety, and shifts in dietary preference ratios, indifference functions, or similar behavioral economic parameters can be explored. We have found that having two diet choices increased daily palatable diet intake under ad libitum access conditions and binge-like intake under intermittent, short access conditions. We have not, to date, detected effects of choice during intermittent, extended access, however [53].

4.5 Non-operant Compulsivity Measure

If a laboratory does not have access to an operant self-administration apparatus, a commonly used alternative measure of compulsive-like behavior is to determine the willingness of rodents to approach and eat their preferred diet despite the food being placed in an aversive, brightly lit, potentially dangerous novel environment such as an open field or light/dark box [259, 260]. This assessment pits the rats' innate avoidance of lit, exposed environments against their acquired compulsion to seek and consume the preferred diet. Preweighed preferred diet is placed in a cup in the brightly illuminated compartment of an unfamiliar test arena, and the rat is placed in the dark compartment to begin the trial. The latency to enter and total time spent in the illuminated arena have been operationalized as measures of impulsive risk-taking behavior. The amount of food that is consumed within the brief test (e.g., 10 min) has been defined as a measure of compulsive-like behavior.

4.6 Ponderal and Metabolic Outcomes

Although not emphasized in this chapter, the model also can be used to investigate the metabolic and health effects of diet cycling with palatable, high-sucrose foods, because the procedure produces rapid oscillations in food intake, body weight, and energy balance. A consistent finding is higher feed efficiency, with the experimental group reliably gaining more weight per calorie that they ingest over the course of the study. For example, in the "weekend" model, despite eating fewer calories, weekend-cycled rats actually became heavier and fatter and exhibit reduced locomotor activity during their active cycle. Relevant to the inflammation associated with metabolic disorders, they also show greater proinflammatory adipokine levels than chow controls [66].

Higher feed efficiency in diet-cycled rats may be related to the decrease in thermogenic brown adipose tissue (BAT) that we have seen in MWF model females. BAT function is known to decrease to conserve energy, such as during lactation, hibernation, and fasting. Our findings suggest that BAT mass itself may decrease as part of an energy conservation strategy. Indeed, indirect calorimetry revealed negative energy balance and rapid reductions of energy expenditure in MWF rats that were self-restricting themselves from chow [67]. Consistent with this interpretation, the magnitude of the reduction in BAT mass strongly correlated with the degree to which diet-cycled rats underrate chow ($r = 0.81$). Decreased BAT mass/function, by decreasing thermogenic capacity, could potentially explain the greater vulnerability to regain weight once access to palatable food resumes [261–267]. The fat-sparing respiratory exchange ratio phenotype of compulsive female rats that is seen during indirect calorimetry may represent another such acquired obesity risk factor [67].

4.7 Diet Schedule Variations

Many aspects of the cycling schedule could be varied to address specific questions. For example, we historically have renewed access to palatable food diet at the onset of the rats' active cycle, which in human terms is akin to breakfast time. Binges and overeating occur more often at other times of day in humans, however. Thus, schedules that renew access during the inactive phase of activity (e.g., night eating) or later in the dark cycle may be of interest. Additionally, although the diet-cycled animals dramatically reduce their caloric intake when left with chow, their meal pattern may still not resemble that of human dieters. For example, many dieters engage in outright meal skipping or day-long fasting, whereas only 10–20% of MWF model females completely fast themselves on nonaccess days. Thus, there may be value to impose periods of discrete food deprivation to simulate practices such as breakfast skipping that are practiced by many people. Finally, many individuals report being triggered to overeat by food cues or negative moods. Such triggers can be experimentally introduced by frustratively priming subjects with only 2–3 pellets of food [52] or, worse, with the smell and sight of the food [268, 269], before later testing them for consummatory and emotional behavior [93].

4.8 Conclusion

Compulsive eating, a component of food addiction, is a major public, mental health problem that is overrepresented in cardiac, diabetes, psychiatry, and bariatric surgery clinics in the developed world. Common in obesity, bulimia nervosa, and BED, compulsive eating also is highly comorbid with negative emotional conditions, including depression, anxiety, suicidal ideation, and post-traumatic stress. Compulsive eating may share competing neural substrates with substance use disorders; understanding it may also provide into the neurobiology of addiction in general. The above models

based on intermittent, extended access to palatable food provide paradigms to better understand the risk factors, etiology, pathophysiology, adverse health effects, and potential therapy of compulsive eating.

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Chapter 9

Animal Models of Binge Eating: Hedonic Feeding and Alcohol Intake

Julianna N. Brutman, Sunil Sirohi, and Jon F. Davis

Abstract

Binge eating is a behavioral component of some eating disorders associated with the development of obesity and associated metabolic pathologies. Over the past two decades, it has become increasingly clear that the intake of food and drugs is controlled by a shared set of neuronal circuitry. In this regard, the excessive food intake, present in binge eating, has been linked with development of addictive behavior. This chapter describes an animal model of fat bingeing in combination with assessment of alcohol intake, which enables investigators to measure the behavioral, physiologic, and genetic impacts of pathological feeding on substance abuse. This model of binge eating has been shown to reduce alcohol intake and alter anxiety-like behavior and central genetic changes linked with regulation of alcohol intake. Overall, these data support the hypothesis that some palatable food exposure may have the potential to alter the intake of drugs of abuse.

Key words Binge eating disorder, Alcohol use disorder, Hedonic feeding, Ghrelin, Hypothalamus, mPFC

1 Introduction

1.1 Eating Disorders and Alcohol Use Disorder in Humans

In the United States, roughly two-thirds of the adult population and one-third of the youth population are considered overweight or obese, and this number continues to rise both domestically and internationally [1]. Repeated bouts of caloric overconsumption are thought to contribute to the development of an obese phenotype [2–4]. More specifically, the development of obesity has been linked to binge eating disorder (BED), a condition defined by the DSM-V as repeated instances of caloric overconsumption within a discrete (2 hr) time period [4, 5]. Clinical BED diagnoses are more prevalent in women relative to men, 3.5% versus 2.0% lifetime prevalence rate, respectively [6]. However, the prevalence of binge eating behavior, including subthreshold binge eating, is nearly equally prevalent in women (4.9%) and men (4.0%) [6].

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An emerging body of clinical literature implicates eating disorders (EDs) as a predisposing factor for initiation of substance abuse disorders (SUDs), namely, alcohol use disorder (AUD) [7–9]. According to a recent epidemiological study, 61% of patients presenting with bulimia nervosa (BN) and 52.0% of BED patients met the criteria for alcohol use disorder compared to 28.7% of those presenting with nonspecific EDs [10]. Since EDs are documented more frequently in women, studies examining the etiology and onset of feeding pathologies have primarily been conducted in women, although recent work has begun to investigate these parameters in men [7, 11]. In this regard, clinical evidence suggests that comorbid hedonic EDs and AUD emerge independently of each other [7, 12–15]. However, separate clinical studies contend that these disorders have familial roots [7, 8, 11, 16]. Currently, it is unclear whether hedonic EDs precede AUDs or vice versa [1].

1.2 An Animal Model to Investigate the Influence of Binge Feeding on Alcohol Intake

In the literature, numerous preclinical binge-like feeding behavioral models have been designed to isolate key physiologic and neurobiological aspects of BED. Many of these models aim to capture the time-limited hyperphagic feeding component of BED, as this repeated event is thought to contribute to the development of feeding and metabolic pathology [17–20]. Given the shared neuronal circuitry controlling both binge-like feeding behavior and alcohol intake [21, 22], we sought to design a preclinical model with translational relevance that examines the impact of binge feeding on alcohol consumption. Using this model, we have shown that 2 hr intermittent and daily binge-like access to a nutritionally complete high-fat diet (HFD) attenuates alcohol consumption [17]. Notably, animals exposed to this model display anxiolytic behavioral effects and central genetic changes that favor reduced ethanol consumption [17]. The following protocol is intended to serve as a methodological basis for future studies aiming to examine the behavioral and physiologic changes between comorbid binge eating and alcohol use.

2 Materials and Methods

2.1 Housing Conditions

Initial studies from our lab used male rats in this paradigm [17]. While similar binge-like eating can be induced in female rat [2], its effects on alcohol drinking are unknown at present, and these studies are currently being conducted in our lab. Therefore, the protocol presented here outlines the parameters considering male rats. To initiate these studies, it is therefore recommended to order the required number of male Long-Evans rats (n=6–8/group; ~ 300 gm). All animal procedures, treatments, and dietary exposure conditions must be approved by the Institutional Animal Care and Use Committee.

Following arrival, individually house the rats in standard shoebox cages with wire tops in a temperature-controlled (67–70 °F) room with reverse light/dark cycle (lights off at 7:00 AM). Allow the animals to acclimate in the new environment for at least 3–5 days with standard rodent chow and water ad libitum. Food can be provided in the cage wire top compartments or using food hoppers, and standard rat water bottles could be used for providing water and alcohol. Room should be equipped with red light to carry out reading in the dark. Since none of the procedures involve food deprivation, animals can be housed in cages with regular bedding.

2.2 Collecting Baseline Data

All rats should be handled for at least 5 days prior to any experimental manipulations. Body weight (twice a week), food, and water intake measurements (24 h) can also be conducted during this period to assess baseline body weight, food, and water intake data by weighing the food and bottles. All 24 hr measurements should begin 4 hr into the rodent's subjective dark period. Divide the rats into four groups matched for body weight, food, and water intake.

2.3 Diets

All rats should have ad libitum access to chow (Teklad, 3.41 kcal/g, 0.51 kcal/g from fat) throughout the manipulation period. In addition, rats in the experimental group should receive intermittent access to a nutritionally complete high-fat diet (HFD) (Research Diets no. D03082706, New Brunswick, NJ, 4.54 kcal/g, 1.71 kcal/g from fat). Detailed dietary composition of standard rodent chow and HFD has been described previously [23].

2.4 Binge-Like High-Fat Diet Exposure

Experimental rats should receive 2 hr high-fat diet (HFD) access every day (HFD-ED) or every third day (HFD-3D). This treatment should occur 4 hr into the subjective dark period of rodent's light-dark cycle. The control rats should receive standard rodent chow (chow) during these 2 hr sessions to control for novelty. To do this, provide a pre-weighed amount of HFD or chow to the respective groups at the beginning of binge session, and weigh the remaining food at the end of session. We find that having pre-weighed amounts of food makes this a much more efficient process. In addition, we find that it is important to also weigh the standard rodent chow present in each rat cage before the binge session to assess 24 hr chow intake the following day.

In order to calculate the energy intake in kilocalories (kcal), multiply the food intake in grams with 3.41 for chow and 4.41 for HFD. Record body weight on Monday and Thursday, except on alcohol testing days (*please see below*), when rats are weighed daily. This feeding regimen is maintained for 5 weeks. Analyze the body weight and 24 hr food intake data using a mixed-model two-way ANOVA (days as within group and diet exposure as between group variable). During 2 hr binge sessions, rats in the chow and intermittent HFD-3D and HFD-ED groups typically consume ~6 kcal

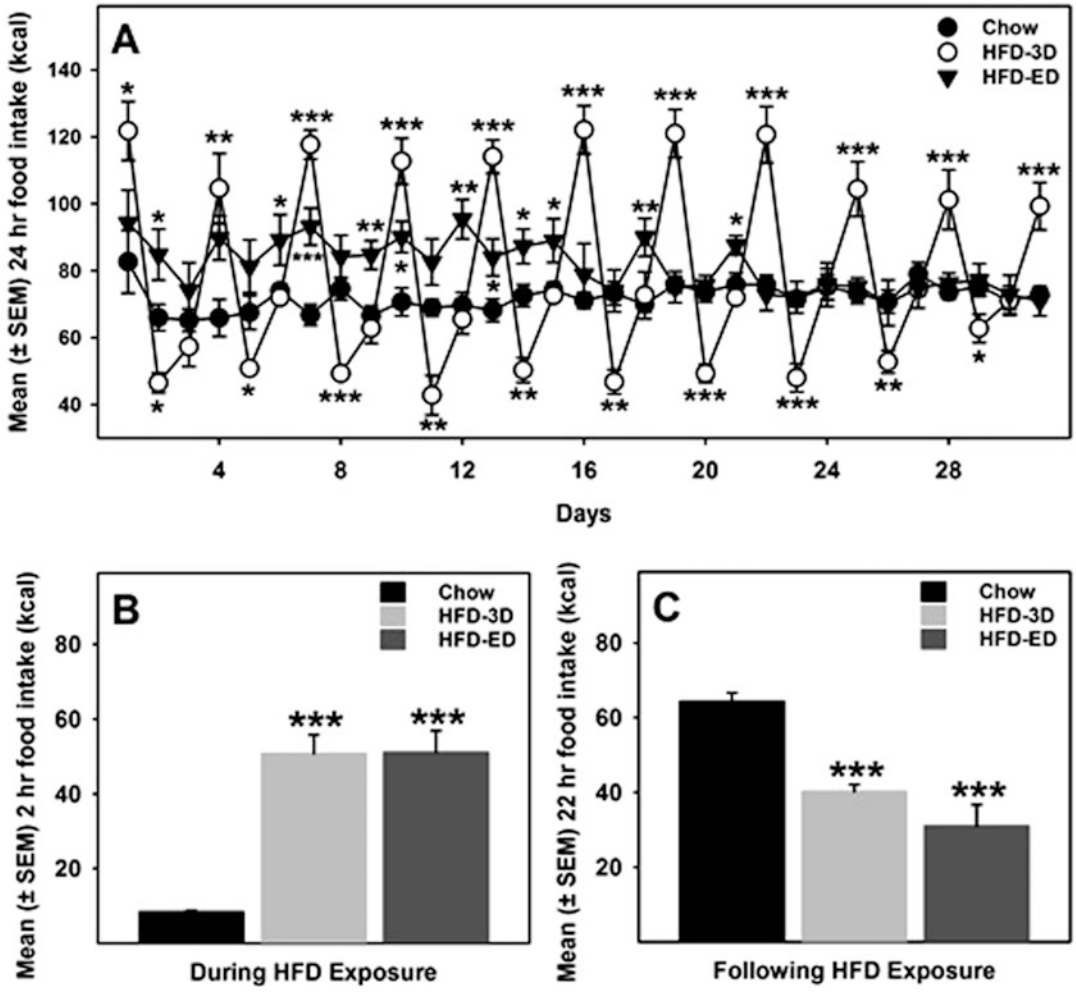

Fig. 1 Daily energy consumption during 5 weeks of binge feeding. (**a**) Mean (±sem) total caloric intake (kcal) (chow + HFD) in control rats and HFD-3D and HFD-ED rats is presented. Rats in the HFD-3D group displayed a significant ($p < 0.05$) 24 hr caloric overconsumption on HFD exposure days followed by voluntary restriction ($p < 0.05$) the following day relative to control rats. Rats in the HFD-ED group also significantly ($p < 0.05$) increased their 24 hr caloric intake on HFD exposure days, and this effect eventually dissipated by the end of HFD exposure period. ***$p < 0.000$, **$p < 0.01$, and *$p < 0.05$ relative to control rats. (**b**) Both HFD-3D and HFD-ED rats displayed significant increases in caloric intake during the 2 hr HFD exposure period and (**c**) significant voluntary restriction from chow 22 hr following HFD exposure. ***$p < 0.000$ compared to chow controls. (Reprinted from Sirohi et al. [17] with permission from Elsevier)

(~10% of 24 hr intake) and ~52 kcal (~60% of 24 hr intake), respectively [17].

Following the binge session, HFD-3D rats compensate by underconsuming the regular chow the following day [17] (*see* Fig. 1). Overall, 24 hr intake in the HFD-ED rats gradually declines and reaches equivalent to chow controls; as a result, both HFD-3D

and HFD-ED rats do not differ in body weight from controls during this period.

2.5 Impact of Binge-Like Intake of High-Fat Diet on Alcohol Drinking

Following 5 weeks of exposure to the binge protocol, all rats are tested in a two-bottle-choice alcohol drinking paradigm. During alcohol testing, rats receive one unsweetened ethanol (20% v/v) and one water bottle in their home cages during the same time of the day when HFD is presented. Bottles are typically filled to the 250 mL maximum. These alcohol testing sessions should occur on the day following binge exposure, and each session should last 2 h. Whereas HFD exposure continues during these alcohol testing sessions, alcohol and HFD are never present concurrently. To avoid any conditioning effects on alcohol drinking behavior, the position of alcohol and water bottles should be switched across testing sessions.

To evaluate alcohol intake, bottles are gently removed and weighed before and after each alcohol drinking session. Alcohol intake can be converted into g/kg by multiplying with ethanol density (20%; 0.166 g/ml) and dividing with rat body weight (kg). The amount of alcohol consumed during initial 2 hr alcohol testing sessions should be low ($\leq$ 0.5 g/kg) which can make it difficult to observe any between group differences. Importantly, short-term alcohol deprivation following the alcohol drinking period produces an alcohol deprivation effect (significant increase in alcohol drinking post abstinence) [24]. Therefore, to obtain measurable amounts of alcohol, it is necessary to impose a brief alcohol deprivation period prior to testing. In our lab, a (1–2 weeks) deprivation period induces increased alcohol intake ($\geq$1.0 g/kg) in the chow control rats over the 2 h sampling period. Importantly, the ability of animals to acquire increased alcohol intake is significantly attenuated in HFD-3D and HFD-ED rats (*see* Fig. 2). Thus, alcohol drinking should be significantly attenuated following binge intake of a high-fat diet in both HFD-3D and HFD-ED rats.

2.6 Important Considerations

Prolonged HFD exposure can induce obesity and is capable of attenuating overall brain reward function [25], which could contribute to reduced alcohol drinking in HFD rats. However, body weight does not change following limited HFD (HFD-3D and HFD-ED) access exposure. Therefore, reduced alcohol drinking behavior observed in our case is not associated with an obese phenotype. Furthermore, independent of body weight gain, HFD exposure has been shown to attenuate operant responding for sucrose and reduce dopamine turnover in the brain reward circuitry [25]. Importantly, HFD access exposure here induced anxiolytic effects in both HFD-3D and HFD-ED rats [17], suggesting that reduced anxiety may contribute to the effects of binge intake on alcohol drinking.

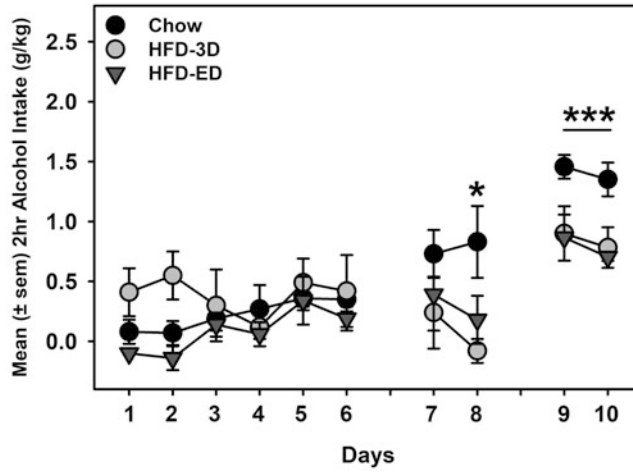

Fig. 2. Binge feeding reduces acquisition of alcohol intake. Data represent mean (±sem) alcohol (20% v/v) consumption (g/kg). When tested following 5 weeks of limited access to HFD, alcohol intake over the first six test sessions was negligible (not significantly higher than zero). A nonsignificant ($p = 0.08$) decrease in alcohol intake was observed in both HFD-3D and HFD-ED rats relative to chow controls when alcohol intake was assessed following release from binge-feeding behavior (days 7 and 8). Following 2 weeks of binge feeding, alcohol consumption was significantly attenuated ($p < 0.001$) in both HFD-3D and HFD-ED groups of rats while maintained on the HFD feeding regimen (days 9 and 10). *$p < 0.05$ and ***$p < 0.001$ a main effect of the exposure. (Reprinted from Sirohi et al. [17] with permission from Elsevier)

We have also evaluated the impact of extended HFD intake on alcohol drinking in male Long-Evans rats. In this paradigm, HFD was provided to rats on Tuesday and Thursday for a 24 h period, and controls received standard rodent chow. In these studies, standard rodent chow and water are available ad libitum to all rats. Following six weeks of HFD exposure, alcohol drinking is evaluated on Monday, Wednesday, and Friday. Using this model, alcohol drinking is significantly reduced by extended HFD exposure [26], an effect observed in the absence of any body weight change. These data suggest that both limited and extended HFD exposure can reduce alcohol drinking behavior. Whether intermittent availability or the diet itself could have contributed to reduced alcohol drinking behavior is currently being investigated.

Lastly, highly palatable foods, rich in sweet and fat, are the typically preferred foods consumed during binge episodes in humans and laboratory animals. Although our initial studies utilized HFD, future studies are needed to examine the impact of binge-like intake of a palatable diet (high in fat and sugar) on alcohol drinking. Notably, intermittent access to sugar can induce withdrawal-like symptoms, and increased alcohol intake has been reported in sugar-dependent rats [27–30]. Therefore, these data

suggest that fundamentally different cognitive-emotional states could be produced following exposure to different macronutrients, a contention which warrants further investigation.

3 Notes

3.1 Frequency of Palatable Food Exposure

The method used to induce binge feeding can vary in frequency. As mentioned above, we have used a schedule where rats receive 2 h exposure to a nutritionally complete high-fat diet (HFD) every three days (Mon, Wed, Fri; termed HFD-3D) or every day (HFD-ED). In each case, experimental rats have ad libitum access to standard rodent chow and water throughout the manipulation period; thus, no experimenter-induced restriction is necessary to induce binge intake in this model. When this model was created [23], we found it important to use the daily HFD-ED access group to contrast the dramatic binge-compensate pattern observed in HFD-3D rats. For example, HFD-3D rats display a caloric binge on days when HFD is offered and a significant decrease in caloric consumption, or compensation, one day following binge exposure. In contrast, HFD-ED rats display binge intake of HFD without compensation. Although it is currently unclear if binge frequency or the total number of binges in a discrete time period is more important for clinical binge diagnoses [DSM-IV, DSM-V], our continued work on this topic suggests that HFD-3D and HFD-ED rats share some similarities. Specifically, both groups display reduced intake of chow prior to binge meals, a phenomenon that we contend reflects anticipation of a binge meal. Both groups display elevated plasma glucose and elevated plasma ghrelin prior to binge meals. Further, both groups display increased acquisition of operant responding for sucrose [3]. Thus, whereas our attempt to model binge feeding in the laboratory may not recapitulate the frequency of binge feeding observed clinically, it does seem to capture some key neuroendocrine and behavioral aspects that likely contribute to clinical BED.

3.2 Anxiety-Like Behavior

Separate from binge frequency, we also examined the impact of binge feeding on anxiety-like behavior. Anxiety has been proposed to be a predisposing factor for alcohol consumption in rodents [31] and patients [32, 33]. Therefore, in our studies, we measured anxiety-like behavior following binge feeding. To do this, we used a light-dark box apparatus (LD), a two-chambered box separated by plexiglass walls. This test relies on the innate ability of rodents to escape from the light to the dark side of the apparatus. Specifically, rats in each group are gently introduced in the light side (600 lx) facing dark side (4 lx) of the apparatus and allowed to freely explore between compartments for 10 min. The total number of entries and time spent in the light side should be recorded by two

independent investigators blind to the treatment groups. In this test, increased time spent in the light side of the apparatus indicates a reduced anxiety phenotype. In addition to the LD box, the elevated plus maze (EPM) could also substitute as a measure of anxiety. In this test, rats are placed in the center of a plus-shaped maze that is elevated off the ground for approximately 18 inches. Two of the arms of the maze have walls, and two of the arms do not. Rats are allowed to freely explore open and closed arms for 5 minutes. Typically, time in the maze is recorded by video tracking, and time spent in the open arms of the maze is associated with reduced anxiety [34]. Thus, there is an option of using a separate method to measure anxiety in binge-exposed rats that could be used in addition to or in lieu of the LD box.

3.3 Conclusion

As feeding pathologies continue to be linked with development and expression of addictive behaviors, understanding the biological underpinnings associated with these phenomena is of utmost importance. The protocol provided herein offers an opportunity to explore the physiologic, behavioral, and neurobiological effects of binge eating on alcohol intake. Further, this model is an effective means to model contingency management of alcohol use in rodents.

Acknowledgments

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Chapter 10

Animal Models of Binge-Eating Palatable Foods: Emergence of Addiction-Like Behaviors and Brain Changes in the Rat

Miriam E. Bocarsly and Nicole M. Avena

Abstract

Binge eating is a behavioral component of some eating disorders, and it is also noted in overweight and obese as well as nonclinical populations. Given its increasing prevalence in society, understanding the behavioral, physiological, and neurochemical components of binge eating is important. Both sugars and fats have been identified as common macronutrients consumed by humans during binge-eating episodes and are thus of interest to study. This chapter describes animal models of sugar and fat bingeing as well as the combination of sugar and fat, which allow for a detailed analysis of these behaviors and their concomitant physiological effects. These particular models of binge eating have been shown to elicit behavioral and neurochemical signs of drug-like dependence in rats, including indices of opiate-like withdrawal, increased intake after abstinence, cross-sensitization with drugs of abuse, and the repeated release of dopamine in the nucleus accumbens following repeated bingeing. These findings support the hypothesis that some palatable foods may have addiction potential when they are consumed in excess.

Key words Binge eating, Dopamine, Food addiction, Nucleus accumbens, Sugar, Rat

1 Introduction

1.1 Binge Eating and Addictive Overeating in Humans

With obesity presently afflicting nearly 40% of the adults in the United States [1], the study of aberrant eating, and specifically overeating, is important. Binge eating, one form of overeating, is a hallmark behavior of binge-eating disorder and is also a common behavior seen in obesity, bulimia nervosa, and anorexia [2–4]. Among individuals seeking treatment for obesity, around 30% report binge-eating episodes. Further, binge eating may also be a predictor of body-fat gain among children, leading to a high risk for adult obesity [5]. In addition, binge eating is associated with increased frequency of body weight fluctuation, depression, anxiety, and substance abuse [6–9]. Taken together, these studies suggest that binge-eating behavior affects a significant proportion of our society and has deleterious consequences, making it important to study from a public-health perspective.

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In addition to the abovementioned detrimental consequences of binge eating, it has been suggested that overeating, and perhaps binge eating, specifically, reflects an addiction analogous to drug abuse [10]. This stems from the idea that binge eating shares behavioral and neurochemical profiles with conventional drug addiction [11] and has been supported clinically by research demonstrating that food craving in normal weight and obese patients activates areas of the brain similar to those indicated in drug craving [12, 13].

While "food addiction" is not recognized as disorder in the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (DSM-V), clinical accounts of food addiction have been reported [14], and surveys, such as the Eating Behavior Questionnaire [15], and clinical interviews [16] have been used to identify addictive eating. However, in 2009, the Yale Food Addiction Scale (YFAS) was first introduced [17]. A psychometrically validated, self-report scale based on DSM-IV substance use disorder diagnostic criteria, this measure was the first self-report scale to operationalize food addiction. This scale has since been updated (YFAS 2.0) based on DSM-V criteria [18], adapted for pediatric populations [19], and translated into several languages. In support of the link between binge eating and food addiction, it has been suggested that up to 92% of individuals with BED met YFAS 2.0 criteria for at least mild "food addiction" compared to 6% of the control population. Further, scores on the YFAS 2.0 selectively predicted binge frequency but held no predictive value over global eating disorder psychopathology [20].

1.2 An Animal Model of Binge-Eating Sugar

Animal models have been proven important in effectively exploring the physiological, behavioral, and neurochemical aspects of binge eating seen in humans. The present chapter discusses an animal model of binge eating that specifically has been used to study addictive overeating. This allows one to discern behavioral and neurochemical indices that are related to addiction within the context of overeating highly palatable foods. Because studies report people binge most frequently on highly palatable foods that are rich in sugar and fat [21–23], this chapter will provide binge-eating models focused on these two macronutrients, independently and in combination.

The DSM-V defines binge eating as a series of recurrent binge episodes during which one eats a larger amount of food than normal during a short period of time [24]. In our model, we impose an intermittent (limited) food access schedule, in which animals have access to a sugar solution (e.g., 25% glucose or 10% sucrose) and chow 12 h daily, followed by 12 h of deprivation for approximately 1 month [25]. We impose a 4-h delay between the onset of the dark cycle and the onset of food access, as rats typically feed at the onset of the dark cycle and thus will be hungry when food is presented. After just a few days of this feeding schedule, rats

develop binge-eating behavior, which we define as distinct, large bouts of intake in discrete periods of time. The most salient binge episode is during the first hour of access, during which animals have been shown to consume approximately 20% of their total daily sugar intake. However, meal analysis throughout the access period reveals that these rats spontaneously engage in binge episodes, in contrast with the continuous consumption of smaller meals seen in control animals (*see* Fig. 1, [26]). Further, sugar-bingeing rats gradually increase their total daily intake of sugar, eventually drinking as much in the 12-h access period as ad libitum-fed rats do in 24 h (70 mL/day).

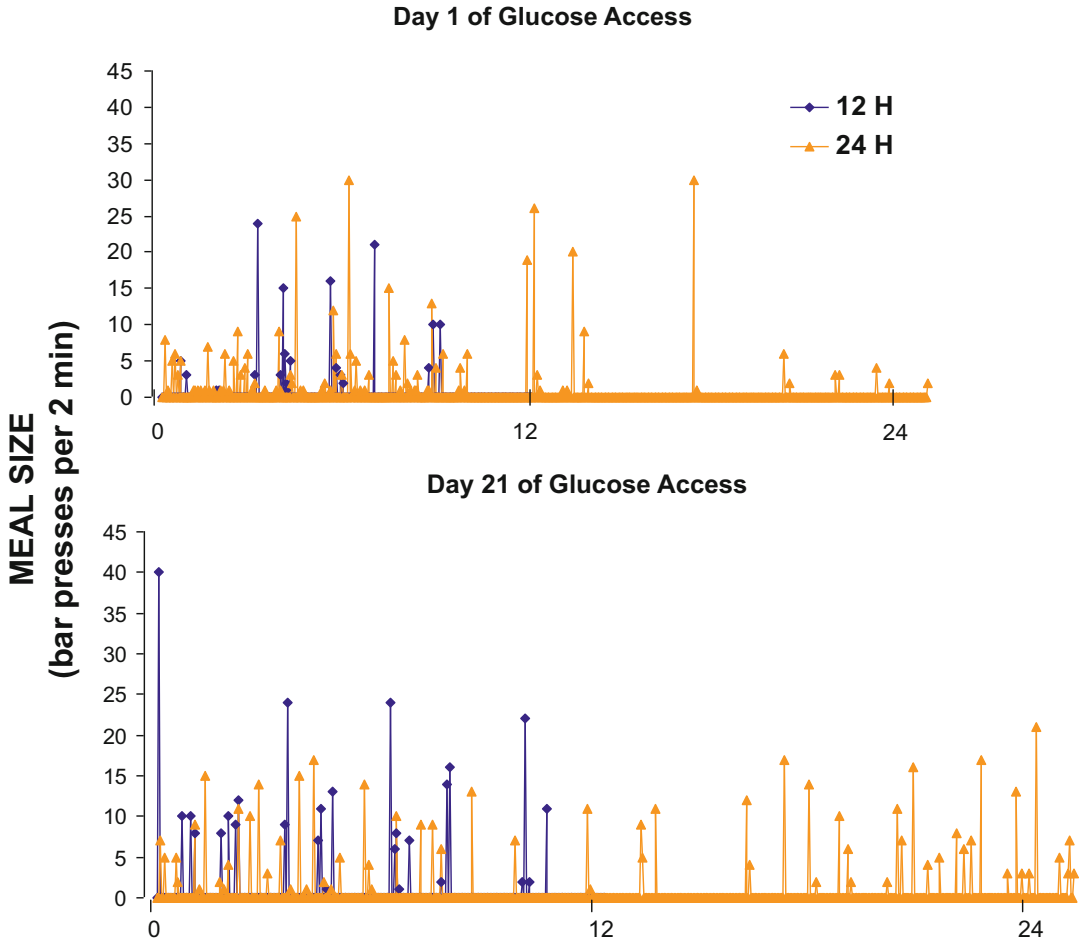

Fig. 1 Meal analysis of two representative rats living in operant chambers. The one maintained on daily intermittent sucrose and chow (blue if viewing in color, black otherwise) had increased intake of sugar compared with the one given ad libitum sucrose and chow (orange if viewing in color/gray otherwise). Hour 0 is 4 h into the dark phase. Each lever press delivered 0.1 mL of 10% sucrose. A sugar meal is defined as ending when the rat does not press for 2 min. Both rats consumed several meals of about equal size on day 1 (top panel). By day 21 (bottom panel), the rat with sucrose and chow available for only 12 h consumed an initial "binge" of sucrose (indicated by the first arrow), followed by fewer but larger meals than the rat with sucrose and chow available ad libitum. (Reproduced with permission from Ref. [26])

What is most interesting and unique about this model of sugar overeating is that it results in both behavioral and neurochemical signs of dependence. Rats maintained on this sugar-bingeing paradigm for 3 weeks show a series of behaviors similar to the effects of drugs of abuse, including the escalation of daily sugar intake and increase in sugar intake during the first hour of daily access. Further, when administered, the opioid antagonist's, naloxone, somatic signs of withdrawal, such as teeth chattering, forepaw tremor, and head shakes, are observed, as well as anxiety as measured by reduced time spent on the exposed arm of an elevated plus maze [27]. Similarly, these signs of opiate-like withdrawal also emerge when all food is removed for 24 h [27, 28].

In the drug abuse literature, animals will self-administer more of the drug when it is made available after an abstinence period. In the sugar-bingeing model, after 2 weeks of forced abstinence from sugar, rats with previous binge access to sugar lever press more than before, suggesting a change in the motivational impact of sugar [29]. Further, in the drug literature, sensitization and cross-sensitization play a role in drug self-administration, and both are typically measured in terms of increased locomotion in response to a drug. Binge-eating rats show locomotor cross-sensitization to a low dose of amphetamine [30]. In addition to its effects on locomotor activity, drug sensitization can lead to subsequent increased intake of another drug or substance. Using this model, we find that rats previously bingeing on sugar drink more 9% alcohol compared to control groups [31].

Concomitant with these behaviors that are similar to those seen in drug dependency, rats maintained on the sugar binge feeding schedule show neurochemical changes similar to those seen in models of addiction. One of the strongest neurochemical commonalities between binge-eating sugar and drugs of abuse is their effect on extracellular dopamine (DA) in the nucleus accumbens (NAc). Using the present model, we show nonabating DA release when animals binge eat sugar, which is similar to the DA response seen with drugs of abuse [32]. This unabated release of DA can be elicited by the taste of sucrose [33] and is enhanced when rats are at a reduced body weight [34]. We have also shown alterations in DA receptor binding and gene expression in the binge model [35, 36]. Again, similar to what is seen in response to drugs of abuse, mu-opioid receptor binding is significantly enhanced in the accumbens shell after 3 weeks of binge sugar access [35]. These animals also have a significant decrease in enkephalin mRNA in the NAc [36].

Lastly, drug withdrawal can be accompanied by alterations in DA/acetylcholine (ACh) balance in the NAc, with ACh increasing while DA is suppressed. This DA/ACh imbalance has been shown during withdrawal from several drugs of abuse [37, 38]. Using our model of sugar bingeing, we have shown that these rats show the

same neurochemical imbalance in DA/ACh during withdrawal precipitated by naloxone [27] or after 36 h of total food deprivation [28]. Thus, multiple addiction-like neurochemical changes can result from drinking a sugar solution in a bingeing manner.

2 Materials and Procedures

2.1 General Notes

The model of sugar bingeing has been developed in Sprague Dawley rats, with studies conducted in both male [27, 28, 31, 32] and female [29, 30] rats, ranging in ages from 6 to 12 weeks at the onset of the study. This is not to say that binge consumption cannot be evaluated in the same manner in other rat breeds or species at ages outside those presented here; however, this protocol has only been validated within these parameters.

Rats should be divided into experimental and control groups (at least $n = 8$–10/group) of similar body weight (<10% variation between groups) and individually housed. There is some variability in chow and palatable food intakes from rat to rat, so it is advisable to use at least eight to ten rats/group. Some potential control groups will be discussed in Subheading 2.4.

Rats should be housed in a rodent vivarium with a 12-h light/dark cycle, maintained at 21 °C. All experimental procedures (including handling, housing, husbandry) must be conducted in accordance with the National and Institutional Guidelines for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee protocols.

2.2 Caging and Animal Preparation

Animals should be individually housed in order to allow for the accurate measurement of food intake throughout the experiment. Use a noncaloric bedding. Consumption of bedding material, which is often caloric in nature, and fecal boli makes it difficult to truly food deprive the rat, which is necessary in this paradigm. Further, gastric distension that results from filling the stomach with bedding or other substances collected in the bottom of the cage can cause the release of feeding peptides and neurotransmitters [39], potentially confounding the results of studies. Cages should be outfitted with removable food hoppers. It is important that hoppers can be easily removed from the cage, allowing for easy facilitation of the 12-h deprivation period without too much disturbance to the rat's environment.

When animals arrive to the housing facility, they should be allowed a minimum of 5 days to acclimate to the new environment prior to experiment onset. Animals should be maintained on standard rodent chow until the start of the experimental paradigm. Water access must be ad libitum for the duration of the study. Water can be provided using bottles with steel-ball tip valves, or it can be made available using automatic watering systems.

2.3 Sugar Diet

Binge behavior has been observed with various sugar solutions, including glucose [29, 35] and sucrose [28, 29, 31, 34, 40]. To prepare the 10% w/v sucrose solution, slowly dissolve 100 g of sucrose (table sugar) in approximately 800 mL of tap water while using a stir bar to stir it. Then, fill the container to 1000 mL with tap water. Prepare only enough sugar solution for each day. Store extra solution at 4 °C for a maximum of 3 days; otherwise, bacteria and mold can begin to grow. Drinking bottles for sugar solutions should be emptied, rinsed, and refilled with new solution each day to avoid microbial growth. Each week, bottles and drinking tubes should be sterilized using a laboratory dishwasher or commercial cage-washing device.

Sugar solution should be presented to animals in 100-mL graduated (in 1-mL increments) drinking bottles (e.g., commercially available glass drinking tubes for rats). Drinking bottles can be mounted to the outside of a wire cage using a spring. Mounting bottles on the outside of the cage allows the researcher to take frequent readings of the fluid volume without disturbing the rat or risking fluid spillage.

Each rat is typically given 100 mL per day of the sugar solution, and those rats that drink almost all of it are given more on subsequent days. Ample amounts of chow should also be provided. Male Sprague Dawley rats consume about 30–35 g of chow each day, nearing 100 kcal, with fluctuation depending on body weight and age. The goal is to always provide more sugar solution and chow than the rats will consume. By the end of 1 month, some rats may increase sucrose consumption to a degree that larger drinking bottles (e.g., 250 mL) are required.

2.4 Sugar Bingeing

The main experimental group with binge access to sugar will have a 12-h deprivation period (no food, water only), followed by 12-h access to a 10% sucrose or 25% glucose solution in addition to standard pelleted rodent chow (e.g., LabDiet no. 5001, PMI Nutrition International, Richmond, IN; 10% fat, 20% protein, 70% carbohydrate, 3.01 kcal/gram) and water. The feeding schedule should be timed such that the access starts 4 h into the dark cycle. Animals typically engage in a large meal at the onset of the dark cycle. By delaying access to chow and sugar until 4 h into the dark period, the rat engages in a binge episode when food is presented. Water is always provided ad libitum, which ensures that sugar solution consumption is not driven by dehydration, but rather palatability and motivation. Maintain rats on this binge-feeding schedule for 21–28 days to elicit the dependence-like signs [27, 28].

At the same time, maintain control groups of rats, which may include the following:

1. Ad libitum sugar solution and chow. This group is highly recommended as a control because it allows for the contrast of behavior and neurochemistry in normal feeding and binge feeding. Further, including this control group allows for the confirmation of binge behavior as indicated by increased first-hour intake in the bingeing animals compared to the free-feeding rats.
2. Intermittent chow. This group has 12-h food deprivation followed by 12-h access to rodent chow only (no sugar solution). This allows for the controls of intermittent access to food coupled with a period of deprivation.
3. Ad libitum chow (without sugar access). Chow intake can also be recorded so comparisons can be made with control animals that do not have access to sugar. Hoppers containing chow can be weighed to determine the amount consumed after returning dropped pieces of the pellets to the hopper to correct for spillage.

Record the 1-h intake of sugar solution after the first hour of access, and record the daily intake before removing the solution at the end of the 12-h access period. Intake data can also be converted into calories. For reference, 1 mL of 10% sucrose solution has 0.4 kcal. 1 mL of 25% glucose solution has 0.97 kcal.

While the time frame for completing an experiment is about 1 month, daily time commitments for routine preparation, administration, and removal of the sugar solution will vary depending on the number of subjects being tested but generally require approximately 1 h/day.

2.5 Variations on the Palatable Food Source: Fat and Sweet-Fat Bingeing

Corwin and colleagues have a well-developed model of binge consumption of vegetable fat *see* Chapter 3 in this volume). We have adapted this paradigm slightly by offering the rats a longer deprivation/feeding period. The main experimental group with binge access to fat has a 12-h deprivation period (no food, water only), followed by 12-h access to a vegetable shortening, in addition to standard pelleted rodent chow and water. Further, as previously described, food should be reintroduced each day 4 h into the dark period. Diets should be maintained for 21–29 days, and intake should be recorded and analyzed as described above. Vegetable shortening contains approximately 9 kcal/g. Control groups, as described above, should also be considered [41].

Various sources of fat can be used. Vegetable shortening can be provided in glass jars (100–125 mL), and cages should be outfitted with springs to hold the jars in place. Vegetable shortening should be replaced every third day or more frequently if needed. Always

provide more shortening than the rat will consume in the access period. Intake will increase over the course of the study, potentially requiring more frequent refilling. Our laboratory has studied various sweet-fat diets, including those that are solid (rodent pellets), emulsions (sugar and oil), and semisolid diets (sugar and butter) [41].

Another variation is to obtain or prepare a nutritionally complete sweet-fat diet (a pelleted diet can be purchased from Research Diets (New Brunswick, NJ, no. D12451; 45% fat, 20% protein, 35% carbohydrate, 4.7 kcal/g)). Again, we use a 12-h deprivation, 12-h feeding model. As described above, the main experimental group will have a 12-h deprivation period (no food, water only), followed by 12-h access to palatable chow and water. Food should be reintroduced each day 4 h into the dark period. Unlike the previous paradigms, because the diets recommended here are nutritionally complete, it is not necessary to provide standard rodent chow with the palatable food. Diets should be maintained for 21–29 days, and intake should be recorded and analyzed as described above. Control groups, as described above, should be considered [41].

3 Notes

3.1 Types of Palatable Food Offered

Although validated only on the presented parameters, this 12-h access method of inducing binge eating is open to a great deal of modification. For example, we have used a variety of palatable foods, ranging in consistency, caloric content, and macronutrient composition with the 12-h deprivation, 12-h access period, and have successfully initiated binge-eating behavior. Some of the diets investigated have been nutritionally complete, such as the pelleted diet suggested above, while others have been sugar or fat supplements to a nutritionally complete rodent chow. Both have clinical relevance, with a great deal of individuals reporting binge eating on "snack"- or "dessert"-type foods (supplements to their diets), while others report binge eating on foods that are calorically dense but nutritionally complete [24, 42]. As previously mentioned, we have also explored a variety of food consistencies, including liquid (sugar solutions), semisolids (vegetable shortening and sugar, "cake frosting"-like diet), emulsions (rich in either fat or fat and sugar), and solid diets (commercially available sweet-fat, pelleted chow). It is of interest to explore varying consistencies, given the differences that have been identified in the consumption of liquids as compared to solid foods [43]. Further, we find it crucial to include a chow-fed control group in order to have a balanced nutritive diet to compare binge-eating groups. Lastly, we have studied various macronutrient combinations. While studying pure sugar or pure fat binges is advantageous in the laboratory and has allowed for the understanding of the specific effects of different

macronutrients [44], it is not representative of the human condition, in which people tend not to consume foods that contain only one macronutrient. For this reason, combination diets are also of importance when exploring binge-eating behaviors. The flexibility of this paradigm allows for further exploration of a variety of foods.

3.2 Body Weight

In addition to specific macronutrients, another variable explored in this method is food access time. With the sugar-bingeing model, we implement a 12-h deprivation period, followed by a 12-h food access period in order to induce binge eating. Though a deprivation period is in place, animals are not calorically deprived and maintain their weight at or above the weights of animals fed an ad libitum diet of standard rodent chow. Animals binge-eating sucrose or glucose do not gain excessive amounts of weight; however, animals binge eating on fat-rich diets do show weight gain [45]. Interestingly, data also suggests that there are some sugars that when given using this paradigm do result in weight gain. Specifically, an 8% solution of high-fructose corn syrup when given for 12 h daily as described here leads to weight gain compared to chow-fed controls [46]. Given the prevalence of high-fructose corn syrup in the American diet, this could lend insight into the obesity epidemic. In addition to overweight conditions, we have explored binge eating at a decreased body weight and have determined that rats with a history of binge-eating sucrose at a low body weight show an exaggerated increase in brain DA and attenuated ACh levels in the NAc. This could indicate an enhanced susceptibility to food "addiction" at a low body weight (potentially similar to a "dieting" state) [34].

3.3 Access Period

The last variable that we have modified in the described method is the length access period. There are various paradigms exploring binge eating, using various periods of palatable food access. For example, Corwin and colleagues have shown that sated rats with ad libitum access to rodent chow will binge on a vegetable fat when it is presented for 2 h each day [47, 48]. This effect is even more dramatic when the fat is offered on a more restricted schedule, for 2 h three times per week. We find that a 12-h deprivation/access period is effective in precipitating binge eating. This schedule is also indicative of food access in the wild, given the fact that rats rarely have unlimited, ad libitum access to food sources. This is also relevant in the clinical realm in that although food might be accessible 24 h each day, we do not engage in consumption during this entire period. Further, in the fat and sugar binge-eating paradigms, rats are given a "choice" between a palatable food and a nutritive food (standard rodent chow), which is similar to the clinical eating condition. Although we focus on a 12-h paradigm, there are important benefits to exploring different access periods, with and without food restriction.

3.4 Age and Gender

While most of the studies completed in our laboratory are done in adult, male Sprague Dawley rats, we have varied the gender (male [27, 28, 31, 32] and female [29, 30]) and age at onset of the binge paradigm. These parameters are of interest for further investigation. Binge eating has been shown to affect both males and females [10–13], making gender studies crucial. Clinically, adolescent females are more likely to demonstrate disordered eating than age-matched males or adults [5, 49]. Further, adolescents have been shown to be more susceptible to addictive behavior [50]. As previously mentioned, binge eating has been suggested as a predictor of body-fat gain among children, leading to a high risk for adult obesity [6], making the exploration of early-onset binge eating of relevance. The described binge-eating model could be used to explore gender and developmental effects of binge eating.

3.5 Macronutrient-Specific Behavior

Not only can different diets allow for the investigation of binge eating with and without the component of obesity, but also these can allow for the determination and study of those factors underlying its pathology. We have, for example, been able to identify differences in binge eating on varying macronutrients, finding different behavioral responses to binge-eating sugars vs. fats, which could lead to different targeted treatments [14]. Namely, when rats are binge-eating diets containing fat, they do not seem to show signs of opiate-like withdrawal, as we see in rats that are binge-eating sugar [41]. Also, certain drugs are effective at suppressing binge eating of fat-rich foods, but not sugar-rich foods [51]. This underscores the different neurotransmitter systems activated by the various diets and also suggests that there may be specific behavioral effects associated with macronutrient-specific "food addictions."

4 Conclusions

Binge eating is increasing in prevalence in the developed world and thus deserves investigation. Further, the link between obesity and binge eating makes its understanding of clinical importance. The above model provides a paradigm that can be used to explore the physiological, biological, and neurological effects of binge eating (*see* Table 1) and provides a model in which potential pharmaceutical interventions can be explored.

Table 1
Findings that suggest similarities between sugar dependence in animal models and substance use disorder

Substance use disorder	Animal model of sugar dependence
A. DSM-V diagnostic criteria	
Substance is taken in larger amounts and/or over a longer period than intended	Spontaneous binge consumption develops over 21 days of intermittent sucrose exposure [26]
Withdrawal	Somatic signs of withdrawal, including teeth chattering and tremors [28] Anxiety-like behaviors on the elevated plus maze [27, 28] Ultrasonic distress vocalizations [27] Depression-like behavior on forced swim test [26]
Tolerance	Escalation of daily sugar intake [35]
Craving or strong desire or urge to use the substance	Enhanced lever pressing for sugar after 2-week abstinence [29] Increased responding for sugar-associated cues during abstinence [52]
Recurrent substance use in situations in which it is physically hazardous	Rats prone to binge eating a sugar-rich diet are more likely to traverse a shock grid to receive the palatable food [53]
Great deal of time spent obtaining, using, or recovering from the effects of the substance	Rats given restricted access to sweetened condensed milk spend more time attempting to obtain the substance [54]
B. Behavioral indications	
Locomotor cross-sensitization to other drugs of abuse	Locomotor cross-sensitization to amphetamine [30]
Proclivity to consume other drugs of abuse	Enhanced voluntary alcohol intake [31]
C. Neurochemical changes	
Repeated release of dopamine	[32, 33]
↑D1 receptor binding	[35]
↓ D2 receptor binding	[35]
↑ D3 receptor mRNA	[36]
↓ Preproenkephalin mRNA	[36]
Dopamine/acetylcholine imbalance during withdrawal	[27, 28]

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Chapter 11

Clinical and Preclinical Bariatric Surgery Approaches to Studying Obesity

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Abstract

Obesity is the largest global public health epidemic. The benefits of bariatric surgery are well established, and bariatric surgery remains as the most common and effective procedure to treat obesity. The present review examined bariatric surgery procedures in humans as well as in rodent models. In addition, we examined different neuroimaging methods of assessing the effects of bariatric surgery on brain chemistry and function. This is especially important given the expanded understanding of gut-brain neurophysiology in studying obesity and ingestive behavior. In addition, given the underlying similarities in vulnerabilities and mechanisms between obesity and drug addiction, the future use of multiple techniques (i.e., imaging, genetics, psychosocial information) could all be utilized in a precision medicine approach to improve long-term patient outcomes.

Key words Obesity, Bariatric surgery, Ingestive behavior, Neuroimaging, Positron-emission tomography, Autoradiography, Reward deficiency syndrome, Addiction

1 Introduction

1.1 Bariatric Surgical Procedures in Humans

The most commonly performed bariatric/metabolic procedures in humans are the vertical sleeve gastrectomy (VSG), Roux-en-Y gastric bypass (RYGB), adjustable gastric band, and biliopancreatic diversion and duodenal switch (BPD-DS). It is worth noting that the last two procedures are rare in children. The Teen-LABS study shows that gastric bypass was the most frequent procedure until 2009 when sleeve gastrectomy started to increase remarkably [1]. Each of these procedures has its own advantages and indications and can be tailored to accommodate individual needs of the patient. Importantly, conversions from one bariatric procedure to another may be necessary over time. All of these procedures are routinely accomplished via minimally invasive approaches. In the

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following few pages, these bariatric procedures and their main potential complications will be briefly described.

1.1.1 Vertical Sleeve Gastrectomy (VSG)

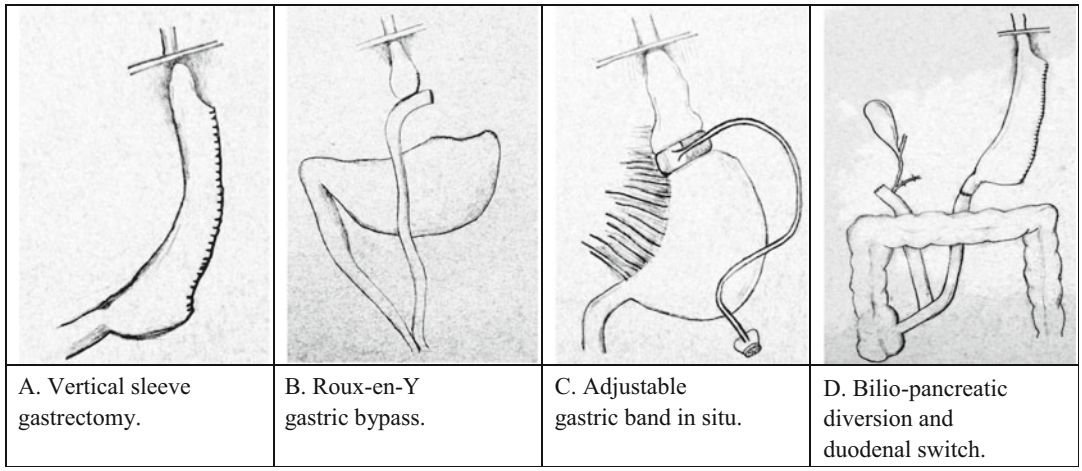
After securing greater curvature of gastroepiploic vascular branches from about 4–5 cm proximal to the pylorus and the gastroesophageal junction, any posterior gastric attachments, often to the pancreas, are divided to expose the left diaphragmatic crus. A bougie (size is debatable) is passed along the lesser curvature and guides the resection of most of the stomach using gastrointestinal staplers and aiming for the angle of His to create the sleeve gastrectomy. The remaining tubularized gastric sleeve should be near 15% of original stomach size (*see* Fig. 1). The procedure is relatively straightforward and safe, but a mortality rate of 0.4% has been described, and primary postoperative complications include staple line leak, bleeding, gastroesophageal reflux, and malnutrition [2, 3].

1.1.2 Roux-en-Y Gastric Bypass (RYGB)

The jejunum is divided approximately 40 cm distal to the ligament of Treitz. The proximal portion is then anastomosed 100–110 cm along the distal segment (Roux limb). A small proximal gastric pouch is created near the gastroesophageal junction and anastomosed to the jejunal Roux limb. The distal stomach remains in place (*see* Fig. 2). The Roux limb length differs based on surgeon preference, patient BMI, and desired weight loss. The mesenteric defect resulting from the jejuno-jejunostomy represents known risk for internal hernia and should be closed routinely. Immediate postoperative care for these patients focuses on gentle diet advancement, as well as early detection of intra-abdominal bleeding and anastomotic leak. The laparoscopic RYGB mortality rate is near 0.45% [2]. The major morbidities include anastomotic leak, gastrointestinal hemorrhage, intestinal obstruction, internal hernias, anastomotic strictures, marginal ulcers, splenic injury, and nutritional deficiencies [3].

1.1.3 Laparoscopic Adjustable Gastric Band (LAGB)

After dissecting the pars flaccida and releasing the angle of His, the fundus is retracted down, and the band is passed around the upper stomach. The band is then locked approximately 1–2 cm below the gastroesophageal junction. The fundus is secured over the anterior aspect of the band to lessen anterior band slide. The silicone tubing is attached to a subcutaneous reservoir/port, fastened subcutaneously to the abdominal wall, and used to adjust the band to the desired level of restriction (*see* Fig. 3). The adjustability of the band permits weight loss control by altering the amount of saline within the band to optimize open diameter, decrease food intake, and accelerate satiety. Band adjustments are typically performed every 6–8 weeks after surgery in the first year. The mortality rate of this procedure is close to 0.06% and stems primarily from venous thromboembolic complications [2]. The most common postoperative complications comprise band slippage/gastric prolapse, band



A. Vertical sleeve gastrectomy.	B. Roux-en-Y gastric bypass.	C. Adjustable gastric band in situ.	D. Bilio-pancreatic diversion and duodenal switch.

Fig. 1 Types of bariatric surgery in humans. (Adopted from Khwaja and Bonanomi [49])

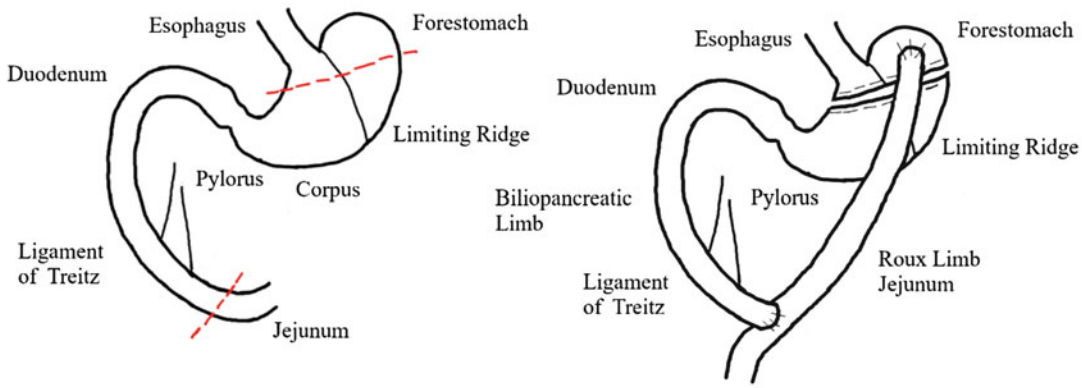


Fig. 2 In rodent RYGB, the stomach is divided to create a small 20% gastric pouch in continuation with the esophagus separated from the bypassed stomach in continuation with the pylorus. The vagus nerve branch supplying the pyloric sphincter is usually severed as a byproduct of the surgery, which helps relax the pyloric sphincter [9]. The jejunum is then divided distal from the ligament of Treitz. The distal segment of the jejunum is anastomosed end-to-side with the gastric pouch to form the gastrojejunostomy. The proximal jejunum is anastomosed end-to-side forming the biliopancreatic/jejunojejunostomy limb [7]

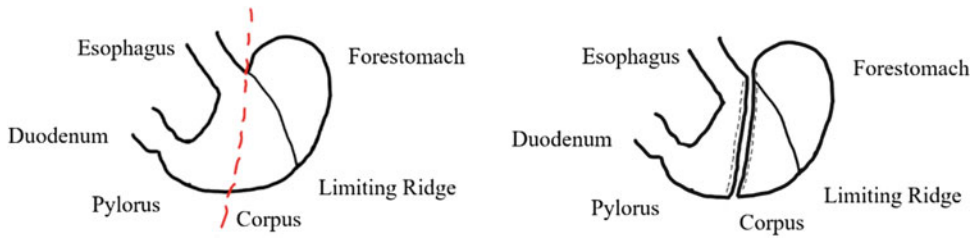


Fig. 3 In rodent VSG, the lateral 80% of the stomach is excised, leaving behind a tubular gastric remnant in continuity with the esophagus and the pylorus [20]. At the conclusion of the respective procedures, the gastrointestinal organs are reintegrated into the abdominal cavity, and the abdominal wall is closed in layers

erosion, pouch dilatation, problems related to port malfunction, gastroesophageal reflux disease, dysphagia, esophageal dilatation, and dysmotility, and reoperation rate is as high as 31% [4, 5]. In contrast to the other bariatric procedures, this approach is thought to have a purely restrictive function.

1.1.4 Biliopancreatic Diversion and Duodenal Switch

This procedure can be performed in one or two stages typically separated by 9–12 months. The first portion of this procedure is identical to a vertical sleeve gastrectomy. The second stage involves a division of the ileum 250 cm from the ileocecal valve and division of the duodenum at the level of its first portion. The distal small bowel limb is anastomosed just distal to the pylorus. A common intestinal channel is created approximately 100 cm proximal to the ileocecal valve by anastomosing the proximal segment of divided ileum (biliopancreatic limb) to the distal segment of distal ileum. The mesenteric defects following duodeno-ileostomy should be carefully closed. The BPD-DS is a technically more demanding procedure and a powerful weight loss tool; however, there are major nutritional consequences, making careful and appropriate patient selection extremely important. The mortality of this procedure is near 0.5% [2]. The most serious complications are gastric staple line, gastric and duodeno-ileostomy leaks, small bowel obstruction, internal hernias of the biliopancreatic limb, and, importantly, nutritional complications such as iron deficiency anemia, hypocalcemia and fat-soluble vitamin deficiency, and protein-calorie malnutrition, which require lifetime supplementation [6].

1.2 Bariatric Surgical Procedures in Rodents

The two most common surgical measures for the treatment of obesity in humans include Roux-en-Y gastric bypass and vertical sleeve gastrectomy. Vertical sleeve gastrectomy (VSG) is a surgical procedure where a large part of the lateral stomach is removed and the remaining stomach tissue is stapled together, leaving behind a sleeve [7]. This contrasts with Roux-en-y gastric bypass (RYGB), where a small pouch is made in the upper stomach and a piece of the jejunum is rerouted between the pouch and the duodenum, forming a "Y" shape [8]. Animal studies give researchers invaluable insight into human bariatric patient outcomes via the ability to control for most variables of the animal's life and investigate only the association in question. Scientific inquiry into outcomes of bariatric surgery has a rich history of using rodent models to test everything from its effect on maternal-fetal outcomes [9], postoperative dietary preferences [10, 11], and new-onset alcohol-seeking behaviors [8, 12, 13]. An analysis of the methods of rodent bariatric surgery provides important insight into human bariatric procedures.

2 Materials

2.1 Animals and Housing

Rat bariatric studies often utilize Sprague-Dawley rat [14] or Long-Evans rats [15]. Mice bariatric studies commonly use C57/BL6J mice [16]. Male rodents are more commonly used, but female rodents are sometimes included as a second cohort in studies. In order to more closely mimic human surgical conditions, rodents in the experimental group are made dietary obese through consumption of high-fat diet ranging from 40% [9] to 60% kcal from fat [10] for a varying number of weeks. Often times, the control group will be fed a standard laboratory rodent diet or will be dietary obese but receive a sham operation. Rodents may be individually housed or group housed in a humidity-controlled environment on a 12 h light-dark cycle with ad libitum access to water and their respective diets and access to veterinary care as needed.

2.2 Surgical Equipment

While rodent RYGB and VSG are two specialized procedures, there is great similarity between the two procedures as far as the equipment required is concerned. All tools that come in contact with the incision site should be autoclaved or chemically sterilized prior to the operation:

Preoperative equipment:: Equipment should include a razor and/or a chemical hair remover, sterile gloves, sterile gauze and sterile cotton applicators, alcohol wipes and iodine solution or chlorhexidine scrub to sanitize the skin, syringes prepared with fluids or pain medicine if specified by the protocol, an anesthesia system whether inhalable isoflurane or injectable ketamine/xylazine, and a heating pad or warm water circulator to maintain rodent body temperature.

Intraoperative equipment:: Equipment should include incision spreaders, forceps and toothed forceps, Babcock clamps, an electrocautery system, hemostats, scissors, scalpels and scalpel holders, a needle driver, suture material, endo-stapling device, and appropriate staple loads. Some studies utilize a permanent metal clip to close off a section of the gastric remnant in place of a staple line [17].

Postoperative equipment:: Equipment should include a recovery environment that minimizes the rodent's ability to eat bedding or fecal material for the first 1–3 days after surgery.

3 Procedures

3.1 Comparison to Surgeries in Humans

Surgeries must be adapted to the small size of the rodent model, and some sacrifices are made because of this. In humans, RYGB surgeries routinely leave the patient with a gastric pouch of around 5% of the former stomach's volume [17]. In rodents, such a feat is technically challenging due to the size and shape of the stomach, and as such, a pouch 20% of the former stomach's volume is often made [16, 18]. However, few studies to date have successfully implemented a 5% RYGB mouse model [17]. In human VSG surgeries, 80–90% of the stomach is excised, leaving behind a narrow tubular lumen 10–20% of its former volume [19]. Rodent models achieve a volume very close to this, at 70–80% excision [16]. Aseptic technique is much the same as with humans, but rodent surgeries are often performed by one researcher at a time, so some alterations to aseptic technique are necessary [20]. Human bariatric procedures are done laparoscopically to minimize complications and recovery time, while this is impossible due to the size of the rodent model.

3.2 Rodent-Specific Techniques

Both VSG and RYGB in rodents start fundamentally the same way. A 4 cm laparotomy incision is made along the midline of the abdomen just below the xyphoid process. Subsequently, the gastric ligaments are severed to free the stomach, and the gastrointestinal organs are exteriorized [21]. From this point, the surgeries begin to diverge.

3.3 Neuroimaging Methods of Assessing Bariatric Surgery: (A) Autoradiography and (B) Positron-Emission Tomography

3.3.1 Introduction

A. Autoradiography is a technique that allows for the visualization and quantification of receptors in tissue samples. In vitro autoradiography using mounted brain tissue has been widely used in preclinical research for decades. Its ability to produce images of high spatial resolution for mapping receptor distribution has made it a highly effective and reliable technique in translational studies. A variety of radioligands are available for the use of autoradiography including ^{125}I, ^{14}C, ^{32}P, ^{35}S, and ^{3}H. As previously described, each radioligand has distinct properties that must be considered when creating a suitable protocol for autoradiography [22]. ^{3}H is particularly conducive to work with, both for its long half-life and due to the fact that it's low-level β-emission travels only a short distance when exposed to x-ray film. This property of ^{3}H provides for the high spatial resolution necessary for precise anatomical measurements of the film.

One of the main advantages of in vitro autoradiography is the ability to detect changes in raw receptor expression as opposed to in vivo approaches like positron-emission tomography [3] which looks at receptor availability. Changes in receptor availability can

result either from changes in receptor expression or by changes in the endogenous ligands that have to compete for receptor binding with the radioligand. Therefore, if the goal is to understand how certain conditions (i.e., diet, obesity) influence receptor expression, then in vitro autoradiography offers a more controlled and precise approach than techniques like PET. In vitro autoradiography in the context of obesity [33, 34] and bariatric surgery has been successfully used in our lab that has extended our understanding of its associated neurochemical changes in dopamine and mu-opioid systems [14, 23]. The disadvantage of in vitro autoradiography, which is a terminal procedure, is that it cannot be employed for longitudinal studies. In this regard, PET would be more advantageous since the animal can be scanned multiple times over the course of the experiment.

B. Positron-emission tomography (PET) is a noninvasive highly sensitive technique that enables the assessment of concentration, distribution, and pharmacokinetics of radiolabeled molecules (short-lived positron-emitting, i.e., radioisotopes) of biochemicals. Compounds involved in normal brain function can be tagged with radioisotopes and then injected into the bloodstream. Among the many examples, probably the most commonly utilized is fluorine-18 (^{18}F), which can be used to label the sugar glucose, the brain's only energy source (^{18}F-fluorodeoxyglucose [^{18}F-FDG]). Other examples include radioactive carbon-11 (^{11}C) and oxygen-15 (^{15}O) which can be used to label water molecules, which can help measure blood flow in the brain. Specialized scanners are designed to contain a ring of detectors around the person or animal being studied, in this case the head of the subject. These detectors are sensitive to measure the signal emitted by these radiotracers (in the nanomolar to picomolar range), which is then sent to a computer that makes a time-stamped three-dimensional image of the brain. Thus, PET can be used to label compounds that are of pharmacological and physiological relevance. These radiotracers then can be used to probe neurochemical and metabolic processes at the relevant physiological concentrations without perturbing the system that is measured.

Unlike other imaging techniques, PET imaging can report metabolic changes occurring in an organ or tissue at the cell level and do so over time for longitudinal changes. This is a major advantage over other imaging methods and is the reason for utilizing this approach when studying cancer or brain function. Unlike other in vivo imaging methods, abnormal function at the cellular level will appear before structural changes, though it is common to employ PET in conjunction with other techniques (CT or MRI). Abnormal eating behaviors are linked to obesity. Prior brain PET

imaging studies have linked the involvement of dopamine (DA)-modulated circuits in pathologic eating behavior(s). Moreover, food cues have been shown to increase striatal extracellular DA (using [^{11}C]raclopride), providing evidence for the involvement of DA in the nonhedonic motivational properties of food [34]. PET imaging showed that food cues resulted in increased metabolism in the orbitofrontal cortex indicating the association of this region with the motivation for food consumption. Thus, PET imaging is a powerful technique in studying obesity and bariatric surgery and has been successfully used to extend our understanding of the neuroscience of obesity and how to map the brain circuit response to food, food cues in obese and nonobese subjects, and the corresponding changes in in vivo functional connectivity associated with bariatric surgery [11, 36–39].

Interestingly, similar to drug-addicted subjects, striatal DA D2 receptor availability is reduced in obese subjects, which may predispose them to seek food as a means to temporarily compensate for understimulated reward circuits [35, 40, 41]. This has been described as reward deficiency syndrome (RDS) and can help explain the underlying common mechanism and vulnerabilities of substance use dependency and obesity [36]. Thus, PET imaging using [^{11}C]raclopride can help examine specific in vivo assessment of dopamine signaling by measuring dopamine D2 availability [33, 41] or other neurotransmitter systems associated with food and anticipation of food. These changes can be examined longitudinally before and after weight loss and before and after bariatric surgery.

3.3.2 Materials and Procedures

A. *Autoradiography*

Sample Preparation: *Following the flash freezing of the brain, a cryostat or microtome can be used to section the brain into thin sections. The thickness of the sections can vary depending on the radioligand that will be used but is typically around 10–20 μm for using ^{3}H. The sections can then be thaw-mounted on slides which are then dried at room temperature for about an hour. Tissue from the same brain can be used for several different binding experiments by mounting the tissue on different sets of slides. Consecutive tissue sections are used to progressively fill the first slot in every set of slides before moving to the second slot and so on. This will produce sets of slides with nearly identical tissue sections on them. When sectioning with a thickness around 10–20 μm, not every section will need to be mounted. Depending on the requirements of the experiment, every second or third section can be taken and mounted, with the others being discarded. Separate slides should be designated for taking sections that will be used to measure the nonspecific binding of the radioligand. In our laboratory, representative sections from five distinct locations in the brain are*

taken for each set. Sections that are typically discarded while filling the regular slides are mounted on the designated nonspecific slides once the appropriate place in the brain is reached. Once the slides are filled with the mounted sections and are dried, they can be stored at −80 °C until the day of the receptor binding.

Receptor Binding: *On the day of the receptor binding, slides from one set are taken out of the freezer and slowly allowed to come to room temperature. Receptor binding for the tissue is typically broken down into three main steps: preincubation, incubation, and wash. The type of buffer, pH, temperature, and the duration of incubation are all critical components for successful binding that are subject to change depending on which radioligand is being used. The purpose of the preincubation buffer is to wash away any endogenous ligands that are on the section which would compete with our radioligand. Following preincubation, slides are dried with a cool stream of dry air and subsequently bathed in the incubation buffer containing the radioligand. The duration of time spent in the incubation buffer corresponds to the time it takes to reach a near equilibrium (or a steady state) between the association of the radioligand and the receptor and the dissociation of the receptor and the radioligand [24]. For measuring nonspecific binding, a separate incubation buffer will be used for the nonspecific slides. This buffer will be identical to the incubation buffer except it will have an added compound in an excessive concentration that is enough to inhibit the binding of the receptor of interest with the radioligand. Since the radioligand won't be able to bind to the receptor of interest, it will only be able to bind to off-target receptor sites. Once the sections have been in the incubation buffer long enough, they go through a series of washes in order to remove the unbound radioligand off of the tissue. This will help maximize the signal-noise ratio of the radioligand. Typically, these washes are performed in the preincubation buffer that is chilled at 4 °C in order to minimize the likelihood of dissociation between the radioligand and the receptor. Slides are then briefly dipped in distilled water to remove any salt residue before being dried immediately in a cool stream. Slides are then left to dry alongside a desiccant for 24 h before being exposed to film (see Table 1).*

Slides and Exposure: *In preparation for film exposure, slides are organized and placed face up on top of poster board using double-sided tape. Each poster board is also accompanied by a calibration standard. In consideration that the use of calibration standards on x-ray films can be prone to inaccuracies [31] – by either the standards themselves or improper training/procedures – balancing the number of slides from each group is an important step that must be taken to help maximize that ability to detect accurate group differences in the data. The poster board with the bound slides is placed into a light-sensitive cassette and apposed to x-ray film – all of which is performed in a dark room. The exposure of the film will vary depending on the*

Table 1
Protocols for in vitro autoradiography adopted from the following references: D1 [24], D2 [24], DAT [25], CB1 [26], GABA(A) [27], μ-opioid [28], peripheral benzodiazepine receptor [29], and NMDA [30]

Receptor	Preincubation	Incubation	Nonspecific	Washes
D1	50 mM Tris 120 mM NaCl 5 mM KCl 2 mM CaCl2 1 mM MgCl2 *Time*: 60 min at room temperature *pH*: 7.4	Preincubation buffer with 40 nM ketanserin and 2.5nM [^{3}H]SCH23390 *Time*: 60 min at room temperature *pH*: 7.4	Incubation buffer with 1 μM flupenthixol *Time*: 60 min at room temperature	1. 2 × 5 min in 4°C preincubation buffer 2. Dip in 4°C distilled water
D2	50 mM Tris 120 mM NaCl 5 mM KCl 2 mM CaCl2 1 mM MgCl2 *Time*: 60 min at room temperature *pH*: 7.4	Preincubation buffer with 40 nM ketanserin and 0.5nM [^{3}H]spiperone *Time*: 60 min at room temperature *pH*: 7.4	Incubation buffer with 10 μM sulpiride *Time*: 60 min at room temperature	1. 2 × 5 min in 4°C preincubation buffer 2. Dip in 4°C distilled water
DAT	30 mM sodium phosphate buffer *Time*: 10 min at 4°C *pH*: 7.4	Preincubation buffer with 0.32 M sucrose and 4nM [^{3}H]WIN 35,428 *Time*: 90 min at 4°C *pH*: 7.4	Incubation buffer with 30 μM cocaine *Time*: 90 min at 4°C	1. 2 × 1 min in 4°C preincubation buffer 2. Dip in 4°C distilled water
CB1	50 mM Tris HCl with 5% bovine serum albumin (BSA) *Time*: 30 min at room temperature *pH*: 7.4	Preincubation buffer with 5 % bovine serum albumin (BSA) and 5nM of [^{3}H] CP55,940 *Time*: 2 hours at room temperature *pH*: 7.4	Incubation buffer with 10 μM cold CP55,940 *Time*: 2 hours at room temperature	1. 1 hour in 4°C 50 mM Tris HCl buffer plus 1% BSA 2. 3 hours in 4°C 50 mM Tris HCl buffer plus 1% BSA 3. 5 min in 4°C 50 mM Tris HCl buffer 4. Dip in 4°C distilled water
GABA(A)	170 mM Tris HCl buffer *Time*: 30 min at room temperature *pH*: 7.4	Preincubation buffer with 2nM [^{3}H]flunitrazepam *Time*: 45 min at 4°C *pH*: 7.4	Incubation buffer with 1 μM clonazepam *Time*: 45 min at 4°C	1. 2 × 1 min in 4°C preincubation buffer 2. Dip in 4°C distilled water

(continued)

Table 1
(continued)

Receptor	Preincubation	Incubation	Nonspecific	Washes
μ-Opioid	50 mM Tris HCl with 0.9% NaCl *Time*: 30 min at room temperature *pH*: 7.4	Preincubation buffer with 5nM [^{3}H]DAMGO *Time*: 60 min at room temperature *pH*: 7.4	Incubation buffer with 10 μM naloxone *Time*: 60 min at room temperature	1. 3 × 30 second washes in 4°C preincubation buffer 2. Dip in 4°C distilled water
Peripheral benzodiazepine	50 mM Tris HCl buffer *Time*: 15 min at room temperature *pH*: 7.4	Preincubation buffer with 1nM [^{3}H]PK-11195 *Time*: 30 min at room temperature *pH*: 7.4	Incubation buffer with 20 μM cold PK-11195 *Time*: 30 min at room temperature	1. 2 × 6 min washes in preincubation buffer 2. Dip in 4°C distilled water
NMDA	50 mM Tris acetate *Time*: 30 min at room temperature *pH*: 7.4	Preincubation buffer with 30 μM glutamate, 10 μM glycine, and 10nM [^{3}H] MK-801 *Time*: 4 hr at room temperature *pH*: 7.4	Incubation buffer with 100 μM unlabeled MK-801 *Time*: 4 hr at room temperature	1. Dip in 4°C preincubation buffer 2. 90 min of 4°C preincubation buffer 3. Dip in 4°C distilled water

radioligand as well as the type of film (typically takes several weeks). Following exposure, the film is taken out of the cassette, developed, fixed, dried, and finally scanned for image analysis.

Image Analysis: *There are many different programs used to aid in quantifying receptor density on the scanned film. One of the most commonly used programs is ImageJ (developed by NIH). Using ImageJ or similar programs, the user can outline regions of interest (ROIs) on the film that correspond with the brain regions seen in the Paxinos Brain Atlas [32]. A brain atlas is crucial for outlining anatomical outlines, as the boundaries between the different regions are often not identifiable from looking at the film alone. After outlining the ROI, the user can obtain a value that is based on optical gray-scale intensity. In many cases, this value is typically converted from gray-scale values to more meaningful units, such as μCi/g or fmol/mg of tissue. Due to the fact that the ligands used do not bind only to the receptor of interest, additional measures are taken to identify the total, specific, and nonspecific binding of radioligands. Total binding represents the binding of the radioligand to the receptor of interest (specific binding) as well as the binding to other off-target sites (nonspecific binding). Specific binding is then calculated by subtracting the values obtained from the nonspecific binding from the total binding.*

B. *Positron Emission Tomography (PET)*

Subject Preparation and Analysis: ***Preclinical PET studies*** Details on procedures for quantification of radiotracer and transmission and emission scans have been published for ^{18}F-FDG and for [^{11}C]raclopride [32, 43]. Briefly, each animal is scanned for 30–60 min, 30 min after 250–500 μCi intraperitoneal injection of the radioligand ^{18}F-FDG. As a glucose analog, ^{18}F-FDG is taken up by cells at a rate correlated to cellular activity. The radioligand remains stable in the cells for at least 60 min following uptake [42], and so the ^{18}F-FDG utilization by the awake animal during the uptake period can be measured during the scan of the anesthetized animal. During the 30 min uptake period, rats are unrestrained and free to move around their environment. During this time, animals can be presented with food cues [37] or receive a pharmacological [41], optogenetic [44], or chemogenetic [45] stimulation. Additional scans can be carried out following ^{18}F-FDG uptake in the same animals and (if desired) in the presence of no cues or stimulation (baseline scan). Behavioral data can also be recorded during this uptake period for later analysis and correlation with the imaging data.

Immediately following the uptake period, animals are anesthetized with 2–3% isoflurane, maintained on 1% throughout the duration of the scan, and secured on the bed of the scanner. Blood glucose levels are measured via tail vein while the animal is anesthetized, and the animal's food is restricted for 8 h prior to the scan to obtain baseline blood glucose levels. PET images are then reconstructed and spatially normalized and can be coregistered to a rat brain MRI template using Paxinos and Watson stereotaxic coordinates using the imaging software PMOD (PMOD Technologies, Zurich, Switzerland). Statistical Parametric Mapping software (SPM) can then be used to identify significant changes in brain glucose metabolism (BGluM) between PET scans. Statistics can then be performed to identify significant contrasts. Activation can then be defined as a statistically significant increase in BGluM in the baseline scan and the experimental scan, while inhibition can be defined as a decrease in BGluM.

4 Notes

Human PET studies: Procedures for human PET imaging obesity studies have been published for dopamine signaling (dopamine D2 availability) by [^{11}C]raclopride [40, 41] and for brain glucose utilization by ^{18}F-FDG [35]. Briefly, for [^{11}C]raclopride, dynamic scans were started immediately after IV injection of 4–10 mCi for a total of 60 min. For ^{18}F-FDG, an emission scan (20 min) was taken

35 min after an IV injection of 4–6 mCi. During the scan, subjects were kept lying in the PET camera with their eyes open; the room was dimly lit, and noise was kept at a minimum. The subjects were monitored throughout the procedure to ensure that they did not fall asleep during the study.

Dopamine D2 receptor availability image analysis consists of a regions of interest (ROI) assessment in the [^{11}C]raclopride images that are obtained for the striatum (caudate and putamen) and for the cerebellum. The ROI is initially selected on an averaged scan (activity from 10–60 min for [^{11}C]raclopride) and then projected to the dynamic scans as previously described [46]. The time activity curves for [^{11}C]raclopride in the striatum and cerebellum and the time activity curves for unchanged tracer in plasma were used to calculate distribution volumes (DV) using a graphical analysis technique [47]. Next, the Bmax/Kd is obtained as the ratio of the DV in the striatum to that in the cerebellum (DV striatum/DV cerebellum), as a model parameter of DA D2 receptor availability. This parameter is unaffected by changes in cerebral blood flow [47]. The correlations between D2 receptor availability and brain glucose metabolism can be computed using statistical parametric mapping (SPM) [48]. For the SPM analyses, the images of the metabolic measures are spatially normalized using a human brain MRI template provided in the SPM package.

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Chapter 12

Preference for Palatable Food, Impulsivity, and Relation to Drug Addiction in Rats

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Abstract

Overall, this review focuses mainly on the behavioral overlap and interaction between drug abuse and excessive behavior directed toward natural rewards. In recent years, rats selectively bred for high (HiS) or low (LoS) saccharin intake and tested for all phases of drug addiction have provided valuable information regarding vulnerability to drug (Carroll et al., Behav Pharmacol 19:435–60, 2008) and food (Avena, Appetite 55:734–7, 2010; Yakovenko et al., Appetite 57:397–400, 2011) dependence/addiction, related affective disorders, and impulsive behavior. The neurobiological bases for this interaction between drug and food rewards have been reviewed by others (Deadwyler, Ann N Y Acad Sci 1187:140–7, 2010; Volkow and Wise, Nat Neurosci 8:555–60, 2005; Olsen, Neuropharmacology 61:1109–22, 2011). The HiS and LoS rats are models of the heritability of maladaptive behaviors, including hallmarks of drug dependence, bingeing, and withdrawal that serve equally well for the understanding of binge eating. The purpose of this chapter is to review recent developments in this area of research, emphasizing that several commonalities between food and drug addiction have been revealed, and to highlight similar connections between other individual differences and their relationships to sweet preference and drug abuse. Impulsivity will also be discussed as a major marker of addiction vulnerability that covaries with sweet preference, as well as other vulnerability factors, such as age (adolescents vs. adults) and sex. New evidence is presented regarding the importance of reactivity to aversive events in predicting drug abuse in HiS and LoS rats and on the importance of other addiction-prone and addiction-resistant phenotypes. Recent data from animal models also suggest that the addiction-prone and addiction-resistant groups (e.g., HiS, LoS) respond in opposite ways when treated for drug abuse. Finally, new evidence shows the importance of self-initiated and self-maintained treatments to reduce vulnerability to behavioral excesses and incubation of craving after termination of their use.

Key words Addiction vulnerability, Individual differences, Selection, Selective breeding, Sweet preference, Impulsivity, Rewarding effects of drugs, Aversive effects of drugs

1 Introduction

In humans, vulnerability to drug dependence is heterogeneous, as its determinants vary widely between individuals. Of these determinants, genetic disposition has substantial influence. Initial studies showed that adopted children born to individuals with histories of drug dependence were more likely to become drug dependent

Nicole M. Avena (ed.), *Animal Models of Eating Disorders*, Neuromethods, vol. 161, https://doi.org/10.1007/978-1-0716-0924-8_12,

[6, 7] than those born to nondependent parents. Subsequent comparisons of drug dependence between monozygotic and dizygotic twins have provided a range of heritability estimates, accounting for between 33% and 79% of the variance in vulnerability between individuals (for review, *see* [8]). Additionally, animal experiments have offered convergent evidence for genetic influence in addiction vulnerability [1, 9, 10].

One approach in the animal literature involves assessing variability in drug-related measures (e.g., self-administration, place preference, locomotor activity) in rats from genetically heterogeneous, outbred stocks. In a seminal study by Piazza et al. [11], outbred Sprague-Dawley rats exhibited significant variation in open-field locomotor activity induced by a novel environment, and this behavior was positively correlated with amphetamine-induced locomotor activity and amphetamine self-administration. The results of this study have been expanded across multiple drug classes and animal models of addiction, with the high-novelty-reactive rats or high responders (HR) displaying drug-prone profiles and the low novelty responders (LR) showing drug-resilient profiles [12].

That approach was refined further by Deroche-Gamonet et al. [13], who modeled multiple criteria established by the *Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition*, (DSM-IV) for human addiction diagnosis in outbred rats using an array of procedures, including progressive-ratio responding, reinstatement, and punishment. The investigators found great variability between the outbred animals in meeting the various criteria, with distributions closely modeling human epidemiological trends, such that only a minority of rats met all three criteria and in humans only a minority was drug abusers. Furthermore, variability in drug-seeking behavior was associated with individual differences in other stable, genetically mediated traits associated with addiction liability in humans. For example, activity level in a novel environment is a stable behavior that corresponds with human characteristics linked to drug abuse liability or protection [10, 14], including sensation seeking [15], stress response [16], and aspects of impulsivity, such as behavioral disinhibition [17] and impulsive choice [18]. These are the behaviors that have been isolated and studied in animal models, as they are often implicated in the devastating effects of drug abuse. Identifying animals with these addiction-prone traits allows us to determine how the maladaptive behavior can be modified and addictive behavior can be treated in the most vulnerable individuals. There have been varied strategies used to establish these traits, including selection by behavioral screening in outbred groups or by selectively breeding rats with extreme phenotypes.

2 Materials and Procedures

2.1 Methods Overview

The methods that will be described to construct rat models of drug addiction are mainly conducted in operant conditioning chambers in which rats are trained to respond on a lever to obtain an intravenous (IV) drug infusion or a food pellet. When impulsivity is evaluated, two levers are available: a response on one delivers a small amount of drug or food immediately, and a response on the other results in a larger amount of drug or food after a delay. In some cases, there is an adjoining compartment where a rat can gain access to a running wheel while maintaining its IV catheter and tether, thereby allowing concurrent access to both exercise and drug self-administration. Testing rats for saccharin preference by measuring 24-h liquid intake occurs in the home cages after the operant conditioning experiments are finished, so that intake of sweetened liquids does not interfere with subsequent drug-motivated responding. The saccharin preference score is obtained from saccharin and water intake to validate the sweet-preferring phenotypes obtained by selective breeding.

2.2 Behavioral Procedures to Model Drug Abuse

Throughout the following discussion of individual differences in saccharin preference and its relationship to impulsivity and drug abuse, several standard procedures have been used to model phases of the addiction process that exist in human addicts. The detailed methods for these essential procedures can be found in the key references cited under each topic; however, the methods described below summarize the main behavioral models that are used to compare individual differences (e.g., HiS vs. LoS, high impulsivity [HiI] vs. low impulsivity [LoI], female vs. male) in drug abuse. The main phases of drug abuse in humans (i.e., phases targeted for interventions) that can be modeled include (1) acquisition or initiation of drug taking in the drug-naïve animal, (2) maintenance of a steady state of drug self-administration under limited- or short-access conditions, (3) escalation of drug self-administration under long-access conditions, (4) extinction of drug-maintained responding following the removal of the drug, and (5) reinstatement of drug-seeking behavior that occurs when the animal is primed with an experimenter-delivered injection of either the previously self-administered drug or a similar drug. Agents that generate stress, such as footshock, restraint, or injection of a chemical agent (e.g., yohimbine), can also be used to reinstate drug seeking, in addition to discrete or discriminative cues that were previously associated with the drug (*see* [1, 19, 20] for a review).

In addition to the rewarding effects of drugs that are indicated by the procedures listed above, new evidence suggests that aversive effects of drugs may accompany the rewarding effects to determine the net motivation to seek drugs. Also, individual differences in

rewarding effects are often related to opposite individual differences in responsiveness to aversive effects (i.e., high vulnerability for reward associated with low vulnerability for aversive effects and vice versa). Several measures have been used recently to show individual differences in aversive effects [21], although unlike methods to compare rewarding effects they have not yet become standardized across laboratories. Aversive effects of drugs of abuse involve symptoms of withdrawal, but aversive events not related to drug administration such as food restriction/deprivation and administration of punishing agents (e.g., histamine) can be used to model reactivity to aversive events. Initial assessment of these results in rats with individual differences in sweet preference will be further described as examples in Subheading 3.

2.3 Acquisition

Methods used for acquisition of drug self-administration in drug-naïve animals have evolved for over 50 years and have recently been reviewed by Carroll and Meisch [22]. In most of the recent research described for individual differences, particularly regarding HiS vs. LoS, HiI vs. LoI, or female vs. male rats, a simple procedure is used. Rats are trained to lever press for IV infusions of cocaine (0.4 mg/kg) under a fixed-ratio 1 (FR 1) schedule of reinforcement during 6-h sessions. Sessions are signaled by illumination of a house light, and each response on the level results in a 2–4 S infusion (drug or saline), depending on the animal's body weight, and the infusion is accompanied by illumination of three stimulus lights above the lever. An inactive lever is used to count nonspecific responses. Responses during the infusion duration are counted as "ineffective responses," and they have no programmed consequences.

Initially, three experimenter-delivered priming infusions of 0.4 mg/kg cocaine are administered periodically throughout the 6-h session, and occasionally, a small amount of ground rat food is placed on the lever to facilitate acquisition. Training is considered complete once the rats earn at least 40 infusions during three consecutive sessions, and then, the session length is reduced to 2 h. This process produces rapid acquisition usually within a week. However, if the goal of the study is to specifically examine the acquisition process, a 2-h session is used instead of a 6-h session to slow the process and allow a more sensitive measure for comparing individual differences (*see* [22]).

2.4 Maintenance

After the acquisition criteria have been met, session length is decreased to 2 h, and behavior is typically monitored for 2–3 weeks until there are no steadily increasing or decreasing trends in infusions earned over five consecutive days. This short-access self-administration is typically stable for long periods of time and often does not increase over several weeks (*see* [23, 24]).

2.5 Escalation

After acquisition criteria have been met, for some rats, the session length is kept at 6 h, which results in escalation of drug intake over approximately 2–3 weeks. For control groups, the session length is kept at 2-h short access, a condition that is not expected to generate escalation of drug intake. Escalation is typically measured by comparing the first 2 or 3 days to the last 2 or 3 days over a 21-day period. This has been a sensitive procedure for comparing individual differences. Typically, HiS and HiI rats escalate their cocaine intake beginning after about 2 weeks, compared to lower and steady intake by their low-performing counterparts [25, 26]. A similar effect is found in female vs. male rats [27].

2.6 Extinction

This is a transition phase between self-administration and reinstatement (relapse) that is used to model abstinence in humans, although in animals it is forced abstinence. The most common method is to substitute the vehicle (e.g., saline) for the drug solution and to continue the self-administration sessions as they were with the cue lights and pump noises accompanying each saline infusion. Typically, there may be a burst of responding for the vehicle on the first day, and that will diminish over several days to very few responses by the end of the 2 weeks. Variations in this process are to stop the drug-paired cues and saline infusions to extinguish cues or to remove the rat from the chamber between sessions to examine renewal or spontaneous recovery of responding when it is placed back in the chamber or context-induced reinstatement when it is placed in the drug chamber vs. a control chamber. However, for examining individual differences, a simple extinction and drug-primed reinstatement procedure has been used (*see* [1, 3, 4]).

2.7 Withdrawal

Another aspect of extinction, especially when large and frequent amounts of drug had previously been self-administered (i.e., as in long-access sessions), is withdrawal. Withdrawal can be detected by monitoring sensitive behavioral baselines with food as a reward [20, 26]. Other measures of withdrawal, such as withdrawal-potentiated startle and taste aversion conditioned during withdrawal, will be discussed in Subheading 3.

2.8 Reinstatement

After extinction behavior has stabilized and all rats demonstrate minimal responding, a reinstatement model is implemented in which an experimenter-delivered drug injection, stress (yohimbine), or drug-paired cues are periodically administered once daily, with one or more intervening days of saline-priming injections or no priming conditions. Sessions are often 2 h in length, and the priming condition is given at the start of the session. Reinstatement responding is measured by the number of responses on the lever previously associated with drug reward. The sensitivity of this method to individual differences has been shown in several studies (*see* reviews [1, 19, 20]).

2.9 Selection and Selective Methods for Studying Individual Differences

Selection and selective breeding methods for understanding individual differences in sweet intake and their relationship to drug abuse have been previously discussed [1, 19, 20]. The selection methods may vary from testing a large group of rats for a specific behavior and then selecting the top and bottom quartile, third and half (median split), or using regression analysis in all the animals to correlate high and low phenotypes and proceed with drug testing. Selective breeding involves breeding high with high and low with low responders and avoiding brother/sister and first cousin matings. These methods and their relationship to sweet intake and drug abuse will be described below.

2.9.1 Selection of Rats for Individual Differences in Drug Abuse Based on Differences in Behaviors Motivated by Nondrug Rewards

In contrast to Piazza et al. [28] and Deroche-Gamonet [29], other investigators have taken a more indirect approach to studying behaviors that are hallmarks of drug abuse. Rats are screened on behavioral measures that serve as vulnerability markers for drug abuse, such as avidity for nondrug rewards (e.g., food, exercise) or impulsive behavior [1, 20]. Typically, there is a direct relationship between assessment of certain drug (e.g., self-administration)- and nondrug (e.g., novel response)-related behaviors, such that high measures on one parameter predict high measures on the other. For example, rats screened for HiI using a delay discounting task for food also acquired cocaine self-administration [24] and escalated cocaine intake at faster rates [25] compared to rats screened for low impulsivity. Using another measure of impulsivity, the five-choice serial reaction time task (5-CSRTT), Economidou et al. [30] showed that rats screened for HiI reinstated drug-seeking behavior for longer periods despite punishment compared to rats screened for LoI. This relationship holds for other procedures that model traits such as high and low responsiveness to a novel environment (HR, LR) [12, 31], attribution of incentive salience to reward-related stimuli (sign trackers (ST)) vs. attention focused on reward location (goal trackers (GT)) [32], and varying interest in natural rewards such as high (HiR) or low (LoR) exercise in a running wheel [33]. Table 1 summarizes individual differences that have been selected with respect to drug-motivated behavior or drug-associated behaviors. Furthermore, Fig. 1 illustrates that there is considerable overlap in these traits that have become associated with drug-seeking behavior. However, since there is no complete overlap, it can be assumed that some traits have unique influences on vulnerability to drug abuse that may operate independently or are dissociable from, but add to, the other factors.

2.9.2 Selective Breeding for Traits Associated with Drug Abuse

Another approach to investigating genetically mediated differences in addiction vulnerability involves breeding rodents based on bidirectional behavioral criteria, as summarized in Table 2. By mating animals that exhibit extremely high or low measures, it can be shown that the behavior of interest is under genetic influence,

Table 1
Selected individual differences and their effects on drug-motivated behavior

Selection criterion (high vs. low vulnerability)	Drug-related behavior	Drug/ reinforcer	References
Novel environment reactivity (HR vs. LR)	Drug-induced locomotor activity (HR > LR)	Amphetamine	[28]
		Morphine	[29]
	Self-administration (HR > LR)	Amphetamine	[28]
Impulsivity-delay discounting (HiI vs. LoI)	Acquisition (HiI > LoI)	Cocaine	[39]
	Acquisition (HiI > LoI)	Ethanol	[38]
	Escalation (HiI > LoI)	Cocaine	[37]
	Reinstatement (HiI > LoI)	Cocaine	[39], Regier et al., unpublished observation)
Impulsivity 5-CSRTT (HiI vs. LoI)	Acquisition (HiI > LoI)	Cocaine	[36]
	Self-administration (HiI > LoI)	Cocaine	[36]
	Reinstatement	Cocaine	[30]
Incentive salience (ST vs. GT)	Self-administration (ST > GT)	Cocaine	[32]
	Reinstatement (ST > GT)	Cocaine	[32]
	Locomotor sensitization	Cocaine	[35]
	Impulsivity (5-CSRTT; GT > ST)	Food	[34, 174]
Wheel running (HiR vs. LoR)	Self-administration (HiR > LoR)	Cocaine	[33]
	Reinstatement (HiR > LoR)	Cocaine	[33]

HR and LR high and low novelty responders, *HiI and LoI* high and low impulsive, *ST and GT* sign trackers and goal trackers, *HiR and LoR* high and low wheel runners

because successive generations show more stable and robust corresponding behaviors. A prominent example of this procedure is found in alcohol research in which breeding for differential drug intake was conducted by selecting animals on measures of high and low ethanol intake [40]. Subsequently, multiple rodent strains have been selectively bred based on varied behavioral and physiological responses to alcohol in humans [41, 42]. Such criteria have included alcohol consumption [43, 44], sensitivity to withdrawal

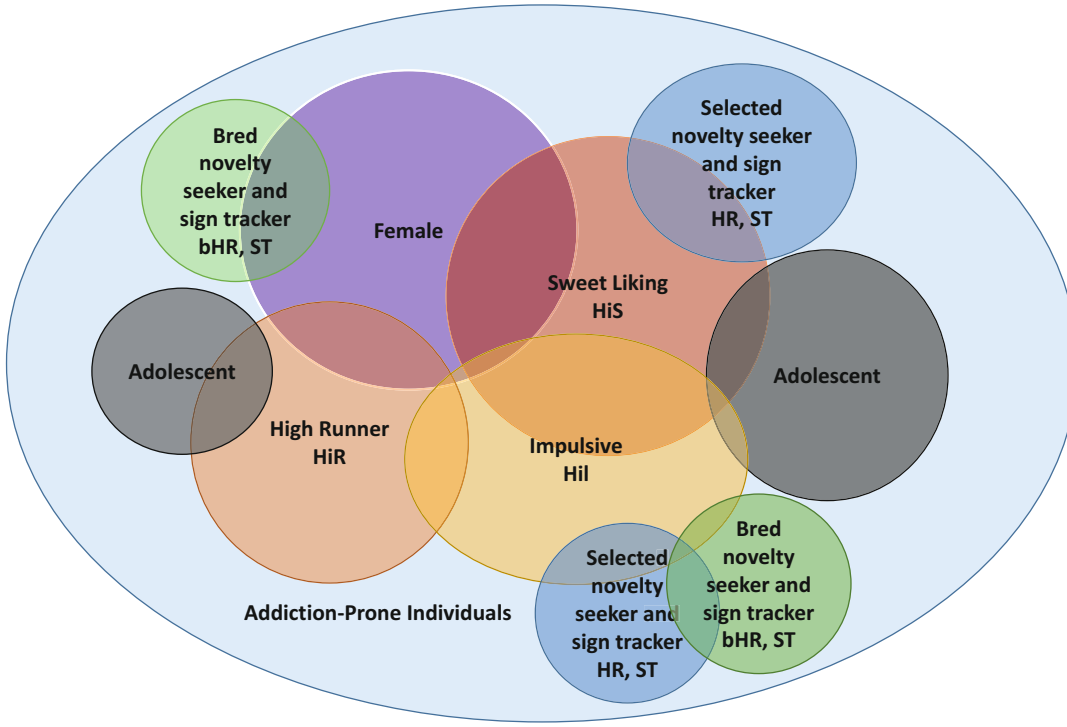

Fig. 1 Hypothetical overlap of addiction vulnerability factors that apply to food, exercise, or drug rewards

[45], ethanol-induced hypothermia [46], and locomotor reactivity [47].

The selective breeding approach was also applied to other drugs of abuse, such as diazepam [48], opiates [49], methamphetamine [50, 51], nicotine [52], and cocaine [53]. Similar to studies that selected outbred animals for behaviors directly or indirectly related to the behavioral effects of drugs, animals selectively bred for high and low addiction vulnerability also showed related behavioral features in measures indirectly related to drug abuse vulnerability. For example, animals bred for high (vs. low) alcohol consumption displayed greater impulsivity using delay and probabilistic discounting procedures [54, 55], as well as tasks of impaired response inhibition [55]. High (vs. low)-alcohol-consuming animals also showed greater avidity for natural rewards, such as exercise [56].

In addition to selective breeding for intake of a substance (e.g., alcohol intake), the procedure of selecting high and low phenotypes has been applied to behaviors that underlie addiction (*see* Table 2). The two behaviors that have received the most attention are bingeing on food (food addiction) [1, 2, 57] and impulsivity for food (*see* reviews in [36, 58, 59]). For example, one criterion is novelty-induced locomotor activity, the same measure shown to have a positive relationship with response to amphetamine by Piazza et al. [28] in outbred rats. Rats selectively bred for high

Table 2
Selectively bred rats that differ on drug- and food-motivated behavior

Selective breeding criterion (high vs. low vulnerability)	Drug-related behavior	Drug/ reinforcer	References
Ethanol consumption (HAC vs. LAC)	Impulsivity (delay discounting; HAC > LAC)	Saccharin	[54]
	Impulsivity (probabilistic discounting; HAC > LAC)	Sucrose	[172]
	Impulsivity (response disinhibition; HAC > LAC)	Sucrose	[55]
	Exercise (HAC > LAC)	Wheel running	[56]
Novel environment reactivity (HR vs. LR)	Acquisition (HR > LR)	Cocaine	[14]
Active avoidance learning (RHA vs. RLA)	Acquisition (RHA > RLA)	Cocaine	[173]
	Locomotor sensitization (RHA > RLA)	Cocaine	
	Impulsivity (delay discounting; RHA > RLA)	Food	[174]
	Impulsivity (response disinhibition; RHA > RLA)	Food	[174]
	Novel environment reactivity (RHA > RLA)	–	
Lewis vs. Fischer 344	Acquisition	Cocaine	[107]
	Self-administration	Cocaine	[175]
		Ethanol	[176]
	Locomotor sensitization	Cocaine	[177]
	Reinstatement	Cocaine	[178]

HAC and LAC high and low alcohol consumers, *HR and LR* high and low novelty responders, *RHA and RLA* Roman high and low avoidance

activity in this paradigm acquired cocaine self-administration more rapidly than those bred for low activity [14]. The fundamental principle unifying the literature described so far is the following: addiction vulnerability and certain types of behaviors reliably covary; therefore, it is probable that they are mediated by common underlying mechanisms. High or low avidity for sweetened dietary substances is another common feature to many of these models, and its interaction with addiction vulnerability is the subject of this chapter.

In some cases, the traits that predict drug abuse are bidirectional; for example, many of the animals screened or selectively bred

Table 3
Selected and selectively bred individual differences for sweet preference and their effects on drug- and food-motivated behavior

Selective breeding/selection criterion (high vs. low vulnerability)	Behavior	Sweet substance/drug	References
Novel environment reactivity (HR vs. LR)[b]	Operant responding (HR > LR)	Sucrose	[60]
Impulsivity (5-CSRTT; HiI vs. LoI)[b]	Operant responding (HiI > LoI)	Sucrose	[61]
Active avoidance learning (RHA vs. RLA)[a]	Consumption (RHA > LHA)	Saccharin	[62]
Ethanol consumption (HAC vs. LAC)[a]	Consumption (HAC > LAC)	Saccharin	[64] [66]
	Consumption (HAC > LAC)	Sucrose	[65]
Ethanol consumption (HAC vs. LAC)[b]	Consumption (HAC > LAC)	Saccharin	[67]
Sweet preference (SL vs. SDL)[b]	Consumption (SL > SDL)	Ethanol	[68]
	Self-administration (SL > SDL)	Amphetamine	[69]
	Self-administration (SL > SDL)	Morphine	[70]

HR and LR high and low novelty responders, *HiI and LoI* high and low impulsivity, *HAC and LAC* high and low alcohol consumers, *SL and SDL* sweet likers and sweet dislikers
[a]Selectively bred; [b]Selected from outbred stocks

to show divergence in drug seeking or drug response also show divergence in avidity for sweet substances. For instance, the selected HR rats respond more robustly for sucrose [60] compared to LR rats, while rats selected for HiI using the 5-CSRTT show more operant responding for sucrose compared to LoI rats [61]. Further, rats selectively bred for high rates of active avoidance learning (RHA) consume more sweetened dietary substances, such as a saccharin solution, compared to those selectively bred for low rates of avoidance learning (RLA) [62, 63].

In many cases, behaviors that are selected for or selectively bred are interchangeable or substitutable behaviors related to addictive behavior. For example, rodents selected from outbred stocks or by selective breeding for high and low alcohol consumption ingest high and low amounts of sucrose and saccharin solutions, respectively [64–67]. Conversely, rats screened for high consumption of sweet substances ingest more ethanol [68], amphetamine [69], and morphine [70] than rats screened for low ethanol intake. In fact,

Table 3 shows that rats selected for an affinity for a variety of substances or events have elevated drug-seeking behavior compared to their counterparts with low selection criteria.

Similar results have been found in clinical studies. Avidity for sweet consumption is positively related to drug abuse in humans. For instance, cocaine [71], nicotine [72, 73], opioid [74], and alcohol [75–77] users experience greater hedonic effects of sweetened dietary substances than nonaddicts.

Similar to addiction vulnerability, response to sweets also has a heritable influence [78–82]. It has been proposed that these differences in sweet preference are not predominantly mediated by genetic variation in coding for peripheral taste processing (i.e., taste receptors), but for differences in reward processing that are related to central nervous system function [83, 84]. Further, both alcohol-naïve individuals and alcoholics with familial histories of alcoholism display greater sweet preference than those without family histories of alcoholism [85]. Taken together, these results display a clear relationship between sweet intake and addiction vulnerability that strongly implicates some shared genetically mediated biological mechanisms. Selectively breeding rats for differential saccharin intake has allowed us to more directly examine this relationship.

2.9.3 Selective Breeding for High or Low Saccharin Intake (HiS, LoS)

While investigating genetic influence on variability in response to sweets, Nachman [86] was the first to illustrate the heritability of saccharin preference by employing a selective breeding program in which rats were mated based on consumption of a saccharin solution. Subsequent experiments using inbred strains of mice further supported the heritability of saccharin preference [87–90]; however, their avidity for drug intake was not investigated. Later, Dess and Minor [91] selectively bred rats (Holtzman Sprague-Dawley, Indianapolis, Indiana, USA) for high and low saccharin intake based on extreme saccharin phenotype scores that were derived from this measure:

$$Saccharin\ phenotype\ score = \frac{24\text{-h}\ \ saccharin\ intake(ml) - 24\text{-h}\ water\ intake(ml)}{Body\ weight(g) \times 100}$$

Originally, the resultant high- and low-saccharin-consuming lines, now called Occidental (Occidental College, Los Angeles, CA) HiS and LoS rats, were used to investigate the interaction between genetically mediated sweet preference and measures of emotionality. Subsequently, ethanol intake was compared in HiS and LoS rats, and as predicted, the HiS rats consumed more ethanol than LoS rats in free-choice and forced-consumption tests [57]. These results prompted further investigation into differences

Table 4
Summary of results from studies on selectively bred HiS and LoS rats and drug-related behavior

Behavioral model	Drug/ reinforcer	Phenotype effects	Sex	References
Acquisition	Cocaine	HiS > LoS	M only	[23]
Adoles vs. adult	Cocaine	HiS > LoS	M only	[179]
Maintenance	Heroin	HiS > LoS	F > M	[23]
	Cocaine	HiS = LoS	M = F	[95, 180]
Escalation	Cocaine	HiS > LoS	F only	[26]
Impulsivity (delay discounting)	Food	HiS > LoS	F > M	[97]
	Cocaine	HiS = LoS	F = M	[97]
Impulsivity (response disinhibition)	Food	HiS = LoS	F = M	[96]
	Cocaine	HiS > LoS	F > M	[96]
Extinction	Cocaine	HiS > LoS		[26]
Reinstatement	Cocaine	HiS > LoS	F only	[26]
Adoles nic exposure/adult coc	Cocaine	HiS > LoS	F only	[94]
Adoles vs. adult	Cocaine	HiS > LoS	F only	Holtz et al., in preparation
Drug-induced locomotor activity	Cocaine	HiS > LoS	F > M (HiS)	[95]
Drug-induced locomotor sensitization	Cocaine	HiS > LoS (F)	F > M	[95]
Dysregulation of dose selection	Cocaine	HiS > LoS	F only	[180]
Treatment – Baclofen on escalation	Cocaine	LoS > HiS	F only	[92]
Progesterone on escalation	Cocaine	LoS > HiS	F only	[103]
Histamine punishment	Cocaine	LoS > HiS	F only	Holtz et al., in preparation
Neurobiological studies c-Fos	Cocaine inj	LoS > HiS	M only	Regier et al., unpublished observation
Orexin cell labeling in hypothal	Coc/Sal inj	HiS > LoS	M only	Holtz and Carroll, in preparation

F female, *HiS* high saccharin, *LoS* low saccharin, *M* male

in drug self-administration with the HiS and LoS lines. For example, Carroll et al. [23] showed that HiS rats and females acquired IV cocaine self-administration faster and in more animals per group than LoS rats or males.

A second colony of HiS and LoS rats was established from the Occidental HiS and LoS lines at the University of Minnesota, in which the primary interest was cocaine self-administration across various phases of the addiction model, although other drugs (e.g., heroin) and assays of drug vulnerability (e.g., delay discounting, drug-induced locomotor activity) have been employed. These experiments have largely shown the HiS and LoS rats to have consistent drug-prone and drug-resilient profiles, respectively, across several phases of drug abuse (*see* review in [1]). For example, as shown in Table 4, HiS rats exceed LoS rats in all phases of drug abuse, from acquisition to maintenance [23], escalation [26, 92, 93], and reinstatement [26, 94].

In addition to drug self-administration, the HiS and LoS rats also display differential behavioral profiles on other addiction-related measures, such as novelty reactivity [91], cocaine-induced behavioral sensitization [95], impulsivity for food and/or cocaine during a motor impulsivity task [96], and a delay discounting choice task [97]. Given that these are common features in human and animal addiction vulnerability research, the HiS and LoS rats provide an exemplary animal model of genetically mediated addiction proneness and protection. A review of experiments conducted on HiS and LoS rats (*see* Table 4) substantiated the drug addiction proneness and resistance of the HiS and LoS rats, respectively, and also showed that these characterizations may interact with other vulnerability factors such as sex, age, and impulsivity [1].

2.9.4 Monitoring and Selecting for Impulsivity: A Trait that Is Closely Associated and Additive with Sweet Preference and Drug Abuse

As indicated in Fig. 1, another factor that has emerged as a strong determinant of drug addiction is impulsive behavior [59, 98–101].

Impulsive Choice-Delay Discounting

Impulsivity is a second individual difference to be discussed in this review as a determinant of vulnerability to drug abuse. The method that has been used most often to select animals for HiI and LoI is delay discounting, which is a choice between a small, immediate reward vs. a large, delayed reward. There are other variations, such as immediate high probability of reward vs. delayed low probability of reward. In the delay discounting procedure, the sessions consist of 15, four-trial blocks and last for 2 h or until rats complete 60 trials, whichever is first. The first and second trials are forced on the immediate and delay lever; the third and fourth trials are choice trials. Different stimulus conditions are associated with each lever, and the delay and immediate sides are reversed daily or every other day. At the start of the experiment, the delay of the large reward is set at 6 s, and thereafter, responses made on the

delay vs. immediate lever result in an increase or decrease, respectively, by 1 s of the delay on the delayed side. Thus, the animal adjusts its delay by responding on either side, and at the end of the session, the delays on all of the 30 choice trials are averaged to yield a mean average delay (MAD) for the session. The MAD is the main measure of impulsivity, and based on a distribution of over 180 rats, empirical evidence suggested a bimodal distribution of delays. Thus, high impulsivity was defined as less than or equal to 6 s, and low impulsivity was defined as greater than or equal to 13 s. Typically, a food reward is used to screen rats for high and low impulsivity [24], with the total daily food allotment being adjusted after the delay discounting session, but cocaine infusions (small vs. large doses) have also been used as rewards [24].

Selecting rats for HiI and LoI choice on a delay discounting task [24, 59, 98] or on premature responding on a 5-CSRTT [58, 102] has led to the same degree of predictability of addiction-prone (HiI) and addiction-resistant (LoI) behavior as described for the bred HiS vs. LoS lines (e.g., [24]). For example, male and female HiI rats acquired cocaine self-administration faster and with more animals per group than their LoI male and female counterparts [39]. During long access to cocaine, HiI rats showed significant escalation, while LoS did not change [25]. However, during the reinstatement paradigm, different results occurred with HiI and LoI compared to HiS and LoS rats. The HiI and LoI groups did not differ during the 2-h maintenance phase; however, there were differences during extinction opposite to the HiS and LoS rats, with the LoI rats being more resistant to extinction than the HiI rats [39].

When HiS and LoS rats were tested on delay discounting tasks for food or IV cocaine infusions [24], HiS males and females were more impulsive than LoS males and females, and females were more impulsive than males. Thus, vulnerability factors of saccharin preference (i.e., HiS), impulsivity (i.e., HiI), and sex (i.e., female) together form the most vulnerable condition. However, when cocaine was the reward in the delay discounting task, there were no sex or saccharin phenotype differences. This is probably due to floor effects, since the MAD scores were in the HiI range in all groups. Using the Go/No-Go or signaled reward and non-reward task, the HiS rats exceeded the LoS, and females were greater than males on the measure of impulsive action, responding during signaled non-reward (No-Go) [96]. Using this task for food reward revealed no differences in impulsive action in HiS vs. LoS or males vs. females. Although it depends on the sensitivity of the impulsivity task, it appears that there is some overlap between saccharin preference and impulsivity.

The results obtained with HiS and LoS rats are similar to those found with another vulnerability factor, age. Adolescent rats showed higher Go/No-Go responding than adults [103] when

tested as adolescents and later as adults and compared to adults that had been tested twice as a control. Adolescents, like HiS rats, also acquire cocaine self-administration faster, escalate their intake at a higher rate, show resistance to extinction, and reinstate more readily to a cocaine-priming injection than adults or LoS rats. With regard to natural rewards, adolescent rats exhibited greater positive taste reactivity to [104] and consumed more of 6% [105] and 10% [104] sucrose solutions compared to adults. Thus, adolescence and high sweet preference are both prominent vulnerability factors and may produce even more pronounced effects on drug seeking.

Ineffective Responding as a Possible Measure of Impulsivity in HiS Versus LoS Rats

In our laboratory, rats typically self-administer IV drug solution under fixed-ratio schedules of reinforcement wherein a single active lever press results in a single infusion. Thus, active lever responses are followed by an infusion delivery interval during which responses are recorded but produce no additional infusions. These responses during the infusion delivery are considered unreinforced responses [106] or ineffective responses [107]. In recent studies of individual differences such as HiS vs. LoS, HiI vs. LoI, and adolescents vs. adults, an additional behavior that has been noted in the more vulnerable genotypes and phenotypes is responding during the drug infusions. Ineffective responses were found by Kosten et al. [107] who found them at low cocaine doses (0.0625, 0.125 mg/kg) in Fischer 344 rats but not at higher doses (0.25–1 mg/kg) or in Lewis rats. They suggested that since their Fischer rats maintained cocaine-reinforced behavior at higher levels than Lewis rats (although Lewis rats acquire faster), this might be a reflection of more craving in Fischer rats. Cummins and Leri [106] found ineffective responding with heroin self-administration but not at a 0.5 mg/kg dose of cocaine, and they interpreted the result as an elevation in motivation to obtain the drug. Ineffective responding has been found to be greater in HiS than LoS rats in our laboratory (with FR 1 schedules of 0.4 mg/kg cocaine reinforcement), in adolescents than adults, and in HiI than LoI rats (*see* Fig. 2). Since ineffective responding occurs in groups that self-administer more cocaine than their lower vulnerability counterparts and elevated responding is not consistently found on the inactive lever in the high-vulnerable rats, it is assumed that this behavior is directly related to the reinforcing effects of the drugs and stimulus properties of the manipulanda (lever) associated with drug reward. In this sense, it may be a form of sign tracking that is associated with elevated drug self-administration in rats selected for behavior directed toward the CS (sign – lever) [108]. Other potential explanations suggested increased motivation because ineffective responding increases at lower drug doses (that may have a priming effect), under PR schedules [106, 107] that assess motivation, and during food restriction that increases motivation [106, 109, 110]. Ineffective responding may also be an indicator of elevated

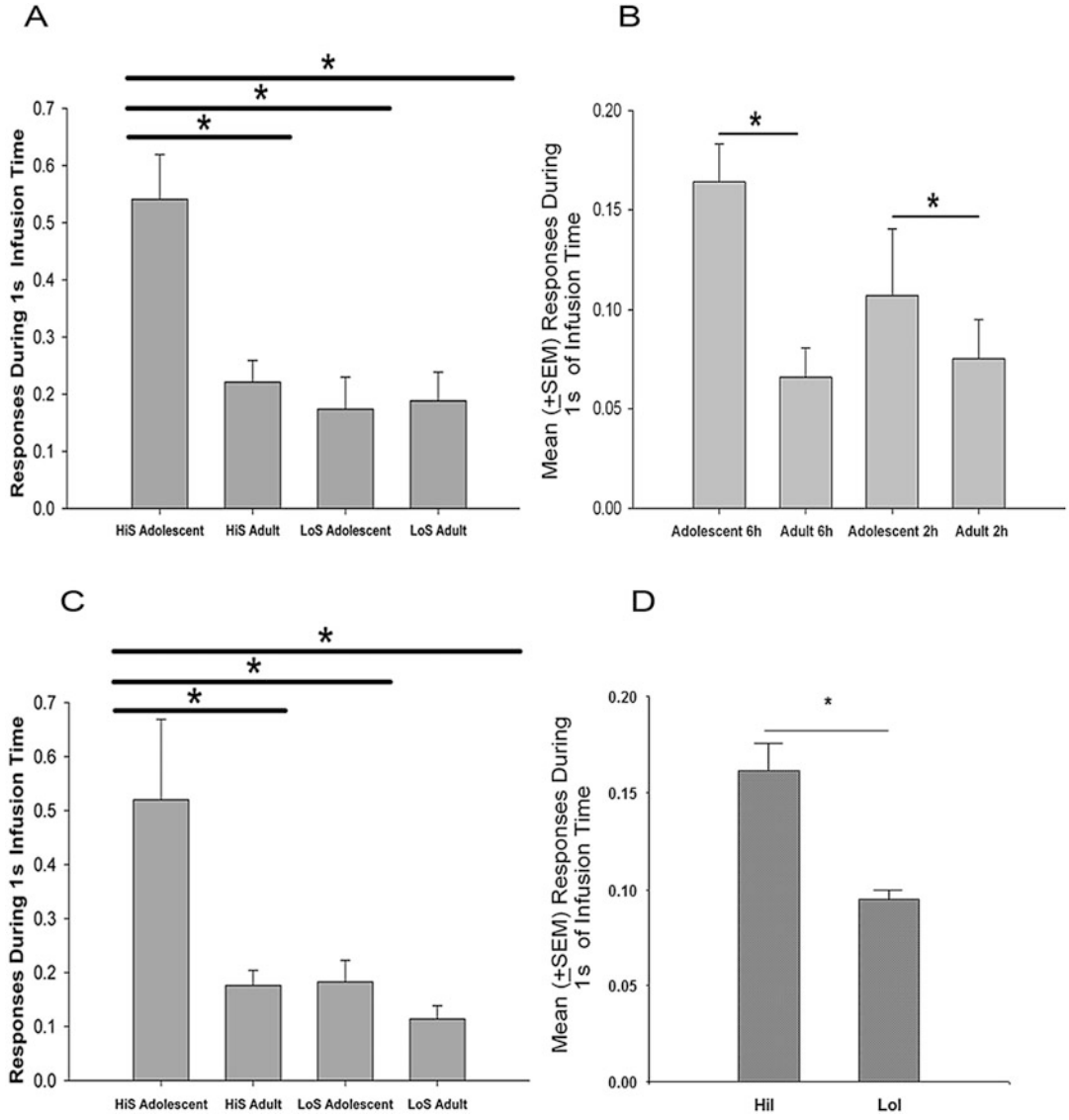

Fig. 2 Responses on the active lever during infusions per s of infusion time. $p = 0.05$. (A is from Holtz et al., unpublished observation; B is redrawn from [185]; C is from Holtz and Carroll, unpublished observations; and D is from Regier et al., unpublished observations)

impulsivity, as it is higher in rats selected for high (vs. low) impulsivity (*see* Fig. 2d), and rats selected on other vulnerable features, such as HiS [24] or adolescents [93], also show elevated impulsivity.

Additional evidence that the HiS rats may have higher motivational status than LoS rats is from a neurobiological analysis of HiS and LoS rats that examined the number of orexin-positive cells in the lateral hypothalamus. Figure 3 indicates that these cell counts were higher in the HiS than the LoS rats (e.g., ineffective

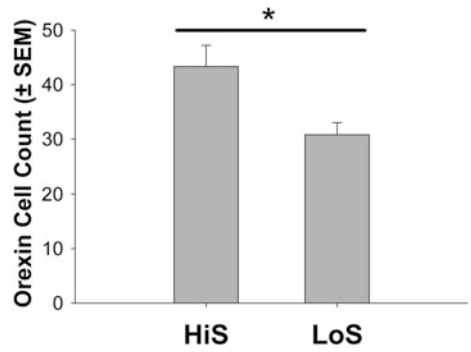

Fig. 3 Orexin cell counts in the hypothalamus. $p = 0.05$. (From Holtz et al., unpublished observation)

responding) supporting the behavioral data that suggest higher motivation levels. Orexin-1 is a neuropeptide that stimulates the motivation to ingest preferred substances and mediates dopamine release that affects motivation for cocaine and other highly valued rewards. Interestingly, orexin administration increased saccharin intake in rats, and mRNA expression of orexin increased following saccharin consumption [111]. Further, the orexin-1 antagonist blocks motivation for highly preferred rewards, such as high-fat chocolate in rats [112] and craving and reinstatement [113]. These data suggest that differences in the distribution of orexinergic neurons between the lines may contribute to a variety of differences in reward-seeking behavior, including the phenomenon of ineffective responding.

3 Notes

3.1 Aversive Effects of Addictive Drugs

3.1.1 Assessing Responsiveness to Aversive Effects of Drugs in HiS Versus LoS Rats

Previous work has shown that LoS rats display greater negative reactivity, or emotionality, compared to HiS rats under stressful conditions [91, 114]. For example, LoS rats show greater latency of emergence and increased defection in the novel open field and more stress-induced anorexia [91] and analgesia than HiS rats [114]. Further, compared to HiS rats, the LoS animals display increased reactivity of the hypothalamic-pituitary-adrenal (HPA) axis, a system that is integral to the stress response and linked to a multitude of psychiatric disorders, including drug addiction [115, 117].

As stress is a powerful liability in many aspects of substance dependence [117], the HiS and LoS model may have special utility in investigating a more broad construct of emotionality that can, along with results from other high- and low-vulnerable animal models (*see* Table 5), provide an overarching framework with predictive and translational value. The following studies are examples

Table 5
Relationship between high and low responders for drug and nondrug rewards and response to aversive conditions (adapted from [20])

Groups	Positive effects	Negative effects	References
HiI vs. LoI (impulsivity)	HiI > LoI	LoI > HiI cocaine extinction	[39]
		LoI = HiI	Regier et al., unpublished observation
		Heroin withdrawal	[102]
		Cocaine withdrawal	[102]
		LoI > HiI Cocaine devaluation	Carroll et al., unpublished data
HiS vs. LoS (saccharin intake)	HiS > LoS	LoS > HiS Ethanol withdrawal Glucose withdrawal	[126] [2]
		LoS > HiS Food-deprivation-induced wheel running	Dess et al. (2007)
		LoS > HiS acoustic startle	[114]
		LoS > HiS Food deprivation + methylphenidate-induced startle	
		LoS > HiS Punishment of cocaine intake	Holtz et al., unpublished observation
HR vs. LR (novelty reactivity)	HR > LR	LR > HR fear, anxiety, and emotionality	[31, 181, 182]
HAC vs. LAC (ethanol intake)	HAC > LAC	Lac>HAC Ethanol withdrawal	[183]
LEW vs. F344 (inbred strains)	LEW > F344	F344 > LEW Fear, anxiety, emotionality F344 > LEW	[184]
		Taste aversion (morphine)	[21]

HiI and LoI high and low impulsivity, *HiS and LoS* high and low saccharin, *HR and LR* high and low responders, *HAC and LAC* high and low alcohol consumers, *LEW and F344* Lewis and Fischer F344

of the interactions between genetic differences in drug abuse vulnerability (e.g., HiS vs. LoS) and several measures of stress reactivity.

3.1.2 Food Restriction-Induced Hyperactivity

One stressful condition, acute food restriction, increases wheel running more in LoS rats compared to HiS rats ([114]; Zlebnik and Carroll, unpublished observations). In the rat's natural environment, this increased activity is considered paradoxical because

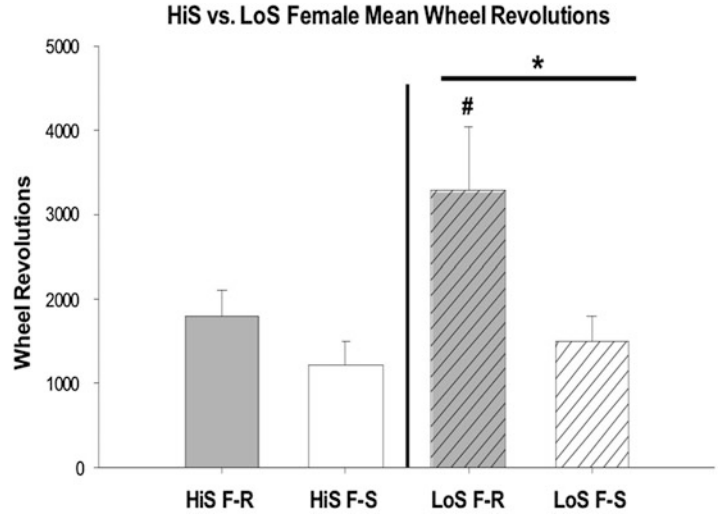

Fig. 4 Punishing effects of adding histamine to IV cocaine self-administration solution in (**a**) HiS and LoS and (**b**) HiI and LoI rats. $p = 0.05$. (From Holtz et al., unpublished observation)

the animal is expending extra energy when caloric resources are scarce. However, hyperactivity also promotes foraging behavior and increases the probability of discovering new sources of food. Recently, McLaughlin et al. investigated the interaction of restriction-induced hyperactivity in the running wheel and the effects of methylphenidate, a stimulant that activates the mesolimbic dopamine system and increases HPA activation [118] and increases wheel running [119]. The LoS food-restricted group treated with methylphenidate showed the most wheel running compared to HiS and vehicle-treated groups. These results indicated that methylphenidate enhanced the relatively elevated restriction-induced locomotor activity in the LoS rats, but not in the HiS rats. Similarly, wheel running was elevated in chronically food-restricted LoS rats compared to HiS and food-satiated groups ([114], Zlebnik and Carroll, unpublished data, *see* Fig. 4). This enhancement of the already elevated emotional reactivity in the LoS rats may be attributable to the augmentation of HPA functioning.

3.1.3 Food Restriction- and Withdrawal-Induced Acoustic Startle

The startle response is a defensive reflex that follows an acute, salient stimulus (e.g., brief, loud noise; acoustic startle), and its amplitude reflects internal affective states, such as anxiety [120]. Startle can be modulated by environmental and intrinsic variables or exogenously administered (pharmacological) agents [121–123]. Recent studies have used this paradigm to associate the differential reactivity to aversive conditions in the HiS and LoS rats to their respective differences in drug abuse vulnerability. An initial study by Dess et al. [114] showed that LoS rats exhibited greater startle amplitude in response to brief, intermittent bursts of

white noise (acoustic startle). A recent study expanded these findings by investigating the effects of stress in the form of inescapable footshock on startle amplitude between the phenotypes [124]. Greater aversive effects in LoS (vs. HiS) rats were also indicated in another recent experiment from McLaughlin et al. that found that food-deprived LoS rats treated with methylphenidate had greater acoustic startle compared to those treated with saline, while these results were not found with the HiS rats. The studies illustrate that rats with distinctive drug vulnerability profiles also show divergent responses to stressful events, suggesting that emotional reactivity may modulate the aversive aspects of drug administration (toxicity, withdrawal) and strongly impact addiction liability.

Another approach to assessing the aversive aspects of commonly abused drugs is to compare withdrawal effects between HiS and LoS lines. Withdrawal is a negative, anxiety-like affective state that involves, in part, the activation of the HPA axis [125]. Dess et al. [126] investigated the effects of withdrawal 24 h following 2 weeks of chronic, forced ethanol exposure on an acoustic startle response in the HiS and LoS rats. The LoS rats exposed to ethanol had greater startle amplitude than LoS rats with access only to water and HiS rats exposed to ethanol or water.

Withdrawal-like responses in the HiS and LoS rats were recently extended to the effects of forced glucose abstinence [2]. Because drug dependence, dysregulated food intake, and food addiction show strong parallels [127–129], strain differences could provide deeper insight into variance in emotionality by examining nondrug rewards. In this experiment, rats were given extended access to a glucose solution and then glucose deprived for 1 day until the acoustic startle response was measured. While there were no line differences in startle between rats that received glucose, escalation of glucose intake was correlated with increased startle in the LoS group. Combined, these results are in line with the previous experiments showing that HiS rats displayed elevated intake (bingeing) of ethanol and other drugs, while LoS rats showed more severe withdrawal effects and were more sensitive to the aversive aspects of chronic drug exposure, providing a partial explanation of their relative reduction in drug-seeking behavior.

Recent preliminary work with punished drug self-administration has provided additional evidence to this end. In this study, HiS and LoS rats maintained a period of stable IV cocaine self-administration, and then, histamine was added directly into the cocaine solution. Systemic histamine administration is an effective punisher that reduces cocaine self-administration in monkeys [130, 131]. Therefore, to investigate strain differences in drug-seeking behavior in spite of aversive consequences, a defining behavioral characteristic of addiction, a primary function of histamine in this experiment, was to act as a contiguous,

interoceptive punishing agent. Following this assessment, the cocaine/histamine solution was replaced with a histamine-free cocaine solution, and a third self-administration phase commenced. Preliminary results indicate that, compared to baseline, histamine reduced cocaine infusions administered equally in both strains. However, when histamine was terminated, HiS rats returned to higher levels of cocaine self-administration at a faster rate, while LoS rats remained significantly suppressed for up to 20 days (*see* Fig. 5a). These data suggest that the LoS rats are more sensitive to

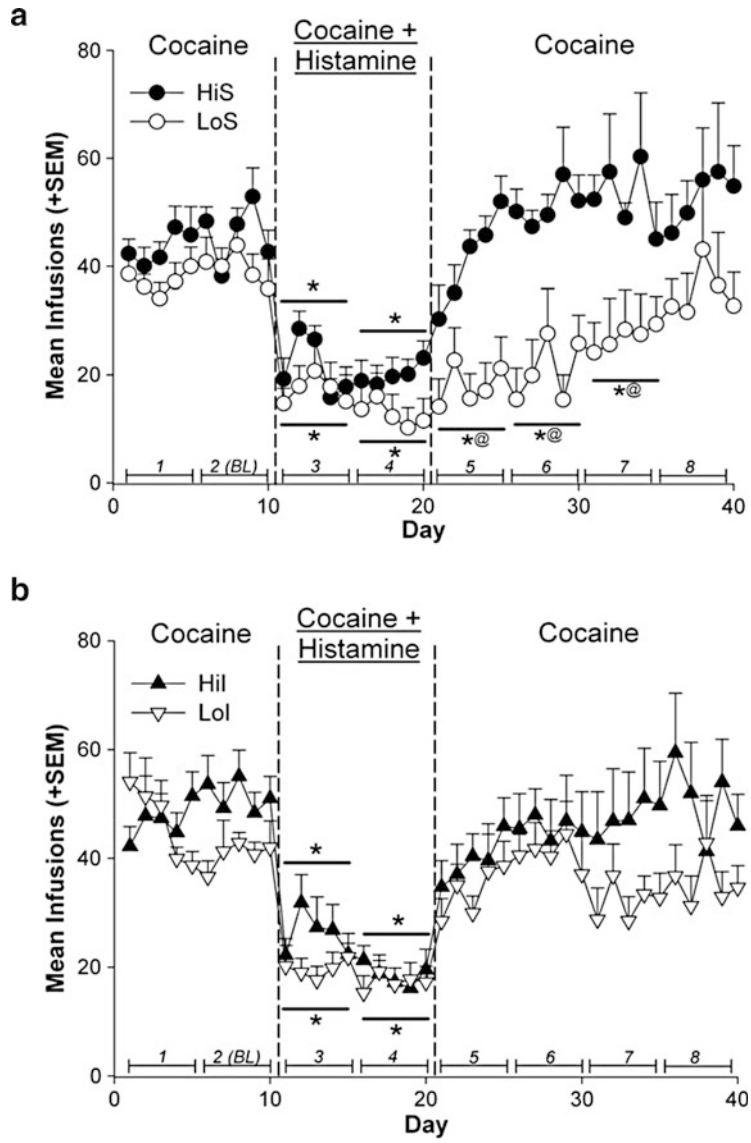

Fig. 5 Wheel revolutions in HiS and LoS rats that were fed restricted (F-R) or food satiated (F-S). *$p < 0.05$, LoS F-R vs. LoS F-S, and #$p < 0.05$, Los F-R vs. HiS F-R. (From Zlebnik and Carroll, unpublished observation)

histamine's punishing effects than HiS rats. This same experimental procedure was also conducted with rats selected for high and low impulsivity (HiI vs. LoI rats), and preliminary results indicate a similar long-term punishing effect of histamine on LoI rats compared with HiI (*see* Fig. 5b), suggesting that sensitivity to aversive events may be a common factor mediating drug-protected phenotypes.

3.2 Novel Approaches to Treating Addiction

3.2.1 Examining Individual Differences in Responsiveness to Treatment

Recent evidence suggests that the individual differences that have been reported for many aspects of drug abuse also extend to differences in responsiveness to treatment. Most treatment studies regarding individual differences involve males and females, as summarized in Table 6. For example, spiradoline, a κ-opioid agonist, produced a greater reduction in cocaine-induced locomotor activity in female mice compared to males [132], and a similar drug, bremazocine, decreased phencyclidine self-administration under an FR schedule to a greater extent in female rhesus monkeys compared to males [133]. Furthermore, the gamma-aminobutyric acid B ($GABA_B$) agonist, baclofen, had a greater effect of lowering acquisition rates of IV cocaine self-administration in female rats compared to male rats [134], and ketoconazole, a corticosterone synthesis inhibitor, suppressed heroin self-administration more in female than in male rats [135]. Sex differences in responsiveness to

Table 6
Individual differences in sex and age and treatment effects

Behavioral model	Drug	Treatment	Treatment effect	References
Locomotor activity	Cocaine	Spiradoline	F > M	[132]
Acquisition	Cocaine	Baclofen	F > M	[134]
Self-administration	Heroin	Ketoconazole	F > M	[19]
	Cocaine	Exercise	F > M	[136]
	Phencyclidine	Bremazocine	F > M	[133]
	Phencyclidine	Saccharin	F > M	[137]
	Cocaine	Histamine	F = M	Holtz et al., unpublished observation
Escalation	Cocaine	Exercise	Adolescents > adults	Zlebnik et al., unpublished observations
Reinstatement	Cocaine	Cocaine hydrolase	F = M	[103]

F female, *M* male, *Adoles* adolescents

treatment for drug abuse also extend to non-pharmacological interventions. For example, access to a running wheel significantly decreased cocaine self-administration in female rats but not in males [136], and access to saccharin reduced phencyclidine intake to a greater extent in female compared to male monkeys [137].

Further studies have shown consistent findings that many aspects of drug abuse are reduced in female rats, monkeys, and humans by treatment with progesterone or its metabolite allopregnanolone, and there are no effects in males, as these are ovarian steroid hormones (*see* reviews in [19, 138–140]). While the scope of individual differences in these examples is limited to sex, together, they offer an experimentally tractable link between neurobiological differences that underpin both addiction severity and treatment sensitivity.

3.2.2 Treatment for Drug Abuse Modeled in HiS and LoS Rats

While the HiS and LoS rats also represent groups that consistently differ in responsiveness to the positive and negative effects of drugs and vulnerability to addiction, little is known about how these treatments affect rats with these individual differences. In a previous study, Garbutt et al. [141] showed that individuals with a preference for high concentrations of sucrose responded more favorably to naltrexone treatment of alcoholism than those preferring lower concentrations. Recently, selectively bred HiS and LoS and selected HiI and LoI rats have been tested with treatments that have been shown to reduce drug intake without affecting food-maintained behavior (e.g., baclofen, progesterone) or treatments that involve punishment of drug-maintained responding (e.g., histamine).

In the initial studies, a long-access (6 h) escalation procedure was used to examine the effects of treatments in HiS and LoS rats. This procedure is considered an animal model of drug bingeing in humans, and it is sensitive to individual differences, including sex [27, 142], and saccharin intake, such as that displayed by the HiS vs. LoS rats [26], and impulsivity in selected HiI and LoI rats [25]. In these studies, females exceed males, HiS rats exceed LoS rats, and HiI rats exceed LoI in escalation of their cocaine intake over a 21-day period. Escalation is considered to be a critical component in the transition from controlled drug use to uncontrolled use and addiction; it is mediated by dramatic shifts in mesolimbic reward system functioning [125], and it is considered to be target for treatment approaches. However, only a few studies have addressed the use of treatment agents during this phase [143, 144], and none have evaluated individual differences using selective breeding models.

In two studies conducted recently, pharmacological treatments were administered to female HiS and LoS rats during the escalation phase of cocaine self-administration. In one study by Holtz and Carroll [92], chronic baclofen treatment was administered over a

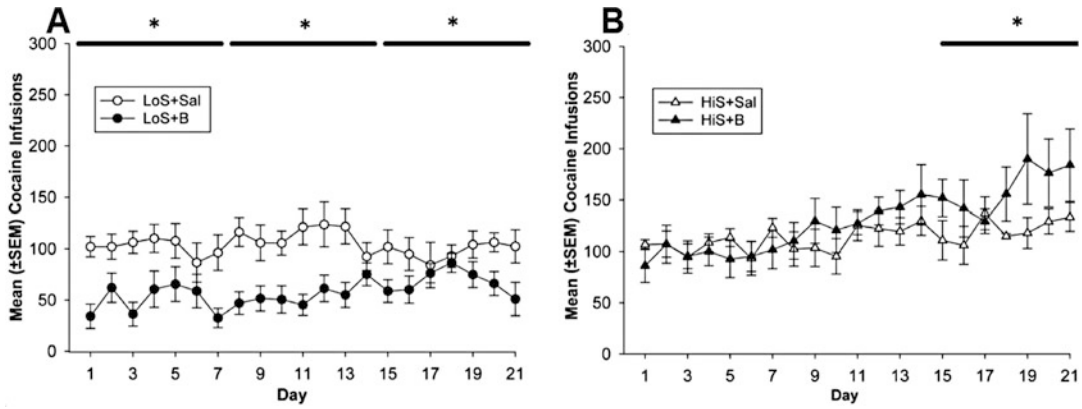

Fig. 6 Mean (±SEM) cocaine infusions are presented over a 21-day period when rats were allowed access to 0.4 m/mL cocaine for 6 h sessions 7 days per week. (**a**) baclofen (filled circles)- and saline-treated (open circles) rats selectively bred for low saccharin intake (LoS). (**b**) baclofen (filled triangles)- and saline-treated (open triangles) rats selectively bred for high saccharin intake (HiS). Horizontal lines and * indicate blocks of 7 days when there were significant group differences. (Reproduced, with permission, from Ref. [92])

long-access period in which rats self-administered and typically escalated their IV cocaine infusions. Baclofen, a potent agonist at $GABA_B$ receptors, has been used for alcohol and cocaine dependence in humans and modeled as a potential treatment for cocaine abuse in the animal laboratory [145]; for review, *see* [146–148]. Baclofen suppresses cocaine-induced dopamine increases in the nucleus accumbens [149]. Animal models have generally supported baclofen's efficacy as a treatment for drug addiction [150–152]; however, clinical studies have yielded equivocal results [153]. Compared to vehicle injections, baclofen potentiated the escalation of cocaine self-administration in the HiS rats, while it suppressed cocaine intake in the LoS rats over the entire long-access period (*see* Fig. 6 and Table 7). The effect on LoS rats was similar to the suppression of binge eating of a pure fat diet by baclofen in Sprague-Dawley rats [154].

In another recent study by Anker and coworkers [155], a similar protocol was used to examine the treatment effects of progesterone on the escalation of cocaine self-administration in HiS and LoS female rats. Females are particularly vulnerable to substance abuse when circulating estrogen levels are high, and progesterone attenuates these elevations [19, 155]. Furthermore, exogenous progesterone administration has been investigated as a potential pharmacological treatment for substance abuse disorders [156, 157]. When HiS and LoS female rats were treated with progesterone during periods of long access, similar to baclofen, progesterone attenuated cocaine intake in the LoS rats but had the opposite effect in HiS rats by increasing intake during the first half of this phase (*see* Fig. 6 and Table 7). Together, these results suggest that some pharmacological treatments may be more

Table 7
Differences in pharmacological and behavioral treatment effects between rats selectively bred for high (HiS) and low (LoS) saccharin intake or selected for high (HiI) and low (LoI) impulsivity

Behavioral model	Drug/ reinforcer	Treatment	Treatment effect	References
Maintenance FR and PR	Sucrose	Naltrexone	HiS = LoS	[158]
	Cocaine	Histamine	LoS > HiS	Holtz et al., unpublished observation
	Cocaine	Histamine	HiI = LoI	Holtz et al., unpublished observation
Escalation	Cocaine	Baclofen	LoS > HiS	[92]
	Cocaine	Progesterone	LoS > HiS	[138]
	Cocaine	Exercise	Adoles > adult	Zlebnik et al., unpublished observation
Reinstatement	Cocaine	Baclofen	HiS = LoS	[92]

effective in individuals with lower vulnerability than in those who are highly vulnerable to drug abuse. That these treatments exacerbated drug seeking in addiction-prone individuals is a serious concern for medication development, and these data highlight the importance of testing potential treatments in individuals differing in vulnerability to addiction. The recent animal results may explain some equivocal results found in clinical assessments of medications for stimulant addiction and may inform treatment approaches by ruling out specific agents for severe addicts that continue to abuse drugs during treatment.

3.2.3 Treatment for Overconsumption of Palatable Food

To determine the selectivity of the HiS vs. LoS phenotypes for drug addiction, it is important to compare treatment effects in these rats with excessive behavior rewarded by a nondrug substance. In another study, the relationship between drug addiction and food bingeing was investigated with HiS and LoS rats by Gosnell et al. [158]. In addition to divergent saccharin ingestion and drug-seeking behavior, the HiS and LoS rats also ingest differential amounts of natural sugars and sweeteners such as sucralose [159–161], suggesting a strong role of the endogenous opioid system in the HiS and LoS line differences. Furthermore, while HiS rats also show escalation of cocaine intake over extended periods, they also exhibit binge-like behaviors when given access to fat- and sugar-rich foods [2]. In this study of short access to sucrose pellets under progressive and fixed-ratio schedules of reinforcement, responding of HiS and LoS rats was compared with and without naltrexone treatment, an opioid receptor antagonist that decreases operant responding for food and ethanol [162]. The HiS rats earned

more sucrose pellets under all schedules of reinforcement; however, naltrexone attenuated responding comparably in HiS and LoS rats. While the HiS rats did not escalate their sucrose consumption, these results replicated previous findings showing that HiS rats were more susceptible to excessive consumption behaviors, and they support the efficacy of naltrexone as a treatment for addiction despite individual differences. The potential differential effects of naltrexone on operant responding for food in HiS and LoS rats may have been revealed under transitional phases like escalation and reinstatement that are more sensitive to individual differences and treatment interventions. Nonetheless, the results from these studies support the notion that phenotypic markers of addiction vulnerability, such as sweet preference, may predict treatment outcomes. These data indicate that it is not always those that have low vulnerability that are more responsive to treatment (e.g., LoS baclofen, progesterone), but it depends on the type of individual difference and phase of the addiction process. The sensitivity of the measures should also be considered (e.g., treatment during withdrawal to prevent reinstatement may be reflecting floor effects).

3.2.4 Genetic Determinants of Addiction: A Basis for Pharmacotherapy

Throughout the discussion of individual differences in response to food, drugs of abuse, and other rewards, there is a strong genetic determinant that needs to be considered in the development of treatment strategies. Phenotypic markers, such as sweet preference and impulsivity, mediated by variance in autosomal genomic regions are related to substance abuse behaviors, and pharmacological treatment outcomes for addiction may likewise be influenced by these genetic factors. This assumption drives the developing field of pharmacogenetics, which proposes that polymorphisms within an individual's genome may predict treatment outcome and can therefore guide treatment choices. The application of pharmacogenetics has been investigated for a number of psychiatric illnesses including schizophrenia [163], depression [164], attention-deficit/hyperactivity disorder [165], and substance abuse disorders [166]. Initial clinical evidence showed that the Asp40 allele in the gene encoding for the μ-opioid receptor (OPRM1) predicted the efficacy of naltrexone administration on abstinence and reduction in alcohol craving in alcoholics [167, 168], as well as the reduction of positive subjective response to and self-administration of alcohol in individuals not diagnosed with alcoholism [169]. These findings have also been supported by preclinical work in which a single nucleotide polymorphism in the OPRM1 gene was associated with the dose-dependent effects of naltrexone on the reduction of alcohol intake in rhesus monkeys [170]. Our understanding of how to predict addiction severity and customize treatments based on individual vulnerability should lead to better management of drug abuse treatments.

3.2.5 Novel Self-maintained Treatments for Incubation of Drug Craving

Initial work in our lab established that drug self-administration was increased by food restriction and reduced by food satiation in rats [187, 188] and monkeys [188]. This established the principle that nondrug rewards are effective self-administered treatments for drug addiction, and this method has been widely studied with self-administered rewards such as palatable substances in monkeys [189] and rats. We conducted similar studies with physical exercise as a self-maintained, rewarding treatment for several aspects of drug addiction, such as predicting drug abuse vulnerability [190], initiation and maintenance [191], and escalation [192] of cocaine self-administration and relapse [193] associated with previous cocaine self-administration. Recently, we found that self-maintained physical exercise blocked incubation of cocaine craving that accelerates over weeks to trigger drug seeking and relapse in cocaine-abstinent rats [194, 195]. Finally, consistent with these findings, Zlebnik et al. reported that chronic wheel running affected cocaine-induced c-Fos expression in brain reward areas in rats [196]. Overall, these findings indicated that physical exercise reduced drug seeking with results similar to our initial use of rewarding, palatable substances to reduce drug seeking [197]. Self-maintained physical exercise was also used in humans in residential treatment to reduce methamphetamine craving and addiction [198].

Recent studies in rats have used positive rewards to reduce incubation of drug craving, and similar to our finding with self-maintained physical exercise [194, 195], there is a wide range of self-administered and self-maintained nondrug rewarding events that block incubation of craving in rats. For example, recently, palatable food [199], social interaction [200], and operant self-administered social reward [201] have been shown to reduce incubation of stimulant and opioid craving in rats. Results of these recent novel treatments suggest that key features of their success are the rewarding aspect (e.g., palatable substances, physical exercise, social access to conspecifics), as well as self-initiation, self-maintenance, and self-sustainability for long durations that allow time for incubated craving to diminish. Self-maintained treatments shift responsibility for maintaining the long-term treatment from the provider to the affected individual, and involvement in the inherent rewards of self-recovery likely adds to the individual's positive experience. It would be useful to examine this approach on other addiction-like behaviors such as binge eating palatable foods and food addiction in humans.

4 Conclusion

In this review, we have discussed findings from our studies on selectively bred, high- and low-saccharin-consuming rats (HiS and LoS), and innate sweet preference is a major predictor of drug addiction. In addition, other predictors of addiction, such as sex,

age, and impulsivity, have similar and additive effects with sweet preference on drug and food addiction. Together, these factors describe an addictive profile that could be useful for predicting problems with drug addiction in the human population. Studies reviewed in this chapter suggest that vulnerability to addiction is not only determined by both the positive and negative effects of drugs, but these factors may also be predictive of the outcome of treatment attempts. It will also be important to take into account the close relationship of reactivity to food rewards and impulsivity in future work on treatment for drug abuse, as impulsivity is a major factor in addiction. Several other factors such as motivation, impulsivity, and sweet preference are closely related to characteristics of drug addiction (e.g., *see* Fig. 1). Recent data, based on animal research and clinical examples, also indicate that key features of successful treatments for drug addiction are that they are self-initiated, self-maintained, and self-sustained and occur over long durations that outlast incubation of craving (weeks to months) after substance use has ended. It would be useful to examine this novel treatment approach for other excessive behaviors, such as binge eating in humans.

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Chapter 13

Impact of a History of Caloric Restriction and a Frustration Stress Manipulation on Binge-Like Eating Behavior in Female Rats: Preclinical Results

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Abstract

Medications, at present, play a limited role on eating disorders. Currently, only two drugs were approved by the Food and Drug Administration, fluoxetine and lisdexamfetamine dimesylate, respectively, for bulimia nervosa and binge eating disorder. Meanwhile, eating disorders are a growing public health problem, and the pharmacological management is extremely important, in support of the recommended cognitive behavioral therapy. In this context, animal models are essential to facilitate the study of human conditions and validate potential therapies. In this chapter, we will describe a preclinical model of binge-like eating, triggered by yo-yo dieting and frustration stress on palatable food. It represents a tool to investigate the underlying behavior, physiological mechanisms and the involvement of neural circuitry on binge-like eating behavior with the final aim to develop pharmacological approaches. Corticotropin-releasing factor 1 receptor antagonists, orexin receptor type 1 antagonists, and A_{2A} adenosine receptor agonists will be discussed as promising therapeutic treatments that may have clinical implications.

Key words Binge eating, Stress, Food restriction, Palatable food, Female rats, Corticotropin-releasing factor 1 receptor antagonists, Orexin receptor type 1 antagonists, A_{2A} adenosine receptor agonists

1 Introduction

Eating disorders are a growing public health problem, characterized by severe disturbances in eating behavior and consequently in body weight, that significantly impact the physical health and psychosocial functions [1]. They are highly prevalent in adolescents and specially in young women, and their rates are increasing worldwide [2, 3]. Moreover, they are associated to serious psychiatric complications, such as depression or anxiety, that precipitate the quality of life [3–5] and increase mortality [6, 7] especially in individuals that suffer from anorexia nervosa (AN) [8]. The urge to compulsively overeat certain food in a discrete period of time, leading to an episode of binge eating, is a key symptom in major

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types of eating disorders, including bulimia nervosa (BN), binge/purging subtype of AN, and binge eating disorder (BED). Differently from the other two eating disorders, individuals with BED do not engage in regular compensatory behaviors after binging, such as induced vomiting, laxative misuse, prolonged fasting, or excessive exercise for controlling weight gain [1]. Therefore, it is often associated with overweight or obesity [9, 10], and it increases the risk to develop co-occurring pathology such as diabetes or metabolic syndrome [11]. It is estimated that 2–5% of the general adults suffer from BED [12], representing the most prevalent eating disorder. Although there is high prevalence of eating disorders, with their psychiatric or medical comorbidities, they continue to be underrecognized and undertreated [13, 14]. Thus, the pharmacological management of eating disorders is extremely important, in support of the recommended cognitive behavioral therapy (CBT), as well as of the intrapersonal therapy (IPT) [15–17]. Major advance was the first Food and Drug Administration (FDA)-approved medication to treat moderate to severe BED in adults in 2015: lisdexamfetamine dimesylate (LDX, Vyvanse®), a prodrug of d-amphetamine [18]. However, the adverse reactions include insomnia, dry mouth, diarrhea, nausea, anxiety, anorexia, agitation, increased blood pressure, hyperhidrosis, restlessness, and decreased appetite [18–20]. Despite the effect on food intake, it is not approved for weight loss. To avoid serious side effects, patients with hypertension, coronary artery disease, diabetes, psychiatric disorders or substance abuse were excluded from the treatment. Moreover, the Drug Enforcement Administration categorized LDX as a schedule II medication; therefore, the risk of abuse and dependence needs to be monitored. Currently, there are no FDA-approved medications for AN; thus, the drug treatments are extremely limited. Only olanzapine and other second-generation antipsychotics may be efficacious [21–23]. Since 1994, only fluoxetine was approved for BN [24–26]. To date, the most effective treatments for BN are CBT and 60 mg fluoxetine, even if the remission rates continue to be low [23, 27, 28]. Even though alternative medications are using "off-label" to treat eating disorders, this overview highlights the need to investigate and to find novel pharmacotherapeutic options.

The aims of this chapter are (I) to describe environmental conditions that facilitate the episode of binge eating; (II) to provide a summary of current therapeutic approaches under study, using a preclinical model of binge-like eating behavior, induced by yo-yo dieting and frustration stress; and (III) to discuss and extend these approaches with other animal models to better understand eating disorders, characterized by excessive intake of palatable food in a short time.

2 Materials

2.1 Binge Eating Episode in Rodents: Role of Hedonic Food, Stress, and Caloric Restrictions

Animal models should be consistent with the known clinical features of eating disorders and reflect the changes in dietary patterns and food availability in many developing countries [29] to facilitate the discovery and the validation of new therapeutic targets. Thus, several environmental conditions, such as repeated cycles of food restriction and refeeding, acute or chronic stress, food deprivation, and limited access to palatable food, are used in different models to lead binge-like eating episodes in rodents [30].

2.1.1 Hedonic Food

Commonly, binge eating protocols in rodents need tasty food with low nutritional values in order to induce overconsumption, for example, sugar solutions [31], chocolate [32], cookies [33], or chocolate-high sucrose pellets [34]. In fact, binging does not occur on standard chow [35], but on energy-dense food, under intermittent access of food [36, 37] or after caloric restriction and stress [33, 38], inducing changes in the central reward pathway, especially in dopamine (DA) release [39, 40]. Similarly, in women, it was reported that types of food selected during the binge episode include hedonic foods: ice cream, cookies, potato chips, etc. [41–43]. Consumption of palatable food is a rewarding experience that increases the neuronal activity marker c-Fos compared to chow intake, within the mesocorticolimbic reward circuit [44, 45], whereas striatal DA D2 receptors are downregulated in obese rats [46]. The decrease of DA D2 receptors levels induces a severe reward hyposensitivity that consequently promotes the development and maintenance of compulsive-like eating [47–49], to mitigate the persistent state of reduced reward, contributing to overeating and obesity. The same brain reward deficit is caused by drug abuse, and it is considered a primary trigger in the progression from normal to compulsive intake of drugs or food, supporting the "food addiction" hypothesis, in humans [50–53] and animals [54–57]. Further to the hedonic value and addictive potential, eaten palatable food may become a "comfort food," sustaining binge eating, even without hunger, in order to alleviate anxiety and stress responses [58]. Thus, individuals may lose control on food intake to struggle their negative affective conditions, reducing the hypothalamic-pituitary-adrenal (HPA) axis activity, with the downregulation of corticotropin-releasing factor (CRF) [58–60].

Likely drug abuse, especially during dietary withdrawal, this relief from negative states could drive to overconsumption of energy-dense food [60, 61] and promote the "dark side" of food addiction [62].

2.1.2 Stress

Stress is strongly involved in the etiology of eating disorders, and it represents also a risk factor for maintaining and exacerbating the maladaptive feeding behavior in both animals and humans [58, 63]. Stress affects not only the course of eating disorders but also the outcome and relapse of the therapies [64–66]. There are various kinds of stress experiences that change in duration and intensity. For example, severe acute stress, activating the HPA axis, suppresses appetite [63] while mild to moderate stress increases the ingestion of palatable food [67, 68], and it is strongly correlated to binge eating [69]. Indeed, repeated stressors overstimulate the HPA axis, which is the hallmark of the stress response, and contribute to pathological states, including eating disorders and obesity [70]. In fact, obese individuals with BED show hyperactive HPA axis: their cortisol levels are higher compared to obese individuals without BED [71, 72]. In addition, the severity of binge eating in obese women with BED is linked to the increased nocturnal levels of salivary cortisol [73] and to the higher blood cortisol levels in response to a novel laboratory stressor [74]. These results suggest a dysregulation on HPA axis during the binge episode, and then the cortisol reactivity may be a useful marker in order to evaluate the vulnerability on stress-induced eating [74]. In this context, palatable food intake is recruited to blunt activation of the HPA axis [75, 76], persisting also beyond the exposure of hedonic food [75]. As mentioned before, the eaten food may represent a kind of self-medication in conditions of stress, in accordance with the "comfort food" hypothesis. In fact, in humans, chronic stress increases the preference for high carbohydrate and high saturated fat foods [77], and in animals, different stress manipulations are used to motivate the consumption of palatable food [78].

2.1.3 Caloric Restriction

Dieting refers to intentional efforts to achieve or maintain a desired weight through the reduction of caloric intake [79]. The availability of palatable food (limited access or short exposure with periods of restriction) represents a risk factor to develop binge eating in nonclinical populations [80, 81], and chronic caloric restriction is a strong predictor of overeating in stress condition [81, 82]. In fact, a synergistic relationship between stress and caloric restriction is reported, aggravating the qualitative and the quantitative aspects of aberrant eating pattern [33, 38, 62, 63]. Studies revealed that dieting periods are really common during the life of binge eaters, although hunger per se appears not to be enough to induce binge eating in the absence of stress and negative affective states [83, 84]. Other works indicated that caloric restriction was able to reprogram stress and orexigenic pathways [85, 86], and during the deprivation, the extracellular DA levels in the nucleus accumbens (NAc) were drastically reduced [87]. On the other hand, the following refeeding may restore this state, producing a reward

stimulation by DA releasing [88, 89]. Then, Avena and collaborators showed, using the binge eating "sugar model" [90], that binging is more reinforcing in food restricted rats, caused by an increase in DA and a decrease in acetylcholine (ACh) release [91]. ACh may be a blunt satiation factor, considering that ACh levels in the NAc normally enhance during a meal [92], while diminish when feeding stops [93]. These neurochemical changes resemble the effects of substance abuse. In fact, the persistent release of DA and altered gene expression of DA receptors and opioid peptides in the NAc, detected in binge sugar rats [94], are also observed in response to morphine [95]. This model strongly provides, in several behavioral studies, sugar binging and "food addiction" [57], describing each component of addiction: prolonged binge eating, craving and then withdrawal, and finally sugar/drugs cross sensitization [31, 37, 39, 49]. In other paradigms of binge eating, rats are subjected to modifications in the pattern of distribution of the meals, via chronic food restrictions [33, 38, 96, 97] or limited-access periods to palatable food [78, 98–100] to elicit binge eating. All protocols successfully promote the episode of binge eating in rodents and highlight important commonalities, for example, the availability of hedonic food or caloric restriction.

2.2 History of Caloric Restriction and Stress-Induced Binge Eating Episode

In our laboratory, we developed and characterized an animal model of binge eating to investigate the underlying behavior, physiological mechanisms and the involvement of neural circuitry on binge-like eating behavior [38]. We studied female rats in according with the high prevalence of eating disorders among young adolescents and young adult women [3]. In our model, we consider a "binge eating episode" the significantly higher palatable food consumption in 2 h in the repeated restriction plus frustration stress group than in the other three experimental groups: rats with no food restriction and no stress, only stressed rats, or only restricted rats. The palatable food (3.63 kcal/g) is a paste prepared by mixing Nutella (Ferrero) chocolate cream, ground chow food pellets, and water in the following percent ratio: 52% Nutella, 33% chow pellets, and 15% water. We followed the same feeding schedule of Hagan model [33]: three cycles of chow food restricting/refeeding protocol, totally 25 days (*see* Fig. 1). The rats lost weight during the four restriction days (66% of their chow intake) but completely recovered their body weight after 4 days of ad libitum chow access, simulating the human "yo-yo" dieting [32, 101] (*see* Fig. 2A). Hagan study suggested that at least three cycles of restriction plus stress were sufficient to elicit overconsumption of palatable food, and the body weight changes during restriction period could be considered a physiological stress response that promotes the neurobiological adaptations to elicit binge eating [33]. On day 25, the test day, after the third and last cycle of chow restricting/refeeding

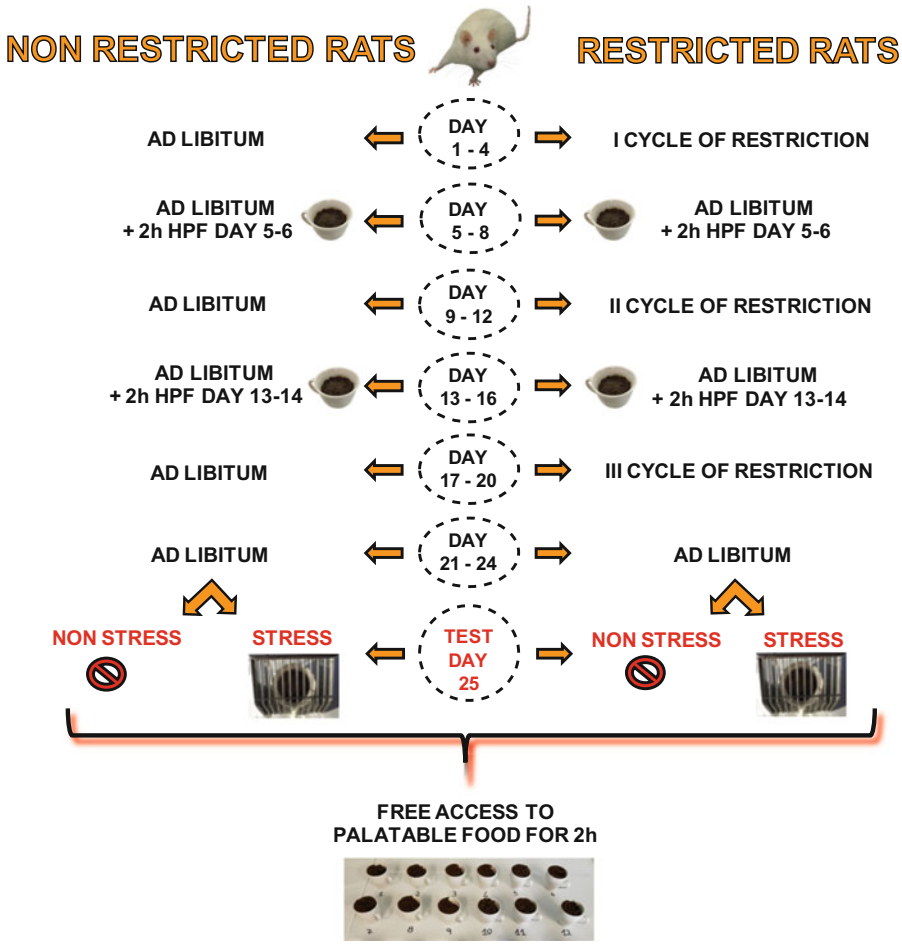


Fig. 1 Timeline of the experimental procedures for the food non restricted (left) and food restricted (right) rats

protocol, rats were stressed. The rats exposed to stress were acclimated in a different room from the other groups without stress. Differently from Hagan model, foot shock was replaced by "frustrative non-reward" or "frustration stress" manipulation [102]. This procedure consists of 15 min exposure to the odor and sight of a familiar chocolate paste, without access to it, just before offering the palatable food for 2 h on day 25. To avoid neophobia, all rats have four free access on days 5–6 and 13–14 for 2 h (*see* Fig. 1). After the frustration stress, food intake was measured, and only the combination of restriction plus stress significantly increased the palatable food intake (binge episode) compared to the other three groups (*see* Fig. 2B). As in drug dependence, the attractive and rewarding environmental cues, like the sight or smell of palatable food, have been associated with binge eating [84, 103]. Indeed, the frustration stress may activate the negative-valence system, especially the HPA axis system and

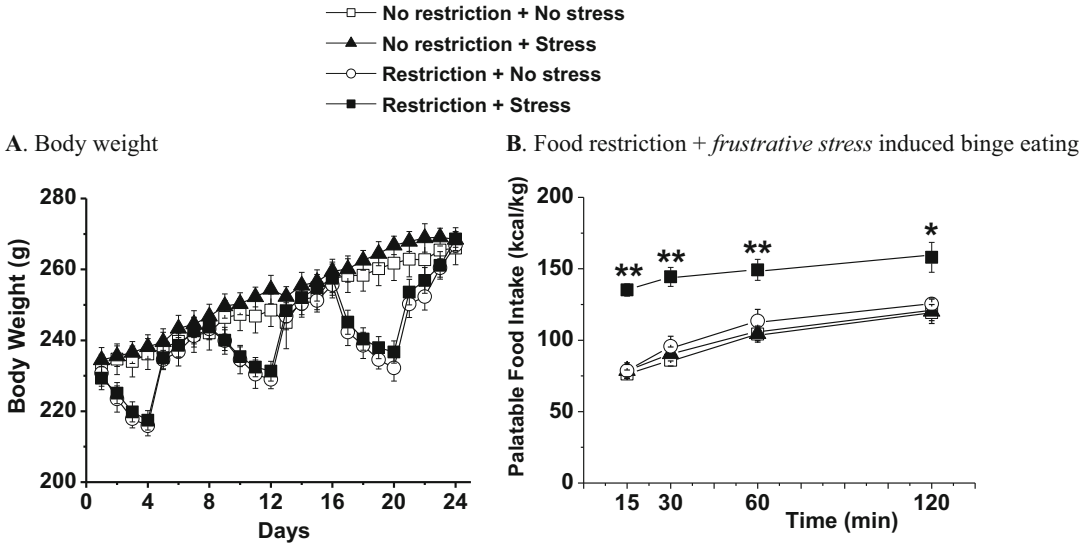


Fig. 2 (**A**) Mean ± SEM. Body weight (g) of female rats exposed or not exposed to repeated intermittent cycles of food restriction/refeeding. (**B**) Mean ± SEM. Palatable food intake (kcal/kg) at 2 h time points during the test day. *$p < 0.05$, **$p < 0.01$ different from the other three groups. (Adapted with permission from [113])

corticolimbic neural circuits, producing negative mood which contributes to binge eating behavior [104]. We found a robust behavioral activation in stressed rats [105], observing repeated movements of the forepaws, head, and trunk of the rats in order to reach the palatable food, closed inside a metallic grid [32], and increased plasma corticosterone levels [38, 106]. Although the frustration stress or foot shock in Hagan model [33] are fundamental triggers for binge eating episodes, rats need to be also food restricted to show binge eating behavior. Moreover, calorie-dense food availability per se did not induce binge eating; however, it becomes a main factor in food restricted and stressed rats, underlying the importance of the diet schedule of access conditions of food. On day 25, the test day, the previously restricted rats were satiated; in fact, restricted rats did not increase their chow intake.

We tested in our model three effective drugs on reducing binge episode in humans [38]: topiramate, sibutramine, and fluoxetine [24, 107, 108], providing evidence for its predictive validity. In fact, all drugs potently inhibited palatable food intake in our model [38]. Further element of face validity is the influence of ovarian hormones on binge-like eating behavior in our model [105, 109], like in women [110, 111]. The episode of binge eating does not occur during the estrus phase of the ovarian cycle in intact female rats or during the treatment with estradiol near-physiological regimen in ovariectomized rats. All rats tested during the estrus cycle phase ate about 20% less than rats tested during diestrus or proestrus, which corresponds to the usual change in food intake in intact

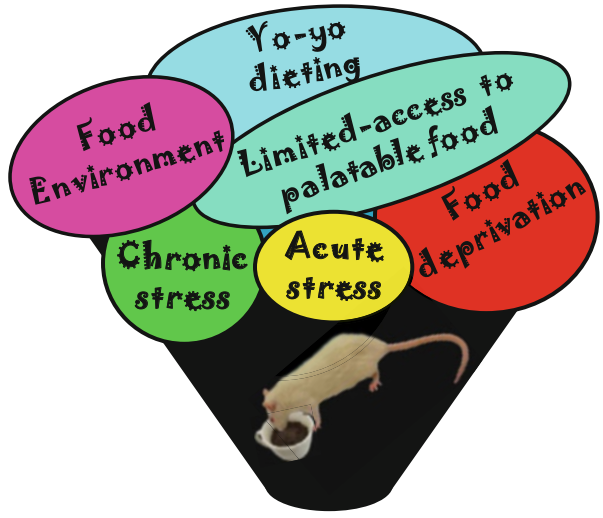
rats [112], while the reduction in binge-like eating in the estrus state was significantly larger than this typical effect. This reduction suggests a distinct phenomenon, not related to the classical ovarian rhythm that affects eating. Indeed, the estradiol treatment was sufficient to suppress binge-like eating in ovariectomized rats, like in Klump study, regarding the inhibitory effect of estrogens and progestins on binge frequency in women [111]. In the same ovariectomized rats [105], the number of cells expressing phosphorylated extracellular signal-regulated kinases (pERK) suggests that the central nucleus of the amygdala (CeA), paraventricular nucleus of hypothalamus (PVN), and dorsal and ventral bed nuclei of the stria terminalis (BNST) contributed to the neural mechanism underlying binge-like eating. The BNST pERK results showed that the neural activation of the BNST in ovariectomized rats subjected to food restriction and frustration stress was negatively regulated by estrogens, extending the involvement of BNST CRF in the binge-like eating studies [113].

2.3 Overview of the Preclinical Results in Selected Neurotransmitter Systems

We studied in our animal model [38] selected neurotransmitter systems with the final aim to identify novel pharmacological strategies to treat binge-like eating behavior (summarized in *see* Fig. 3).

2.3.1 Effect of the CRF1 Receptor Antagonist R121919 on Stress-Induced Binge Eating

As stated above, stress plays a determinant role in the etiology of binge eating in patients suffering from BED or BN. The CRF system activation, precisely the CRF1 receptor, is responsible for starting the neuroendocrine response to stress via the HPA axis and modulates many emotional reactions via extra HPA sites (i.e., CeA, basolateral amygdala (BLA), BNST) [114, 115]. Therefore, we determined the role of CRF1 receptor and HPA axis system, considering the increased plasma levels of corticosterone in our stressed rats [38] and the potential effect of CRF1 receptor antagonist in these conditions. In fact, in previous works, CRF1 receptor antagonist reduced stress-induced palatable food seeking [116] and withdrawal symptoms under intermittent access to palatable food in rats [117]. We found that systemic injections of CRF1 receptor antagonist (R121919; *see* Fig. 4A) and BNST injections of the CRF receptor antagonist (D-Phe-$CRF_{(12\text{-}41)}$) selectively decreased the palatable food consumption in the binge eating group [113], without a general inhibition of food intake, observed for serotonergic drugs [38]. Additionally, food restrictions plus frustration stress induced a selective increase in Fos immunoreactivity in ventral and dorsal BNST [113]. To further investigate the critical role of BNST CRF system on binge eating, maybe not only related to HPA activation, we focused on extrahypothalamic mechanisms. We recently reported that metyrapone, a corticosterone synthesis



Binge-like eating behavior

Potential pharmacological targets:

CRF-1 receptor antagonist	N/OFQ system	A_{2A}AR agonist
Micioni Di Bonaventura et. al 2014 Micioni Di Bonaventura et. al 2017	Micioni Di Bonaventura et al. 2013 Pucci et al. 2016	Micioni Di Bonaventura et al. 2012 Micioni Di Bonaventura et al. 2019
Cottone et al. 2009 Iemolo et al. 2013 Parylak et al. 2012	Hardaway et al. 2016 Statnick et al. 2016	
OX-1 receptor antagonist	**Sigma-1 receptors**	**Endocannabinoid system**
Piccoli et al.2012	Del Bello et al. 2019	Pucci et al.2019
Alcaraz-Iborra et al. 2014 Rodríguez-Ortega et al. 2019 Vickers et al. 2015	Cottone et al. 2012	Satta et al.2018 Scherma et al. 2013

Works using preclinical model Cifani et al. 2009: light blue. Other animal models: white. Abbreviations: CRF: cortocotrophin-releasing factor; Nociceptin/Orphanin (N/OFQ) FQ peptide; A_{2A}AR: A_{2A} adenosine receptor; OXR-1: orexin receptors type 1.

Fig. 3 Potential novel pharmacological targets studied on binge-like eating behavior in female rats, induced by restriction plus frustration stress

inhibitor, did not reduce palatable food intake in any experimental groups (*see* Fig. 4B), and the replacement of frustration stress with corticosterone injection failed to elicit binge eating (*see* Fig. 4C) [118]. Moreover, we found a significant upregulation of crhr1 mRNA levels in the dorsal portion of the BNST and in the CeA only in binge eating rats, whereas no changes were detected in the BLA and in the PVN in any groups of rats [118]. Then, considering these in situ hybridization data, we decided to administer, into the

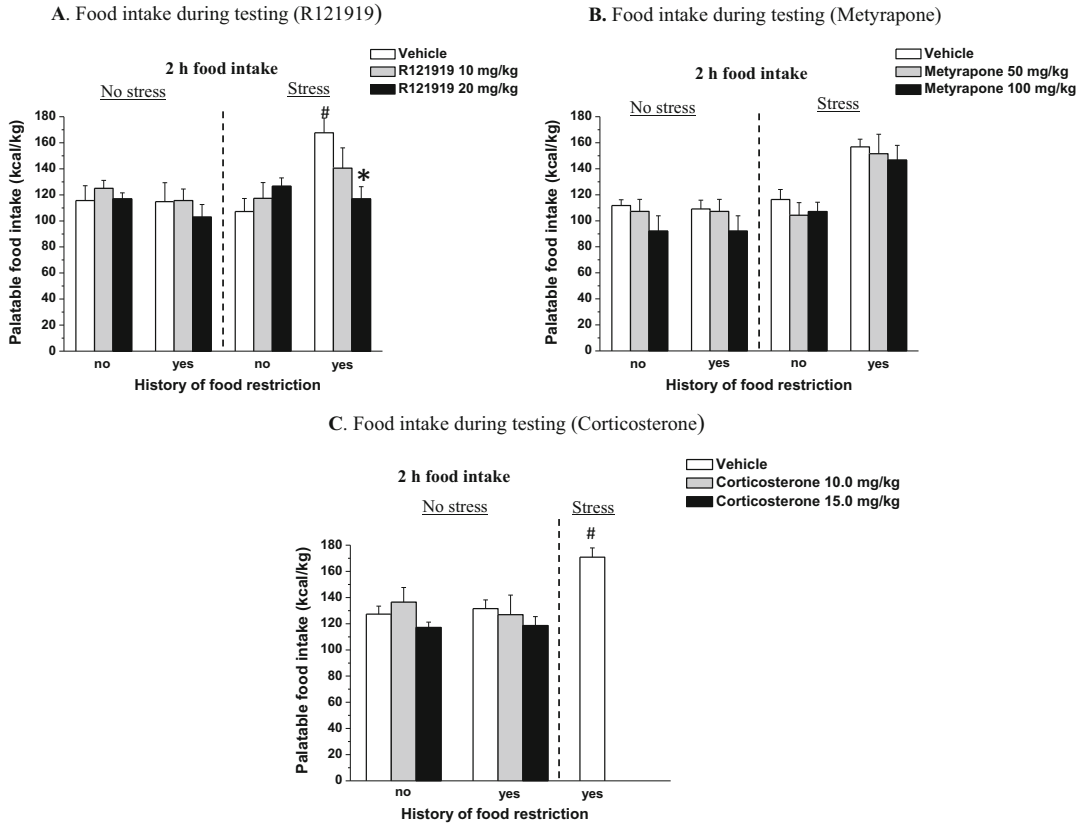


Fig. 4 Mean ± SEM. Effect of systemic injections of (**A**) CRF1 receptor antagonist R121919, (**B**) metyrapone, and (**C**) corticosterone on binge-like eating behavior in female rats. $^{*}p < 0.05$, different from the vehicle condition. $^{\#}p < 0.05$, different from the other vehicle groups. (Adapted with permission from [118])

CeA, a nonselective CRF receptor antagonist D-Phe-$CRF_{(12\text{-}41)}$ that completely blocked the episode of binge eating [118]. Together, these findings suggest an independent role of extrahypothalamic CRF system on binge eating in female rats, from the known role of CRF within the HPA axis, extending other works on the effect of CRF1 receptor antagonists in drug abuse [116, 119–122].

2.3.2 Nociceptin/Orphanin (N/OFQ) System: Functional Antagonists of CRF

The activation of nociceptin/orphanin (N/OFQ) FQ peptide receptor (NOP) increases food intake in rats (*see* recent review [123]). Since this discovery, many NOP agonists and antagonists were developed and tested in rodents [124–126]. Interestingly from these studies, N/OFQ system revealed to act as a functional antagonist of CRF, with an anxiogenic-like effect [127–129], and NOP antagonists showed antidepressant like effects in rodents [130, 131]. Thus, we decided to investigate this system on disorders associated with hyperphagia. In our binge model, we found that N/OFQ reduced palatable food intake but at a low and limited

dose (intracerebroventricular injections, 0.5 nmol/rat), taking into account that N/OFQ induces hyperphagia at 1.21 nmol/rat [132]. However, at the dose of 1 nmol, N/OFQ already increased food intake in restricted rats, enhancing the animals' sensitivity to its hyperphagic effect for palatable food [86]. Moreover, the food restriction seems to downregulate mRNA levels of N/OFQ and its receptor NOP in the hypothalamus, and these alterations might be due to selective histone modification changes [133]. N/OFQ may stimulate feeding via inhibition of anorexigenic pathways, resulting in the disinhibition of orexigenic mechanisms [134], while epigenetic modifications of N/OFQ and CRF systems in binge rats [133] may contribute to binge eating behavior, as shown in drug abuse [135–137]. Consistent with these findings, the blockade of NOP with selective N/OFQ antagonist LY2940094 (per os) attenuated food intake and consequently weight gain in male mice and rats, in various behavioral models [134]. In the same year, another selective NOP antagonist SB-612111 was able to inhibit the binge eating behavior in mice during 1 h sessions of high-fat (60%) palatable food [138].

2.4 Antagonism of the Orexin-1 Receptor Inhibits Reward-Based Feeding Behavior

Experimental evidence suggests a role for the orexin system on impulsivity and binge-like consumption, regulating not only metabolic signals [139, 140], but also mesolimbic reward circuitry [141–143] and external environmental cues [142, 144]. In line with these findings, several "hedonic hot spots" in the brain that amplify the "liking" for sweetness were linked to orexins: in ventral pallidum [145], NAc [146], and insula [147]. The orexin neuropeptides (orexin-A and orexin-B, also known as hypocretin-1 and hypocretin-2, respectively) are produced by a small subpopulation of hypothalamic neurons. They were identified in 1998 as ligands of two orphan G-protein-coupled receptors, named orexin receptors type 1 (OXR-1) and type 2 (OXR-2) [139, 140]. Orexin-A has high affinity for both orexin receptors while orexin-B shows a higher affinity to OXR-2 than to OXR-1 [140]. OXR-1 seems more involved in modulating motivation for palatable foods [148] while OXR-2 on sleep–wakefulness cycle and arousal [149]. OXR-1, expressed in the areas important to energy homeostasis, but also localized in appetite-regulating neurons, could contribute to the development of overweight, such as those producing neuropeptide Y (NPY)/agouti-related peptide (AgRP), proopiomelanocortin (POMC)/cocaine-amphetamine-regulated transcript (CART), melanin-concentrating hormone (MCH) [150, 151], and orexin itself in the hypothalamus [152]. After the observation that central injection of orexin-A and orexin-B in freely fed rats enhanced feeding behavior [140], especially on high-fat food consumption [153, 154], numerous studies demonstrated a role for orexin system in feeding behavior. Interestingly, orexin neurons in the lateral hypothalamus mediate reward [155, 156],

and these neurons are activated by food or drug reward-associated cues, and their activation reinstates drug seeking in rats [144, 157, 158]. OXR-1 antagonists inhibited the hyperphagic effect of orexin [153, 159] and inhibited also high-fat food and sucrose self-administration [160–162]. Moreover, orexin-A affected food seeking motivated in response to fasting [163] or Pavlovian cues [148]. Indeed, the activation of orexin-containing neurons was also found in response to the injection of neuropeptide S (NPS) [164]. This transmitter modulates arousal, anxiety and food intake [165–168], and it could regulate motivation for palatable food, since its receptors are expressed in hypothalamic and extrahypothalamic regions [169]. Recent studies are more focused on the role of orexins in compulsive, binge-type feeding behaviors in rodents (*see* recent review [170]). In our binge eating model, the OXR antagonists SB-649868 (dual OX1/OX2 receptor antagonist) and GSK1059865 (a selective OXR-1 antagonist) selectively blocked the occurrence of binge eating episodes without affecting normal food intake in rats [171]. Instead, JNJ-10397049, a selective OXR-2 antagonist, failed to affect binge eating of palatable food. The selective antagonism at the OXR-1 could represent an important target for BED and other eating disorders with a compulsive component. Consistently to our results, then OXR-1 antagonists reduced binge-like eating behavior in different models, in both rats [162, 173] and mice [174].

Specifically, in our model, the binge episode is induced by two conditions closely linked with orexin system: chronic restriction and stress induced by food cues. Caloric restrictions induced dysregulation of orexigenic pathways, such as MCH and orexin [85]. Previously restricted mice showed an increase in binge eating episodes, and only in these mice was found a significant elevated expression of these two hormones that interact with the mesolimbic DA system, promoting the consumption of palatable food [160, 175]. SB-334867 (OXR-1 antagonist) decreased lever responding for sucrose during self-administration and dose dependently reduced the number of sucrose pellets in food restricted rats. In the same way, SB-334867 blocked cue-induced reinstatement to sucrose seeking in food restricted rats but not in ad libitum rats [161]. Furthermore, orexin system may regulate adiposity signals and energy expenditure under restriction [157, 176]. Indeed, the ability of transcription factor ΔFosB to increase the effortful operant responding for high fat pellets, under restriction, requires the presence of orexin [177]. Thus, it is possible to speculate that during the food restriction, there is an enhancement of orexin transmission, which in turn increases the motivation for palatable food, driving to binge-like consumption. In our model, the orexin transmission is further stimulated by the odor and sight of a familiar chocolate paste for 15 min (frustration stress), inducing binge eating episode only in previously restricted female rats. In this

context, OXR-1 antagonist effectively worked to block the binge episode and might be a good target for the control of compulsive eating. Recently, it was suggested that environmental enrichment on binge eating might impact the orexin system [178]. In fact, the enrichment was able to blunt the inhibitory effect of SB-334867 on sucrose binge consumption.

2.5 Role of A_{2A} Adenosine Receptor in Binge Eating Behaviors

Although the adenosine system has been extensively investigated for the development of antiparkinsonian agents, based on the antagonism between specific subtypes of adenosine and DA receptors in the striatum [179, 180], limited studies are focused on binge eating behavior. However, A_{2A} adenosine receptor (A_{2A}AR) was involved in drug addiction [181], and many works, using different preclinical models, showed the role of A_{2A}AR on feeding, on goal-directed behavior, and on effort-related behaviors [182–187]. Moreover, it has been described the functional interaction between the DA D2 receptor and A_{2A}AR [188]: a blockade of A_{2A}AR could mimic the action of DA D2 receptor agonists [189]. Adenosine acts through A_1, A_{2A}, A_{2B}, and A_3 receptors, among which A_{2A}AR shows a very high distribution in the striatum, as well as in the cortex, amygdala, olfactory tubercle, hippocampus, hypothalamus, thalamus, and cerebellum [190, 191]. We investigated the role of A_{2A}AR agonist and antagonist, already tested on alcohol intake [192], on binge eating behavior. We found that A_{2A}AR agonists significantly inhibited palatable food intake in female rats [193], whereas the A_{2A}AR selective antagonist completely reverted this effect, confirming that its actions are mediated by A_{2A}ARs [194].

Gene expression studies revealed a selective increase of DA and A_{2A}A receptors' mRNAs in the amygdaloid complex of binge eating rats compared to control group (non stressed and non restricted rats) [194]. Consistently, pyrosequencing analysis revealed a significant reduction of the percentage of DNA methylation but only at the A_{2A}AR promoter region in rats showing binge-like behavior compared to the control rats. Indeed, the A_{2A}AR agonist (VT 7) administration induced a significant increase of A_{2A}AR mRNA levels in binge eating rats. On the contrary, we observed a significant decrease in A_{2A}AR mRNA levels in rats treated with the A_{2A}AR antagonist (ANR 94). In line with these findings, changes in the DNA methylation status of the A_{2A}AR promoter were detected in binge eating rats, after administration of VT 7 or ANR 94 [194]. We highlight the importance of epigenetic regulation of the A_{2A}AR gene, possibly due to a compensatory mechanism to counteract the effect of binge eating. We suggest that A_{2A}AR activation, inducing receptor gene upregulation, could be relevant to reduce food consumption. Thus, these results, together with the others mentioned before on effort-related food choice behavior, strongly supported the adenosine system as the inhibitory mediator of food intake.

3 Conclusions and Future Directions for Research

Eating disorders are characterized by complex underlying mechanisms, which emphasize the importance of predictive and translational animal models in understanding these disorders and promoting the development of innovative drugs. Using a preclinical model of binge-like eating behavior, triggered by yo-yo dieting and frustration stress on palatable food [38], we suggested three drug candidates to treat binge episode: CRF1 receptor antagonist, OXR-1 antagonist, and A_{2A}AR agonist. They could have potential clinical implications considering the promising results that the CRF1 receptor antagonist pexacerfont showed on the anti-craving properties in a clinical study [195]. Unfortunately, this study was precociously stopped for administrative interpretation of US federal law. Further, the last December was announced the initiation of a Phase I study of AZD4041, which is OXR-1 antagonist, for treating tobacco use and dependence (source Eolas Therapeutics).

In the future, it would be important to continue exploring the mutual relationship among CRF stress system, DA, orexin, and also oxytocin on emotional processing, that have been found to trigger binge eating episodes [196–200]. Additionally, we will extend our positive results obtained with a potent sigma-1 receptor antagonist on binge eating [201] and with endocannabinoid system [202–206]. We recently reported a selective downregulation of the hypothalamic fatty acid amide hydrolase gene [202] in the hypothalamus of binge eating groups. This enzyme is responsible for the anandamide degradation [207], and its specific modulation could be a potential biomarker for binge eating episodes.

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Chapter 14

Anorexia and Undereating

Guido K. W. Frank

Abstract

Anorexia nervosa is a severe psychiatric disorder characterized by food restriction and high mortality rate. Research has identified consistently changes in brain monoamine neurotransmitter systems, some of which persist after recovery. There is also a host of neuroendocrine alterations during the course of illness, and it has been hypothesized that state-related changes in stress, gut, and sex hormone expression may contribute to the pathophysiology of anorexia nervosa. Recent human brain imaging research on the reward circuitry has helped us to better understand this illness. Those studies provide empiric evidence to develop models that center around the role of dopamine during development and maintenance of anorexia nervosa and integrate the neuroendocrine system and its interaction with reward processing. Those new models together with advanced basic science research provide hope that we will find treatments that can target directly the disease mechanism of anorexia nervosa and treat the disorder more effectively.

Key words Anorexia nervosa, Dopamine, Brain, Circuitry, Reward, Endocrine, Treatment

1 Introduction

The Diagnostic and Statistical Manual of Mental Disorders fifth Edition recognizes eating disorders that are associated with undereating, most notably anorexia nervosa (AN) and avoidant restrictive food intake disorder (ARFID) [1]. AN is the third most common chronic illness among adolescent females. AN has the highest mortality rate among the psychiatric disorders, and most deaths occur between 16 and 29 years of age [2]. AN is associated with severe emaciation from self-driven food refusal and a perception of being overweight despite severe underweight [1]. Etiologically, it is thought that a complex interplay between neurobiological, psychological, and environmental factors contributes to developing AN [3]. ARFID is characterized by an apparent lack of interest in eating or food, an avoidance based on the sensory characteristics of food or concern about aversive consequences of eating that lead to weight loss or need for nutritional supplementation. ARFID is a highly heterogeneous category, and research on the underlying neurobiology is largely lacking. Therefore, this chapter will focus

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on AN, and the discussion of ARFID will be deferred to a later point.

The neurotransmitters serotonin and dopamine have long been associated with psychiatric disorders that include mood, anxiety, eating, reward, and reinforcement regulation, among other processes. Earlier studies in AN that collected cerebrospinal fluid samples showed that levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) as well as homovanillic acid (HVA), the major DA metabolite, were low when underweight [4]. Positron emission tomography imaging to visualize neurotransmitter receptor in the living brain showed higher serotonin 1A receptor binding in AN when ill, and after recovery, normal serotonin 2A receptors in ill AN, but lower serotonin 2A receptors compared to controls when recovered, and receptor binding was frequently associated with anxiety [5]. It is likely that abnormalities in expression of neurotransmitters and or associated receptors are in part trait alterations that could contribute to ED development but also adapt to the effects of ED behaviors and hinder recovery, although the underlying mechanims remain largely unclear [6]. Other chemicals that affect brain function are hormones, produced by endocrine glands, and neuroactive peptides, which are proteins produced and released by neurons or in the periphery such as fat cells [7, 8]. Those include the typical sex hormones such as estradiol and testosterone and the gut hormones such as ghrelin, peptide YY, cholecystokinin, leptin, and insulin [9]. Research on those substances in AN showed inconsistent results for many of those chemicals, but it appears that those substances that regulate body homeostasis are altered during the ill state of EDs and in turn alter the body's energy balance [9]. Recent studies have implicated the brain reward system in ED pathophysiology, providing an important link and possible mechanism on how hormones and peptides may affect ED-specific behavior. Specifically, it has been increasingly recognized how neuroendocrine and neuropeptide alterations may alter brain dopamine function, which could alter food approach in AN [10]. For instance, low levels of the fat cell-derived hormone leptin or high ghrelin levels secreted by the gastric mucosa typically stimulate dopamine release and food intake, but an aberrant leptin and ghrelin response has been hypothesized that reinforces the experience of a thin body instead [10]. Meta-analyses on the role of cytokines, markers of inflammatory processes, indicate a pattern of elevated tumor necrosis factor alpha in AN, while the data on other cytokines in AN are mixed [11]. Whether those markers are indeed relevant for AN illness development, maintenance or recovery remains elusive.

Human functional brain imaging during the past two decades using tasks that test specific behavioral constructs has made much progress in helping us understand brain function. Review articles of the brain imaging literature on AN have repeatedly implicated

reward-processing circuits, aside from pathways involved in cognition or emotion processing; nevertheless, understanding the exact neurobiology of AN has been challenging [12–14].

Our lab has pursued the study of reward circuits in AN and specifically the dopamine-associated prediction error (PE) model. The dopamine PE is a learning signal important for food approach, and animal models suggested enhanced neuronal dopamine activation following food restriction [15, 16]. This led to the hypothesis that brain dopamine circuits are important for the pathophysiology of AN [6]. The so-called PE response, the dopamine neuron's response to ARW17H unexpected receipt or omission of salient or rewarding stimuli, was first identified using single neuron recordings, and it can be studied using human brain imaging and computational models of brain function [17]. In ill and recovered adult AN, randomly applied sucrose taste stimuli evoked higher insular and striatal PE response [18]. Unexpected omission or receipt of monetary or taste reward in adolescent AN also resulted in heightened response in those regions, suggesting that enhanced dopamine reward system response is an adaptation to starvation in AN and could be an illness state biomarker [19, 20]. PE brain response was also related to weight gain in treatment as well as high anxiety, supporting the clinical utility of the PE model in AN [20]. Dopamine and PE-related brain circuitry have also been associated with reversal learning [21], which has important clinical implication for AN as reversal learning is impaired in AN and could interfere with treatment response [22, 23]. Therefore, dopamine system modulation could improve reversal learning and thus treatment [24]. Possibly in support of this hypothesis is a recent retrospective chart review in AN that indicated that the partial dopamine D2 receptor agonist aripiprazole was beneficial for weight gain [25].

The complex interplay between neurobiological, psychological, and environmental factors in AN, including the self-driven food refusal, motivation for weight loss, and a perception of being overweight in spite of a very low body weight in AN, suggests strong both psychological and biological factors that perpetuate illness behavior. We have recently developed a model that integrates our neurobiological knowledge with psychosocial factors to capture illness development and perpetuation, taking into account the conscious motivation to lose weight and the body's response to drive food intake [26]. Animal research provides the opportunity to further develop this model for AN brain pathology and altered brain function and identify molecular mechanisms that drive malnutrition and underweight. For instance, rodents can be subjected to under- or overfeeding and binge eating, and one can measure neurotransmitter receptor changes or the dynamics of neurotransmitter release in those animals. Other studies subject animals to excessive exercising and can relate this behavior to neurotransmitter

changes. Those studies can inform about mechanisms that underlie the AN phenotype, and that could be targeted psychopharmacologically. A caveat has been that basic research has lacked the ability to model the intrinsic drive and motivation to starve that is seen in AN. However, newer studies now suggest that different rodent types may respond more humanlike and also develop food avoidance without running wheel use (Dulawa personal communications) [27].

In summary, research has been increasingly able to better understand the neurobiology underlying AN although much more work needs to be done to develop effective pharmacological interventions. Systematic basic research to model AN brain circuitry is more important than ever to align human with basic studies and take advantage of the complementary information that both directions provide.

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Chapter 15

Activity-Based Anorexia, an Animal Model of Anorexia Nervosa for Investigating Brain Plasticity Underlying the Gain of Resilience

Chiye Aoki

Abstract

Anorexia nervosa (AN) is a mental illness characterized by continuously severe, self-imposed starvation and intense anxiety, manifested as fear of gaining weight. An increasing number of individuals are diagnosed with AN, especially among men. AN is now recognized to include those serving the military as well. With no accepted pharmacological treatments available, coupled with its high mortality and relapse rates, better understanding of the neurobiological basis of this mental illness is needed. This chapter describes the animal model of AN, called activity-based anorexia (ABA), that captures multiple core features of AN successfully, including voluntary food restriction, heightened anxiety, and excessive exercise, culminating in severe body weight loss. Also described in this chapter is how individual differences in vulnerability to ABA can be quantified. This chapter will include examples of synaptic plasticity measurements that may underlie the gain of resilience, quantified as the suppression of two maladaptive behaviors – excessive exercise and voluntary food restriction. Finally, the chapter will describe potential uses of the ABA model for exploring pharmacological treatments to reduce the maladaptive behaviors elicited in the ABA model.

Key words Food restriction, Food-anticipatory activity, Hippocampus, Prefrontal cortex, Dopamine, Serotonin, GABA, Glutamate, Synaptic plasticity, Rodents

1 Introduction

1.1 The Human Condition of Anorexia Nervosa

Anorexia nervosa (AN) is a mental illness characterized by continuously severe, self-imposed starvation and intense anxiety [1], manifested as fear of gaining weight [2]. Approximately 0.3–0.4% of young women are diagnosed with this grave mental illness [3, 4]. The mortality rate for AN is the second highest of all mental illnesses (10–15%) [5], surpassed only by opioid addiction [6]. The mortality rate is more than 200 times greater than the suicide rate in the general population [7] and has a high relapse rate as well (25%) [3, 8]. In spite of these indications of significant clinical burden, there are at present no accepted pharmacotherapy for AN [9]. For example, comorbidity of anxiety and AN is high [1, 10],

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but anxiolytics, such as benzodiazepines, are of limited efficacy for reducing the maladaptive behavior of food restriction. Body dysmorphia is another comorbidity of AN. Antipsychotics that are used to treat hallucinations are of limited efficacy for reducing obsessions that perpetuate food restriction by patients with AN, although it is modestly helpful in weight restoration [11, 12]. Ketamine, an FDA-approved antidepressant, has been reported to ameliorate the obsessive-compulsive thoughts that propagate restricted eating behaviors [13], even though the more traditional antidepressants, such as the serotonin reuptake inhibitors, are of limited efficacy. These observations reflect the paucity of knowledge linking the etiology of the illness to pharmacological treatments.

Although not listed in the Diagnostic and Statistical Manual of Mental Disorders fifth Edition (DSM-5), excessive exercise is one of the core symptoms of AN. Incessant, excessive exercise exacerbates the severe weight loss associated with food restriction [14–16]. One study noted hyperactivity in 25 out of 33 patients, and that all were hyperactive at some time during the course of the illness, and 21 were hyperactive prior to dieting and weight loss [17]. Many of those individuals who successfully restore their body weight continue to stay on their feet, and this activity pattern contributes toward recurrent body weight loss [18]. Amenorrhea is one other commonly observed symptom among individuals diagnosed with AN, although this condition is no longer considered an essential diagnostic criterion [2].

Analysis of the demographics of individuals with AN provides some clues about the etiology of AN. The onset is almost always during early adolescence and much more prevalent among females (previously reported ratio was 9 females to 1 male, but more recent estimates indicate a ratio of 3 to 1) [3, 19, 20]. AN is higher among those individuals with a pre-existing history of anxiety [21, 22]. These demographics suggest that gonadal hormonal surges at puberty are risk factors, perhaps due to changes in brain regions and synapses that are modulated dually by gonadal hormones and stress hormones [23–27]. As noted above, individuals engaged in and able to tolerate strenuous physical training are also at risk. This may be one reason eating disorders, including AN, are prevalent among individuals in military services [28].

1.2 Activity-Based Anorexia (ABA), an Animal Model of AN

First reported in 1954 [29], activity-based anorexia (ABA) is a widely used rodent model of AN. ABA captures multiple clinical features of AN:

Voluntary food restriction.

Severe weight loss.

Excessive exercise that is elicited by hunger [30].

Heightened anxiety [31, 32].

Cessation of the estrous cycle for females.

Heightened vulnerability during adolescence, compared to early adulthood [15, 30] (also reviewed in [27] and unpublished observations).

These parallelisms between humans and rodents indicate that identification of biological mechanisms underlying these behavioral phenotypes in rodents can provide clues for understanding the condition of AN as well as the gain of resilience that prevents relapse. Indeed, this laboratory has used the ABA model to reveal several forms of synaptic plasticity that are induced by the experience of ABA during adolescence. For example, within brains of animals that exhibited signatures of resilience to ABA, such as the suppression of food restriction-induced hyperactivity, we observed increased expression of $GABA_A$ receptor at excitatory synapses on pyramidal neurons in the hippocampus [33, 27, 34, 35], and at excitatory synapses on inhibitory interneurons in the dorsolateral amygdala [36]. Increased GABAergic innervation of pyramidal neurons in the hippocampus [37, 38] and prefrontal cortex [39] was also observed among animals that exhibited abilities to suppress hyperactivity. Conversely, within hippocampus of animals that exhibited increased signatures of vulnerability to ABA – hyperactivity, the expression of NR2B-containing NMDA receptors at excitatory synapses in the hippocampus was significantly increased [40]. In a study that examined the risk factor of sex, we learned that both sexes of laboratory rodents exhibit similar levels of vulnerability to ABA, but the cellular mechanisms underlying the gain of resilience differ across the sexes [41].

Another chapter in a previous edition of this book [42] provides comprehensive coverage of the methodological details of the rat model of ABA. Multiple review articles also exist, pertaining to the revelations of the underlying neurobiology of AN based on data from the rat model of ABA [15, 27, 43–46] (see more references listed under Subheading 1.6). Our lab has invested in developing the mouse model of ABA. This effort was driven by the hopes that a mouse model could provide additional advantages due to the much wider availability of strains, mutants, and transgenic animals as tools for unraveling the genetic, cellular, and molecular mechanisms underlying individual differences in ABA vulnerability and resilience [37]. Thus, this chapter will describe the mouse model of ABA, with added notations of slight differences between the rat and mouse models of ABA. Moreover, this chapter will also describe methods we use to highlight individual differences in vulnerability to ABA. Finally, we believe that the ABA model is particularly useful for revealing individual differences in the gain of resilience when animals undergo repeated exposures to ABA induction. Thus, we describe the protocols that we have used for repeated ABA

inductions and methods we have developed to quantify the gain of resilience.

1.3 Key Steps for Inducing Activity-Based Anorexia That Capture the Symptoms of AN

Activity-based anorexia (ABA) protocol begins by acclimating animals to a wheel within its home cage, for providing ad libitum opportunity for voluntary exercise. Within this environment, voluntary wheel running distance and duration are measured 24/7, with temporal resolution of 1 min. Following this phase of acclimation, starvation is induced by limiting the hours of food access without limiting the amount of food available. The extent of starvation is aimed to be sufficiently severe so as to induce up to 20% body weight loss within 3–5 days, but not so severe as to cause death by food restriction alone. For rats, limiting the food access to 1 h. per day but of unlimited amount meets this condition. For adolescent mice, access to unlimited amount of food for 2 h per day meets this condition. Interestingly, most adult mice that undergo three episodes of ABA gain the ability to eat sufficiently during the 2 h. to retain its body weight, even in the presence of a wheel.

Curiously and most importantly, after losing body weight due to food restriction, adolescent animals increase the extent of wheel running severalfold. Wheel running becomes so excessive as to continue even during the limited hours of food access. Thus, one is able to quantify the emergent behavioral phenotype of voluntary food restriction. This seemingly maladaptive behavior exacerbates body weight loss far beyond the extent of body weight loss due to food restriction, alone. Unless the wheel is removed and the hours of food access are lengthened, these animals can die, even though food restriction, alone, is not lethal. To be sure, the behavioral trait of voluntary food restriction emerges only after the imposition of food restriction. The heightened wheel activity, especially during the hours leading up to the time of feeding, termed food-anticipatory activity (FAA) becomes evident within 24 h. of the imposed food restriction.

1.4 Theories Regarding Individual Differences in ABA Inducibility

As noted above, the greatest change in food restriction-evoked behavior is hyperactivity, especially during the hours leading up to the feeding hour, FAA. There is evidence to indicate that FAA reflects the animals' learning of the hours of food availability [47]. This learning requires the expression of dopamine D1 receptors by GABAergic neurons in dorsal striatum [48]. However, even if FAA indicates that the hours of food availability has been learned, animals continue running during the hours of food availability, albeit less than during the hours preceding food availability. The extent of wheel running (distance and duration) during the hours of food access as well as the hours leading up to feeding (FAA) can be used to quantify the maladaptive anorexic behavior (hence, the name "activity-based anorexia").

While approximately 80% of female mice in mid-adolescence become hyperactive, when food restricted, the proportion drops to 50% when the same ABA-inducing environment is imposed during late adolescence. The greater vulnerability for ABA during mid-adolescence may be due to asynchrony in the maturation of brain regions, causing an imbalance in the interregional connectivity [49, 50]. In addition, individual differences in the gain of resilience during the mid to late adolescent may reflect individual differences in the progression of the last phase of brain maturation. In what ways might brain maturation differ across individuals? Possibilities include the cellular events underlying microcircuit-level fine-tuning, such as synapse pruning, increased myelination of the prefrontal cortical pyramidal cells, and changes in the balance of excitatory-to-inhibitory synapses, among others [27, 51, 52].

The causal-effect relationship between food restriction, anxiety, and exercise remains unclear, but there are at least two prevailing views (reviewed in [27]). One view is that food restriction and excessive exercise are evoked due to a preexisting condition of anxiety and that patients deliberately choose these as anxiolytic behaviors to abate the intense fear of weight gain. In support of this view, preexisting conditions of anxiety and overexercise are common among individuals with AN [21]. Similarly, mice carrying genes that elevate trait anxiety exhibit stronger hyperactivity when stressed [53, 54]. Moreover, results from animal models indicate that exercise can be anxiolytic [55] and stress-relieving [56] through the production of BDNF [57, 58]. Food restriction can also be anxiolytic, through the production of ghrelin [59, 60], for which there are ubiquitous binding sites throughout the brain, including the hippocampus [61].

The other prevailing view is that anxiety and hyperactivity are inevitable behaviors stemming from starvation. There is a wealth of evidence indicating that many species, including healthy humans, rodents, and even pigs, become hyperactive following starvation [62]. Although food restriction-evoked hyperactivity seems paradoxical, it may have an evolutionary advantage of propelling foraging behavior, an innate behavior that is adaptive for organisms encountering insufficient food supply in the wild [62]. However, for animals in captivity, the incessant voluntary wheel running that is evoked by FR is clearly maladaptive, because it exacerbates the negative energy balance without bringing the animal closer to a new source of food. Although the incessant voluntary wheel running appears stereotypical, it is not an artifact of captivity: voluntary wheel running is a behavior that can be elicited repeatedly, even by feral mice [63].

Additional explanation about food restriction-evoked hyperactivity is that it is an animal's attempt to counter the hypothermia caused by food restriction. In support of this idea, elevating ambient temperature from 21 °C to 31 °C effectively reduces body

weight loss caused by hyperactivity of food-restricted adult rats [64]. For the explanation of the incessant wheel running, in spite of the progressive weight loss, it has also been hypothesized that running is an addictive behavior associated with the release of endorphins. In support of this explanation, antagonists of endogenous opioid peptides (naloxone) can elicit withdrawal-like symptoms, such as teeth-chattering much more strongly among animals that had undergone ABA compared to animals that were acclimated to a wheel without food restriction [65].

1.5 ABA as an Experimental Tool for Preclinical Studies

As described above, AN is a grave mental illness without accepted pharmacological treatment. Studies to investigate pharmacological manipulations that reduce ABA vulnerability could provide insight into the efficacy of drugs for treating AN. The ABA model can be of use for quantifying the effect of drugs or environmental factors, hormonal status, sex, earlier sensory and emotional experiences, etc., upon ABA vulnerability (*see* Subheading 3.4 for methods to quantify ABA vulnerability). For such studies, one can compare ABA-induced changes in cognitive and anxiety-like behavior, brain anatomy, synaptic physiology, neurochemistry, gene expression, etc., with and without the treatments that are applied at different time points within the ABA schedule. One of our preclinical pharmacological study was to test whether ketamine could ameliorate the maladaptive behavior of excessive exercise and voluntary food restriction. We learned that a single dose of ketamine administered during mid-adolescent ABA induction could ameliorate both of these maladaptive behaviors during the second ABA induction that followed recovery after the first ABA induction [66]. This gives us hope that ketamine may somehow rewire brain in ways that boost the suppression of maladaptive behavior later in life, when stress-inducing environmental factors recur.

1.6 Video and Review Articles on ABA

This chapter describes details of the procedures, rationale of the procedures, and notes to enhance success. As an additional source for familiarizing with the ABA procedure, a visual description of the mouse ABA procedure is available [67]. Many excellent review articles on the theory of ABA exist [15, 30, 27, 44, 68–70] as well as an up-to-date comprehensive summary of ABA studies [46]. Since the focus of this chapter is not theory or review of the literature but presentation of procedural details, reader is urged to examine these other resources for additional information.

2 Materials

2.1 Animals

A key factor for ABA induction is stress-induced anxiety associated with the imposed food restriction. It is therefore important to

minimize all other sources of stress that may potentially interact with the experimental manipulation of food restriction stress.

For studying the effects of ABA during adolescence, it is best to obtain mice of specified ages, strains, and genotypes through breeding in the home institution's animal facility, so as to minimize the uncontrolled levels of stress associated with shipment [71, 72]. Depending on the extent of stress-associated shipment, acclimation to the new facility could require as long as 4 weeks, precluding the ability to begin ABA induction at a desired developmental stage. One recommendable source of mouse breeders is Jackson Laboratories. If in-house breeding is not possible, then one should consider the possibility that shipment of young rodents can incur more or less stress, depending on the age at the time of shipment. We have aimed for the transportation to occur during the fourth postnatal week for female rats. It is desirable to schedule at least 7 days for acclimation to the animal facility in the absence of wheels or food restrictions. Group-housing during this period is desirable, for minimizing stress associated with isolation. For analysis of ABA vulnerability during mid-adolescence, mice and rats begin to be housed individually in cages with free access to a wheel starting around postnatal day 36, an age that is significantly separated from puberty onset, when gonadal hormone fluctuations are known to influence stress-induced anxiety [23, 34].

2.2 Equipment

The desirable features of equipment for measuring wheel activity are that they be able to record wheel activity of multiple animals synchronously, automatically, and over multiple days. Med Associates sells such a model for rats (ENV-046, *see* Fig. 1) and mice (ENV-044, *see* Fig. 2), but multiple other competing companies also sell equipment with these features. The model sold by Med Associates for mice is the Low-Profile Wireless Activity Wheels (ENV-044), that fits within most mouse cages, including the Optimouse "pizza pie" cages (*see* Fig. 2) and Allentown cages with flat tops. The shown rat wheel is made freely accessible through a guillotine door connected to a plastic shoebox-style home cage.

2.3 Water and Food

Food restriction is imposed through limitations in the hours of food access but not in amount. Since mice and rats are nocturnal, the food access should be scheduled during the dark phase. Setting the hour(s) of food access to be at the start of the dark phase has the advantage of maximizing capture of FAA, which can be contrasted to the circadian behavior of non-food-deprived individuals that are usually asleep during the hours leading up to the first hours of the dark period.

It is recommended that the animals be pre-acclimated to the specific foods planned for feeding during the limited hours of food access, so as to avoid hyponeophagia. Hyponephagia is shyness that healthy animals exhibit toward a newly encountered food [73]. A

Fig. 1 Rat wheel access connected to a home cage. A model from Med Associates

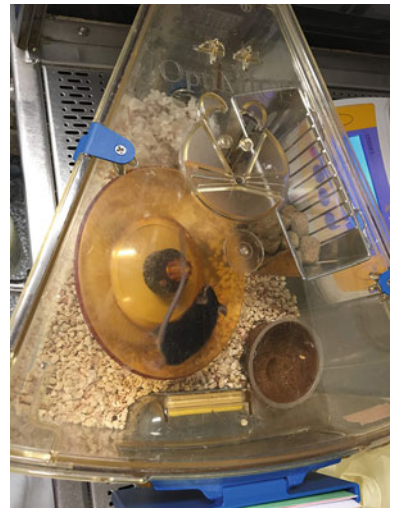

Fig. 2 Mouse wheel (Med Associates) arranged within the cage (Optimouse brand), together with a food hopper that allows easy weighing of dry food. Wet food, kept in the manufacturer's plastic cup (Clear H_2O brand DietGel 76A), is also weighed daily. Nesting material can be found, away from the wheel, to ensure free rotation of the wheel

good time to begin food acclimation is the time animals begin to be acclimated to single-housing and wheel access.

It is recommended that dry food pellet (LabDiet PMI Nutrition Int'l, Brentwood, MO's #5001,10% fat, 20% protein, 70%

Fig. 3 The same mouse wheel arranged within the cage, with an empty dry food hopper and Hydrogel that is 0 cal (Clear H_2O brand)

carbohydrate, 4.07 gross energy kcal/g, 3.02 metabolizable kcal/g; alternatively LabDiet Rodent Diet 20 EXT (5053), 20% protein, 4.5% crude fat, 6% crude fiber, 7% ash, 12% maximum moisture with 4.07 gross energy kcal/g, equal to 3.07 metabolizable energy kcal/g) be dispensed in readily removable food hopper, synchronously with soft wet food (Clear H_2O brand DietGel 76A in plastic cups, 0.998 kcal/g, 4.7% protein, 17.9% carbohydrates, 1.5% fat, 73.4% moisture) which is easier for animals to ingest, but of lower caloric content. During the hours of no food, water gels (Clear H_2O brand Hydrogels Produce #70-01-5022) and empty food hoppers are placed in positions within the cage that are identical to those during the hours of food availability, so as to minimize changes to the context (*see* Fig. 3).

3 Methods

3.1 General Comments about Animals

This study was preceded by works from other labs examining comparisons of ABA vulnerability in other strains of mice that were adult males [53, 54, 74, 75] and mutants that were much younger (anx/anx because they do not survive to adolescence) [76, 77].

If the subjects of the research are to be adolescents, it is recommended that adolescent animals be of similar body weight (<10% variation of mean body weights across groups) and postnatal ages (±5 days) at the start of the acclimation period (however, *see* Subheading 4.1 about animal weights and ages). They need to be individually housed, so as to be able to measure their daily intake of dry and wet food and wheel activity.

Animals should be housed in a rodent vivarium with a 12:12 light/dark cycle maintained at 21 °C and controlled humidity. All experimental procedures (including handling, housing, husbandry,

food restriction) must be conducted in accordance with National and Institutional Guidelines for the Care and Use of Laboratory Animals and University Institutional Animal Care and Use Committee protocols. Since food restriction is a USDA Category E procedure, involving imposition of "more than slight or momentary distress that cannot be treated with anesthetics and analgesics" (USDA Policy #111997 on the use of animals), it is important to provide the strongest scientific justification and to have procedures in place to minimize distress. The procedures for food restriction should include daily monitoring of body weight twice – before and after the hour(s) of food access – and a plan to provide additional food when an animal's body weight decreases to be less than 80% of the baseline body weight that is assessed just before the food restriction period has begun.

It is recommended that animals of a single cohort be divided across ABA and control groups. ABA animals receive the combined treatment of food restriction and wheel access. For the initial establishment of the ABA paradigm, there are three desirable control groups. They are FR (food restricted only for equivalent days and ages), EX (exercise only, with access to the identical wheel model for equivalent days and ages), and CON (no food restriction and no wheel access).

3.2 General Comments About Caging, Acclimation, and Ambient Temperature

Body weight loss associated with restricted food access causes body temperature to decrease. Conversely, raising the ambient temperature reduces wheel running and body weight loss [64, 78]. Based on these observations, it is likely to be important that nesting material be provided, so as to enable individuals to create thermally insulating nests (*see* Fig. 4). If so, then the nesting material should be equalized across cages, so as to minimize differences in body temperature and stress arising from differences in the thermally insulating nesting material.

Fig. 4 A typical nest that an adolescent female mouse creates using three kinds of products: facial tissue, paper strips, and compressed cotton squares

A substantial amount of food remains at the bottom of cages. In order to ensure that food availability is restricted to the hours of the experimental design, the cage bedding must be refreshed completely at the end of the feeding hour, with the bottom of cages wiped clean to remove all dry food crumbs and dust. Water should be freely accessible at all times, with additional hydration available through water gel cups during the food-restricted hours, in the place of wet food gel cups. The water gel assures that animals can remain hydrated while acclimating to the new water delivery system, such as the automatic water delivery systems.

If animals are to be shipped, they should be allowed a minimum of 7 days of acclimation to the new facility [72] while group-housed. Group-housing should strive to maintain same cagemates as found during the shipment, so as to avoid stress due to aggressive social interactions.

3.3 The Daily Schedule of ABA and Control Groups

The environmental treatment of ABA is comprised of a combination of food restriction and exercise. In order to be able to identify behavioral and brain changes elicited mainly by food restriction, mainly exercise or through their interaction, it is useful to set up four groups: ABA (food restricted in the presence of wheel), FR (food restricted, with schedule equalized to that of ABA animals), EX (given wheel access, to which it has acclimated as are the ABA animals), and CON (neither food-restricted nor given wheel access) [34, 37]. Listed below are the procedures scheduled for each experimental phase for animals undergoing one to three bouts of ABA (ABA1, ABA2, ABA3), with ABA2 and ABA3 designed to measure adaptive changes evoked by the previous bout of ABA (*see* Fig. 5).

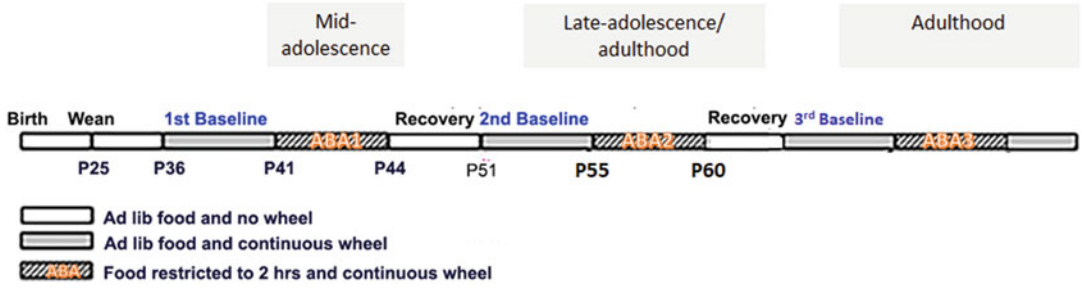

Fig. 5 Timeline for three ABA inductions spanning adolescence to adulthood, each preceded by an acclimation phase and followed by a recovery phase

3.3.1 Acclimation – 4 Days

This is the phase for acclimating animals to single housing, wheel in the cage, and food type. For studies of adolescents, the recommended start time is 6 h prior to the beginning of the dark phase (e.g., 1 pm, for rooms that become darkened at 7 pm) of postnatal day (P) 36, ending at 1 pm on P40. [For the sake of simplicity, postnatal ages will be considered to increase by 1 day at 1 pm. Thus, 1 pm of P40 is equal to the beginning of P41]. Adolescent animals exhibit daily increases in body weight. Wheel acclimation is indicated by daily increments in wheel counts per day, as animals gain speed of running.

Introduction

Wheels

Animals designated for wheel access (EX and ABA) are acclimated to the wheel in their cages starting the first day of acclimation. Be sure that the wheel can rotate freely, not obstructed by other items in the cage. In tightly fitting cages, such as the Optimouse cages, it is important to be vigilant that wheel turning is not obstructed by the food hopper, wet food cup, or nesting material *(see* Figs. 2 and 3). If the wheel counting system relies on battery, be sure that the battery voltage is safely above the cutoff (e.g., >4 volts for the ENV-044, manufactured by Med Associates).

Food

Rodents exhibit hyponeophagia – shyness to new source of food. Hyponeophagia is minimized by introducing the dry and wet foods for a minimum of 4 days, starting on the first day of acclimation.

Bedding and Nesting Material

All animals should receive equal amount of bedding and nesting material. Nesting material can comprise of tissue, shredded paper, and compressed cotton pads, which mice shred to mix with shredded paper and tissue to create thermally insulating nests (*see* Fig. 4). Since animals' body temperature can decrease during food restriction, nesting material can play an important factor of thermal regulation. The nesting material should be equalized across the cages.

Record Keeping

Wet and dry food consumptions, body weight, and wheel activities are recorded throughout the entire experiment. The frequency of recording can be kept to once per day during the acclimation phase, although it should be at least twice per day during the food-restricted, ABA-inducing periods, timed to be just before and at the end of the hours of food availability. It is important to record wheel activities at least once per day, even if the wheel activity system is recording animals' activity every minute automatically. This is because more often than not, the data acquisition system is interrupted unexpectedly. The daylight savings day is one predictable cause for failures, as are the days of power failures.

3.3.2 ABA1–3 Days

General Comments

This is the first phase in which food restriction is combined with wheel access for the ABA group of animals. The FR group of animals begin to be food-restricted synchronously with the ABA animals, in the absence of wheel access. ABA and EX groups continue to receive wheel access, as is done during the acclimation phase. The type of food remains unchanged for all animals, but the food access becomes limited to 2 h per day for mice and 1 h. per day for rats in the ABA and FR groups. For adolescent mice undergoing their first experience of food restriction, 3 consecutive days of food restriction is the maximum duration before body weight loss exceeds 20%. For animals that have begun acclimation on P36, ABA1 begins on P40 and ends at the end of P43 (which equals the beginning of P44).

Details of the Schedule

During the food-restricted days, food access should be scheduled for the first hour of the dark period for rats and the first 2 hours of the dark period for mice. The first food-restricted day of ABA1 (FR1) should begin 6 h prior to the beginning of the dark phase. Thus, all food should be removed 6 h prior to the beginning of the dark phase. For example, for animals housed in rooms with light hours set to be from 7 am to 7 pm, all food is removed from the cage at 1 pm, returned to the cage at 7 pm, and taken away again at 8 pm for rats or at 9 pm for mice.

Details of the Procedure for Food Restriction

Food removal is achieved by removing the wet gel food cup and the hopper containing dry pellets. Bedding material can contain crumbs from the dry pellet. Therefore, the bedding needs to be exchanged with new bedding material at the end of the feeding period and the interior surface of the cage wiped with a dry paper cloth. However, as much as is possible of the nesting material is returned to the cage, in order to minimize changes to the cage interior. As further effort to minimize changes to the cage interior, an empty food hopper and zero-calorie water gel are placed in the same positions as the food hopper with food and wet food cups. Nesting material should be placed in the original position while ensuring that none of the cage objects interfere with free movement of the running wheel. This is the procedure that should be followed at 1 pm of ABA1-FR1 and at the end of the feeding period of FR2 and FR3.

Daily Data Collection

For calculating food consumption, dry pellets and wet gel food are weighed in their containers every time food is removed from the cage and re-weighted every time that food is returned to the cage. For monitoring animal's body weight fluctuation associated with food restriction and feeding, it is recommended that animals' body weight be recorded at the time of food removal and at the time food is returned to the cage. The body weight at the time of food removal of the first day of food restriction (ABA1-FR1) can be considered the animal's baseline body weight (*see* Fig. 6a).

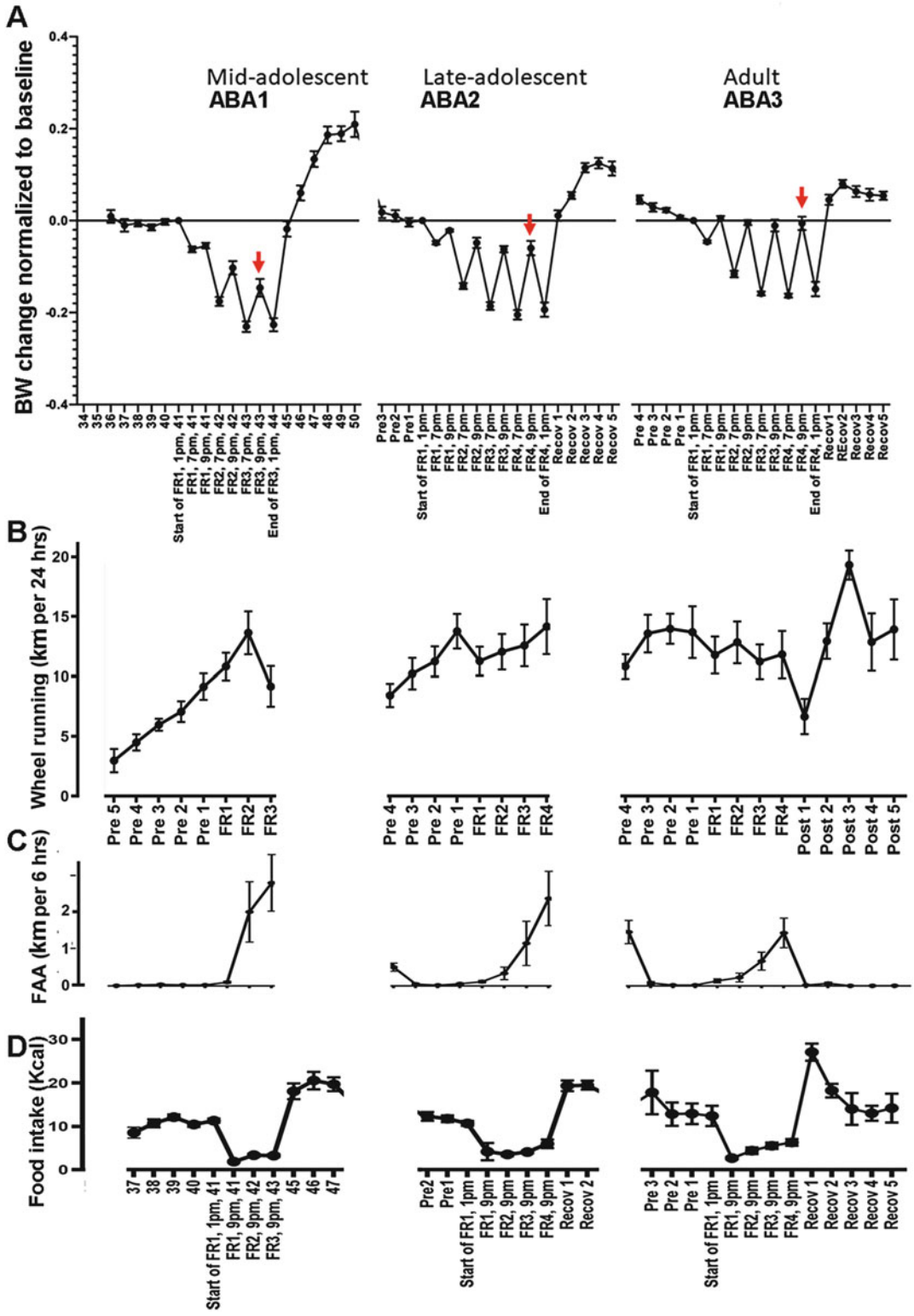

Fig. 6 Group average of body weight change (panel A), wheel running distance per day (panel B), and FAA (food-anticipatory activity), spanning 1 pm to 7 pm on food-restricted days (panel C) and food intake (panel D) of 9 female mice that underwent three ABA inductions. The red arrow in panel A highlights the progressive decrease in body weight, as animals undergo the successive ABAs

Summary and Conditions Requiring Supplemental Feeding

In short, during the first day of the first ABA (ABA1-FR1, P40), body weight, dry food weight, wet food weight, and wheel counts are recorded three times: at 1 pm, 7 pm, and 9 pm. On subsequent days (FR2 and FR3), these measurements are made twice per 24 h: at 7 pm and 9 pm. If, at the scheduled time of food removal (9 pm), measurement indicates that the animal has failed to retain at least 80% of the baseline body weight, and/or if the animal is exhibiting stillness, these behaviors are signs that the animal may not survive to the next feeding time. For them, additional pre-weighed amount of food, roughly equal to half of prior days' food consumption, should be placed at the bottom of the cage, for animals to have the easiest access to them, so as to avoid lethality. Approximately one out of eight mice exhibit gravely reduced locomotion by FR3 and need to receive supplemental food at 9 pm (unpublished observations). This is rarely observed for the FR-only group of animals and is more often observed for the ABA animals with high levels of food restriction-evoked wheel running. Food restriction-evoked wheel running increases most dramatically during the hours preceding the feeding hours and is thus called "food-anticipatory activity" (FAA) (*see* Fig. 6c).

3.3.3 Recovery from ABA1–5 Days

At the end of ABA1, which for the example above is 1 pm of FR3 (beginning of P44 or end of P43), animal's body weight is measured, as are dry and wet food, just before returning them to the cage, and the wheel is removed from the cage. During the recovery days, body weight and food consumption are measured once per day. Almost all animals restore their body weight within 1 day. Adolescent animals resume the daily body weight gain, as shown during acclimation. For animals that enter the experiment on P36, the ages of recovery is the beginning of P44 to the beginning of P49.

3.3.4 Reacclimation – 4 Days

For animals scheduled to return to a second ABA (ABA2) or EX, wheels are returned to the cage at the end of recovery so that animals can reacclimate to the wheel. Feeding condition remains ad libitum during this period for all groups. Most animals exhibit wheel activity that is similar to the extent observed on ABA1-FR1, indicating that they retain the state of acclimation to the wheel (*see* Fig. 6b). Body weight, food consumption, and wheel counts are recorded once per day during reacclimation. For animals that enter the experiment on P36, the age of reacclimation is the beginning of P49 to the beginning of P54. The average of wheel running during the last 2 days of reacclimation is considered the baseline wheel activity, to be used for calculating food restriction-evoked increase in wheel running during the succeeding ABA2.

3.3.5 ABA2–4 Days

ABA2 is similar to ABA1, except that the duration of food restriction can be extended to 4 days or longer. This may be due to behavioral adaptation, such as more efficient eating during the hours of food access, decreased wheel running during the hours of food access, and/or increased baseline body weight, all of which are questions that can be addressed experimentally for pursuing cell biological and neurochemical understanding.

ABA2 begins by removing food at 1 pm, returning the food to the cage for 1 or 2 h at the beginning of the dark phase, and removing the food after 1 h (for rats) or 2 h (for mice). The body weight at 1 pm at the beginning of ABA2 is considered the new baseline body weight. The average of wheel running during the last 2 days of reacclimation is considered the baseline wheel activity, to be used for calculating food restriction-evoked increase in wheel running during ABA2.

As was cautioned for ABA1, bedding material should be exchanged completely, and the interior surface of the cage should be wiped to avoid residual food availability during the hours of food deprivation. During the first day of the second ABA (ABA2-FR1), body weight, dry food weight, wet food weight, and wheel counts are recorded three times: at 1 pm, 7 pm, and 9 pm for mice and at 8 pm for rats. On subsequent days (FR2, FR3, FR4), these measurements are made twice per 24 h: at 7 pm and 9 pm for mice and at 7 pm and 8 pm for rats. If at the scheduled time of food removal body weight measurement indicates that the animal has failed to retain at least 80% of the baseline body weight, and/or if the animal is exhibiting inability to locomote, additional pre-weighed amount of food, roughly equal to half of prior days' food consumption, should be placed in the cage, so as to avoid lethality. However, this is rarely observed during ABA2. For animals that enter the experiment on P36, the age of reacclimation is the beginning of P54 to the beginning of P59, which is considered a stage for transitioning into adulthood.

The wheel activity observed during ABA1 typically increases multiple folds relative to the level observed during acclimation (~80% of the population of C57BL6-J strain). In contrast, *group average* of total wheel activity observed during ABA2 is only minimally increased relative to the level recorded during reacclimation (*see* Fig. 6b). Interestingly, closer examination of individuals reveals that some of the animals increase wheel running in response to food restriction during ABA2, while others reduce wheel running in response to food restriction, yielding a group average value that suggests no overall change (*see* Figs. 7 and 8). This observation fits with previous reports that studied ABA behavior of adult mice, indicating that C57BL6 mice are not vulnerable to ABA while other strains with known traits of heightened anxiety are [53]. However, individual differences revealed during ABA2 is an opportunity to identify brain and physiological changes that

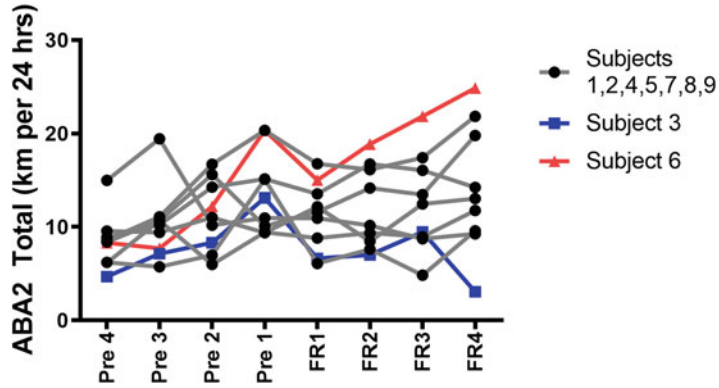

Fig. 7 Individual records of wheel activity per day, during ABA2 of the same nine mice used to calculate the group average shown in Fig. 6a. Subject 6 exhibited progressive increase in wheel running during the days of food restriction (FR1 through FR4), while subject 3 exhibited no significant increase in food restriction-evoked wheel running. These individual differences are examples of ABA vulnerability and resilience, respectively

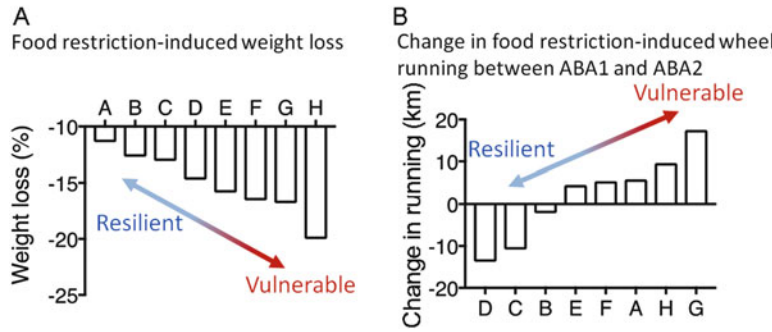

Fig. 8 The relationship between body weight loss and wheel activity was examined using another cohort of adolescent female mice that underwent ABA1 and ABA2. Each alphabet corresponds to datum from an individual mouse. The severity of food restriction-induced body weight loss (panel A) aligns somewhat, but not exactly with hyperactivity (panel B), presumably because differences in food intake during the limited hours of food availability and individual differences in energy metabolism contribute to body weight losses as well

correlate with persistence versus suppression of food restriction-evoked hyperactivity which, in turn, contributes toward ABA vulnerability versus resilience, respectively. Indeed, comparison of brain circuitry across vulnerable versus resilient mice and rats has revealed individual differences in the GABAergic synapses and $GABA_A$ receptor subunit expressions, with the resilient mice showing enhancement of these measures, compared to CON. Strong correlations ($r > 0.8$, $p < 0.05$ by Pearson correlation test) between wheel activity and these measures in dorsal

hippocampus [35, 38, 41, 79] and prelimbic area of prefontal cortex [39] have also been revealed. Conversely, this approach has revealed strong positive correlations between body weight loss and NMDAR levels at postsynaptic density of excitatory synapses in dorsal hippocampus (higher levels of NMDARs, the more they had lost body weight), while the levels of NMDARs sequestered in the postsynaptic spine cytoplasm correlated with suppression of hyperactivity [40]. These correlation analyses provide the data necessary for forming working hypotheses about the cellular and molecular basis of ABA vulnerability/resilience, which can be tested for causality using pharmacological and molecular approaches, such as knockin/knockdown of genes of interest within specific brain regions.

3.3.6 Recovery from ABA2–5 Days

For animals that are scheduled to continue onto ABA3, 5 days of recovery is recommended. The details of the procedure are as described for recovery from ABA 1 (*see* Subheading 3.3.3).

3.3.7 Reacclimation – 4 Days

For animals that are scheduled to continue onto ABA3, 4 days of reacclimation is recommended. The details of the procedure are as described above (*see* Subheading 3.3.4).

3.3.8 ABA3–4 Days or longer

For animals that are scheduled to continue onto ABA3, 4 days of food restriction is recommended. The details of the procedure are as described for ABA2 (*see* Subheading 3.3.5).

3.3.9 Euthanasia

The end point of the experiment should be decided based on the experimental question. For studies intending to correlate antemortem behavior with neurochemistry or brain anatomy, euthanasia may be scheduled on the last day of food restriction of the ABA period [34, 36, 40, 80]. On the other hand, for capturing brain mechanisms underlying the stable gain of resilience versus the persistent vulnerability to ABA, a better time point for euthanasia may be the end of the recovery phase that follows ABA [41]. We have also attempted to capture brain changes evoked during ABA, for which animals were euthanized on FR2 of ABA1, when body weight had begun to decrease and food restriction-evoked hyperactivity was on the rise but not yet maximal [35].

For optimal brain preservation for light and electron microscopic immunocytochemistry, see previously published methodological papers on transcardial perfusions to preserve synaptic structure [81, 82].

3.4 Quantification of ABA Vulnerability

As was remarked in Introduction, ABA is useful for capturing major characteristics of AN, consisting of body weight loss, voluntary reduction of food consumption, hyperactivity, and increased anxiety-like behavior. Individual differences in ABA vulnerability within and across cohorts can be quantified by making

measurements of these variables. Examples of procedures used to measure these changes are described below.

3.4.1 Body Weight Loss

Adolescent rodents on ad libitum food access gain weight continuously, but this body weight gain is severely interrupted when food access is reduced to 1 or 2 h per day (*see* Fig. 6 for an example of mouse data; *see* Fig. 8 for an example of rat data). Interestingly, body weight loss due to food restriction (in grams or percent of baseline) is less during ABA2 than observed during ABA1 (*see* Fig. 6), with body weight gain (a turning point) exhibited by the fourth day, in spite of the continuation of restricted food access. Analysis of individual animals' daily body weight changes can reveal differences, with some animals showing less weight loss and earlier turning points. The degree of weight loss is not paralleled exactly by individual differences in wheel activity (*see* Fig. 8), suggesting that individual differences in food consumption during the restricted hours of food availability and intrinsic differences in metabolism may contribute additionally to weight loss.

3.4.2 Reduction of Food Consumption and Suppression of Running during the Hours of Food Availability

Although the imposition of food restriction causes inevitable reductions of food intake, the extent to which animals reduce food intake can still vary. This is due, at least in part, to some animals "choosing" to run more than others during the hours of food availability. During ABA1 and ABA2, almost all animals run maximally during the first night of food restriction. Their running decreases progressively during FR2 to FR3, presumably reflecting their having learned the restricted feeding schedule. Some animals "learn" better than others, evidenced by their greater suppression of wheel running during the feeding hours (*see* Fig. 9). This is one other variable that can be used to quantify individual differences in ABA vulnerability (i.e., less suppression of running equals greater vulnerability).

3.4.3 Food Restriction-Evoked Increase in Wheel Running

In our experience, food restriction-evoked increase in wheel running is the variable that provides the greatest precision for quantifying individual and group (e.g., with versus without drug) differences in ABA vulnerability. By comparing the extent of voluntary wheel running during the days before versus the days after food restriction (i.e., acclimation versus ABA), one is able to quantify wheel activity associated with the introduction of the environmental stressor – food restriction. The comparisons may be of the total activity over a 24-hr period, or focused upon specific time bins of interest, such as the hours of FAA (food-anticipatory activity, when an animal must make a choice between foraging and conserving energy), the hours of food availability (when an animal must make a choice between running and eating), and the postprandial hours. Similar measurements can be compared just prior to versus just following drug administration (see an example of the analysis of the

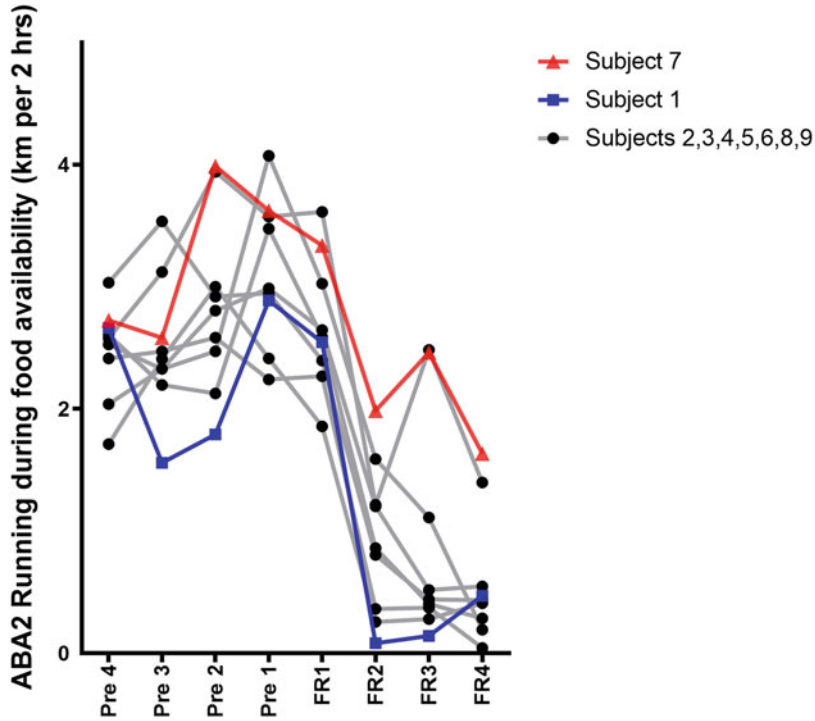

Fig. 9 Individual records of wheel activity during the 2 h of food availability during ABA2 of the same nine mice whose data are shown in Figs. 6 and 7. The red and blue lines depict the extreme examples of hyper- and hypoactivity, respectively

influence of ketamine upon ABA vulnerability, *see* Figs. 10 and 11). Additionally, combinations of time segments, such as the light versus dark hours, may be useful for examining the impact of ABA and/or of drugs upon circadian rhythm. Individual differences or treatment effects may be more evident when comparing wheel running in terms of distance run, duration run, or velocity of running. Finally, treatment effects (e.g., drug versus vehicle) may require analytic approaches that eliminate preexisting individual or group differences, such as of wheel running or of body weight. This comparison can be achieved by normalizing the degree of change evoked by the treatment, relative to pretreatment levels (e.g., body weight loss following food restriction during ABA minus baseline body weight measured just prior to the start of ABA divided by baseline body weight; *see* Figs. 10a and 11).

One powerful use of the ABA model is to identify potential cellular substrates for individual differences in ABA vulnerability. Figure 12 shows an example of a strong correlation revealed between wheel running during the food-restricted period (ABA2) and sizes of GABAergic axon terminals forming axosomatic inhibitory synapses onto layer 5 pyramidal neurons in the Cg1 area of prefrontal cortex. In contrast, the wheel activities of the same

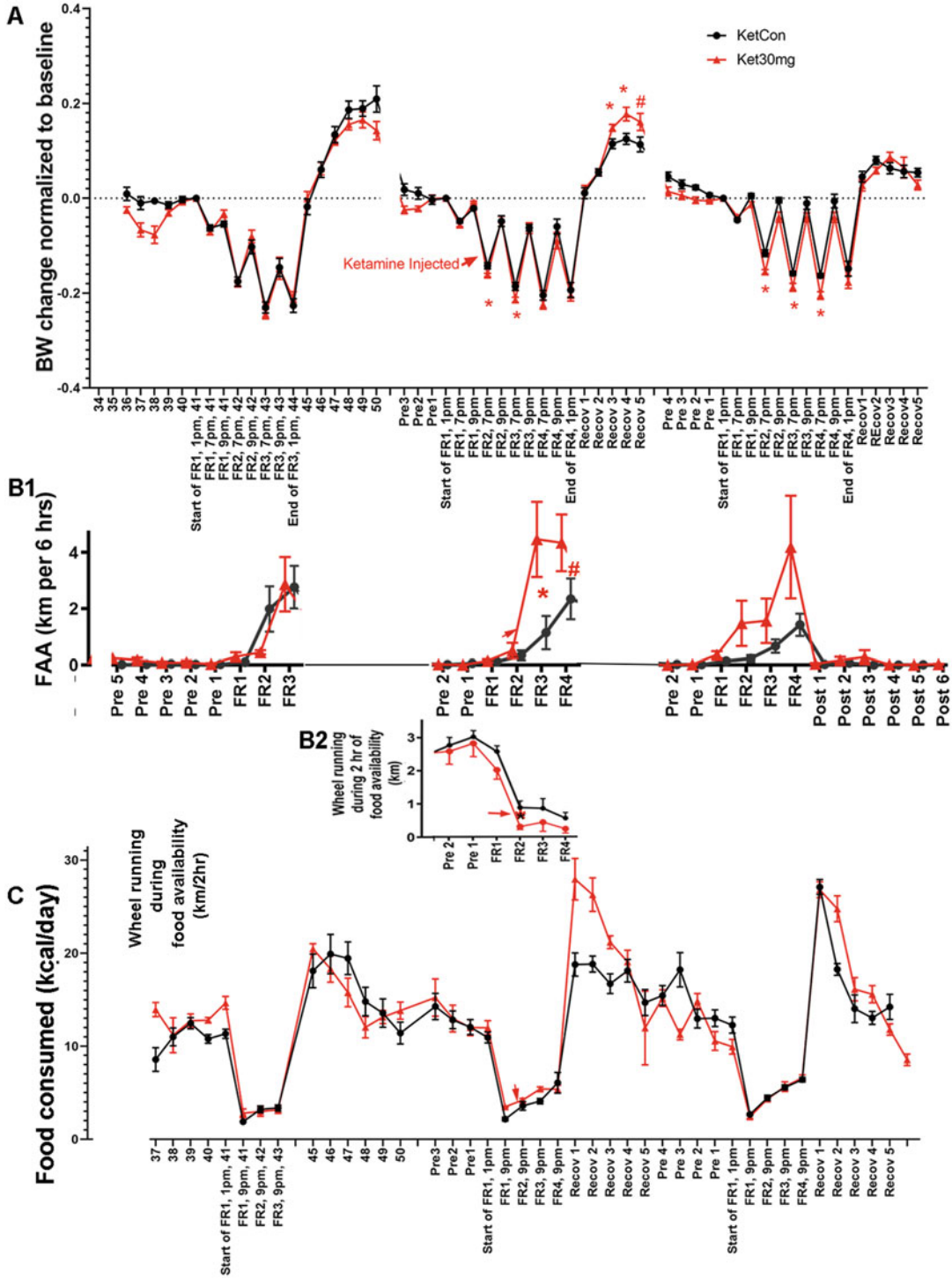

Fig. 10 An example of the use of the ABA paradigm to explore drug effects on ABA vulnerability/resilience. Data shown here suggest that ketamine exacerbates body weight loss, when administered during ABA2 in late adolescence. We had previously shown that a single injection of ketamine (30 mg/kg body weight) during

animals during acclimation/baseline showed no correlation with GABAergic axon terminal sizes. Also, note that the GABAergic axon terminal sizes within the corresponding brain region of control animals that experienced neither wheel activity nor food restriction were significantly smaller, indicating that the ABA experience stimulated enlargement of GABAergic axon terminal sizes. This finding has prompted us to prepare for the next phase of study, which will be to modulate GABAergic interneurons in the region using DREADD technology, for determining whether boosting the activity of these neurons can suppress the maladaptive wheel running during FAA and during the 2 h of food availability, thereby minimizing body weight losses.

3.4.4 Increase in Food Restriction-Evoked Anxiety-like Activity

A major feature of AN is anxiety. All animals that undergo food restriction express food restriction-evoked stress, as is indicated by the rise of circulating cortisol, but the extent to which animals increase anxiety-like behavior differs across individuals [31]. Specifically, individual differences in anxiety (before versus during food restriction) correlate significantly with the extent of increase in wheel running ($p = 0.004$, $R^2 = 0.56$) [31] (*see* Fig. 13), indicating that wheel running can be used to assess changes in anxiety. Unlike anxiety tests, which cannot be repeated and must be limited in number (we used open field before and elevated plus maze after food restriction), wheel running can be used to monitor changes in food restriction-evoked anxiety continuously.

3.4.5 Altered Cognition

Individuals diagnosed with AN are often characterized as high achievers but with rigidity in their decision-making [83]. Do

Fig. 10 (continued) mid-adolescence (P42) could increase food intake and decrease food restriction-evoked hyperactivity, leading to reduced body weight loss [66]. Encouraged by this result, the follow-up study shown here was to assess the influence of 30 mg/kg ketamine injection that is delayed by 10 days, corresponding to an age during late adolescence. Ketamine was injected on FR2 (second day of food restriction) of *ABA2* at 6 pm, corresponding to 1 h. preceding the time of feeding. Preliminary results indicate that ketamine treatment at this age does not yield protection against body weight loss.
The red line depicts group average of body weight changes of the cohort that received 30 mg/kg of ketamine on the second day of food restriction of ABA2. The black line depicts group average of the control group that received vehicle injection (saline), instead of ketamine, on the injection day. Asterisks indicate significant group difference ($p < 0.05$, unpaired t-test), while # depicts a trend. Red arrows point to the time of ketamine/vehicle injections. The exacerbated body weight loss is evident during FR2 and FR3 of ABA2 (panel A) and is persistently exacerbated even during ABA3 (panel A). FAA, calculated as the extent of wheel running from 1 pm to 7 pm, is increased on days following the ketamine treatment (panel B1, red asterisk). This increase in FAA is likely to be causal to the exacerbated weight loss. In contrast, ketamine's effect of reducing wheel running during the hours of food availability (7 pm to 9 pm) (panel B2) and the slight increase in food intake during those hours did not protect animals from body weight loss. Ketamine-treated animals consumed significantly more food, and especially so during the days of recovery from ABA2 (panel C), but this did not protect them from the exacerbated weight loss during the subsequent ABA3

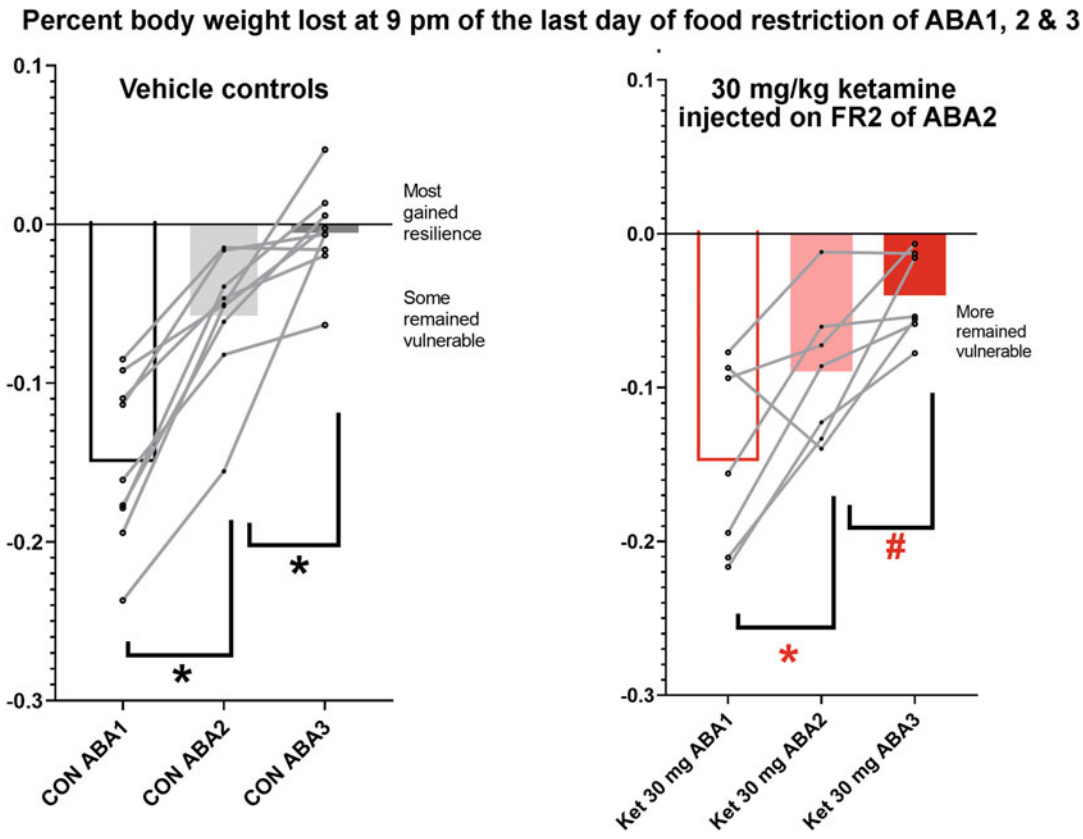

Fig. 11 Individualized analysis of food restriction-evoked body weight losses following ketamine treatment on FR2 of ABA2. Each data point reflects body weight measurement of one animal at one time point, with each time point taken at 9 pm of the last day of the food restriction period, corresponding to FR3 of *ABA1,* FR4 of ABA2, and FR4 of ABA3. These data were taken from the same cohort of animals whose group averages are shown in Fig. 10. Ketamine (30 mg/kg, right graph with red bars) or vehicle only (saline, left graph with gray bars) was injected intraperitoneally at 6 pm on FR2 of ABA2. All but one animal exhibited progressively less body weight loss, as the ABA induction was repeated. For both ABA2 and ABA1, ABA vulnerability, measured as the extent of body weight loss, tended to be more for the individuals of the ketamine group, compared to the group average of the values obtained from the CON group

animals that have undergone ABA also exhibit inflexibility or impairments in updating memory? Does the experience of ABA interfere with spatial memory formation? Our unpublished observations indicate that the answer is "no." We observed that ABA animals exhibit superior cognitive abilities in the active place avoidance (APA), a hippocampus-dependent spatial memory task [84], relative to CON with no experience of food restriction or wheels [85, 86]. Moreover, ABA animals with the greatest FAA were also the best performers in APA but only if the APA was delayed by 9–10 days after the end of food restriction [85, 86]. These observations suggest that the experience of ABA does not impair but rather enhances hippocampus-dependent cognition.

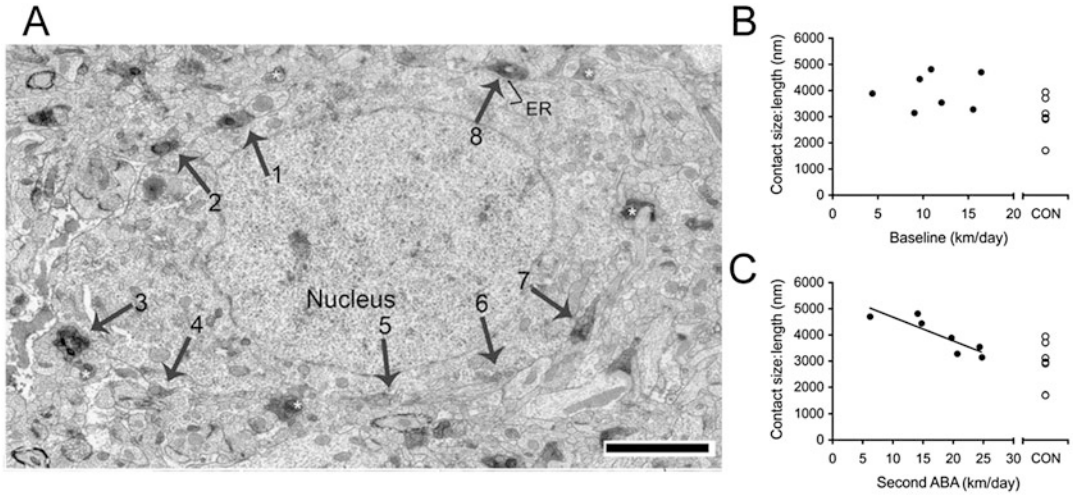

Fig. 12 Individualized analysis of food restriction-evoked change in wheel running revealed a strong correlation with the extent of GABAergic inhibitory synaptic input to pyramidal neurons in Cg1 area of medial prefrontal cortex. Panel A shows an example of electron micrographs used to assess GABAergic synapse lengths formed onto cell bodies of layer 5 pyramidal neurons in Cg1 of medial prefrontal cortex. Calibration bar = 2 μm. Panels B and C show correlation analyses of these synaptic lengths with the individual animal's extent of wheel running prior to (panel B) and during (panel C) ABA2 induction. (From Chen et al. [39], reproduced with permission)

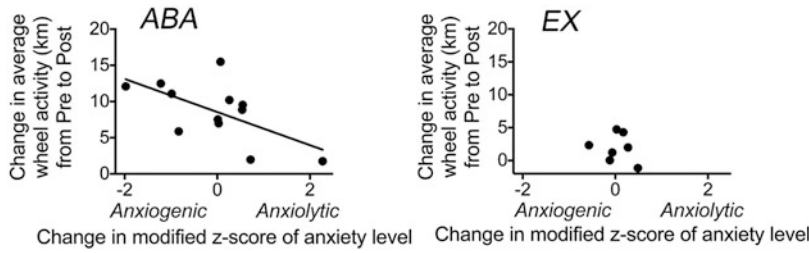

Fig. 13 Food restriction-evoked increase in wheel activity correlates with food restriction-evoked increase in anxiety. Anxiety of adolescent female mice that had acclimated to the wheel was measured before and after food restriction (ABA, left panel) and compared to those that acclimated to the wheel without the experience of food restriction (EX, right panel). In the absence of food restriction, the animals' anxiety-like measurements were similarly ranked during the first open field test and the second elevated plus maze test conducted 21 h later. For those that underwent food restriction, the two anxiety-like tests revealed wide individual differences. Those that exhibited more anxiety-like behavior (anxiogenic) were the same ones that had increased their wheel running the most. (Adapted from Ref. [31], *see* Fig. 4b)

4 Notes

4.1 Notes on Animals

In Subheading 3.1, it was advised that the baseline body weight and age be kept to within 10% variance. However, both of these parameters remain to be explored as potential risk factors. Do animals

with lower body weight but of equal postnatal age exhibit greater vulnerability? Are animals undergoing puberty onset more at risk than those that are mid-adolescent? For addressing these questions, experiments can be conducted in which the schedule is kept identical, for running correlation analyses between the baseline body weight or the age of entry into the experimental schedule and the extent of food restriction-evoked wheel running, increase of anxiety-like behavior, or change in cognition.

Sex of the animal is also an important parameter that should continue to be explored. Most published studies have used adult male rats. We have examined ABA inducibility (vulnerability) of adolescent males versus females and shown that both sexes are vulnerable, but resilience is attained using molecularly distinct mechanisms across the sexes [41]. In experiments examining the efficacy of certain drugs for reducing ABA vulnerability, differences in the outcome across the sexes could also shed lights upon the differences in the mechanisms underlying the gain of resilience.

4.2 Notes about Caging, Acclimation, and Ambient Temperature

We have run the mouse ABA paradigm using a variety of cage models. Obviously, it is desirable for the caging to remain unchanged throughout the ABA paradigm and for comparisons across cohorts. One parameter that the experimenter should strive to keep constant is the amount of nesting material, since this could affect the thermal insulation provided to the animal. It has been shown that elevated ambient temperature reduces the food restriction-evoked hyperactivity [64, 78], suggesting that animals (in this study, the subjects were adult rats) may increase wheel running so as to raise body temperature under the starvation mode. Based on this observation, one must consider the possibility that the degree of thermal insulation provided by the nesting material may also interact with animals' food restriction-evoked wheel running.

4.3 Notes about the ABA Schedule

Variations can be introduced easily into the ABA schedule, for addressing many different questions. The schedule described under Subheading 3.3 was for a preset duration of food restriction. An alternative might be to let the number of days of food restriction to be a set according to an individual animal's body weight change, such as 80% of baseline. The number of consecutive days of food restriction could be prolonged, as animals undergo repeated ABA sessions and the baseline body weight increases with maturity. Alternatively, although our earlier study indicated that many of the mid-adolescent mice do not survive when food access is limited to 1 h. per day, this shorter duration may be possible for late adolescence and adulthood. Conversely, increasing the number of hours of food access to be greater than 2 h for mice or greater than 1 h. for rat will definitely reduce hyperactivity, as animals will be able to maintain their baseline body weight sufficiently.

The duration of wheel acclimation is recommended based on the baseline wheel activity that is usually attained for adolescent mice and rats. It is helpful to wait until an animal's pre-food restriction wheel running attains a steady level, for enabling measurements of food restriction-evoked increases. For adults, a much longer acclimation period may be required, compared to animals described here that undergo two sessions of ABA during adolescence.

5 Conclusion

Anorexia nervosa is increasing in prevalence, especially among the male population. Although recognized to be more prevalent among athletes, dancers, and models, the Department of Defense recognizes that those serving the military may also be at risk. This author agrees with the viewpoint of the DoD since individuals serving the military are selected based on their ability to tolerate the combination of extreme stress and strenuous exercise, the two environmental risk factors for AN and reliable factors that induce ABA in rodents. The animal model of ABA promises to serve as a useful preclinical trial tool for exploring pharmacological treatments that ameliorate the addictive, maladaptive aspects of excessive exercise and food restriction and for understanding the neurobiological basis for brain plasticity that enables suppression of these maladaptive behaviors.

Acknowledgments

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Chapter 16

The Anorectic Phenotype of the *anx/anx* Mouse Is Associated with Hypothalamic Dysfunction

Ida A. K. Nilsson, Tomas Hökfelt, and Martin Schalling

Abstract

The anorectic *anx/anx* mouse, mimicking the core characteristics of anorexia nervosa (AN), i.e., reduced food intake, emaciation, and premature death, is an interesting and useful model for studies of mechanisms involved in the regulation of food intake as well as the development and maintenance of AN. The anorexia (*anx)* mutation arose spontaneously at the Jackson Laboratory in 1976 and has been mapped to a 0.2 cM interval on chromosome 2 (Chr. 2). Although the mutation is still unknown, it has been associated with a mild hypothalamic mitochondrial complex I dysfunction and a downregulation of the complex I assembly factor *Ndufaf1*, a gene located in the mapped interval. Aberrances in several neuropeptidergic and neurotransmitter systems important for the regulation of food intake, particularly in the hypothalamus, have been documented, as well as signs of hypothalamic neuroinflammation and neurodegeneration and pancreatic dysfunction.

Key words Anorexia, Neuropeptides, Hypothalamus, Neuroinflammation, Neurodegeneration, Oxidative stress, *Ndufaf1*, Mitochondrial dysfunction

1 Introduction

The regulation of food intake is a vital task of the brain. Derangement of the systems involved in this process can have serious consequences. The hypothalamus integrates peripheral signals reporting on the nutritional status of the body and initiates a proper response—to eat or not to eat. It is thus considered to be the major feeding center of the brain [1–18]. When this sensitive and complex system malfunctions, it can lead to disturbed eating behavior and/or appetite, which in turn impact an organism's well-being.

Over the past 20 years, our knowledge and understanding of the genetic regulation of food intake and energy expenditure have increased, in part through the use of genetic rodent models of obesity [19–29]. Many of these models have been critical for the elucidation of pathways and molecules important for the regulation of food intake also in humans, such as the leptin-signaling pathway

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identified in the obese *ob/ob* mouse [30]. Significantly fewer genetic models of anorexia have been developed. One explanation could be that the genetic changes leading to anorexia become lethal or affect the fertility of mice to a greater extent than the ones resulting in obesity.

The *anx/anx* mouse is a unique anorectic genetic mouse model with several characteristics in common with patients with anorexia nervosa (AN), including emaciation, starvation, premature death [31, 32], changed blood lipid profile [33], and low leptin [34]. This mouse is therefore a unique resource in research on the (neuro)biology of AN and is also of value for research on food intake regulation in general.

2 Materials and Procedures

2.1 Phenotypic Characteristics

The lethal, recessive, *anorexia* mutation (*anx*) arose at the Jackson Laboratory in 1976 and was first described by Maltais and colleagues [35]. Mice homozygous for the mutation, *anx/anx*, are mainly characterized by reduced food intake, which results in an emaciated appearance. Maltais and coworkers showed that from postnatal day 5 (P5), *anx/anx* mice eat less than their normal littermates (+/+ and +/*anx*) despite free access to the dam, food, and water. As a result of their low food intake, *anx/anx* mice start to deviate significantly from the normal growth curve beginning around P9. By P21, they weigh half as much as their normal littermates, approximately 4 vs. 8 g (*see* Fig. 1). In addition, *anx/anx* mice occasionally show other phenotypic traits, e.g., hyperactivity and several neurological problems, such as head weaving, tremors, and uncoordinated gate. They die prematurely around 3–5 wks of age, possibly due to the severe starvation. This is in

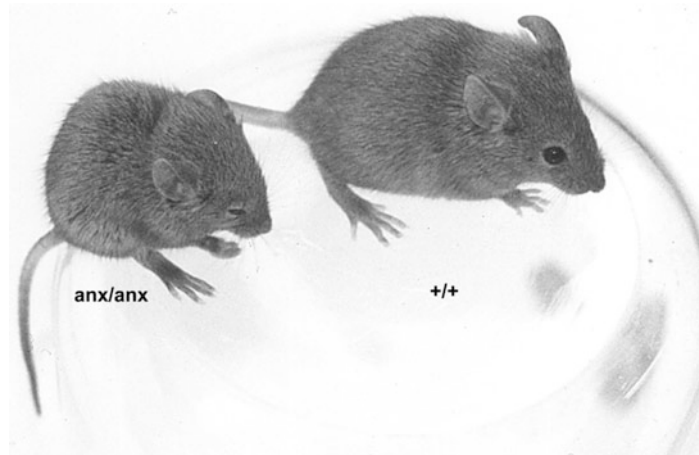

Fig. 1 An anorectic *anx/anx* mouse and a +/+ mouse, age 17 days

line with AN being the most lethal of the psychiatric disorders with around 10% mortality [36, 37].

Histological analyses have neither revealed any abnormalities in the gastrointestinal system or blood parameters, e.g., total red blood cell count, hematocrit, or hemoglobin, nor have any abnormalities in other organs been found using routine histological staining techniques [35].

2.2 Genetics

The *anx* mutation arose spontaneously in the second filial generation (F2) of a cross between dwarf-J and an inbred strain, which was derived from a cross between an inbred Swiss stock and mus musculus poschiavinus. The male *anx* carrier was crossed with a B6C3H-a/a F1 female, and the mutation has since been maintained on this background [35]. The *anx* interval was initially mapped to a region approximately 20 cM proximal to the agouti locus, on chromosome 2 (Chr. 2) [35]. By fine mapping, the locus was later narrowed down to a 0.2 cM interval between markers *D2Mit133* and *Jojo5*,[1] and the *anx* mutation was found to co-segregate with markers *D2Mit104*, *D2Mit395*, and *Jojo8* (*see* Fig. 2) [38]. The interval includes approximately 40 identified genes. To date, several sequencing efforts have been unable to show any unique sequence alteration. Of note is that the background of the *anx/anx* mouse includes five different strains (see above), making de novo assembly challenging. The difficulties with pinpointing the unique genetic variant might also indicate that the mutation is situated in a regulatory element outside the interval. Work by Kim and colleagues identified a point mutation in the gene *Tyro3* which they however concluded is not the *anx* mutation but rather a strain-specific modifier of *anx* phenotypes [39]. Thus, despite the 40 years that have passed since the "birth" of the *anx/anx* mouse model, the mutation is still undefined. It is our hope that novel techniques will be able to shed light on this mystery.

2.3 Expression and Sequencing

Using an Affymetrix microarray analysis of the Arc, we found that a gene corresponding to one of the assembly factors for Complex I in the oxidative phosphorylation system (OXPHOS), *Ndufaf1*, is downregulated in the *anx/anx* mouse [38]. The downregulation was confirmed both with Western blot in the brain and with real-time PCR in addition to pancreas, liver, and lung. Since *Ndufaf1* is mapped to the *anx* gene interval, we considered it a strong *anx* gene candidate. However, when sequencing the gene, both genomic (exons) and cDNA, no unique alterations in the *anx/anx* mice could be identified (see above), still not ruling out the possibility of the mutation being located in a regulatory element. Gene editing techniques such as CR ISPR-Cas9 [40] might aid in clarifying a potential causality of the downregulated *Ndufaf1* gene in the *anx/anx* mouse.

[1] Chr 2: bp 118,889,896-120,175,108; www.ensembl.org

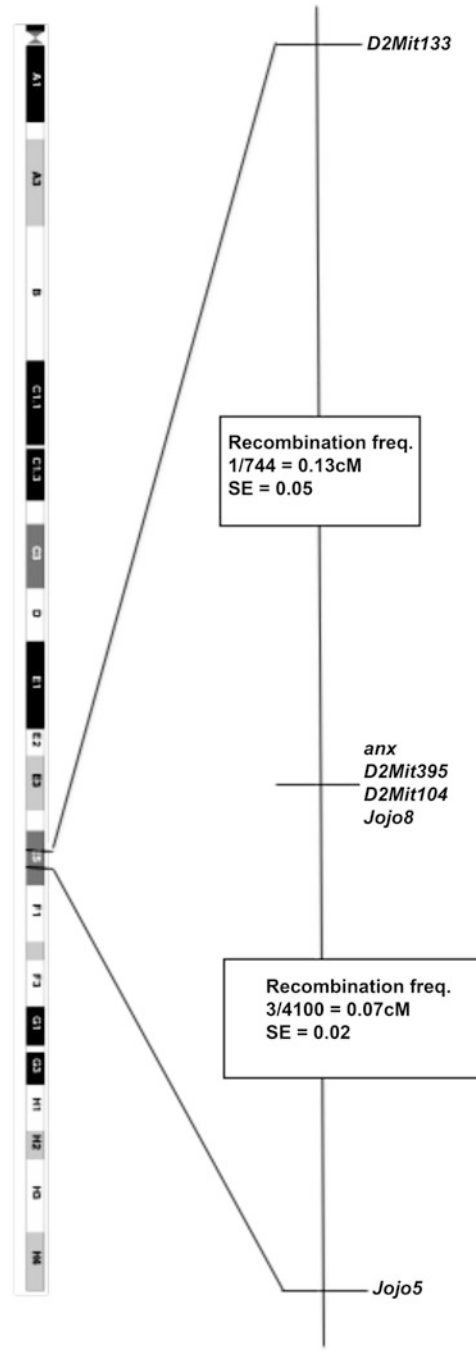

Fig. 2 Mapping of the *anx* mutation

2.4 Neurochemical Aberrances

Histochemical studies of the anorectic *anx/anx* mouse have revealed several aberrances in transmitter and neuropeptidergic systems, particularly in systems important for the regulation of food intake and energy metabolism, i.e., energy homeostasis (summarized in Table 1) [41–49]. The Arcuate nucleus (Arc) is central in the energy homeostatic processes [50–59], together with the lateral hypothalamus (LHA) [60] and the parabrachial nucleus that harbors neurons expressing calcitonin gene-related peptide (CGRP) neurons [18]. Arc harbors, in particular, two neuronal populations important for the regulation of food intake and energy metabolism (*see* Fig. 3). One Arc population coexpresses the orexigenic neuropeptides neuropeptide Y (NPY) and agouti gene-related transcript (AGRP). Whereas AGRP is selectively synthesized in these Arc neurons, NPY is in addition expressed in multiple, widely distributed cell groups [49, 61–63]. Thus, AGRP is an ideal marker for studies on the distribution of AGRP/NPY projections in the brain. The other population coexpresses the anorexigenic cocaine- and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC), a precursor protein generating, i.e., alpha-melanocyte-stimulating hormone (α-MSH). The AGRP/NPY neurons are known to partly be gamma-aminobutyric acid (GABA)-ergic [64], and the POMC/CART cells are glutamatergic [65]. Both the AGRP/NPY and POMC/CART neurons can sense peripheral hormones, e.g., leptin and insulin, which are able to enter the Arc via a partly permeable blood-brain barrier in the Arc-median eminence complex. The signal is propagated further via extensive projections from these Arc neurons to other hypothalamic areas, as well as to extra-hypothalamic regions, in fact even in

Table 1
Neurohistochemical aberrances in hypothalamus of the *anx/anx* mice, at P21

Marker	Immunohistochemistry		In situ hybridization
NPY	↑ cell number/intensity	↓ fiber density	↑/no change mRNA
AGRP	↑ cell number/intensity	↓ fiber density	↑ mRNA
ACTH	↓ cell number		
α-MSH		↓ fiber density	
CART			
POMC			↓ mRNA
Serotonin		↑ innervation of arc	
Y1	↓ cell number	↓ dendrite density	↓ mRNA
Y2			↓ mRNA
Y5			↓ mRNA

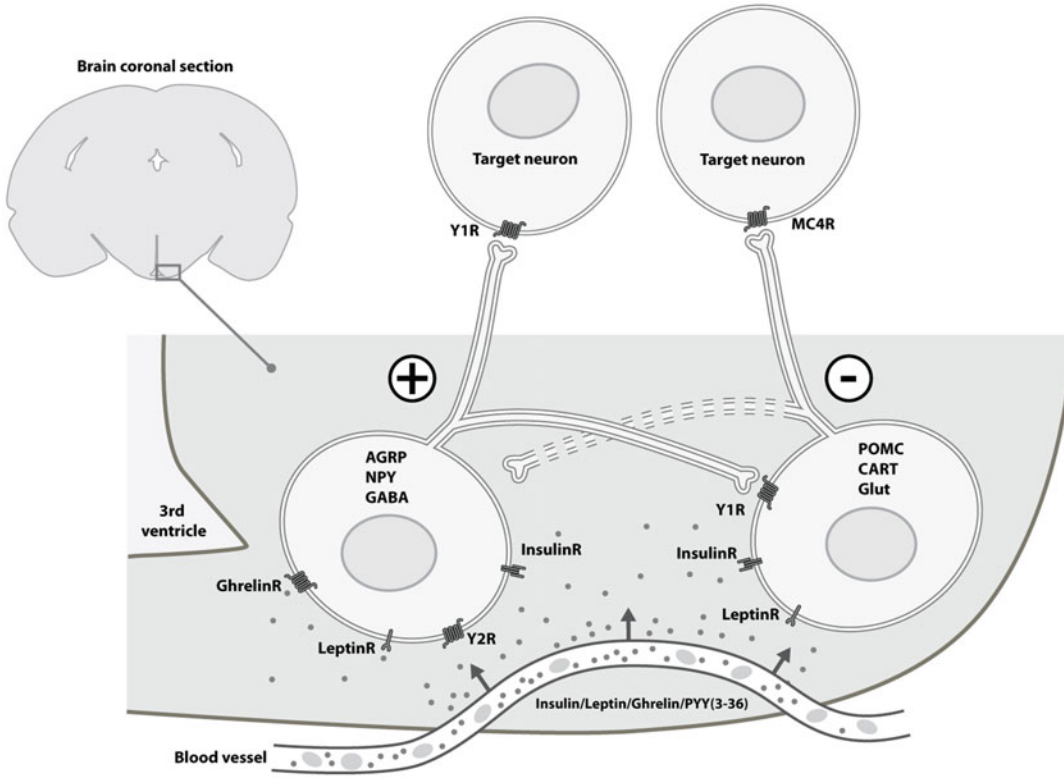

Fig. 3 The Arc is located in the most ventral, medial aspects of the hypothalamus, adjacent to the third ventricle and the median eminence, and ventral to the ventromedial nucleus. It harbors a large number of neuronal populations characterized by different neurochemical markers, in many cases coexisting [53, 139]. There are two prominent cell groups which have received much attention due to their involvement in control of food intake: the NPY/AGRP neurons (ns), in part GABAergic (NAGns), and the POMC/CART neurons, in part glutamatergic (PCGns). In contrast to many other neurons in the Arc, they do not project to the external layer of the median eminence, but their axons are directed into the brain, targeting both more closely located hypothalamic nuclei such as the paraventricular nucleus, and the perifornical area, harboring neurons expressing orexin/hypocretin and the melanin-concentrating hormone (MCH), but also sending axons to the lower brain stem such as the solitary tract nucleus, an input station for the vagus nerve and targets for blood-borne hormones like cholecystokinin, conveying both catabolic and anabolic information. The NAGns stimulate food intake (+), whereas the PCGns exert the opposite effect (−). The NAGns also innervate the PCGns, releasing NPY acting on inhibitory postsynaptic Y1 receptor (Y1Rs) on the PCGns. A reciprocal innervation, i.e., PCGns innervating the NAGns, is probably of less importance. Both NAGns and PCGns express leptin (cytokine receptor-type) and insulin (tyrosine kinase-type) receptors, whereas Y2 and ghrelin receptors are only found on NAGns. Since the Arc partly is outside the blood-brain barrier, leptin, insulin, ghrelin, and PYY [3–36] can, even if large molecules, access the Arc neurons. However, active transport molecules may also be involved. This is a highly simplified drawing. For more detailed information and references, *see* [1, 2, 4–6, 12, 13, 15, 16, 140–152]

the lower brain stem [49]. Leptin and insulin bind to their respective receptors on the AGRP/NPY and POMC/CART neurons, affecting their activity by inhibiting the former and activating the latter [2, 12, 66]. It has also been established that the GABA/

NPY/AGRP neurons innervate the POMC/CART neurons [64]. Due to their roles in the regulation of food intake, both the AGRP/NPY and POMC/CART neurons have been extensively studied in anorectic *anx/anx* mice [42, 43, 45, 46, 48, 67].

Immunohistochemical analyses of the *anx/anx* mouse, using antibodies targeting NPY or AGRP, have revealed a reduced density of immunoreactive fibers in all projection areas studied, including the paraventricular nucleus of hypothalamus (PVN), the LHA, the dorsomedial hypothalamic nucleus (DMH), and the Arc, when comparing these mice and +/+ mice at P21 [49, 67]. In addition, both peptides show a dramatic increase in number of cell bodies and in their staining intensity in Arc, resembling what in wild-type mice is only seen after colchicine injection. Colchicine is a mitosis inhibitor and arrests centrifugal axonal transport, resulting in accumulation of molecules and organelles synthesized in the cell body and transported centrifugally in the axon [68]. In situ hybridization studies of NPY and AGRP mRNA levels have been conflicting [47, 48, 67]. Broberger et al. concluded [67] that there was no difference in mRNA levels of NPY in *anx/anx* when compared with +/+ mice at P21, which was confirmed by Jahng et al. [47]. However, a later study showed increased levels of both AGRP and NPY mRNA in *anx/anx* mice of the same age as in the previous studies [48]. One explanation for the aberrances in the AGRP/NPY system is that loss of AGRP (and NPY) in the nerve terminals is due to increased release, which is then compensated for by increased synthesis/transcript levels. However, increased transcript levels can also be due to axonal degeneration, followed by feedback-induced increase in neuropeptide synthesis.

Immunohistochemistry with antibodies against α-MSH, one of the peptides generated from the precursor protein POMC, shows markedly attenuated immunoreactive fibers in *anx/anx* hypothalamus. This is accompanied by reduced numbers of immunoreactive cell bodies for ACTH, another fragment of the POMC precursor [46]. Furthermore, immunohistochemistry for the NPY Y1 receptor, which decorates the soma and dendrites of POMC/CART neurons [69], confirms these results by showing a reduced density of both immunoreactive dendrites and cell bodies in *anx/anx* hypothalamus, suggesting atrophy or degeneration (*see* Fig. 4) [46].

In addition, two studies have reported serotonergic hyperinnervation of Arc, as well as the olfactory bulb, frontal cortex, hippocampus, and cerebellum in *anx/anx* mice [41, 47]. Serotonin decreases food intake, and accordingly, increased serotonergic innervation of Arc fits the anorectic phenotype of these mice. It is also possible that the reported alterations in the serotonergic system affect the abnormal motor behavior of the *anx/anx* mouse, e.g., head weaving, tremors, and uncoordinated gait. In fact, 15-day-old normal mice that are treated with a serotonin precursor display these type of behaviors, and treating *anx/anx* mice with a

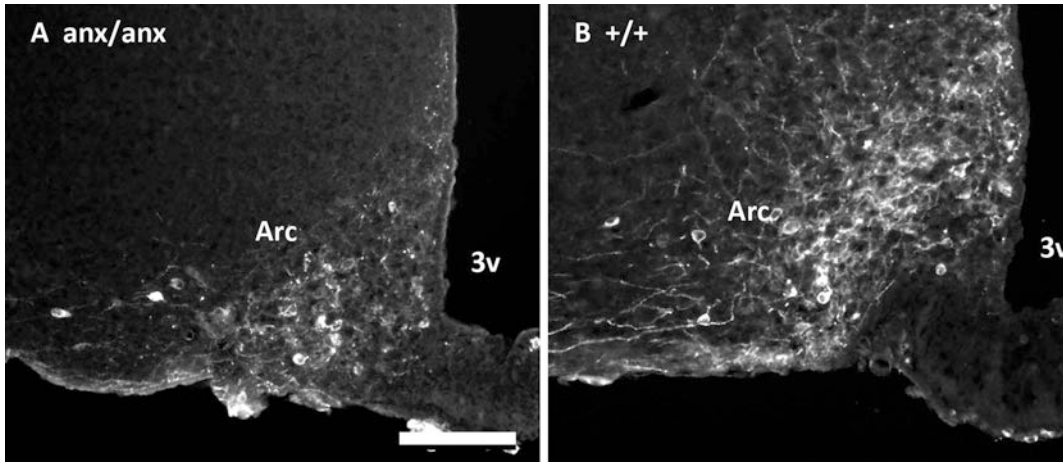

Fig. 4 Immunohistochemistry for *Y1* a marker for POMC/CART neurons in Arc of an *anx/anx* (A) or +/+ mouse (B). Note the reduced density of Y1-ir cell bodies and dendrites in the *anx/anx* mouse. 3v, third ventricle; *Arc* arcuate nucleus. Scale bar = 10 μm

serotonin antagonist has been shown to stabilize their motor abnormalities [35].

Abnormal dopaminergic neurotransmission has also been demonstrated in the *anx/anx* mouse [44]. In this study, a decrease of dopamine and its metabolites was detected in the striatum of *anx/anx* mice. The Na^+, K^+-ATPase activity, which is normally inhibited by dopamine, was upregulated in striatum of the *anx/anx* mouse, and isolated neostriatal neurons failed to respond to exogenous dopamine [44]. Changes in dopaminergic, as well as serotonergic systems, are rather well documented in AN [70–72]. Finally, increased expression of neurotrophin tyrosine kinase receptor 3 (*NTRK3*) has been detected in the hypothalamus, but not in the cortex, of the *anx/anx* mouse. Interestingly, the *NTRK3* gene has also been associated with eating disorders in humans [73].

3 Notes

3.1 Postnatal Development of Food Intake-Regulating Systems in the Anx/Anx *Mouse*

Using AGRP as a marker for the Arc NPY neurons, we have studied the postnatal development of this orexigenic system in the *anx/anx* mouse. The AGRP/NPY system is known to develop postnatally in mice, i.e., there is a continuous increase in fiber density during the first 3 weeks of life, reaching adult appearance by P21 [56, 74, 75]. In *anx/anx* mice, this system initially appears to develop as in wild-type mice, but around P12–P15, the normal increase in fiber density ceases. Between P15 and P21, the amount of fluorescent fibers is reduced in the *anx/anx* mouse (exemplified by PVN in Fig. 5 A, B, E, and F) [43]. Whether this represents increased release of AGRP and NPY, abrupted development and/or degeneration remains to be determined, even though findings to be presented below support the latter.

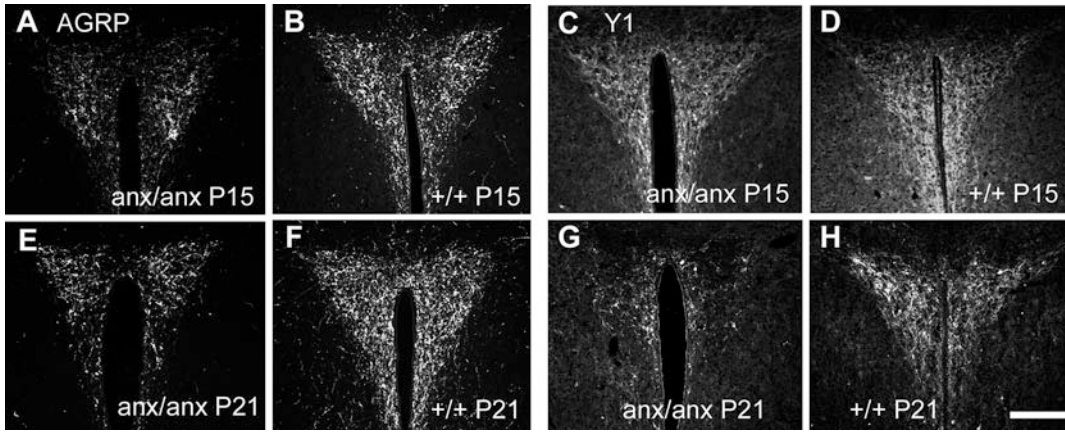

Fig. 5 Immunohistochemistry for AGRP (**a**, **b**, **e**, and **f**) and Y1 (**c**, **d**, **g**, and **h**) in PVN of *anx/anx* (**a**, **c**, **e**, and **g**) and +/+ mice (**b**, **d**, **f**, and **h**) at P15 (**a**–**d**) and P21 (**e**–**h**). Note that a reduced density of AGRP-ir fibers in the *anx/anx* mice is visible already at *P15* while such a reduction in Y1-ir processes is present first by P21. *AGRP* agouti gene-related protein, *Y1* neuropeptide Y receptor 1. Scale bar = 200 μm

The development of the anorexigenic POMC system was also studied, using the NPY receptor Y1 as a marker. Here, we concluded that the POMC system develops as in normal mice until after P15; thus, a reduced density of Y1-positive fibers was detected first by P21 (exemplified by PVN in Fig. 5 C, D, G, and H) [42]. In fact, the POMC neurons do not show signs of degeneration until P21, thus succeeding the disappearance of AGRP/NPY fibers, which are already showing reductions at P12–P15 [43].

From P8, *anx/anx* mice have significantly reduced serum leptin levels [45] which is not surprising, keeping in mind that this is an adipocyte-derived hormone. Based on leptin's role in the postnatal development of the food intake-regulating neurons in Arc [56, 76], one can speculate that the low levels of leptin contribute to the aberrances seen in these circuits in the *anx/anx* mouse, including neurochemical, neurodevelopmental, and neurodegenerative processes. Bouret et al. showed leptin's neurotrophic effect on the Arc neurons in studies on the leptin-deficient *ob/ob* mouse [77]. In fact, the *ob/ob* mouse shows an abnormal development of Arc projections, resembling the pattern seen in *anx/anx* mice. By postnatally injecting leptin in *ob/ob* mice, it was possible to normalize the development of these projections [77]. Individuals with AN have decreased levels of leptin [78].

3.2 Hypothalamic Neuroinflammation

Several studies have indicated an association between hypothalamic inflammation and the anorexia of *anx/anx* mice. Laucher and colleagues, using gene expression analyses of the hypothalamus, concluded that an inflammatory response is related to the phenotype [79]. In a second gene expression study of the *anx/anx* hypothalamus, Mercader and colleagues also concluded that the

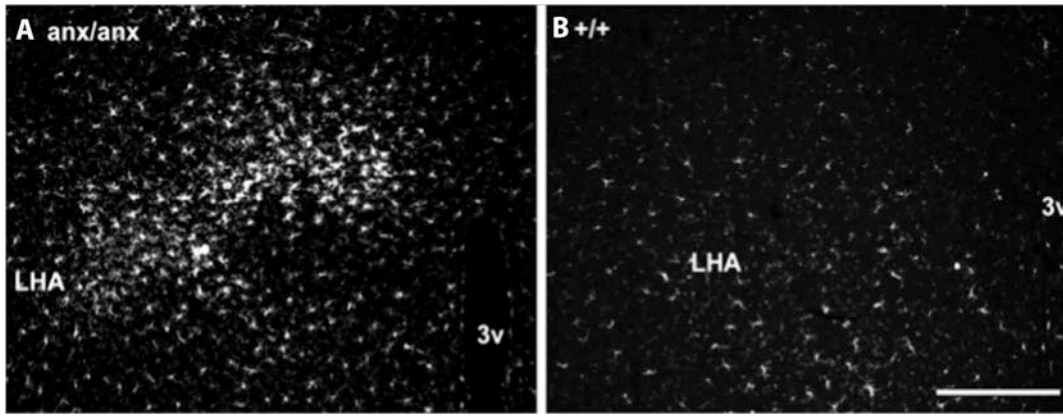

Fig. 6 Activated microglia cells, visualized by ionized calcium-binding adapter 1 (Iba1) *IHC* in hypothalamus of an *anx/anx* (**a**) or +/+ mouse (**b**). *LHA* lateral hypothalamic area, 3v, third ventricle. Scale bar = 200 μm

phenotype is associated with changes in several inflammatory genes [80]. The same team also showed altered microRNA machinery expression in the *anx/anx* hypothalamus (as well as cortex), which they concluded is consistent with its involvement in inflammatory/cancer-associated anorexia-cachexia [81]. In parallel, we have shown a strong activation of microglia cells in *anx/anx* mice, specifically in regions to which the food intake-regulating AGRP neurons project, in particular hypothalamic regions. Signs of activated microglia in these regions have been documented from P12, with a progressive increase reaching strong activation at P21, exemplified by LHA in Fig. 6 [43]. We also showed that the activated microglia express toll-like receptor 2 [43].

Microglia cells are normally activated in the central nervous system in response to neuroinflammation and/or neurodegeneration [82–84], and the results indicate that such processes occur in the hypothalamus of the *anx/anx* mouse. In addition, we have provided evidence of expression of major histocompatibility (MHC) complex I by glia cells located in the areas to which the AGRP neurons project (LHA, PVN, and DMH) as well as by Arc neurons [42]. MHC class I is well known to be expressed by activated microglia in response to inflammatory stimuli and by neurons during pathological conditions, such as acute inflammation [85–89].

Inflammatory mechanisms in the hypothalamus have been related to the impaired signaling seen after feeding with high-fat diet [90], in obesity [91], and in cachexia [92]. Thus, it seems that conditions of both excess and shortage of energy are coupled with hypothalamic mechanisms involving inflammation [93]. This appears paradoxical, and a critical evaluation of the complex relation of the energy balance to the hypothalamic inflammatory

progression in the different conditions is urgently needed. It is possible that studies of the *anx/anx* mouse may help resolve this paradox.

3.3 Hypothalamic Neurodegeneration

In addition to the signs of neuroinflammation and the loss of AGRP and NPY in nerve terminals, several observations have indicated ongoing neurodegenerative processes in the hypothalamus of the *anx/anx* mouse. Initially, Broberger and coworkers observed a reduced staining of POMC cells with an apparent retraction/shrinkage of Y1-immunoreactive dendrites, suggesting a degeneration of the POMC/CART system of *anx/anx* mice at P21 [46]. It should be noted that this view was based on data with a chemical marker, the Y1 receptor protein. Thus, no "structural" degenerative changes were demonstrated, and a downregulation of Y1 receptor synthesis could not be excluded. Interestingly, as said, the loss of AGRP/NPY in terminals precedes the signs of degeneration seen in the POMC system. It is thus possible that the POMC system degenerates as a consequence of the lost input from the AGRP/NPY neurons.

The finding of a strong activation of microglia cells, overlapping both in time and region with the reduced density of AGRP immunoreactive fibers [43], gave further support to the neurodegeneration hypothesis in the *anx/anx* Arc. These results agree with results from Wu et al., showing that ablation of AGRP neurons results in gliosis in the target areas [94] and anorexia [95]. Moreover, the expression of MHC I by Arc neurons [42] may also support involvement of such a process, since MHC class I has been shown to be expressed by degenerating [96, 97] or lesioned [98] neurons. We have detected an increased number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive neurons in *anx/anx* hypothalamus, as well as possible signs of what Ribak et al. call "microglia-associated cell death" [42, 99]. This type of cell death seems to be different from both apoptosis and necrosis, whereby the microglia cell appears to embrace a neuron and create "holes" in the plasma membrane, resulting in a watery cytoplasm and nucleoplasm and damaged organelles. We have also detected NPY-positive fibers expressing activated caspase 6 [42], a marker for axonal degeneration [100]. Taken together, all of these results strongly indicate degenerative processes in the hypothalamus of the *anx/anx* mouse.

With regard to neurodegeneration, it should be mentioned that bromodeoxyuridine- and TUNEL-labeling studies have shown increased cell proliferation and apoptosis in dentate gyrus of *anx/anx* mouse [101]. A differential display analysis of the whole brain identified, among other genes, the apoptotic protease activating factor 1 as being regulated by the *anx* gene [102]. Last, our microarray analysis of the Arc region, mentioned previously, indicated that several genes related to apoptosis/cell death/degeneration are dysregulated in the *anx/anx* mouse (*see* Table 2).

Table 2
Genes related to degeneration/cell death/apoptosis with altered expression in the *anx/anx* Arc

Symbol	Full gene name	Fold change
HLA-C	Major histocompatibility complex, class I, C	7.5
USP18	Ubiquitin-specific peptidase 18	5.6
STAT1	Signal transducer and activator of transcription 1, 91 kDa	5.6
IFIH1	Interferon induced with helicase C domain 1	4.6
B2M	Beta-2-microglobulin	3.3
GFAP	Glial fibrillary acidic protein	2.8
CTSS	Cathepsin S	2.3
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	2.2
PIM3	Pim-3 oncogene	2.1
BCL2L11	BCL2-like 11 (apoptosis facilitator)	2.1
HMGB1L1	High-mobility group box 1 pseudogene 1	2.0
GSTM5	Glutathione S-transferase mu 5	1.8
CNP	2′,3′-cyclic nucleotide 3′ phosphodiesterase	1.7
SOD1	Superoxide dismutase 1, soluble	1.7
VAMP3	Vesicle-associated membrane protein 3 (cellubrevin)	1.7
HINT1	Histidine triad nucleotide binding protein 1	1.6
EGR1	Early growth response 1	1.6
MBP	Myelin basic protein	1.6
ZNF622	Zinc finger protein 622	1.6
NDN	Necdin homolog (mouse)	1.6
GLO1	Glyoxalase I	1.5
MT2A	Metallothionein 2A	1.5
CSF1R	Colony-stimulating factor 1 receptor	1.5
FKBP1A	FK506 binding protein 1A, 12 kDa	1.5
DTYMK	Deoxythymidylate kinase (thymidylate kinase)	1.5
LAMP1	Lysosomal-associated membrane protein 1	1.5
RPS6	Ribosomal protein S6	1.5
HK1	Hexokinase 1	1.5
CTTN	Cortactin	1.5
GJA1	Gap junction protein, alpha 1, 43 kDa	1.5

(continued)

Table 2
(continued)

Symbol	Full gene name	Fold change
PAK3	P21 protein (Cdc42/Rac)-activated kinase 3	−1.5
PNP	Purine nucleoside phosphorylase	−1.5
ABCD2	ATP-binding cassette, subfamily D (ALD), member 2	−1.5
GABRB2	Gamma-aminobutyric acid (GABA) A receptor, beta 2	−1.5
CXCL12	Chemokine (C-X-C motif) ligand 12	−1.5
LGALS1	Lectin, galactoside-binding, soluble, 1	−1.5
MAP2K4	Mitogen-activated protein kinase kinase 4	−1.6
GRM3	Glutamate receptor, metabotropic 3	−1.6
TNFAIP8	Tumor necrosis factor alpha-induced protein 8	−1.7
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	−1.7
TUBB3	Tubulin, beta 3	−1.9
PLA2G7	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	−1.9
SLC1A2	Solute carrier family 1 (glial high-affinity glutamate transporter), member 2	−2.1
NDUFAF1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	−2.2
FSTL1	Follistatin-like 1	−2.3
IGFBP4	Insulin-like growth factor binding protein 4	−2.8

3.4 Hypothalamic Mitochondrial Dysfunction

By using mRNA microarray analysis of Arc in *anx/anx* mice, followed by Ingenuity Pathway Analysis (IPA) of the microarray dataset, we identified oxidative phosphorylation, mitochondrial dysfunction, and oxidative stress as the most likely pathways to be involved in the phenotype of the *anx/anx* mice. In addition, as mentioned above, the microarray analysis also identified the Complex I assembly factor gene, *Ndufaf1*, to be downregulated in *anx/anx* mice [38]. Blue native polyacrylamide gel electrophoresis revealed lower levels of fully assembled Complex I in *anx/anx* hypothalamus compared with +/+ mice, and in-gel staining showed lower activity of the same complex. By assessing mitochondrial respiration in the hypothalamus from *anx/anx* and +/+ mice, using high-resolution respirometry, we gained further proof of mitochondrial dysfunction, in particular related to Complex I [38]. We have also detected increased reactive oxygen species (ROS) and upregulation of antioxidative molecules in the hypothalamus of these mice [38]. Increased levels of ROS represent a common phenomenon accompanying dysfunction of Complex I. ROS are known to act as signaling molecules affecting

hypothalamic neurons, leading to decreased appetite [103–105], but is better known as a cause for oxidative stress and subsequently neuronal degeneration [106]. Thus, it is possible that increased levels of ROS are involved both directly in the anorexia or loss of appetite and in the neurodegenerative phenotype of *anx/anx* mice. Altered mitochondrial function and increased oxidative stress have been documented in individuals with AN [107]. In conclusion, we suggest that the anorexia and premature death of the *anx/anx* mouse are related to a downregulation of the Complex I assembly factor, *Ndufaf1*, followed by hypothalamic mitochondrial dysfunction, specific for Complex I.

Interestingly, knockout of *Ndufs4*, one of the subunits of Complex I, results in a mouse with several phenotypic traits in common with the *anx/anx* mouse, e.g., gliosis, induction of apoptotic pathways, retarded growth, and early death [108]. In addition, the mechanism of neurodegeneration related to Complex I dysfunction resembles events occurring in dopaminergic neurons in parkinsonism or Parkinson's disease [109–111]. In fact, it has been shown that the degeneration of the dopaminergic neurons requires a combination of Complex I dysfunction and disruption of microtubule dynamics. It would thus be interesting to study microtubules in the Arc neurons in the *anx/anx* mice [112].

3.5 Hypothalamic Metabolism

Mitochondrial dysfunction is generally associated with a stressed metabolic profile, and hypermetabolism [113–115], likely in an attempt to maintain adequate ATP levels. In some cases, however, the outcome is the opposite, i.e., mitochondrial dysfunction is associated with hypometabolism and reduced glucose uptake. This occurs in, e.g., Alzheimer's disease and epilepsy [116, 117] and is similar to what we have seen in the *anx/anx* hypothalamus. We showed lower glucose uptake, decreased lactate, and elevated phosphocreatine (PCr) as well as reduced activation of AMP-activated kinase (AMPK) under basal conditions [118]. This is comparable to the hypometabolic state of hibernation [119] and likely reflects lower neuronal activity [120]. Based on the subtype of ATP-sensitive potassium channel (K-ATP) expressed by different neuronal populations, the response to this type of metabolic stress will differ [121]. A specific subtype of K-ATP channel consisting of Kir 6.2 and SUR1 subunits becomes activated by the increased ROS levels and/or reduced levels of ATP seen upon mitochondrial CI dysfunction. This in turn results in reduced electrical activity, hyperpolarization, and ceased firing, aiming at protecting the cell from energy deficiency [122]. Firing of action potentials and neurotransmitter release are activities that require large amounts of energy, and therefore, inhibition of these processes conserves energy during conditions when energy is limited [123, 124]. This specific type of K-ATP channel is expressed by the hypothalamic POMC/CART and AGRP/NPY neurons but

also by a small number of other cell populations including the dopaminergic neurons in substantia nigra and the pancreatic beta cells [50, 125–127]. In addition, uncontrolled elevation of ROS can cause reduced firing of the AGRP/NPY neurons, thereby resulting in a reduced drive to eat [105, 128].

3.6 Pancreatic Dysfunction and Deviant Levels of Fat-Derived Molecules

In addition to the hypothalamic aberrances, the *anx/anx* mouse also displays pancreatic phenotypes [129]. More specifically, the model is distinctly glucose intolerant and releases less insulin when exposed to the glucose tolerance test. This is associated with both elevated serum free fatty acid (FFA) concentrations and increased macrophage infiltration of *anx/anx* islets [129]. The latter is indicative of inflammation [130, 131]. Increased levels of FFAs are known to inhibit glucose-induced insulin secretion [132]. On the contrary, isolated *anx/anx* islets that are cultured in the absence of FFAs release more insulin upon glucose stimulation, compared with islets from +/+ siblings, and show no signs of inflammation. Thus, the pancreatic phenotype of the *anx/anx* mouse is likely related to the elevated FFAs and inflammation of pancreatic islets. This is interesting in the light of a few studies showing increased incidence of eating disorders in young women with diabetes [133, 134], as well as the finding of increased levels of circulating FFAs in AN [33, 135]. In addition, analogous to individuals with AN, the *anx/anx* mouse has high levels of serum cholesterol [35, 45, 78, 136].

4 Conclusion

Thus, the *anx/anx* mouse have several characteristics in common with eating disorder patients, in particular AN. These include emaciation, starvation, premature death, pancreatic dysregulation, increased FFA and cholesterol, and reduced leptin. For some of the other phenotypical traits of the mouse, it remains to be fully established if they also occur in patients, i.e., neurodegeneration, neuroinflammation, and mitochondrial dysfunction, potentially in the hypothalamus, even though some support for the role of these mechanisms in AN does exist [107, 137, 138]. Taken together, the *anx/anx* mouse is a very valuable tool for further evaluating and increasing the knowledge about the (neuro)biology and etiology of AN.

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Chapter 17

Exploring the Neural Underpinnings of an Antidepressant and Rewarding Action of Early Anorexia

Valérie Compan

Abstract

Organisms do not make the decision to feel hungry, but they can decide to satisfy, or to not satisfy, hunger. Consuming foods then maintains energy balance and can favor rewarding effects related to motivation to obtain food ("wanting"), defining eating behavior. In this context, this chapter describes part of the neural basis of eating behavior, focusing on critical action of serotonin (5-hydroxytryptamine, 5-HT) 4 receptors (5-HT_4Rs) under stressful conditions. We found that 5-HT_4Rs, located in an adaptive-decisive system (voluntary nervous system), including the medial prefrontal cortex and the nucleus accumbens, may favor rewarding and antidepressant effects of restrictive food intake (anorexia-like behavior). Here, we describe experimental procedures which have been associated in order to study a part of the neural bases underlying food intake following intracerebral infusion of pharmacological and nucleic treatments (siRNA, virus) in freely moving mice treated or not with a recreational drug of abuse ("ecstasy"). It includes the description of a micropunch technique required for analyzing specific downstream molecular events (cAMP: FRET, pCREB: Western blot, mRNA: RQ-PCR, binding sites: radioautography). Our conclusion introduces that processes within the voluntary nervous system (underlying decision, motivation) could be modified to prevail over a cerebral autonomous control (hypothalamus) of hunger, compromising survival. The 5-HT_4Rs could be targeted with antagonist/inverse agonist combined to psychological approach to better cope with the stressors related to anorexia and drug dependence.

Key words Anorexia, Addiction, Animal and genetic models, Pharmacology, Microsurgery, Restraint stress, Forced immobilization

1 Introduction

The description of some characteristics of eating disorders related to insufficient or excessive food consumption, including complex devastating mental diseases such as anorexia nervosa and bulimia nervosa, can be found in a myriad of texts from the past six centuries. To the best of our knowledge, the most illustrious example is that of Catherine de Siena (1347–1380) [1]. However, only in the seventeenth century did anorexia became the subject of a clinical description by Thomas Morton (1689), and the first scientific

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accounts by Charles Lasègue in France and William Gull in England date from the beginning of the nineteenth century [2].

To simplify, bulimia is characterized by impulsive and repeated phases of high food intake, whereas the main characteristic of anorexia is a self-imposed food restriction despite the energy requirements [3]. The patients, usually women, may suffer from either anorexia or bulimia or from both disorders simultaneously [3]. These disorders often correlate with deregulations of neural circuits [3] and often coexist with other mental diseases, such as pathological anxiety [4] and depression [5]. In particular, a study has further strengthened the notion that anorexia-like behavior includes an addictive component [6].

In industrialized countries, patients suffering from anorexia nervosa have the highest mortality rate among people with mental diseases (4.5% to 5.9%; 0.56% per year) [7, 8]. At least 36% die within 20 years from diagnosis (i.e., between 30 and 35 years of age as the disease usually begins during adolescence). Associated symptoms include emaciation, amenorrhea, and, less known although quite frequent, overexercise, and hyperactivity [3]. Understanding such a complex disease obviously requires deciphering the associations between nonvoluntary and voluntary brain controls, biological and environmental factors, genetic and epigenetic influences, and thus the collaborative effort of psychologists, psychiatrists, and neurobiologists.

The central nervous system controls the organism energy balance by regulating food intake and energy expenditure. The neurobiological abnormalities related to anorexia nervosa are poorly understood. Hypothalamus plays a central influence in the regulation of feeding behaviors, but motivation disorders in which patients self-impose food restriction despite the energy demand (anorexia) may also involve prominent disturbances in the nucleus accumbens (NAc) and in the medial prefrontal cortex (mPFC), key cerebral structures of the reward system [6, 9]. Among the numerous neural and hormonal messengers involved in feeding disorders, altered 5-HT transmission appears to be at the forefront of the investigations. It is generally admitted that increased activity of 5-HT neurons and higher 5-HT expression in brain trigger reduced food intake and body weight loss [10]. Accordingly, treatment-induced increases in the levels of 5-HT release in brain provoke anorexia-like behavior (self-imposed food deprivation despite the energy requirement) [11]. For instance, fenfluramine increases extracellular 5-HT levels and lowers the consumption of food in humans and rodents [12, 13]. Similarly, amphetamine and 3,4-*N*-methylenedioxymethamphetamine (MDMA, ecstasy) diminish food consumption in rats [14] and humans [15] and reduce deprivation-induced eating in mice [16].

The 5-HT neurons and receptors (5-HTRs) are distributed throughout the brain, including all anatomical regions classically

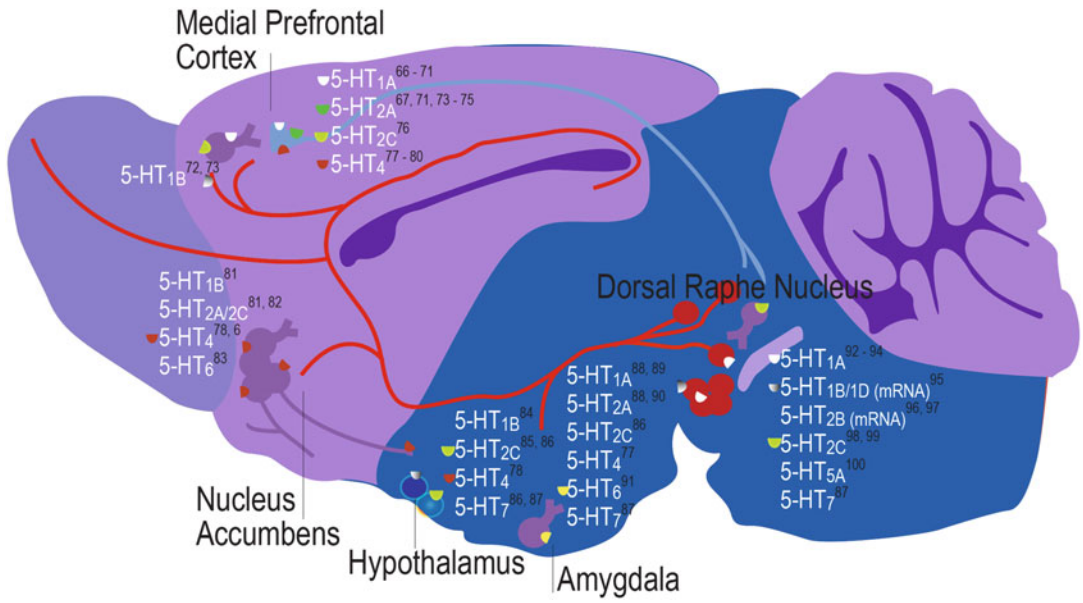

Fig. 1 Schematic representation of (A) 5-HT receptor subtypes present in brain regions involved in feeding behavior as reported by several authors [6, 17–51]

involved in feeding behavior (*see* Fig. 1). Over the past three decades, seven families of 5-HTRs have been identified with 18 5-HTR subtypes, without including mRNA editing and splice variant isoforms. The implication of 5-HTRs in behavior has been widely described in studies using mice dispossessed of one 5-HTRs: 5-HT_{1A} [52], 5-HT_{1B} [53], 5-HT_{2A} [54], 5-HT_{2B} [55], 5-HT_{2C} [56], 5-HT_3 [57], 5-HT_4 [58], 5-HT_{5A} [59], 5-HT_6 [60], or 5-HT_7 [61]. Both hypothalamic and NAc neurons express different 5-HTRs. In the NAc, 5-HT_4Rs were the first example of a 5-HTR, described for their influence on food in fed and food-deprived mice [6]. If one considers that anorexia nervosa is related to motivational disorders to consume food, 5-HT_4Rs are potential targets for pharmacological treatment, and the same is likely for bulimia. For now, both of these diseases remain not well controlled with pharmacological treatments.

Anorexia and bulimia nervosa in humans are extremely complex compared to the "simplicity" of the animal models. However, animal models represent, so far, the first required step to propose molecular therapeutic targets and alternatives. Obtained results in animal models may further ensure the "anecdotal evidence that anorexia sufferers might be hooked on the self-starvation and self-control involved in the disorder" [62].

Using animal models, we postulated that anorexia might involve altered signaling events within the NAc and the mPFC, brain structures involved in reward; it might favor a rewarding and then an antidepressant action of early anorexia [6, 63]. To

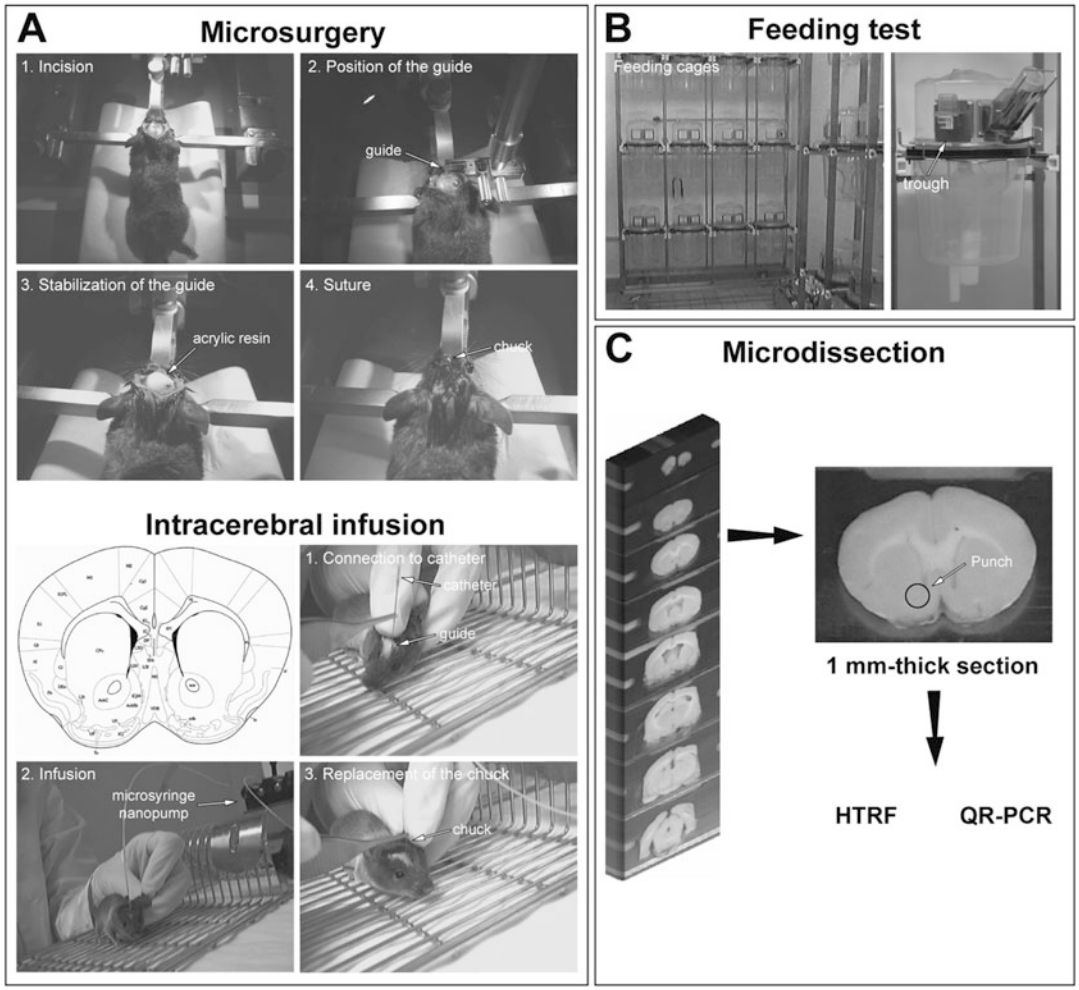

Fig. 2 Overview of the experimental strategy: from molecule to behavior analyses. (**a**) Microsurgery for intracerebral infusion of compound (agonist, antagonist, inverse agonist, siRNA, transformed virus) in freely moving animals. Five days before any infusion of compounds into a specific brain area, a steel guide is unilaterally implanted at coordinates from the bregma according to the brain atlas (described in details in Subheading 2.1). (**b**) Following classic measurement of the intake of food (excluding the spillage), animals are sacrificed at a determined and precise time period following the treatment, which are then infused at 7 min interval between each mouse. The brains are quickly frozen in isopentane cooled with liquid nitrogen (or dry ice) and stored at −80 °C. (**c**) Brains are sliced into 1 mm, and tissue samples were microdissected at −20 °C. Tissue samples are then treated using the HTRF technique (described in details in Subheading 2.2) or QR-PCR

address this possibility, we examined the effects on food intake of directly stimulating or inactivating 5-HT_4Rs in the NAc (*see* Fig. 2, microsurgery in freely moving animals). The 5-HT_4Rs have been selected because we have previously observed attenuated feeding responses to the restraint stress (forced immobilization) in 5-HT_4R knockout (KO) mice compared with wild-type (WT) mice (*see* Fig. 2) [58]. We also investigated whether 5-HT_4Rs are further

invo(lved in the anorectic effect of MDMA by using a combination of pharmacological, biochemical (homogeneous time-resolved fluorescence-based, HTRF), immunocytochemical, and molecular biology techniques (quantitative real-time PCR, QR-PCR) that include intracerebral injection of siRNA-mediated 5-HT_4R (si5-HT_4R) knockdown into the NAc (siRNA-mediated knockdown in adult mouse). Using 5-HT_4R KO mice, we further determined that 5-HT_4Rs contribute to the appetite-suppressant effect of MDMA [6, 64]. In addition, the generation of 5-HT receptor KO mouse required to use different and specific complex methods, as previously and extensively reviewed [65–67]. Additional experiments revealed that the 5-HT system in the NAc mediates reduction in motivation for food in food-deprived mice, mediating anorexia-like behavior through the activation of addictive signaling (cyclic adenosine monophosphate [cAMP]/protein kinase A [PKA])/cocaine- and amphetamine-regulated transcript [CART]) under the control of 5-HT_4Rs [6], suggesting commonalities between restrictive food intake and addiction [64]. Indeed, in neurons of the NAc, activation of a cAMP signaling is a means of transforming an immediate reduction of drugs' rewarding effect into a durable dependence. Drugs of abuse, such as cocaine and amphetamine, trigger adaptive responses, including an increased activity of a cAMP/PKA signaling pathway in the NAc [68–70]. The resultant phosphorylation of the cAMP-responsive element binding (CREB) dampens rewarding effects. In consequence, the sensitivity to subsequent drug exposures decreases (tolerance) with increased activity of reward pathways (dependence) to the point that drugs removal triggers declines in motivation, mimicking depressive-like behavior [71]. Anorexia-like behavior is induced by local stimulation of a cAMP/PKA/CART pathway, in the NAc, induced by stimulation of the NAc-5-HT_4Rs [6]. This signaling pathway is also implicated in anorexia induced by MDMA [6]. The ability of cocaine addiction-related animal models, as 5-HT_{1B}R KO [72], to self-impose restrictive food intake despite an early period of food deprivation further depends on a gain-of-function of 5-HT_4Rs with CART overexpression in the NAc [64]. Considering the involvement of CART in motivational properties of cocaine [73, 74], these findings evidence a "shared neural signal foul-up" between drug dependence and anorexia, consistent with deficits in neural networks underlying addiction in patients with anorexia nervosa [75, 76]. The rewarding effect of anorexia has been described in humans at the onset of anorexia nervosa symptoms [77]. Indeed, the brain can implement food restriction until death, as the result of maladaptive decision-making. Since the prospect of receipt of a positive reward is capable of inducing risky and potentially lethal behavior, potential neural deficits that restrict food intake to a lethal point could be included in those of dependence. Interestingly, cocaine administration increased the phosphorylated

CREB (pCREB/CREB ratio) in the NAc in WT mice, but not in 5-HT_4R KO animals [3], suggesting that 5-HT_4Rs enhance CREB phosphorylation. Considering that inhibition of the transcription factor CREB in the NAc has been associated with anxiety-like behavior [78], anorexia induced by stimulation of 5-HT_4Rs in the NAc could favor the "anxiety-reducing character to dietary restraint" [79–81]. In contrast, reduced activation of 5-HT_4Rs could enhance anxiety [58, 82] that is provoked by overeating.

Accordingly, activation of mPFC-5-HT_4Rs reduced food intake through an antidepressant pathway [63]. The ascending serotoninergic inputs from the dorsal raphe nucleus (DRN) to the mPFC, known to be implicated in adaptive goal-directed behavior (decision) to avoid adverse effect of stress and then depressive states [83–85], are involved in eating behavior under basal and stressful conditions [63]. The neural underpinnings of stress-induced hypophagia and the mechanisms by which the brain prevents the transition from transient to persistent hypophagia were undetermined. This study [63] shows the involvement of a network governing goal-directed behavior (decision). This network consists of the ascending serotonergic inputs from the DRN to the mPFC. Specifically, adult restoration of 5-HT_4R expression in the mPFC (viral gene transfer in 5-HT_4R KO mice) rescues hypophagia and specific molecular changes related to depression resistance in the DRN (5-HT release elevation, 5-HT_{1A}R, and 5-HT transporter reductions) of stressed 5-HT_4R KO mice. The adult mPFC-5-HT_4R knockdown (induced by local infusion of siRNA into the mPFC) mimics the null phenotypes. When mPFC-5-HT_4Rs are overexpressed and DR-5-HT_{1A}Rs are blocked in the DRN, hypophagia following stress persists, suggesting an antidepressant action of early anorexia. This later recent study introduces that processes within the voluntary nervous system (underlying decision, motivation) could be modified to prevail over a cerebral autonomous control (hypothalamus) of hunger, compromising survival. This possible primary mechanism could support the onset of a persistent hypophagia, "an early anorexia," whereby individuals shift from adaptive to persistent maladaptive, restrictive food choice as in anorexia nervosa [86]. These findings could be relevant for a better understanding of the neural underpinnings of decision-making to eat [3] and is consistent with a recent study indicating that citalopram (a selective serotonin reuptake *inhibitor, an antidepressant*) favors choices of more healthy foods over less healthy foods [87].

2 Materials

2.1 Microsurgery

Intracerebral infusion of the compound into the NAc or any other brain area, as the mPFC, in freely moving mice required the implantation of a permanent sterile stainless steel guide (internal diameter,

0.405 mm; external diameter, 160 mm). A chuck is required to temporarily fill the guide until injection. The length of the guide and chuck is strictly equal and has to be determined according to the coordinates to the brain atlas [88]. The coordinates are adapted for mice of 30 grams. In any case, the precise location of the site of injection has to be assessed using a classic cresyl violet staining.

The guide is stabilized and maintained on the bone surface with a nontoxic acrylic resin (liquid/powder mixture). These products have to be stored in a cool place away from direct sunlight. The liquid is highly flammable and has to be stored to avoid sources of ignition. The liquid/powder mixture reaches a dough state 15 s after mixing. The manipulation should be then finished before 2 min after mixing when setting starts.

Mice are deeply anesthetized by intraperitoneal (i.p.) injection ketamine (60 mg/kg) and xylazine (15 mg/kg). Ketamine is an anesthetic and xylazine takes a double advantage to be sedative and analgesic. Lachrymal frost is systematically used in order to protect the eyes of each animal over the surgery period from light. This mixture of both compounds is then recommended in the *Guide for Care and Use of Laboratory Animals* established by the Centre National de la Recherche Scientifique (CNRS, France). Each compound used are stored and maintained systematically at 4 °C until use and are dissolved in NaCl (9‰) on the surgical day. The dissolved solution is maintained at 4 °C for all the surgical session.

On the treatment day, each compound is infused at a precise rate (1 μl/min) into the target brain area using a microsyringe nanopump. The guide is connected to a catheter on one side while the other extremity is linked to a syringe placed on the microsyringe nanopump.

2.2 Restraint Stress or Forced Immobilization

Animals were handled and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (European Communities Council Directive of 24 November 1986 (86/609/EEC). Mice were housed three to six per cage and allowed to acclimatize for at least 5 days after their arrival. All animals were then housed in individual cages and handled daily for 5–7 days before the experiment *per se* to minimize handling-associated stress; this period is thought to correspond to the complete adaptive process [89]. Some mice were subjected to acute immobilization stress or restraint stress according to a well-established protocol [90, 91], as described in details below. In any case, the animals were randomly assigned to stress procedure or control groups. Two ways could be used for the execution of immobilization stress: immobilization-induced stress with restrainer device and immobilization-induced stress without restrainer.

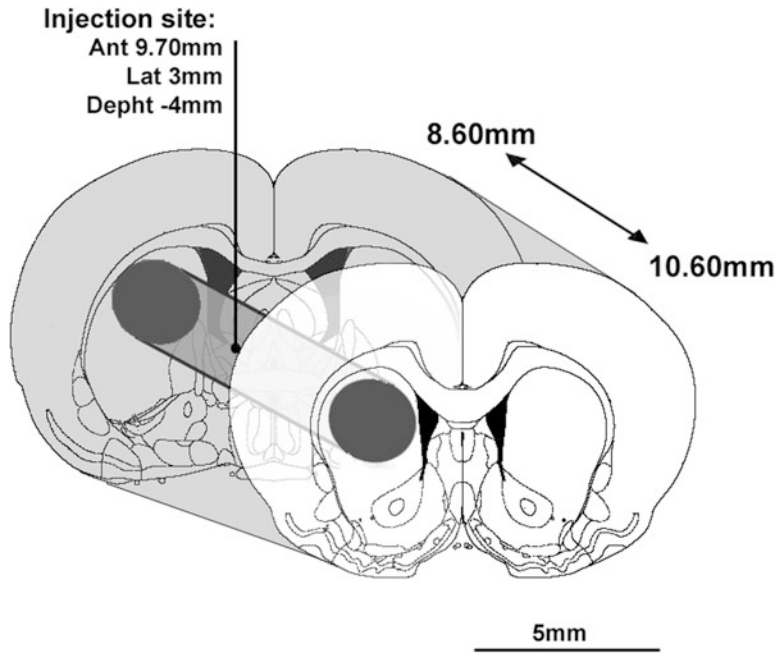

Fig. 3 Illustration of the punch technique. Two millimeters striatal sections obtained at −20 °C using a specific slicer designed for the present investigation. Inside sections, a cylinder (2 mm diameter) was dissected with a micropunch allowing to obtain cylindric striatal samples of 6.28 mm^3 according to the coordinates of the stereotaxic atlas of Paxinos and Watson (1997) [92]: anterior, 8.60–10.60 mm from interaural line. Punched samples included the treatment (e.g.) injection site located at anterior: 9.70 mm from the interaural line

2.3 Micropunch Technique

Animals are sacrificed (e.g., 3 h) after the various treatments and the brain area (e.g., NAc, 1.2 mm^3; hypothalamus, 2 × 3.9 mm^3) microdissected from 1 mm-thick sections at −20 °C using a micropunch following the landmarks of the stereotaxic atlas (NAc, A +1.6 mm; hypothalamus, A +0.58 and −1.58 mm) from bregma [88]. Figure 3 illustrates the micropunch technique [6, 93] and illustrated here in rat for the striatum. The 1-mm-thick brain sections are performed using a brain slicer matrice allowing the precise sectioning of brain.

From these tissue samples, total mRNA can be then isolated for example and the mRNA of your interest can be analyzed, as described [93, 94].

3 Methods

3.1 Microsurgery

One night before the surgery, mice are placed in the surgery room in order to adapt and avoid any stress related to the novelty of the surgery room compared to their usual room. Mice have to be

operated over the morning until 4 p.m. in order to approach their active night phase. They have further to recover sufficiently before the active period.

Mice are anesthetized by i.p. injection of ketamine (60 mg/kg) and xylazine (15 mg/kg) and placed in a stereotaxic frame. Over the head, the skin is precisely cut in only one movement from the top to the bottom in sterile conditions. Only the bone is treated with a diluted solution of H_2O_2 (1/10). Do not touch the skin with the H_2O_2 solution. Really small incisions at the surface of the bone are performed in order to fix the resin in a next following step. The bone is drilled in order to implant the guide. A saline solution is used to remove all dusts of bone. The guide is placed slowly according to the coordinates. The resin is then applied in order that the mixture surrounds the guide using a small spatula that does not touch in any case the guide. Experimenter has to wait for 3 min: the resin is then solid and the guide is permanently implanted. Using sterile thread of surgery, the skin is stitched. The localization of the injection sites was systematically assessed for each mouse.

3.2 Restraint Stress and Forced Immobilization

In the restraint stress paradigm, the animal has to be immobilized individually in adjustable semicylindrical, acrylic restrainer with air holes (Plexiglas restrainers) for 110 min, in the morning. Other designs with device variations have been proposed. Several models proposed, for example, to restrain the animal inside cylindrical wire mesh restrainer that was clamped at both openings and was placed inside his home cage during the restraint stress session. In another alternative, mice subjected to acute restraint stress are placed individually in a well-ventilated polypropylene tube (40 mm in diameter and 90 mm in length). The method ensures minimum movement including that of tail and involves no pain. Control mice were left undisturbed in their home cages. Body weight of each animal was measured daily, beginning 1 week before stress and up to sacrifice.

In the immobilization paradigm, mice are placed on wooden boards in prone position by taping their limbs and shoulders to metal mounts at 9:30 a.m. for 60 min, after which they were returned to their home cage. Head movements could be restricted with an appropriate loop around the neck. For the chronic stress, the group of mice is subjected to immobilization daily for 1–10 days, accordingly; control mice are handled each day for the same time period. All stress procedures begin at the same time of the day, between 09:30 h and 10:30 h, 3–4 h after the start of the light cycle, to reduce as far as possible the circadian rhythm-associated variations in stress hormone levels. The body weight and 24 h-cumulated food intake of each animal are measured daily (at 07:30 and 15:00 h) beginning 1 week before the chronic stress period and up to sacrifice. Depending on the assay used, mice are sacrificed by decapitation following stress; controls are sacrificed at identical time of day.

Immobilization stress differs from the restraint stress. Restraint stress is widely and commonly used as an appropriate stress paradigm model for the induction of acute stress. Immobilization stress is believed to be the most severe type of stress in rodent models and has comparative effects in humans. Immobilization stress, which prevents any body movement because of the taping of the legs and trunk to a wooden apparatus outside the home cage of animal, is considered a stronger stress than other paradigms [95] such as restraint stress, in which the mouse is restrained in its home cage within a cylindrical wire mesh net that prevents locomotion but is flexible and allows some body motility.

3.3 Micropunch Technique

Following sacrifice, brain should be frozen in powder dry ice because brains have to keep their form of origin. Blocks of dry ice would alter the form of brain and then of structures; tissue samples will not contain the target area. We commonly used the micropunch technique in mouse, but it can also be used in rat. The brain matrice, micropunch, slides, pincer, and tubes for collecting tissue samples should be strictly put into the cryostat and maintained at −20 °C. Otherwise, tissue samples could melt.

4 Notes

1. The troubleshooting of microsurgery is that experimenters have to be extremely precise. For each mouse, the site of injection has to be precisely assessed. If the guide is not placed in the expected location, experimental value cannot be taken into account. It then requires precisely analyzing the location of the site of injection on frontal brain sections using cresyl violet staining. When the aim is to evaluate the intake of food, it requires assessing before any period of treatments that feeding behaviors are similar compared with a non-operated group of mice.
2. Following surgery, we recommend to place the mice on a warmwater plastic pocket in order to preserve the life of the animals following surgery.
3. At the end of the surgery session, we recommend to hydrate the mice with one i.p. injection of NaCl (9‰; 800 μl/30 g). Food is directly placed inside the cage for each isolated mouse.
4. Micropunch technique requires being extremely systematic to dissect the tissue samples at the same location. The sample should be maintained at −20 °C. The cryostat chamber is then appropriated to perform this technique.
5. Prior to the restraint stress or immobilization and particularly before beginning the handling, ensure that there are no animals

presenting weight loss, abnormal locomotor activity, or wounds.

6. If several mice display abnormally weight loss during handling procedures, it would be very desirable to exclude them. Be sure also that strain(s) used were not sensitive to handling.
7. It is important to minimize the impact of environmental conditions by keeping constant the dark/light cycle, the temperature, the humidity with water and standard mouse chow ad libitum, and the same experimenter, for at least 1 week before and during the whole experimental period.
8. Make sure that mice are stressed in an isolated room (avoiding noise influences).
9. Since the procedure used in immobilization implies taping limbs, it may be necessary to remove gently the tape in order to avoid nociception.

4.1 Results

Using animal model such as the restraint stress, mimicking anorexia-like behavior, we found that 5-HT_4R knockout KO mice displayed attenuated feeding responses to acute restraint stress [58]. We next further explored the molecular mechanisms behind anorexia-like behavior and discovered similarities to ecstasy's effects. Ecstasy mimics the appetite loss characteristic of anorexia. We surmised that these effects might be centered in the NAc, one of the brain's reward centers. Stimulating these receptors in mice reduced their drive to eat and increased production of the same transcripts stimulated in response to cocaine and amphetamines (CART, *see* Fig. 4). Blocking the receptor with RNA interference increased food intake. Mice lacking 5-HT_4Rs displayed attenuated feeding responses to the appetite-suppressant effects of ecstasy (*see* Fig. 4).

5 Conclusion

Anorexia and bulimia are the sum of complex symptoms in humans. Exploring these correlations in simpler transgenic animal models only makes possible the study of molecular and behavioral phenotypes in isolation and has revealed the conservation of specific molecular mechanisms in humans ([96], reviewed in [97]). Overall, there are benefits in combining different methodological approaches (stress, microsurgery and molecular techniques) in order to analyze mechanisms underlying eating disorders such as anorexia. Adaptive techniques allowing the visualization of molecular cascade of events in human brain are not available yet. The use of the stress paradigms proposed then appears appropriate and takes advantage to mimic the psychological stress and associated behavioral changes like anorexia.

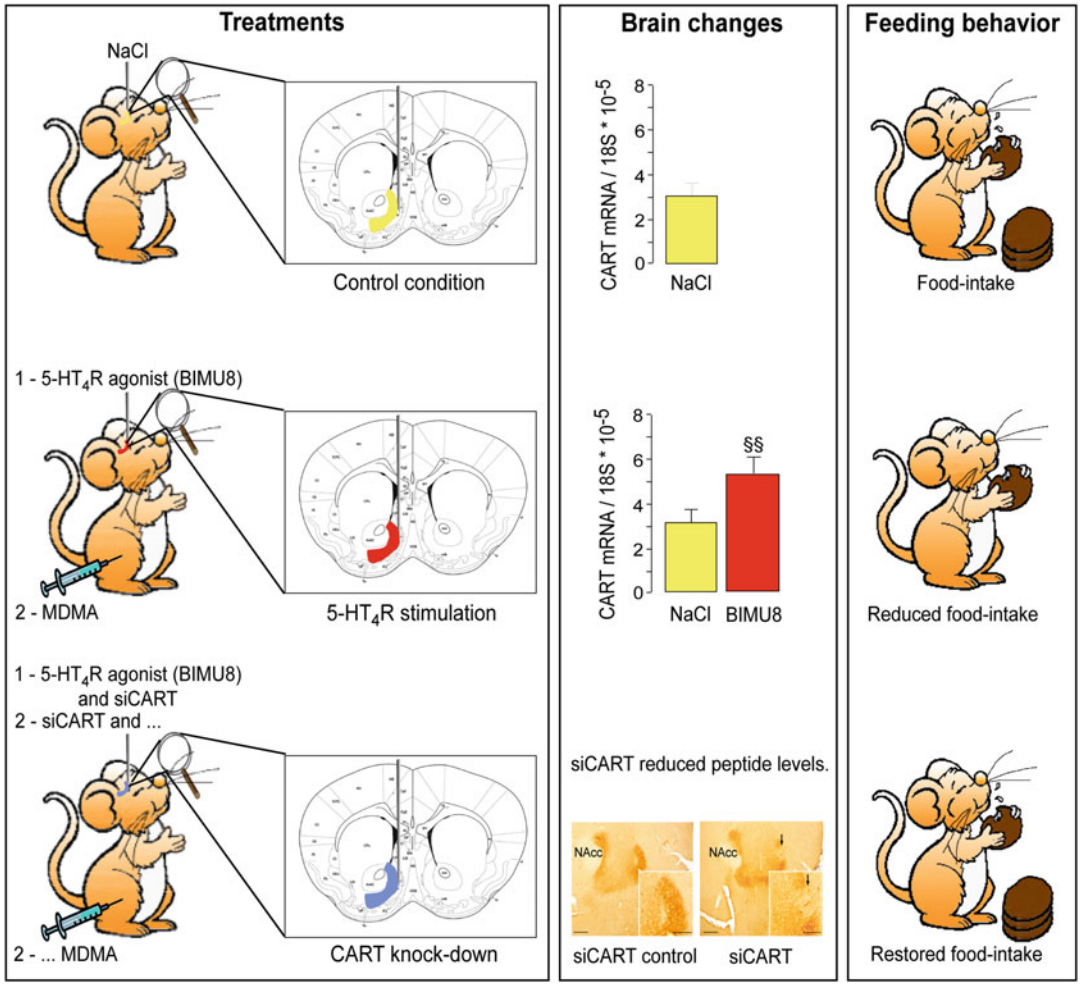

Fig. 4 Schematic representation: stimulating 5-HT_4 Rs in mice reduced their drive to eat and increased production of the same transcripts (CART) stimulated in response to MDMA (psychogenic compound of ecstasy)

The hope to detect therapeutic targets using animal model toward human can be however established (melanocortin 4 receptors and 5-HT_{2C} Rs for obesity [98]). Our animal models are highly predictive toward human clinical applications because, in agreement with our previous studies [6], findings of the G. Knudsen's team has further observed changes in the 5-HT_4R binding site density in the NAc and in the mPFC in humans suffering from eating-related disorders [99], but also from hyperphagia [100]. Consistently with decades of our findings seen in animal models [6, 58, 63, 64] (reviewed in [3, 10, 101]), the US Federal Drugs and Administration has further recently revealed that prucalopride (Motegrity™), a selective agonist of 5-HT_4Rs used for treating chronic idiopathic constipation, provoked

reduction in appetite (1–10%) and, less frequently, anorexia (0.1–1%) in patients without eating disorders, which further encourages investigation of the implication of 5-HT_4Rs in eating disorders.

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Chapter 18

Food-Anticipatory Activity: Rat Models and Underlying Mechanisms

Roger A. H. Adan

Abstract

In Western societies, the prevalence of obesity continues to increase, and hence, the need to unravel pathways and mechanisms that regulate (un)healthy food intake increases concurrently. This chapter focuses on animal models of food-anticipatory activity (FAA). In rats, FAA occurs when they have time-restricted access to food or a palatable snack. It includes increased locomotor activity and arousal prior to food access. These models can be used to shed more light on research questions, like "What happens in the brain when we think about food?" Three animal models of FAA will be discussed, namely, the activity-based anorexia model, a restricted feeding schedule model, and a palatable feeding schedule model. Descriptions of how these models are run in our lab will be provided. In addition, the potential mechanisms underlying FAA, with a special focus on leptin, dopamine, and ghrelin signaling, will be discussed.

Key words Food-anticipatory activity, Activity-based anorexia, Ghrelin, Leptin, Dopamine

1 Introduction

1.1 Relevance for Studying Food-Anticipatory Activity (FAA)

Weight gain has increased tremendously over the past decades. In many Western societies, the majority of the adult population is considered overweight. A person is classified as overweight if his or her body mass index (BMI) is between 25.0 and 29.9 kg/m^2; a person with a BMI $\geq$ 30 kg/m^2 is defined as obese. The increased prevalence of obesity raises many health issues, since it is associated with an increased risk of diabetes mellitus, cardiovascular diseases, and several cancers [1].

Obesity occurs when energy intake exceeds energy expenditure over a period of time and excess energy is stored as fat. It is considered a multifaceted disease to which both heritability and environmental factors contribute. The estimated heritability of BMI is between 50% and 90% [2, 3]. Genome-wide association studies have identified multiple genetic loci implying obesity susceptibility [4]. However, the rapid rise in the prevalence of obesity cannot be attributed solely to genetic factors, since our genes have

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not changed considerably in this short time period. On the other hand, our environment has changed significantly. Nowadays, food is available abundantly, and physical activity levels in the population have dropped. Hence, the interaction of genetic predisposition with exposure to this obesogenic environment is likely to contribute to the onset of obesity.

In an environment with an overload of cheap palatable and energy dense foods, hunger and satiety determine the decision to eat or not to eat to a limited extent. The sight or smell of food triggers neural circuits that urge us to eat in the absence of hunger. On the other hand, staying lean is considered healthy and attractive. Thus, when confronted with palatable food, one has to decide to take an immediate short-term reward by consuming the palatable food or to suppress this and go for the delayed reward of staying lean and more healthy. This important decision is made in the brain.

Not only obese people are faced with this dilemma; it is also a crucial problem for patients suffering from eating disorders, such as bulimia nervosa, binge eating disorder, and anorexia nervosa (AN). Restricting-type AN patients are the extremes in refusing food in order to be lean. AN occurs predominantly in females and has a strong genetic origin. The average prevalence of AN is around 1% in teenagers [5], and a high mortality rate ($\geq$10%) has been reported [6, 7]. AN is characterized by several criteria as described in the Diagnostic and Statistical Manual of Mental disorders, fourth edition (DSM-IV): (1) refusal to maintain a normal body weight for age and height (BMI $\leq$ 17.5 kg/m^2), (2) intense fear of gaining weight or becoming fat, (3) disturbances in body perception, and (4) amenorrhea in women (this last criterium has been dropped in DSM5). Although not mentioned in the DSM-IV and DSM5 criteria for AN, hyperactivity is frequently considered as a symptom of the disorder [8–12].

With regard to eating disorders and obesity, an important question is: What happens in the brain when we are triggered to think about food? This chapter focuses on the behavior of FAA and its underlying mechanisms, as demonstrated through feeding models in rodents. When an animal knows when to expect food, FAA often occurs. FAA is expressed when a rodent has time-restricted access to food or a palatable treat and involves hyperactivity preceding mealtime.

A better understanding of the processes underlying FAA is clinically relevant to eating disorders, including AN and obesity, in several ways. First, the hyperactivity observed in these models reflects hyperactivity in AN patients and might share common regulatory mechanisms [8]. Second, many studies have shown that metabolism and circadian rhythms are tightly coupled [13]. Meal timing plays a pivotal role in integrating behavioral and physiological rhythms. Deficiencies in FAA could diminish this circadian organization and, in this way, hamper metabolic

function [14]. Third, conditioned cues can elicit feeding in sated rats [15] and humans [16]. As a result, entrainment to a daily treat could lead to increased vulnerability to overconsume palatable food in a specific time period or in a specific context.

Three animal models that elicit FAA will be discussed, namely, the activity-based anorexia (ABA) model, a restricted feeding schedule (RFS) model, and a palatable feeding schedule (PFS) model. In contrast to the first two models, rats on a PFS are not food-restricted but have ad libitum access to chow and limited access to a palatable treat. The descriptions of the rat models are according to how these models are performed in our lab. The potential roles of the fat-derived hormone leptin, the gut peptide ghrelin, and dopamine signaling in the regulation of FAA observed in these models will be discussed as well.

2 Materials and Procedures

2.1 ABA

In this chapter, we first focus on the ABA model to investigate the underlying mechanisms of AN. First described in 1954 [17], ABA models important characteristics of AN. Rats (or mice) are housed in cages with a running wheel and have restricted access to food usually in the beginning of the dark phase. Over time, these animals become more active, particularly in the light phase, eventually leading to excessive wheel running. In combination with decreased food intake, this results in dramatic body weight loss of more than 20% in 1 week [17, 18]. In addition, hypothermia, stomach ulceration, and a loss of estrous cycle take place [19–21]. Eventually, ABA rats will die of emaciation. One feature of the observed hyperactivity is the onset of FAA, hyperactivity preceding access to food. Some studies describe the ABA model as starvation-induced hyperactivity [22], semi-starvation-induced hyperactivity [23], or self-starvation [24] because the animals consume less food when restricted in the presence of running wheels than without them.

Several parameters contribute to the development of ABA, including species, gender, age, initial body weight, baseline activity, and period of food availability. This chapter will focus on the ABA model in rats, but ABA has also been described in other species. Mice, hamsters, and guinea pigs develop ABA, whereas hibernators and genetically obese rats are less susceptible to this model [8, 25, 26]. Furthermore, susceptibility to the ABA model depends on the genetic background within species, which has been shown in mice [27]. The majority of ABA studies have been conducted in female rats. Hyperactivity is more evident in female rats [20], whereas male rats are more susceptible to body weight loss [28, 29]. The latter could be explained by the fact that female rats have more body fat, meaning they have more energy stored and might tolerate the ABA procedure better. Young animals, which have a lower body weight,

are more susceptible to develop ABA than older, heavier rats [29]. Development of ABA is more progressive when rats were adapted to the running wheel prior to the ABA model [30]. Furthermore, high baseline levels of activity result in faster development of ABA than low baseline levels of activity [20]. Finally, the length of the period of food availability is negatively correlated with the progression of ABA. ABA is more severe in rats with 1-h daily access to food than in rats that have 2-h access to food [31]. Mice typically need at least 2 hours access to food to survive the model.

2.1.1 Experimental Setup and Procedure

Female outbred Wistar WU rats weighing 160 g upon arrival (or about 6 weeks after birth) are individually housed in a temperature- and humidity-controlled room under a 12:12 h dark:light cycle. They are allowed to acclimate under ad libitum food and water conditions. One week after arrival, rats receive transmitters (TA10TA-F40, Data Sciences International, St. Paul, Minnesota) in the abdominal cavity under fentanyl/fluanisone (0.2 mg/kg fentanyl, 10 mg/kg fluanisone, i.m.; Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium) and midazolam (2.5 mg/kg, i.p.; Dormicum®, Roche, Woerden, the Netherlands) anesthesia in order to allow continuous measurements of general locomotor activity and body temperature. After surgery, carprofen (5 mg/kg, s.c.; Rimadyl®, Pfizer Animal Health, Capelle a/d Ijssel, the Netherlands), as an anti-inflammatory agent, and saline (3 mL, s.c.), to prevent dehydration, are administered to the rats. After 2 weeks of recovery, rats are housed in novel cages containing a running wheel with a circumference of 1 m. After 10 days of free running, rats have established stable daily running wheel activities. Food is removed 1 h after the onset of the dark phase. From then on, rats have restricted access to food during the first hour of the dark phase. Body weight and water intake are measured just before the onset of dark phase. When body weight loss exceeds 20% or when body temperature is lower than 33 °C, the experiment is terminated just before onset of the dark phase for ethical reasons.

Once they acquire stable running wheel activity, female rats typically run 8000–9000 revolutions per day under ad libitum conditions. Rats over days of exposure to ABA increase their 1 hour food intake from 2–3 gr on the first day to about 7 grams at day 6–7 [32].

On the first day of the ABA model, a rat's consumption is limited to approximately 25% of its normal daily caloric intake. Although food intake will increase over the course of the ABA model, it will not reach the level of daily food intake. In addition, it will be lower compared to rats that have 1-h access to food but lack a running wheel. Body weight will decrease in ABA rats, since food intake reduces and running wheel activity increases, as depicted in Fig. 1a. It is noteworthy that the amount of fat tissue is strongly reduced. In our studies, experiments are terminated when body weight drops below 80% of the initial body weight. In

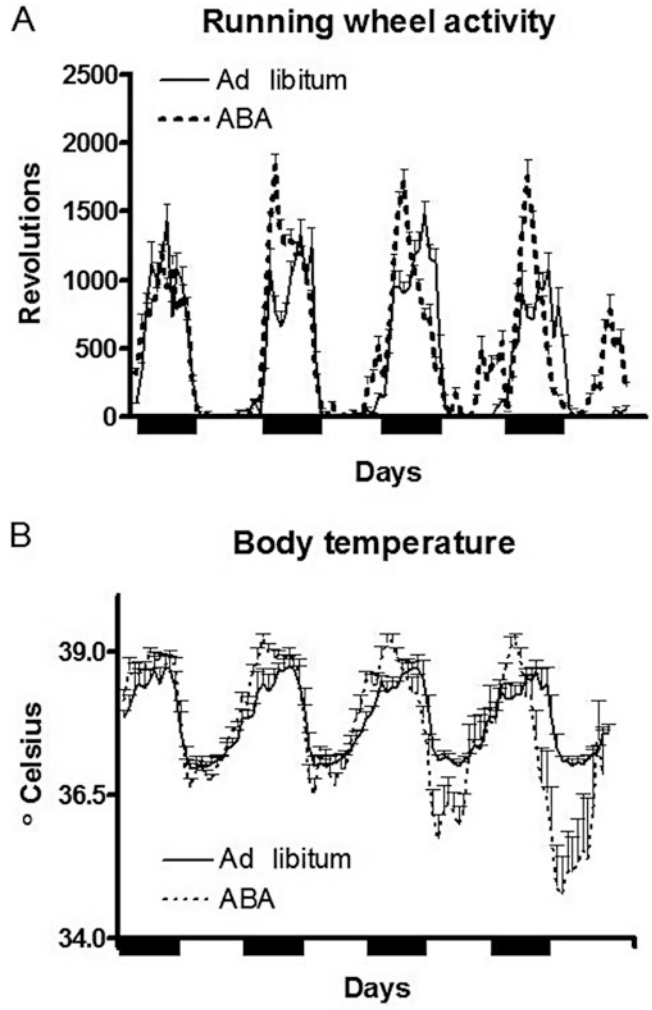

Fig. 1 Female Wistar rats were housed in running wheel cages and had ad libitum access to chow and water (filled line, $N = 8$) or were subjected to an activity-based anorexia (ABA) model (dotted line, $N = 8$) with 1-h access to food at the onset of the dark period. (**a**) depicts average running wheel revolutions (averaged per hour per group) during the 4 days of the experiment. Rats were sacrificed at the start of the dark period of day 5. (**b**) represents the body temperature rhythms (averaged per hour per group) of rats during ad libitum feeding or ABA

general, this threshold is reached within 5–7 days. The nadir in body temperature during the light phase decreases during the ABA model, reflecting starvation-induced hypothermia, plotted in Fig. 1b. Running wheel activity will increase in the dark period in the course of the ABA model but will increase in particular in the hours preceding food availability. The hyperactivity in the ABA model could reflect a starvation response, and be an expression of increased foraging behavior. In addition, rats display anticipatory

running wheel activity, which can usually be observed from the second day of ABA on, as shown in Fig. 1a.

Thus, the increase in locomotor activity preceding access to food is driven by FAA and starvation-induced hyperactivity (foraging). When food is given at the onset of the dark phase, there may even be increased locomotor activity due to anticipation of the dark phase, the period in which rats naturally start eating.

2.2 RFS

As mentioned previously, rats on an RFS have limited access to food and lack access to running wheels. Due to this intervention, circadian rhythms alter and FAA occurs [33–35]. Rats are not the only species that exhibit FAA. FAA in response to an RFS has also been reported in various other species, including mice, fish, birds, hamsters, and rabbits [33]. Several parameters determine the course of an RFS model.

Although the majority of RFS studies in rats have provided rats with food for 2 h in the light phase, FAA occurs under different conditions as well. Food availability in the dark period will also result in FAA [36, 37]. However, as the dark period is their normal active period, the difference between FAA and "normal" activity is more difficult to distinguish than when food is offered during the light phase. Furthermore, FAA can be observed in rats that are housed under 24-h light [38–40] or 24-h dark [41] conditions.

The development of FAA depends on a diurnal schedule. Rats on a 12:12 h light:dark cycle did not show FAA when daily meals were separated by 19 h or 29 h [33, 42, 43]. The range of daily mealtime intervals that will elicit FAA is thought to be 23–26 h [33]. Nevertheless, the onset of FAA does not demand a single daily meal. Studies showed that rats anticipated two, but not three, meals per day, which were at least 5 h apart from each other [36, 44]. Furthermore, durations of food access up to 12 h could induce FAA [45], although the amount of FAA was reduced with longer durations [46].

2.2.1 Experimental Setup and Procedure

Male outbred Wistar WU rats are individually housed in a temperature- and humidity-controlled room under a 12:12 h dark:light cycle. They are allowed to acclimate under ad libitum food and water conditions. One week after arrival, rats receive transmitters as described in Subheading 2.1.1. in order to allow continuous measurements of general locomotor activity and body temperature. Following 2 wks of recovery, rats are subjected to an RFS. On the first day, food is removed at zeitgeber time (ZT), i.e., 8 h after the lights come on. From then on, rats have daily restricted access to food from ZT6–ZT8. Body weight and water intake are measured just before ZT6. When body weight loss exceeds 20% or when body temperature is lower than 33 °C, the experiment is terminated just before access to food.

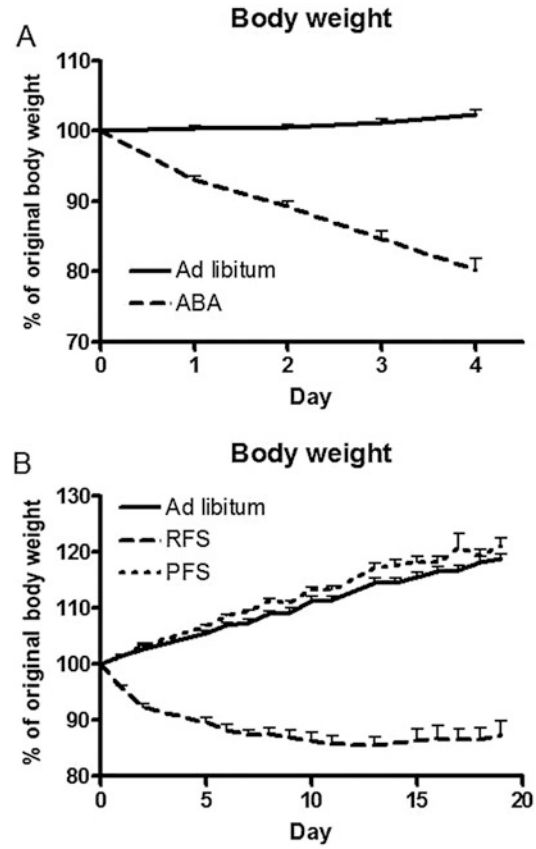

Fig. 2 Relative body weight gain, expressed as percentage of original body weight, of rats subjected to an activity-based anorexia model (ABA) (**a**), restricted feeding schedule (RFS), or palatable feeding schedule (PFS) (**b**) compared with ad libitum-fed rats

At first, rats on an RFS will not be able to consume their baseline food intake during the 2 h of food access. However, as the model continues, they gradually increase their food intake. Rats eat on average two to three large meals of 5–6 g.

At the beginning of an RFS, rats will lose weight due to reduced food intake. As shown in Fig. 2b, weight loss stabilizes [47], but, to a large extent, the amount of weight a rat will lose depends on the amount of locomotor activity it performs. When rats have access to running wheels, hyperactivity will cause a rapid decline in body weight. On the other hand, when rats are housed in standard cages, body weight loss will not be severe, and rats have time to adjust to the RFS.

In response to an RFS, rats will alter their rhythms of locomotor activity and body temperature [47–49]. Anticipatory peaks of these parameters arise, and values of locomotor activity and body temperature are reduced during the dark phase [49]. In general,

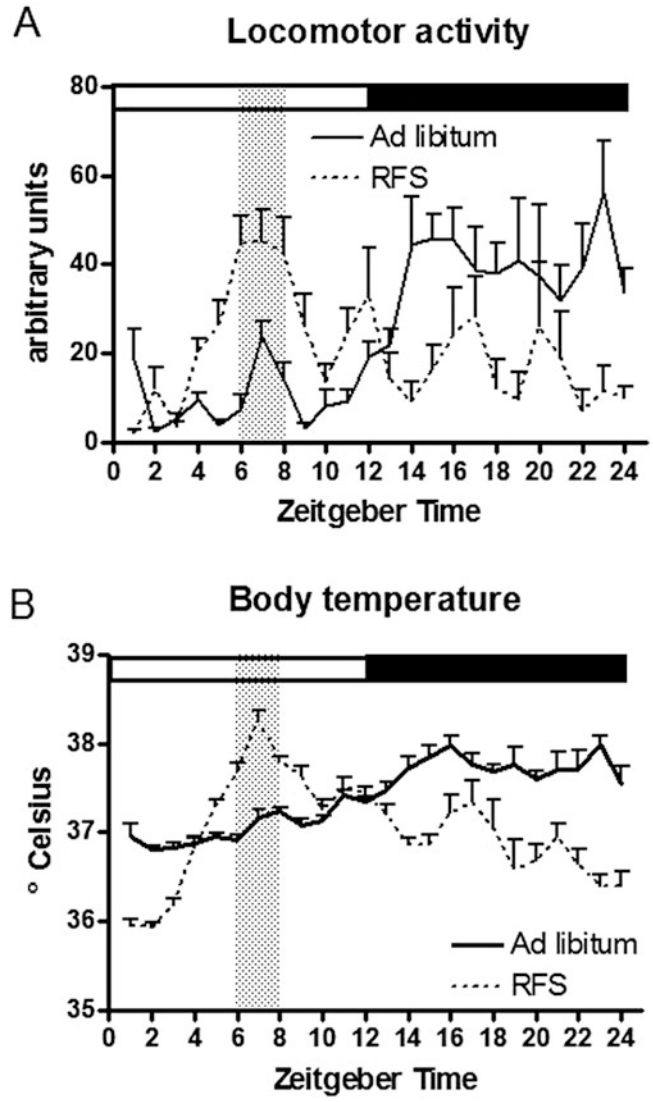

Fig. 3 Measures of locomotor activity (**a**) and body temperature (**b**) (averaged per hour) of male Wistar rats ($N = 6$) were taken during ad libitum feeding (filled line) and when rats were subjected to a restricted feeding schedule (RFS, dotted line) with food available from ZT6 to ZT8, as indicated by the gray vertical bar

FAA can be observed within 3 days of the onset of an RFS. Subsequently, the amount of FAA increases in amplitude and duration in the course of RFS [33]. FAA usually ceases when rats are provided with ad libitum food access and reestablishes when rats are food deprived [33, 50]. An overview of the changes in circadian rhythms of locomotor activity and body temperature is provided in Fig. 3.

While ABA is a severe model mimicking features of AN, the RFS model can be maintained longer and is more suitable to identify neural mechanisms underlying FAA.

2.3 PFS

The development of anticipatory locomotor activity is not limited to animal models of food deprivation. Ad libitum chow-fed rats were shown to anticipate a daily palatable treat as well [51]. Unlike the RFS model, rats anticipating a small palatable treat will not show an anticipatory increase in body temperature. However, due to the relatively large meal ingested in the middle of the light period, they will show postprandial hyperthermia, potentially diet-induced thermogenesis. In response to the palatable feeding schedule, rats will not shift their circadian rhythm of locomotor activity, unlike rats on RFS and ABA. However, a small anticipatory peak in locomotor activity can be observed in the hour preceding availability of the palatable treat [35, 50, 52, 53]. Figure 4 depicts the changes due to a PFS in locomotor activity and body temperature.

The ability of a palatable meal to evoke FAA depends on several parameters. First, the palatable meal needs to have some nutritive value, since a palatable mash without caloric content did not induce anticipatory wheel running [51]. Studies in rats indicate that carbohydrates, but not fat, have properties to induce a phase shift in the circadian food-entrained clock [54]. Remarkably, FAA to a palatable treat in mice was only observed in males, and only when given a high-fat treat, but not a chocolate treat [55]. In rats, chocolate [50, 52, 53]; a palatable mash consisting of chow, vegetable oil, chocolate syrup, and icing sugar [51], chocolate Ensure [56], and sucrose [57] have been used to evoke palatable meal-induced FAA.

Second, palatable meal size has to be reasonably large. The anticipated palatable meal was suggested to have to exceed a certain caloric threshold to be able to induce FAA, either in absolute value or relative to the total caloric intake [58]. Access to a 32% sucrose solution resulted in FAA in 85% food-deprived rats, but not in ad libitum chow-fed rats [57]. A 4-g palatable meal was able to induce FAA in only a minority of rats, whereas the majority of rats with a 2-h window of access to the palatable food, of which they consumed on average 9 g, exhibited FAA [51]. This study used running wheel activity as a read-out parameter for FAA. Another study using this read-out parameter showed that only 37% of the rats anticipating a palatable treat exhibited FAA. However, studies that assessed FAA with general locomotor activity measurements, e.g., using infrared motion sensors, showed that 5 g of chocolate was able to evoke FAA in rats [50, 52, 53]. Whereas FAA in rats on an ABA or RFS schedule started 2–3 h prior to mealtime, palatable meal-entrained rats showed a brief increase in anticipatory locomotor activity 15–30 min before access to the palatable snack [50, 53]. Interestingly, once the palatable feeding schedule was discontinued and rats had ad libitum access to regular chow, FAA was still observed around palatable mealtime for several days [50].

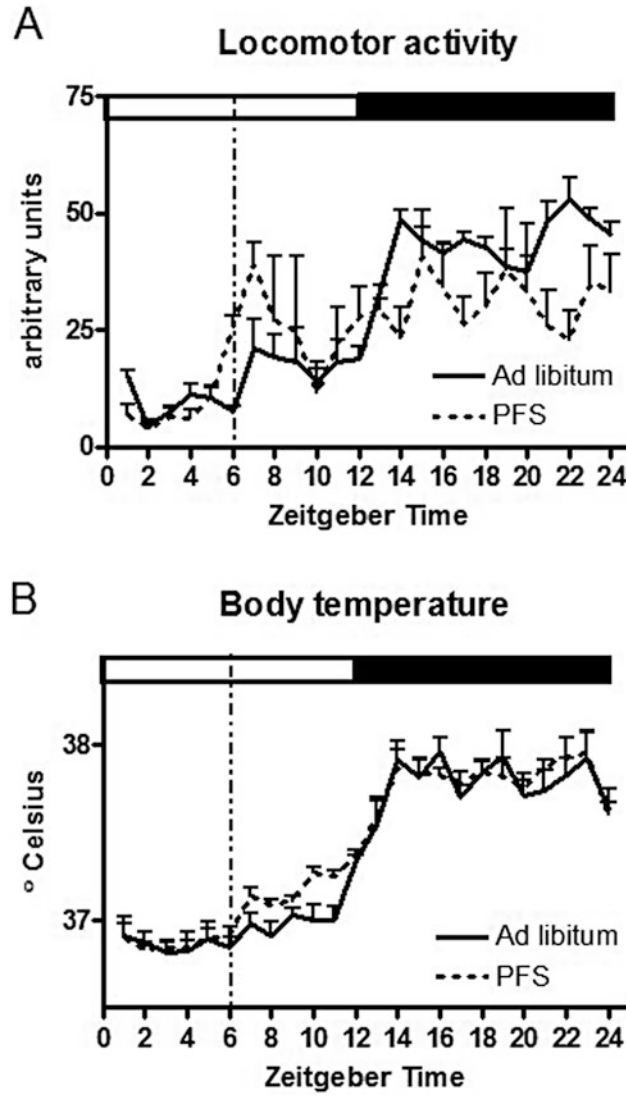

Fig. 4 Measures of locomotor activity (**a**) and body temperature (**b**) (averaged per hour) of male Wistar rats ($N = 6$) were taken during ad libitum feeding and when rats were subjected to a palatable feeding schedule (PFS, dotted line) with 5 g of chocolate available at ZT6 in addition to ad libitum chow access, as indicated by the dotted vertical line

2.3.1 Experimental Setup and Procedure

Male outbred Wistar WU rats are individually housed in a temperature- and humidity-controlled room under a 12:12 h dark:light cycle. They are allowed to acclimate under ad libitum food and water conditions. One week after arrival, rats receive transmitters as described in Subheading 2.1.1. in order to allow continuous measurements of general locomotor activity and body temperature. Following 2 weeks of recovery, rats are subjected to a PFS. They have ad libitum access to normal rat chow and are, in addition,

provided with 5 g of milk chocolate (Droste®, Vaassen, the Netherlands; per 100 g, 562 kCal, 35.4 g fat, 6.1 g protein, and 54.7 g carbohydrates) at ZT6. Body weight, chow, and water intake are measured just before ZT6.

Rats will decrease chow intake via a decrease in meal frequency in response to the extra palatable meal, especially during the light phase. When the palatable meal consists of a small chocolate bar (5 g), total caloric intake will increase slightly in the first week, but in the course of the palatable feeding schedule, total caloric intake will return to baseline levels. Others have shown as well that in free-fed rats, daily 2-h access to a dietary fat did not induce increased caloric intake [59]. The body weight of the rats will not change compared to ad libitum chow-fed rats, as illustrated in Fig. 2b.

Altogether, rats anticipate a palatable treat but to a lesser extent and at a later time of onset than rats anticipating a restricted meal. The caloric value of the palatable treat determines the amount of FAA that can be observed. Not all rats run in anticipation to a palatable treat, but general measures of locomotor activity seem to be sensitive enough to detect this form of FAA.

3 Notes

3.1 Assessing FAA

FAA can be assessed in various ways [34]. Running wheel activity has been used in numerous studies and has several advantages. It is a self-reinforcing behavior [60] and provides a reliable, precise measurement of anticipatory locomotor activity. One has to take into account that rodents need to adapt to the running wheel before being put on an RFS; otherwise, the running will become obsessive and rodents will run themselves to death [30]. Under ad libitum conditions, wheel running occurs almost exclusively during the dark period. Hence, the signal-to-noise ratio for increased activity during the light period is very good. On the downside, running wheel cages can be expensive and occupy more space than standard cages.

Measurements of general locomotor activity are also extensively used. They include noninvasive approaches such as tilt floors, infrared motion sensors, and photobeam systems, as well as transponders (which emit radiofrequency pulses) placed intraperitoneally. Recently, some debate arose on the validity of transponders versus infrared motion sensors to measure FAA [61–65]. One study compared the ability to detect FAA of these two measurements and revealed identical locomotor activity patterns [66].

All these measurements have a worse signal-to-noise ratio than running wheels, since general locomotor activity is not exclusive to the dark phase in ad libitum-fed rats. Furthermore, these devices record not only locomotor activity but also unrelated behaviors,

such as grooming, which makes them less sensitive and precise than running wheels.

To improve the signal-to-noise ratio, nest boxes or dark pipes can be added to the home cage. Rats will seek shelter in their rest period, which reduces the background measurements of unrelated behaviors. However, when assessing FAA with running wheels or general locomotor activity, any kind of manipulation that interferes with locomotor activity might seem to reduce FAA. Others have resolved this issue by looking at food bin-directed behavior [67].

Intraperitoneal transponders require invasive surgery but have the advantage that locomotor activity and body temperature can be assessed continuously, thereby providing additional information on the physiological state of the rat. Body temperature has been used previously as an indication of FAA. Since body temperature is strongly associated with locomotor activity (hyperactivity will result in an increase in body temperature), it is not an independent parameter. However, some studies have reported dissociated effects on anticipatory locomotor activity and body temperature [68, 69].

3.2 Assessing Underlying Mechanisms of FAA

Since there is not a clear consensus on the driving force of FAA, many studies seek to explore the underlying mechanisms of this behavior. Different strategies have been used to tackle this research question. Some of these techniques will be described in this section.

3.2.1 Observational Approaches

The immediate early gene *fos* is frequently used as a marker of neuronal activity due to its low baseline levels and short half-life. *Fos* protein levels rise 60 minutes after the event that evoked neuronal activation. Hence, *fos* immunohistochemistry (IHC) of brain slices of a rat sacrificed at ZT6 reflect the neuronal activation at ZT5. To investigate whether the rhythmicity of a certain brain area is uncoupled from the suprachiasmatic nucleus (SCN)-driven circadian rhythm due to food entrainment, IHC of the clock genes *Per1* and *Per2* can be applied. Another way of examining potentially involved brain areas in FAA includes studying the uptake of 2-deoxyglucose as a marker for cerebral glucose utilization [70]. Activation of brain areas can also be investigated with in vivo electrophysiology. This technique has an advantage over *fos* IHC because neuronal activity of single neurons within a brain area can be measured directly over time, instead of one time point per rat. In addition, this approach can be combined with pharmacological interventions. Multiple unit activity has also been applied to record activity of a brain area during FAA [71, 72]. The limitation of in vivo electrophysiological recordings is that only one brain area can be investigated per rat.

3.2.2 Interfering Approaches: Lesion, Genetic, and Pharmacological Studies

Genetic models have been used to study FAA. Several knockout mice have been examined to determine whether certain genes play a role in FAA. Unfortunately, knockout models are scarce in rats. Another disadvantage of knockout models is that the gene is lacking from development onward, thereby enabling compensatory mechanisms. Conditional knockout models in which genes can be switched off after development overcome this problem. Viral vector-mediated knockdown or overexpression of genes enables the manipulation of local gene expression in rats.

Involvement of brain areas in the regulation of FAA has been intensively studied by lesioning or ablating brain areas. Lesions can be performed in multiple ways. First, the use of radiofrequency current as an electrolytic lesion will destroy all cells and passing fibers in a brain area. Second, neurotoxins, such as ibotenic acid, will destroy cells but spare fibers of passages.

To determine whether a certain neurotransmitter or other signaling peptide is involved in the control of FAA, pharmacological manipulation has been applied. Acute or chronic administration of specific agonists or antagonists of a receptor can reveal the role of that receptor in FAA. Chronic application is achieved by daily injections or by an implanted osmotic mini-pump. Additionally, it is possible to distinguish central and peripheral effects by injecting via a canula in a brain area or ventricle, intraperitoneally, subcutaneously, or intravenously. The involvement of specific neurotransmitters in FAA can also be investigated using intracerebral microdialysis [73]. With this approach, the release of neurotransmitters and their metabolites in a brain area can be measured during FAA and food intake.

3.3 Underlying Mechanisms of FAA

Under normal conditions, daily circadian rhythms in all kinds of behaviors, such as locomotor activity, are entrained by the master clock of the brain: the SCN. This hypothalamic area receives direct input from the retina and synchronizes other brain areas and peripheral tissues to the light:dark cycle via neuroendocrine and autonomic output pathways [74–77]. Rats are nocturnal animals, and their feeding behavior is also coupled to the SCN-controlled circadian rhythm. Not only is food intake controlled by the circadian system, food intake can, in return, affect circadian rhythms. When access to food is restricted to a few hours in the light period (i.e., the normal resting phase for nocturnal animals), the circadian rhythm of behavior is disengaged from the central clock and cycles in relation to feeding time. Animals will develop hyperactivity preceding the feeding time, hence FAA when they would normally not be active [33–35]. This FAA is reflected in several features, including general hyperactivity, exploratory behaviors, increased instrumental behaviors to obtain food, and food bin-directed behaviors. Moreover, the circadian rhythm of body temperature alters, as well as the rhythms of metabolic parameters, such as glucose,

hormones, and free fatty acids [78, 79]. Numerous studies aimed to identify the food-entrainable oscillator (FEO), the brain structure, or signaling pathway that drives FAA.

3.3.1 Involvement of Peripheral Regulation in FAA

The peripheral digestive system and the brain can communicate with each other to regulate FAA, as food intake might be a stimulus for this behavior. However, transsection of the vagus nerve, which innervates the peripheral organs and sends sensory input to the brain about the state of the organs, does not prevent the corticosterone shift observed during FAA [80]. Subdiaphragmatic transsection of this nerve does not impact FAA measured by running wheel activity in SCN-lesioned rats [81]. Moreover, disruption of nonvagal visceral input to the brain by intraperitoneal capsaicin injections does not hamper the development of FAA [82]. Therefore, (para)sympathetic innervation is not likely to be the essential route of communication between the gut and the brain for the regulation of FAA. This implies that humoral signaling could play a role in the development of FAA. However, adrenalectomy, which prevents the secretion of corticosterone, does not attenuate FAA [83]. In addition, diabetic rats with destroyed insulin-producing cells [84] and rats with a mutated leptin receptor [85] still exhibit FAA. Hence, to date, the pathway via which the brain and the periphery communicate to regulate FAA remains to be elucidated. A potential candidate is ghrelin signaling, since ghrelin levels rise prior to mealtime [86–88], and ghrelin receptor knockout mice show attenuated FAA [86, 89–91]. The roles of leptin and ghrelin in FAA will be discussed in more detail in Subheading "Leptin".

3.3.2 Involvement of Neural Circuits in FAA

Peripheral feeding-related input arrives at the brain at various locations. The gastrointestinal system sends information via the vagus nerve to the nucleus of the tractus solitarius (NTS) in the hindbrain. A blood-brain barrier is absent at the nearby area postrema (AP), which enables the AP to detect humoral signals. Both the NTS and AP project to the parabrachial nucleus (PBN). Electrolytic and neurotoxin-induced lesions of the latter structure result in attenuated FAA as measured by food bin approach behavior [92]. However, lesions of the AP [93] or NTS [94], the main inputs to the PBN, do not affect FAA. *Fos* immunoreactivity in these three brain areas is increased only after consumption of the anticipated meal, not during FAA [95].

Hindbrain

Hypothalamus

The hypothalamus has been implicated in the homeostatic regulation of energy balance and autonomic behaviors and is hence a logical candidate for mediating FAA in rats on an RFS. It has been demonstrated that dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), tuberomammillary nucleus (TMN), and perifornical area (PeF) showed increased *fos* expression during FAA [49, 96–100]. *Per1* rhythms shifted or changed in DMH, arcuate

nucleus (Arc), PeF, paraventricular nucleus (PVN), and ventromedial hypothalamus (VMH) [50, 101–103] in rodents on an RFS. Per2 rhythms shifted or changed in DMH, PVN, and VMH in rodents on an RFS [56, 101–103]. On the other hand, rats subjected to PFS with restricted access to palatable food in addition to ad libitum access to chow did not show any hypothalamic increase in *fos* during FAA [52], and *Per2* rhythms did not change in DMH [56]. *Per1* rhythms were shown to change in this paradigm in SCN, DMH, and PeF [50, 53].

The SCN, which contains the light-entrainable oscillator, is located within the hypothalamus. In 1979, Stephan and colleagues demonstrated that this brain area is not the FEO as SCN lesions did not impair FAA [83]. Lesions of the PVN and LH did not attenuate FAA either [67]. Although PVN lesions decreased FAA in general locomotor activity measurements, anticipatory food bin approaches were still intact [67]. At first, ablations of the VMH seemed to attenuate or even abolish FAA [104, 105]. However, later studies revealed that this effect was only transient and that FAA recovered eventually [106, 107]. Additionally, a weak correlation between the size of VMH lesion and the reduction in FAA was reported [49]. The Arc lacks a blood-brain barrier, like the AP. Interestingly, lesions of the Arc induced by neonatal monosodium glutamate increased FAA [108].

A body of literature describes the role of DMH in FAA [49, 61, 62, 66, 109, 110]. Electrolytic lesions of this brain structure did not impair FAA as detected by general locomotor activity and food bin-directed behavior [110]. In contrast, neurotoxin-induced lesions, which spare passing fibers, were reported to attenuate FAA as measured by wakefulness, body temperature, and general locomotor activity [49]. This was also observed in mice with a large mediobasal hypothalamic lesion, which included DMH [111]. Differences between these studies include parameters assessed, lesion type, and cage configuration (e.g., use of dark pipes in cage). Replication of all parameters from the Gooley study [49], apart from type of lesion, by Landry and colleagues [109] still revealed no impact of DMH lesion on FAA [109]. In two studies, FAA persisted after 48 h of food deprivation in DMH-lesioned rodents, indicating that the food-entrainable rhythm is not impaired [103, 109]. The discussion focuses on the correct way of assessing FAA and the best approach to lesion a brain area [61, 62, 66] and has yet to be resolved. Interestingly, a recent paper showed that DMH ablation diminished FAA. However, when in addition the SCN was lesioned, FAA returned. This suggests that the DMH has a role in silencing output from the SCN to permit FAA [99]. In line with the modulating role of DMH in FAA, it was shown that DMH lesions might attenuate FAA to a daytime meal, but when food is provided at nighttime, FAA is intact [112].

Orexin neurons are predominantly located in the LH, and orexin is known for its orexigenic and arousal-stimulating properties. Orexin neurons are activated during FAA in rats expecting a chow meal and those that anticipate a palatable meal [113–116]. Orexin knockout mice still exhibit FAA, although reduced or with a delayed acquisition [113, 116–119]. Interestingly, the effect of the lack of orexin signaling on FAA seems to be dependent on the circadian phase, since anticipation to RFS in the light period was impaired to a larger extent than anticipation to RFS in the dark phase [113]. However, specific ablation of orexin neurons in LH did not impair FAA [120].

Reward-Related Brain Areas

Food intake is not just a matter of balancing energy intake with energy expenditure but motivational and rewarding aspects of food influence consumption as well. Corticolimbic areas involved in the reward-related effects of food intake were shown to play a more important role in FAA in rats anticipating a palatable treat. During FAA in PFS rats, *fos* activation was increased in several corticolimbic areas, including the paraventricular nucleus of the thalamus (PVT), central amygdala (CeA), nucleus accumbens (NAc) core, NAc shell, and prefrontal cortex (PFC) [52, 53]. In rats on an RFS, *fos* levels increased also in these brain areas, although to a lesser extent [52, 121, 122]. In addition, circadian rhythmicity of these brain areas changed during FAA in rats on a PFS and an RFS, as observed by altered *Per1* rhythms [50, 53, 121].

Although these brain areas were activated during FAA, lesioning studies did not implicate any corticolimbic area in the development of FAA. The PFC is suggested to organize adaptive autonomic alterations to an expected situation [123]. Although lesions of the infralimbic cortex, which is part of PFC, prevented anticipatory and postprandial rises in body temperature, FAA remained unaffected [68]. A similar effect, loss of anticipatory body temperature increase, but intact FAA, was observed after ablation of the pituitary [69]. The PVT receives projections from the brain stem and hypothalamus and projects to corticolimbic and hypothalamic areas that play a role in the regulation of reward and arousal [124, 125]. Lesioning of the PVT resulted in decreased FAA as measured by general locomotor activity [126]. However, when examining food bin-directed behavior, rats with PVT ablations still showed robust FAA [127]. Large lesions covering the hippocampus, involved in learning and memory, and a large part of the amygdala, implicated in emotional memory, did not prevent the development of FAA either [128]. Discrepancies exist in the effects of NAc ablations on FAA. NAc receives dopaminergic input and could mediate increases in locomotor activity. One study determined FAA as food bin-directed activity and reported no reduction on FAA [128], whereas another study looked at general locomotor

behavior and observed a reduction in FAA in NAc core-lesioned rats, but not in NAc shell-lesioned rats [122].

Taken together, the results of various lesion studies suggest that the regulation of FAA is distributed over a network of brain nuclei. When one node of this network is lesioned, FAA still persists or is only temporarily attenuated.

3.3.3 Hormones and Transmitters Implicated in FAA

Leptin

The adipose tissue-derived hormone leptin circulates in the blood and enters the brain to provide information about body fat content [129]. Leptin suppresses food intake and stimulates thermogenesis and locomotor activity, thereby increasing energy expenditure [130]. At least part of this effect can be attributed to leptin signaling via its receptors in the hypothalamus, mainly the Arc [131]. Leptin receptors have also been identified on dopaminergic neurons in the ventral tegmental area (VTA), a brain area which has been implicated in reward and locomotion [132].

ABA rats exhibit reduced leptin levels [73, 133, 134], and peripheral [8, 23] and central [32, 135] leptin treatments suppress hyperactivity in this model. Leptin might exert its effect on hyperactivity via the VTA, since administration of leptin in the VTA reduces hyperactivity in the ABA model [135], whereas knockdown of leptin receptors in the VTA results in increased food intake, hyperactivity, and higher sensitivity to palatable food [136]. Leptin treatment in the ABA model reduces hyperactivity not only in the dark period but also in the hours preceding food access, hence FAA [8, 32, 137]. Plasma leptin levels are low preceding the anticipated meal and peak after food availability in rodents subjected to an RFS [138, 139]. In line with the data obtained in ABA rats, AN patients suffer from low leptin levels, which correlate negatively with physical activity levels [23, 140, 141]. Hence, a lack of leptin signaling could lead to hyperactivity and increased FAA.

Leptin-deficient ob/ob mice show augmented FAA [142]. However, mice do not exhibit the complete repertoire of FAA [118], which could be due to their general hypoactivity [118, 143]. Thus, if a lack of leptin signaling elicits hyperactivity and increased FAA, this should also be represented under conditions of leptin resistance. Obese people [144, 145] and diet-induced obese rodents [146–149] have high leptin levels. However, they do not respond to these high levels of leptin by reducing food intake or increasing energy expenditure, meaning they could be considered as leptin resistant. Indeed, obese Zucker rats, which are leptin resistant because they have a point mutation in the gene encoding the leptin receptor, still exhibited strong FAA to an RFS [85]. Interestingly, rats on a high-fat diet, which have high leptin levels, showed attenuated FAA [150].

Altogether, this suggests high leptin levels reduce FAA. The lack of leptin signaling, as in Zucker rats, in ABA and RFS models might drive hyperactivity and FAA, probably via the VTA [151].

Dopamine

Dopamine is known for its involvement in the regulation of reward and motivation via the mesolimbic dopamine system [152]. This system originates in the dopaminergic neurons of the VTA and substantia nigra, which projects to the NAc, which in turn projects to many other limbic areas. Ingestion of palatable food resulted in augmented dopamine release in the NAc [153, 154], which attenuates during food intake following habituation and transferred to the food-signaling cue [155–158] in anticipation of the predicted reward. Dopamine may increase the motivational drive to forage food which is expressed as FAA. Dopamine is thus a potential candidate for the regulation of hyperactivity and FAA in the RFS and PFS models, since local injections of dopamine in the NAc induced locomotor activity [159]. Moreover, disruption of dopaminergic neurotransmission in the NAc prevented nicotine- and amphetamine-induced increases in locomotor activity [160], suggesting that dopamine is an essential signal to increase locomotor activity.

Obese and AN individuals show alterations in reward signaling, especially related to dopamine. Those that are obese exhibit increased activation of brain areas involved in motivation and reward in response to pictures of high-caloric food [161]. In addition, decreases in dopamine release and striatal dopamine receptor 2 (D2R) density were reported in obese individuals and rodents [162–165]. AN patients show lower levels of the major dopamine metabolite homovanillic acid after recovery of the disease [166, 167]. D2R polymorphisms have been associated with AN [168, 169]. AN patients exhibited increased peripheral expression of the dopamine transporter (DAT), whereas D2R expression is decreased, accompanied by epigenetic dysregulation of DNA methylation of these genes [170]. Furthermore, recovered AN patients exhibit increased D2R and D3R binding in the anteroventral striatum [171]. Thus, in AN, there is evidence for increased striatal dopaminergic activity, whereas in obese people, there may be decreased activity.

In mice exposed to the ABA model, D2R mRNA expression in the caudate putamen region is increased [172]. Dopamine depletion reduces hyperactivity in restricted rats with periodic food access [173]. This is in line with the finding that dopamine antagonism reduces hyperactivity in the ABA model, although levels of FAA remain stable [174]. In addition, in ABA rats, dopamine release in the NAc increases during food intake, but not during FAA [73]. This is in contrast to previously mentioned observations that dopamine is initially released upon food intake but transfers to

the food-signaling cue (anticipation). However, in these studies [73, 174], the ABA model lasted for a few days, which might be too short for this transfer to have taken place. Others have shown that dopamine antagonism or NAc ablation did not affect FAA in restricted rats anticipating normal chow [128, 175]. However, dopamine antagonism in rats anticipating a palatable meal reduces FAA [175–177].

In summary, dopamine is suggested to play a role in the hyperactivity observed in the ABA and RFS models. Although its role in FAA is less clear, data indicate that dopamine signaling could be important for FAA, particularly in anticipation of a palatable food source. However, studies on the role of dopamine, where dopamine signaling is reduced pharmacologically or by lesions, are compromised by the role dopamine plays in general locomotor activity and in all motivational behaviors.

Ghrelin

Ghrelin is an orexigenic hormone secreted by the oxyntic cells of the stomach and subsequently released in the blood [178]. Post-translational modification by ghrelin O-acyltransferase (GOAT) is essential for ghrelin to bind the growth hormone secretagogue receptor 1a (GHS-R1a) [179, 180]. Apart from stimulating growth hormone release [178], ghrelin acts as a potent stimulator of appetite when injected peripherally [181, 182] or centrally [181–186] and can cause an increase in fat mass [185]. Ghrelin plasma levels are increased in AN patients and after fasting [187], while weight gain and obesity reduce plasma ghrelin levels [187, 188]. The central effect of ghrelin on food intake is at least partly mediated via GHS-R1a on neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons in the Arc of the hypothalamus [183, 189]. In addition to the Arc, GHS-R1a is expressed in other hypothalamic regions such as the VMH, DMH, and VTA [190–192].

Ghrelin stimulates the consumption of and motivation to work for palatable food, probably via the VTA [184, 192–196]. Furthermore, ghrelin induces locomotor activity and accumbal dopamine release via its presynaptic and postsynaptic binding to GHS-R1a in the VTA [192, 197, 198]. Alcohol-, nicotine-, and cocaine-induced stimulation of locomotor activity and dopamine release in the NAc were prevented in GHS-R1a -/- mice and by GHS-R1a antagonism [199–201]. Based on these studies, ghrelin could play a role in FAA. Indeed, ghrelin plasma levels showed entrainment to habitual meal patterns in humans and rats [87, 88, 202]. Plasma ghrelin levels were increased in ABA and RFS rats and correlated with FAA [86, 88, 134]. Central administration of ghrelin increased FAA in RFS rats [90], whereas a GHS-R1a antagonist reduced anticipatory locomotor activity in ABA rats [86]. Moreover, GHS-R1a -/- mice showed attenuated FAA in RFS and ABA models, without affecting general locomotor activity [86, 89–91]. In contrast, ghrelin -/- mice exhibit normal levels of FAA

[118, 203], which could suggest that ghrelin signaling does not play a pivotal role in FAA and that a still unknown ligand of GHS-R1a can modulate FAA via this receptor. Taken together, GHS-R1a signaling likely contributes to the development of FAA.

4 Conclusion

FAA reflects the physiological adaptation to restricted access to food in the ABA and the RFS models. Circadian rhythms in behavior and neuronal activation in hypothalamic areas uncouple from the light:dark cycle. In addition, altered plasma levels of hormones and increased expression of orexigenic neuropeptides signal hunger. In contrast, the PFS model elicits temporary increases in locomotor activity and arousal, without changing circadian rhythms in behavior or hypothalamic activation. As corticolimbic structures are activated during FAA in PFS rats, FAA might be mediated via these areas. The magnitude of FAA is less in the PFS than in the RFS or the ABA models, suggesting that FAA in the RFS and the ABA involves hunger and motivational components, whereas FAA in the PFS models is mainly driven by motivational aspects.

However, to date, no brain area, hormone, or signaling molecule has been proven to be both essential and sufficient to mediate FAA. Therefore, a redundant, distributed network likely mediates this behavior. Lack of leptin signaling and increased ghrelin signaling (hence hunger) augment anticipatory behavior. Hunger signals arriving in the hypothalamus could be the initiator of anticipatory behavior in ABA and RFS rats. The dopaminergic VTA-NAc projection is a prominent candidate to execute FAA. As the hypothalamus is not activated during FAA in PFS rats, initiation of FAA is probably mediated via corticolimbic systems directly in this paradigm.

Further research is required to determine whether common regulatory pathways are involved in FAA in response to the RFS, ABA, or PFS models. In addition, it remains unknown whether and how the hypothalamus communicates to other brain areas to regulate FAA. Could this be via an LH (orexin)-VTA (dopamine)-NAc connection? Other areas of research needing further attention include whether ghrelin is the endogenous ligand that drives FAA or whether a still unknown ligand of GHS-R1a is involved; which brain area GHS-R1a is involved in FAA, within the hypothalamus or the VTA; and the possibility that different neural circuits mediate FAA due to the RFS, ABA, and PFS. Answering these questions will help us understand the regulatory pathways that can shift our metabolism and circadian rhythms to the presence of food and the neural circuitry that makes us ready to eat when starved or take a palatable snack when satiated.

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Chapter 19

In Search for Perfection: An Activity-Based Rodent Model of Anorexia

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Abstract

Anorexia nervosa represents a disorder with the highest mortality rate among all psychiatric diseases, yet our understanding of its behavioral, physiological, and neurochemical components still remains incomplete. Clearly, societal pressures (i.e., cultural values of thinness and fitness), obsessional temperament, and perfectionism cannot be illustrated by any animal model, but on the other hand, some strong similarities between self-starvation in rodents and anorexia nervosa can be noted. An activity-based rodent model of anorexia provides a phenomenon (i.e., excessive running, reduced food intake, and subsequent weight loss) that can be easily implemented and effectively used to explore the pathophysiological consequences of semi-starvation and hyperactivity, which are characteristics of anorexia nervosa. To date, it is thought to be the best-fitting animal model analogous to anorexia nervosa.

Key words Activity-based anorexia, ABA, Wheel running, Hyperactivity, Anorexia nervosa, Rat

1 Introduction

1.1 Anorexia Nervosa: Food Restriction and Hyperactivity in Humans

Anorexia nervosa (AN) is a severe eating disorder predominantly affecting adolescent girls and young women and presents with a loss of appetite, underweight with BMI values below 17.5 kg/m^2, as well as endocrine alterations including amenorrhea. A patient must show a restriction of energy intake inducing low body weight, a fear of gaining weight or behavior preventing weight gain, as well as a disturbance of body image or lack of understanding of the danger of low body weight to fulfill the diagnostic DSM-V criteria of AN [1].

Food restriction and subsequent malnutrition in AN directly lead to severe multiorgan complications, such as gastrointestinal, cardiac, pulmonary, hematologic, musculoskeletal, neurologic, and dermatologic conditions. Most of them are treatable after weight gain, effective medical interventions, and psychotherapy, especially given the relatively young age of onset of AN [2]. Those complications resemble simple starvation (semi-starvation) from the

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pathological point of view, yet, underlying mechanisms responsible for the development of AN are still poorly understood. The high prevalence of pressures for thinness and the low prevalence of AN (0.3–0.7% of females in the general population), together with clear evidence of AN occurring at least several centuries ago, its stereotypic presentation, substantial heritability, and developmentally specific age-of-onset distribution, suggest biological accountabilities. From the neurobiological point of view, a disturbance of brain serotoninergic networks predates the onset of AN and should contribute to premorbid symptoms of anxiety, behavioral inhibition, and a vulnerability for restricted eating. What is more, puberty-related female gonadal steroids or age-related changes may exacerbate serotonin dysregulation, and stress and/or cultural and societal pressures (i.e., cultural values of thinness and fitness) may contribute by increasing anxiety, obsessional temperament, and the strive for perfectionism [3]. However, there is only minimal to moderate evidence that available psychiatric medications are effective [2–4]. For example, Kaye et al. suggested that starvation-induced abnormalities in serotonin receptors (especially 5-HT1A) and extracellular serotonin concentrations should be responsible for poor response to antidepressants [3, 5]. Altogether, brain neuropeptides together with monoamine systems, especially serotoninergic and dopaminergic, are of most interest in AN, yet our understanding of the pathophysiologic role of those systems in patients is still rather limited.

According to literature, up to 40–80% of patients with AN show excessive levels of physical activity. Thus, hyperactivity plays a fundamental role in the development and maintenance of the disorder, may precede food restriction and accelerate body weight loss once food restriction has been initiated, and obviously interferes with the recovery process [6–8]. Unfortunately, it is unclear to which extent activity levels remain high after recovery from AN [7]. The nature of this feature remains uncertain, although it was already recognized and described by Gull and Lasègue in the nineteenth century [9, 10]. Rewarding properties through an activation of dopaminergic reinforcing pathways, hypoleptinemia, and thermoregulatory compensation due to hypothermia have been hypothesized as the leading causes of hyperactivity [7, 8, 11, 12]. Starving or overly active humans have increased activity of the hypothalamic-pituitary-adrenal axis with high blood levels of cortisol. Glucocorticoids can cause euphoria as well as dependence and exert rewarding effects mediated by an enhanced mesolimbic dopamine release through cortisol [13]. Yet, it should be stressed that mental alertness and continued increased activity levels in the presence of a negative energy balance and weight loss are usually viewed as unique to AN as compared to individuals with semi-starvation due to other causes [6]. Rewarding activation upon reduced energy intake has been proposed as the gateway into AN

[14], but it is also highly possible that genetic factors regulating activity levels independent of that pathway may contribute to the development of AN upon restricted food intake [7].

1.2 An Activity-Based Rodent Model of Anorexia

Also referred to as an exercise-induced anorexia, or a food restriction-induced hyperactivity, activity-based anorexia was introduced by Routtenberg and Kuznesof as a result of the observation that rodents have a tendency to self-starvation when exposed to restricted feeding schedule and voluntary physical activity. Animals increase their activity (wheel running) and reduce food intake leading to self-starvation with hyperactivity further aggravating their weight loss [15]. Hyperactivity observed upon exposure to the model has been explained in terms of foraging behavior, anticipation, reward, and stress [16–19], while hyperactivity and reduction of food intake have been associated with dopaminergic dysfunction [20, 21], together with an increased ghrelin and decreased leptin signaling [22]. Although these mechanisms remain uncertain, the core pathophysiological features of an activity-based rodent model of anorexia (ABA) and anorexia nervosa are alike (*see* Table 1).

Female rodents subjected to ABA, in addition to excessive activity and reduced food intake, present with weight loss and the cessation of the estrous cycle (*see* Fig. 1), and thus, the ABA model successfully reproduces the typical clinical manifestations present in AN.

1.3 Pathophysiology Underlying an Activity-Based Rodent Model of Anorexia

The typical ABA model [15] exposes rodents to severe level of stress that in the long term stimulates adaptive response by an increased secretion of corticosterone [23, 24]. A thorough analysis of the ABA phenomenon (i.e., excessive running, reduced food intake, and subsequent weight loss) is largely focused on the metabolic circuits in the hypothalamus, including mainly arcuate nucleus, dorsomedial hypothalamus, lateral hypothalamus, paraventricular nucleus, and ventral medial hypothalamus, which are considered to be part of a major feeding regulation center in the central nervous system. The expression of orexigenic hormones—neuropeptide Y (NPY) and agouti-related peptide (AgRP)—in ABA rats is elevated as a compensatory physiological reaction for starvation [25]. Their orexigenic activity is enhanced by elevated levels of ghrelin and corticosterone. The high concentration of ghrelin stimulates physical activity, increases hunger, and triggers reward pathways. So why does this seemingly proper adaptive metabolic response (hyperghrelinemia) in rats, as well as in patients with anorexia nervosa, not play a protective role? One hypothesis is a ghrelin resistance as a central and/or peripheral unresponsiveness (or altered signaling) to elevated levels of this hormone. Indeed, a significant contribution of the ghrelin pathway to the development of food-anticipating behaviors is observed since ghrelin receptor blockade (GHS-R1A)

Table 1
Pathophysiological features of an activity-based rodent model of anorexia (ABA) and anorexia nervosa (AN)

		Additional features	
	Common features	**ABA**	**Anorexia**
Alterations in neurotransmitters involved in hyperactivity	↓ Dopamine, ↓ serotonin (striatum or CSF) ↑ endogenous opioids	↓ Histaminergic neuronal activity	↓ Adrenergic neurotransmission (noradrenaline)
Hormonal imbalance	↑ Ghrelin, ↓ leptin, ↑ CRH, ↑ ACTH, ↑ corticosterone, ↑ ADH, ↓ LH, ↓ FSH, ↓ estradiol, ↓ oxytocin, ↓ testosterone	↑/↔ IGF-2	↓/↔ IGF-2, ↑ GH, ↓ IGF-1, ↓ T_3
Alterations of energy homeostasis	↓ Plasma glucose, ↓ insulin, ↓ free fatty acids, ↓ protein synthesis	↑ Hypothalamic protein synthesis	
Gastrointestinal alterations	Modification of gut microbiota composition, delayed gastric emptying	↑ Colonic permeability	↓ Permeability of small intestine
Alterations of the immune system	↑ Inflammatory cytokines, production of neuropeptide-reactive antibodies	↑ Anti-α-MSH IgM	↑ Anti-α-MSH IgG

↑ increase; ↓ decrease; ↔ unvarying

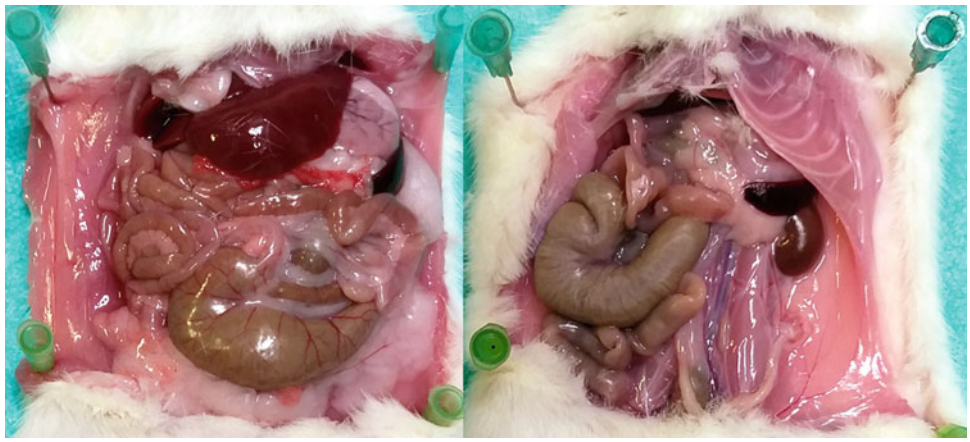

Fig. 1 A female rat subjected to ABA (right) in comparison with a healthy female animal (left). (ABA, an activity-based rodent model of anorexia)

has been shown to suppress rodents' hyperactivity before food intake [26]. Projections of NPY/AgRP neurons to second-order neurons stimulate production of the neuropeptide orexin, which was reported to induce food-seeking hyperlocomotion [27]. Analogously, the number of anorexigenic POMC and CART neurons is decreased as well as levels of peripheral peptides, such as leptin, that activate those neurons [25, 28].

The maladjusted hormonal balance represented by a failure of increased orexigenic signaling in protecting against the development of ABA may incline the role of addiction in this phenomenon. Food deprivation and physical activity are known for their ability to activate reward pathways [29] involving a complex dopaminergic and serotoninergic cooperation with orexins [20, 27, 29]. Restricted feeding in rats leads to a decrease in the level of the hypothalamic serotonin, which in turn would be expected to enhance food consumption [30]. The release of serotonin is significantly lower in the nucleus accumbens, where it regulates the motivation of ABA rats to eat [31]. The concentration of dopamine in the ventral striatum also presents a decreasing trend upon exposure to ABA, with an expected peak during food consumption. Indeed, dopamine appears to play an important role since a nonselective blockade of its receptors results in a reduced activity and an increased food intake, counteracting the effects of ABA [32]. Moreover, leptin receptors are expressed in the ventral tegmental area (VTA), and their stimulation leads to a decreased dopamine release from dopaminergic neurons. In healthy rodents, leptin in VTA, a region strongly associated with a mesocorticolimbic dopamine circuit responsible for reward-directed behavior, causes a significant decrease in food consumption as well as physical activity via activation of signal transducer and activator of transcription-3 (STAT3) [33, 34]. Lack of such direct suppressive activity on reward-related pathways might be an essential component of hyperactivity in ABA animals. In addition, high concentration of ghrelin, which positively correlates with a food anticipatory activity (FAA), directly activates dopaminergic neurons in VTA [26, 35]. Although neither dopamine nor serotonin signaling was shown to be clearly responsible for an FAA in the ABA model, in a restricted feeding paradigm, FAA seems to be under control of dopaminergic system, especially D1 and D2 receptors [36]. However, an administration of selective serotonin reuptake inhibitors reduces FAA in genetically modified ABA mice, and an addition of olanzapine increases their survival [37]. Pathophysiological contributions to the development of FAA in the ABA model have also been attributed to an excessive activation of the hypothalamic-pituitary-adrenal axis, with high cortisol levels and a dysregulation of negative feedback mechanisms controlling a corticotropin-releasing hormone secretion. Hypercortisolemia may act directly through receptors located on dopaminergic neurons in VTA, causing further stimulation of

the reward pathways [38]. The combined effect of an altered neurotransmission of dopamine, serotonin, and noradrenaline in the reward system increases susceptibility to addictive stimuli. The role of endogenous opioids in this process is unclear, but both plasma and hypothalamic concentrations of β-endorphin are elevated in ABA rats [39, 40]. In general, reward mechanisms similar to those observed in patients with AN are disturbed in ABA rodents, but current state of knowledge cannot provide us with an unequivocal description of the affected pathways.

An alternative concept for a neuroendocrine signaling dysfunction in AN has also recently been proposed. Based on reports revealing the presence of autoantibodies against feeding-related hormones, it assumes pathological autoimmune reactions, the source of which is the molecular mimicking of the intestinal microflora, to peptides involved in the regulation of altered behaviors [41]. Upon exposure to the ABA model, the composition of gut microbiota changes and the intestinal permeability increases, resulting in the translocation of bacterial proteins that cross-activate the production of autoreactive antibodies, which in turn might modify the activity of numerous peripheral and central neuropeptides [42, 43].

2 Materials and Procedures

2.1 General Notes

ABA is an example of a biobehavioral phenomenon, and thus, it is critical to minimize the amount of unpredictable stress and maximize comfort for experimental animals. For example, handling of animals should be kept to a minimum and, if possible, one person should be responsible for animal handling throughout the experiment. All experimental procedures have to be always conducted in accordance with National and Institutional Guidelines for Care and Use of Laboratory Animals.

2.2 Caging and Animal Preparation

Animals upon their arrival should be housed under controlled conditions with a 12 h/12 h light/dark cycle and temperature of 22 ± 2 °C. A red incandescent bulb is usually used to provide illumination during the dark phase. Animals should be allowed a minimum of 5 days of the acclimatization period prior to an experiment onset. All animals should be maintained on standard rodent chow with water access ad libitum (*see* Subheading 2.3).

In general, to perform ABA experiments, cages have to be equipped with a voluntary running wheel (with the diameter of 35 cm for rats or 11 cm for mice) with either semiautomatic or fully automatic (computerized) monitoring systems of in-cage activity. While simple digital counters with a magnetic switch allow monitoring of only the number of wheel turns (rotations/revolutions), the more advanced systems allow monitoring of additional

parameters, such as the exact run distance, run duration, or the exact timing of the exercise. In addition, cages should be outfitted with removable food hoppers. It is important that hoppers can be removed from a cage without too much disturbance during food deprivation period. Alternatively, removable blockers, located in the opening of the feeding niches, can be used to block access to food during food restriction. Water can be provided using bottles with steel-ball tip valves or using automatic watering systems.

After acclimatization period, animals should be divided into the main experimental ABA group with a restricted-feeding schedule and unlimited access to a running wheel as well as other appropriate control groups. Animals should be of similar initial body weight (<10% variation between groups). There is some variability in wheel running activity so it is advisable to use at least eight to ten animals per group. All experimental groups are discussed in detail in Subheading 2.4. Animals have to be individually housed to allow the accurate measurement of food intake and running wheel activity throughout the experiment. However, cages should be transparent and placed adjacent to each other to provide animals with sight, acoustic, and odor contact. A noncaloric bedding should be used in order to truly food deprive the rat, which is necessary in this phenomenon. Gastric distension that results from filling the stomach with bedding can cause the release of neurotransmitters and thus confound results of the studies.

2.3 Diet

A standard rodent chow is used in the model, with limited (a restricted-feeding schedule) or unlimited access, depending on an experimental group (details are given in Subheading 2.4). It should be noted that the limitation concerns the time frame during which animals have access to food pellets (feeding schedule) but not the amount provided during that time (i.e., an animal can eat as much as it wants during a single meal per day). Unlimited access to food pellets is provided for all experimental groups during acclimatization period only. Food intake data can be easily converted into calories for further comparisons. Water is always provided ad libitum throughout the experiment.

2.4 Experimental Groups

The main experimental group, an ABA group, has unlimited access to a running wheel and limited access to standard rodent chow, usually between 1 and 2 h/day (90 min/day is optimal according to our own experience) at the onset of the dark phase [15]. The development of ABA is directly proportional to the amount of wheel access [14]. The protocol is pursued until a weight loss criterion, usually 25% loss of the initial body weight, is reached in the ABA group [15]. Food intake, body mass, and wheel running have to be recorded so comparisons can be made with appropriate control groups.

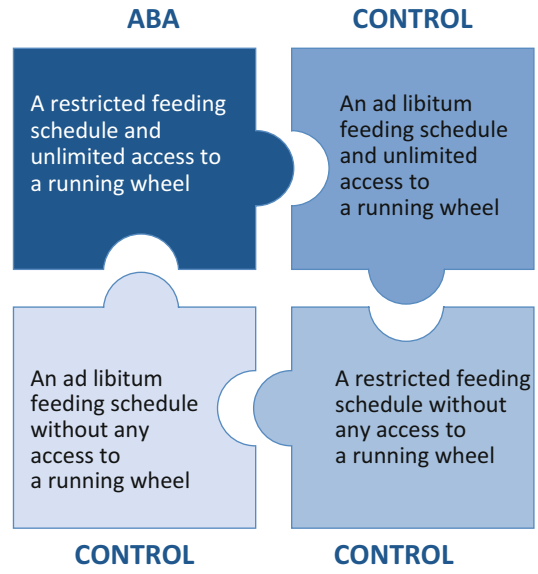

Fig. 2 All experimental groups in an activity-based rodent model of anorexia (ABA)

The following control groups should be maintained in the ABA experiment (*see* Fig. 2):

1. An ad libitum feeding schedule and unlimited access to a running wheel. This group is highly recommended as a control because it allows for the contrast of behavior and neurochemistry in normal feeding and increased voluntary physical activity. Food intake, body mass, and wheel running have to be recorded so comparisons can be made with other group of animals.
2. A restricted feeding schedule (identical to the ABA group) without any access to a running wheel. This group has 90 min access to standard rodent chow at the onset of the dark phase (food deprivation is identical to the ABA group). This group is highly recommended as a control because it allows for the contrast of behavior and neurochemistry in restricted feeding without any access to a running wheel. Food intake and body mass have to be recorded so comparisons can be made with other group of animals.
3. An ad libitum feeding schedule without any access to a running wheel. Food intake and body mass have to be recorded so comparisons can be made with other group of animals in the ABA model.

The time frame for completing an ABA experiment (*see* Fig. 3) is about a week (excluding acclimatization period), primarily depending on the initial weight of animals, the duration of food

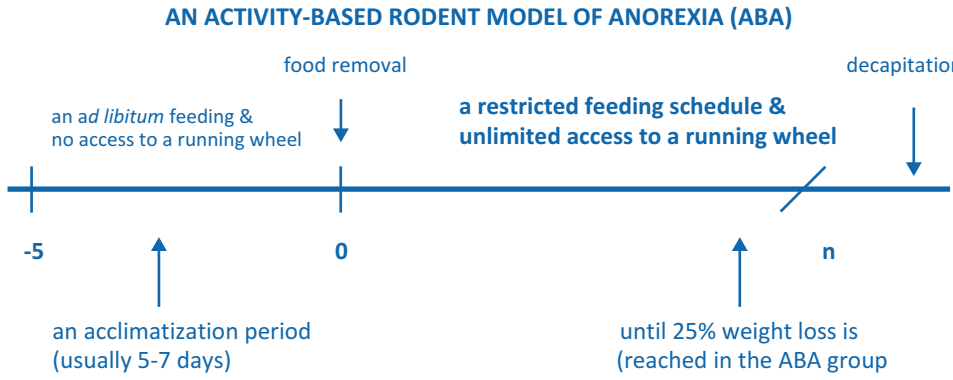

Fig. 3 An activity-based rodent model of anorexia (ABA) was introduced by Routtenberg and Kuznesof [15]. Rodents are exposed to a restricted feeding schedule and are housed in cages with an unlimited access to a running wheel, after an acclimatization period. Animals voluntarily increase their locomotor activity and reduce food intake leading to self-starvation with hyperactivity further aggravating their weight loss. The protocol is pursued usually until 25% weight loss of the initial body weight is reached in the ABA group of rodents. Food intake, body mass, and wheel running have to be regularly recorded so comparisons can be made within all experimental groups in the ABA model

access period, and prior access to a running wheel during acclimatization period. Body weight of all animals is usually measured just prior the dark phase or just before a mealtime in the food-restricted experimental groups. Daily commitments for routine measurements as well as administration and removal of food pellets vary depending on the number of animals being tested and generally require approximately 2–3 h/day.

2.5 Variations Within the Model

Any kind of stress prior to an ABA experiment could impact its results [44]. Thus, there is a great heterogeneity of the protocols used to induce ABA. What is more, animals always show individual variability in their wheel activity, eating behavior, and rate of weight loss. While this individual variability might make it difficult to obtain statistically significant differences, it opens an avenue of analysis by correlation. According to Perez-Leighton, the differences in food intake explain the rate of weight loss, whereas running wheel activity influences the amount of body weight loss [45]. Dixon et al. suggested that some of the variability may be due to individual differences in energy balance on the day in which restricted feeding is introduced, which is particularly relevant for studies involving female rats [46]. Energy balance is known to fluctuate across the estrous cycle. Female rats decrease food intake and increase locomotor activity during estrous compared to non-estrous phases [47–49].

A well-established variation of the ABA protocol is a gradual reduction of food access over several days [50]. There are several more protocols available for prolonged endurance of food restriction and a running wheel exposure even over a period of 55 days [25, 26, 44, 51, 52].

3 Notes

3.1 Body Weight

The protocol is pursued until a weight loss criterion, defined between 15% and 30% weight loss, is reached in the ABA group [15]. At the end of the experiment, animals have to be removed from the experimental conditions; otherwise, self-starvation and death occur. Indications of excessive starvation in rodents include a hunched posture and an inability to move around the cage. An animal may be cold to the touch and fail to eat during the scheduled food access period. Animals should be also removed from the experiment when their body temperature is lower than 33 °C. It should be mentioned that if animals were given unrestricted access to food after the end of an experiment, they should soon recover weight and remain healthy [44, 53].

3.2 Age and Gender

Rodent males and females exhibit different patterns of physical activity after limitation of food access [45]. However, female rats and mice are preferred due to the high prevalence of anorexia nervosa in female adolescents and young women. In general, the effect of ABA is much stronger in younger animals [53]. Rats given brief handling when pups receive more maternal interaction and, several weeks later, lose weight more slowly when subjected to an ABA procedure [54]. To develop ABA in an adult rodent population takes considerably longer than in younger animals, probably resulting from the fact that—besides absence of significant body weight gain during this period—intensification of running is lower in adults compared to adolescent rats. As a consequence, adult animals can often be maintained for up to several weeks without reaching the maximum weight loss criterion, while younger animals are more vulnerable to ABA and cannot be maintained for more than several days regardless of sex [52]. ABA has been performed in the same manner in various rodent breeds or species, in most cases either Sprague-Dawley or Wistar rats. Physical activity levels play an important role in ABA susceptibility in different rodent species with genetically diverse background [55].

It was also reported that wheel running is significantly greater and weight loss occurs more quickly in female rats than in male rats [15, 56–58], which might be related to higher rates of wheel running in female rats [46, 59, 60].

3.3 Access Period to Food

It is critical to always provide more chow than animals are able to consume. Otherwise, when food access is not restricted in time but a fixed amount of food is given in the so-called semi-starvation-induced hyperactivity model, no (further) reduction in food intake can be observed as indicated by similar intake of food in food-restricted animals without any access to a wheel and the ABA animals [61]. However, according to Verhagen, a restricted feeding

group without any access to a wheel should be pair-fed with the ABA group, and thus, rodents should receive an average amount of food eaten by the ABA rats the day before [26]. In general, the longer the feeding period, the slower the weight loss and the less likely that self-starvation occurs [44].

It is expected that at least some animals from the ABA group may not eat at all after a week as food intake declines at a scheduled mealtime when a running activity increases. Control animals, which are food deprived and without any access to a running wheel, adapt to the feeding schedule within several days and remain healthy throughout the experiment. Thus, pre-exposure to a restricted feeding schedule attenuates the ABA effect by providing animals with the opportunity to adapt to the new feeding schedule before gaining access to a running wheel. Animals pre-exposed to food restriction lose less weight, eat more, and run less that the nonexposed group when food restriction and wheel access are combined [56, 62]. What is more, more frequent but shorter feedings mitigate the effect of a restricted feeding schedule as rats tend to eat frequently but in small amounts. Such feeding conditions approximate a natural feeding pattern of rodents [63].

Rodents, which are nocturnal animals, typically engage in eating at the onset of the dark phase [64], and a meal in the ABA model is usually scheduled during that time. Food access scheduled in the middle of the day (during the light phase) presents an additional difficulty in adjusting to a restricted feeding schedule, especially when paired with an access to a running wheel [56, 65, 66].

3.4 Access Period to a Running Wheel

When a running wheel is available, an animal can walk, run, or sit or exhibit any other behavior in a cage. There are no programmed activities. However, when food restriction and the opportunity for wheel running co-occur, animals begin to run voluntarily. They increase running over days, even though there is no requirement to do so, and a typical rat in an ABA model may run even up to 15 km per day [67]. In general, the shorter the time per day that a rat can spend in a running wheel, the slower the weight loss and the less likely that self-starvation occurs [44]. The introduction of a running wheel causes a transient decrease in food intake in male rats with an ad libitum feeding schedule and unlimited access to a running wheel [68], even when the access to a running wheel is reduced to 2 h/day [69]. Increased running upon exposure to the ABA model is displayed throughout the dark phase as well as in the light phase preceding food access and is referred to as food anticipatory activity (an increased activity preceding mealtimes) [18, 70].

The animals usually have access to a running wheel inside the cage for 24 h/day; however, Pierce and Epling suggested that experimental animals should be given continuous access to a running wheel except during the feeding period in order to avoid competition between running and eating [67]. It should be noted that due to the spontaneous variability in activity, about 20–30% of rats are not interested in wheel running [71, 72]. It was also reported that in-cage preadaptation (during or after acclimatization period) to a running wheel may accelerate the development of an activity-based anorexia [46, 62]. Animals pre-exposed to a running wheel lose more weight, eat less, and run more that the nonexposed group when food restriction and wheel access are combined [56, 62]. Low running activity during acclimatization/preadaptation period may suggest low susceptibility to ABA [45, 55].

3.5 Ambient Temperature

Most ABA experiments are run at a temperature of 22 ± 2 °C, but when the temperature is raised to around 30–32 °C, both female and male rats introduced to the ABA procedure run less and lose weight more slowly [72–74].

4 Conclusions

AN represents a disorder with the highest mortality rate among all psychiatric diseases [75, 76], and ABA is thought to be the best animal model analogous to AN [19, 73, 77]. The ABA model provides a phenomenon that can be easily implemented and effectively used to explore the pathophysiological consequences of hyperactivity and malnutrition in AN. Nowadays, brain imaging techniques such as computerized tomography scan (CT), magnetic resonance imaging (MRI), or positron emission tomography (PET) can be also successfully employed in rodents and thus hold the promise for accurate characterization of complex neurocircuits involved in ABA and their relationship to behavior in living animals. The flexibility of the ABA phenomenon allows for further modifications and explorations in search for possible pharmacological interventions.

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