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Animal Models of Eating Disorders

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Foreword

The salient features of the most common human eating disorders are simple to describe and, in practice, not difficult to recognize. The relentless pursuit of thinness accomplished by 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 well over 100 years ago. More recently, clinicians have come to appreciate 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 are clearly abnormal and are sufficiently robust that they have been examined objectively in laboratory studies. However, it has proven exceedingly difficult to understand precisely how these disturbing behaviors arise, and, once they have become established, why they are frequently so persistent. These questions are among the most important facing clinical researchers in this field.

In many areas of medicine, it has been possible to examine critical features of illnesses in animal models. For example, mechanisms underlying the development of inflammatory diseases, cardiovascular illnesses, and malignancies have been successfully probed in nonhuman species, and such investigations have led to major advances in understanding critical pathological processes and to the development of effective treatments. The development of animal models to study mental disorders has been much more difficult, as these disorders usually involve cognitive and emotional disturbances that are extremely difficult to confidently express in animals. In recent decades, however, significant progress has been made in probing the neural circuitry of disturbances, which may be critical to understanding mental disorders. For example, the mechanisms responsible for fear learning and avoidance behavior in animals may be of substantial relevance to the pathophysiology of anxiety disorders in humans. Similarly, disturbances of working memory, the function of which can be elegantly probed in animals, may play an important role in the functional impairment of individuals with schizophrenia.

The current volume is a testament to the burgeoning use of animal models to probe core facets of human eating disorders. Part I focuses on binge eating, the salient feature of both bulimia nervosa and binge eating disorder. The chapters in this section 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. Such manipulations have clear parallels to the impact of external parameters and of internal emotion state on the fluctuation of symptoms in human eating disorders. In addition, several chapters highlight how the potential utility of medication can be explored in such models. The section also includes a chapter describing the direct translation of sham feeding, a very useful procedure to examine the control of animal eating behavior, to humans with eating disorders. In addition, deep-brain stimulation in a binge eating model is discussed.

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 section 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. Attempts to elucidate the parallels between eating disorders and substance abuse by focusing purely on descriptive human studies have yielded limited clarity, and the attempt to bring insights from animal models is most welcome.

The self-imposed food restriction that is the salient feature of anorexia nervosa is challenging to study both in humans and animals. Part II of this volume is comprised of chapters describing a range of innovative approaches which may provide insights into this striking behavioral syndrome. 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 development. Almost all cases of anorexia nervosa and bulimia nervosa begin during adolescence. While adolescence is a time of enormous psychological and biological change and stress, whether and how such factors contribute to the vulnerability to develop eating disorders is unknown. The chapters in this section identify behavioral and biological parameters and the impact of stress during early development that may set the stage for the development of eating disorders.

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 line of research is a most welcome new addition to the attempts to decipher the mysteries of anorexia nervosa, bulimia nervosa, and binge eating.

New York, NY, USA *B. Timothy Walsh M.D.*

Preface to the Series

Under the guidance of its founders Alan Boulton and Glen Baker, the Neuromethods series by Humana Press has been very successful since the first volume appeared in 1985. In about 17 years, 37 volumes have been published. In 2006, Springer Science + Business Media made a renewed commitment to this series. The new program will focus on methods that are either unique to the nervous system and excitable cells or which need special consideration to be applied to the neurosciences. The program will strike a balance between recent and exciting developments like those concerning new animal models of disease, imaging, in vivo methods, and more established techniques. These include immunocytochemistry and electrophysiological technologies. New trainees in neurosciences still need a sound footing in these older methods in order to apply a critical approach to their results. The careful application of methods is probably the most important step in the process of scientific inquiry. In the past, new methodologies led the way in developing new disciplines in the biological and medical sciences. For example, Physiology emerged out of Anatomy in the nineteenth century by harnessing new methods based on the newly discovered phenomenon of electricity. Nowadays, the relationships between disciplines and methods are more complex. Methods are now widely shared between disciplines and research areas. New developments in electronic publishing also make it possible for scientists to download chapters or protocols selectively within a very short time of encountering them. This new approach has been taken into account in the design of individual volumes and chapters in this series.

Wolfgang Walz

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 U.S. and in other developed countries continues to pose a problem, and clinicians continue to struggle with 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. I would also like to thank Ms. Cindy Kroll for her invaluable editorial assistance, as well as Ms. Miaoyuan (May) Wang for her assistance with the images and Ms. Susan Murray, Monica Gordillo and Hana Shin for their help with formatting and editing. I would also like to thank Wolfgang Walz (Series Editor) and Springer for their guidance and interest in this important topic.

Gainesville, FL, USA — *Nicole M. Avena*

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Part I

Binge Eating, Bulimia, and Hedonic Overeating

Chapter 1

Introduction: Binge Eating, Bulimia Nervosa, and Hedonic Overeating

Sarah Shafer Berger and Marian Tanofsky-Kraff

Abstract

Binge eating, bulimia nervosa, and hedonic overeating share a critical common component; namely, overeating that involves a lack of healthy restraint. However, these constructs are distinct from one another and are related to differential correlates and outcomes in human beings. Notably, all three behaviors can be modeled in animals, thus providing important insights to inform human research.

Key words: Overeating, Loss of control, Compensatory behaviors

1. Binge Eating and Binge Eating Disorder

Binge eating is characterized by the consumption of an objectively large amount of food accompanied by a feeling of loss of control (i.e., the sense that one cannot control what or how much one is eating) over eating (1). Binge eating is the hallmark behavior of binge-eating disorder (BED). BED is defined as recurrent episodes of binge eating with associated impairment and/or distress regarding the eating episodes (1). The prevalence of BED is estimated to be about 3% with the disorder being somewhat more prevalent in women (2). It is estimated that 76% of adults and 85% of adolescents with BED also have psychiatric comorbidities (e.g., anxiety, mood disorders, substance abuse) (2, 3) or suicidal ideation (3). Functional impairment in work, home, or personal life is also reported among individuals with BED compared to non-obese individuals without BED (2).

Approximately 35% of those who regularly binge eat are overweight or obese (2) and may be at higher risk for hypertension, dyslipidemia, or type 2 diabetes (4–7). However, individuals with BED differ from obese adults without the disorder. In laboratory

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investigations of eating and studies using self-report surveys, individuals with BED consume significantly more total energy than obese adults without BED (8–12). During weight loss treatment, some studies show less weight loss, more rapid weight regain, or more attrition from treatment for those individuals with BED compared to individuals without BED (8, 13).

2. Bulimia Nervosa

Similar to BED, bulimia nervosa involves recurrent binge eating, impairment, and/or distress. However, bulimia nervosa also involves compensatory behaviors (e.g., vomiting, laxative use, excessive exercise) following binge episodes in order to prevent weight gain (1). The rates of bulimia nervosa are estimated to be lower than BED, with 1.5% of women and 0.5% of men reporting lifetime prevalence (2). Bulimia nervosa is associated with psychiatric comorbidities like suicidal ideation (53% reported ideation and 35% reporting attempts) and functional impairment (2, 3). Unlike BED, there are associated features that differ; individuals with bulimia nervosa tend to eat fewer meals, nibble more, and have higher levels of dietary restraint (14). Persistent bulimia nervosa is associated with body image disturbances, general psychopathology, and impaired sexuality and social relationships (15).

3. Hedonic Overeating

Hedonic overeating is the over consumption of highly palatable foods in the absence of current energy needs (16). It is often discussed in the context of food addiction (17), which remains a controversial topic (18). Nevertheless, hedonic overeating may be an important area for understanding atypical eating patterns and for weight-loss treatment development (19). It is assumed that when consuming highly palatable or "liked" foods, brain signals activated are the same as those triggered by drugs of abuse (20, 21). Hedonic overeating is also linked with obesity, but there are little data on other adverse correlates (16, 22–25).

4. Animal Models of Binge Eating, Purging, and Hedonic Overeating

Animal models of binge eating, bulimia nervosa, and hedonic overeating offer a unique opportunity to carefully elucidate these behaviors that may ultimately benefit human beings. For example,

animal models allow for isolation of the aberrant eating pattern from body weight, thereby eliminating the potential confound of obesity influencing physiology and/or behavior (26). Furthermore, animal models of bulimia nervosa allow for examination of changes in neurochemistry (27) that would not be safe or ethical in human samples. Similarly, hedonic overeating can be modeled in rats to elucidate brain changes in the reward pathways (28) that would be too difficult and invasive in human beings.

In conclusion, binge eating, bulimia nervosa, and hedonic overeating involve unhealthy eating patterns that can have serious physical and psychological consequences. Animal models are an innovative research strategy that provide valuable preclinical knowledge and additional information about the biopsychosocial nature of eating disorders and obesity. In collaboration with human research, these two approaches have the potential to yield effective interventions.

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Chapter 2

Binge-Prone Versus Binge-Resistant Rats and Their Concomitant Behavioral Profiles

Mary M. Boggiano

Abstract

Binge eating is a recalcitrant symptom of bulimia nervosa, binge-eating disorder (BED), and the binge/purge subtype of anorexia nervosa. Binge eating is rooted in gene–environment interactions, but the biology of these interactions is largely unknown. This chapter describes a simple and reliable animal model of binge eating that is based on such an interaction: a significant inherent difference in eating patterns when palatable food (PF) is encountered in the environment. Roughly one-third of rats exhibit a binge-like pattern of intake of PF despite normal intake when only chow is available. The PF intake of these binge-eating prone (BEP) rats is significantly and consistently greater than that of binge-eating-resistant (BER) rats. Also described are subsequent experimental manipulations that reveal additional parallels between BEP rats and human binge-eating behavior, including preference for and abnormal intake of PF when stressed, binge eating in the absence of hunger and despite evidence of satiety, motivation to obtain and eat PF despite punishing consequences, and age of onset shortly after puberty. The model also dissociates binge eating from obesity proneness such that four subgroups can be obtained that resemble bulimia nervosa (binge eating with compensatory restriction to prevent obesity), BED (binge eating with propensity for obesity), frank obesity (obesity proneness without binge eating), and healthy controls (non-binge-eating, obese-resistant rats). These behavioral profiles render the BEP/BER model a useful tool to uncover some of the genetic and epigenetic substrates distinguishing BEDs. It can also be used to develop and test more targeted treatments against these life-threatening conditions.

Key words: Binge eating, Diet-induced obesity, Eating disorder, Obesity, Rat

1. Introduction

This chapter describes an animal model of binge-eating prone (BEP) versus binge-eating resistant (BER) rats. Binge eating in humans is a highly distinct pattern of overeating that is characterized by the intake of an abnormally large amount of food in a discrete period of time and is accompanied by a sense of lack of control over the ability to limit the amount of food eaten or to stop eating (1).

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Binge eating is a stubborn symptom in the binge/purge subtype of anorexia nervosa, of bulimia nervosa, and of binge-eating disorder (BED) (1), which collectively afflicts approximately 8% of the U.S. adult population (2). For brevity, these three disorders will be referred to here as "binge-eating disorders."

In its most basic form, the BEP rats most closely model BED out of all of the eating disorders. This is because caloric restriction and/or weight loss are not required to produce BEP rats. However, this chapter describes subsequent manipulations of the model that can be easily conducted to yield behavioral responses with parallels to features of bulimia nervosa and binge/purge anorexia. The BEP/BER model originated from the observation that, while rats of the same age and sex eat relatively equal amounts of standard lab chow, their intake will vary when offered highly palatable food (PF). The PF is high in sugar and/or fat and is given intermittently (e.g., 2–3 times per week vs. daily) to simulate the "forbidden" regard of these foods and diagnostic frequency in clinical binge eating (1, 3, 4). The key observation is that approximately one-third of the rats *consistently* eat the highest, and one-third eat the lowest, amount of the PF (5). This stable pattern is consistent with the established chronic and stable nature of binge eating in BED (6). Importantly, the expression of binge eating in the BEP rats is not observed until they come in contact with PF. That is, BEPs and BERs are indistinguishable until exposed to an environment containing PF. BEPs also do not have to learn to overeat PF; they inherently overeat during the first exposure. This is important because it represents an example of a gene–environment interaction. Gene–environment interactions are pathogenic of eating disorders, yet are not researched as aggressively as they need to be (7, 8).

Our work suggests that the only environmental factor needed to elicit the BEP/BER model is PF. The salience of PF in human binge-eating behavior cannot be understated. Since these foods are typically high in refined sugars and fat, they are rewarding and calorie dense, and are therefore regarded as "forbidden foods" outside of binges. They are obsessed over, craved, and overconsumed during binges (4, 9, 10). Intake of just a morsel of PF or simply the smell of PF is known to trigger relapses back to binge eating (11–13). It is not possible to estimate the extent to which exposure to PF is necessary for the development of BEDs because of its ubiquitous nature in the modern world. We are exposed to these foods from childhood and even as neonates (14, 15). Nonetheless, the reliance of the BEP/BER model on PF exposure does not devalue the model as a research tool in BEDs, especially considering the salient role of PF in these disorders.

The numerous parallels between BEP rat behavior and clinical binge eating validate its use as a preclinical tool. These parallels go beyond binge eating in a discrete period of time and the stable nature of the binge-eating pattern. Here we describe additional parallels that emerge when the rats are subjected to factors relevant

to life experiences of those with BEDs. These factors include stress, hunger, tolerance of painful consequences in order to binge on PF even when sated, and exposure during puberty. In all cases, BEP rats respond with striking similarity to individuals with BEDs (5, 16, 17). The BEP/BER model also recapitulates the independence between binge eating and propensity to develop obesity. Given the large weight range of patients with BEDs, it is not surprising that familial studies confirm a strong genetic contribution for binge eating that is independent of the obesity phenotype (18, 19). Here too is described how it is possible to obtain four subgroups from the BEP/BER model that are behaviorally similar to bulimia nervosa, BED, non-BED obesity, and healthy controls (5). These subgroups could prove useful in the discovery of genes that distinguish propensity for each of these conditions. In so doing, more targeted treatments can be designed. Lastly, the model promises to help identify the exact physiological changes that take place when modern "super-hedonic" food ingredients and predisposing genes intersect. This type of research is needed to learn how to best prevent or decrease the recidivistic nature of BEDs.

2. Materials and Procedures

2.1. Animals

Young adult female Sprague–Dawley rats (Harlan, IN and WI) are used in keeping with the higher female incidence and age of onset of BEDs (2, 7). The rats may be older in age but not pre-pubertal (17). The typical results presented here are those obtained with 60-day-old rats. Regarding the number of rats required at the onset, the BEP/BER model relies on *extreme amounts* of PF consumed. These extremes are best observed from an initial group that is three times the number of rats desired in the BEP and BER group. For example, for $N=10$ BEPs and $N=10$ BEPs (a typical N per group in the author's and others' rodent studies), one would start with $N=(10\times3)$ or 30 rats. Of course, the number of BEP and BER rats required will depend on the complexity of the study design; e.g., drug versus control conditions may require 20 rats per group if working with a between-groups design in which case a good start number would be (20×3) or 60 rats. Multiple studies confirm the reliability of the "$N\times3$" formula because roughly one-third of female rats will meet BEP and one-third will meet BER criteria (5, 16, 17, 20, 21). The rats can be single or pair-housed when not being tested and should be acclimated to standard colony conditions with a 12:12-h dark/light phase. Lights should be timed to turn off at the onset of the feeding tests; e.g., if feeding tests wish to be conducted at 1000 hours then lights should go off at 10 a.m. (1000 hours). This captures intake during the initial dark period. Rats should be well acclimated to any new light/dark schedule as for any other study.

2.2. Diet

The rats are maintained on ad libitum water and standard rat chow (e.g., Harlan Teklad Global Diets, IN; 3.3 kcal/g) throughout the studies. To identify BEP and BER groups, a PF must be introduced. Most studies have used Oreo Double-Stuf® cookies (4.8 kcal/g; Nabisco, NJ) (5, 16, 20). Oreos® have worked well in other models of binge eating (5, 20, 22–24) and include the high-fat and sugar contents that are typically craved and overeaten by humans who are binge eating (3, 4). Other PFs have been used successfully (5, 17, 21), but the results given here are those resulting with the use of Oreos®. The use of other PF types is discussed in Sect. 3. The PF is always given alongside standard rat chow.

2.3. Identifying BEP and BER Rats

BEP and BER rats are identified by a series of "feeding tests." All rats are first allowed to overcome neophobia by introducing a few grams of the PF (e.g., half a cookie) in home cages prior to the feeding tests. The rats are then subjected to four measured feeding tests. Each test consists of placing a generous premeasured amount of the PF (e.g., two Oreo® cookies, ~29 g) and chow pellets (e.g., 10 g) inside of or on the lid of the home cages just prior to lights out. Intake is measured after 4 h under red or dim lighting. The cookies remain in the cage for 24 h. Care should be taken to include any spillage, although it tends to be minimal with these types of foods. The 4-h interval is a discrete period of time that provides measurable differences in intake between groups in this and other models of binge eating (20, 22–25). Food intake can be measured at any other intervals up to 24 h, but is not necessary for the identification of BEP/BER status. Body weights can be recorded periodically to confirm no change in weight between the groups over time. However, body weights are also not necessary in determining BEP/BER status. Importantly, the feeding tests are separated by at least 1 day of only ad libitum chow. Typically the PF and chow feeding tests occur 2–3 times per week. This renders PF intake as an intermittent event, simulating the two times per week criteria for binge eating (1) and the "forbidden food" regard for PF in BEDs (4, 9, 10). Periodic 24-h measures of chow intake on the chow-only days serve to confirm that there is no significant difference in amount of this food eaten between BEP and BER rats. Differences are only observed with PF. Once BEP/BER status is established using the criteria described in Sect. 2.4, the feeding tests can occur less frequently (e.g., one time per week) for subsequent experimental manipulations.

2.4. Criteria Used to Classify BEP from BER Rats

For each of the four feeding tests, the kcal intake of PF of each of the rats is grouped into tertiles. That is, the values and their corresponding rat identification are evenly distributed into three groups: a lowest, a middle, and a highest PF-intake group. Rats in the lowest PF-intake tertile across all four of the feeding tests, or in three out of the four tests, are assigned BER status. Those in the highest PF-intake tertile across all four, or three out of the four

tests, are assigned BEP status. How consistently a rat appears in a particular tertile is more important in determining status than the absolute kcal value of PF consumed—extreme as it might be if the rat appears in more than one tertile. Rats falling into the middle tertile do not need to be retained for further BEP versus BER tests unless one wishes to maintain the rats on chow throughout as a chow-control group. This is an appropriate control because all rats, regardless of BEP or BER or "middle" status eat equivalent amounts of chow when only chow is available.

Alternatively, the middle group can be treated as a third "middle PF-eating" group, but the caveat must be considered that some of the rats making up this group were placed in the group because of their inconsistent amount of PF intake. Another option, and one used by the author, is to retain the middle rats to pilot test any experimental variables before they are tested on the BEP and BER rats. This is useful given that the rats are of the same age, sex, and body weight as the BEP and BER rats. But again, if time, labor, and cost are an issue, and if the study aims permit, the middle tertile rats need not be used at all. At the end of the feeding tests, there should be an equal number of BEP and BER rats. Subsequent PF + chow feeding tests can be conducted to confirm the stability of the BEP/BER patterns, but these should be preceded by at least 1 day of only chow with no experimental manipulations. The typical BEP/BER intakes one can expect are described in the Sect. 3.

2.5. Time Required

If the feeding tests are administered 2–3 times per week, BEP and BER rats can be identified within two weeks' time.

2.6. Data Analysis

Frequency descriptive statistics set at 33.3% percentiles will yield tertile groups of PF intake for each feeding test. Cronbach's alpha can be used to verify consistency of high versus low PF intake within rats across the feeding tests. Student's *t*-test or ANOVA is used to compare differences between BEPs and BERs on PF intake, chow intake, and body weights. Bonferroni or Tukey post hoc tests are used if more than two groups are compared, e.g., if the middle group is analyzed. The alpha level for all comparisons is 0.05. More complex designs have been used, such as mixed within-subject and repeated measures ANOVAs (20), and mixed linear models when measuring changes over time (17). All food intake data should be converted to and analyzed as kilocalories, especially when combining measurements of intake of various energy-dense foods.

3. Notes and Anticipated Results

The following variables are *not necessary* to obtain the BEP/BER model, but they yield additional behavioral profiles in BEP rats that parallel features of binge eating. These can serve as additional

"models" according to the characteristics one wishes to explore further. Given the simplicity of obtaining BEP and BER groups, these subsequent manipulations do not require much additional time to perform. All of the variables described below were tested in a between-groups design starting with $N=60$ rats for $N=20$ BEP and $N=20$ BER young adult female Sprague–Dawley rats, except where noted. If rat labor and upkeep, but not time, are issues, half the rats per group (e.g., $N=8$–10/group) is acceptable for most within-subjects designs. The results of these manipulations and their relevance to understanding binge-eating behavior in humans are described below, as are alternate methods of conducting these tests.

3.1. Effect of Acute Food Deprivation on BEP Versus BER Rats

Once rats are classified as BEP or BER and following at least 2 days of chow-only feeding, half of the rats from each group are given 50% of their normal 24-h chow intake at lights out. The reduced amount is 50% of the mean of all the rats' previous day's 24-h chow intake. The other half of the rats of each group remain on ad libitum chow. On the following day just prior to lights out, all rats are given a premeasured amount of PF and chow, as in the feeding tests, and intakes are recorded after 1, 4, and 24 h. More frequent recordings can be taken if needed.

3.2. Effect of Stress on BEP Versus BER Rats

Stress typically has anorectic effects on laboratory rats (26, 27). The exception is when stress is combined with a "history of dieting," which the author used to develop a different model of binge eating (22, 24) (also see Chap. 3 for innovative variations on this model). The BEP/BER model does not require caloric restriction or dieting simulations, so the rats are not expected to overeat when stressed. Nonetheless, stress evokes interesting and clinically relevant differences between the BEP and BER rats. Once the rats are classified as BEP or BER and following at least 2 days of chow-only feeding, half of the BERs and half of the BEPs are individually subjected to four 3 s bouts of 0.6 mA of scrambled foot shock in a shock alley apparatus, prior to lights out. The other half of the rats in each group is placed in the shock alley for the same amount of time without shock.[1] The rats are then returned to their home cages while lights are still on, with a premeasured amount of PF and chow as in the feeding tests. Intakes are recorded after 2 and 4 h of feeding.

3.3. Effect of Suffering Consequences for PF in BEP Versus BER Rats

This test of motivation for PF uses the same foot shock apparatus as in the stress procedures above. Here $N=10$ BEP and $N=10$ BER rats naïve to foot shock are allowed to eat ad libitum amounts of chow in their home cages during the first 2–4 h in the dark. This precludes hunger from confounding this test, which is intended to

[1] Additional details on this manipulation are in (5); shock apparatus details can be found in (22).

measure motivation for the rewarding versus metabolic properties of PF intake. The rats are then allowed to individually roam in the shock alley under red light to acclimate to the space and to learn that one end of the alley is baited with PF. Plain M&M's® candy (Mars, McLean, VA) has been used, but it is likely that another PF such as Froot Loops (Kellogg, MI) or small flavored pellets (Research Diets, NJ) would work as effectively (28). Acclimation to the alley is confirmed when all rats take at least one bite of an M&M® during the first minute after being placed into the shock-free end of the alley. This typically occurs after three 10-min sessions in the alley (over 3 days). On the first day of actual testing, the rats are placed in the alley for 10 min, but with no shock, in order to obtain a baseline measure of PF intake under these conditions. On the second day, the lowest level of shock (0.10 mA) is administered for 3s immediately following retrieval of an M&M®. The candy must be completely removed from the food hopper by paw or mouth before shock is delivered. This level of shock is readministered for as many times as the rat returns and retrieves an M&M® during a single 10-min session. In each 10-min session thereafter (on the following days), the shock level is increased by 0.05-mA increments until the rat no longer retrieves PF. On the test day following a session where the rat chooses not to retrieve an M&M®, the rat is given a last chance to retrieve and if there is no attempt within the 10-min session, the rat is no longer put into the alley for the duration of the study. When placed into the alley, the rats are always placed in the end of the alley that is not baited with food or wired to shock.[2] Measures recorded include number of M&M's® retrieved, amount of M&M's® kilocalories consumed per session at each shock level, and the highest shock level tolerated per rat.

3.4. Effect of a High-Fat Diet on the Propensity of BEP Versus BER Rats to Develop Obesity

Clinical binge eating occurs in individuals that maintain a wide range of body weights, e.g., binge eating occurs in underweight anorexia nervosa, normal weight bulimia nervosa, and overweight or obese BED patients (1). Hence, the susceptibility to develop obesity should be independent of binge-eating status. To test for this, $N = 20$ BEP and $N = 20$ BER, which never significantly differ in body weight, are subjected to a traditional diet-induced obesity (DIO) protocol (29, 30). Under the DIO protocol, all the rats are provided with a daily sole ad libitum diet of 35% fat pellets (Research Diets, Diet # D12266B, New Brunswick, NJ) in their home cages for a minimum of 14 days. Body weights and 24-h food intakes are recorded daily or at minimum on days 1 and 14. During statistical analyses, body weights on day 14 of half of the rats that gain the most weight are compared to weights of the other half of the rats that gain the least weight, regardless of BEP/BER status.

[2] Refer to (16) for additional details.

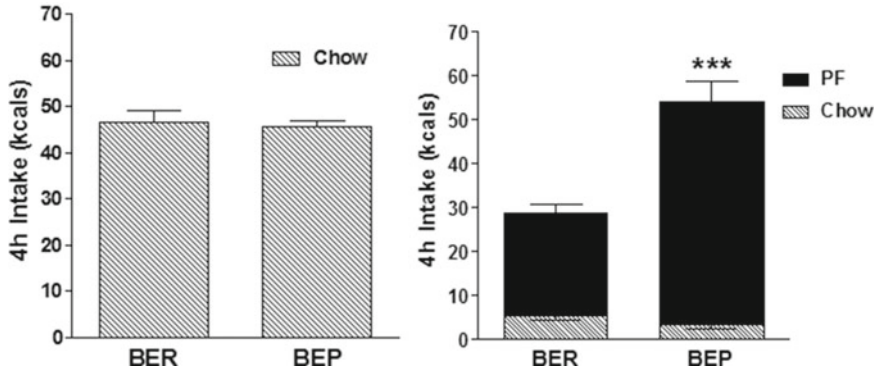

Fig. 1. Typical 4-h intake patterns of binge-eating resistant (BER) versus binge-eating prone (BEP) rats; $N=20$/group. (**a**) When only chow is available the groups are indistinguishable by their intake (ns). (**b**) When PF is available with chow, BEPs consistently consume >40% more PF kilocalories than BERs (***$p<0.001$). The effect is also observed with a smaller $N=8$–10 rats/group (16, 17, 20, 21). Reproduced, with permission, from (5).

A statistically significant difference in the means will confirm the success of the DIO protocol to identify obese-prone from obese-resistant rats. Then a chi-squared test can be used to determine if there are a different number of BEP versus BER rats in the obese-prone versus obese-resistant groups. Equal numbers of BEPs and BERs are expected in each weight group given the independence of binge eating from obesity proneness.

3.5. Effect of Developmental Factors on the Expression of BEP Versus BER Patterns

One may wish to use this model to examine environmental or physiological correlates of early-life experience, puberty, or aging on binge eating. Klump and colleagues investigated the age of onset and effect of estradiol removal on BEP/BER patterns in female Sprague–Dawley rats. The PF for the feeding tests was 15–20 g of Betty Crocker Vanilla Frosting (General Mills, MN) in a dish suspended inside the cage. More was added as needed with the rats' growth over time. The feeding tests were conducted as described above and occurred three times per week from postnatal day P23 through P69. Puberty onset occurred at P34-P39 (defined as vaginal opening), during which time feeding tests were conducted only one time per week. In sum there were six feeding tests in pre-early puberty, four in mid-late puberty, and five in adulthood. Mixed linear models analyses were used to compare PF intake, chow intake, and body weight during age development in BEP versus BER rats (17). In a separate study, adult BEP and BER rats were ovariectomized (OVX) at age P70 or P71 and subjected to four additional feeding tests on day P79 through P86. A second study controlled for any effects due to the surgery by including sham-operated rats (21).

3.6. Typical/ Anticipated Results

The feeding tests yield two groups of rats that never differ in the amount of plain chow intake if they only have access to chow (Fig. 1), but that differ consistently (Cronbach's alpha =0.86) and significantly in the amount of PF they consume. As shown in Fig. 1,

the BEP group typically consumes >40% more PF kilocalories by 4 h (55% shown here) than do the BERs. The statistical difference in PF intake can actually be observed as early as the first hour of eating (5) (not shown) but the 4-h period assures that rats have eaten to satiety and it is also a time interval when BEP/BER differences are the largest. By 24 h, the BERs approach but do not quite match the BEPs' PF intake (5). Replications of the model have obtained similar BEP/BER differences with as few as $N = 8–10$ rats per group (16, 17, 20, 21). Tests using the middle PF eaters show that they eat an amount of PF intermediate with that of the BER and BEP groups (5). There is never a significant difference in body weights between the two groups due to the intermittent access to PF. In line with the stable nature of clinical binge eating (6), the BEP/BER patterns are stable. Consistent patterns have been observed even after multiple manipulations, some noxious, including acute and cyclic food deprivation, foot shock, contextual-cue conditioning, exposure to other PFs (5, 16, 20), and surgeries (21). Eating a larger amount of food than normally expected, within a discrete period of time, and with a sense of lack of control to limit intake are diagnostic features of binge eating (1). Likewise, BEPs consume an amount of food clearly larger than normal, in a discrete period of time. This is not only due to the fact that their intake is being compared to the extreme lowest PF-eating rats because they can also eat a significantly greater amount of PF than the middle PF eaters (21). They also seem unable to regulate the amount of PF they consume despite the fact that, when only chow is available, they consume as much as BERs, which hints of normal satiety function. Hence, exceeding this level of food intake suggests that they ignore satiety signals when bingeing on PF. The results from the hunger test below support this assumption.

3.6.1. Effect of Acute Food Deprivation on BEP Versus BER Rats

As shown in Fig. 2, a period of caloric restriction causes BERs to eat significantly more food then when sated. This increase consists of greater chow intake, which is typical of rats hungry from metabolic deficit (25, 31). BEPs, too, eat proportionally more chow than when sated and so appear to respond normally to hunger. Also, because hungry BEPs do not eat more total kilocalories than hungry BERs, it can be implied that they also have normal satiety. However, as also shown in Fig. 2, the amount of total kilocalories that BEPs eat under restricted conditions matches the amount of calories they take in under sated conditions. This behavior is clearly abnormal especially given the behavioral indices of normal hunger and satiety cues in these rats. The responses just described are observed in the 4th hour of feeding but can all be observed as early as after the 1st hour of feeding (5). Clinical binge eating also appears to be unaffected by hunger and satiety cues. Indeed, hunger is one of the weakest triggers of binge eating and binges are diagnostically defined as occurring in the absence of hunger (1, 32–34). In humans, a more potent trigger is stress (34–38).

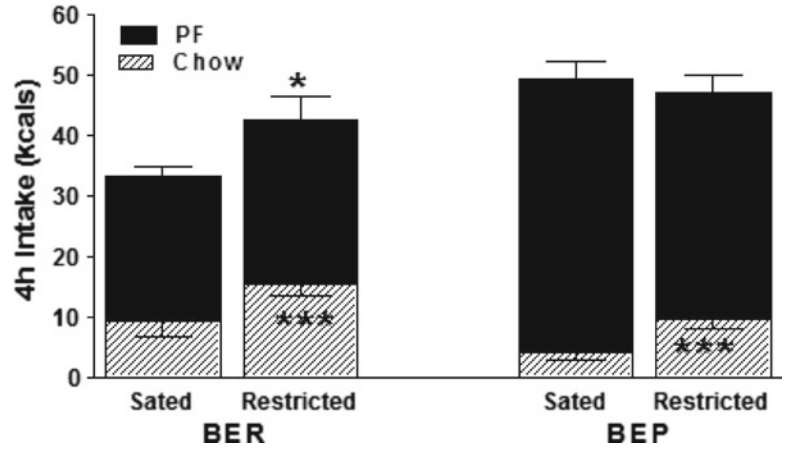

Fig. 2. Amount of chow and PF consumed by BEPs and BERs ($N=10$/per condition) under calorically restricted or hungry versus ad libitum or sated conditions. BERs eat more total kilocalories after a period of deprivation,*$p<0.05$, and both groups eat proportionately more chow kilocalories under food deprived than sated conditions, ***$p<0.001$. However, BEPs eat as many kilocalories when sated as when they are hungry after a period of food deprivation (ns). Reproduced, with permission, from (5).

3.6.2. Effect of Stress on BEP Versus BER Rats

As seen in Fig. 3, at 2 h following foot shock, stressed BERs consume less food than when not stressed. This is the expected and normal response of rats to laboratory stressors (26, 27). However, stressed BEPs fail to display this normal hypophagia and in fact appear to be completely unaffected by shock. By 4 h, stress causes BERs to remain hypophagic. Notably, they are eating less PF, not less chow. By this time BEPs appear somewhat affected by the stress, but their decrease in intake is due to forsaking the healthy chow over PF. Their PF intake remains abnormally elevated. By 24 h, the intake of each group normalizes to match their counterparts' intake under nonstressed conditions. The behavior of BEPs under stress resembles that of human binge eating in that stress triggers overeating versus undereating and is associated with increased consumption of PFs (34–37). In human binge eating, stress may actually make PFs more rewarding (38). Just how rewarding BEP rats find PF can be seen by how much punishment they are willing to tolerate for it.

3.6.3. Effect of Suffering Consequences for PF in BEP Versus BER Rats

As shown in Fig. 4, BEPs make significantly more M&M® retrievals than BERs. This difference reaches significance at shock levels of 0.25 mA and higher. At 0.40 mA and higher, only one BER versus 8 BEP rats braved shock for M&M's®. Only BEP rats continue to cross at 0.60 mAs (Fig. 4). As also seen in Fig. 4, the retrieved M&M's® are consumed as evidenced by the 2-fold greater kcal intake of M&M's® by BEPs versus BERs across all shock levels (16). In sum, the BEP rats' willingness to tolerate increasing pain and anxiety associated with foot shock models the addictive-like

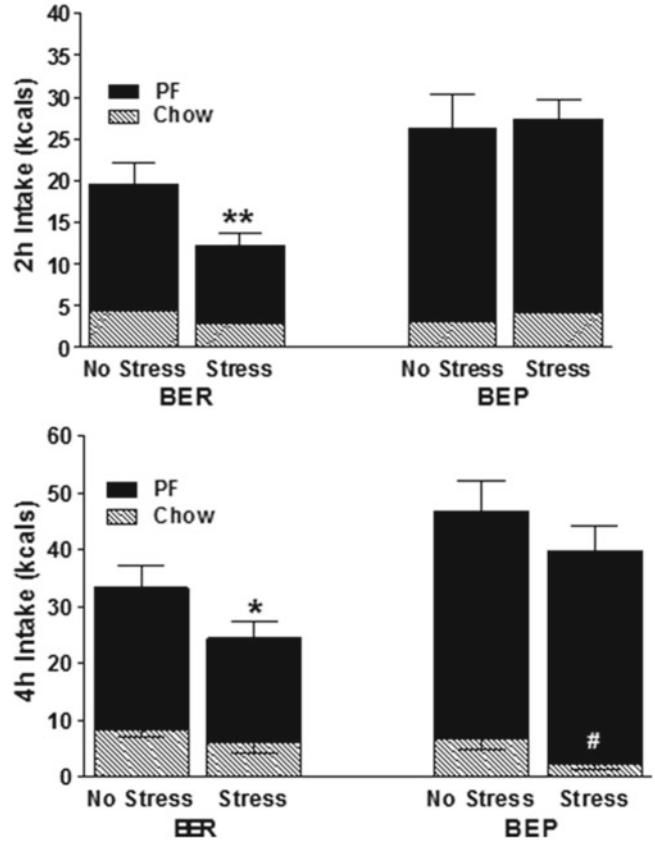

Fig. 3. Amount of chow and PF consumed by BEPs and BERs following foot shock stress or no stress ($N=10$/condition). (**a**) In the first 2 h, only BERs show a normal anorectic effect to stress, $^{**}p<0.01$ versus unstressed BERs. At this time, BEPs are not affected by stress. (**b**) By 4 h, there is evidence of a stress-induced anorectic effect in BEPs but the decreased intake is on chow, not PF intake ($\#p<0.05$). Conversely, at 4 h, BERs forsake PF, not chow, when stressed ($^{*}p<0.05$). Reproduced, with permission, from (5).

nature of human binge-eating where motivation to binge-eat persists despite the mounting psychological and physical consequences directly associated with this behavior (1, 39–41).

3.6.4. Effect of a High-fat Diet on the Propensity of BEP Versus BER Rats to Develop Obesity

When BEP and BER rats are fed a high-fat diet for 2 weeks, exactly half of the BERs and half of the BEPs develop obesity while the other half of each BEP and BER group resist weight gain. The obese-prone rats gain approximately 8.3% of initial body weight vs. a 1.9% gain by the obese-resistant rats ($p<0.01$) (5). This is due to the obese-prone rats' failure to decrease their normal volume of food when forced to eat the more calorie-dense high-fat diet. Importantly, BEPs are as likely to do this as BERs. Each group is also as likely to voluntarily restrict the amount of the high-fat diet which results in maintaining normal weight.

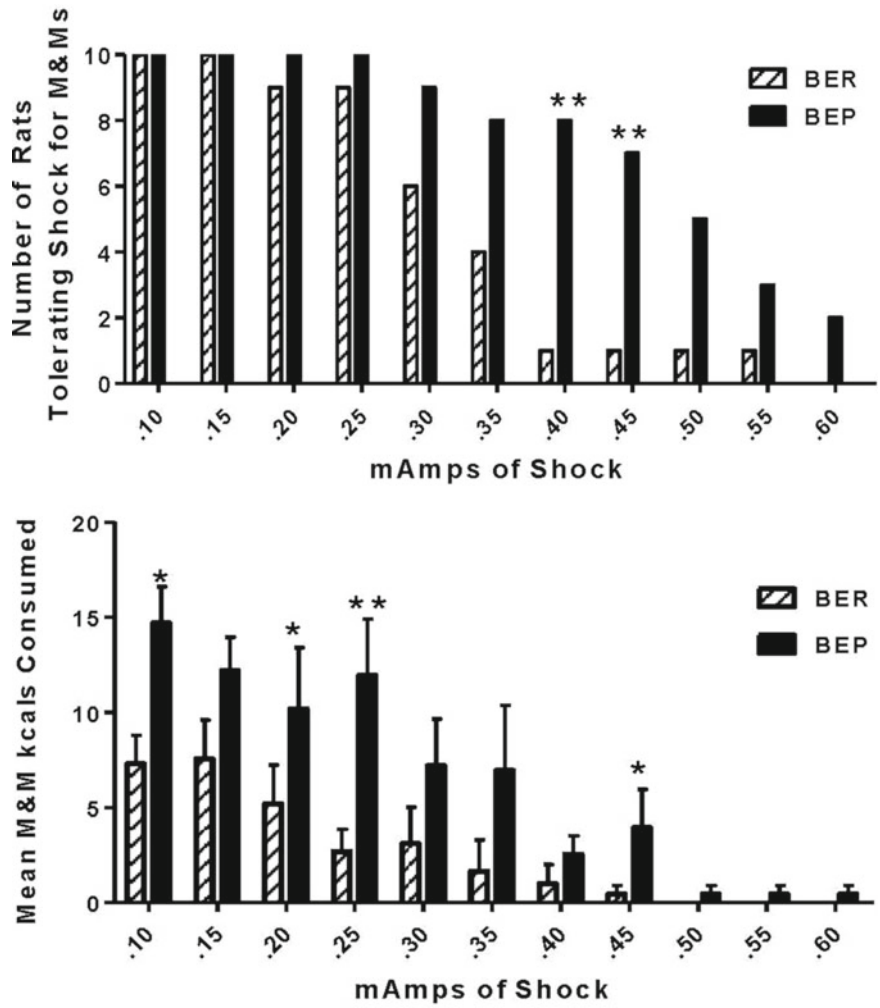

Fig. 4. (**a**) More BEP versus BER rats are willing to cross incrementing levels of foot shock to obtain M&M® candies; $^{**}p<0.01$; $N=10$/group. (**b**) The retrievals are also consumed by the BEPs for a final greater intake of M&M's® vs. BERs across all levels and statistically significant at some of the levels denoted by $^{*}p<0.05$; and $^{**}p<0.01$. Reproduced, with permission, from (16).

At the end, the DIO protocol yields four subgroups that can be used to investigate possible biological differences between bulimia nervosa, BED, non-binge-eating obesity, and healthy controls (see Table 1). For example, some individuals with bulimia or BED resist obesity through compensatory behaviors, including limiting caloric intake (1, 42). Similarly, the obese-resistant BEP rats reduce their volume of high-fat intake, thereby reducing total kilocalories consumed to maintain normal body weight. Not all human motivations to restrict caloric intake can be modeled in rats, but there may be a common physiology between bulimia nervosa patients and BEP-obese-resistant rats that enables them to reduce caloric intake amidst PF, a physiology possibly compromised in BEP-obese-prone rats and obese individuals with BED.

Table 1
Clinical conditions represented by the four subgroups that result from placing BEP and BER rats on a traditional diet-induced obesity (DIO) protocol

	BER	BEP
Obese-Resistant	Healthy These rats do not have a binge pattern on intermittent PF and when placed on a forced high-fat diet stay lean by voluntarily eating less	Bulimia Nervosa These rats have a binge pattern on intermittent PF but remain lean when placed on a forced high-fat diet by voluntarily eating less or by "compensating" for the increased calories
Obese-Prone	Frank obesity These rats do not have a binge pattern on intermittent PF but gain weight on a forced high- fat diet because they fail to adjust their intake for the additional calories of that diet	Binge-eating disorder These rats have a binge pattern on intermittent PF and gain weight on a forced high-fat diet because they fail to compensate for the additional calories of that diet

BER binge eating resistant, *BEP* binge eating prone rats based on difference in intake of palatable food (PF) during feeding tests used to identify the groups (see text for procedures). Obese-Resistant and Obese-Prone groups ($p < 0.01$ difference in weight gain) emerge from switching BEP and BER rats to a no-choice high-fat diet. Exactly ½ of BEP and ½ of BER rats develop obesity while the other ½ of BEP and BER rats resist weight gain. $N = 10$ per subgroup (5)

3.6.5. Effect of Developmental Factors on the Expression of BEP Versus BER Patterns

As is typical of the BEP/BER model in the author's hands, Klump and colleagues found that roughly one-third of an initial group of thirty rats could be clearly classified as BEPs and one-third as BERs based on their significant difference in amount of PF intake (17). Of major importance is that when they observed PF intake patterns across time, they found that the onset of the BEP phenotype does not appear until mid-late puberty (P39-P58). This seminal change in PF intake occurred as chow intake during chow-only days, and body weights, remained the same for both groups. Only PF intake differed. The emergence of PF binge eating shortly after puberty was replicated in a separate squad of $N = 36$ rats that were exposed to more frequent feeding tests during puberty (three times per week vs. one time per week) (17). The results are a compelling parallel to the age of onset for eating disorders, which is after puberty (1, 2, 43). Results from the OVX study revealed that OVX caused an expected increase in general food intake and body weight across all hormone-depleted rats, regardless of BEP or BER status. However, the OVXed BEP rats still continued to eat significantly more PF than the OVXed BER rats (21). The fact that BEP/BER patterns remained stable despite removal of estradiol indicates that other—yet unknown—signals activated at puberty are needed to express binge eating. While these results were at first surprising given that the BEP pattern appears after and not before puberty, they are consistent with the fact that a significant number of men, and not only women, develop BEDs (2).

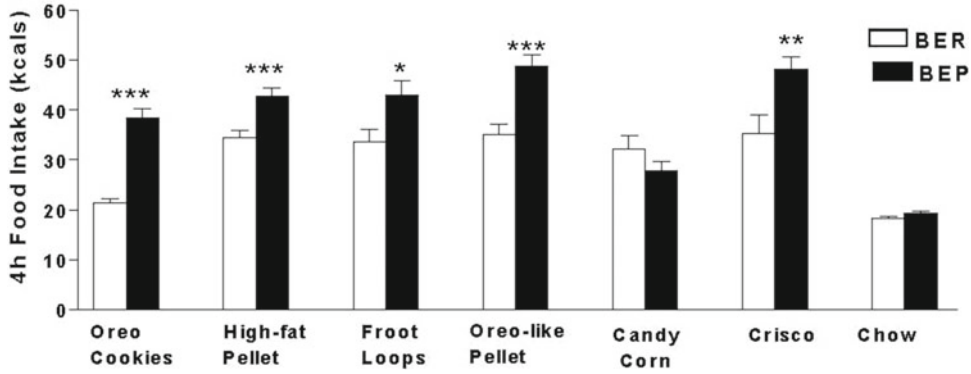

Fig. 5. The BEP/BER patterns generalize to other PFs including those containing mainly sugar (Froot Loops)®, mainly fat (Crisco)®, or combinations of both (cookies, pellets). All are preferred by both groups ($N = 20$/group) over chow but, with the exception of candy corn, BEPs eat significantly more of them than do BERs; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Reproduced, with permission, from (5).

3.7. Troubleshooting and Guidelines if Altering Variables

3.7.1. Identifying BEP/BER Status

In the rare event that not enough rats meet the four-out-of-four or three-out-of-four feeding test criteria for consistency in PF intake, additional tests should be conducted. When choosing BEP rats, if, after selecting the most consistent eating animals, the choice must be made between selecting a rat that ate in the highest quartile three times and once in the lowest quartile versus a rat that ate three times in the highest quartile and once in the *middle* quartile, the latter should be chosen into the BEP group because the middle values are closer to the high end of intake. The same applies to selection of BER rats (middle intake is closer to the lowest tertile than the highest tertile). There is no specific amount of kilocalories that determine BEP or BER status. As pointed out by Klump et al., this also parallels the method by which clinical binge-eating was first defined; it was based on comparing women in the high vs. low ends of the binge eating distribution (1, 17).

3.7.2. Using Other Palatable Foods to Identify BEP/BER Status

Most studies have used either Oreo cookies (5, 16, 20) or Betty Crocker® Vanilla Frosting (General Mills, MN) (17, 21). Figure 5 illustrates that rats first identified as BEP/BER with Oreos®, exhibit the same patterns of intake on other PFs, including high-fat pellets (Research Diets, Diet # D12266B, NJ), Oreo-flavored pellets (Research Diets, NJ), Froot Loops® (Kellogg, MI), Crisco® (Proctor & Gamble, OH) (5), and M&M's® (16). Hence, it may be possible to identify BEP and BER rats with nonfat sugary (e.g., Froot Loops) or nonsugar fatty PFs (e.g., Crisco®) and not just mixed macronutrient PFs like Oreos® and frosting. Still, one is warned to first test any new PF. For example, Fig. 5 illustrates that one of the PFs tested, Candy Corn (Brach's Confections, TN), failed to yield a significant difference between groups although it

was still preferred over chow by both groups (5). Hence not all PFs may dissociate BEPs from BERs. It may be that some PFs produce negative alliesthesia more quickly than others and BEPs may be as sensitive to this as BERs. Salty snack foods have not been tested, but are predicted to discern BEP/BER groups given that rats find them rewarding (44).

3.7.3. Using Alternate Modes of Stress-Induction

If foot shock is not practical, there is no reason that other standard laboratory stressors, including immobilization, cold temperature or water exposure, social defeat, noise, or emotional stress, should not yield the same differences in responses to stress observed in the BEP and BER rats. A particular "human-like" stressor, to the extent that craving can be regarded as stressful, is to dangle the PF in front of the rats without allowing them access to it. This has been used successfully in replications of the author's *stress+dieting model* of binge eating, which originally used foot shock (45).

3.7.4. Intermittent Versus Chronic Access to PF

The intermittency of PF used in this model is integral. If instead, rats are allowed *daily* access to PF and chow, the model is compromised because over time (within 2 weeks) the PF intake of BEPs and BERs become comparable. This is due to an eventual decrease in PF intake among the BEPs (5). The model relies on the intermittent, not daily, access to PF, which closely models how individuals with BEDs eat (1). PF is regarded as "forbidden" (4, 9, 10) and there is evidence that sporadic access to PF may exacerbate binge eating by increasing its rewarding quality (46).

3.7.5. Using Male Versus Female Rats

Currently there are no published studies using male rats with this model. However, Klump et al. report that age-matched males do not ingest as much PF (vanilla frosting) as females, and hence do not exhibit the wide range of PF intake needed to be classified as BEPs or BERs (personal communication, October 9, 2011). While male rats still prefer the PF to chow, they eat proportionately more chow during meals than do young females, likely because of increased protein needs. The lower incidence of male rats to achieve BEP status may offer future explanations for the lower male-to-female ratio in BEDs. However, the effects in the male rats may be confounded by the type of PF used. Gender is known to influence PF preferences. Male rats and humans prefer palatable fat/protein or "savory" combinations, and female rats and humans prefer carbohydrate or "sweet" combinations (47–49). Therefore, attempts to replicate this model with male rats should first test the PF to be used; it should yield a range of consistent intakes wherein the highest and lowest amounts consumed are statistically different.

3.7.6. Conducting Pharmacological Tests

Drug studies have not yet been conducted with this model. Since the BEP/BER patterns remain stable and robust even after surgical procedures and aversive manipulations like foot shock, it is not

expected that drug administration procedures will compromise the model so long as the rats are first acclimated to the procedures. Acclimation to injection procedures should be assured and followed with another "feeding test" to confirm the BEP/BER patterns prior to any drug testing.

3.7.7. Using Other Rat Strains or Species

Only Sprague–Dawley rats have been used so far with this model, but it is expected that results replicate in other inbred or selectively bred strains of rats. Mice have not been used, but since they show clear preferences for PF, including food used in the BEP/BER model here with rats (50), it may be possible to use mice if their patterns of PF intake are determined to be stable.

4. Conclusion

The BEP/BER model offers a simple, quick, and reliable method by which to study human binge eating. Pavlov posited that a simple reflex could give clues as to the mechanisms behind some of the most complex reactions between humans and their environment (51). Eating disorders are certainly complex reactions to the environment. The BEP/BER model was developed by targeting one simple "reflex-like" symptom: that of overeating once PF enters the mouth. Once rats with an inherent penchant to do this (BEPs) are identified and discerned from those without (BERs), it is discovered that there are many more behavioral parallels to human binge eating than eating abnormally larger amounts of food in a discrete period of time. Like clinical binge eating, BEPs binge in the absence of hunger, the binges override satiety, PF intake remains high under stress, BEPs tolerate aversive consequences for PF, and only some are prone to obesity. Also as is typical of human BEDs, the age of onset for the BEP binge pattern is shortly after puberty.

The BEP/BER model also offers a tool with which to investigate a variable that warrants much more attention in eating disorders research, namely the biological changes that take place to explain how factors in the environment interact with predisposing genes to express eating disorder symptoms (7, 8). PF is ubiquitous in the environment, as are stress and dieting, yet not all develop eating disorders when subjected to these. The BEPs never learn to overeat PF, but instead do this upon first encountering PF. Therefore, binge eating is a preexisting disposition that is expressed when exposed to PF. The identification of genetic and epigenetic markers that confer the BEP versus BER phenotypes once PF is eaten (and once puberty sets in) should help clarify the physiology of binge eating. Similarly, identification of gene markers in the four subgroups obtained from subjecting BEPs and BER to a high-fat diet should shed light on the physiology that predisposes some

who binge eat to develop BED vs. bulimia nervosa, and to develop obesity with and without binge eating.

Lastly, the BEP/BER model attests to the incredible value of animal models in eating disorder research. BEP rats display behaviors such as willingness to cross painful shock for M&M's®, inability to limit PF intake despite normal hunger–satiety cues, and in some, an ability to restrict calories and prevent weight gain despite the stable trait to binge eat. They also do not start bingeing until they reach puberty. These are responses that in humans with BEDs are commonly attributed to processes only capable in humans (e.g., cognitive dysregulation, irrational thinking, concern with body weight and shape, judgment by peers and the opposite sex). Clearly, researchers cannot mimic all motivations that drive human binge eating in rats, but it is clear that there is a more basic "reflexive" biology underlying binge eating when many of these complex behaviors are observed in rodents. This biology can be exploited with the help of the BEP/BER model, as well as with other animal models in this book, to prevent the expression of eating disorders altogether, or, at minimum, to develop superior treatments for the millions that suffer from them.

Acknowledgments

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Chapter 3

Binge Eating in Female Rats Induced by Yo-Yo Dieting and Stress

Carlo Cifani, Maria Vittoria Micioni Di Bonaventura, Roberto Ciccocioppo, and Maurizio Massi

Abstract

Preclinical models are needed to investigate the neuro- and psycho-biology of binge eating (BE) and to identify innovative pharmacotherapeutic strategies. A new model, based on the combination of cyclic caloric restriction and acute stress, has been recently developed in our laboratory to induce BE of highly palatable food (HPF) in female rats. Rats were exposed to three cycles of food restriction/refeeding and then stressed on the test day. Acute stress was elicited by exposing rats to HPF, but preventing them from accessing it for 15 min. This experimental procedure induces a marked binge-type intake of HPF. Interestingly, in this model BE does not occur during the estrus phase of the ovarian cycle; if data from female rats in estrus are not included in the statistical analysis, the variability of the BE response is very low. Topiramate, sibutramine, and fluoxetine potently inhibited HPF intake in this model, providing evidence for its predictive validity. The model has been used to investigate the effect of drugs targeting stress mechanisms. The corticotrophin-releasing factor (CRF)-1 receptor antagonist R121919 selectively inhibited BE, indicating that CRF is involved in the BE response. Its effect is likely exerted in extra-hypothalamic sites rather than in hypothalamic sites controlling the hypothalamic–pituitary–adrenal axis. In addition, orexin-1 receptor antagonists selectivity inhibit BE; studies are under way to evaluate whether their effects are related to influences on stress or on reward mechanisms. This preclinical model appears to be highly reliable and reproducible; it may represent a valid model to identify novel pharmacological treatments of BE disorder and bulimia nervosa.

Key words: Binge eating, Stress, Food restriction, Highly palatable food, Female rats, Ovarian cycle, CRF-1 receptor antagonists, Orexin receptor antagonists

1. Introduction

1.1. Background Information

Episodes of binge eating (BE) in humans are characterized by compulsive, nonhomeostatic consumption of an unusually large quantity of highly palatable food (HPF) in a short period of time. Even though not hungry, subjects eat more rapidly than normal

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until feeling uncomfortably full. As described by the DMS-IV-TR (1), these episodes are accompanied by a subjective sense of loss of control over eating, and are associated with feelings of distress, disgust, depression, being guilty about overeating and eating alone because of embarrassment.

BE represents a central feature of bulimia nervosa (BN), in which episodes of BE are followed by behaviors aimed at avoiding weight gain, such as self-induced vomiting. Intense and persistent BE episodes represent a typical phenomenon occurring also in subjects suffering from binge-eating disorder (BED) (2). BED, described for the first time by A.J. Stunkard (3), is probably the most prevalent eating disorder (4). It is characterized by repeated episodes of BE in the absence of compensatory behaviors to avoid weight gain. The DMS-IV-TR (1) indicates that among diagnostic criteria for BED, BE episodes should occur at least 2 days per week for 6 months. BED is associated with significant medical and psychiatric comorbidity (5–7). It is estimated that BE afflicts approximately 5% of the US adult population at some time in their life (8) and it contributes to obesity and associated pathologies (4, 9–11).

Medications that have been reported to reduce BE in clinical studies, like topiramate (12, 13) or sibutramine (14–16), are associated with a variety of adverse side effects, which represent a serious problem during chronic treatment (13, 17, 18). For example, sibutramine, due to increased risks of serious cardiovascular side effects, has been recently withdrawn from the European market. Fluoxetine has been approved by the Food and Drug Administration for BN, but evidence for its efficacy is inconclusive (19). Treatment of BED and BN cannot simply rely on pharmacological agents aimed at reducing food intake in general (i.e., serotonergic drugs). Hence, BED and BN represent a still largely unmet medical need. In this regard, our research group has activated a research program with the aim to develop new pharmacological treatments for BED and BN.

1.2. Existing Experimental Models of BE

To date, several preclinical models to study the neuro- and psychobiology of BE have been proposed (see for review (20)), and these models have been used in attempts to develop innovative pharmacological treatments. A large body of evidence suggests that dieting, stress, and negative affective states represent possible triggers of BE in patients (21, 22). Indeed, dieting periods are a common finding in the history of binge eaters, although hunger per se appears not to be enough to induce BE in the absence of stress and negative affective state (23, 24). Considerable evidence suggests that BE may be caused by a unique interaction between dieting and stress; thus, a history of cyclic food restriction and of environmental stress may be responsible for its precipitation and maintenance (25–27). Accordingly, recurring food restriction is consistently the strongest predictor of overeating in response to stress (21). Also typical of BE in humans is the preference for HPF.

Craving, preferential selection, and ultimate overconsumption of HPF are common in BE disorders (28) and are considered to play an important binge-triggering role in animal models (20, 29, 30).

In line with the hypothesis that dieting and stress are key etiological determinants of BE (4, 22), the model proposed by Boggiano and colleagues (and described in Chap. 2) (31–33), combines cycles of food restriction/refeeding and acute stress to evoke BE for sweet HPF. In this model, rats are submitted to cyclic caloric restriction and stressed with electric foot-shock; stress is delivered to animals that are not energy deficient, in keeping with the idea that BE episodes usually occur in conditions of satiety and normal body weight (34). Rats are submitted to three consecutive 8-day cycles of food restriction/refeeding, followed by the final test on day 25, since the hyperphagic response to stress was found to be contingent upon a minimum of three cycles. In response to stress, a selective increase in HPF intake over chow is observed. Rats, which cycled through food restriction and refeeding, in response to stress, fail to show hyperphagia when only chow is available.

Several epidemiologic studies suggest that BE episodes are more common in females than in males (1, 4, 35–37). American women are approximately one-and-a-half times more likely to develop BED than men, and they are three times more likely to develop BN than men (4). In Norway, the female-to-male ratio is 1.7:1 for lifetime prevalence of BED, and 3:1 for lifetime prevalence of BN in adolescents (37). In consideration of the higher prevalence of BE in adolescent and young adult women, young female rats are used. This method is considered to have strong construct validity and face validity as an isomorphic model (38) that presents elements of similarity with the human symptomatology (20, 33).

2. Materials and Procedures

A new experimental model of BE was recently developed in our laboratory, modifying the Boggiano model, and aiming to increase its reliability and face validity (39).

2.1. The Stressful Procedure

The most relevant change compared to the Boggiano model was related to the stressful procedure. In our previous studies concerning stress-induced reinstatement of alcohol-seeking behavior (40), large variability in the sensitivity of rats to electric foot-shock was observed; animals highly sensitive to this stressful procedure exhibited freezing behavior that prevented them from engaging in ingestive behavior. Several reports have shown that electric foot-shock suppresses food intake (41), and in a few instances it has been reported to induce either no effect or a small increase in intake (42, 43). Thus, electric foot-shock stress was substituted with a stressful

Fig. 1. The stressful procedure: rats were prevented from accessing HPF, but were able to see and smell it.

procedure characterized by exposure of the animals to HPF, but preventing them from accessing it. On the test day, animals were prevented from getting access to HPF for 15 min before testing, even though they were able to see and smell it (Fig. 1). This expedient was adopted to generate a mild stressful condition characterized by a temporary lack of control over the environmental circumstances (39).

In this 15 min period, rats engaged in repeated movements of the forepaws, head, and trunk aimed at obtaining the HPF without being able to reach it. Rats underwent the stressful procedure between 1000 and 1200 hours. After 15 min, the cup was placed inside the cage, so that HPF became accessible to the stressed rats.

As shown in Fig. 2, exposure to HPF without access to it, increased corticosterone (CORT) levels in serum samples obtained from rats sacrificed 15 min after the beginning of the stressful procedure, both in rats submitted to food restriction ($R+S$) and in rats not submitted to food restriction ($NR+S$). These findings provide evidence that the procedure was indeed able to evoke a stressful response in the animals. This response was rather short lasting, since 60 min after removal of the HPF cups from the wall of the cage (without allowing the rats to access HPF), CORT levels returned to control ($NR+NS$) levels.

This type of stress offers several advantages over the electric foot-shock stress. First, it is a mild stress that, unlike the electric

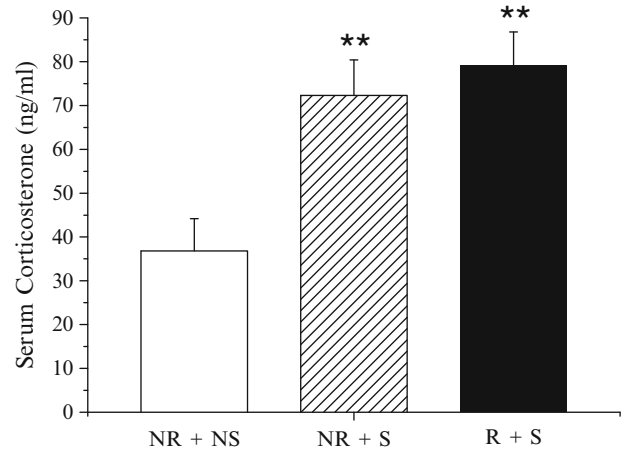

Fig. 2. CORT levels in control rats (*NR + NS*) and in rats exposed to stress with (*R + S*) or without (*NR + S*) cycles of food restrictions. In *R+S* and *NR+S* rats CORT levels were measured 15 min after the beginning of the stressful procedure. The values shown are the mean ± SEM. Statistical differences from controls (*NR+NS*): $^{**}p < 0.01$.

foot-shock, never induces fear and freezing, but elicits a robust behavioral activation. Second, the stressful experience implies a relationship with HPF that resembles the approach-avoidance stress over "forbidden" food that is very common among human binge eaters, thus providing a further element of face validity.

2.2. The HPF

Rather that using sweet biscuits, which usually generate a large amount of spillage, a HPF formulated as a paste was employed. It was obtained by mixing (a) Nutella (Ferrero, Alba, Torino, Italy) chocolate cream (5.33 kcal/g; 56%, 31%, and 7% from carbohydrate, fat, and protein, respectively), (b) grounded food pellets 4RF18 (Mucedola, Settimo Milanese, Italy), and (c) water in the following percent ratio: 52% Nutella, 33% food pellets, 15% water.

Rats exhibited a pronounced intake of HPF, since the first exposure to it on day 5 of the first cycle (about 5.5 g per rat); the intake increased on the following day (about 9 g per rat). On days 13 and 14 of the second cycle HPF intake did not increase further.

2.3. The Experimental Procedure

Before tests, female rats were divided into four weight-matched experimental groups (usually $N=9$ per group):

Group 1: nonrestricted and not exposed to stress (*NR + NS*)

Group 2: restricted and not exposed to stress (*R + NS*)

Group 3: nonrestricted and exposed to stress (*NR + S*)

Group 4: restricted and exposed to stress (*R + S*)

Rats were maintained on 3 consecutive 8-day cycles, followed by the final test on day 25, as follows:

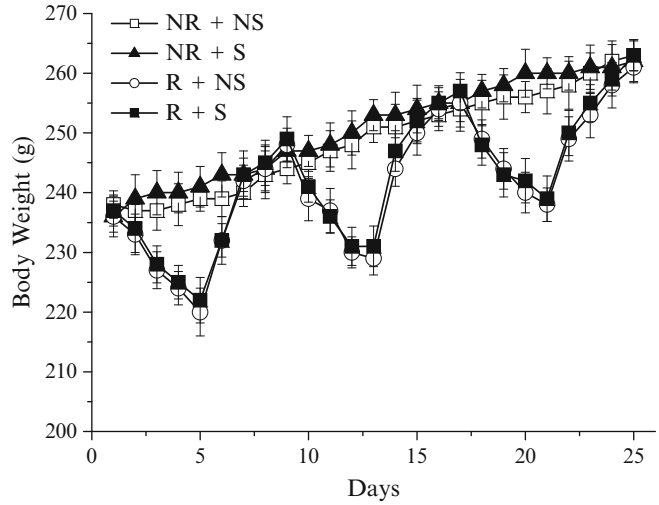

Fig. 3. Effect of the three restriction/refeeding cycles on body weight (g) of the four groups of female rats. The values are the mean ± SEM.

Rats in *Group 1* (*NR + NS*), the control group, had ad libitum access to chow for 4 days, on days 5–6 they received chow ad libitum + HPF for 2 h; on days 7–8 they had chow ad libitum; on day 25 they were not exposed to stress.

Rats in *Group 2* (*R + NS*) had chow restricted to 66% of the normal intake for 4 days; they were offered chow ad libitum and HPF for 2 h on days 5–6, and only chow on days 7–8; on day 25 they were not exposed to stress. Restriction to 66% of normal chow intake was defined for the first cycle on the basis of the intake measured in the 6 days before the beginning of the experiment. For the second and third cycle, 66% restriction was defined on the basis of the intake of nonrestricted rats during the last 2 days of the cycles, in which rats received only normal chow ad libitum.

Rats in *Group 3* (*NR + S*) had chow and HPF as controls, but were exposed to stress on the test day (day 25).

Rats in *Group 4* (*R + S*) had food available similar to *Group 2*, but were exposed to stress on day 25.

The 8-day cycle was repeated three times, but in the third cycle the animals did not have access to HPF on days 21 and 22. On day 25, free access to HPF and chow was offered and the intake was measured in the first 2 h, taking care to collect any spillage. A minimum of three caloric-restriction and refeeding cycles were run, as in previous studies (31–33).

Body weights and food intake were recorded daily. Food intake was expressed as the mean kcal/kg ingested ± S.E.M. By the last day of refeeding, body weight and food intake of restricted rats were not statistically different from those of nonrestricted rats (Fig. 3), thus eliminating the potentially confounding influence of hunger or energy deficit.

3. Anticipated Results and Notes

3.1. Anticipated Results

The experiments were initially carried out in four different groups of rats; the overall ANOVA revealed a statistically significant difference in 2-h HPF intake in the different groups of rats in all of the experiments.

As shown in Fig. 4, HPF intake in the *R + S* group was markedly higher than in the control (*NR + NS*) group at the different times of observation. The intake of HPF by *R + S* rats began immediately after access to it; these animals never engaged in competing behaviors, but continuously remained over the cup containing HPF and focused their attention on its intake. HPF intake was very pronounced in the first 15 min of access to it. After the first 15 min, the additional intake of the four groups was similar, thus at 2 h the cumulative intake of the *R + S* group remained higher than that of the other groups. HPF intake of the groups *NR + S* or *R + NS* were neither significantly different from that of controls. As far as the intake of food pellets is concerned, the ANOVA revealed no significant difference among groups. The intake of food pellets was affected neither by food restriction, stress, nor the combination of both.

The rats' body weights were markedly reduced during the 4 days of food restriction, but rapidly recovered to levels of controls by the end of each cycle. On the test day, the body weights of the four groups of animals, as well as their food intake in the previous 24 h, were not statistically different.

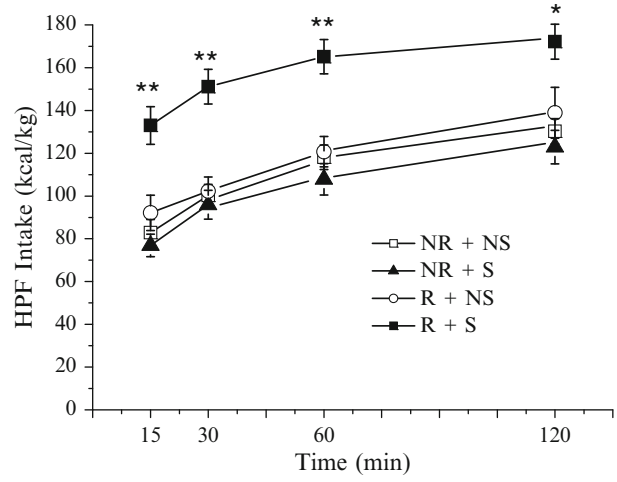

Fig. 4. HPF intake at different times after access to it on the test day (day 25). The values shown are the mean ± SEM. Statistical differences from controls (*NR + NS*): *$p < 0.05$; **$p < 0.01$; where not indicated, the difference was not statistically significant.

Although the ANOVA gave a statistically significant effect in all the experiments carried out with this model, a considerable interanimal variability was evident: while in some animals the increase in HPF was very pronounced, almost doubling the intake of *NR* + *NS* rats, a small percentage of rats had a HPF intake strictly similar to that of *NR* + *NS* animals.

3.2. Influence of the Ovarian Cycle in This Model of BE

Searching for the reasons accounting for the observed variability, we thought it would be interesting to evaluate whether it might be related to the ovarian cycle of the female rats. Indeed, menstrual cycle and gonadal hormones are known to influence eating behavior in healthy females. Reductions in food intake and meal size occur in the peri-ovulatory phase, following the rise of estradiol secretion, while food intake generally increases in the luteal phase, when plasma progesterone levels are high (44–47).

A large body of evidence shows a significant association between changes in ovarian hormone levels and BE episodes during the menstrual cycle in women with BN. For instance, several studies have found an increased number of BE episodes in the premenstrual compared to the menstrual period (48–50). Moreover, a relatively recent longitudinal study reported that the increase in estradiol and decrease in progesterone prospectively predict reduction of binge frequency (51). This negative association has been replicated by Klump et al. (52) in two community samples of women, in which decreases in estrogens were associated with overeating and greater likelihood of feeling "out of control."

The ovarian hormone estradiol is involved in the physiological control of feeding via interactions with anorexigenic peptides like cholecystokinin (53–58), insulin, and leptin (59), as well as with orexigenic peptides like neuropeptide Y (60–62), ghrelin (63–65), and melanin-concentrating hormone (66, 67). Estradiol also influences serotonin release and degradation, as well as activation of its receptors (68). Estrogens modulate dopaminergic systems, and this modulation may involve environmental estrogen exposure (69).

In female rats, the estrous cycle is usually 4 days in length, with four distinct phases (70):

1. Proestrus (P): estradiol rises to the highest levels and progesterone levels are low at the beginning and rapidly rise and descend toward the end.
2. Estrus (E): estradiol and progesterone levels rapidly decline.
3. Metestrus (D1): estradiol levels are low and progesterone levels begin to rise.
4. Diestrus (D2): estradiol levels are rising and progesterone levels decline.

During the estrus phase rats, like other animals, show a transient decrease in food intake while they eat most during the diestrus

phase (71, 72). Most physiological estradiol effects have a latency of 12 h or more, ~30 h in the study by Asarian and Geary (73), since the activation of estrogen receptors stimulates transcription factors. Therefore, decreases in food intake during the estrus phase may be caused by the preceding increase in estradiol secretion in the proestrus phase.

Bilateral ovariectomy (OVX) produces a rapid increase in food intake, body weight, and adiposity (74, 75), removing estrogen's inhibitory effect. These responses to OVX can be normalized by a regimen of estradiol treatment with a single subcutaneous injection of estradiol (76) that produces hormonal changes in plasma similar to those observed in cycling rats. Estrogens regulate body adiposity and fat distribution through the ER alpha (ERα) and beta (ERβ) receptors (77, 78). However, only ERα has been proposed to have a major influence on energy homeostasis (79). The effects of estradiol on food intake appear to be mediated by ER within the hypothalamus, particularly ventromedial and paraventricular nucleus (PVN) (54, 65, 80).

Little is known about the role of estrogens on BE in rats. Yu et al. (81) have shown that estradiol reduces binge size in female rats, in which a highly restricted schedule of access to fat led to binge-like intake of fat in a 1-h test. However, this effect was evident at the start of the study but not at the end, when bingeing was fully established.

In our study, the ovarian cycle was monitored by examination of vaginal smears obtained with a moistened cotton swab and warm physiological saline; afterwards, the samples were transferred onto glass slides for microscopic analysis. Following examination of vaginal smears on the test day, immediately after the HPF intake test, statistical analysis revealed that HPF intake was significantly lower during the estrus phase both in $NR+NS$ and $R+S$ rats (82). HPF intake of $R+S$ rats was significantly higher than that of $NR+NS$ rats during proestrus, metaestrus, and diestrus; however, during estrus it was only slightly higher in $R+S$ rats, and the difference between the two groups was not statistically significant (Fig. 5). These findings indicate that BE in our model does not occur during the estrus phase and that the observed variability in the BE response can be almost completely abolished if female rats in estrus are not included in the statistical evaluation. These findings encourage further investigations of the mechanisms by which ovarian hormones, particularly estradiol, control BE.

3.3. Drugs so Far Tested in This Model

3.3.1. Drugs to Evaluate the Predictive Validity of the Model

To test the predictive validity of this preclinical model of BE, the following three drugs have been tested: sibutramine, fluoxetine, and topiramate. They were chosen because clinical studies have reported that they may be effective in the treatment of bingeing-related eating disorders (12–17, 83–88). Sibutramine (Reductil®) is a centrally acting serotonin–noradrenaline reuptake inhibitor,

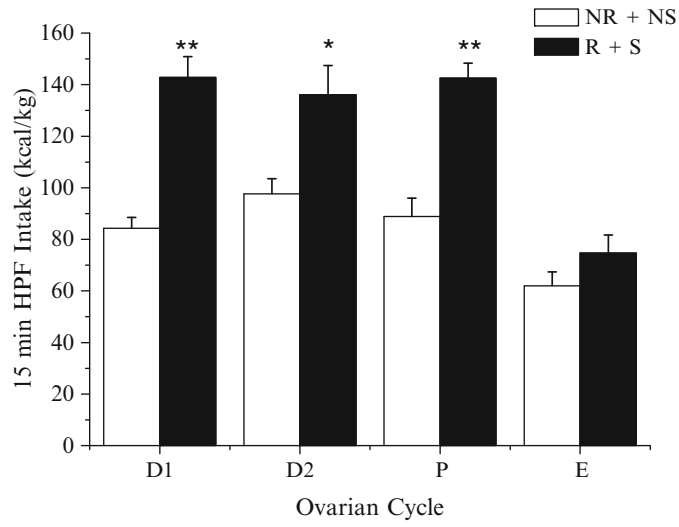

Fig. 5. HPF intake on the test day (day 25) in *R + S* and in *NR + NS* female rats in the different phases of the ovarian cycle: D1 (Metestrus), D2 (Diestrus), P (Proestrus), E (Estrus). The values shown are the mean ± SEM. Statistical differences from controls (*NR + NS*): *$p < 0.05$, **$p < 0.01$; where not indicated, the difference was not statistically significant.

which has been reported to reduce food intake and to increase energy expenditure (89). Fluoxetine (Prozac®) is a selective serotonin reuptake inhibitor; it reduces food intake by affecting appetite and satiety. In addition, fluoxetine appears to have positive effects on the control of impulsive and compulsive symptoms associated with psychiatric conditions like obsessive–compulsive disorder, BN, and hypochondriasis (86). Topiramate (Topamax®) was developed as an antiepileptic drug; however, clinical trials have shown that it inhibits BE (12, 13). Sibutramine reduced HPF intake not only in the *R + S*, but also in the other experimental groups, suggesting that it exerts a rather general inhibitory effect on food consumption. Fluoxetine, at the intragastric dose of 3 mg/kg, significantly reduced HPF intake only in the *R + S* group. At the dose of 10 mg/kg, the effect of fluoxetine was not selective and it significantly reduced HPF intake in all four groups. On the other hand, topiramate, even at the highest dose (60 mg/kg) given by gavage, selectively reduced HPF intake only in *R + S* rats.

These findings, particularly those obtained with topiramate, suggest that the preclinical model described by Cifani et al. (39) exhibits interesting elements of predictive validity. In addition, further elements of face validity for this model derive from the finding that the occurrence of BE in the female rats employed is influenced by the ovarian cycle, as it happens in women.

3.3.2. Drugs Targeting Stress Mechanisms

As stated above, a large body of evidence suggests that stress may represent a key determinant of BE in patients suffering from BED or BN (21, 22). The important role of stress in the etiology of BE is emphasized by the finding that obese individuals with BED

exhibit activation of the hypothalamic–pituitary–adrenal (HPA) axis, and their cortisol levels are higher in comparison with those of obese individuals without BED (90, 91). Moreover, salivary cortisol levels are positively correlated with BE severity (92), and higher blood cortisol levels in response to stress predict greater intake of sweets (93). Therefore, we thought it would be interesting to evaluate the effect of pharmacological agents directed at stress mechanisms in our BE model.

Corticotropin-releasing factor (CRF) is a 41 amino acid polypeptide that represents the major mediator of the stress response at both hypothalamic and extrahypotalamic levels. Hypothalamic CRF, which is synthesized in PVN neurons, controls the production and release of adrenocorticotropic hormone (ACTH) from pituitary corticotropes through activation of CRF-1 receptors. In turn, ACTH released into the blood stream stimulates glucocorticoid synthesis and secretion from the adrenal cortex (94, 95). The brain extrahypothalamic CRF systems (including amygdala, extended amygdala, and medial septum) appear to be involved in emotional and motivation processes, and CRF-1 receptors are the main mediators of these effects (95). CRF-1 receptor antagonists have been reported to inhibit negative emotional states in drug dependence, and to block stress-induced reinstatement of drug seeking for cocaine, opiates, ethanol, and nicotine (40, 96–102). Apparently, the bed nucleus of the stria terminalis, the median raphe and the ventral tegmental area are important sites for the effect of CRF-1 receptor antagonists on stress-induced relapse in drug abuse (103–106). Interestingly, CRF-1 receptor antagonists have been reported to reduce stress-induced palatable food seeking in rats (101, 107), and to reduce withdrawal symptoms in conditions of intermittent access to palatable food (108).

In addition to CRF, glucocorticoids have received great attention in relation to motivational responses to stress. Increased CORT levels represent a hormonal marker of BE in rats in the cyclic food restrictions + stress models (33, 39, 109). Palatable food intake has been shown to blunt activation of the HPA axis (110–112), suggesting that it may be used, at least in part, as self-medication from stress. It is noteworthy that CORT levels are also increased following high-fat diet removal (113), as they are during withdrawal from addictive drugs (112, 114). CORT appears to be implicated in the motivation to seek rewarding substances (107, 115–119), probably by evoking dopamine release in the nucleus accumbens (120, 121).

3.4. Examined Components of the Stress Response

3.4.1. CRF-1 Receptor Antagonist

In the experiments carried out by our group, and published as an abstract at the 2011 Meeting of the Society for the Study of Ingestive Behavior (122), the role of CRF on BE was investigated by means of the highly selective CRF-1 receptor antagonist, R121919 (123–125), provided by Dr. Kenner Rice, NIDA/NIH, Bethesda (USA).

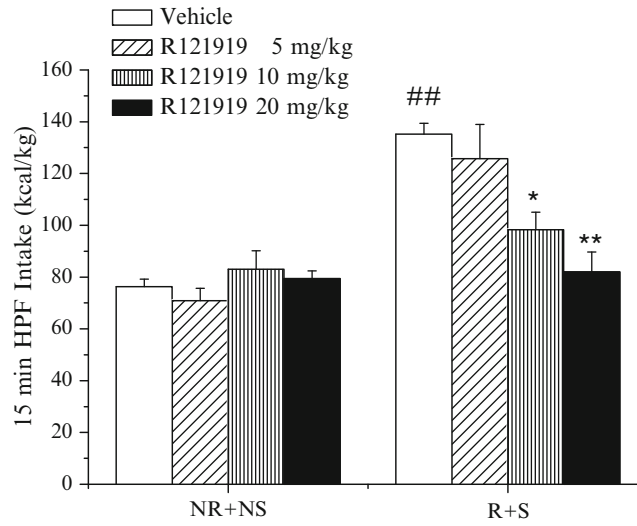

Fig. 6. The effect of subcutaneous administration of the CRF-1 receptor antagonist R121919 on HPF intake on the test day in *R+S* and *NR+NS* rats. The data are the mean ± SEM. Statistical differences: $^{\#\#}p<0.01$ vs. *NR + NS* vehicle; $^{*}p<0.05$ and $^{**}p<0.01$ vs. *R + S* vehicle.

As shown in Fig. 6, the subcutaneous administration of this CRF-1 receptor antagonist significantly reduced the increase in HPF intake of the *R+S* group at the dose of 10 mg/kg and completely suppressed it at 20 mg/kg. No effect was observed at 5 mg/kg. While suppressing the increase in HPF intake in the *R+S* group, doses of 10 or 20 mg/kg of R121919 did not significantly reduce HPF in the control group (*NR+NS*). Thus, the effect of R121919 is clearly selective, and it is not producing a general inhibition of food intake, like that observed for serotonergic drugs, such as fluoxetine or sibutramine (39).

3.4.2. CORT

As already reported (39), the stressful procedure adopted in our model induces a marked increase in serum CORT levels. CORT has been implicated in the motivation to seek rewarding substances (107, 115–119), raising the question whether it might be involved in the BE response. To address this issue, stress exposure was substituted with an intraperitoneal injection of CORT in rats already submitted to cycles of food restriction/refeeding. If CORT mediates the effect of stress to evoke BE, *R+NS* rats should express BE following treatment with CORT. Contrary to this prediction *R+NS* rats, exposed to a wide range of CORT doses, failed to show an increase in HPF intake in comparison to *NR+NS* rats (122).

3.4.3. Metyrapone

Metyrapone is CORT synthesis inhibitor. When it was administered by intraperitoneal injection at different doses, it failed to prevent BE in *R+S* rats and did not modify HPF intake in *NR+NS* rats (122).

Taken together, these findings do not support a direct role of CORT in the etiology of BE. Studies concerning drug abuse have shown that high circulating levels of glucocorticoids can "sensitize" the CRF systems in extrahypothalamic sites that are involved in behavioral responses to stressors (126–130). The finding that BE is observed only after three cycles of food restriction/refeeding might imply the occurrence of processes of brain plasticity/sensitization; activation of the HPA axis may lead to subsequent sensitization of extrahypothalamic stress mechanisms, as described for drug addiction (131–133).

Thus, the effect of CRF on BE is probably exerted not through activation of the HPA axis in the hypothalamus, but in extrahypothalamic sites involved in emotional and motivational control. Many of these effects of CRF have been localized in the extended amygdala, a macrostructure composed of basal forebrain structures, including the central medial amygdala, the bed nucleus of the stria terminalis, and a transition zone in the posterior part of the medial nucleus accumbens, the posterior shell (134). Key elements of the extended amygdala include not only neurotransmitters associated with the positive reinforcing effects of drugs of abuse, but also major components of the brain's stress systems associated with the negative reinforcement of dependence (132).

Several pieces of evidence suggest similarities between drug addiction and compulsive eating disorder. For example, CORT is increased during high-fat diet removal (113), as it is during withdrawal from addictive drugs (114). This may set up a vicious addiction-like cycle of eating palatable food when stressed, then suffering the consequences of palatable food withdrawal, a stressor in itself (108). Cottone et al. (108) found that rats with intermittent access to palatable food elicit symptoms of withdrawal when the palatable food is not available, symptoms reversed by CRF-1 receptor antagonists. Thus, the results of this study provide evidence that CRF-1 receptors are involved in the control of BE and CRF-1 receptor antagonists may represent interesting tools for the pharmacotherapy of bingeing-related eating disorders.

3.4.4. Rhodiola rosea Dry Extracts

Another study of our group was aimed at investigating the effect of a dry extract of *Rhodiola rosea* and of its active principles in the same model of BE in female rats. *R. rosea* is a plant commonly used in traditional medicine for its ability to increase body resistance to physical, chemical, or biological stressors. The antistress properties have been attributed to modulation of the activity of the sympathetic system, of the HPA axis, as well as to influence on molecular chaperons like Hsp70, stress-activated c-Jun N-terminal protein kinase (JNK1), Forkhead Box O transcription factor DAF-16, cortisol, and nitric oxide (135, 136). Studies evaluating the effect of *R. rosea* at CRF receptors are not available, but *R. rosea* extracts have been reported to reduce behavioral responses evoked by central CRF administration, such as CRF-induced anorexia (137).

Administration by gavage of a dry extract of *R. rosea*, 10 mg/kg, significantly reduced the increase in HPF intake in the *R + S* group, while 20 mg/kg completely abolished it (138). While suppressing the increase in HPF intake in the *R + S* group, 20 mg/kg of *R. rosea* extract did not reduce HPF intake in the control group (*NR + NS*), the *NR + S* group, or the *R + NS* group; moreover, it did not modify the intake of food pellets in food-sated or food-deprived rats. The same doses of *R. rosea* abolished the increase in serum CORT levels, suggesting that it abolishes the stress response.

While in the present study *R. rosea* extract inhibited BE, Mattioli and Perfumi (137) have shown that *R. rosea* antagonizes the anorectic effect of stress and of CRF, increasing food intake in conditions in which it was suppressed by restraint stress or by central CRF injection. Indeed, stress is well known to be responsible for both stimulation and inhibition of feeding (41, 43), apparently depending on the intensity of the stress itself. Thus, the different effects of *R. rosea* on feeding behavior may likely be the consequence of its primary influence on the stress mechanisms, rather than a direct effect on orexigenic or anorexigenic mechanisms. By influencing the response to stress, *R. rosea* extracts can either suppress stress-induced BE or stress-induced anorexia.

R. rosea roots contain a variety of biologically active compounds, including organic acids, flavonoids, tannins, and phenolic compounds. Phenylpropane and phenylethane phenolic glycosides, such as salidroside, rosavin, syringin, and triandrin, are considered the most important active principles (135). Extracts of *R. rosea* are usually standardized for rosavin and salidroside; therefore, the effect on BE of purified rosavin and salidroside was evaluated in the *R + S* group, which is the animal group that exhibited BE. As shown in Fig. 7, salidroside (administered in amounts similar to those present in 20 mg/kg of the extract) was able to significantly reduce BE. On the other hand, the effect of rosavin (again in amounts similar to those present in 20 mg/kg of the extract) induced a lower, not statistically significant reduction of BE. These findings indicate that salidroside may represent the main active principle of *R. rosea* in suppressing BE.

3.5. Drugs Targeting Orexin Mechanisms

A large body of evidence supports a role of the orexin (OX) system in feeding behavior, particularly in the control of reward-based food intake (139). Interestingly, OX neurons in the lateral hypothalamus have been proposed to mediate reward ((95) for review); these neurons are activated by cues associated with rewards, such as food or drugs, and their activation reinstates drug seeking in rats (140–142). OX-1 receptor antagonists inhibit the hyperphagic effect of OX (143, 144) and inhibit also high-fat food self-administration (145).

Several reports have implicated OX peptides in stress responses. OX neurons in the perifornical–dorsomedial hypothalamus have

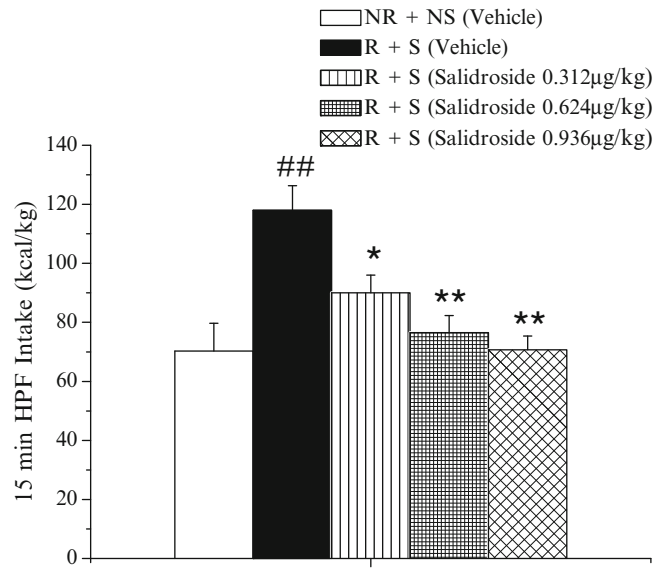

Fig. 7. The effect of intragastric administration of salidroside or vehicle on HPF intake on the test day in *R + S* rats. The data are the mean ± SEM. Statistical differences: $^{\#\#}p < 0.01$ vs. *NR + NS* vehicle; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. *R + S* vehicle.

been proposed to mediate stress (95, 146) through the activation of CRF-expressing neurons in the PVN of the hypothalamus and in the central nucleus of the amygdala (147). Accordingly, OX-1 receptor antagonists inhibit electric foot shock-induced reinstatement of cocaine seeking (148, 149), as well as ethanol and sucrose seeking induced by the pharmacological stressor yohimbine (149). Recent reports also support a role for OX signaling in drug reinforcement and drug abuse-induced plastic changes in the brain (150–153). Consistent with these findings, blockage of OX-1 receptors decreases ethanol, cocaine, and nicotine self-administration (141, 151, 154).

Evidence is accumulating that excessive intake of certain foods under specific conditions produces behaviors and changes in the brain that resemble an addiction-like state (155–160). Neural systems that motivate and reinforce drug abuse have been proposed to underlie behaviors associated with compulsive food seeking and food intake (161–165). These findings raise the question of whether the OX system has a role also in eating disorders characterized by compulsive binge-type episodes, such as BN and BED.

Therefore, the effects of GSK1059865, a selective OX-1 receptor antagonist, JNJ-10397049, a selective OX-2 receptor antagonist, and SB-649868, a dual OX-1/OX-2 receptor antagonist, were investigated in our experimental BE model in female rats. The results obtained, published in (166), showed that none of the OX antagonists affected feeding in *NR + NS* rats. However, in *R + S* rats SB-649868 and GSK1059865 selectively reduced

HPF intake. No effect was observed for JNJ-10397049. Altogether these findings suggest the involvement of OX-1 but not OX-2 receptors in BE.

Further studies are underway to evaluate whether OX-1 receptor antagonists reduce BE by interfering with stress or reward mechanisms; however, their ability to evoke pronounced and selective suppression of BE suggests that targeting OX-1 receptor system could represent an interesting pharmacotherapeutic approach for the treatment of BE-related disorders.

4. Conclusion

At present the preclinical model of BE described in this chapter (39) has been employed by our group in at least 15 groups of female rats and, in most of these groups, the BE test has been replicated for up to five consecutive times, without evidence of development of tolerance. Following the observations related to the influence of the ovarian cycle on the BE response, and the consequent exclusion of data coming from female rats in the estrus phase of the ovarian cycle, the model proved to be highly reliable and reproducible.

The results obtained with topiramate, sibutramine, and fluoxetine indicate that the model has interesting elements of predictive validity. It exhibits evidence of face validity related to the very prompt and pronounced intake of HPF in a short period of time, which is repeated over time when animals experience the stressful procedure. Moreover, like in women, in the female rats used in this model BE is tightly linked to the hormonal fluctuations of the ovarian cycle. Lastly, the model exhibits strong construct validity, since BE is evoked by yo-yo dieting and stress, which are also considered key determinants of BE in humans.

Our BE model may be particularly suitable to investigate the role of stress mechanisms in the arousal and maintenance of the binge-type behavior. The interesting results so far obtained with the CRF-1 receptor antagonist R121919 clearly support the involvement of CRF mechanisms in BE. Further studies are needed to explore at a more systematic level the significance of CRF mechanisms not only on the expression of the BE episode, but also on the processes leading to its emergence. Studies are ongoing in our group to explore the involvement of the CRF system in the processes of brain plasticity/sensitization occurring during restriction/refeeding cycles.

We find the results obtained with the OX-1 receptor antagonists particularly interesting. The mechanisms by which OX-1 receptor antagonists suppress BE remain to be investigated.

However, considering that excessive intake of certain foods produces behaviors and changes in the brain that resemble an addiction-like state (155–160), it is reasonable to speculate that OX mechanisms may be common to both drug abuse and compulsive eating behaviors. Targeting this system may offer a strategy to develop pharmacotherapeutic agents potentially effective for both addiction and eating disorders.

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Chapter 4

Binge-Type Eating Induced by Limited Access to Optional Foods

Rebecca L.W. Corwin and Francis H.E. Wojnicki

Abstract

Binge eating is characterized by the consumption of more food within a discrete period of time than would normally be consumed within the same time period under similar circumstances, accompanied by a sense of loss of control. This form of consummatory behavior is common, and it is accompanied by comorbidities that make treatment difficult. Animal models of bingeing have been developed in order to examine mechanisms as well as to develop potential therapeutic interventions. In this chapter, the limited access model of binge eating is described. This model makes use of established criteria for binge eating in humans and it has good face and construct validity. Recent clinical data suggest predictive validity, as well. Results obtained with the limited access model complement those obtained with other models, and indicate that fatty and sugary foods in-and-of themselves are not "addictive." However, the consequences of binge eating are nonetheless profound. Specifically, repeatedly engaging in intermittent bouts of behavioral excess over extended periods of time appears to promote behavioral and neurological changes that are difficult to treat and that may predispose an individual to other forms of dysfunctional behavior. In short, when it comes to fatty and/or sugary foods, *how* one eats may be more important than *what* one eats.

Key words: Binge eating, Bulimia, Animal models, Psychiatric disorders, Food intake, Ingestive behavior

1. Introduction

Modeling psychiatric disorders in animal models is challenging, and the available models can, at best, only provide insight into selective aspects of these complex problems. Binge eating is no exception, as it is often accompanied by psychopathology as well as a host of psychiatric comorbidities (1, 2). However, without animal models, cause and consequence are difficult to distinguish and novel treatment modalities cannot be tested. We developed the limited access model of binge-type eating in order to study the behavior of bingeing, to examine some of its causes and consequences, and to test

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potential therapeutic interventions. The model has predictive validity, as indicated by clinical trials (3, 4), as well as good face and construct validity (5).

Binge eating can be defined clinically as consuming more food in a discrete period of time than would normally be consumed during the same period of time under similar circumstances. In addition, bingeing is accompanied by a sense of loss of control, i.e., once a binge has been initiated, people feel like they cannot stop eating or even control what they are eating (1). Bingeing, therefore, is a behavior (eating a large amount) that can be modeled in animals, accompanied by feelings (loss of control) that are more difficult to model in animals. Frequent bingeing (i.e., several times a week for several months) characterizes several eating disorders, including bulimia nervosa and binge eating disorder (1), but also can occur even when criteria for an eating disorder are not met (2). About 1 in 20 Americans binge eat at some point during their lives, and the 12-month prevalence (2.1%) is similar to that of illicit drug dependence (2.8%) (2, 6). In spite of the large amount of energy consumed during a binge, most people who binge (~65%) do not become obese (2) due to compensatory behaviors such as purging or subsequent energy restriction. Although most people who binge do not become obese, binge prevalence is higher among those with a higher body mass index (BMI) (7) and binge frequency is associated with less weight loss and greater weight regain after gastric bypass surgery (8). Bingeing also is associated with psychological disturbances; about 76% of adults and 85% of adolescents who binge eat also experience comorbidities such as anxiety, mood, impulse control, or substance use disorders (2, 9). In short, bingeing is common and is associated with comorbidities that complicate treatment. In addition, since most people present clinically after the behavior has already been established, distinguishing the causes and consequences of bingeing has proven difficult.

Several animal models of binge-type eating have been developed over the past decade to address the challenges described above (see (5, 10) for reviews). In developing the limited access model, we made use of the Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition (DSM-IV) criteria (1) the bingeing rats must consume more than controls during a discrete period of time and (2) the conditions of the control group and the binge group must be similar. In addition, we wanted (1) the binge behavior to be maintained for extended periods of time and (2) compensatory behavior to be initiated by the rat, rather than imposed by the investigator (11). The limited access model builds on previous research showing that limiting access to a variety of ingestible substances, including ethanol and saccharin, will increase their consumption during discrete bouts of availability, even if the animals are not deprived of food (12–17). In this model, all groups have continuous

access to chow and water and are not food deprived. The typical experimental protocol consists of two groups, i.e., a bingeing group and a control group. The bingeing group is given limited (usually 1-h) access to an optional fatty and/or sugary food on an intermittent (INT) basis (three noncontiguous access periods per week), with days of access typically separated by at least one day of nonaccess. We usually provide access to the INT group on Mondays, Wednesdays, and Fridays (MWF) of each week. The control (nonbingeing or Daily) group is given limited access to the same optional food during the same time period as the INT group, but access is provided 7 days per week. The Daily group, therefore, is consuming the same food, during the same period of time, under similar circumstances and therefore serves as the control against which bingeing in the INT group can be compared. Most of our studies have used vegetable shortening (a partially hydrogenated vegetable oil used in the preparation of baked goods) as the limited item, though we also have reported results with liquid sugar (18), solid fat emulsions (19), and solid fat/sugar mixtures (20). More recently, we have also been testing emulsions with different concentrations of fat or sugar or their combination thickened with various biopolymers (manuscript in progress).

The INT and Daily groups consume the same amount of the optional food during the first week. For example, each group generally consumes about 2 g of shortening during the first week, whereas higher intakes of other optional foods often are seen. Regardless of the initial intake, the intake in the INT group increases over a period of several weeks to a greater extent than intake in the Daily group. As a result, the INT intakes gradually become significantly greater than the Daily intakes. Intakes generally escalate more rapidly in the INT group than in the Daily group, an effect that has been reported in both females and males (21). Bingeing in this model is operationally defined when INT consumption of the optional food becomes significantly greater than Daily. The goal in our studies is to distinguish the "normal" response to a palatable food from bingeing on a palatable food. We conceptualize the Daily intakes as being representative of "normal" consumption of the optional food, whereas the INT intakes represent bingeing. This approach has relevance to human bingeing, as eating in the absence of hunger has been associated with bingeing in humans (22), and binge episodes often include "forbidden foods" rich in fat and sugar (23).

After several weeks on the protocol, intake of shortening in the INT rats becomes quite large, typically being comparable to that of rats that have continuous access (24 h per day, 7 days per week) to shortening (24–26). In spite of the large amount that is consumed during the limited access period, INT rats do not become obese since they reduce their chow intake. The net energy consumption, therefore, is not only the same as the Daily rats, but also

is the same as that of Chow-only control rats with no shortening access (27, 28). This is similar to human reports showing that most people who binge do not become obese (2). Although the INT rats reduce chow intake, the high intake during the limited access period is not due to self-imposed energy restriction during the pre-binge period; rats will binge even if chow intake prior to the binge is not reduced (29).

In addition, the INT rats respond more than the Daily rats under a progressive ratio 1 (PR1) schedule of shortening reinforcement (30), as well as other schedules of reinforcement. The PR data are consistent with the idea that motivational differences between bingers and nonbingers can result from repeated intermittent brief bouts of excessive consumption. In addition, it appears that a prior history of bingeing can have long-term effects on the response to other rewarding substances. Specifically, rats that previously were maintained on the INT shortening access protocol, but no longer had access to shortening, responded more for cocaine under a PR schedule than did rats previously maintained on the Daily protocol, chow only, or continuous access to shortening (26). Recent data suggest that these behavioral differences may be due to alterations in dopamine signaling in the prefrontal cortex (31, 32).

2. Materials and Procedures

2.1. Rats

The majority of our research has been conducted with Sprague–Dawley rats (obtained from Harlan Laboratories) that are 60 days of age at the start of the study. We have also reported bingeing in a strain other than Sprague–Dawley, as well as rats of different ages (33) and in both males and females (21, 27, 28).

2.2. Chow

We typically use pelleted rodent chow (LabDiet 5001 Rodent Diet, Purina Mills), but also have used powdered chow in some studies (34).

2.3. Vegetable Shortening

We use Crisco® All-Vegetable Shortening (J.M Smucker Co., Orrville, OH), rather than generic shortening in order to maintain consistency of the product. Shortening works well as binge food in this protocol for two reasons: (1) Rats will readily consume it (35) and differences between groups can be assessed. Foods that are more acceptable than shortening will stimulate large intakes in INT as well as Daily rats and "binge" intake cannot be distinguished from "normal" intake. (2) When using shortening intakes are high enough to be able to show reductions with pharmacological probes, but are low enough for stimulation to also be seen (36). Fresh shortening is provided on at least a weekly basis in a glass

bowl that is clipped to the inside front of the hanging-wire home cage. The glass bowls containing shortening are weighed prior to presentation to the rats, and immediately after the limited-access period. The weights are subtracted to calculate how much shortening was consumed. Bowls are then placed in a safe place until the next presentation period. The can of shortening does not need to be refrigerated to maintain freshness.

We have also used optional foods other than shortening, with mixed success. When consuming liquid sucrose solutions, INT rats consumed more than Daily rats, but only when concentrations of 3.2% or 10% were used with a specific type of delivery tube (18). We think this may have been due to the effort required to obtain the sucrose from the tube. INT and Daily intakes of semisolid fat emulsions at concentrations of 18%, 32%, and 56% do not reliably differ from one another (19), but this may have been due to the presence of starch in the biopolymer powder (thickener) that was used (37). When starch was a component of the biopolymer powder, both INT and Daily rats consumed large amounts of emulsion (19). Less emulsion is consumed, however, when starch is absent (37). When vegetable shortening and sugar are whipped together, INT rats consumed more than Daily rats when 3.2% or 10% sugar was mixed into shortening, but not when 32% sugar was mixed in (20), a result mimicking that of liquid sucrose. One reason for the failure to see differences in intake with some of these optional foods may be ceiling effects on intake. If so, then differences might be expected to emerge under conditions in which gastric fill would not be a limiting factor, such as under progressive ratio schedules of reinforcement. We previously have shown that INT rats binging on shortening earn more shortening reinforcers under a PR1 schedule of shortening reinforcement, even if home cage intakes in the INT and Daily rats are matched (30). Current studies suggest similar results with other types of optional foods.

2.4. Housing

We house our rats individually in hanging wire cages. This allows us to accurately measure food intake in individual rats and to collect any food spillage that may accumulate beneath the cage. The cages are equipped with a food hopper for the pelleted chow and a metal clip that can hold a glass bowl containing shortening. In addition, water bottles are clipped to the outside of the cage so that water is freely available. Optimal temperature within the vivarium appears to be about 21°C with relative humidity at about 40–45%. Males and females are housed in separate rooms to avoid any influence of pheromones.

2.5. Lighting

Prior to the arrival of the rats at the vivarium, we set the light cycle timer to the times that will be used for the duration of the experiment. We use a 12:12 light/dark cycle, but the cycles can start and stop at the convenience of the investigator. However, once the

start and stop times are chosen, they need to be maintained throughout the study. We usually allow a minimum of 5 days for the rats to adjust to the change in the time shift. The binge period should be timed as close to lights out as possible (we start about 2 h prior to dark onset), without actually running into the dark cycle. Rats naturally eat a large meal when the lights go out (38). The goal is to avoid having them fill up on chow prior to the binge. If the binge takes place shortly before lights out, the animals will most likely be awake, but will not normally have consumed large amounts of chow yet. Thus, the light cycle needs to be set such that the investigators can provide the shortening at a fixed time each day. A red light should also be set up in the rat room within easy reach, so that if any procedures need to take place after the vivarium lights are out, the investigators will still be able to see, but the circadian rhythm of the rats will not be disturbed. Avoid the use of red ink, as it will not show up under red light illumination. To avoid disrupting the circadian rhythm of the rats, it is critical to avoid shining any bright lights in the room once the vivarium lights go off. It is also recommended that the cages be arranged in the vivarium such that if one needs to enter the room during the dark cycle, the light from the hallway will not shine directly into the rats' eyes.

2.6. Groupings

When the rats arrive, immediately place them into their individual cages making sure they have water bottles and chow readily available. We do not disturb them for the first 5 days after arrival other than daily inspections to make sure that chow and water are being consumed. Rats are usually weighed on day 6 after arrival and weighed at least weekly thereafter. During the first weighing the rats are handled for 30–60 s. During subsequent weighings rats are handled for 10–15 s. Before initiating the binge procedure, the rats need to be matched on several different variables to assure, as much as one can, the similarity of the INT and Daily groups. The variables we use are (1) body weight, (2) average 3-day chow intake, and (3) overnight shortening intake.

Body weight is the weight of the animals just prior to assigning them to groups. The 3-day chow measurements take place about 6–7 days after the arrival of the rats in the vivarium. We take three 24-h measurements and average them for each rat. For these measurements, a clean spill pad (we use paper towels) is placed beneath each cage daily and any spillage is taken into account when calculating chow intake. After the 3-day chow intake measurements are complete, each rat is given a bowl containing at least 15 g of shortening for a single overnight period. The shortening is pressed up against the side of the bowl so that the rats cannot pick it up and play with it. The overnight access allows the rats to get familiar with the shortening and avoids neophobia during the brief periods

of access required of the limited access procedure. The bowls are weighed before presenting them to the rats and again the next day. For rats to be included in the study, they must have at least sampled the shortening.

The rats are then assigned to groups such that the groups do not differ statistically on any of the three variables (body weight, 3-day chow average, and overnight shortening). Arrange the groups in the hanging racks in a manner that distributes variables such as light access, airflow, etc. equally across the groups. For example, an equal number of INT and Daily rats should be housed at the top and bottom of the racks, in the front and back of the room, etc. Furthermore, the INT rats should be arranged so that they can observe the Daily rats being given access to the optional food.

2.7. Limited Access Procedure

Once the rats have been assigned to groups, the limited access procedures can be initiated. This usually is done about 12 days after the rats arrive at the vivarium, and about 2–3 days after the overnight shortening exposure. The limited access procedure is quite straightforward: simply place a preweighed jar of shortening into the cage (using the clip to stabilize it) for the amount of time that you want it provided, and then remove it and weigh it. We originally used a 2-h access period, but have had equal success with 1 h. If access for less than 1 h is desired, start out with 1 h during the first week, and then gradually shorten it over the next couple of weeks, so that the rats have time to learn the procedure. For instance, in a recent study a 20-min access period was desired (21). Therefore, shortening was provided for 1 h during the first week, for 40 min during the second week, and for 20 min thereafter. Daily rats get the shortening every day during the assigned limited access time period, and the INT rats get the shortening for three noncontiguous days a week at the same time of day and for the same length of time as the Daily rats. We traditionally have used MWF for the INT rats, but have used other schedules with equal success (29). If you are also measuring chow, be sure to put spill pads under the cage so that spillage can be incorporated into the intake calculations.

2.8. Experimental Variables

The primary dependent variable is intake. We measure intake in grams (mL if liquid is being used), but often convert it to energy. If studies are long in duration, we normalize energy intake to body weight (39) when comparing intake at different time points. Daily intakes are measured, but in our current work we generally only use the MWF data to make statistical comparisons between INT and Daily groups. Weekly average intakes or 2-week intakes have also been used for comparative purposes.

3. Notes

3.1. Shortening Intake During the Limited Access Period

Intake during the limited access period should be the same in the INT and Daily groups within the first day or so. However, during the initial few weeks of the study, the intake of the INT rats should increase such that intakes exceed those of the Daily group after about 4 weeks (Figs. 1 and 2a). However, in some studies we have seen a significant difference between the INT and Daily group within the first week (24). There will be individual variability in the INT and Daily groups with a range of intakes possible; as a result, overlap between the groups is to be expected.

3.2. 24-h Chow Intake

Chow intake in the INT and Daily groups is generally less than that of rats that have continuous access to chow but no shortening access (Fig. 2b).

3.3. Total Energy Intake

Daily 24-h energy intake in the INT groups differs from that of chow-maintained controls on most days (Fig. 3a). On the days that shortening is provided, chow intake is not sufficiently reduced to compensate for the energy provided by the shortening. INT rats, therefore, consume more energy on shortening days than chow-only controls. However, on the days that shortening is not provided (Tuesdays, Thursdays, Saturdays, and Sundays, in the study shown), INT rats consume less energy than chow controls. The net result of this "overeat/undereat" pattern is that cumulative

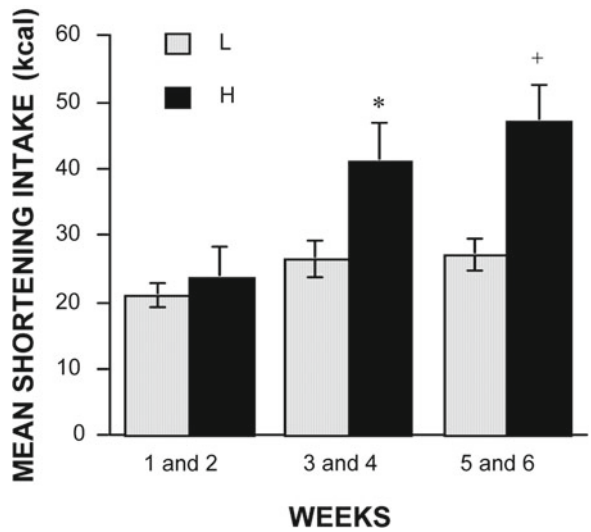

Fig. 1. Mean shortening intake during the 2-h access period. *Bars* represent mean shortening intake for the three 2-week periods of the study. *Gray bars* represent rats with Daily 2-h access to shortening (designated "L" for "low restriction" in this study); *dark bars* represent intermittent (INT) rats that had 2-h access to shortening on Monday, Wednesday, and Friday (MWF) (designated "H" for "high restriction" in this study). $*p<0.05$; $+p<0.01$. *Vertical lines* indicate SEM. Reproduced with permission from (27).

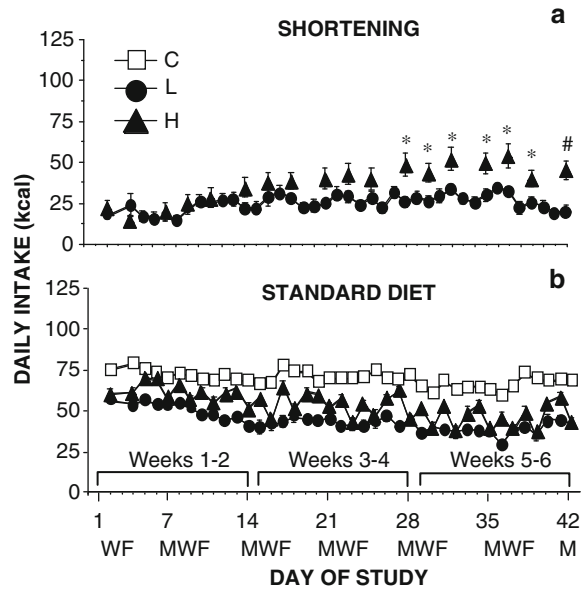

Fig. 2. Effects of restricting access to shortening, without restricting access to the standard chow diet, on daily shortening (**a**) and standard chow diet (**b**) intake in male rats. *Open squares* represent chow controls "C." The C rats had continuous access to the standard chow diet but no access to shortening. *Closed circles* represent rats with Daily 2-h access to shortening (designated "L" for "low restriction" in this study) and continuous access to the standard chow diet. *Closed triangles* represent INT rats that had 2-h access to shortening on MWF, and continuous access to the standard chow diet (designated "H" for "high restriction" in this study). *Vertical lines* indicate standard error of the mean (SEM). If no error bar is visible, then it is included in the symbol. (**a**) Shortening intake. *Symbols* indicate H > L: $*p < 0.05$; $\#p < 0.001$. (**b**) Standard chow diet intake. Symbols indicating significance are not shown for clarity. L < C on all days ($p < 0.001$); H < C on all of the MWF shortening days ($p < 0.05$); H < C on 12 of the Tuesday, Thursday, Saturday, Sunday non-shortening days ($p < 0.05$). Reproduced with permission from (27).

total energy intake across the study does not differ from that of chow controls (Fig. 4).

In the Daily rats, daily 24-h energy intake does not differ from that of chow-only controls on most days (Fig. 3b). Thus, the cumulative total energy intake of the Daily rats also does not differ from that of chow controls (Fig. 4). Note that although total energy intake does not differ among the groups, cumulative energy from fat does. Specifically, even though the Daily group consumes less fat within the 1-h access period (Fig. 1), the Daily group usually consumes more cumulative fat over the course of a study than the INT group, because the Daily group has access to it 7 days per week.

3.4. Troubleshooting

3.4.1. Presence of Daily Group

The Daily group provides a standard against which bingeing in the INT group can be compared. It is essential that bingeing be compared to some control group that has access to the same optional food, for the same period of time, under similar circumstances, in order to clearly distinguish binge-type consumption from that which would normally occur in response to fatty and/or sugary

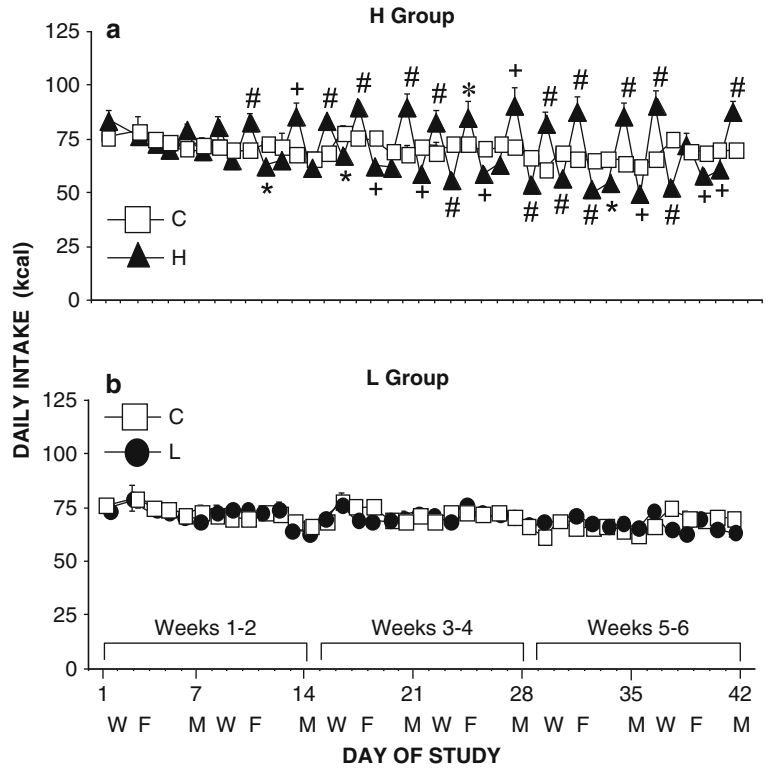

Fig. 3. Effects of restricting access to shortening, without restricting access to the standard chow diet, on daily total energy intake in male rats. Group designations are as described for Fig. 2. (**a**) Total intake in the H (INT) group differed significantly from C (Chow controls). $^{*}p<0.05$, $^{+}p<0.01$, $^{\#}p<0.001$. (**b**) Total intake in the L (Daily) group never differed from C. Reproduced with permission from (27).

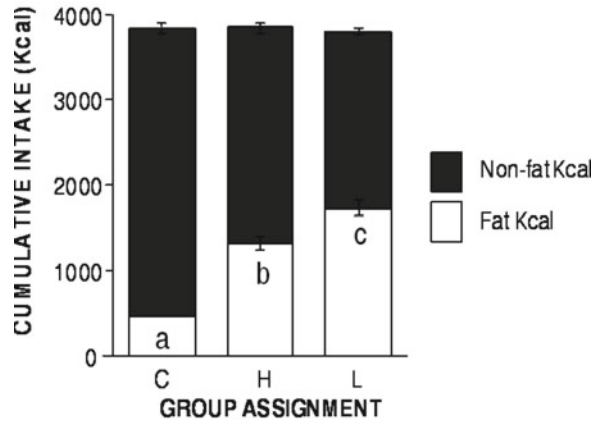

Fig. 4. Cumulative intake across the entire study. Total calories consumed did not differ among the groups; however, total fat increased as the fat became more available. Group designations are as in Fig. 2. *Lower case letters* indicate that the groups were different from each other ($p<0.05$). *Vertical lines* indicate SEM. Reproduced with permission from (27).

foods. In addition, recent work indicates that the presence of the Daily group in the same room is critical for stimulation of progressive ratio responding in the INT group (manuscript in preparation), an effect possibly due to the introduction of uncertainty regarding when the shortening will be provided (40).

3.4.2. Failure to Sample the Shortening on Overnight Exposure

Seldom does a rat fail to sample the shortening on overnight exposure. When this occurs, we leave the jar of shortening in the cage for a full 24 h, which is usually enough time for the rat to start consuming the shortening. A rat that refuses to consume shortening after a full 24-h exposure is eliminated from the study.

3.4.3. Inability to Match Rats on More than One Variable

We have always been able to match the groups (up to four groups) by body weight, average 3-day chow intake, and overnight intake of shortening by first matching the overnight shortening intake. Once the groups are matched on one variable, the other variables generally fall into place. If not, a minimal amount of "tweaking" (switching a couple of rats among the groups) is required.

3.4.4. Failure to Consume Shortening During Limited Access Period

We occasionally get an animal that will consume only 0.1–0.3 g of shortening during the access periods in the first week. We have found that instead of putting the jar in the jar clip, putting the jar in the middle of the cage will engender greater amounts of consumption. Once the rat reliably consumes shortening, the jar can be put in the clip by the third week.

3.4.5. Chow Spillage/ Shortening Hording

When measuring chow intake, paper towels are placed below the cage under the chow hopper outside the reach of the rat. It is necessary to make sure that the rat cannot reach the paper towel, lest you find a nest built in the cage the following day. It is also necessary to make sure that the cages near a door do not have enough change in air pressure to move the paper towel from its place when opening and closing the door. Spillage is placed into a weigh bowl by rubbing the paper towel together to make sure the entire spill is obtained. When removing the shortening from the cage after the access period, make sure to check under the cage and in the cage for balls/masses of shortening. Some rats have a propensity to scoop the shortening out of the jars. If an unusually large amount of shortening appears to be consumed, check the cage again by opening it and looking inside while the rat is moving. In a couple of studies, at least one rat would scoop out the shortening and then lie upon it, perhaps to "hide it from the investigator," a strategy that is quite successful if the investigator is not vigilant.

3.4.6. Biting/Nipping

When using shortening as the optional food, rats will seldom bite or nip the fingers of the experimenter. When they do, it is usually when the shortening jar is removed from the cage. In a couple of studies one or two rats have actually jumped at the jar when being placed in the cage and became aggressive upon its removal. A clipboard placed between the rat and jar clip solves the problem.

When using sucrose as the optional food, either in liquid or semisolid form, rats seem to nip more often. With the liquid form, rats will nip for no apparent reason when filling chow hoppers or when the rats are being weighed. With the semisolid form of sucrose, rats will nip upon removal of the jar from the jar clip.

3.4.7. Eating at the End of the Access Period

Generally, all rats finish their consumption within 30 min and then retire to the back of the cage. Occasionally, one will encounter a rat that will resume eating when the jars are being pulled out of other cages. We generally pull the jar on this rat last as the duration of consumption is very short.

3.4.8. Fresh Versus Old Shortening

Jars are filled with fresh shortening weekly on the same day each week. Changing the shortening every session is both costly and unnecessary. We have been able to obtain consistent results throughout a week using shortening that was placed into the jars at the beginning of the week. We have used "old" shortening in a couple of pilot studies to save costs, only to find that the rats generally consume less if the shortening has remained in the jars for 2 weeks or longer.

3.4.9. Unanticipated Changes in Intake

Usually once or twice in an 8-week study, there will be a dramatic increase or decrease in optional food consumption on a particular day with a resumption of normal intakes the following day. We have no explanation for why this occurs.

3.4.10. Type of Limited Access

When rats are first exposed to the limited access procedure, it is critical that they be allowed to eat as much as desired within the limited access period. In short, it appears that they need to learn how to binge. In one study (24), we "clamped" intake by limiting the amount of shortening that rats with MWF and Daily 1-h access were allowed to consume during the first 5 weeks of the study. Results were then compared to rats with MWF and Daily access that were allowed to consume as much as desired during the 1-h access period. After 5 weeks, all groups were allowed to consume as much as desired during the 1-h access period for an additional 5 weeks. Binge size during the second 5-week period was reduced in rats with MWF access whose intake previously had been clamped, relative to the MWF rats that previously had been allowed to eat as much as desired. In contrast, intake in the previously clamped Daily rats was slightly increased (though not significantly so) relative to Daily rats that previously had been allowed to consume as much as desired. The net result was that the intake of the previously clamped MWF and Daily groups did not differ from each other, i.e., bingeing could not be assessed if only comparing these two groups. In contrast, the MWF and Daily groups that had not been clamped responded in our standard manner, i.e., MWF rats binged relative to Daily.

3.4.11. Hanging Cages Versus Shoe Box Cages

We use hanging wire cages for our studies rather than shoebox cages in order to more accurately measure food intake. Hanging cages allow for measurement of spill age, whereas shoebox cages do not. In addition, the bedding in the shoebox-type cages gets kicked into the shortening bowl, which introduces measurement error. Throughout the 20-plus years that we have been using hanging cages, health problems related to the cages have not been an issue.

3.4.12. Transfer of Rats to Clean Cages Mid-Study

Once rats are placed in their cages upon arrival at the vivarium, they should remain in those cages for the duration of the study. The one time we transferred rats into clean cages midway through a study, the intakes were altered (decreased) for over 10 days, but eventually recovered. We have never encountered any health problems with keeping rats in their original cages for the duration of a study.

3.4.13. "Enriched" Environment

Except for one pilot study, we have never examined the effects of enrichment on binge size. The results of the pilot study were inconclusive and hence, we have decided not to change our established protocol.

3.4.14. Temperature/ Humidity

Large sudden shifts in outside temperature and/or humidity can significantly, but temporarily alter intakes. Large buildings generally have large heating/cooling systems that do not respond immediately or quickly enough to sudden changes in outside temperature. Additionally, air handlers for vivaria will draw in fresh air from the outside. One can expect a change in intakes for a couple of days in the spring and/or fall when there are sudden shifts in outside temperatures that the heating/cooling system is not able to accommodate. The altered intakes have always been temporary in nature. The temperature and humidity of the vivarium are also critical to obtaining consistent results. During one of our studies, the heating system malfunctioned causing the humidity in the vivarium to reach 70–84%. This resulted in significant decreases in both chow and optional food intake. We have found that a constant room temperature between 20.8 and 22.2°C and relative humidity between 37 and 45% are ideal to generate reliable intake of the optional foods.

3.4.15. Time of Day

As discussed above, the 12:12 light/dark cycle should be set prior to the arrival of the animals. The optional food should be provided 2.0–2.5 h prior to the dark cycle to allow the rats time to consume the optional food and the investigator to collect the data.

3.4.16. Binge Food: Use Something that Allows You to Distinguish the Groups

As mentioned above, it is critical that the optional food be one that allows intake differences to occur. If large (or small) intakes occur in both groups, bingeing cannot be distinguished from "normal" intake.

3.4.17. Schedules of Reinforcement

Because intake measurements are limited by gastric capacity, we also have examined behavioral outcomes using operant procedures. The schedule of reinforcement used in a study must be appropriate to the context of the study and the question(s) being asked. We initially used an exponential progressive ratio (PRe) schedule that has been used in cocaine self-administration studies (41), but were not able to distinguish any differences between the groups (42). However, a recent study found that a PR1 schedule, but not a PR3 schedule, was able to distinguish the groups, i.e., INT rats earned more reinforcers than Daily rats when the PR1 schedule was in effect (30). Apparently, the slope of the ratio increase of the PRe and PR3 schedules was too steep, thereby limiting the number of reinforcers that were earned by each group. While the PRe may be appropriate for cocaine self-administration, it is not appropriate for non-food-deprived rats working for shortening deliveries. Our laboratory has recently completed a study that replicated our previous results (30), again showing that a PR1 schedule is best able to distinguish the INT and Daily groups.

Although our studies use non-food-deprived rats, we have found it necessary to deprive them of food for 24 h in order to establish lever pressing for the optional food. In addition, once lever pressing is established under food-deprived conditions, we conduct at least two more 24-h food-deprived sessions over a week's period to make sure that lever pressing is well established. Once established, further sessions can be conducted without the use of food deprivation, except when there is a change in operant contingencies. When one schedule has been in effect for several sessions and the next several sessions require a change in the operant contingencies, we have found it helpful to conduct the first session of the change when the rats are deprived of food for 24 h.

3.4.18. Operant Equipment

When using optional foods such as whipped shortening or semi-solid emulsions in operant studies the shortening or emulsion needs to be loaded into a 20-mL glass syringe with a metal slip lock tip. The emulsions are roughly the consistency of pudding. The syringe is mounted onto a syringe pump on the side of the chamber, with the metal tip extending into the chamber. Shortening or emulsion is delivered onto a small shelf mounted just below the tip. The operation of the syringe pumps should be calibrated so that a consistent amount of the optional food is delivered per ten deliveries. We calibrate our system to deliver about 0.1 g of reinforcer per delivery. This amount minimizes the possibility of satiation during operant sessions, while still being of sufficient magnitude to maintain behavior.

It is critical to minimize air pockets in the syringe so that each reinforcer delivery consists of shortening or emulsion, not air. To do this, first scoop the optional food into a plastic bag that can be sealed with a zip-type closure. Seal the bag, cut a tip off the bottom

of one of the corners and squeeze the contents into preferably a 60-mL syringe. Then squeeze the contents of the 60-mL syringe into a 20-mL glass syringe. It is also critical that glass syringes with glass plungers be used instead of plastic disposable syringes as the delivery system in operant sessions. The plungers of the disposable syringes have rubber tips that degrade over time as a function of exposure to the shortening. When using highly concentrated (e.g., 64%) semisolid sucrose as the reinforcer, moisten the plunger with water prior to insertion in the syringe to avoid jamming. Highly concentrated sugar-based emulsions are very sticky and can jam the syringe like glue. When using this type of reinforcer, relatively short sessions (30–60 min) are also advisable.

4. Conclusion

Limiting access to fatty or sugary food may seem like a strange way to model bingeing when most people who binge eat do so within environments where food is plentiful, not limited. However, many people within such environments limit their access to fatty and sugary foods in what often become failed attempts to control energy intake. Human studies have shown that people often binge on the very foods that they have tried to restrict (23) and cognitive behavioral therapy treatment programs for bingeing include the incorporation of forbidden foods back into the diet (43). Furthermore, under controlled laboratory conditions, restricting access to a snack food increased consumption of that food (44). Thus, while fatty and sugary foods are readily available in environments of food abundance, people often limit access to them, which appears to promote bingeing on those very same foods.

What, then, can be done? Clearly, round the clock free access to fatty and sugary foods would not seem to be the best strategy. Many rat studies have shown that such feeding protocols only serve to promote obesity. In contrast, the data generated by our rat model indicate that the consumption of small amounts of optional foods each day under predictable circumstances does not promote binge-type consumption of those foods or obesity. In human subjects, the introduction of certainty into daily eating patterns is used in therapeutic interventions for binge eating (43) and appears to provide some protection against the development of bingeing in adolescents (45, 46). In addition, our data indicate that only being allowed to consume a small amount of the optional food upon initial exposure protects against subsequent bingeing to some extent (24).

We recognize that a rat model is exactly that: a model. No animal model can provide a comprehensive understanding of the complexity involved in human psychiatric disorders, and we do not claim to do so with ours. Even with their limitations, however,

animal models can inform our thinking about human disorders and can provide opportunities for elucidating mechanisms and testing interventions that otherwise would not be possible. Several animal models of bingeing have been developed, each of which has made important contributions to our understanding of binge behavior (see (4, 10, 47) for reviews). The limited access model has good face and construct validity (5), and recent clinical trials with baclofen suggest predictive validity, as well (3, 4). The model is simple and inexpensive to use and does not include potential confounds introduced by food deprivation and/or obesity. Although the various models of bingeing differ in many ways, converging evidence from studies using them indicates that the consequences of binge eating can be profound. Specifically, repeatedly engaging in intermittent bouts of behavioral excess over extended periods of time appears to promote behavioral and neurological changes that are difficult to treat and that may predispose an individual to other forms of dysfunctional behavior. While these reports provide reassuring evidence that fatty and sugary foods are not addictive in-and-of themselves, the results are perhaps even more sobering: when it comes to fatty and/or sugary foods, *how* one eats may be more important than *what* one eats.

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Chapter 5

Assessment of Stress-Independent Binge-Like Eating Behavior in Mice

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Abstract

In humans, binge eating is central to the harmful effects of bulimia and binge-eating disorder (BED). The development of preclinical mouse models of binge-like eating behavior has proved to be challenging, as minor stressors can significantly inhibit food intake in this species. Herein, we present two reinstatement models that can reliably induce binge-like eating behavior in mice. Both utilize a schedule of intermittent access to a palatable, high-energy-dense diet (HED). We describe the typical procedures for inducing binge-like eating behavior with daily 1-h or weekly 24-h free-choice access to the HED. No investigator-initiated caloric restriction or exogenous stressors are imposed to induce bingeing using either paradigm. We compare the results obtained with the two models and the unique features of both. The intermittent access to HED allows one to obtain reproducible binge-like eating behavior that can be maintained consistently for several weeks. Furthermore, these two hedonic feeding paradigms are highly reproducible across the common C57BL/6 and 129SvEv inbred mouse strains. Therefore, the models described herein may prove useful in the analysis of feeding behavior in genetically engineered mouse strains.

Key words: Binge-eating behavior, Genetically engineered mice, Intermittent access schedule, C57BL/6, 129SvEv, Palatable energy-dense diets, Neuropeptide Y, Cannabinoid receptor 1

1. Introduction

Recently, many weight-loss drug trials have likely failed because the studies have predominately tested patient populations without regarding the etiology of their obesity (1, 2). Currently, drug candidates are tested for efficacy based on their ability to reduce free-access food intake and body weight in diet-induced obese mice. Therefore, translation of efficacy in rodent models is based on the assumption that there are normal circadian feeding patterns in all obese patients. Identifying drug candidates that reduce food consumption in specific models of abnormal food intake might lead

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to a better success rate for developing weight-loss therapies. It is estimated that approximately 3–5% of the population binge eats and approximately 30% of obese people meet the criteria for a diagnosis of binge-eating disorder (BED) (3). Thus, targeting the dysregulated feeding by reducing the frequency and duration of binge eating will likely reduce weight specifically in patients with BED.

Dieting and stress are well-known precipitating factors inducing binge eating in humans. Developing a mouse model of binge-like eating behavior using exogenous stressors has proven difficult, since relatively minor stressors can significantly inhibit food intake in this species (4). The mouse, however, offers several advantages as a preclinical research model, including smaller size, which reduces housing costs and the amount of drug needed for the experiment. Moreover, the large repertoire of existing genetic models provides a method to study the involvement of single genes in binge eating. At least two independent laboratories have demonstrated that a combination of stress and dieting can induce binge-like eating in the mouse. One group described that a history of repeated exposure to forced swim stress paired with food restriction increased the consumption of Nabisco Oreo® cookies (5). However, once initiated, this behavior was not maintained past three cycles and therefore is likely not useful to determine behavior and/or metabolic effects associated with chronic bingeing. A second model employed exposure to an unpredictable daily stressor for 17 days, which increased total caloric intake (4). Moreover, exposure to this chronic variable stress (CVS) led to a specific increase in fat intake when mice were given free-choice access to macronutrient-specific diets. However, when a high-fat diet was restricted to 1 h per day, CVS exposure did not increase intake above that of control mice (4). In fact, total caloric intake over the 17 days was reduced. It should be noted that the mice used by Teegarden and Bale (4) were on a B6:129 mixed genetic background, which may have influenced the study results. A subsequent study by the same group demonstrated that prior caloric restriction (75% of average daily caloric intake for 21 days) enhanced intake of a high-fat pelleted diet when presented for 10 days during exposure to CVS (6). Thus, there is a complicated interaction between caloric restriction, stress, and access schedule of energy-dense diets that must be considered when evaluating binge-like eating behavior in mice.

In order to study mechanisms driving the initiation and maintenance of binge-like eating behavior, it is necessary to isolate the behavior from potential confounds. Both stress and food deprivation can quickly lead to physiological changes which, by themselves, likely alter food intake as noted above. Food restriction by itself reduces circulating leptin levels, increases hepatic gluconeogenesis, reduces energy expenditure, causes hyperlocomotion, and increases circulating corticosterone levels (7–10). In addition, the previous models require a significant time commitment from the

investigator because they require daily manipulations and only produce significant binge-eating patterns following several weeks of the protocol. Furthermore, there are significant limitations with stress models since stress is variable and the physiological consequences of stress can be dependent upon animal husbandry conditions, vendor sub-strains, and other environmental variables that are difficult to control across institutions. Thus, lab–lab reproducibility might also be an issue with the previously described mouse models as was recently reported in rat models (11).

2. Materials and Procedures

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. 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 ≤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 there is an automatic watering source to supply the mice with water or a water bottle is utilized. If there is an automatic watering source then the standard metal cage tops will suffice. Otherwise, an additional divider/barrier needs to be created in the food area, or food hoppers that hang into the cage need to be used. 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, 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.

The present methods describe two access schedules where mice have either daily 1-h or weekly 24-h access to an HED. It should be noted that during assessment of binge-like feeding there is free-choice access to standard chow and a nutritionally complete diet high in fat and sucrose (i.e., the HED). Teklad 95217 diet induces robust binge-like eating with both daily 1-h and weekly 24-h access schedules (12, 13). The consumption of an energy-dense diet high in fat and sucrose increases extracellular dopamine (DA) levels in

the nucleus accumbens (NAcc) specifically associated with the feeding behavior (14). Activation of the mesolimbic DA pathway has been previously demonstrated under various hedonic feeding paradigms (14–17). Interestingly, the elevation in extracellular NAcc DA levels was associated with consumption and not anticipation of palatable food on an intermittent access schedule. Moreover, the increased extracellular DA did not habituate following daily access to HED (14).

2.2. Intermittent Access Schedules

2.2.1. Daily 1-h, Free-Choice Access Schedule

Mice are exposed to a nutritionally complete, palatable diet high in fat and sucrose (i.e., the HED) (39.8% fat, 41.4% carbohydrate, 4.3 kcal/g; TD95217, Teklad, Madison, WI) at the same time each day for 1 h over a period of multiple days. Standard rodent chow (13% fat, 67% carbohydrate, 2.9 kcal/g; TD2014, Teklad, Madison, WI) is available at all times, including the 1-h HED access period. The studies are conducted in the light phase to minimize the impact of nocturnal feeding bouts. During the testing phase, mice receive the HED diet for exactly 1 h each day at the same time every day (Fig. 1). Mice should be randomized into experimental groups for pharmacological studies (e.g., drug- and vehicle-treatment groups) based on intake of the HED during the access period on the day preceding drug treatment. Drug treatment can begin as early as 4 days after initiation of 1-h limited access to the HED.

As the mice have free access daily to chow and HED, this method can be used to access palatability cues in knock-out mice (18). One limitation of the model is that access to HED is predictable and likely entrains anticipatory activity to the HED access. Moreover, the mechanism driving the feeding behavior on a daily intermittent access schedule is likely distinct from the feeding in response to caloric restriction used in previous models. Interestingly, once stable 1-h HED intakes are established, this intake is hard to extinguish. Even after a 3-week period where no HED is presented, the 1-h HED intake will reestablish itself back to similar levels upon return of HED access (DK Sindelar, unpublished observations). Thus, once the behavior is established, wash-out periods following pharmacological manipulation can be performed without having to retrain or use a naïve group of mice, allowing the investigator to perform multiple experiments within the same cohort of mice. It should be noted that investigators should be cautious when working with genetically engineered mice (GEM) until caloric intake studies are performed and baseline results compared to appropriate wild-type control mice.

2.2.2. Weekly 24-h, Free-Choice Access Schedule

The procedure used to initiate and maintain binge-eating behavior with once weekly access is outlined in Fig. 2 (13). Adult mice should be randomized by body weight into one of three experimental groups. The chow-only and continuous HED access groups

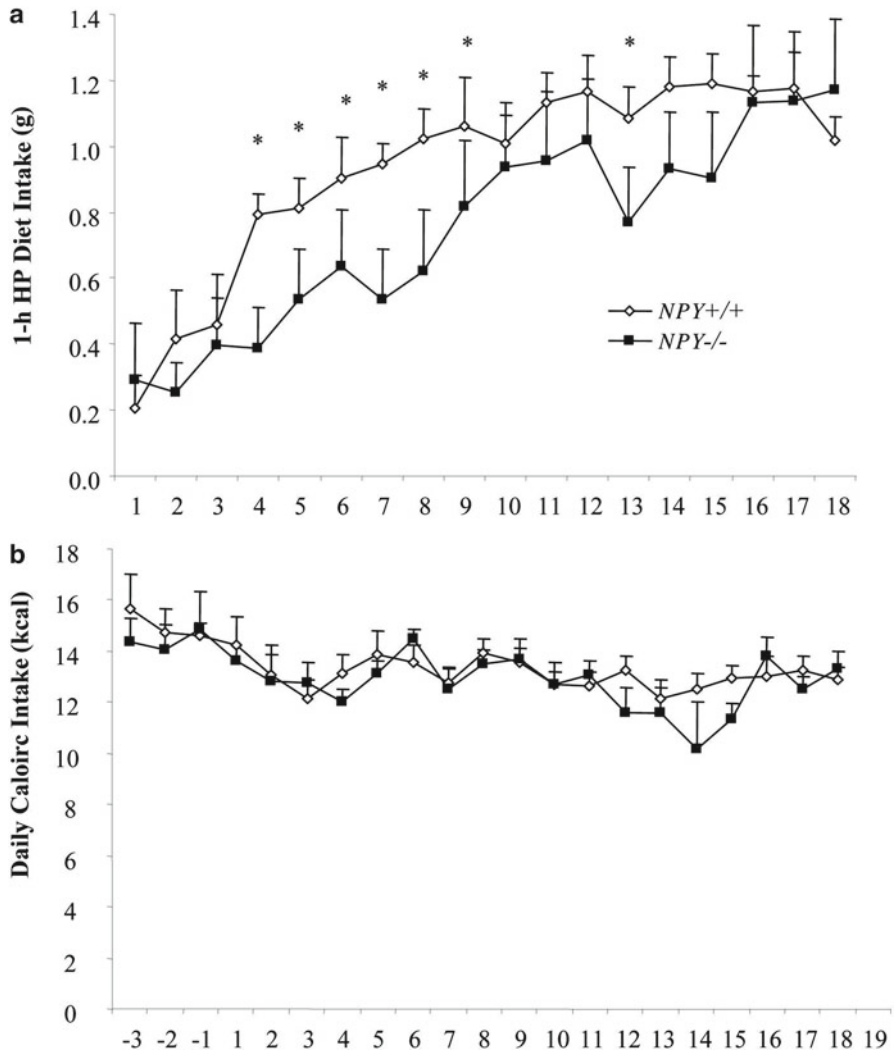

Fig. 1. Daily 1-h free-choice access to a palatable, energy dense diet induces binge-like eating in mice. (**a**) In 129SvEv mice, 1-h intake of a highly-palatable diet (labeled HP on *y*-axis) increases steadily with each subsequent exposure, until it reaches a plateau after approximately 10 days. (**b**) However, consumption of the standard chow diet declines so that 24-h caloric intake remains similar to chow-only fed controls. Also shown are results in Neuropeptide-Y (NPY) knockout mice. NPY knockout mice take longer to reach maximal 1-h intakes compared to wild-type controls, suggesting that this strain has deficits in acquiring this binge-like eating behavior. Reprinted with permission from (18).

serve as controls. 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 95217, 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.

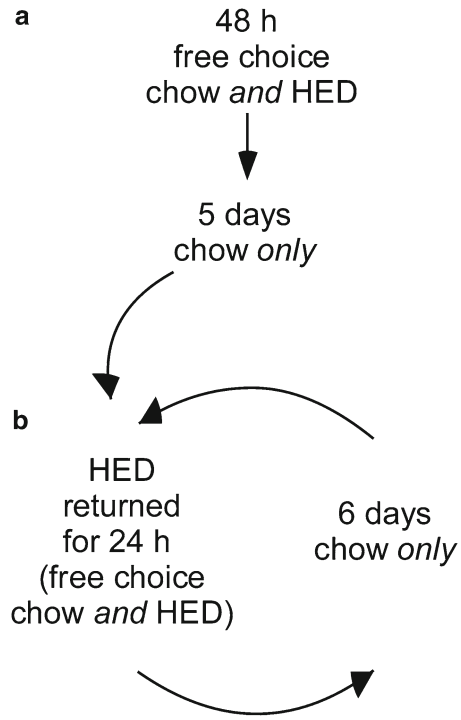

Fig. 2. Method to induce binge-like eating in mice with weekly 24-h free-choice access. (**a**) Beginning of binge cycle #1: initial 48-h access to the high-energy diet (HED), then 5 days of ad libitum chow only before access to the HED for 24 h. (**b**) Successive binge cycles: mice in the intermittent group have free choice access to standard chow 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 (not shown). Reprinted with permission from (13).

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 binge-eating period. The 24-h time point is used to monitor any compensatory changes in caloric intake that may occur following the binge eating episode. Following the 24 h of free-choice access to both chow and the 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

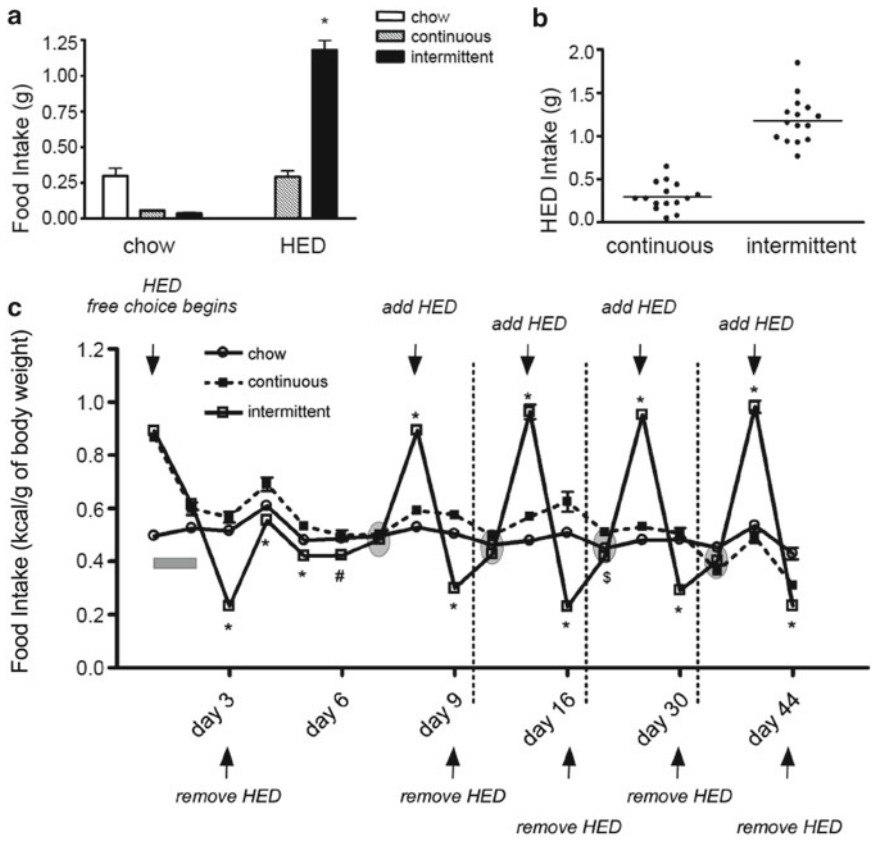

Fig. 3. Typical 2.5-h and 24-h food intakes after presentation of a palatable, energy dense diet (HED) in mice with weekly 24-h free-choice access. (**a**) Mice consume approximately 33% of their total daily caloric intake in the 2.5 h following presentation of the HED. (**b**) Scatter plot showing uniform preference of the HED in all mice. (**c**) Shown are 24-h kcal intakes. Stable binge eating was able to be obtained for at least 6 successive weeks. The *shaded horizontal rectangle* indicates initial 48-h exposure to the HED and *shaded ovals* highlight the 24-h chow intake immediately preceding access to the HED. Data are from male C57BL/6 mice. (Bonferroni post hoc tests $^{*}p<0.001$, $^{\$}p<0.01$, $^{\#}p<0.05$; continuous vs. intermittent binge groups). Reprinted with permission from (13).

the mice and not the investigator. With this weekly access paradigm, we have been able to obtain reliable binge-like eating during the first binge cycle. 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 are shown in Fig. 3. During this time period, mice consume approximately 33% of their typical daily caloric intake. We have been able to maintain consistent binge-like eating behavior for at least 6 weeks in male mice using this model (Fig. 3)

2.3. Time Required

When using the daily 1-h free-choice access schedule, several days are necessary to obtain reliable binge eating. In some cases, particularly when working with GEM, it may be necessary to have an initial 24 h exposure to the HED to reduce neophobia to the diet

1 week prior to initiating daily access. This prior exposure should significantly increase HED intake once daily 1-h access begins. Within 3 days of 1-h daily access, HED intake is significantly increased compared to the first day (Fig. 1). On day 4, testing of pharmacological agents can commence in those studies testing for validation of compound mechanism or efficacy. When utilizing the weekly 24-h free-choice access schedule, 7 days are required to complete one binge cycle. At least one binge cycle should be completed before testing pharmacological agents. Other than measuring chow intake, daily manipulations are not required with the weekly 24-h free-choice access method.

2.4. 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-eating group) should be done with repeated measures ANOVA followed by post hoc analysis.

2.5. Experimental Variables

It is important to note two major differences that exist between the two models that could affect the interpretation of experimental results. Reduction in chow intake after removal of HED is inevitable. When utilizing the weekly 24-h access model, mice are monitored daily until their chow intake returns to pre-HED levels before each subsequent HED binge-eating episode. However, chow consumption in the daily 1-h access group is typically reduced so that a majority of their daily calories comes from the 1-h HED binge period (12, 18). Reduced chow intakes are likely driven by entrained food anticipatory activity associated with the HED access. However, in the weekly 24-h access model 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 (13). 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 male mice, we have consistently observed no change in body weight gain in either model. It is likely that compensatory reductions in chow intake correct for the total caloric intake over the course of several weeks maintaining body mass. Therefore, these models may not be suitable for monitoring how binge-eating leads to overweight and obesity. In both intermittent access models described herein, only male mice have been utilized. Therefore, the behavioral and metabolic effects using the models in female mice are unknown and may differ from results obtained in male mice.

We have assessed the effects of mouse strain on establishing binge-like eating with intermittent access to a nutritionally complete HED. We found that both C57BL/6 and 129SvEv mice will binge using the methods described herein (13, 18). 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.

An initial 24–48 h exposure to the HED is necessary to remove possible neophobia as a variable in both models as described in Sect. 2.3. This presentation (free-choice, ad libitum access to both chow and the HED) occurs 1 week prior to measuring binge-like feeding. A nutritionally diverse, energy-dense diet more closely mimics human "binge" foods, increasing the likelihood of translation into a clinical setting. However, it is not necessary to use a pelleted macronutrient diet as the palatable food source. In mice, similar binge-like patterns can be obtained with sweetened condensed milk offered in a sipper bottle for 1 h each day (19).

All mice should be bred on and maintained on a standard low-fat rodent chow diet limited to no more than 13% calories from fat (e.g., TD2014, 13% fat, 67% carbohydrate, 2.9 kcal/g, Teklad, Madison, WI). Note that some "breeder" chow diets typically used in breeding cages may be used as maintenance diets in some vivariums and can contain higher than desired fat contents (e.g., PicoLab 5058, 21% calories from fat, LabDiet, St. Louis, MO). Controlled experiments need to be performed to determine if there are differences in the levels of binge-eating on the HED as the macronutrient content of the standard chow changes. Furthermore, the source of the fat for the HED as well as the total fat content should be considered. Both protocols have been tested utilizing both standard chow and energy-dense diets where the primary source of fat calories are derived from soybean oil and hydrogenated vegetable shortening, respectively. It should be noted that other dietary choices such as animal fat (lard)-based, high-fat diets are sometimes utilized as highly palatable food sources in rodent binge models. Lard-based or vegetable/plant fats may result in variations in study results. To control for variations in HED composition, the source of fat in both the chow and HED should be similar. Small but significant differences in food consumption and body weight gain may be seen when altering ratios of macronutrients in the diet (20). How this may affect binge-like eating has not been determined. Thus, detailed comparisons of plant and animal fat-based diets may be necessary to assess palatability and macronutrient preference in specific mouse strains.

Background strain can also influence macronutrient preference and should be considered when selecting an appropriate mouse strain to study (21–24). For example, the obesity-prone C57BL/6 mice will consume more fat when presented with a choice of protein, carbohydrate and fat-rich diets compared to the obesity-resistant C3H/Hej (22) and 129/J (25) inbred strains.

When investigating GEM, it is important to discern the effects of the genetic mutation on preference for specific macronutrients.

If a reduction of binge eating 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. For the daily 1-h free-choice access model, measuring the rate of acquisition of binge-like eating will be informative and likely can be differentiated between GEM strains. For example, in the neuropeptide Y (NPY) knock-out mouse, the rate of acquiring binge-like eating is slower than wild types, but once established the size of the binge is not different between NPY knock-outs and wild-type mice (18). Conversely, mice lacking the cannabinoid receptor-1 have a blunted binge-like eating response when compared to wild types (12).

In those studies examining pharmacological intervention and behavioral testing, the timing of compound administration is important. One can 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 1 h or 2.5 h intake of the HED in the prior binge cycle to account for inherent preferences that might exist for the HED amongst a given cohort of mice. It is necessary to determine the question being addressed: e.g., preventing development of binge eating, reversing binge-like feeding, or reducing intake by blocking initiation or cessation when eating the HED during the test session. As an example, if we are trying to prevent development of binge-like eating, 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.

3. Anticipated Results and Notes

3.1. Daily 1-h, Free-Choice Access

Within the first week, one can expect approximately 40–50% of total daily calories consumed to come from HED intake during the 1-h access period (18). Meanwhile, 24-h chow consumption will be significantly reduced. Expect the HED consumption levels to plateau after approximately 2 weeks in order to establish stable binge intake.

3.2. Weekly 24-h, Free-Choice Access

Maximal binge-eating can be observed in the first cycle of 24-h free-choice access. 129SvEv and C57BL/6 mice in the intermittent binge group consume approximately 0.85 and 1.2 g of HED, respectively, in the initial 2.5 h after reinstatement; this is approximately

33% of their daily caloric intake (measured relative to the day prior to presenting the HED) (Fig. 3). Over 24 h of access to the HED, one can expect intakes of mice in the binge group to be double that of chow only and continuous access controls. In addition, 100% of the calories consumed by mice in the binge group will be from the HED. A significant compensatory reduction in chow consumption in the 24 h following HED access in the binge group can be expected. With the weekly access paradigm, we were able to obtain binge-like eating during the first binge cycle (Fig. 3).

We have not observed in the weekly 24-h access model any evidence of entrained food intake or any change in serum corticosterone levels. In fact, we have found no behavioral or biochemical evidence for stress in either model (13, 18). It should be noted that the presence or absence of a stress component may alter the findings for pharmacological treatments. For example, the literature describes differentiation of non-stress models and those containing a stress component in the differential actions of fluoxetine and topiramate (13, 26). In contrast to rats, no binge-resistant mice have been identified. Indeed, the individual levels of binge-like eating within each cohort of mice evaluated were quite similar. None exhibit any significant preference for chow intake, with all animals preferring the HED once it was presented. In both models, no change in body weight is expected since both exhibit compensatory reductions of standard chow intake when the HED is removed. For daily 1-h access, there is typically no overall change in 24-h kcal intake. With weekly 24-h access, each 24-h kcal intake is cyclical depending on when the HED was presented last. The two models can be distinguished by the increase in total 24-h caloric intake when HED is presented in the binge group.

3.3. Specific Conditions to Consider When Troubleshooting

The following outlines some practical considerations for both intermittent access models that will help investigators to obtain the most reliable and reproducible food intake measurements.

1. If the HED diet is in more of a powder form, feeders can be made from labware items such as 50-mL conical tubes with a hole cut in the side and the top of the conical tube which is then clipped to the side of the cage with a paper clip or similar device. With this presentation of the HED, the powder can be packed into the tube. However, increased spillage can occur and bedding material can easily stick to the powdered HED if wet with saliva.
2. The investigator should consider limiting the number of food pellets offered to each mouse to 5–6 full-sized pellets of each diet during the days that food intake is being monitored. This limits spillage and also facilitates weighing.
3. 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. In addition, there is the issue of palatability and novelty between fresh new pellets and stale older pellets. We cannot rule out the effects of palatability and novelty on consumption. Both chow and HED should be "refreshed" consistently across all groups. There is also an issue with evaporation of moisture from the fresh diet. This can be simply avoided by allowing refrigerated diets to come to room temperature 1 or 2 days prior to use.

4. The composition of standard chow diet that is being offered should be considered if significant HED consumption is not noted. All mice should be bred on, and maintained on, a standard low-fat rodent chow diet limited to no more than 13% of the total calories derived from fat. In addition, 24–48-h initial exposure to the HED should occur 1 week prior to initiating HED limited access using either paradigm.

With the daily 1-h free-choice access schedule, including an initial 24-h exposure 1 week prior to starting the experiment should be considered. With the weekly 24-h free-choice access schedule, body weights should be obtained before mice are separated into experimental groups so that initial body weights are similar. Furthermore, food intake data should be normalized to body weight as mice that have continuous access to the HED will gain more body weight over the course of a single binge cycle. It is also important to note that 1 week between presentations of the HED allows for chow consumption to return to preexposure levels. Chow consumption should be monitored prior to measuring the binge-like eating.

4. Conclusion

Our nutritionally diverse, energy-dense diet more closely mimics human "binge" foods, increasing clinical relevance. Moreover, our weekly intermittent access schedule can be used to determine how more infrequent and "casual" binge-eating behavior could progress to BED.

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Chapter 6

Predicting and Classifying Rats Prone to Overeating Fat

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Abstract

The availability and overconsumption of palatable foods rich in fat likely contribute to the worldwide obesity epidemic. With environmental factors and genetic predisposition each playing important roles in this serious health problem, it is crucial to identify 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 behavioral or physiological factors that are positively associated with and can predict future fat consumption and obesity. The methods for classifying and subgrouping these animals, which are described herein, focus on three early predictive measures, namely, 5-day fat intake, novelty-induced locomotor activity, and fat-induced triglyceride levels, which have all been successfully used to identify outbred rats that are prone (i.e., have higher propensity) or resistant to overeating fat. The early identification based on these three factors 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

During the last several decades, the consumption of palatable foods, particularly those rich in fat, has increased dramatically (1) and may be one contributing factor to the worldwide obesity epidemic (2, 3). Such unhealthy eating patterns may be a result of environmental factors, such as increased availability of high-fat junk food, and also genetic factors that predispose individuals to either overconsume or gain weight on fatty foods (4–7). Clinical evidence has suggested that food preferences and feeding patterns at a young age are associated with and can identify individuals prone to overeating and gaining weight in adulthood (8). It is especially important to identify these "predisposed" individuals

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

Studies with rodent models have been particularly informative for understanding the inherent driving forces of unusually high consummatory behavior. In particular, outbred rat strains such as Sprague-Dawley (SD) are especially appropriate for understanding mechanisms of human feeding behavior, as like humans they are genetically heterogeneous and show a wide range of behaviors, allowing them to be subgrouped based on inherent differences in their behavioral patterns. Using outbred SD rats as a model, recent 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. The measures that are predictive of such behavior include early consumption patterns during exposure to a high-fat diet (9–12), novelty-induced locomotor activity prior to diet exposure (13), and circulating triglyceride (TG) levels induced by a fat-rich meal (14). 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, methods and procedures for classifying animals based on early predictor measures, such as consummatory patterns, locomotor activity, and fat-induced TG levels, into those that are prone or have an increased propensity to overeat fat and those that are resistant or have a reduced propensity for overeating fat.

2. Materials and Procedures

2.1. Animals

To examine patterns of fat consumption, a variety of strains including outbred and selectively bred rodents as well as genetically altered and inbred mouse lines have generally been used (9, 12, 15–18). For translational purposes, however, the outbred animal for several reasons seems to be the most appropriate model of human fat overconsumption, as illustrated in recent studies of feeding behavior in SD rats (9, 12, 13). These animals, usually purchased from Charles River or Taconic Laboratories, are easy to handle and do not show any abnormal behavioral traits, such as excessive anxiety or aggression, as previously reported in other outbred strains (19, 20). Also,

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)	*1,000*
Energy density (kcal/g)	5.02
% Macronutrient (kcal)	
Fat	50
Carbohydrate	25
Protein	25

Ingredients expressed by weight (g)
Reprinted with permission from (13)

SD rats are genetically heterogeneous and thus more accurately represent the individual genetic variability seen in humans. Most importantly, these animals naturally overconsume a high-fat diet when initially exposed to it, while exhibiting considerable variability in their intake, thus allowing them to be rapidly and easily classified as high or low fat consumers for further analyses (9, 12, 13).

2.2. Diet Recipe

To examine natural patterns of fat consumption, different types of high-fat diets have been employed in various laboratories, including a diet rich in animal fat, such as lard (9), or vegetable fat, such as Crisco All-Vegetable Shortening® (21), as well as a common palatable junk food diet, such as one including Oreos® (22). From our past experience using lard as the main fat source, we find that the optimal amount of fat in the diet that accurately predicts future fat intake is 30–60% (9). With this information, subsequent studies in our laboratory have utilized a diet composed of 50% fat containing a total of 5.02 kcal/g of diet (Table 1). More specifically, 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, 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 routinely tested in an open field. The historically used apparatus for this is a 182.9 cm by 182.9 cm box constructed from plywood painted black with the bottom composed of 16 equal size squares. However, open field measurements can now be recorded using the more advanced computerized open field activity chamber, consisting of a Plexiglas box (43.2 × 43.2 cm) with a white floor, opaque walls, and infrared photocells which detect both vertical and horizontal movement (MED Associates, St. Albans, VT, USA). The latter approach provides a more precise analysis of locomotor behavior. With the use of other parameters such as vertical counts, distance traveled, average velocity, and peripheral versus center movement, the computerized open field activity chamber can more accurately detect additional behaviors related to exploration and anxiety.

2.4. Lipid Determinations

For determination of circulating lipid levels in rat serum samples, an enzyme kit from Sigma-Aldrich (St. Louis, MO) is commonly used. The Serum Triglyceride Determination Kit utilizes a 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 wave length of 540 nm.

2.5. Predicting Fat Intake Based on Initial Consumption Patterns

Several important studies examining patterns of fat consumption have demonstrated that initial fat preference and weight gain on a high-fat diet have a strong relationship to long-term consumption of fat, body weight gain, and body fat accrual (23–25). Building on this naturally occurring phenomenon, subsequent publications have successfully utilized measures of early fat preference and initial weight gain on this diet to identify animals with a greater propensity to overconsume a high-fat diet over the long term as well as gain more weight and accumulate heavier fat deposits (9, 10, 16, 26). These studies performed in rats have demonstrated that animals preferring fat over other macronutrients, such as carbohydrates or proteins, are more likely to eat greater amounts and gain weight on this diet. Likewise, rats 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 order to have accurate measures of feeding behavior, factors affecting the animals' stress levels should be kept to a minimum, and measurements should be consistent from day to day. Animals should be maintained on a 12 h light cycle, preferably with the dark or active period occurring during the evening when there are few people in the laboratory. They should be acclimated to standard housing conditions, handled daily for at least 1 week prior to diet exposure, and acclimated to the diets themselves prior to ad libitum feeding in order to avoid effects of neophobia. To prevent the diet from spoiling or becoming less palatable, it should be prepared fresh every week and kept refrigerated until served, and the animals should receive fresh diet every 2–3 days. Measurements of food intake should be recorded daily at the same time each day, preferably immediately after the end of the dark cycle.

In more recently published articles, a modified version of the fat preference or weight gain model has been used to more easily and accurately classify rats based solely on their initial consumption of a fat-rich diet (12, 13). 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 the 1-week acclimation period to laboratory conditions, chow intake should be monitored for 3 days and then the high-fat diet introduced. This introduction to the new diet should occur over 3 consecutive days, with a 15 kcal high-fat meal given to the animals along with their daily chow. By the end of the third day of exposure, all subjects should have learned to consume the entire high-fat meal by consuming it at least once. After this acclimation, chow is removed, and the animals are allowed to consume the high-fat diet ad libitum for 5 days, with measures of fat intake and body weight taken 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 rather than chow, and the bottom third forming the Control rats, which tend to consume equal calories from both diets. Using this classification procedure, HFC rats have been shown to be more prone to consuming excessive fat over the long term, both during chronic high-fat diet access as well as during reexposure after a 2-week withdrawal from this diet (12). Further, when maintained on the diet chronically, these HFC rats ultimately gain more weight and develop larger fat pads (12). This model is also validated by other studies showing similar measures of energy intake and weight gain to be accurate indicators of long-term patterns of fat consumption and mild obesity (9, 11, 16, 26, 27).

2.6. Predicting Fat Intake Based on Activity Measures

Locomotor activity has been closely associated with food seeking and is particularly high immediately prior to scheduled palatable meals (28). Several studies have related high activity levels to the consumption of palatable foods and other reinforcing substances

(13, 29, 30), suggesting that locomotor activity may serve as a good indicator of future patterns of fat intake. The procedures commonly employed to classify animals by their locomotor activity are described here.

Locomotor activity is usually tested within specialized open field chambers (described above), in which either an observer blind to the study or a computerized program records behaviors related to horizontal and vertical motion. Testing should be carried out during the animals' waking time (i.e., the dark cycle), as this is when their baseline activity is highest (31). 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 pure locomotor activity or novelty-induced locomotor activity, respectively. Several published articles have suggested that novelty-induced activity is a more reliable predictor of subsequent intake of reinforcing substances, including fat (13, 32, 33). Another important factor to consider for novelty-induced locomotor activity is the duration of the test. Optimally, activity measures within the first 5–15 min of exposure to the arena are examined, a time when the novelty of the environment appears to have the greatest impact (31, 34). The specific measurements frequently used to represent locomotor activity are either line crossings when scored manually or ambulatory distance in the case of computerized programs. For novelty seeking, an additional measure of rearing behavior (manual) or vertical counts (computerized) can be recorded.

Using these measures, SD rats have been successfully characterized as HFC and Controls based on their levels of novelty-induced locomotor activity (13). 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. Between tests, the apparatus needs to be thoroughly cleaned with 70% EtOH and allowed to dry. After the activity testing, animals can be given the high-fat diet for classification into HFC and Controls as described above. These initial measurements of line crossings have been found to be greater in the 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. In recent studies, this same measure of novelty-induced activity has also been found to strongly and positively correlate with ethanol consumption, suggesting that this behavioral trait may be important for identifying animals prone to over consuming drugs of abuse as well as fat (13, 35).

2.7. Predicting Fat Intake Based on Meal-Induced TG Levels

Circulating lipid levels such as serum TG, which are elevated by the consumption of a high-fat diet (14, 36), are a good indicator of metabolic activity and efficiency. In the body, these lipid molecules can either be stored in adipose tissue for future use or broken down

for immediate energy in the form of fatty acids that are known to produce a feeling of satiety (37). When they accumulate in serum, however, they may signify inefficient metabolism and a disruption in energy homeostasis (38). Based on this information, it is likely that animals showing exaggerated exaggerated TG levels after a fatty meal may find this diet less satiating and therefore go on to overconsume the diet. This principle has recently 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 (39). Based on this idea that exaggerated fat-induced TG levels may be an indicator of metabolic inefficiency and reduced feeding satiety, it has been suggested that these lipid levels may serve as a valid predictor of future fat overconsumption as well as obesity.

According to a recent publication, measurements of fat meal-induced TG levels can be used to predict increased caloric intake and dietary obesity in rats (14). In order to use TG levels to identify these 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% high-fat diet (see Table 1), given for 30 min each day over 3–4 days until all subjects have consumed 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 by exposing them to a similar small 15 kcal meal of high-fat diet once daily over 3 nonconsecutive days and collecting their tail vein blood 1 h after each exposure for measurements of serum TG levels using the Serum Triglyceride kit described above. During these tests, 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. On the basis of their 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 fat-eating (Control) counterparts, with the middle group omitted.

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 as a result to 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 three test meals of a high-fat diet, which is a metabolic rather than behavioral parameter. Although 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 whether they in fact represent the same subgroups of animals. However, with TG levels

also increased in the HFC rats predicted 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 can predict future weight gain (40) and that lipid-lowering drugs reduce food intake in obesity-prone rats (41).

2.8. Experimental Variables

When using 5-day fat consumption to identify HFC rats, there are two main variables to monitor, namely, diet consistency and day-to-day consumption patterns. Since this prediction is based on the animals' consumption of this diet over a short 5-day period, it is important to keep all ingredients and the texture of the diet identical for each animal. When a 50% high-fat diet is used, this diet should have a completely smooth texture, while other lower fat diets may have a more powdered consistency. The smooth texture allows the diet to be made into a small 15 kcal ball and placed right in the rat cage for training purposes, and it is also important for keeping the diet in a metal bowl or glass jar during periods of ad libitum feeding, rather than having it spilled by the animals. Unaccounted-for spillage will surely lead to incorrect rank ordering of animals and thus unreliable results. Additionally, it is important to keep the fat concentration at 50% since animals seem to 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 (9, 26), more recent studies have found a 50% fat diet, which is closer to the human diet, to yield reliable and replicable results (12, 13). Aside from the fat consistency, it is important to carefully examine and correlate the rats' fat consumption each day. The 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, 13). The lack of a stable consumption pattern could signify a problem with either the animal's health or the diet consistency and therefore should be investigated prior to classifying animals as prone or resistant.

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 the animals' stress level. Standardizing the measurements is especially important for manual recordings, as some behaviors may be hard to capture and track with the human eye. For example, line crossing should be counted only if the animal has both paws and torso in a new square, and rearing behavior should be counted if the animal is fully on his hind paws for a minimum of 2 seconds. If the observer has any doubts about these measures, use of a camcorder may be advisable to videotape the test session and then analyze the behavior later based on the recording. Also,

as animals may be anxious upon exposure to a new environment, it is important to note any particular anxiety measures in the open field. One measure of such behavior is the amount of time spent in the periphery versus the middle of the open field, with greater peripheral time representing an anxious state. Since anxiety can mask increases in novelty-induced locomotor behavior, it is important that the animals are well handled and gently placed into the open field apparatus for testing.

In order to use circulating TG levels as a predictor of fat consumption, variables involving high-fat meal consumption must be well controlled. It is important that the animal consumes 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 stays consistent for each animal, there are three steps one could take. First, the animals must be trained to expect the meal at the same time each day and therefore wait to consume this palatable fatty food. Second, in order to increase the motivation to consume the meal quickly, the rats should be food deprived for several hours before they are given access to the high-fat meal. Lastly, each cage should be thoroughly checked for any remaining high-fat diet prior to collection of tail vein blood, as animals that do not consume their daily meal cannot be used for the analysis. By following these three steps, elevations in fat-induced TG levels should accurately and reliably identify animals prone to fat overeating.

3. Anticipated Results and Notes

3.1. Anticipated Results

Results from a typical experiment using two of the discussed predictors of fat consumption are shown in Tables 2 and 3. From the first table (Table 2), it is clear that animals characterized as HFC show a significant increase in fat consumption within the first 5 days and that this persists for an additional 2-week period and, although not shown in this table, is still evident after reexposure 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 (9, 26).

The data presented in Table 3 additionally describe the typical characteristics of animal subgroups predicted by their fat-induced TG levels. These data demonstrate that animals showing a higher TG response to a fat meal go on to consume more chow in a subsequent test meal, and after 4 weeks on a high-fat diet, they have greater body weight, consume more calories each day, have larger fat deposits and even 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

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 per day)		
Chow (before high-fat diet)	91 ± 6	89 ± 4
High-fat diet (5 days)	95 ± 4	121 ± 10*#
High-fat diet (2 weeks)	94 ± 5	118 ± 10*
Body weight (g)		
Chow (before high-fat diet)	302 ± 10	300 ± 8
High-fat diet (5 days)	371 ± 9	392 ± 12#
High-fat diet (2 weeks)	427 ± 15	475 ± 19*#

Data are mean ± S.E.M. *$p < 0.05$ vs. control; #$p < 0.05$ vs. chow
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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 ± 12.0*
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

*$p < 0.05$ vs. Low-TG responders
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predict animals prone to overeating fat as well as demonstrating the beginning of more chronic metabolic disturbances.

3.2. Troubleshooting

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

1. For the early consumption predictor model, it is very important to properly adapt rats to the high-fat diet with a minimum

of three daily exposures. This is to prevent the occurrence of neophobia when the new diet is introduced 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 their home cage and by a failure to consume the entire 15 kcal meal during the training days, should be omitted from the study.

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 rats from eating their daily meals, these measurements should be performed during the light cycle, when animals are resting. Since animals may vary in their individual patterns of feeding behavior, taking a measurement during either a time of high consumption or low consumption may provide unreliable results and therefore reduce the reliability of this model.
3. For the novelty-induced locomotor activity measure to predict fat intake, a standardized measurement system for recording activity is essential. This is especially important if a computerized system is not available and line crossings are manually recorded. The observer must have a uniform way to measure line crossing and other behaviors such as vertical counts. Sometimes, if several measurements are taken, it is best that a single researcher be assigned to this task as the animals may vary in their behaviors when exposed to a new person.
4. Since rats are naturally anxious animals, the room conditions during testing should be well controlled. For example, during novelty-induced locomotor behavior, it is also 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 could be achieved either by having a constant low level background noise or complete silence in the room with a red light continuously on in the room. Once the animal is placed in the chamber, the observer should always stand at an angle where the animal cannot see him or her, and if using a computerized program, the person should leave the room immediately after placing the animal into the field.
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. This is due to the fact that TG levels show circadian fluctuations which 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 either be cleared from plasma or broken down with prolonged periods of time. To capture the immediate TG response to fat, blood should be collected within 1 h of high-fat meal exposure.

4. Conclusion

In summary, the procedures described above for using initial consumption patterns, locomotor behavior, and circulating lipid levels have been successfully and reliably used to classify animals into those prone and resistant to overeating a palatable high-fat diet. These models are well supported by clinical literature in which early patterns of food consumption, hyperactivity, or dyslipidemia have been associated with a greater probability of consuming excess amounts of fat or developing obesity in adulthood (8, 42, 43). In animal studies, these predictive measures have been instrumental to our understanding of the neurochemical mechanism underlying excessive fat intake and also obesity. Specifically, by thoroughly examining animals prone to overconsuming fat, several recent studies have shown that these animals also exhibit elevated levels of orexigenic hypothalamic peptides, suggesting that disturbances in these central systems may contribute to the overeating phenotype of these animals (9, 12–14). With these unique patterns in behavior and possibly 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 7

Modeling Binge Eating in Nonhuman Primates

Richard W. Foltin

Abstract

Human and nonhuman primates are opportunistic feeders and are prone to both obesity and binge eating when food is abundant. This chapter describes procedures for studying binge eating using a foraging model that engenders large meals in nonhuman primates. Baboons have access to food 24 h each day, but they have to complete a two-phase operant procedure in order to obtain it. Responding on one lever during a 30-min "seeking" phase is required before they can start a "taking" phase, where responding on another lever will lead to food delivery, i.e., a meal. Three days per week, baboons received candy during the first meal, and then food pellet meals were available the remainder of the day. Initially we compared a chocolate sugar-coated candy (M&M's®) to a jelly sugar-coated candy (Skittles®). All baboons ate as much candy in the single candy meal as they did food pellets throughout the remainder of the day. Five baboons developed an aberrant behavior when they had access to M&M's® in that they began to suck the candy coating off and throw away the chocolate center. This behavior rarely occurred with Skittles®, and it never occurred with food pellets.

This procedure can also be used to determine the effects of pharmacological agents on binge eating. For example, we examined the effects of the serotonergic drug dexfenfluramine and the dopaminergic drug D-amphetamine on food and candy consumption by determining complete dose–response functions for each drug when given on days prior to when Skittles® were available for the first meal and on days when only food pellets were available. The two drugs had different behavioral profiles of action. Both drugs decreased candy and food pellet consumption, but had varying effects on the latency to the first meal. Amphetamine increased the latency to the first candy and food meal, while dexfenfluramine only increased the latency to the food pellet meal. Thus, periodic access to a highly palatable candy food engendered large amounts of candy consumption in a single meal by all baboons, providing a behavioral baseline for assessing the effects of manipulations on binge eating, as well as regular food consumption, in the same animals.

Key words: Nonhuman primates, Baboon, Binge eating, Foraging, Palatable foods, Amphetamine, Dexfenfluramine

1. Introduction

A vast amount of research on mechanisms underlying both normal and abnormal eating patterns has used laboratory rodents, primarily rats. Such studies have provided the basis for understanding neural

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and hormonal factors underlying appetitive behavior. Far less work has been conducted with nonhuman primates, and recently the work has focused on behavioral factors affecting eating behavior. All studies conducted with laboratory animals need to meet strict criteria about the potential utility of the work for understanding behavior of animals and the implications of the work for understanding human behavior. Careful oversight by the local Institutional Animal Care and Use Committees (IACUC) assures that all studies are carefully conducted and attention is paid to the humane treatment of the laboratory animals during the study. Research with nonhuman primates must meet even more stringent animal-welfare criteria and reporting requirements than work with rodents in the United States. The standardization of both animal-welfare regulations and oversight by local institutions has improved both the experimental experience of the nonhuman primate and the consistency of data obtained in research. All of the studies conducted by the author and described below were reviewed by the local IACUC and met all United States Federal and New York State local animal welfare requirements.

Nonhuman primates are opportunistic feeders and are prone to obesity when food is abundant. The popular media is full of images of obese macaques (a Google® search for obese monkey pictures yielded 85,000 hits in November of 2011; though many had no monkeys in them) and troops of macaques and baboons that live near humans are known to steal food from humans and even appear to have "coordinated" attacks. Recently in South Africa I witnessed a troop of baboons raid a safari lodge and try to carry off all the food they could hold, including what had been a delicious ham roast. Also in South Africa, the baboons that live on the cape near Cape Town have become quite aggressive in taking food and other items from the many tourists. Many baboons have learned to open car doors in search of food and other mischief. The environment on the cape is not rich in plants, as fierce winds and poor soil limit growth. Thus, foraging for food among humans is a better way of finding food than foraging in the natural environment.

The popular images of obese monkeys eating refined foods designed for human consumption find scientific support in a report by Jeanne Altmann and colleagues (1). They compared body morphology between a troop of baboons living in their natural ecology to a troop of baboons living near a human garbage dump. Female baboons that foraged in a garbage dump weighed approximately 17 kg compared to approximately 11.5 kg for wild-foraging females. The differences were smaller for males; those that foraged in a garbage dump weighed approximately 30.5 kg compared to approximately 26 kg for wild-foraging males. Baboons that foraged in the wild were necessarily more active than those who foraged in the garbage dump. Clearly, ready access to calorically dense food supports the development of obesity.

The over consumption of calorically dense food also occurs in the natural ecology. Knott recorded daily energy intake in wild orangutans in Indonesia across one year and compared intake during a brief period when fruit trees were bearing fruit to when less fruit was available (2). Caloric intake of males more than doubled when fruit was in season, from approximately 3,800 to 8,400 kcal per day, and caloric intake of females *quadrupled* when fruit was in season, from 1,800 to 7,400 kcal per day. In the natural ecology, over consumption provides for the development of fat stores to be utilized when the quality and quantity of food is less.

This chapter focuses on modeling a type of aberrant eating behavior known as binge eating. In humans, the occasional consumption of a large meal occurs in normal populations, but it can also characterize several eating disorders. For some, the consumption of large amounts of preferred foods becomes a repeated pattern (3) known as a "binge." Consumption of a binge can be followed by compensatory behavior such as vomiting or exercise as in bulimia nervosa, or not, as in binge-eating disorder (4), which is defined as the recurrent consumption of large amounts of food in a brief time frame, and psychologically characterized by feelings of loss of control over eating, and feeling distressed after overeating (4). Corwin and colleagues (also see Chap. 4) have developed a model in rats of excessive eating of a single food based on limiting access to a preferred food, but not limiting access to chow (5). Rats given access to fat for 2 h per day (2 h before the dark cycle) only 3 days per week develop a binge-type eating pattern of fat intake during those 2 h (6–8). Human children with restricted access to preferred foods also overeat when preferred foods are available (9). Thus, restricted access to a preferred food leads to a large intake of that food in a short time frame. We developed a model of binge eating in nonhuman primates by providing highly palatable foods on an intermittent schedule, similar to that described by Corwin et al. (7).

2. Materials and Procedures

2.1. Animals

The current studies used male and female baboons (*Papio cynocephalus anubis*), the largest nonhuman primate commonly used in research. Animals were singly housed with an apparatus containing response levers and food dispensers attached to each cage. All animals had visual access to other animals, but were not permitted to touch each other. Smaller animals could be used, as well as group-housed animals. If animals are group housed, they will need to be trained to enter a chamber in order to work for food at set times during the day. The ability to work and receive food independently is essential in order to accurately measure food consumption and motivation to acquire food.

2.2. Schedule of Intermittent Access to Palatable Foods

We modeled natural foraging behavior, i.e., time spent moving from one food source to another, time spent acquiring food within a food source, and the comparably small amount of time actually eating (10), based on a model of foraging behavior developed by George Collier and colleagues (11, 12). Operant behavior of four male and four female baboons was studied under a schedule of reinforcement that simulated food seeking and food taking. In the natural ecology, food seeking occurs in the context of environmental cues that are associated with food (i.e., certain trees at certain times of year; for example see (13)). To simulate this aspect of food seeking, responding during the seeking component was reinforced by stimuli paired with food using a second-order schedule, i.e., light flashes that also occurred with reward delivery (14). After completion of a 30 min foraging phase, baboons then received one reward after every ten responses: the lights associated with the reward also flashed at that time. Baboons could eat as much as they wanted by continuing to pull the lever ten times. A meal terminated after the baboon did not respond for 10 min. Thus, baboons controlled when they started foraging and how much they ate in a single eating bout or meal. Foraging for a meal was possible 23.5 h per day: 0900 hours one day until 0830 hours the next day. The 30-min interval without food was used to clean the cages and test all equipment.

In our first study (15), we limited access to preferred highly palatable food to a single meal in the morning, 3 days per week, for 8 or 9 weeks. Baboons were not food deprived and had access to food pellets for the remainder of the day. Thus the baboons determined the timing and size of each candy and food pellet meal, but candy was only available as a single meal on Monday, Wednesday, and Friday mornings. The effect of food type on "binge eating" was evaluated by comparing consumption of a chocolate sugar-coated candy (M&M's®; chosen because it contains both fat and sugar (38% of calories derived from fat)), and a jelly sugar-coated candy (Skittles®; 9% of calories derived from fat).

During the initial 3.5 weeks of M&M's® candy access, some baboons began to waste a small number of candies each day. By "wasting" we mean that they did not eat the entire candy. When it became clear that this was a pattern for some animals, not random wasting of a few M&M's®, the number of uneaten candies were counted each day after the candy meal ended. Beginning in week 6, a natural dichotomy became obvious such that three baboons wasted few M&M's® (<25; "Eaters") and five baboons wasted a large number of M&M's® (>100; "Wasters"). Three of the five "Wasters" were males. There was one male and two female "Eaters."

As shown in the left panel of Fig. 1, M&M's® deliveries nearly doubled between the first and second day of candy access in the baboons in the Wasters group (defined by behavior after 5 weeks of M&M's® access) then remained stable for the first 4 weeks (15).

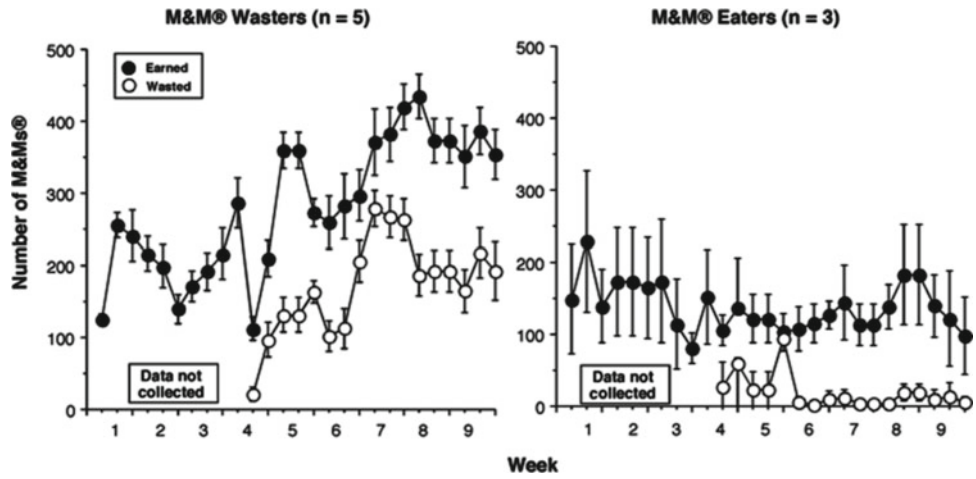

Fig. 1. Mean total daily number of M&M's® earned and wasted each candy session for the five animals who wasted M&Ms® (*left panel*) and the three animals who ate nearly all of the M&M's® earned (*right panel*) as a function of week of access to candy. Although small numbers of M&M's® were wasted during the first 3.5 weeks of M&M's® access, the number wasted were not accurately counted until week 4. Although shown sequentially, candy was available on Mondays, Wednesdays, and Fridays. *Error bars* on the data points represent ±1 SEM.

The Wasters increased the number of candies earned during week 5, but this increase was accompanied by an increase in the number of M&M's® thrown away. M&M's® deliveries (number earned) and M&M's® wasted remained stable across all 9 weeks of access to M&M's® in the group that wasted few candies (Fig. 1, right panel). We had originally planned for 8 weeks of M&M's® access, but because there was a decrease in the number of M&M's® earned during week 8 in the Wasters group, we extended the period of M&M's® access for another week for a total of 9 weeks. The five baboons that wasted M&M's® did so by throwing some untouched candy on the floor, but most were initially placed in the mouth, then spat out, i.e., baboons sucked off the candy coating and threw the chocolate part away.

When Skittles® replaced M&M's®, the baboons that were in the Wasters group (Fig. 2, left panel) and the Eaters group for M&M's® (right panel) nearly *doubled* Skittles® deliveries between the first and third or fourth day of candy access, but then Skittles® deliveries remained stable for the remaining 8 weeks of candy access (15) for both groups. There was no significant difference between the M&M's® Wasters and Eaters in the number of Skittles® earned or wasted. All baboons wasted few Skittles®. No baboon wasted food pellets.

In summary, when given periodic opportunities to "forage" for a highly-palatable food item, free-feeding nonhuman primates worked for and consumed a large quantity of the preferred item. When candy was available, the number of candies consumed in the first meal of the day was similar to or larger than the total number

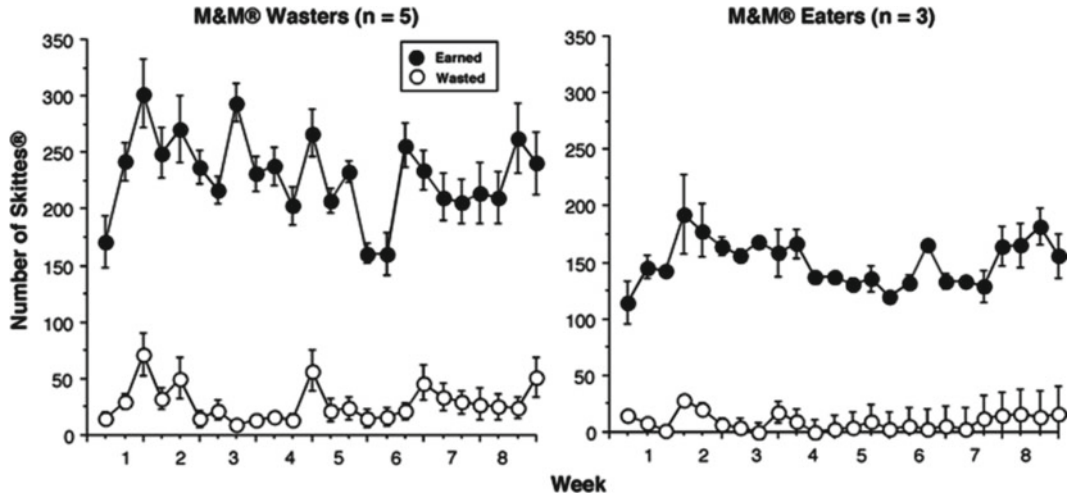

Fig. 2. Mean total daily number of Skittles® earned and wasted each candy session for the five animals who wasted M&M's® (*left panel*) and the three animals who ate nearly all of the M&M's® earned (*right panel*) as a function of week of access to candy. Although shown sequentially, candy was available on Mondays, Wednesdays, and Fridays. *Error bars* on the data points represent ±1 SEM.

of food pellets consumed over the rest of the day. Due to the greater caloric content of candy, baboons also consumed more calories on days that candy was available. Candy intake increased during the first week of candy access, but then remained stable across the period of access to candy.

Candy meals, based on a priori definition, lasted longer than pellet meals at the same time of day. Individuals with binge-eating disorder increase their caloric intake generally by having a *longer* meal (16, 17). All eight baboons ate one less food pellet meal on the days candy was available, thereby reducing total food pellet intake on the days when candy was available. Clinical data indicate that when humans eat a large binge meal, that meal substitutes for a small meal rather than being an additional meal that day (18–20). These similarities between human and nonhuman primates suggest that the present procedures may provide a model for large meals.

Five (three males and two females) of the eight baboons developed the unexpected behavior of earning then not fully eating M&M's®, i.e., they did not eat the chocolate center. This wasting pattern generally occurred toward the end of the M&M's® meal. Minimal tasting and wasting behavior occurred when baboons had access to Skittles®. This may have been due to the fact that Skittles® have five flavors while M&M's® have only one flavor (six colors). Perhaps the wasting behavior, which only occurred in the laboratory when sugar-coated M&M's® were available, reflects normal behavior observed when baboons are in a dense patch of preferred food. Of course, Skittles® are also coated in sugar, but they have a fruit-flavored, not chocolate-flavored interior.

The large candy meals in the current procedure parallel the consumption of excessive amounts of food within a single meal that is a defining characteristic of disordered eating in patients with bulimia nervosa and binge-eating disorder.

2.3. Effects of Pharmacological Manipulations on Binge Eating

The current procedure provides a behavioral baseline for evaluating potential pharmacological interventions (e.g., fluoxetine (21)) for these disorders. The specificity of an intervention on eating of a large meal of preferred items compared to "normal" eating can be evaluated by comparing the effects of drugs on days when a preferred food is available, and eaten during a large meal, to days when only food pellets are available, and eaten in multiple smaller meals. Drug effects are determined by administering a dose of drug prior to the first meal of the day, which in our case was available when the computers were restarted at 0900 hours.

We initially looked at a serotonergic and a dopaminergic drug based on evidence that these two neurotransmitters affect different aspects of feeding behavior. An increase in serotonin, as caused by dexfenfluramine (22), has been hypothesized to be vital for the development of satiation (see (23) for a review). An increase in dopamine, as caused by amphetamine (24), has been hypothesized to decrease hunger. Four doses of each drug plus placebo were tested, with each dose–response function requiring approximately 3 weeks for determination: placebo was tested on 25% of the test days to control for conditioned responses. At each dose–response determination, half of the baboons were tested with the smaller doses first and half were tested with the larger doses first. Dose order was systematically varied within and across baboons. Doses were given intramuscularly at 0900 hours when the computers were restarted for the day. At that time, completion of the first 30 min seeking interval resulted in the initiation of the first meal of the day that was either a Skittles® candy meal or a food pellet meal. Latency to the first candy or food delivery provided a measure of food seeking and was defined as the number of minutes between 0900 hours and the first food or candy delivery; the minimal interval was 30 min, including the duration of the seeking interval. Separate dose–response functions were determined when candy was available as the first meal of the day followed by food pellets meals and when food pellet meals were only available.

Dexfenfluramine did not affect the latency to the candy meal, but produced dose-dependent decreases in candy intake during the candy meal (Fig. 3; (25)). By contrast, dexfenfluramine dose-dependently increased the latency to the food pellet meal and decreased the number pellets consumed in the first food pellet meal. Amphetamine dose-dependently increased the latency to the food pellet and candy meals and decreased the number pellets and candies consumed in the first meal (26).

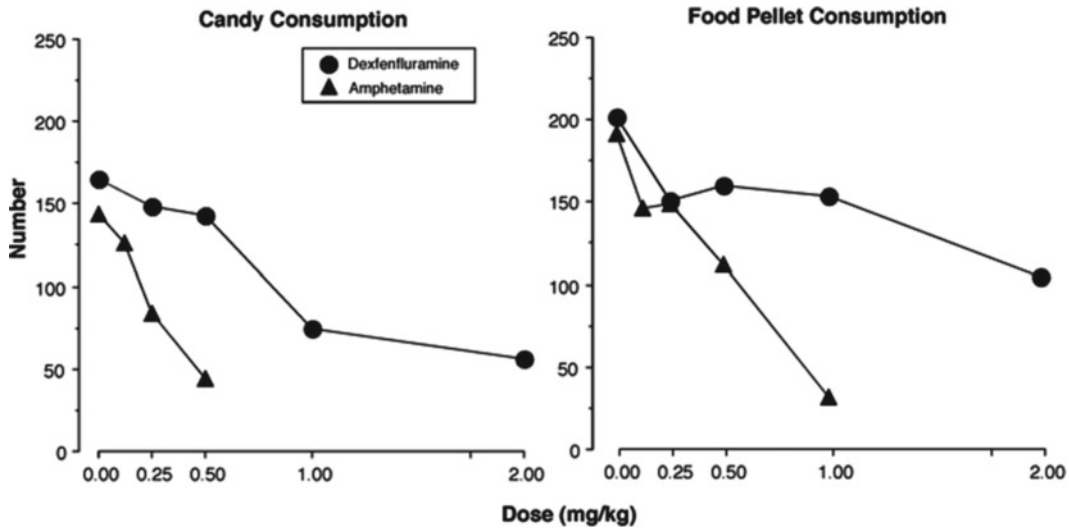

Fig. 3. Mean number of Skittles® consumed during the candy meal (*left panel*) and mean total number of food pellets (*right panel*) consumed over the entire session as a function of dose of D-amphetamine and dexfenfluramine ($N=8$: four female and four male baboons).

The absence of a significant effect of dexfenfluramine on candy seeking, coupled with a decrease in candy taking, supports the concept that dexfenfluramine, presumably by increasing serotonin, increases satiety. The data obtained with food pellets are a bit more difficult to align with the satiation hypothesis. Perhaps dexfenfluramine increased the satiety value of the food already consumed, thereby increasing the latency to the first food pellet meal. This effect was not seen on candy days, as animals were more motivated to respond to candy than to food. This assumes baboons learned the sequence of candy on Mondays, Wednesdays, and Fridays. Regardless, this dissociation between candy and food pellet seeking and taking was *not* observed for amphetamine, arguing that different behavioral mechanisms were involved for the two drugs. Thus, the behavioral baseline of 3 days access to a single candy meal before food pellet meals and 4 days access to only food pellet meals allowed for the investigation of the behavioral specificity of pharmacological agents.

The number of rewards earned during the seeking phase of the candy meal and food pellet meals was not affected by the drugs, indicating that responding during the 30-min intervals was not a sensitive measure of commodity seeking. This suggests that a simpler approach to providing food or candy meals could be used to provide a behavioral baseline. For example, the second taking component could be used alone.

3. Notes

One may be tempted to simplify the approach by providing monkeys free-access, i.e., a generous amount of the food in the home cage, to a supply of preferred food on an intermittent basis and determine intake by weighing the food before and after a limited period of access. When given free access to food, monkeys are wasteful in that they will pick and eat little bits of food and throw other bits away, not unlike the tasting and wasting described above. By adding a response cost, even a minimal one, e.g., ten lever pulls that take 2–3 S to accomplish, monkeys are generally less wasteful. The addition of the operant requirement allows for better measurement of intake (see (27) for an excellent history of the development of operant techniques to study feeding behavior). Although not presented, we collected data on rate of lever pulling and have examined the pattern of cumulative intake within a meal.

The type of food also influences how easy it is to keep good records of eating behavior. We chose M&M's® and Skittles® candies as they could easily be dispensed using the same devices that we use for standard 1 g food pellets (the feeder plates required that the holes that contain the food item prior to delivery be made slightly larger). Also, the use of hard candies makes delivery of the palatable item easier than delivery of a softer palatable food. Manufacturers do make palatable food pellets by adding flavorants, but the basic "feel" of the food pellet remains that of a compressed grain-based product. Thus, hard-cased candies varied from the standard 1 g food pellet in many ways.

4. Conclusion

Clearly the excessive consumption of candy in our model of binge eating is motivated by the highly palatable taste of sweet, high-sugar candies in nonhuman primates and most likely, based on data in laboratory rodents, dependent upon the limited-access schedule. The model does not address the psychological issues reported by patients with eating disorders. We are not privy to the emotional state of our animals, so we must rely on behavioral similarities in developing our models.

Social factors have been shown to affect the response of nonhuman primates to palatable food. For example, in our experience, subordinate animals are less likely to try a new food until after they have seen a more dominant animal eat the food. Arce et al. (28) compared consumption of a highly palatable food between subordinate and dominant ovariectomized female rhesus monkeys. Submissive

animals ate more palatable food than dominant animals. This finding parallels the work of Morgan et al. (29) showing that submissive cynomolgus monkeys self-administered more intravenous cocaine than dominant monkeys. Recently, we examined the effect of social status (social rank was assigned by three independent observers) on Skittles® consumption in a group of eight male baboons. Confirming the results of Arce et al. (28) with female monkeys, the four submissive animals consumed on average 18% more candy than the four dominant baboons. Thus, while our model of excessive eating behavior is based on palatability and pattern of food access, actual levels of binge eating are influenced by social factors as well, further supporting the validity of the model.

In conclusion, providing baboons limited access to a preferred, highly palatable food item for a single "meal" 3 days per week engendered significant consumption of that item, with animals consuming as many calories during the single candy meal as they consumed over the rest of the day. On days of candy availability baboons' energy intake was mostly derived from sugar. Providing baboons with candy-coated fruit-flavored jellies (Skittles®) led them to respond to and eat nearly all the candies earned. By contrast, when baboons had access to candy-coated chocolate (M&M's®) during the single candy meal, five of eight baboons developed an aberrant behavior pattern where they licked the sugar coating off the chocolate and tossed the chocolate away, i.e., tasting and wasting behavior. We hypothesize that both the binge consumption of Skittles® and the wasting behavior associated with M&Ms® model disordered human eating behaviors.

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Chapter 8

Psychosocial Stress and Diet History Promote Emotional Feeding in Female Rhesus Monkeys

Vasiliki Michopoulos, Carla Moore, 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 that is high in fat and sugar (HFSD) is added to the LCD environment, subordinate females become significantly hyperphagic and exhibit significant increases in fat mass within a 2-week period. These studies also suggest a significant effect of diet history whereby subordinate animals previously exposed to the HFSD continue to be hyperphagic when returned to a LCD-only condition. Future studies are warranted to explore the long-term 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,

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34% of adults in the United States were classified as overweight (2). While obesity can be explained in biological terms as the 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 towards 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 (54). 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 (55), show diminished glucocorticoid negative feedback as assessed by a dexamethasone suppression test (Fig. 1b), and exhibit diminished cortisol reactivity in response to ACTH administration (Fig. 1c) (56). Thus, the psychogenic,

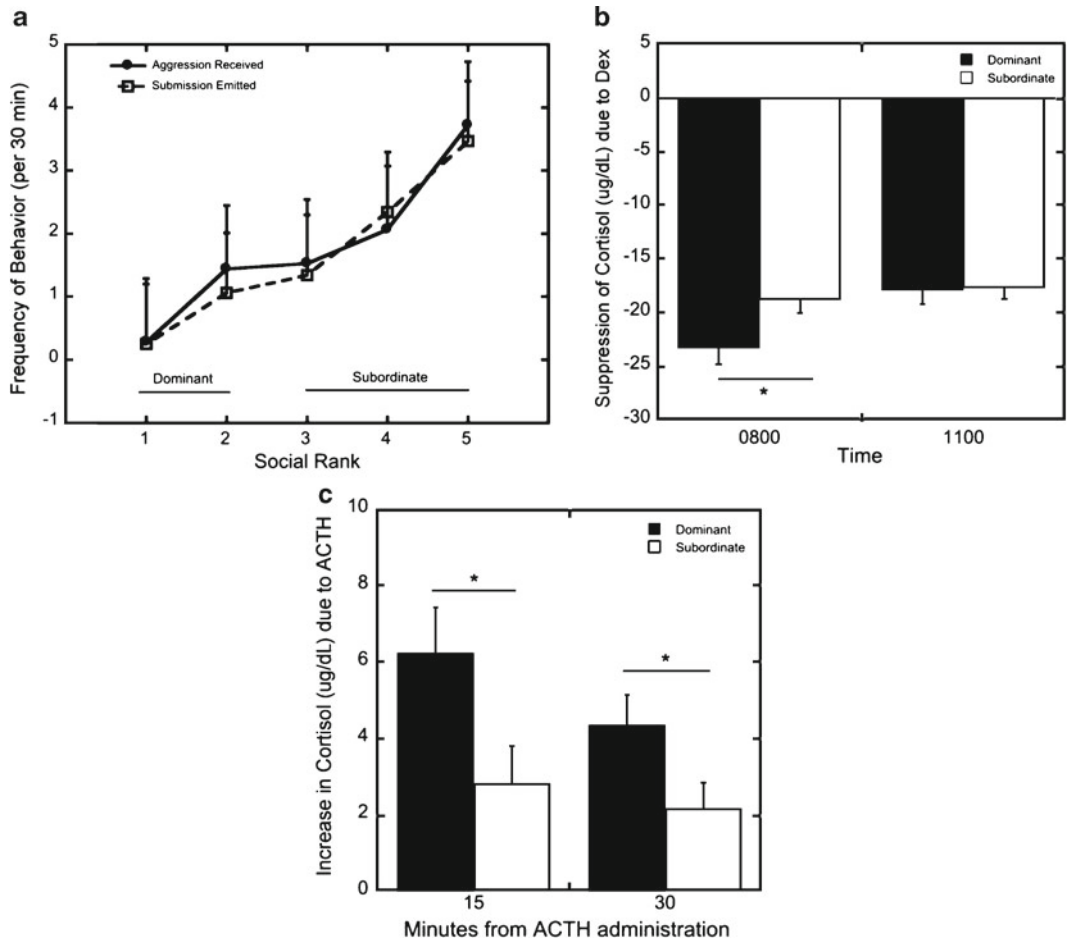

Fig. 1. (**a**) Mean ± SEM rates of agonistic behavior for animals categorized as dominant (ranked 1 and 2) and subordinate (ranks 3 thru 5). Dominant females receive less aggressive behavior (*closed square*) than those categorized as subordinate, while subordinate animals emit more submissive behaviors (*open square*) than dominant animals. (**b**) Mean ± SEM change of serum cortisol at 0800 and 1100 hours following dexamethasone administration at 1750 hours 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 from (53).

uncontrollable, unpredictable nature of the imposition of social rank in macaque social groups leads to a dysregulation of the 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 (47, 50, 53, 57–63).

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

Recent 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 (53, 65), subordinate females weigh significantly less than dominant animals (Fig. 2a). This decreased body weight is associated with reduced fat, but not lean, mass (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

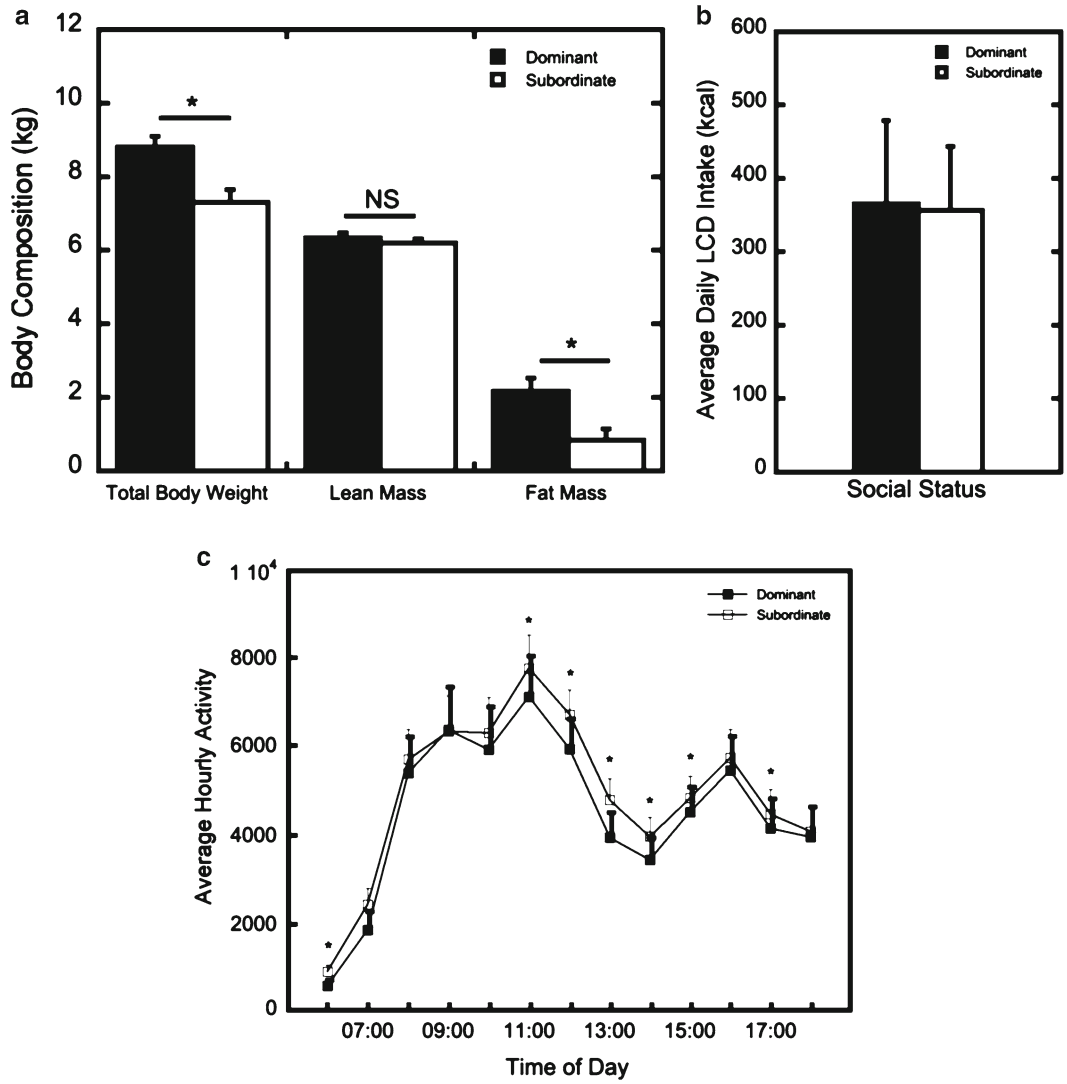

Fig. 2. (**a**) Body weight and body composition (kg) in dominant (*dark bar*) and subordinate (*open bar*) females. *Asterisks* denote increased body weight due to increased fat mass in dominant compared to subordinate females. (**b**) Average daily caloric intake (kcal) of a standard low calorie diet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

females corroborates findings of diminished body weight in rodents exposed to repeated restraint stress (66), chronic variable stress (67), or exposure to a predator (68). These results in rodents are consistent with other data from rodents showing that exposure to chronic stressors (69, 70) or CRH administration (71–74) 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 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 a LCD than dominant females in this dietary environment (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 (75), 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 (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 (47) reduces body weight, and these lower body weights are sustained over time (76). 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 caloric diet that is high in fat and sugar (HFSD)? 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. Availability of a Diet Choice Including Highly Palatable Food

Availability of a HFSD in combination with a LCD for just 2 weeks has a profound impact on caloric intake, most notably for subordinate females. Although both dominant and subordinate females prefer to consume the HFSD 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 HFSD is offered concurrently with a LCD option. This augmented intake of calories upon HFSD availability in subordinate animals is greater than both subordinate baseline

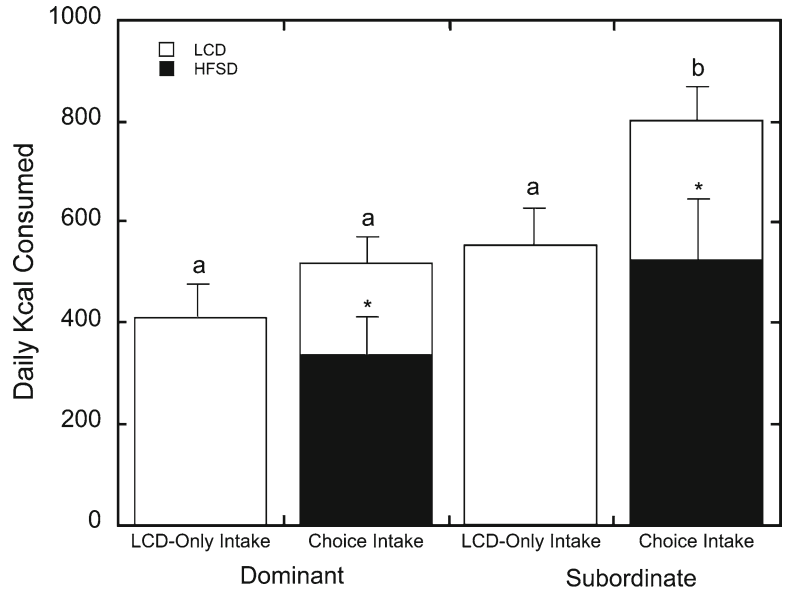

Fig. 3. Average daily caloric intake (kcal) in dominant and subordinate females during a no choice and choice diet condition. *Open bars* represent intake of the low calorie diet (LCD) and *closed bars* indicate caloric intake of the high-fat and -sugar diet (HFSD) diet. *Asterisks* denote overall preference 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 a HFSD increase their total caloric intake to levels higher than those seen in subordinates on a LCD diet only and to dominant females, regardless of diet condition. Reproduced from (56).

intake during the LCD-only condition and dominant female intake under the choice condition (Fig. 3). These data in monkeys corroborate findings from a number of rodent models (77–79) and data from humans (3, 5, 6), 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 (45). 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 (45), 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. These 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).

2.3. Long-Lasting Effects of HFSD 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 previous 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 a LCD upon the removal the HFSD was quantified (56). 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 HFSD phase (Fig. 4b). While caloric intake was lower than observed during the choice phase when HFSD was available, it was significantly higher than the previous LCD phase before any exposure to a HFSD (Fig. 4b). These data suggest that a background of chronic, psychosocial stress can interact with diet history (and exposure to a HFSD) to increase caloric intake even in a healthy dietary environment (56).

The lasting effects of HFSD exposure on appetite regulation in subordinate female macaques could be due to changes in specific satiety signals. While intake of a HFSD 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 HFSD is removed (56). The finding that leptin levels are increased only in subordinate females following HFSD exposure during the subsequent period of increased LCD-only intake (56) further supports the notion that satiety signaling

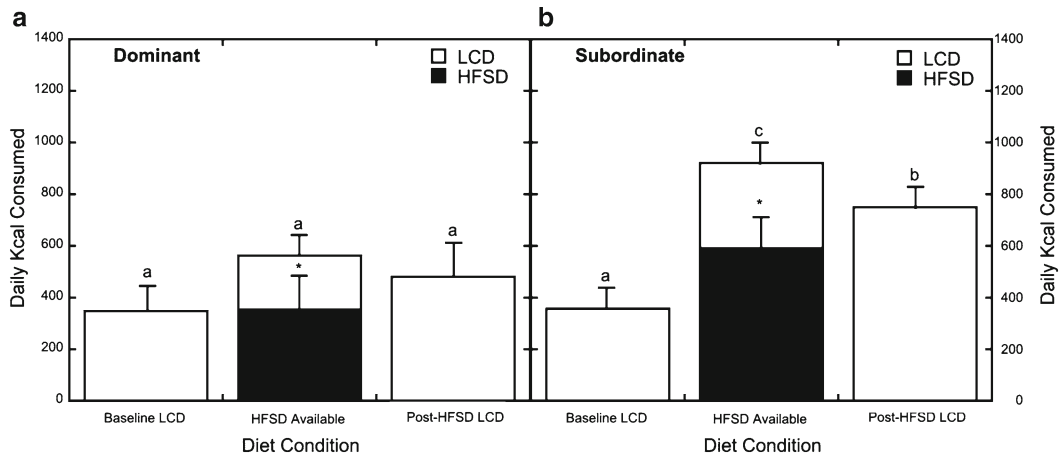

Fig. 4. Average daily caloric intake in dominant (**a**) and subordinate (**b**) females. *Open bars* represent intake of the low calorie diet (LCD) and *closed bars* indicate caloric intake of the high-fat and -sugar diet (HFSD). *Asterisks* denote overall preference of the HFSD over the LCD during 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 a HFSD than they did prior to HFSD exposure (**b**), whereas prior HFSD exposure does not affect caloric intake of a LCD in dominant animals following removal of HFSD. Reproduced from (56).

might be altered in subordinate females (56). Studies in rodents have shown that glucocorticoids can lead to both weight gain and increased feeding by inducing leptin insensitivity (80, 81). 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 (82). A consequence of these physiological changes include augmented food intake and increased body weight as described in individuals suffering from excess cortisol levels due to Cushing's disease (83).

The diet interventions we have used in our rhesus monkey model were tested for 2–3 week durations, too brief to increase fat mass. However, longer HFSD exposure can lead to insulin resistance and reduce satiety signaling in subordinate females (84), which could possibly contribute to excess calorie consumption. Taken together, these data suggest that the exposure to a HFSD 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 exposure account for a significant proportion of variance in total caloric intake when a HFSD 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 HFSD intake during its availability (Fig. 5), whereas females categorized as subordinate show a greater amount of variance in HFSD 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 (85). 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 (85). Likewise, a polymorphism in the dopamine D2 receptor gene is linked to increased incidence of obesity (86). 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 (87–89). Furthermore, epigenetic changes in gene expression due to life experiences and the environment likely facilitate individual variability in emotional feeding and other stress-induced phenotypes (90). Importantly, social subordination in female rhesus monkeys

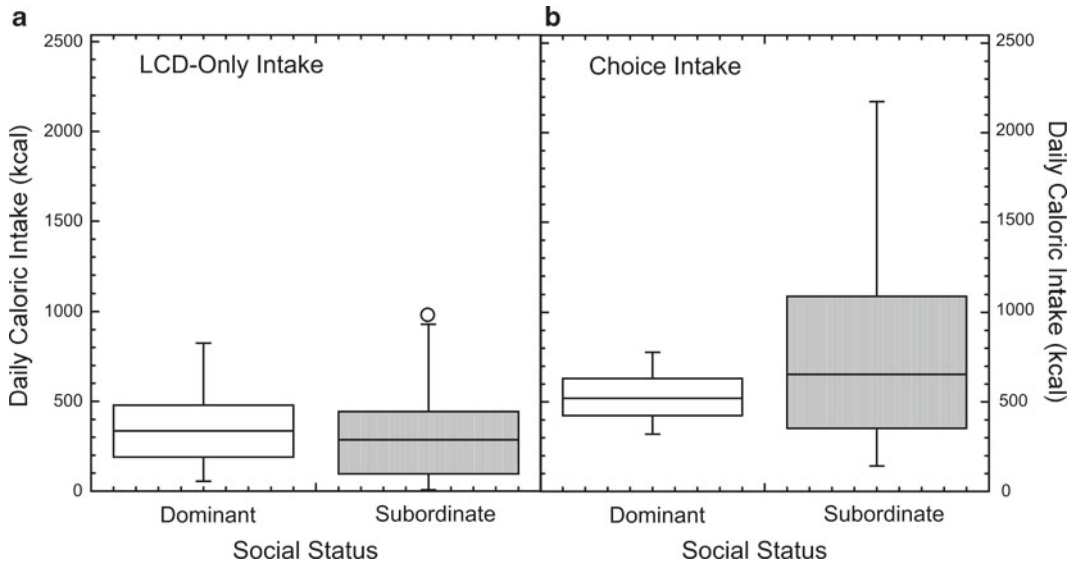

Fig. 5. *Box plots* depicting variability of overall caloric intake and emotional feeding within animals of each social status categorization during (**a**) the LCD-only with no previous history of HFSD and (**b**) a diet choice condition where HFSD was available.

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 a 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 (91). Leptin signaling might also be disrupted by social subordination as HFSD intake in subordinate females increases circulating leptin levels while still maintaining increased caloric intake (56). 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 of 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 (22, 78, 92, 93) and in high-stress premenopausal women (94).

However, because glucocorticoids are a critical component in initiating emotional feeding (95–97), a reduction in stress hormone responsivity by comfort foods is likely not a sustaining factor that maintains this phenotype. In contrast, the availability of a HFSD in female macaques increases the diurnal rhythm of cortisol (45) and HFSD consumption augments cortisol responsivity to an acute social separation stressor, regardless of social status (45, 56). These data in subordinate female monkeys not only support data from rodents with access to a HFSD (98–102), but also are consistent with clinical data linking enhanced LHPA activity to measures of central adiposity (103–105). This increased LHPA activity linked to ingestion of HFSD 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 through an increase in glucocorticoids and LHPA activation.

Because ingestion of HFSDs is linked to the activity of the LHPA axis and LHPA axis activity can modulate behavior (53, 91), 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 a HFSD reduces aggression and anxiety-like behavior (45, 92, 101, 106, 107), other data from monkeys show that HFSD availability has no effect on socioemotional behavior (56). Additionally, while the lack of behavioral effects upon HFSD availability could indicate that HFSD intake does not affect behaviors, it is more plausible that the increased LHPA activation associated with HFSD 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 HFSD 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 (108), including psychostimulant abuse (109), by reducing dopamine D2 receptors in mesolimbic regions and producing a hypodopaminergic condition in corticolimbic regions of the brain that are important for reward processing (110–112). Intake of calorically dense diets reduces dopamine D2 receptors in these reward regions of the brain in obese humans (113, 114) and in some animals (113). Additionally, studies in rats and in humans indicate that HFSD

availability activates reward circuitry in forebrain structures (115–117). Because social subordination in macaques also results in a reduction in dopamine D2 receptor binding potential (61, 118), it is likely that emotional eating in subordinate animals may act as a self-prescribed treatment for increasing the activation of an already dysfunctional reward system (56). Furthermore, the long-lasting effects of HFSD exposure on caloric intake among subordinate monkeys in a healthy dietary environment could be based on a continuing drive to engage a hypoactive dopaminergic system in the absence of a HFSD (56).

4. Conclusion

The ongoing studies of food intake in socially subordinate 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: What is the underlying neurobiological mechanism that initiates and sustains emotional feeding? Does hyperphagia in a healthy dietary environment persist indefinitely following HFSD exposure, and if so, what are the mechanisms that maintain this feeding phenotype? Do satiety signals become ineffective at curbing emotional feeding? How does HFSD exposure alter LHPA activity and how do these changes affect socioemotional behavior? How does long-term (greater than 2 weeks) availability of HFSDs affect LHPA activity, metabolism, and food intake superimposed on a background of social chronic stress? What accounts for individual variability in stress-induced changes in feeding and socioemotional behavior and physiology? Are there behavioral interventions or pharmacotherapies that might rescue or curtail emotional feeding in vulnerable individuals?

The animal model of stress-induced emotional eating described in this chapter will allow us 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 (Fig. 6).

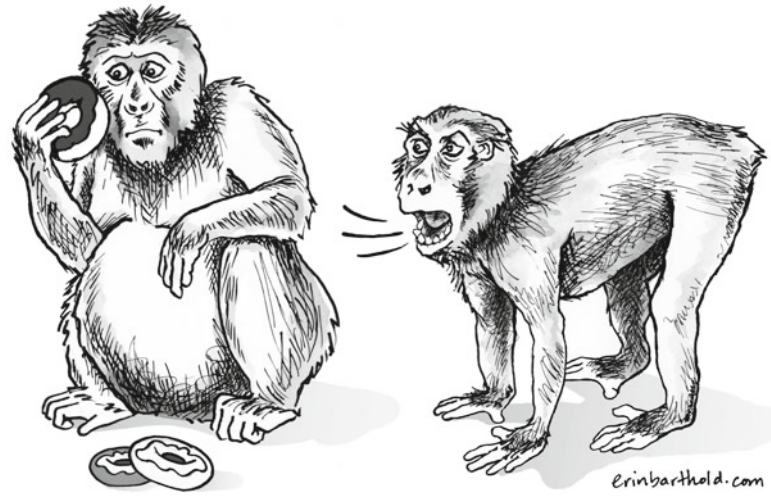

Fig. 6. Cartoon of stress-induced emotional feeding in female monkeys.

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Aqui!!!

Chapter 9

Stressful Experiences in Early Life and Subsequent Food Intake

Jeong Won Jahng

Abstract

A number of studies have indicated a strong correlation between traumatic events during early life and the development of behavioral abnormalities later in life, including psychoemotional disorders such as anxiety and depression. Patients with eating disorders frequently exhibit symptoms of depression and/or anxiety, as well as reporting experiences of childhood abuse, a type of early-life trauma. Dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis is implicated in the pathophysiology not only of anxiety and depression, but also of eating disorders. Neonatal maternal separation and isolation rearing in rodents are well-known animal models of stressful experiences in early life. Many studies have demonstrated their impacts both on the activity of the HPA axis and on the development of psychoemotional disorders later in life. This chapter reviews research using animal models of eating disorders associated with stress in early life. Results suggest that neonatal maternal separation leads to the development of binge-related eating disorders when it is challenged with social or metabolic stressors later in life, in which dysfunctions in the HPA axis and the brain monoaminergic systems may play important roles. Also, social isolation in adolescence induces hyperphagia and depression-like behaviors in female rats, but not in males; a tonic increase of plasma corticosterone seems to be implicated in its underlying mechanism.

Key words: Neonatal maternal separation, Social isolation in adolescence, Eating disorders, Stress in early life, Hypothalamic-pituitary-adrenal axis, Corticosterone, Anxiety, Depression

1. Introduction

1.1. Neonatal Maternal Separation

Studies have indicated a strong correlation between traumatic events during early life and the development of behavioral abnormalities later in life. Maternal Separation (MS) in rodents is an animal model of stressful experiences in childhood. MS can permanently modify characteristics of the HPA axis in offspring (1–6). Many studies have also demonstrated the impact of MS and other traumatic events early in life on the development of depression- and anxiety-like behaviors later in life (7–14).

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Experience of childhood abuse, a type of early life trauma, is prevalent among patients with eating disorders (15), and patients with eating disorders frequently exhibit symptoms of depression and/or anxiety. Results from both clinical and preclinical studies suggest that long-term dysfunction of the HPA axis due to stress in early life may represent a key factor for increased vulnerability to psychiatric diseases (16, 17). Dysfunction of the HPA axis, which is one of the major pathophysiological alterations observed in patients suffering from mood disorders (18), is implicated in the pathogenesis of eating disorders (19–21).

In order to establish an animal model of eating disorders, a series of studies has been performed in my laboratory using an animal model of MS. The MS model used (i.e., 3 h of daily MS during the first 2 weeks after birth) may comprise not only the daily 3 h of maternal deprivation and pup-cooling, but also a disruption in infant nursing patterns. Studies in animals have demonstrated that the early relationship between mother and infant is critical for optimal development of the offspring. Separation of neonatal rat pups from their dams during the early postnatal period results in a variety of physiological changes, the nature of which are dependent on the specifics of the separation experience, the environmental conditions, and the duration of separation (22). Our MS pups showed anxiety- and depression-like behaviors, as well as hyperphagia following social or physiologic stress challenges in later life. Many patients with eating disorders, particularly those with bulimia nervosa, have reported to have been abused in childhood (23–26). In addition, reported sexual abuse in childhood is higher in patients with bulimia nervosa than in patients suffering from anorexia nervosa (27). Bulimia nervosa patients often have an adverse family background (28, 29). Our MS rat model showing hyperphagia can be used as an animal model to study the underlying neural mechanisms of bulimia nervosa.

1.2. Postweaning Social Isolation

Interactions with peers during adolescence are thought to be of principal importance for social development in humans, since individuals spend more time interacting with peers during adolescence than at any other developmental period (30, 31). The importance of peer relationships in the lives of adolescent girls has been well documented (32, 33). Among young women with bulimia nervosa, higher levels of negative interactions and conflict have been observed (34). Feelings of alienation from peers have also been associated with other problems among both adolescents and adults, including depression (35), drug use, and suicide (36). A strong association between psychosocial stressors in early life and increased risks for depression, anxiety, and substance abuse in adulthood has been reported in women (37). Early disruption of social interactions between conspecifics may affect brain development, leading to prolonged aberrant behavior (38–40).

Postweaning isolation rearing of rodents is one procedure that models some of the behavioral consequences of adverse early-life experiences in humans. A large number of studies employed chronic (longer than 1 week) postweaning social isolation, also termed isolation rearing, as a rodent model for adverse early-life experience or social deprivation (41–44). Studies of postweaning social isolation in rodents have manipulated rearing conditions during different periods of the developmental window between pre- and late adolescence. In both humans and rodents, neurotransmitter systems implicated in modulation of emotive behaviors, such as serotonin, dopamine, and corticotropin releasing factor, do not mature fully until early-to-late adolescence (45–47). Clinical and preclinical studies suggest that perturbations to these neurochemical systems during early life appear to have long-lasting consequences on emotional behavior in rodents and on mental health in humans (41, 42, 44, 48, 49).

The reported behavioral effects of postweaning isolation in rats include changes in learning and memory (50, 51), increased anxiety (52–56) and aggressiveness (57), and enhanced cocaine self-administration (58). Isolation-reared rats showed alterations in monoaminergic neurotransmission in regions of the brain, such as the hippocampus and the nucleus accumbens (NAc) (59–65), and dysfunction of the HPA axis activity responding to stress (55, 66–69).

As mentioned above, dysfunction of the HPA axis has been implicated in the pathogenesis of eating disorders (19–21), and increased serum cortisol levels in anxiety (70), depression (13), and binge eating disorders (19, 21). Symptoms of anxiety and depression are associated with the pathophysiology of eating disorders (71), especially with binge-like eating disorders (72, 73). Isolation rearing in male rats, despite its profound long-lasting neurochemical, endocrinological, and psychoemotional impacts, does not cause consistent alterations in body weight and food intake from age-matched controls (41, 53–55). However, isolation rearing in female rats results in hyperphagia with increased depression-like behaviors (74). Male and female rats differ in numerous neuroendocrine and behavioral parameters, and vulnerability to stress is gender dependent (75–78). Social isolation during adolescence is stressful and reported to activate the HPA axis, but does so differently in adolescent male and female rats (68, 79).

This chapter introduces animal models of eating disorders associated with stressful experiences in early life. The behavioral changes observed in the rats subjected to our MS or isolated rearing protocols, as stressful experiences in early life, are reviewed with the underlying neural mechanisms, and their relevance to bingeing-related eating disorders in humans is discussed.

2. Materials and Procedures

2.1. Neonatal MS

The effects of MS differ depending on variables such as duration of each episode of separation, the number of episodes experienced, and the timing of episodes during development. The wide variations in results from studies employing MS protocols may in part be explained by the use of different rat strains, timing and length of MS, rearing condition of the control group, and the age of the offspring at the time of assessment (80). Our MS protocol was designed to clarify the effects of childhood stress on feeding behavior as a stress response in later life (Fig. 1).

Nulliparous females and proven breeder males were used for breeding in the laboratory, and the pups were reared in a controlled manner to minimize and standardize unwanted environmental stimulation from in utero life. Twelve hours after confirming delivery (postnatal day (PND) 1), pups were culled to five males and five females per litter. Each litter was assigned to either the MS group or the nonhandled (NH) group. The MS group was removed from their dam and home cage and placed closely together in a new cage bedded with woodchips for 180 min, and then returned to their home cage and dam. No additional treatment to keep the pups warm during the separation period, other than placing them closely together, was offered, i.e., pup-cooling during MS was expected. MS was performed during 0900–1200 hours daily from PND 1–14, and then the pups were left with their dam undisturbed until weaning on PND 22. The NH group remained undisturbed until weaning except for routine cage cleaning.

2.2. Adolescence Social Isolation

Breeding was performed in our animal facility as described above. Twelve hours after confirming delivery (PND 1), pups were

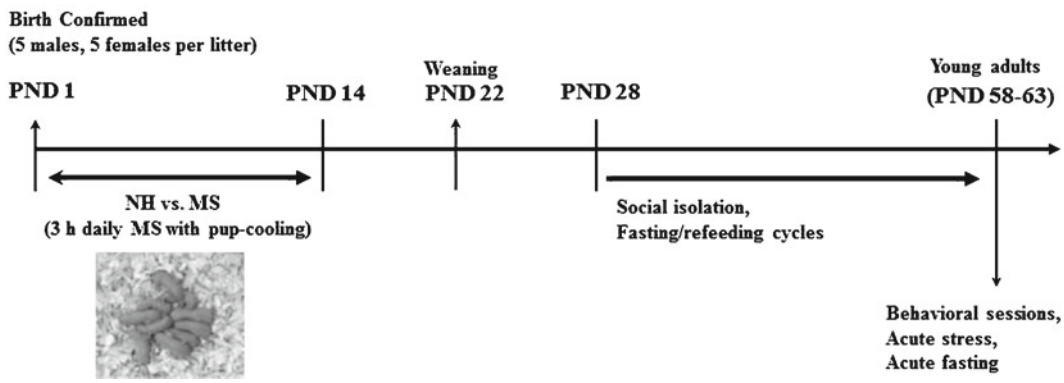

Fig. 1. *Experimental paradigm*. Rats were subjected to 3 h of maternal separation (MS) daily during PND 1-14, or left undisturbed (NH). MS and NH rats received social isolation stress or metabolic stress, such as fasting/refeeding cycles, during the adolescent period (from PND 28), or underwent behavioral sessions examining their psycho-emotional behaviors and neuroendocrine measurements with/without acute stress or fasting at young adulthood (PND 58-63).

culled to five males and five females per litter, and then left with their dam undisturbed until weaning, except for routine cage cleaning. On weaning day (PND 22), five female pups were caged together; 1 week later (PND 28), they were weighed and caged either in groups of three littermate pups (group-caged) or singly (isolates). Isolates and group-caged rats were housed in the same holding room so that isolates had visual, auditory, and olfactory social contact.

In the vast majority of postweaning social isolation studies, rats remain in isolation for 4–6 weeks or more (41, 44) and are then tested while still in isolation-housed conditions. Thus, rats are tested in a state of social deprivation in addition to being reared in isolation during postweaning development (43, 57). In our protocol, rats were socially isolated from early adolescence (PND 28), subjected to the behavioral tests at late adolescence (PND 50–54), and received a stress challenge at early adulthood (PND 59). In the rat, PND 21 (earliest day of weaning) to PND 28 corresponds to preadolescence, PND 28–34 corresponds to early adolescence, PND 34–46 corresponds to mid-adolescence, PND 46–56 corresponds to late adolescence, and PND 56 can be considered early adulthood (45, 46, 81).

3. Notes

3.1. Depression- and Anxiety-Like Behaviors

Stress in early life, such as that induced by MS, child physical, sexual, and emotional abuse, and general neglect, has been associated with serious psychiatric impairment in adulthood (82). Specifically, early parental loss, a stressful life event, is related to unipolar and bipolar depression, as well as anxiety disorders, beyond familial or genetic factors (11, 12, 83, 84). Many human studies have reported that syndromal major depression and anxiety disorders are frequent in adults with a history of childhood abuse (85–87). Women with histories of childhood abuse display abnormal responses of the HPA axis with signs of depression (13, 14). A deregulation of the HPA axis activation is the most common and consistently reported symptom of depression (88). Neonatal MS, a well-established animal model of stressful experience early in life, results in permanent alterations in the HPA response to stress later in life (2, 4, 6). Our MS model that showed dysfunctions in the HPA axis activity (89, 90) also produced depression- and anxiety-like behaviors in young adulthood (54, 91), in accordance with reports by others following a similar separation paradigm (7–10, 17). That is, ambulation and rearing decreased, immobility during a forced-swim test increased, and time spent in the closed arms of elevated plus maze increased in our MS rats compared to NH control rats.

3.2. Serotonergic (5-HT) Neurotransmission in Brain Regions

Dysfunction in 5-HT neurotransmission is implicated in a variety of psychiatric disorders, including major depression (92–94) and anxiety (95, 96). It has been reported that periodic MS during the preweaning period results in persistent alterations in 5-HT concentrations (97) and 5-HT functions in selective brain regions, including the hippocampus (10, 98–100). The hippocampal 5-HT content was decreased in our MS rats exhibiting depression- and anxiety-like behaviors (91). The hippocampus is known to regulate the HPA axis activity via mediation of glucocorticoid negative feedback, and serotonin neurotransmission in the hippocampus is believed to be involved in the regulation of HPA axis activity throughout life. In vitro studies demonstrate that exposure to 5-HT significantly increases mRNA levels of glucocorticoid receptor in the hippocampal neurons (101). The HPA axis response to stress challenges was altered and the basal plasma level of corticosterone was elevated in our MS rat model (90, 102). Together, it is concluded that decreased 5-HT neurotransmission in the hippocampus may have a role in the pathophysiology of depression- and/or anxiety-like behaviors, possibly in relation to dysfunction of the HPA axis activity in our MS model.

Pharmacologic inhibition of 5-HT reuptake transporter (5-HTT) with selective 5-HTT inhibitors, such as fluoxetine, enhances 5-HT neurotransmission and decreases depression symptoms (103). Reduced 5-HTT binding was observed in the raphe nuclei of depressed patients with single photon emission computed tomography (104). In rodents, decreased expression or lack of 5-HTT appears to correlate with the development of depression-like behavior (105, 106). Chronic treatment with selective 5-HTT inhibitor improved depression-like behavior in an animal model of depression in Flinder Sensitive Line rats with or without neonatal maternal separation (8). 5-HTT expression levels in the dorsal raphe nucleus of our MS rats decreased (91), suggesting that decreased expression of 5-HTT mRNA is related to behavioral depression by MS experience. Most 5-HT neurons innervated to the entire brain, including the hippocampus, are localized in the raphe nucleus. 5-HTT takes up 5-HT from the synaptic cleft immediately after its release and ceases 5-HT neurotransmission, and 5-HTT mRNA expression in the raphe has been reported to be altered by brain 5-HT levels (107, 108). Thus, it is likely that the decreased mRNA levels of 5-HTT in the raphe nucleus of our MS rats is related to the decreased 5-HT levels in the hippocampus.

3.3. Food Intake and Weight Gain by MS Experience

Few studies have focused on the association of feeding behaviors in later life with stress in childhood (109–111). The separation protocol used by Matthews et al. (109) and Iwasaki et al. (111) was daily 6-h MS for 3 weeks using Lister Hooded rats and Wistar rats, respectively. Iwasaki et al. (111) reported that there was no significant

difference in normal daily food consumption and weight gain, except a transient decrease in body weight shortly after the separation period, both in male and female offspring. Rebound hyperphagia following a time-restricted scheduled feeding was significantly increased in 6–9-week-old female MS rats, but no difference was observed in males. That is, postnatal MS enhanced rebound hyperphagia of female rats in later life. These results indicate that postnatal MS made female rats more vulnerable to the development of abnormal feeding behavior in response to food restriction in later life. McIntosh et al. (110) used a protocol of daily 3-h MS during the first 3 weeks after birth using Sprague–Dawley rats. They reported that palatable snack consumption was increased in MS females, but not in MS males.

Previous studies have reported that repeated MS during the neonatal period transiently alters bodyweight gain in offspring. Iwasaki et al. (111) reported that both MS males and females are slightly lighter than their NH counterparts shortly after the separation period, and thereafter MS pups tended to be heavier than NH pups, but without statistical significances. Kalinichev et al. (9), using a daily 3-h MS for 2 weeks in Long-Evans rats, reported that MS males are slightly lighter, but MS females are heavier, than their NH counterparts shortly after the separation period, and thereafter body weights of MS pups did not significantly differ from NH pups. In our separation model using Sprague–Dawley rats, we did not measure body weights of the pups until weaning (PND 22) to minimize handling effects in the NH control group. Although we do not know whether or not the body weights of MS pups were lighter shortly after the separation period (PND 2–14), both MS males and females were slightly heavier than their NH counterparts shortly after weaning, and thereafter the weight difference between NH and MS became nonsignificant (54, 89, 109). Daily chow intake of MS males did not differ from NH males (54), and a transient increase was observed in MS females on PND 36 compared with NH females (112). Interestingly, when the weaning male pups were singly housed, significant increases in body weight gain were detected in MS pups from PND 36, and the weight difference between single-caged NH and MS rats persisted until sacrifice (54). Increased chow intake in single-caged MS males appeared to contribute to their increased weight gain. Contrarily, postweaning isolation (isolation rearing) did not affect weight gain and food intake of MS females (unpublished observation).

Collectively, it is concluded that repeated experience of MS during the preweaning period in rats may not permanently affect food intake and body weight gain of the offspring. However, stressful challenges, such as time-restricted scheduled feeding (111) and isolation rearing (54), or exposure to palatable food (110) may evoke disordered eating behaviors in MS offspring, with gender differences.

3.4. Stressful Challenges Following MS Experience

3.4.1. Activity-Based Anorexia in MS Rats

Activity-based anorexia (ABA) is a syndrome that can occur in humans and other animals, characterized by suppressed food intake, below-normal body weight, and hyperactivity (113, 114). Detailed descriptions of the ABA model can be found in Chaps. 16 and 17 of this volume. In brief, in rats, ABA develops when animals are provided with a limited (1–2 h) daily period of food access and otherwise unlimited (22–23 h) access to a running wheel. Under these conditions, rats fail to consume enough calories during the once-daily meal to compensate for energy expended during wheel running. Body weight drops and running increases progressively across days, whereby reduced eating results in a vicious cycle that can lead to death by starvation (115, 116).

Few studies have examined early life environmental regulation of stress reactivity and later susceptibility to ABA. Recently, Carrera et al. (117) examined the effects of early postnatal handling of male and female rats on wheel-running rates, food intake, and weight loss in the ABA paradigm. Handling blunts stress reactivity (118) and should, therefore, make rats less susceptible to ABA-induced effects. Handling did not, however, affect any of these measures in young adolescent males. In adult females, handled runners required more days to reach the study removal criterion (i.e., a dangerously low body weight) than did animal facility-reared runners, but no differences were observed in weight loss and food intake. Hancock and Grant (119) examined the effects of prolonged periods of free wheel running, in combination with a restricted feeding schedule, on food consumption, weight loss, and running rates in male and female rats that experienced handling or MS repeatedly during PND 1–14. In comparison to handled rats, MS rats with 22-h daily access to a running wheel, in combination with a 1-h daily restricted feeding schedule, exhibited a faster rate of body weight loss, lower levels of food intake, greater daily increases in wheel running, and faster attainment of the removal criterion (119). In order to examine the differential effects of postnatal treatment on food intake and weight loss in the ABA paradigm depending on gender and developmental age, Hancock and Grant (120) used a milder version of the ABA paradigm, comprising 2-h daily running wheel access followed by 1-h food access. Handled and MS rats in both genders were tested either in adolescence or adulthood. Compared to handled females, MS females demonstrated greater increases in wheel running and a more pronounced running-induced suppression of food intake during adolescence, but not in adulthood. In contrast, it was only in adulthood that wheel running produced prolonged anorexic effects in MS more so than in handled males. These findings highlight the interplay between early postnatal treatment, gender, and developmental age on running, food intake, and rate of body weight loss in a milder version of the ABA paradigm.

Hyperactivity of the HPA axis is a marked feature of anorexia nervosa (121, 122). Hancock and Grant (119) hypothesized that

the differential effects of postnatal treatment on food intake and weight loss during the ABA paradigm might result from differences in the HPA axis reactivity. Running-induced suppression of food intake and weight gain in rats is concurrent with increased expression of the hypothalamic corticotropin-releasing factor (123), and circulating adrenocorticotrophin (124) and corticosterone (124, 125). In MS rats, the HPA axis activity is already increased (1) and likely to be exacerbated by wheel-running stress. As such, increased weight loss and decreased food intake in MS rats subjected to the ABA paradigm may result from heightened release of stress hormones during running, which may account for the increased anorexic effect in these rats.

3.4.2. Hyperphagia by Social Isolation in MS Rats

Interactions with peers during adolescence are thought to be of principal importance for social development in human adolescents, since individuals spend more time interacting with peers during adolescence than at any other developmental period (30, 31). In rats, early disruption of social interactions, such as isolated rearing, may affect brain development and produce profound, long-term neurochemical, endocrinological, and behavioral effects (38–40, 43, 126). Isolation-reared rats showed alterations in hippocampal neurotransmission (59), disregulation of the HPA axis activity responding to stress (55, 66), and anxiety-like behaviors (53, 55, 56). As described above, food intake and weight gain were significantly increased in our MS males, but not in NH, by isolation rearing (54). Increased serum cortisol levels are implicated in anxiety (70), depression (13), and binge eating disorders (19, 21). We have found that the basal plasma level of corticosterone was elevated in isolation-reared MS males compared with their counterpart NH (102), although it did not differ between group-housed MS and NH males (90). Thus, it is likely that increased food intake and/or body weight gain in our MS pups by isolated rearing may be related to a tonic increase in the plasma corticosterone level, which may worsen their psychoemotional behaviors.

Dysfunction of the HPA axis has been implicated in the pathogenesis of eating disorders (19–21). Symptoms of anxiety and depression are associated with the pathophysiology of eating disorders (71), especially with binge-like eating disorders (72, 73). The behavioral scores of our MS rats, such as ambulatory counts, rearings, defecation scores, immobility duration during the swim test, and the arm stays and entries of elevated plus maze test, did not seem to be further worsened by isolation rearing, i.e., those scores of single-housed MS rats did not differ from group-housed MS rats per se (54). However, further analyses revealed an interaction between MS and postweaning isolation in the time spent in both open and closed arms of the elevated plus maze test, suggesting an impact of postweaning isolation on anxiety-like behaviors in MS pups. In addition, they showed an interaction in defecation scores, representing emotional status (127–129), between MS and isolation rearing

conditions. Thus, isolation-induced increases in food intake and weight gain observed in our MS males are likely to be related to their impacts on the psychoemotional behaviors representing anxiety.

However, isolation rearing did not affect food intake and weight gain in our MS females (unpublished observation), contrary to its significant effect observed in MS males. Plasma corticosterone level of isolated MS females showed a trend toward being increased compared to group-housed MS females. The HPA axis response to stress is known to be affected by gender, and the effects of gender or sex steroid hormones on the HPA axis vary with species and stressors. Currently, we do not have a clear explanation of the mechanisms underlying the differential gender effect of isolation rearing on food intake and weight gain in MS rats.

3.4.3. Response to Food Deprivation in MS Rats

Glucocorticoids, stress hormones released by activation of the HPA axis, are known to be involved in the regulation of energy balance (130, 131). It has been demonstrated that food deprivation markedly elevates the plasma levels of corticosterone, representative of glucocorticoids in rodents (132–135). In adulthood of our MS model, food deprivation significantly elevated the plasma corticosterone levels of NH males, but not MS males, and the basal plasma levels of corticosterone did not differ between NH and MS rats (89). This result suggests that the responsiveness of the HPA axis to fasting, a stressful episode, is altered by the experience of neonatal MS in our model. It was reported that 3-h daily MS during PND 2–14 blunts the release of adrenocorticotrophic hormone (ACTH) responding to restraint stress in adult rats (10). Together with our result, this supports the conclusion that the experience of neonatal MS may blunt the HPA axis activation, stress response, of the offspring in adulthood. However, the patterns of plasma ACTH (5) and corticosterone (1) responses after restraint stress were not changed by the experience of 3-h daily of MS during the first 2 weeks of life, while foot-shock stress increased the plasma corticosterone level only in the MS group, but not in the NH group (136). MS effects on the characteristics of the HPA axis activation in offspring appear to vary depending on stressors. Also, response characteristics of the HPA axis to stressful stimuli at later ages are reported to vary depending on the timing of separation during the postnatal period (4).

Some of the central effects of glucocorticoids are believed to be mediated by hypothalamic neuropeptides (137, 138), and the hypothalamic mRNA expression (139–141) and release (142) of neuropeptide Y (NPY), a potent orexigenic peptide, increases during food deprivation. MS experience blunted the fasting-induced increase not only of plasma corticosterone, but also of the arcuate NPY mRNA expression in our MS males (89). There are some reports suggesting that glucocorticoids may control the hypothalamic NPY expression, and that the arcuate NPY

neurons contain glucocorticoid receptors (143). NPY mRNA expression was increased by glucocorticoids in vitro (144, 145), and adrenalectomy down-regulated the hypothalamic NPY expression (146–148). Furthermore, elevated plasma corticosterone was necessary for fasting-induced increase of NPY mRNA expression in mice (133, 149). However, other studies have suggested that the hypothalamic NPY may influence the level of plasma glucocorticoids, i.e., NPY increases the mRNA expression (150), content and release (151) of corticotropin-releasing hormone in the hypothalamus, and the pituitary release of ACTH (152). Also, it was reported that fasting-induced increase of NPY expression requires neither an elevation of plasma corticosterone (153) nor the existence of endogenous glucocorticoids (148). Therefore, although the causal relation between plasma corticosterone and the hypothalamic NPY expression is controversial, reports support that the blunted NPY expression in our MS males during food deprivation may be related to the blunted response of plasma corticosterone involved with fasting.

In our female MS model, the plasma corticosterone increases responding to 48 h of food deprivation tended to be greater in MS than in NH females, although a significant difference was not found in statistics (112). Desbonnet and colleagues (154) have suggested that MS stress may result in a more reactive neuroendocrinological stress system in females than in males, i.e., acute swim stress increased the plasma corticosterone level in MS females but not in MS males, and the corticotropin-releasing factor response to stress was enhanced in MS females relative to males. This supports our findings that the HPA axis response to food deprivation is blunted in MS males (89), but not in MS females (112). Whereas most of the studies investigating long-lasting effects of MS have used male subjects, those that studied both males and females have revealed significant gender differences in the MS effects on psychoemotional behaviors (110, 155) and HPA axis status (156).

In the arcuate nucleus of our MS females, increased NPY expression and decreased pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript expressions responding to 48 h of food deprivation were exaggerated compared to NH females (112), while the arcuate NPY expression responding to food deprivation was reduced in MS males compared with NH males (89). These results support the idea that MS stress may result in a more reactive neuroendocrinological stress system in females than in males (154). Male and female rats differ in numerous neuroendocrine and behavioral parameters, and vulnerability to stress is gender dependent (75–78). Thus, it is likely that MS experience may increase stress vulnerability in female rats and exaggerate the feeding peptides expression in the arcuate nucleus responding to a metabolic stress, food deprivation, but currently molecular mechanisms underlying the exaggerated response of the hypothalamic feeding peptides expression in MS females to food deprivation are not clear.

3.4.4. Repeated Fasting/Refeeding Cycles in MS Rats

Hypothalamic NPY expression responding to food deprivation was blunted in our MS males in young adulthood, which was accompanied by a blunted response of plasma corticosterone (89). NPY potently stimulates food intake (157–159), and increased NPY expression in the hypothalamus appears to be implicated in the induction of hyperphagia (160, 161). Rats display compensatory hyperphagia when food is returned with ad libitum access following food deprivation. Thus, it is likely that compensatory hyperphagia following food deprivation may be diminished, or at least reduced, due to blunted increases of the hypothalamic NPY and/or the plasma corticosterone in our MS males.

In order to determine if MS rats show different feeding responses to fasting trials, we subjected the NH and MS male pups to 24 h of fasting and 24 h of refeeding ad libitum repeatedly during the adolescent period (90), especially because the symptoms of eating disorders mostly start with dieting and the incidence of eating disorders is higher among adolescents and youth (162). Not only NH pups, but also MS pups showed compensatory hyperphagia on PND 30 following the first fasting trial, with no difference in the amount of foods consumed (90). The arcuate NPY expression responding to fasting on PND 29 was blunted in our MS pups; however, the plasma corticosterone levels increased after the first fasting trial not only in the NH but also in the MS group, in accordance with previous reports that food deprivation elevates the plasma level of corticosterone (132–135). Adrenal glucocorticoids, corticosterone in rodents, have been implicated in the regulation of energy homeostasis (130, 131), and centrally administered glucocorticoids increase food intake and weight gain in rodents (138). Thus, it is concluded that elevated plasma corticosterone during the first fasting trial on PND 29 might have contributed to compensatory hyperphagia in both NH and MS pups. The findings also suggest that increased expression of the arcuate NPY is not necessary to induce compensatory hyperphagia following food deprivation, nor for the orexigenic action of corticosterone.

Compensatory hyperphagia responding to 24 h of food deprivation diminished after the second fasting/refeeding cycle in NH males; however, it persisted in MS males throughout the entire experimental period (90). It has been reported that repeated weight cycling (or fasting/refeeding cycling) may reduce metabolic rate and, consequently, reduce the need for energy intake (163–165). Thus, it seems that diminished hyperphagia in male NH pups on repeated fasting/refeeding cycles may be due to a reduced metabolic rate, and experience of neonatal maternal separation may affect the metabolic adaptation to repeated weight cycling.

Interestingly, both the arcuate NPY expression and the plasma corticosterone level were increased in satiated MS pups that still showed compensatory hyperphagia following the six sets of fasting/refeeding cycles, contrary to the fact that neither NPY nor plasma

corticosterone increased in NH pups that did not show hyperphagia in the same condition (90). Plasma corticosterone is implicated in the hypothalamic NPY expression (133, 143, 146, 149). Thus, it is likely that a chronic increase of plasma corticosterone during the repeated fasting/refeeding cycles might have contributed to a tonic increase of NPY expression in MS rats, and the tonic increases in the plasma corticosterone and NPY expression in our MS rats on repeated fasting/refeeding cycles may partly be in charge of the sustained compensatory hyperphagia.

Repeated fasting/refeeding cycles resulted in a marked weight loss both in NH and MS males (90), in accordance with previous reports that 24 h of fasting and 24 h of refeeding cycles lead to a significant suppression in weight gain (163, 164, 166, 167). Interestingly, MS pups appeared to lose more weight during food deprivation, and gain more during refeeding, than NH pups (90), suggesting that experience of repeated MS during the preweaning period may lead to an exaggerated response to caloric challenges in weight gain of the offspring later in life. Notably, the increased weight loss of MS pups on each fasting day was less significant than the increased weight gain on each refeeding day; consequently, total weight loss after the six sets of fasting/refeeding cycle was significantly reduced in MS rats compared to NH rats (90). This appeared to be related to the increased feeding response (sustained hyperphagia) in MS rats during repeated fasting/refeeding cycles. Compared to NH males, the plasma corticosterone levels responding to either acute fasting or repeated fasting/refeeding cycles were further elevated in MS males (90). Thus, it is concluded that in our male MS model, experience of neonatal MS may lead to an exaggerated feeding response to repeated fasting/refeeding challenges at adolescence, possibly, due to increased responsiveness of the HPA axis.

Altered emotional and mood states, including depression and anxiety, affect eating behavior and food choice. Depression and anxiety can be linked to compulsive behaviors such as drug use and craving for palatable food, which induce feelings of pleasure (168, 169). Studies of humans showed that most subjects reported a preference for palatable food rich in fat and sugar during negative emotions (170). It is hypothesized that sustained hyperphagia during repeated fasting/refeeding cycles observed in our MS males may, at least partly, be related with their psychoemotional status. That is, depression- and anxiety-like behaviors were increased in MS males compared with NH males (54, 91), and repeated fasting/refeeding cycles, metabolic stress challenges, not only resulted in sustained hyperphagia but also improved depression-like behaviors of our MS males (unpublished observation). It was reported that rats consuming palatable foods after exposure to a stressor displayed reduced signs of stress (171, 172). Stress-stimulated consumption of palatable food is proposed as reward-based eating, which indirectly blunts the stress response (173).

3.5. Alterations in the Reward System by MS Experience

Childhood trauma and neglect appear to affect future adult vulnerability to substance abuse (174–176). Indeed, altered sensitivities to opioids, psychostimulants, and alcohol have been reported following MS (9, 97, 177, 178), suggesting a link between early adverse experience and dysfunctions in the reward system.

Anhedonia is a core symptom of major depressive disorders. Development of anhedonia has been ascribed to dysfunction of the reward pathway, in which the NAc plays a pivotal role (179, 180). Palatability and hedonic value of food play central roles in nutrient intake, and recent studies have demonstrated that the NAc is strongly implicated in the motivational mechanisms for feeding (181–183) and the hedonic property of palatable food ingestion (184–186). Our MS rat model showed anhedonia with a reduced intake of palatable food during the adolescent period (187), in accordance with previous reports showing decreased consumption of sucrose in MS adult male rats (188, 189) and palatable food in an animal model of depression (190).

The dopaminergic system has been of particular interest, as dopamine in the NAc has been shown to be associated with motivation, reward, and hedonia (191). Reduced dopaminergic function within the NAc may cause anhedonia in rodents (179, 192), and the striatal dopaminergic activity was suggested to be associated with the severity of anhedonia in depressed patients (193). Previous studies have reported that long-term exposure to various unavoidable stress factors may suppress the mesolimbic dopamine function (179, 194, 195). Dopamine transporter was decreased in the NAc of adult rats that experienced daily 3 h of MS during the first 2 weeks after birth, a type of long-term exposure to unavoidable stress (196). In our MS males, the basal activity of the mesolimbic dopamine system did not appear to be affected, i.e., not only the basal dopamine contents in the midbrain and the NAc, but also the basal expression of tyrosine hydroxylase (TH), a rate limiting enzyme of dopamine biosynthesis, in the ventral tegmental area of our MS pups did not differ from NH pups (102).

Acute exposure to different forms of stress activates the mesolimbic dopaminergic pathway and increases dopamine release in the NAc (197–199). The stress-induced dopamine increase was blunted not only in the midbrain dopaminergic neurons but also in the NAc of our MS pups at adolescence (102, 200). Also, the stress-induced TH expression was blunted in MS pups both in the ventral tegmental area and the substantia nigra. These results suggest that experience of neonatal MS may lead to a long-term suppression in the mesolimbic dopamine system, perhaps the nigrostriatal as well, responding to stressful stimuli in the male offspring later in life, which may comprise an epigenetic control, such as the suppressed TH expression in the midbrain.

Both the shell and core of NAc receive a dense afferent dopaminergic innervation from the ventral mesencephalon (201), and acute restraint stress induces *c-fos* expression, a conventional marker

for neuronal activation, in the NAc core and shell (202, 203). We have demonstrated that acute restraint increases not only dopamine contents in the NAc but also *c-fos* expression in the NAc core and shell in NH pups (102, 200), suggesting that increased dopaminergic input in the NAc by acute restraint contributed to the neuronal activation, *c-fos* expression, in the NAc. Previous reports have shown that stressors stimulate the secretion of dopamine over the NAc in proportion to cortisol responses (204, 205). However, neither the dopamine contents nor *c-fos* expression in the NAc of our MS rats was increased by acute restraint, despite a significant increase in the plasma corticosterone level (102). This suggests that a putative interaction between the HPA axis and the mesolimbic dopamine system responding to stress is dysregulated in our MS male model.

It has been suggested that dopamine release within the NAc is regulated by 5-HT transmission (206) and malregulation of dopaminergic activity in the NAc by 5-HT is involved in a depressive phenotype (207). Chronic antidepressant treatment normalized the 5-HT-dopamine interaction as well as depressive behavior in the forced swim test (208). Depression-like behaviors observed in our MS males were accompanied by reduced 5-HT activities in the raphe and the hippocampus (91) and decreased 5-HT contents in the raphe nucleus where most of 5-HT neurons in the brain are located (102). The NAc, both the core and shell, receives a dense 5-HT innervation from the raphe nucleus (209). Reduced pleasure seeking in an animal model of depression has been suggested to comprise a blunted 5-HT response in the NAc (210). Short duration immobilization stress altered 5-HT levels in the NAc shell (211), and olfactory bulbectomized rats, a model of depression, displayed a blunted 5-HT response to a challenge with a metabolic stressor (212). Also, chronically stressed rats, a model of depression, showed a reduced 5-HT response in the NAc shell to cocaine (210), indicating blunted pleasure stimulation. However, a reduced pleasure seeking behavior observed in our MS males (187) appeared not to involve reduced serotonergic function in the NAc, i.e., statistical analysis did not show main effects of MS or restraint on the NAc serotonin level (102).

3.6. Social Isolation During Adolescence

3.6.1. Food Intake and Body Weight Gain

Rearing rodents in persistent social isolation from weaning, to deprive them of social play, is a relevant paradigm for studying early life stress and produces a large array of consistent long-lasting neuroendocrinological and behavioral alterations compared with group-housed controls (41, 43, 126). The reported behavioral and neuroendocrinological effects of postweaning isolation in rats, which have been mostly studied in males, have strongly suggested its tentative impact on feeding behaviors; however, isolation rearing in male rats did not cause consistent alteration in body weight and food intake from age-matched controls (see Sect. 1.2). In particular, the effect of social isolation in adolescence on body weight and food intake of female rats has rarely been reported. We have

demonstrated that adolescence social isolation may promote food intake and weight gain of female rats (74). The stress-induced elevation of the plasma corticosterone was blunted in isolated females, and furthermore, the basal plasma level of corticosterone was elevated in the isolates compared with group-caged ones. Increased serum glucocorticoids have been implicated in binge-like eating disorders (19, 21, 90), and central administration of glucocorticoids increased food intake and weight gain in rodents (138). Notably, isolated females showed a selective increase in cookie intake when they had access to cookies in addition to standard chow (74). This result is in accordance with previous studies showing that binge eating in animal models is evident in the selective increase in palatable food intake, resulting in an overall increase in caloric intake (213–215).

3.6.2. Anxiety- and Depression-like Behaviors

In our study, isolated young females showed hyperactivity in accordance with previous reports (75, 216, 217). Hyperactivity observed in isolation-reared female Hooded Lister rats was accompanied by strong and stable preferences for their most preferred food (216). Increased food intake, especially palatable food intake, in chronically stressed rats has been suggested to correlate with anxiety-like behaviors (218). Although the behavioral scores of isolated females during elevated plus maze test did not differ from group-housed ones, number of rears and repetitive standing with two forepaws up were increased in isolated females during the activity test (74). Increased rearing activity in rats has been reported to reveal an anxiety-related behavior responding to stress, as a proactive emotional coping behavior (219, 220). It has been suggested that increased food intake responding to stress is a stress coping behavior and consumption of palatable food dampens psychological and physiological responses to stress (221, 222). The stress-induced elevation of plasma corticosterone was blunted in our isolated females that ate more food than group-housed ones. Therefore, increased consumption of food in isolated females may be a stress coping behavior, likely in relation to anxiety-related behaviors, dampening psychological and physiological responses to chronic social isolation stress.

Loss of social contact and behavioral withdrawal are associated with the etiology of depression in men. However, little change was observed in immobility or struggling time during the Porsolt forced swim test in isolation-reared male rats (54, 223, 224). Women are almost twice as likely to suffer from depressive disorders as men (225). In several putative animal models of depression, female rather than male rats show a greater response to stressors (226). Teenage girls, in particular, report more stressful experiences than do teenage boys or older women and men (227, 228). Immobility duration in the Porsolt swim test was significantly increased in isolated females compared with group-housed controls (74), revealing that adolescence social isolation may increase depression-like

behaviors in female rats. Association of binge-like eating disorders with symptoms of anxiety and depression has been reported (72, 73). Previous studies have suggested that increased serum cortisol levels are implicated in depression (13) and binge eating disorders (19, 21). Taken all together, it is concluded that social isolation in adolescence increases food intake and depression-like behaviors in female rats, and a tonic increase of the HPA axis activity responding to chronic isolation stress may play a role in its pathophysiology.

3.6.3. Gender-Specific Effects of Social Isolation

Male and female rats differ in numerous neuroendocrine and behavioral parameters, and vulnerability to stress is gender dependent (75–78). For example, compared to males, female rats are more active in the open arms of the elevated plus maze (229, 230) and the open field (75, 231), indicative of higher levels of general arousal and exploration, as well as decreased anxiety among females (76). The anxiogenic property of postweaning social isolation has been found in male rats with elevated plus maze test (54, 55), but not in female rats (55, 74). In male rats, isolation rearing did not increase depression-like behaviors (54, 223, 224), nor cause any consistent alterations in food intake (41, 53–55).

Women with anorexia and bulimia nervosa have been found to display high levels of social insecurity and social isolation (232). The consistent relationship between disordered eating behaviors and negative friendship qualities (friend alienation, friend conflict) was reported in adolescent girls (233), and depressed mood was associated with poor body image and disordered eating symptoms (233–235). Obese girls were more likely to report more serious emotional problems, hopelessness, and a suicide attempt, when compared to their normal weight peers (236). Depression and obesity were positively associated in females and the entire sample, but not in males (237). Here, we propose that social isolation in adolescence of female rats can be used as an animal model to study the pathophysiology of binge-like eating behaviors associated with symptoms of depression in young females.

4. Conclusion

Our MS rats showed depression- and anxiety-like behaviors in adulthood with dysregulated 5-HT neurotransmission in the brain regions. MS procedures in common do not appear to affect food intake and weight gain of the offspring as long as the pups are group housed with ad libitum access to food. The hypothalamic feeding peptides expressions responding to acute food deprivation in adulthood appeared to be blunted in MS males, but exaggerated in MS females. Postweaning isolation stress following our MS protocol promoted hyperphagia and weight gain in male rats, but not

in females, with impacts on anxiety-like behaviors. Repeated fasting/refeeding cycles during the adolescent period of our MS males induced a binge-like eating disorder, in which increased activity of the HPA axis responding to such metabolic challenges appeared to play a role, at least partly, in mediation with the hypothalamic NPY. Anhedonia, a major symptom of depression, was observed in our adolescent MS males with decreased activity of the mesolimbic dopamine system responding to stress challenge. Isolation rearing did not cause any consistent alterations in food intake nor increase depression-like behaviors in male rats; however, it induced a binge-like eating and depression-like behaviors in young female rats. Studies have not yet reported sexual dimorphism of any neurochemical consequences of isolation rearing.

Currently available animal models of bingeing-related eating disorders are isomorphic, sharing the common feature of binge episodes that occur repeatedly over extended periods of time, but vary in their similarities to human-disordered binge-type eating (238). For an example, the Corwin model with limited access to highly palatable food, which is also described in detail in Chap. 4 of this volume (239), is relevant to bingeing in the absence of hunger in humans (240). Our MS rats on repeated fasting/refeeding cycles may have specific relevance to the hypersensitive response of the HPA axis in patients with bingeing-related eating disorders (21, 241), which is associated with childhood trauma (242). A history of dieting and overeating is thought to contribute to future binge eating in some people (243), and stressful events early in life have been suggested to increase vulnerability to the development of bingeing-related eating disorders in humans (244, 245). Also, adolescent social isolation of female rats can be used as an animal model to study the pathophysiology of binge-like eating behaviors associated with symptoms of depression in young females.

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Chapter 10

Sham Feeding in Rats Translates into Modified Sham Feeding in Women with Bulimia Nervosa and Purging

Diane A. Klein and Gerard P. Smith

Abstract

Bulimia nervosa (BN) is a psychiatric illness characterized by repeated binge eating and purging episodes that can be associated with significant psychosocial impairment and chronicity. Mechanisms maintaining this maladaptive set of behaviors remain poorly understood, but several lines of evidence support the presence of enhanced responsiveness to orosensory cues in people with BN. Sham feeding (SF) in the rat is an animal model of binge eating and purging that has been used extensively for the investigation of the orosensory excitatory controls of eating. We translated SF in the rat into modified sham feeding (MSF) in humans to investigate the orosensory excitatory control of eating in patients with BN and purging. BN women sham fed significantly more sweet and unsweetened solutions than control subjects or women with anorexia nervosa. This result validates the utility of the SF rat as an animal model of BN and purging and establishes MSF as a heuristic technique for the analysis of the orosensory controls of ingestion in women with BN and purging.

Key words: Bulimia nervosa, Purging, Sham feeding, Animal model, Modified sham feeding, Orosensory control of eating, Self-report, Sweetness intensity, Hedonic intensity

1. Introduction

1.1. Bulimia Nervosa: Definition and Clinical Features

Bulimia nervosa (BN) is a psychiatric disorder characterized by repeated episodes of binge eating and postprandial purging. It typically begins during adolescence and affects some 1–3% of women in Western cultures (1, 2). Treatment affects a significant remission rate (3), but relapse is common. Outcome studies suggest that approximately 30% of individuals have a chronic relapsing/remitting course of the disorder (4, 5). Rarely, individuals with BN die, usually from electrolyte disturbances produced by purging. More common sequelae of BN are dental erosion, esophageal reflux (6), and impaired quality of life.

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Table 1
DSM-IV-TR criteria for bulimia nervosa

A. Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the following:
1. Eating, in a discrete period of time (e.g., within any 2-h period), an amount of food that is definitely larger than most people would eat during a similar period of time and under similar circumstances.
2. A sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating).
B. Recurrent inappropriate compensatory behavior in order to prevent weight gain, such as self-induced vomiting; misuse of laxatives, diuretics, enemas, or other medications; fasting; or excessive exercise.
C. The binge eating and inappropriate compensatory behaviors both occur, on average, at least twice a week for 3 months.
D. Self-evaluation is unduly influenced by body shape and weight.
E. The disturbance does not occur exclusively during episodes of Anorexia Nervosa.
Purging Type: during the current episode of Bulimia Nervosa, the person has regularly engaged in self-induced vomiting or the misuse of laxatives, diuretics, or enemas.
Nonpurging Type: during the current episode of Bulimia Nervosa, the person has used other inappropriate compensatory behaviors, such as fasting or excessive exercise, but has not regularly engaged in self-induced vomiting or the misuse of laxatives, diuretics, or enemas.

DSM-IV-TR criteria for Bulimia Nervosa. Reprinted, with permission, from (7)

The hallmark clinical features of BN are episodes of binge eating followed by purging to rid the body of ingested food and its unwanted calories. A binge-eating episode involves consumption of an unusually large amount of food with subjective loss of control over intake. The number of calories consumed during a binge-eating episode range from a few hundred to over ten thousand. Purging typically involves self-induced vomiting, but laxatives, diuretics, and stimulants (e.g., amphetamines) are also commonly used. Current diagnostic criteria for BN are given in Table 1.

Additional diagnostic features of BN are excessive concern about food, body weight, and body shape. Patients are typically normal weight: emaciation takes diagnostic priority and thus an underweight individual exhibiting binge eating and purging would be considered to have anorexia nervosa (AN). Historically, only a small proportion of patients with BN have been found to be overweight (e.g., 4.2% in one 1990 study (8)), though in a recent study, the prevalence of overweight people in a community-residing population with BN was 64% (9). Thus, the percentage of individuals with BN who are overweight or obese is not well established.

Risk factors linked to the development of BN include a history of parental obesity or of childhood obesity, early parental criticism

about eating and body weight (10, 11), dieting, and a history of AN (12). Initiating factors for the disorder may include any of a number of circumstances that precipitate dieting behavior or dietary restriction. Dieting usually precedes binge eating, though a significant subgroup reports binge eating prior to the onset of dieting (13). As with other eating disorders, factors that maintain the behavioral cycle of BN may be distinct from factors that initiate BN (14).

Factors likely to initiate a binge meal and thus perpetuate the behavioral cycle of BN include cognitive, affective, and learned factors such as binge-eating episodes preceded by negative mood states. This suggests that some binge episodes are attempts to relieve such aversive states. Learned factors probably play a prominent role in binge eating. For example, there is behavioral (15) and self-reported (16) evidence for heightened responsiveness to binge food stimuli, "cue reactivity," in this population. Dietary restriction in between binge-eating episodes is common, and resultant hunger may serve to "fuel" subsequent overeating (17, 18). A typical pattern of eating may feature skipping breakfast, consuming a minimal lunch, and binge eating in the evening.

Laboratory meal studies have provided the preponderance of the information about the pathophysiology of binge eating in BN. Typical meal studies involve patients and controls in a laboratory setting where either multiple-item or single-item meals are available and specific instructions are provided. These studies showed that when instructed to "let yourself go and binge" (with knowledge of access to a restroom for purging after the study) patients with BN ate larger meals than control subjects did and BN subjects rated the meals as typical of an actual binge (19–22).

An abnormally large meal is the result of decreased inhibitory controls of eating, increased excitatory controls of eating (23), or both. Early meal studies focused on possible *decreased inhibitory* controls of eating, i.e., decreased satiation during the meal. These studies identified candidate abnormalities in gastrointestinal and abdominal vagal afferent functions that are involved in satiation. Gastrointestinal abnormalities in BN include delayed gastric emptying (24, 25), reduction in gastric relaxation following food ingestion (26), diminished sensitivity to gastric distension (27), enlarged gastric capacity (25), and decreased release of cholecystokinin (24, 28, 29). Vagal afferent abnormalities in BN include alterations of somatic pain detection thresholds modulated by vagal afferents (30, 31), and normalization of pain thresholds and of bulimic symptoms following treatment with ondansetron, a serotonin type-3 receptor antagonist known to decrease vagal afferent activity (30, 32).

Each of these deficits is presumably a result of active eating-disordered symptoms, and at least some evidence suggests that certain abnormalities improve with treatment (e.g., tricyclic antidepressants improve postprandial cholecystokinin responses and satiety (28)). Each deficit could also conceivably contribute to

further binge-eating and purging episodes. For example, delayed gastric emptying could be experienced as an uncomfortable sense of fullness even after a normal eating episode that would promote self-induced vomiting. The degree to which these deficits normalize with successful treatment of BN remains unknown.

A separate line of investigation supports the presence of *increased excitatory* controls of eating in people with BN. The excitatory controls are activated by orosensory food stimuli. Evidence for increased responsiveness to orosensory food stimuli includes:

1. Individuals with BN have been found to continue to eat despite reporting maximal fullness (33). While this may be accounted for by a failure to experience maximal fullness as satiating or aversive, it may also be explained by increased responsiveness to orosensory stimulation.
2. Patients with BN reported persistent urges to eat (34) and/or hunger (35) in the postmeal period to a greater extent than controls.
3. Patients with BN increased the rate of food consumption as a meal progressed, in contrast to controls whose eating slowed as more food was consumed (36). This abnormal acceleration suggests an enhanced excitatory drive to eat, as rate of eating in animal feeding studies has been very closely linked with appetitive drive (37).
4. Individuals with BN rated concentrated sweet solutions as more pleasing than controls did (38, 39); this suggests an increased hedonic value of sweet orosensory stimuli in BN.
5. Reports of sham-feeding (SF)-like behaviors, including chewing and spitting out food, have been linked to BN as well as other eating disorders (40–42).
6. Individuals with BN reported consumption of larger quantities of sweet nonnutritive products (e.g., chewing gum (43)) than non-eating-disordered controls, consistent with a heightened drive for, or heightened reinforcing value of, orosensory stimuli.

These observations suggest that the excitatory controls of eating are hyperresponsive in people with BN and are responsible, at least in part, for binge eating. Investigation of this possibility would be facilitated by an animal model. A review of the literature revealed that the SF rat was a good model of BN and purging.

1.2. SF Rat: An Animal Model of Bulimia Nervosa and Purging

As we have seen, intermittent binge eating and purging characterize BN. The combined effect of these two behaviors is to provide orosensory stimulation of palatable food, while minimizing its postingestive digestive, metabolic, and caloric consequences. This is similar to SF in animals. Reports of subjects with BN chewing

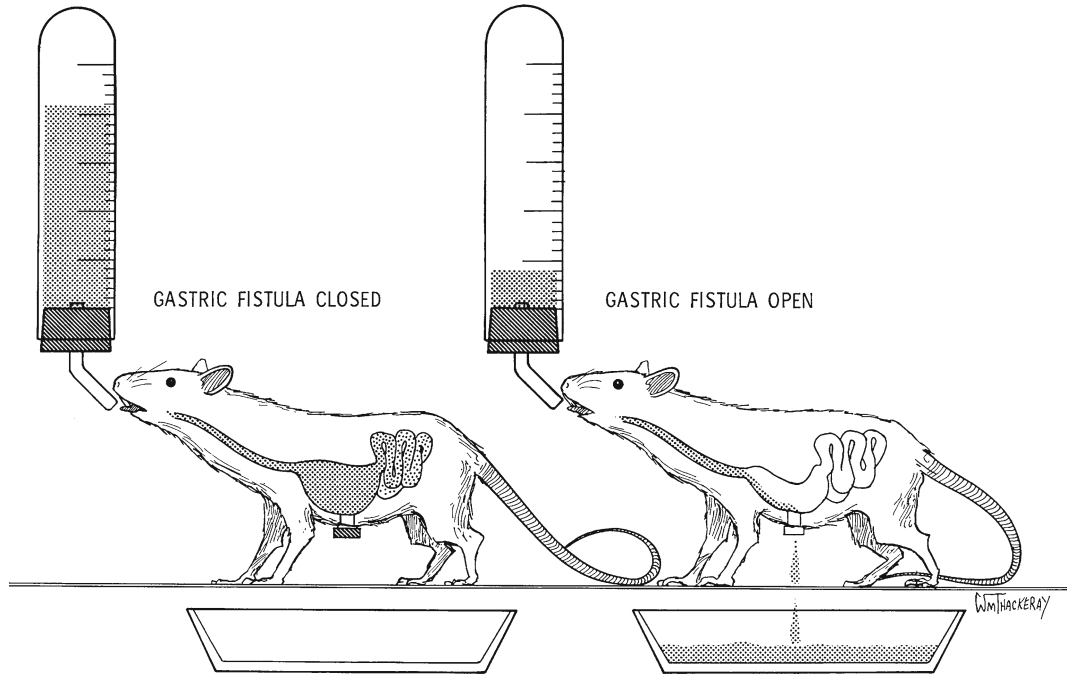

Fig. 1. Chronic gastric fistula rat preparation for sham feeding. When the screwcap is removed from the cannula, ingested liquid drains out of the stomach (*visual right*). This prevents postingestive negative feedback effects on intake from the stomach and small intestine. This is sham feeding. When the screwcap is in place and the cannula is closed, ingested food accumulates in the stomach and is emptied into the small intestine where digestion and absorption occurs (*left*). This is real feeding. Reproduced, with permission, from (45).

and spitting out food and ingesting abnormally large quantities of nonnutritive products (e.g., chewing gum (43)) reinforces that impression (40–42).

The best current SF preparation is the chronic gastric fistula rat (44) (Fig. 1). When the screwcap of the fistula is closed, the ingested liquid food produces orosensory stimulation, is swallowed, accumulates in the stomach, empties into the small intestine, and is absorbed into the metabolic pathways. This is real feeding (RF). When the screwcap is open, ingesting the same liquid food produces the same orosensory stimulation, is swallowed, enters the stomach and immediately drains out of the stomach through the open cannula. Thus, drainage out of the cannula prevents all of the usual postingestive effects of the ingested liquid food. This is SF (See Sect. 2.1 for details of the surgical implantation of a gastric cannula and of SF tests).

For the purpose of animal modeling of binge eating, the critical fact about SF is that under a wide variety of deprivation and experiential conditions, and with many different liquid foods, rats eat larger meals when they are SF than when they are RF (46). In fact, when rats are given a palatable liquid food after overnight

deprivation, they eat for hours with only short pauses between episodes of SF (47). This is binge eating in the rat.

When rats are deprived of food for 3 h (an interval within the range of the rat's spontaneous intermeal intervals (IMI)), they SF a larger meal than they RF. Then they stop eating and display the sequence of behaviors that is the signature of postprandial satiety in the rat (48). However, they return to SF much sooner than after they RF. Thus, SF increases meal size and shortens the postprandial IMI. The shortening of the postprandial IMI probably models the more frequent reports of persistent urges to eat (34) and/or hunger (35) in the postmeal period in patients with BN than in controls.

These two effects have been analyzed in the rat into excitatory and inhibitory components. The larger size of the meal is accounted for by the excitatory effects of orosensory stimulation provided by SF and the lack of postingestive inhibitory negative feedbacks of hormones and visceral afferent nerves produced by draining the ingested liquid food out of the stomach. Thus, the larger meal is the result of orosensory excitation acting in the absence of postingestive inhibition. The only inhibition of eating that occurs in the SF rat is due to learned orosensory inhibition (49). It quickly extinguishes when repetitive episodes of SF occur ((50) and see below).

The shorter postprandial IMI is the result of a similar combination of orosensory stimulation and the loss of postingestive stimulation. The IMI after SF stops is about 50% shorter than after RF (51). This is produced by orosensory stimulation acting alone. That the IMI after RF is twice as long as after SF is due to the synergistic inhibitory actions of orosensory and postingestive stimuli for the control of the IMI. Note that the controls of meal size and of IMI are distinct.

The importance of the postingestive stimuli for the IMI has also been demonstrated in RF by draining the stomach of ingested food after eating has stopped (52). This models postprandial purging. When the gastric contents are drained, the IMI shortens. The abdominal vagus nerve is necessary for this effect because drainage of the stomach does not change the IMI in abdominal vagotomized rats. One of the strengths of the SF rat model of BN is that there is a wealth of information available, especially for the controls of meal size, about the peripheral and central mechanisms that are activated by orosensory excitatory stimuli and postingestive inhibitory stimuli (23). Description of these central and peripheral mechanisms, however, is beyond the scope of this chapter.

1.3. Modified SF in Women with BN and Purging

Modified SF (MSF) has been used to study orosensory control of autonomic, neuroendocrine, and metabolic mechanisms (cephalic reflexes). MSF excludes the postingestive effects of food by having subjects chew food and then spit it out without swallowing any (53–55). We modified MSF for the ingestion of liquid food

(see Sect. 2.2). MSF had the following advantages over test meals in which the postingestive effects of ingested food occur: (1) MSF simulated the key features of the SF rat, in that the effects of excitatory orosensory controls of eating are measured, while the postingestive effects of food do not occur; (2) the sipping and spitting out of test solutions in the MSF technique is simple enough that all subjects learn it readily; (3) it is noninvasive and well tolerated by subjects; (4) MSF measures the effect of changing the palatability of liquid food (e.g., sweetness intensity) easily; and (5) various measures of ingesting can be quantified, such as amounts sipped and spit out, self-reports of sensory and hedonic aspects of the liquid ingested, rate of sipping, and intersip interval.

2. Methods

2.1. Methods for SF in the Rat

2.1.1. Materials

1. Anesthetic: ketamine and xylazine or chloropent, a mixture of chloral hydrate and pentobarbital
2. Anesthetic jelly
3. General surgical instruments: toothed forceps, iris scissors, hemostats, wound clips, abdominal spreader, and scalpel with #10 blades
4. Special surgical materials: gastric cannula with screw-cap, collecting tube connector, cannula bullet, cannula pliers, and Marlex mesh cut into 20 mm diameter circles. The cannula, bullet, pliers, and tube connector are not available commercially, but are easily fabricated in a machine shop. Contact gpsmith@med.cornell.edu for details. All instruments and materials must be sterilized by autoclaving
5. Silk sutures 3-0 and 4-0
6. Test cage with a slot cut through the middle of the floor from the front to the back. The cut must be wide enough to accommodate the collecting tube and to permit it to move freely as the rat moves around the cage during a test

2.1.2. Surgery

Preparation of the Animal and Cannula Placement

1. Deprive the rat of food, but not water, overnight.
2. Anesthetize the rat by intraperitoneal injection of an anesthetic that has a rapid onset of action and maintains a level of anesthesia for abdominal surgery for 1 h, e.g., ketamine (70 mg/kg) and xylazine (4.5 mg/kg) or chloropent (3 mL/kg).
3. Shave the ventral surface of the rat from the costal margin to about 1 in. above the pelvis. Incise the midline with a #10 blade through the skin and the linea alba of the abdominal muscles. Make a small incision at the center of the midline incision and grasp the edges with toothed forceps. Insert the blunt

blade of body scissors into the cavity beneath the midline. Use iris scissors to cut along the midline to expose the abdominal organs.

4. Insert an abdominal spreader into the wound and open it against the sides of the wound. Leave it in place during the rest of the operation.
5. Grasp the lower part of the stomach with toothed forceps. Put a 3-0 silk suture through the greater curvature of the stomach and clamp the free ends of the suture with a hemostat. Arrange the hemostat to provide sufficient downward tension on the stomach to keep it in position throughout the surgery.
6. Note that the proximal part of the stomach is lighter in color than the distal part. The curved dividing line between these two parts is called the limiting ridge. For proper drainage of stomach contents, the cannula must be placed at the point where the limiting ridge meets the greater curvature of the stomach. Grasp the proximal part of the stomach just to the right (visual right) of the limiting ridge and cut a small (~5 mm) hole through the wall of the stomach. While holding the left edge of the incision in toothed forceps, insert the circular base of the cannula with a clockwise twist using gentle pressure.
7. When the base of the cannula is completely inside the stomach, insert gauze into the lumen of the cannula to prevent leakage of gastric contents. If leakage occurs, flush the peritoneal cavity immediately with isotonic saline (37 °C) to prevent peritoneal irritation by gastric acid.
8. Place a purse-string suture (3-0 or 4-0 silk) through the wall of the stomach around the base of the cannula ~5 mm from its shaft. Tie the free ends of the suture and invert the ends of the stomach wall into the stomach so that the outer (serosal) surface of the stomach fits tightly against the shaft of the cannula.
9. Cut a small hole in the center of a Marlex mesh circle with iris scissors. Slide the mesh down over the cannula and spread the mesh out flat over the purse-string suture on the surface of the stomach. Tack the outer edge of the mesh to the stomach with 4-0 silk sutures that are spaced equidistant from one another.

Exteriorization of the Cannula Shaft

1. Remove the spreader. Push the pointed end of the cannula bullet through the right abdominal wall at a point that holds the cannula in its present position when it is exteriorized.
2. After the hole has been made through the abdominal wall, retract the bullet. Unscrew the pointed end of the bullet and expose the threaded end. Insert this end through the abdominal wall from the outside in.
3. Grasp the shaft of the cannula with the cannula pliers, screw the threaded end of the bullet into the lumen of the cannula.

When the cannula is firmly fixed to the bullet, remove the pliers and pull the shaft of the cannula through the bullet hole until resistance occurs due to the base of the cannula pressing against the peritoneal surface of the abdominal muscles. Hold the outer end of the cannula with the pliers and unscrew the bullet. Remove the gauze plug from the lumen of the cannula shaft and screw in the screwcap.

4. Coat the area of skin around the base of the cannula shaft with anesthetic jelly. Screw down the nut that threads onto the shaft of the cannula until it is flat against the skin.
5. Remove the spreaders and close the incision through the muscle layer with interrupted 3-0 silk sutures. Close the skin with wound clips.

Postoperative Care and Maintenance

1. Return the rat to its home cage immediately after surgery. When the rat is completely awake, give it water to drink. About 6 h later, give it back its maintenance food.
2. Remove the nut around the cannula shaft 48 h after surgery. Remove the wound clips from the midline incision 10 days after surgery. When the rat is fully recovered and with no signs of infection, testing can begin.

2.1.3. SF Test

1. Remove the rat from its cage. Testing can be performed after some or no deprivation. Grip the cannula with cannula pliers and loop one finger-hole of the pliers over a finger of the hand holding the rat. With the free hand use a stubby screwdriver to unscrew the cap.
2. Flush the stomach with isotonic saline (37 °C) by inserting a 20-mL syringe into the lumen of the cannula; inject the saline quickly and with slight pressure. Flush until no food particles are in the gastric drainage after two consecutive injections of saline.
3. Attach the collecting tube by threading it into the cannula. Gently lower the rat into its cage while directing the collecting tube through the midline slot in the floor of the cage so that its tip hangs into a translucent plastic shoe box placed approximately 25 cm below the cage. Make sure that the tip of the tube stays in the box when the rat moves around the cage. Measure the volume of gastric drainage collected as well as the volume of liquid diet SF.
4. Use three measures to evaluate the completeness of gastric drainage during SF of any liquid food. First, gastric drainage of ingested liquid must begin within the first 15 s of ingestion. Second, the total volume of gastric drainage must equal or exceed the volume ingested. Third, when the collecting tube is removed from the cannula at the end of a test, a significant

volume of drainage does not occur. When these criteria are satisfied, gastric drainage is complete. If all three criteria are not met, drainage is incomplete and the results of that test must be discarded. If this occurs repeatedly, remove the rat from the experiment.

5. At the end of the test, replace the screwcap and return the rat to its cage. Restore maintenance food and water.

2.2. Modified SF in Humans

2.2.1. Materials

Solutions

Test solutions are prepared fresh 18–24 h prior to the experimental day, using aspartame in concentrations of 0, 0.145, 0.3, 0.75, and 2.8 g/L, or 0, 0.01, 0.03, 0.08, and 0.28% wt/wt, respectively. Cherry-flavored Kool-Aid® (1.902 g/L) is added to make the solutions more palatable and more comparable to beverages commonly consumed in the United States. Each sip container contains 1,900 mL of the test solution (2 L are prepared with 100 mL drawn off for the taste-test samples).

The rinse solution is prepared using 23.7 g of baking soda dissolved in 1,000 mL of distilled water and served in a pitcher located on the table along with a cup.

Visual Analogue Scales

Visual Analogue Scales (VAS) consist of pencil-and-paper assessments including the following questions: "How SWEET did what you just tasted seem to you?" and "How much did you LIKE what you just tasted?" in addition to assessing hunger, desire to binge, desire to vomit, and anxiety. Beneath each question is a 10-cm horizontal line, anchored at one end by "Not at all" and at the other end by "Extremely." Subjects are asked to indicate their answers to these questions by placing a vertical mark along the horizontal line to estimate their experiences. Separate sheets of paper are used for each time point and subjects do not have access to their previous responses.

Additional Supplies

1. Digital scale, readability 0.1 g, e.g., Acculab L-Series 7200 scale (Acculab, Edgewater, NY)
2. Second timer used for timing of procedures (e.g., Cole Palmer model 5500 digital timer)
3. Tone to signal subjects to begin and stop sipping and spitting (e.g., wireless doorbell and chime, Radioshack)

2.2.2. Subjects

Participant eligibility is determined and informed consent is obtained prior to study participation. Subjects are told they are participating in a study designed to test the response of people with and without eating disorders to the taste of food without swallowing it. Participants are instructed to consume, on the day of the study, a standardized breakfast of approximately 300 kcal

featuring an English muffin, 6 oz of apple juice, and a pat of butter. They should remain fasting except for water until the time of study participation, which occurs 3–4 h following the breakfast and between 1100 h and 1300 h.

2.2.3. Study Procedures

Standardized instructions are read to the subjects just before the MSF test. Subjects are instructed to sip from the container on their left and to spit into the container on their right, without holding it in their mouths, swishing it around, or swallowing it. Subjects are told that the rate at which they sip and spit is entirely up to them and that there is no requirement or expectation for them to sip all of the solution presented. Subjects are cued to begin and stop sipping and spitting by a tone controlled by a research assistant in the next room.

Each solution is presented in an identical, opaque, unmarked, closed container that prevents visualization of the volume of solution during the 1-min trial. Identical containers are used to collect the liquid spit out. Subjects sip solutions through a straw and spit out the oral contents immediately into a funnel in the top of the spit container. The order in which solutions are presented is determined by randomizing the five solutions within each of the three blocks of trials prior to the study.

A taste test of each of the five solutions presented provides exposure to the solutions prior to MSF. Subjects taste and spit out each of the solutions and make ratings on VASs located below each solution. This should take no longer than 5 min to complete and is not timed.

Subjects are given a 1-min adaptation to the sipping and spitting process with water prior to the test. Then they are presented with one of the five solutions for 1 min. There are a total of 15 trials.

There is 1 min between presentations of the solutions. During this interval between each trial of sipping and spitting, subjects are asked to first make ratings on VASs (detailed above) to report the perceived intensities of sweetness and liking of the solution they just SF. After the test, VAS responses are measured to the nearest millimeter using a centimeter ruler.

Subjects then rinse their mouths with a baking soda-and-water solution to cleanse their palate. During this intertrial interval, the research assistant replaces the sip and spit containers that have been just used with fresh ones. In a separate room hidden from the subjects' view, the sip and spit containers are weighed before and after each trial to the nearest 0.1 g. Total solution sipped and spit out is calculated. If the total amount spit out is significantly more than the total amount sipped, it may be concluded that no solution was swallowed and the MSF test is valid. Total study duration is 1 h.

3. Notes and Anticipated Results

3.1. SF Results

One of the clinically important aspects of binge eating and purging is that it gets worse with experience. Figure 2 shows the results of using the SF rat to model that. Rats were tested for 11 weeks. During the first week, they were offered a milk diet to eat normally (RF) for 1 h once a day after 3 h of deprivation from their maintenance diet of pelleted chow and water. During the second through the seventh week, RF and SF tests were given for 1 h on alternate days. SF meals were significantly larger than RF meals and they increased in size in the second, third, and fourth weeks and then plateaued in the fifth to seventh weeks. During this time, RF meal size did not increase. In the eighth through the eleventh weeks, only SF tests were given and the size of the SF meal increased further.

The increase in meal size during SF across the weeks is due to extinction of conditioned orosensory satiating effects produced during RF (49) and to a conditioned increase of meal size produced during SF (57). Note that, as in BN patients, body weight remained normal throughout the 11 weeks because the rats had access to the maintenance diet and water except for the 3 h of food deprivation, and the 1 h SF or RF tests.

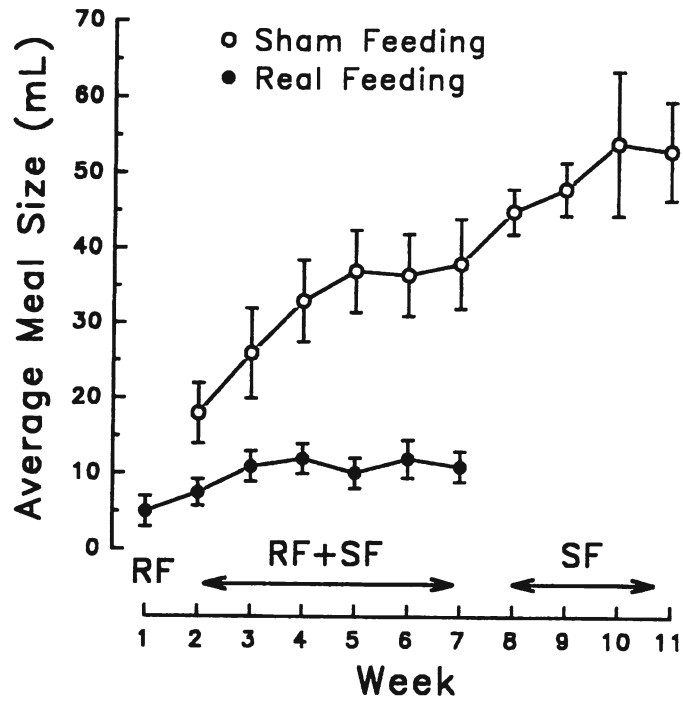

Fig. 2. The average meal size of a milk diet by 13 male rats during real feeding (RF) and sham feeding (SF) tests over 11 weeks. See text for details. Reproduced, with permission, from (56).

3.2. SF Trouble-shooting

There are two technical issues that require attention when using the SF rat. The first is incomplete drainage from the cannula. This can occur from not placing the gastric cannula at the most dependent part of the stomach during surgery. It can also occur if the pretest flushing of particles of food or hair from the stomach was incomplete. Some investigators do not use the collecting tube and just allow the gastric contents to drain out of the cannula. We do not recommend this because the drainage is acid and can irritate the skin over which it flows, and we have had more incomplete collections of gastric contents when we did not use a collecting tube of the type described.

The second issue is that in a minority of rats, the gastric cannula begins to be extruded. We have not been able to repair this surgically despite trying numerous procedures. Thus, we recommend that you remove the rat from the experiment when this occurs.

3.3. MSF Investigations

To investigate the feasibility of MSF, we measured the intake of a series of sucrose-sweetened solutions flavored with cherry Kool-Aid® 4 h after a standardized breakfast in 10 healthy women with no history of eating disorders (58). Subjects were presented twice with five solutions (0%, 2.5%, 5%, 10% and 20% sucrose) in distilled water, flavored with a constant concentration of cherry Kool-Aid®, in a random order (see Sect. 2.2 for details). Intakes of 5%, 10%, and 20% sucrose solutions were significantly larger than the intake of 0%, but intake of the 2.5% solution was not (Fig. 3). Despite this effect of sweetness on intake, there was no significant difference among the effects of 5%, 10%, and 20% sucrose on intake and only 10% sucrose produced a larger intake than 2.5% sucrose.

The effect of sweetness on intake was the result of only orosensory stimulation because subjects spit out significantly more solution than they sipped. This is evidence that subjects did not swallow the ingested solutions. Thus, there were no significant postingestive effects of the solutions—the critical criterion of MSF was satisfied.

In contrast to intake, there was a significant effect of sucrose concentration on the perceived intensities of sweetness and liking (Figs. 4 and 5). When the intake, sweetness, and liking data were averaged for the subjects across the five sucrose concentrations, there were significant correlations between intake and sweetness, intake and liking, and sweetness and liking.

The task of sipping and spitting was well tolerated by the subjects. Only one of the ten participants was unable to comply with the instructions to spit out all of the solution—her data were dropped from analyses. We concluded that the sip-and-spit modification of MSF was feasible in normal women and that it provided a quantitative measurement of the ingestion of solutions and their accompanying perceived intensities of sweetness and

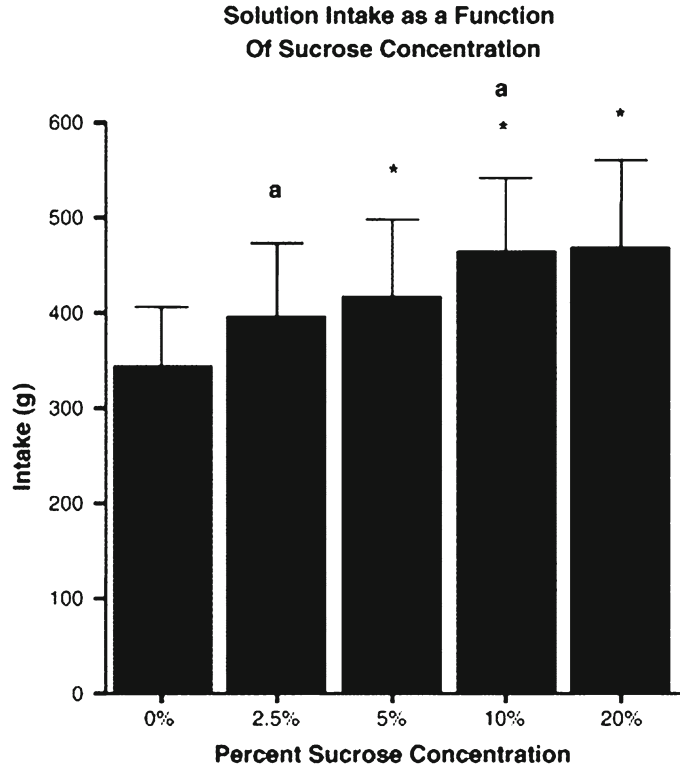

Fig. 3. Data are mean ± S.E.M. intake (g/2 min) from 9 subjects. Sucrose concentration produced a significant effect on intake. *Significantly larger than intake of 0% sucrose, $p < 0.05$. [a]Intake means differ significantly from each other, $p < 0.05$. Reproduced, with permission from (58).

liking produced by orosensory stimuli acting alone in the absence of postingestive effects of the solutions. Reassured by these results, we investigated the usefulness of MSF in women with BN and purging.

Use of the sip-and-spit MSF paradigm in women with BN required replacing the sucrose solutions that we had used in control subjects with aspartame, a low-calorie sweetener, because in preliminary experiments two of the three BN subjects reported liking the sucrose solutions very much, but they ingested little for fear of caloric absorption, despite spitting out all of the solutions. This highlights the importance of minimizing aversive misconceptions of MSF in BN subjects.

Thus, BN subjects were tested with five cherry-flavored Kool-Aid® solutions sweetened with aspartame in concentrations matched to the perceived sweetness of the sucrose solutions used in the feasibility study of normal women (0, 0.01, 0.03, 0.08, 2.8% aspartame, respectively). A taste test of each of the five solutions was done prior to the MSF test. This gave subjects experience with each of the test solutions before the test trials began.

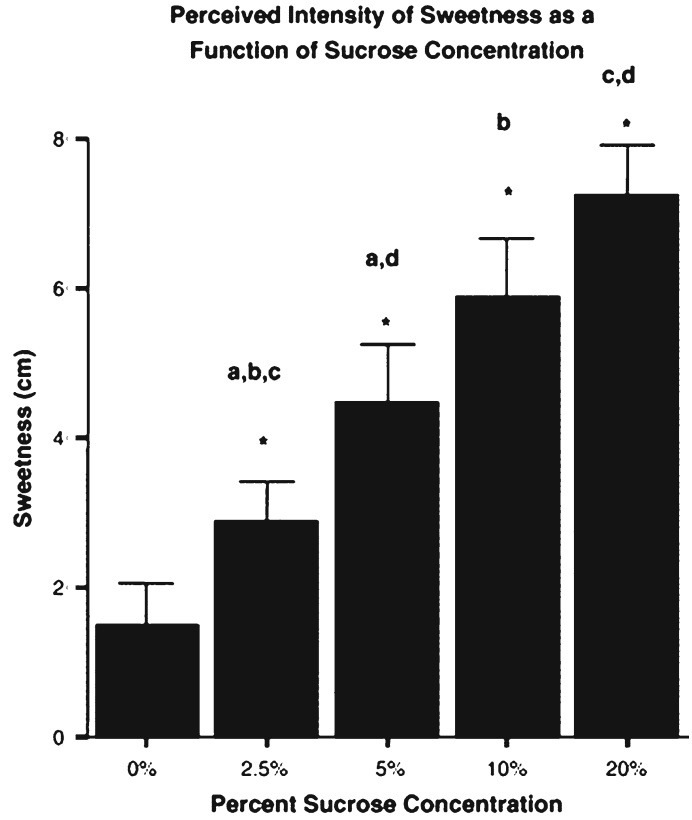

Fig. 4. Data are mean ± S.E.M. centimeters from 7 subjects. Sucrose concentration produced a significant increase in perceived intensity of sweetness. *Significantly larger than the perceived intensity of 0% sucrose, $p < 0.005$. [a–d]Pairs of means that share letters differ significantly from each other, $p < 0.05$. Reproduced, with permission from (58).

To increase the intraoral exposure to each solution and to investigate the possibility of changes of intake as a result of repetitive MSF tests, five 1-min MSF trials of the five solutions were given three times. BN subjects ingested 40–53% more of all the solutions than normal control (NC) subjects with no history of eating disorders run under identical conditions (Fig. 6). Although MSF intake of sweet solutions was a function of sweetener concentration, sweetness was not necessary for the increased intake in BN compared to NC because BN subjects also ingested significantly more of the unsweetened solution than NC subjects. In fact, when the difference in intake of the unsweetened solution was taken into account by expressing the intake of each sweetened solution as a function of difference from unsweetened intake, there was no significant difference between the intakes of sweet solutions by BN and NC (Fig. 7).

The self-reports of sweetness, liking, and wanting of the unsweetened and sweetened solutions were equivalent in BN and

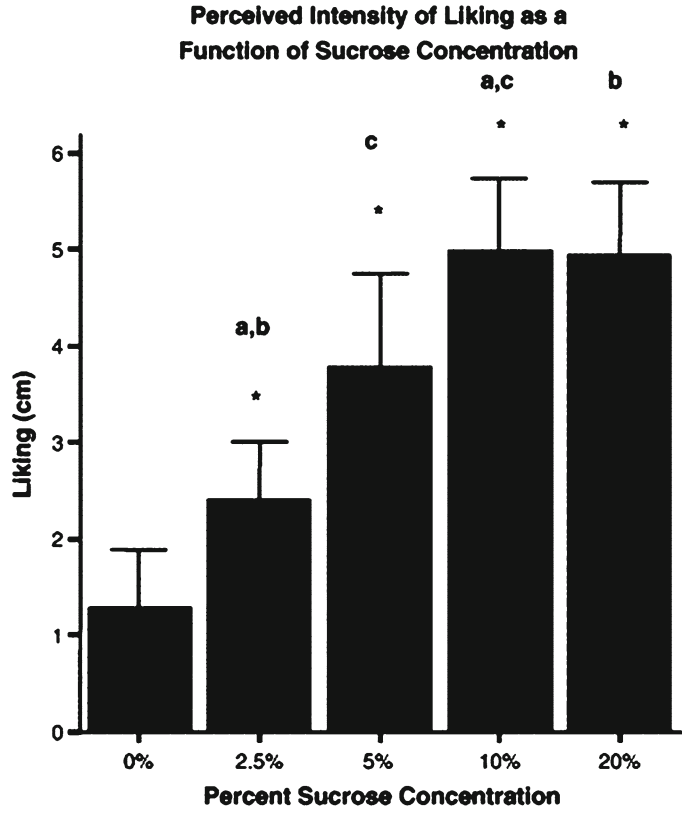

Fig. 5. Data are mean ± S.E.M. centimeters from 9 subjects. Sucrose concentration produced a significant increase in perceived intensity of liking. *Significantly larger than 0%, $p < 0.05$. [a,b,c]Pairs of means that share letters differ significantly from each other, $p < 0.05$. Reproduced, with permission from (58).

NC (Figs. 8, 9, and 10). This was consistent with the lack of difference of the transformed intakes of sweetened solutions as a function of aspartame concentration (Fig. 7). The lack of difference in the self-reports of the unsweetened solution, however, did not explain the increased intake of that solution by BN compared to NC (Fig. 6).

These results show the heuristic value of MSF in BN. BN subjects ingested more of all of the solutions than NC. This was the result of orosensory stimuli without any contribution from postingestive stimuli. This is the first demonstration that orosensory stimulation *alone* can produce hyperphagia in women with BN and purging.

Although the hyperphagia was observed with sweet solutions, sweetness was not necessary for hyperphagia because BN subjects also ingested more unsweetened solution than NC. This is a novel observation that requires replication. If it is robust, its reliability as a behavioral marker of BN and purging and its neuroscientific mechanism will invite further research.

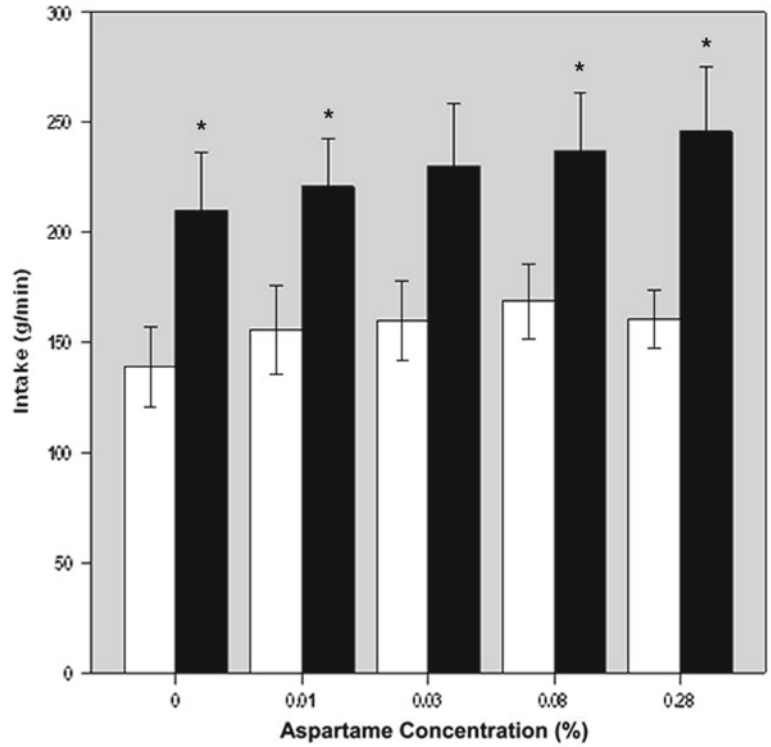

Fig. 6. *Bars* represent mean ±1 S.E. (error bars) grams of solution sipped, averaged over the three 1-min trials at each aspartame concentration. *Blank bars* are for normal control (NC) and *black bars* are for Bulimia Nervosa (BN) subjects. *Significantly greater intake by BN compared with NC subjects, $p < 0.05$. Reproduced, with permission from (59).

The hyperphagia of BN in MSF is apparently specific because it did not occur in patients with AN. AN patients showed decreased intake of the same unsweetened and sweetened solutions under identical MSF test conditions (60). Note that the majority of these AN patients had binge eating and purging. Thus, the increased or decreased intake of solutions during MSF correlates with the diagnosis of these two eating disorders, not just the prior occurrence of binge eating and purging.

3.4. MSF in People with and Without Eating Disorders

Several challenges were encountered with the human subjects. First, we found it necessary to conduct experimental procedures such that subjects were under constant observation by research staff, ideally through a one-way mirror or closed-circuit television. This was critical to ensure compliance with procedures, such as subjects' rinsing their mouths between solutions, and filling out VAS forms prior to rinsing. Several subjects in our studies needed reminders to do so after study procedures had begun.

Compliance with spitting solutions should also be checked by comparing weights of both spit containers and sip containers before and after each trial, as noted above in Sect. 2.2. Due to salivary

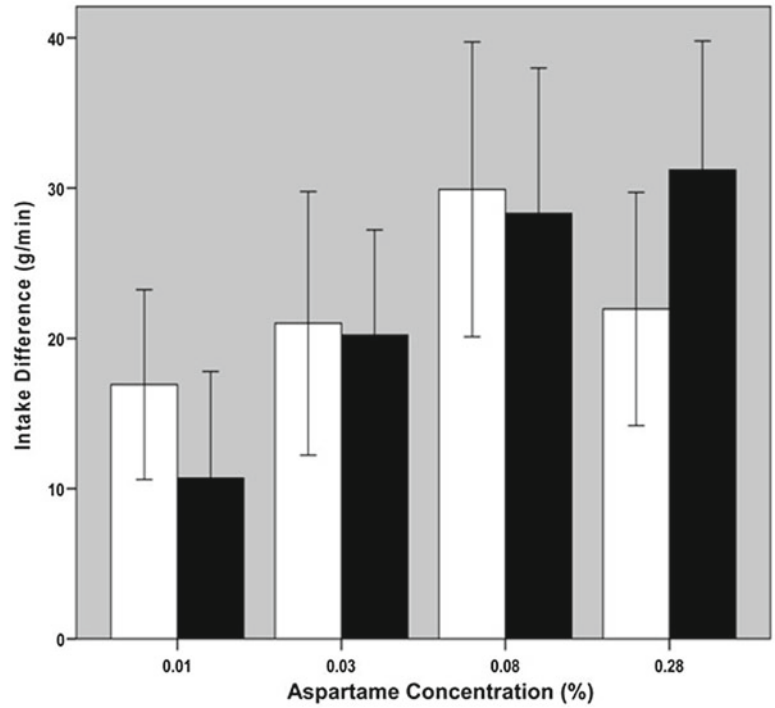

Fig. 7. *Bars* represent mean (±1 S.E.) difference in intake of each of the sweetened solutions (0.01–0.28%) and the 0% aspartame solution, in grams, averaged over the three 1-min trials. *Blank bars* are for normal control (NC) and *black bars* are for Bulimia Nervosa (BN) subjects. Reproduced, with permission, from (59).

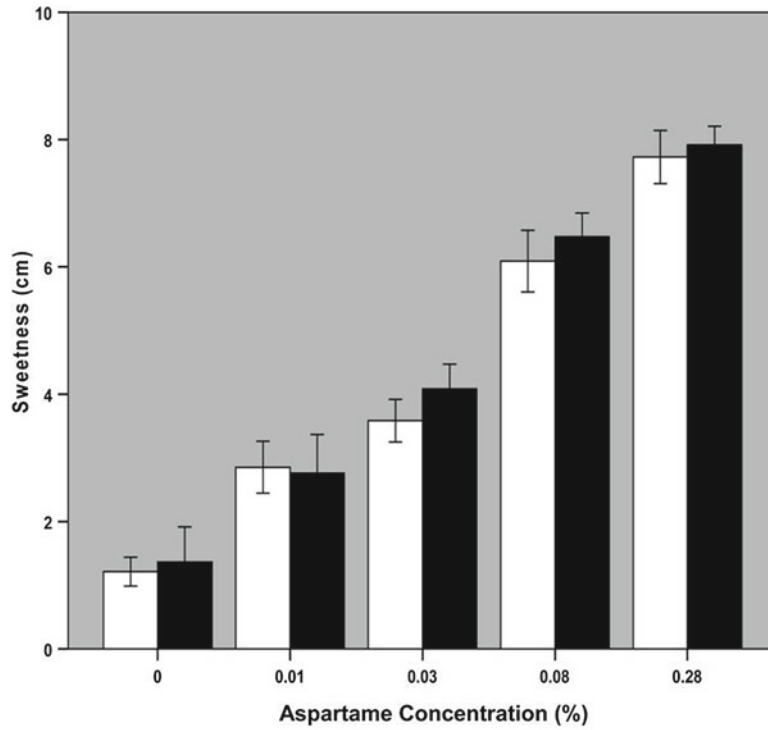

Fig. 8. *Bars* represent mean ± S.E. of self-reported sweetness ratings of solution at each aspartame concentration averaged over the three trials for 11 bulimia nervosa (BN) and 10 normal control (NC) participants. *Blank bars* are for NC and *black bars* are for BN subjects. Reproduced, with permission, from (59).

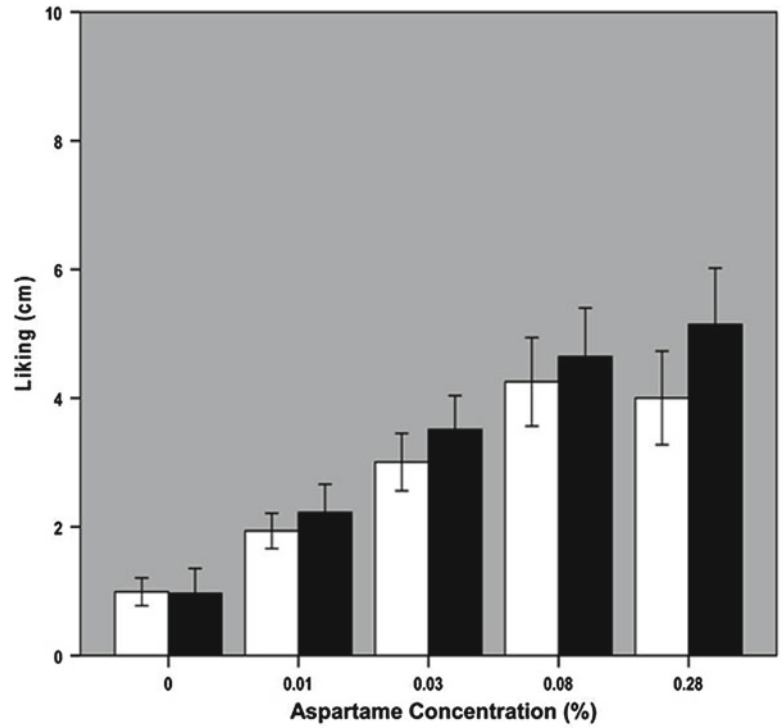

Fig. 9. *Bars* represent mean ± S.E. of self-reported liking ratings of solution at each aspartame concentration averaged over the three trials for 11 bulimia nervosa (BN) and 10 normal control (NC) participants. *Blank bars* are for NC and *black bars* are for BN subjects. Reproduced, with permission, from (59).

production, the amount spit should slightly exceed the amount sipped; we excluded subjects' data when total volume sipped exceeded total volume spit by 10% or more, due to possible postingestive effects from swallowed solution.

Subjects, particularly those with eating disorders, need to be told on more than one occasion that the test solutions contain no sugar. This we found to be critical while piloting the original sucrose MSF procedure with women with BN, two out of three of whom reported liking the solutions very much but sipped minimal amounts, reporting fear of absorption of sugar. We also encountered at least one pilot participant with AN who refused to consume aspartame solutions until being reminded that they contained no calories. Thus, in addition to including in the informed consent process the information that solutions are noncaloric, we found it important to specifically remind subjects immediately prior to study procedures that all solutions are prepared with aspartame and contain no sugar and zero calories.

We found it necessary to have at least one staff member dedicated to observing subjects during the intertrial interval and tim-

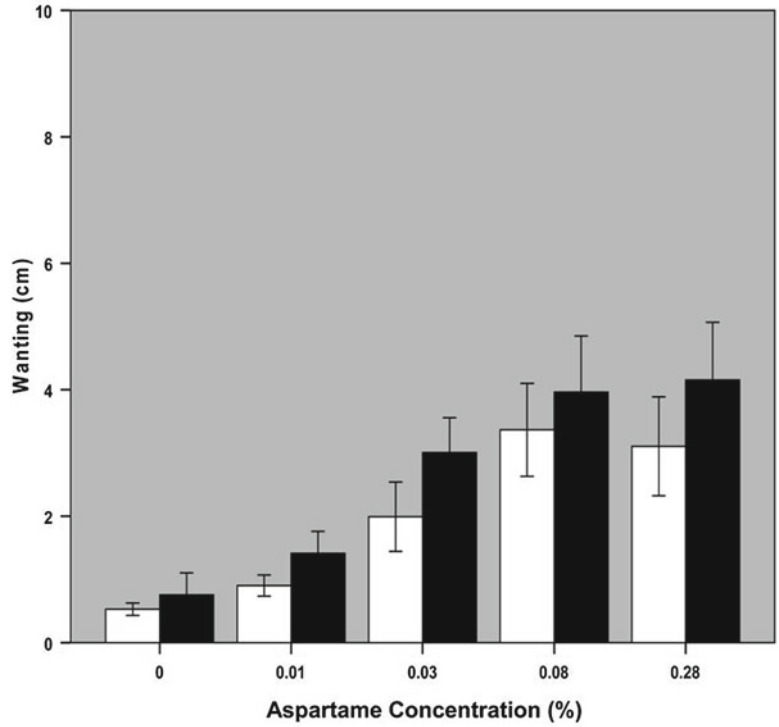

Fig. 10. *Bars* represent mean ± S.E. of self-reported wanting ratings of solution at each aspartame concentration averaged over the three trials for 11 bulimia nervosa (BN) and 10 normal control (NC) participants. *Blank bars* are for NC and *black bars* are for BN subjects. Reproduced, with permission, from (59).

ing the sipping and intertrial intervals, and a second member dedicated to removing the sip and spit buckets and record the weight of each of these in a separate room, removing the VAS scale and labeling the trial number, and then presenting the subject with the next set of sip and spit containers.

4. Conclusion

In summary the MSF results are important in three ways. First, they validate the usefulness of the SF rat as an animal model of binge eating and purging in women with BN. Second, they demonstrate a successful translation of that animal model into a heuristic technique for the study of BN, AN, and other eating disorders. Third, they are the first evidence that the phenotypical changes of food intake in BN and AN can be elicited by orosensory stimulation by food in the absence of concomitant postingestive stimulation. This shows that the various visceral abnormalities proposed as important in these clinical syndromes are not necessary for

obtaining the characteristic changes in intake under laboratory conditions. Thus, the extensive literature on the mechanisms of SF in the rat can now be mined for their importance in BN and AN.

Acknowledgements

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Chapter 11

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 the 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 allows 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 the potential to produce a "food addiction" 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 33% of the population (1), the study of aberrant eating, and specifically overeating, is important. Binge eating is a form of overeating that has been linked to obesity as well as bulimia nervosa and binge-eating disorder (BED) (2–4). Subclinical binge eating has also been identified and has been suggested to affect 10.8% of adolescent females (5). Binge eating may also be a predictor of body-fat gain among children, leading to a high risk for adult obesity (6). In addition, binge eating is associated with increased frequency of body weight fluctuation, depression, anxiety, and substance abuse (7–9). Unique among disordered eating is the epidemiological finding that instances of binge-eating are as prevalent in males as in females in both clinical

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and subclinical populations (10–13). 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.

In addition to the above-mentioned detrimental consequences of binge eating, it has recently been suggested that overeating, and perhaps binge eating, specifically, may result in a state that resembles an "addiction" to food (14). This stems from the idea that binge eating shares similarities with conventional drug addiction (15) 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 (16, 17).

Clinical accounts of food addiction have been reported (18), but it was not until recently that validated measures were designed to test the prevalence of food addiction. In doing so, the criteria in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) pertaining to substance dependence have been adapted to develop diagnostic criteria for "palatable food dependence." Surveys, such as the Eating Behaviors Questionnaire (19) and clinical interviews (20), have been used to identify addictive eating. However, the most specific measure of addictive eating is the Yale Food Addiction Scale (YFAS) (21), which corresponds to the criteria for substance dependence contained in the DSM-IV-TR, and assesses clinically significant impairment or distress from eating. In support of the link between binge eating and food addiction, the above diagnostic measures indicate that 52–92% of BED patients fit the criteria for being "food addicts" (20, 22).

1.2. An Animal Model of Binge Eating Sugar

Animal models of binge eating have proven important in effectively exploring the physiological, behavioral, and neurochemical aspects of binge eating seen in humans (see Chapter 1 of this volume). 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 that people binge most frequently on highly palatable foods that are rich in sugar and fat (23–25), this chapter will provide binge-eating models focused on these two macronutrients, independently and in combination.

The DSM-IV-TR 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 (usually within any 2-h period) (26). 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 one month (27). 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

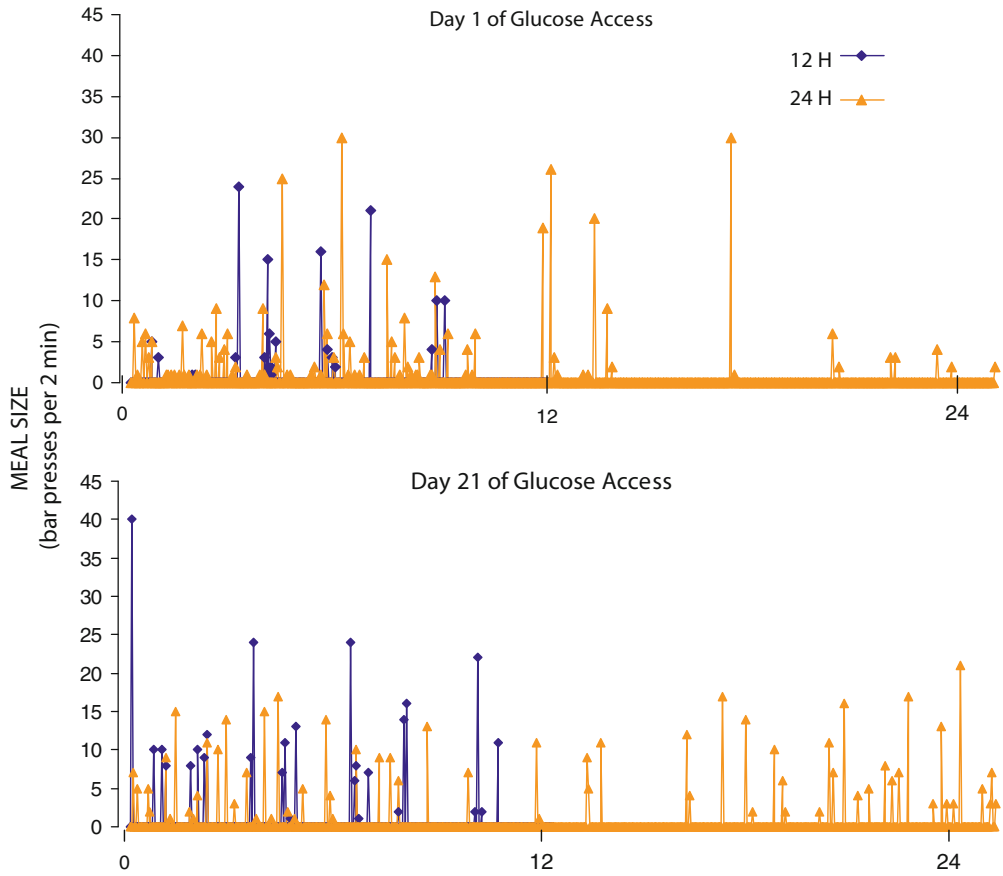

Fig. 1. Meal analysis of two representative rats living in operant chambers. The one maintained on daily intermittent sucrose and chow had increased intake of sugar compared with the one given ad libitum sucrose and chow. Hour 0 is 4 h into the dark phase. Each lever press delivered 0.1 mL of 10% sucrose. 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, followed by fewer, but larger meals, than the rat with sucrose and chow available ad libitum. Reproduced with permission from (28).

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 (Fig. 1, (28)). 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 per day).

Table 1
Findings that suggest multiple similarities between sugar addiction in animal subjects and drug addiction

Substance dependence	Animal model of sugar dependence
A. DSM-IV-TR	
Tolerance	Escalation of daily sugar intake (37)
Signs of withdrawal	Somatic signs (teeth-chattering, tremor) Anxiety measured by plus-maze Ultrasonic distress vocalizations(29, 30)
Consuming more than intended	Deprivation effect (31)
B. Behavioral signs	
Locomotor cross-sensitization	Amphetamine (32)
Proclivity to consume other drugs of abuse	Alcohol (33)
C. Neurochemical changes in the NAc	
Repeated release of DA	(34, 35)
↑ D1 receptor binding	(37)
↓ D2 receptor binding	(37)
↑ D3 receptor mRNA	(38)
↓ Preproenkephalin mRNA	(38)
DA/ACh imbalance during withdrawal	(29, 30)

Reproduced with permission from (40)

What is most interesting and unique about this model of sugar overeating is that it results in signs of dependence. This model has identified both behavioral and neurochemical commonalities between binge eating and drug use (Table 1). 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, 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 (29). Similarly, these signs of opiate-like withdrawal also emerge when all food is removed for 24 h (29, 30).

In the drug abuse literature, animals will self-administer more of the drug after an abstinence period, if the drug is made available again. In the sugar binge model, after 2 wks of forced abstinence from sugar, rats with previous binge access lever press for the receipt of sugar more than before, suggesting a change in the motivational impact of sugar (31). 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 (32).

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 (33).

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 (34). This unabated release of DA can be elicited by the taste of sucrose (35) and is enhanced when rats are at a reduced body weight (36). We have also shown alterations in DA receptor binding and gene expression in the binge model (37, 38). 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 (37). These animals also have a significant decrease in enkephalin mRNA in the NAc (38).

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 (39). 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 (29) or after 36 h of total food deprivation (30). 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 (29, 30, 33, 34) and female (31, 32) rats, ranging in age 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 $n = 8–10$ per group) of similar body weight (<10% variation between groups) and individually housed. There is some variability in chow and palatable food intakes between rats, so it is advisable to use at least 8–10 rats per group. Some potential control groups will be discussed in Sect. 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, etc.) 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.

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. Wire bottom cages (or wire inserts added to solid bottom cages) are preferred because solid bottom cages retain the animals' feces and urine, which introduces confounding factors into the experiment. If using solid bottom cages, use a noncaloric bedding. Consumption of bedding material, which is often caloric in nature, and fecal boli make 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 (41), potentially confounding the study results. 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 (31, 37) and sucrose (28, 30, 31, 33, 36, 42). 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 1,000 mL with tap water. Prepare only enough sugar solution for each day. Store extra solution at 4 °C for a maximum of three days, otherwise bacteria and mold can begin to form. Drinking bottles for sugar solutions should be emptied, rinsed, and refilled with new solution each day to avoid bacterial 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 tubes (e.g., glass drinking tubes (Lab Products, Inc.) or tubes made from 100-mL polyethylene graduated cylinders (Fisher Scientific) by cutting off the flange and filing the top flat). Tubes should be sealed with rubber stoppers

with steal ball tips (e.g., Lab Products, Inc.). These are preferred for providing fluid to the rats because they prevent unintentional fluid spillage. Drinking tubes 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 one month, some rats may increase sucrose consumption to a degree that larger drinking tubes (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 #5001, PMI Nutrition International, Richmond, IN; 10% fat, 20% protein, 70% carbohydrate, 3.01 Kcal/g) 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 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 (28).

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

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 binge 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 control 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 one 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 requires approximately 1 h per day.

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

Corwin and colleagues have a well-developed model of binge consumption of vegetable fat (see Chap. 4). 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 21–28 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 (43).

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, #12451; 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 sweet-fat chow and water. Food should be reintroduced each day 4 h into the dark period. Diets should be maintained for 21–28 days and intake should be recorded and analyzed as described above. Control groups, as described above, should be considered (43).

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) (43).

3. Notes

3.1. Types of Palatable Foods Offered

Although validated only using the presented parameters, this 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, 44). As previously mentioned, we have also explored a variety of food consistencies, including liquids (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 differences that have been identified in the consumption of liquids as compared to solid foods (45). 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 to. Lastly, we have studied various macronutrient compositions. 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 (46), it is not representative of the human condition, as 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 using this method is the effect of food access on body weight. 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 (47). 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 (48). 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) (36).

3.3. Access Period

The last variable that we have modified in the described method is the length of the 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 (49, 50). 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 less palatable, but healthier alternative (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 (29, 30, 33, 34) and female (31, 32)) 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, in similar proportions (10–13), making gender studies crucial. Clinically, adolescent females are more likely to demonstrate disordered eating than age-matched males or adults (5, 51). Further, adolescents have been shown to be more susceptible to addictive behavior (52). 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 these can also 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 (43). Also, certain drugs are effective at suppressing binge eating of fat-rich foods, but not sugar-rich foods (53). 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. Conclusion

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 and provides a model in which potential pharmaceutical interventions can be explored.

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Chapter 12

Deep Brain Stimulation for the Treatment of Binge Eating: Mechanisms and Preclinical Models

Casey H. Halpern, Mark Attiah, and Tracy L. Bale

Abstract

The prevalence of obesity continues to rise despite advances in behavioral, pharmacological, and surgical treatments. This is likely in part due to the overabundance of highly caloric food, which has extremely rewarding properties associated with dopaminergic neurotransmission in the ventral striatum where the nucleus accumbens (NAc) is located. The NAc has been repeatedly implicated in reward-seeking disorders, including binge eating, a common feature of obesity. Altered expression of dopaminergic receptors in the NAc has been associated with binge eating both in animals and humans. The application of deep brain stimulation (DBS) to the NAc to suppress binge eating in mice may further implicate the dopamine system in aberrant eating behavior. Molecular, biochemical, and optogenetic studies of the mechanism of DBS may also shed light on future treatment strategies for binge eating. Furthermore, given that DBS is a commonly used surgical therapy for multiple neurological disorders, this work may also pave the way for expanding the application of DBS to obesity.

Key words: Deep brain stimulation, Binge eating, Obesity, Nucleus accumbens, Reward

1. Introduction

Obesity affects more than 30% of adult Americans and 300 million people worldwide (1, 2), conferring an increased risk for multiple medical comorbidities associated with a lower quality of life and decreased life expectancy (3, 4). First-line treatments such as behavioral and lifestyle modifications frequently result in suboptimal weight loss and dietary relapse with subsequent weight gain (5). Clinicians have thus turned to cognitive-behavioral therapy or pharmacotherapeutic agents (6, 7). To date, such strategies have only provided temporary benefit, though future investigation will certainly better define the role these approaches will play in obesity management (5, 8). Currently, the most effective surgical treatment for obesity is Roux-en-Y-gastric bypass, which is associated

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with a substantial loss of mean excess weight of at least 45% (9). However, a significant number of these patients will regain weight in long-term follow-up, and features of metabolic syndrome frequently return (10, 11). The treatment-refractory nature of obesity therefore mandates careful consideration of novel therapies, most notably involving modulation of brain regions associated with feeding behavior and overconsumption, including reward neurocircuitry.

The hypothalamus is important in regulation of feeding behavior, and evidence from neuroimaging corroborates these findings in humans (12). In addition to eating for basic caloric need, a significant contributor to the obese state is the consumption of calorically dense, highly palatable food (13). The reinforcing properties of such a diet are predisposing toward overconsumption, and are largely mediated by the dopaminergic system, in which dopaminergic cell bodies originating in the ventral tegmental area of the midbrain project to the limbic system via synapses in brain regions including the nucleus accumbens (NAc) (14). This neural circuitry of brain reward has been previously well described to underlie addiction, suggesting common neural and molecular pathways underlying the reinforcing properties of substances such as drugs of abuse and highly palatable food (15). Studies in rodents have revealed significant elevations in levels of various downstream markers of dopamine signaling in the NAc following exposure to a chronic high fat diet (15). As a demonstration of the powerfully rewarding properties of a high fat diet, mice acutely withdrawn from this diet opted to endure an aversive environment in order to access this highly palatable food despite having a lower fat, less preferred diet available in a safer environment (16). Significant downregulation of striatal dopamine receptor availability has also been demonstrated in rodents chronically exposed to high fat food, suggesting chronic increases in dopaminergic transmission may induce downregulation of postsynaptic dopamine receptors (17). Using functional neuroimaging techniques, increased activity in the NAc has also been found in humans in anticipation of and in response to visual cues depicting a high fat stimulus (18, 19). Subsequent work in obese subjects by Wang and colleagues demonstrated decreased striatal uptake of a dopamine type 2 receptor radioligand, findings that complement evidence of decreased dopamine receptor expression from animal studies (20). These findings from positron emission tomography imaging suggest that obese individuals have a relative "dopamine deficiency," which may be a predisposing factor in pathologic eating to compensate for decreased dopamine receptor availability.

Clinical trials have been conducted and are ongoing to study the role of surgical neuromodulation in treating medically refractory psychiatric conditions such as depression and obsessive–compulsive disorder using deep brain stimulation (DBS) (21, 22). Of

note, DBS is a surgical technique currently approved by the US Food and Drug Administration to treat various human neurologic conditions such as Parkinson's disease (23). While the precise mechanism is not clear, DBS appears to reversibly modulate neural circuits (24). Clinically, the effects of high-frequency DBS mimic precursor ablative procedures. However, there is evidence to support both stimulatory and inhibitory actions (25, 26). This paradox may be explained by computational models of basal ganglionic circuitry, which have suggested that at high frequencies, DBS effectively interrupts information within neuronal circuits (27). Nevertheless, the ability to adjust, titrate, and even reverse the "lesioning" effect of DBS makes it not only a more attractive therapy than its precursor ablative procedures, but also safer to test in clinical trials (28, 29). There is a current growing body of scientific and radiologic evidence motivated by medical need to support expanding the application of DBS to other neurologic and neuropsychiatric disorders (30). Below, we discuss the potential application of DBS to certain patients suffering from treatment-refractory obesity.

1.1. Obesity and Binge Eating

Addressing the neural basis of the rewarding value of highly palatable food with DBS may provide benefit to those obese subjects who have failed all available treatments. However, to date no clinical or preclinical studies have investigated targeting the dopaminergic system with DBS to suppress overconsumption and lead to weight loss. Binge eating is a very common feature of obesity and is associated with alterations in dopamine signaling in preclinical studies and in humans (31, 32). Binge eating is characterized by a "loss of control" and defined as significant food intake in a short period of time not driven by metabolic need (33, 34). The potential to modulate dopamine levels with DBS presents an interesting approach to studying this very common feature of obesity. Inhibition of binge consumption with DBS in animals would also provide the opportunity to study contributing DBS mechanisms at molecular and biochemical levels. In addition, the availability of gene-targeted transgenic and knockout mouse lines provide valuable tools in pursuing such investigation allowing for more rigorous, mechanistic work, prior to advancing this research into larger animals.

Examination of NAc DBS to suppress binge eating in mice would provide insight into the direct modulation of dopaminergic release in this region. Binge eating in humans has previously been shown to be related to multiple factors, including caloric restriction, limited access to highly palatable food, and stress and reward dysregulation (35, 36). Thus, studies have examined binge eating in rodents in these contexts.

Food restriction has been shown to both increase the risk of binge eating in humans and prolong this aberrant feeding behavior (37, 38). Moreover, repeated cycles of food restriction are predisposing to binge eating in mice (39). Caloric restriction also enhances sensitivity

to stressors, which in turn also promotes binge eating (40). The stress associated with caloric restriction alone leads to long-term alterations in genes critical in feeding and reward circuitry that influence food intake. Specifically, Pankevich and colleagues revealed that caloric restriction was associated with long-term changes in expression and epigenetic regulation of the stress hormone, corticotropin-releasing factor, in the bed nucleus of the stria terminalis, a brain region with direct projections to the NAc (40).

The limited access model of binge eating is based on the findings that human subjects tend to binge on "forbidden" foods not consumed during typical meals in the absence of real hunger (41, 42). This limited access protocol does not utilize caloric restriction, but rather time-restricted access to highly palatable food. Regular or intermittent access where limited quantities of highly palatable food are available on a regular or an irregular schedule (i.e., daily vs. every other or every third day) are two strategies to approach this model (43). The limited intermittent access approach produces a marked increase in binge eating over regular limited access in rats (43). However, such a model takes a significant time investment to produce stable binge eating patterns (44, 45). The regular limited access approach is simple to execute and rapidly induces more binge eating in mice than those given an ad libitum diet (46). This effect is maintained over several weeks without habituation (46). Daily limited exposure may also mimic clinical experience more relevant to humans who binge eat on a daily basis (47).

In humans, increased stress levels have been associated with a predisposition to binge eat (35, 48). Indeed, increasing levels of stress in daily life influence eating behavior and the adaptation of highly palatable food as "comfort food" (48, 49). Behavioral studies in rats have shown that stress from foot shock induces binge eating (14, 50). Moreover, stress-sensitive mice deficient in one of the corticotropin-releasing factor receptors, corticotropin-releasing factor receptor-2, showed an increase in binge eating in a limited access paradigm following a chronic stress protocol (51). Links between stress and reward pathways may underlie this increased sensitivity to a highly palatable diet, as consumption of a high fat diet has been shown to reduce expression of the stress hormone, corticotropin-releasing factor (15).

2. Materials and Procedures

2.1. Binge Eating Suppression with Deep Brain Stimulation

NAc DBS is hypothesized to suppress binge eating. To test this hypothesis, a diet-induced obesity-prone strain of mice such as C57Bl/6 can be used. Stereotactic implantation of a bipolar electrode into the NAc allows for precise targeting of this very small

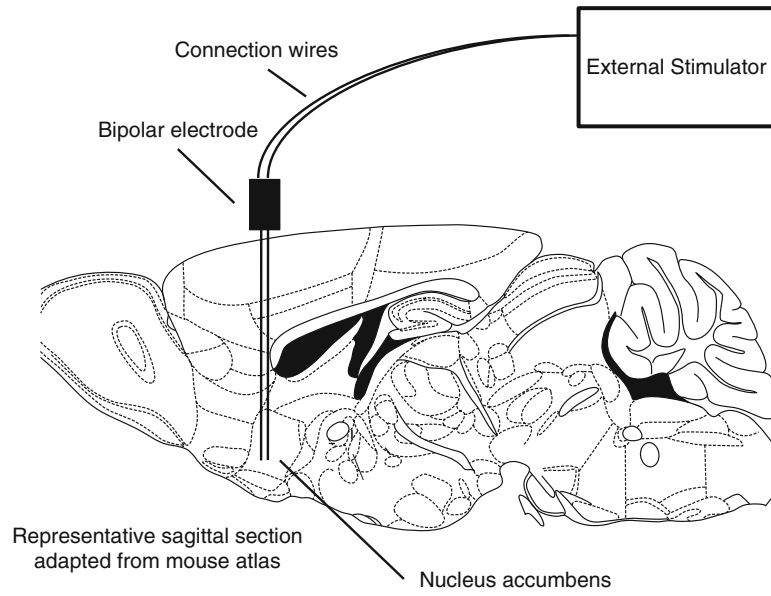

Fig. 1. Schematic representation adapted from the Paxinos and Watson atlas of a bipolar electrode implanted into the mouse nucleus accumbens and externally connected to a stimulator.

region, which is approximately 1.5 mm^3 in mice according to the Paxinos and Watson atlas. Following a recovery period after surgery, mice are given regular limited access to a very high fat diet (60% fat content) food for 1 h each day for 7 days. An incremental increase in consumption is expected until a stable level of binge eating is seen prior to initiating DBS. Binge eating should be defined as greater than 25% of daily caloric intake within this 1-h period. It is important to observe stable binge eating so that a stimulated response can be easily interpreted.

Mice undergoing DBS are attached externally to a stimulator by connection wires, which will be connected to the implanted electrodes (Fig. 1). Mice will be connected to these wires for this 1-h period daily, but disconnected at the end of the binge. DBS is turned on once mice reach a stable level of binge eating over the course of 7 days. On days when stimulation is turned on, a current of 150 μA (60 μs, 160 Hz) is administered. These stimulatory parameters have been used frequently in both preclinical and clinical studies (52, 53). To minimize and control for additional stressors, mice should be stimulated in their home cage. To assess biochemical and molecular changes associated with DBS using immunohistochemistry, western blotting, and polymerase chain reaction, mice are killed immediately after the 1-h stimulation period.

3. Notes

While DBS allows us to study the role of various brain regions in aberrant behavior, it remains unclear which cells are modulated by DBS or how (24). Ninety percent of the neurons in the NAc are medium spiny neurons (54). As previous studies have demonstrated abnormal expression of dopamine D2 receptors in human subjects who binge eat (55, 56), studying the role dopamine plays in perpetuating binge eating is important. Testing whether DBS modulates the expression of dopamine receptors on medium spiny neurons has potential implications on future pharmacologic approaches to obesity management. One way to target such receptors in a cell-specific manner is by incorporating optogenetic techniques (57). Combining biochemical and molecular assays with optogenetic neuromodulation using various transgenic mouse lines expressing Cre-recombinase or viral vectors with Cre-inducible opsins, for example, would provide substantial insight into the mechanism of action of DBS.

4. Conclusion

The future implications of this work may have profound clinical relevance. DBS has evolved over the past two decades as the treatment of choice for well-selected patients with medically refractory movement disorders such as advanced Parkinson's disease, essential tremor, and dystonia (23). The success and safety profile of DBS in treating movement disorders has increased the investigation into and expansion of its application to other neuropsychiatric conditions such as depression, substance abuse, and Tourette's syndrome (58, 59). Pilot studies are underway for obesity, and our studies may provide further impetus for larger animal studies and eventual translation to the human condition.

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Chapter 13

Saccharin Preference in Rats: Relation to Impulsivity and Drug Abuse

Marilyn E. Carroll, Nathan A. Holtz, and Natalie E. Zlebnik

Abstract

In recent years, rats selectively bred for high (HiS) or low (LoS) saccharin intake have provided valuable information regarding vulnerability to drug and food dependence, related affective disorders, and impulsive behavior. The HiS and LoS rats are models of the heritability of maladaptive behaviors, including hallmarks of drug dependence, bingeing, and withdrawal, which 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 will be presented regarding the importance of reactivity to aversive events in predicting drug abuse in HiS and LoS rats and other addiction-prone and -resistant phenotypes. Recent data from animal models also suggest that the addiction-prone and -resistant groups (e.g., HiS, LoS) respond in opposite ways when treated for drug abuse. Thus, assessing the severity of vulnerability may be an important consideration in designing treatment strategies for drug abuse in humans. This review focuses mainly on the behavioral overlap between drug abuse and excessive behavior directed toward natural rewards. The neurobiological bases for this interaction between drug and nondrug rewards have also been reviewed by others.

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 (1). 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

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dependent (2, 3) 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 (4)). Additionally, animal experiments have offered convergent evidence for genetic influence in addiction vulnerability (5–7).

One approach in the animal literature involves assessing variability in drug-related measures (e.g., self-administration, place preference, locomotor activity, etc.) in rats from genetically heterogeneous, outbred stocks. In a seminal study by Piazza et al. (8), 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 (9).

That approach was refined further by Deroche-Gamonet et al. (10), 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 are 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 (7, 11), including sensation seeking (12), stress response (13, 89), and aspects of impulsivity, such as behavioral disinhibition (14) and impulsive choice (15), binge eating (189), and sign tracking vs. goal tracking (192). 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. These interrelated behaviors and how they relate to drug abuse have been previously reviewed (5, 17, 189–191). 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, adolescents vs. adults) 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 foot-shock, 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 (5, 16, 17) for reviews).

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 influence 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 (18), 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 also 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 Sect. 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 (19). 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. For example, 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 lever 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, 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 0.2 mg/kg cocaine dose is used instead of 0.4 mg/kg, 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 (19)).

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 the last 5 consecutive days. This short-access self-administration is typically stable for long periods of time and often does not increase over several weeks (see (20, 21)).

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, LoS or LoI, respectively (22, 23). A similar effect is found in female vs. male rats (24, 107, 174) and adolescent vs. adult rats (118).

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 this process and 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 remove the rat to another environment during the extinction period to examine context-induced reinstatement when it is placed back 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 (5, 25, 26)).

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 alterations in sensitive behavioral baselines with food or a nondrug substance (17, 23). Other measures of withdrawal, such as withdrawal-potentiated startle and taste aversion conditioned during withdrawal, will be discussed in Sect. 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 (5, 16, 17)).

2.9. Selection and Selective Breeding 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 (5, 16, 17). 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, 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, 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. (27) and Deroche-Gamonet (28), 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 (5, 17). 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 (21) and escalated cocaine intake at faster rates (22) compared to rats screened for low impulsivity. Using another measure of impulsivity, the five choice serial reaction time task (5-CSRTT), Economidou et al. (29) 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) (9, 30), attribution of incentive salience to reward-related stimuli (sign trackers (ST)) vs. attention focused on reward location (goal trackers (GT)) (31), and varying interest in natural rewards such as high (HiR) or low (LoR) exercise in a running wheel (32). Table 1 summarizes individual differences that have been selected with respect to drug-motivated behavior or 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 not 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, 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

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

Selection criterion (High vs. low vulnerability)	Drug-related behavior	Drug/ reinforcer	Reference
Novel environment reactivity (HR vs. LR)	Drug-induced locomotor activity (HR > LR)	Amphetamine	(27)
		Morphine	(28)
	Self-administration (HR > LR)	Amphetamine	(27)
Impulsivity: delay discounting (HiI vs. LoI)	Acquisition (HiI > LoI)	Cocaine	(33)
	Acquisition (HiI > LoI)	Ethanol	(34)
	Escalation (HiI > LoI)	Cocaine	(35)
	Reinstatement (HiI > LoI)	Cocaine	(33, 36)
Impulsivity: 5CSRTT (HiI vs. LoI)	Acquisition (HiI > LoI)	Cocaine	(37)
	Self-administration (HiI > LoI)	Cocaine	(37)
	Reinstatement	Cocaine	(29)
Incentive salience (ST vs. GT)	Self-administration (ST > GT)	Cocaine	(31)
	Reinstatement (ST > GT)	Cocaine	(31)
	Locomotor sensitization	Cocaine	(38)
	Impulsivity (5-CSRTT; GT > ST)	Food	(39, 40)
Wheel running (HiR vs. LoR)	Self-administration (HiR > LoR)	Cocaine	(32)
	Reinstatement (HiR > LoR)	Cocaine	(32)

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

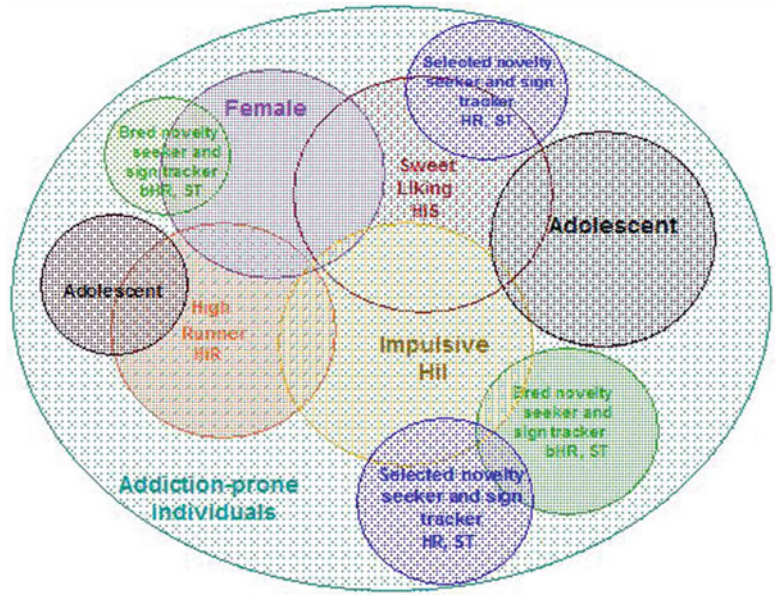

Fig. 1. Hypothetical overlap of addiction vulnerability factors that apply to food, exercise, or drug rewards.

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	Reference
Ethanol consumption (HAC vs. LAC)	Impulsivity (delay discounting; HAC > LAC)	Saccharin	(41)
	Impulsivity (probabilistic discounting; HAC > LAC)	Sucrose	(42)
	Impulsivity (response disinhibition; HAC > LAC)	Sucrose	(43)
	Exercise (HAC > LAC)	Wheel-running	(44)
Novel environment reactivity (HR vs. LR)	Acquisition (HR > LR)	Cocaine	(11)
Active-avoidance learning (RHA vs. RLA)	Acquisition (RHA > RLA)	Cocaine	(45)
	Locomotor Sensitization (RHA > RLA)	Cocaine	(46)
	Impulsivity (delay discounting; RHA > RLA)	Food	(40)
	Impulsivity (response disinhibition; RHA > RLA)	Food	(40)
	Novel environment reactivity (RHA > RLA)	—	(46)
Lewis vs. Fischer 344	Acquisition	Cocaine	(47)
	Self-administration	Cocaine	(48)
		Ethanol	(49)
	Locomotor sensitization	Cocaine	(50)
	Reinstatement	Cocaine	(51)

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

animals that exhibit extremely high or low measures, it can be shown that the behavior of interest is under genetic influence, because successive generations show more stable and robust corresponding behaviors. A prominent example of this procedure is found in an alcohol study in which breeding for differential drug intake was conducted by selecting animals on measures of high and low ethanol intake (52). Subsequently, multiple rodent strains have been selectively bred based on varied behavioral and physiological responses to alcohol in humans (53, 54). Such criteria have included alcohol consumption (55, 56), sensitivity to withdrawal (57), ethanol-induced hypothermia (58), and locomotor reactivity (59).

The selective breeding approach was also applied to other drugs of abuse, such as diazepam (60), opiates (61), methamphetamine (62, 63), nicotine (64), and cocaine (65). 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 (41, 43), as well as tasks of impaired response inhibition (43). High (vs. low) alcohol-consuming animals also showed greater avidity for natural rewards, such as exercise (44).

In addition to selective breeding for intake of a substance (e.g., alcohol intake), the procedure of selectively breeding for high and low phenotypes has been applied to behaviors that are related to drug addiction (Table 2). 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. (27) in outbred rats. Rats selectively bred for high activity in this paradigm acquired cocaine self-administration more rapidly than those bred for low activity (11). The two behaviors that have received the most attention are binging on food (food addiction) (5, 66, 67) and impulsivity for food (see reviews by (37, 68, 69)). 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 focus of the remainder of this chapter.

In some cases the traits that predict drug abuse are bidirectional; for example, many of the animals screened or selectively bred 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 to sucrose (70) compared to LR rats, while rats selected for HiI using the 5-CSRTT show more operant responding to sucrose compared to LoI rats (71). Further, Roman high avoidance (RHA) rats selectively bred for high rates of active avoidance learning consume more sweetened dietary substances, such as a saccharin solution, compared to those selectively bred for low rates of avoidance learning (RLA) (72, 73).

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 (74–77). Conversely, rats screened for high consumption of sweet substances ingest more ethanol (78), amphetamine (79), and morphine (80) 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 (81), nicotine (82, 83), opioid (84), and

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	Reference
Novel environment reactivity (HR vs. LR)[a]	Operant responding (HR > LR)	Sucrose	(70)
Impulsivity (5CSRTT; HiI vs. LoI)[a]	Operant responding (HiI > LoI)	Sucrose	(71)
Active-avoidance learning (RHA vs. RLA)[b] Roman high vs. low avoidance	Consumption (RHA > LHA)	Saccharin	(72)
Ethanol consumption (HAC vs. LAC)[b]	Consumption (HAC > LAC)	Saccharin	(74, 76)
	Consumption (HAC > LAC)	Sucrose	(75)
Ethanol consumption (HAC vs. LAC)[a]	Consumption (HAC > LAC)	Saccharin	(77)
Sweet preference (SL vs. SDL)[a]	Consumption (SL > SDL)	Ethanol	(78)
	Self-administration	Amphetamine	(79)
	Self-administration (SL > SDL)	Morphine	(80)

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]Selected from outbred stocks
[b]Selectively bred

alcohol (85–87) users experience greater hedonic effects of sweetened dietary substances than nonaddicts.

Similar to addiction vulnerability, response to sweets also has a heritable influence (88–92). 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 (93, 94). Further, both alcohol-naïve individuals and alcoholics with familial histories of alcoholism display greater sweet preference than those without family histories of alcoholism (95). 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 (96) 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 (97–100); however, their avidity for drug intake was not investigated. Later, Dess and Minor (101) 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:

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

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 (67). These results prompted further investigation into differences in drug self-administration with the HiS and LoS lines. For example, Carroll et al. (20) 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, etc.) 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 by (5)). For example, as shown in Table 4, HiS rats exceed LoS rats in all phases of drug abuse, from acquisition to maintenance (20), escalation (23, 108, 111), and reinstatement (23, 107).

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 (101), cocaine-induced behavioral sensitization (103), and impulsivity for food and/or cocaine during a motor impulsivity task (106), and a delay-discounting choice task (105). 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 (Table 4) substantiated the drug 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 (5).

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	Reference
Acquisition	Cocaine	HiS > LoS	M only	(20)
Adoles vs. adult	Cocaine	HiS > LoS	M only	(102)
Maintenance	Heroin	HiS > LoS	F > M	(20)
	Cocaine	HiS = LoS	M = F	(103, 104)
Escalation	Cocaine	HiS > LoS	F only	(23)
Impulsivity (Delay discounting)	Food	HiS > LoS	F > M	(105)
	Cocaine	HiS = LoS	F = M	(105)
Impulsivity (Response disinhibition)	Food	HiS = LoS	F = M	(106)
	Cocaine	HiS > LoS	F > M	(106)
Extinction	Cocaine	HiS > LoS		(23)
Reinstatement	Cocaine	HiS > LoS	F only	(23)
Adoles nic exposure/ adult coc	Cocaine	HiS > LoS	F only	(107)
Adoles vs. adult	Cocaine	HiS > LoS	F only	(108)
Drug-induced locomotor activity	Cocaine	HiS > LoS	F > M (HiS)	(103)
Drug-induced locomotor sensitization	Cocaine	HiS > LoS (F)	F > M	(103)
Dysregulation of dose selection	Cocaine	HiS > LoS	F only	(104)
Treatment				
Baclofen on escalation	Cocaine	LoS > HiS	F only	(108)
Progesterone on escalation	Cocaine	LoS > HiS	F only	(110)
Histamine punishment	Cocaine	LoS > HiS	F only	(109)
Neurobiological studies C-fos	Cocaine inj	LoS > HiS	M only	(36)
Orexin cell labeling in hypothal	Coc/Sal inj	HiS > LoS	F only	(109)

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

2.9.4. Monitoring and Selecting for Impulsivity, a Trail that Is Closely Associated and Additive with Sweet Preference and Drug Abuse

As indicated in Fig. 1, another factor that is a strong determinant of drug addiction is impulsive behavior (68, 112–115).

Impulsive Choice-Delay Discounting

Impulsivity is an individual characteristic that has many parallels with differences in sweet preference (e.g., HiS, LoS); however, there are also unique determinants of vulnerability to drug abuse. The method that has often been used 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 adjusted 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 9 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 (21), 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 (21).

Selecting rats for HiI and LoI choice on a delay-discounting task (21, 69, 112) or on premature responding on a 5-CSRTT (68, 116) has led to the same degree of predictability of addiction-prone (HiI) and -resistant (LoI) behavior as described for the bred HiS vs. LoS lines (e.g., (21)). 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 (33). During long access to cocaine, HiI rats showed significant escalation, while LoI did not change (22). 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 (33).

When HiS and LoS rats were tested on delay-discounting tasks for food or IV cocaine infusions (21), 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.

Go/No-go Test of Impulsive Action

In another procedure, the Go/No-go or signaled reward and nonreward task, rats were compared on alternating Go components, when a signal indicated food or drug reward, and No-go components when a different stimulus signaled nonreward. The measure of impulsivity was number of responses that occurred during the signaled nonreward component (No-go) (106). 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 (110) 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 to a cocaine priming injection than adults or LoS rats. With regard to natural rewards, adolescent rats exhibited greater positive taste reactivity to (117) and consumed more of 6% (118) and 10% (117) 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 vs. 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 duration of the infusion are considered unreinforced responses (119) or ineffective responses (47). 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 phenotypes is responding during the drug infusions. Ineffective responses were found by Kosten et al. (47) 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

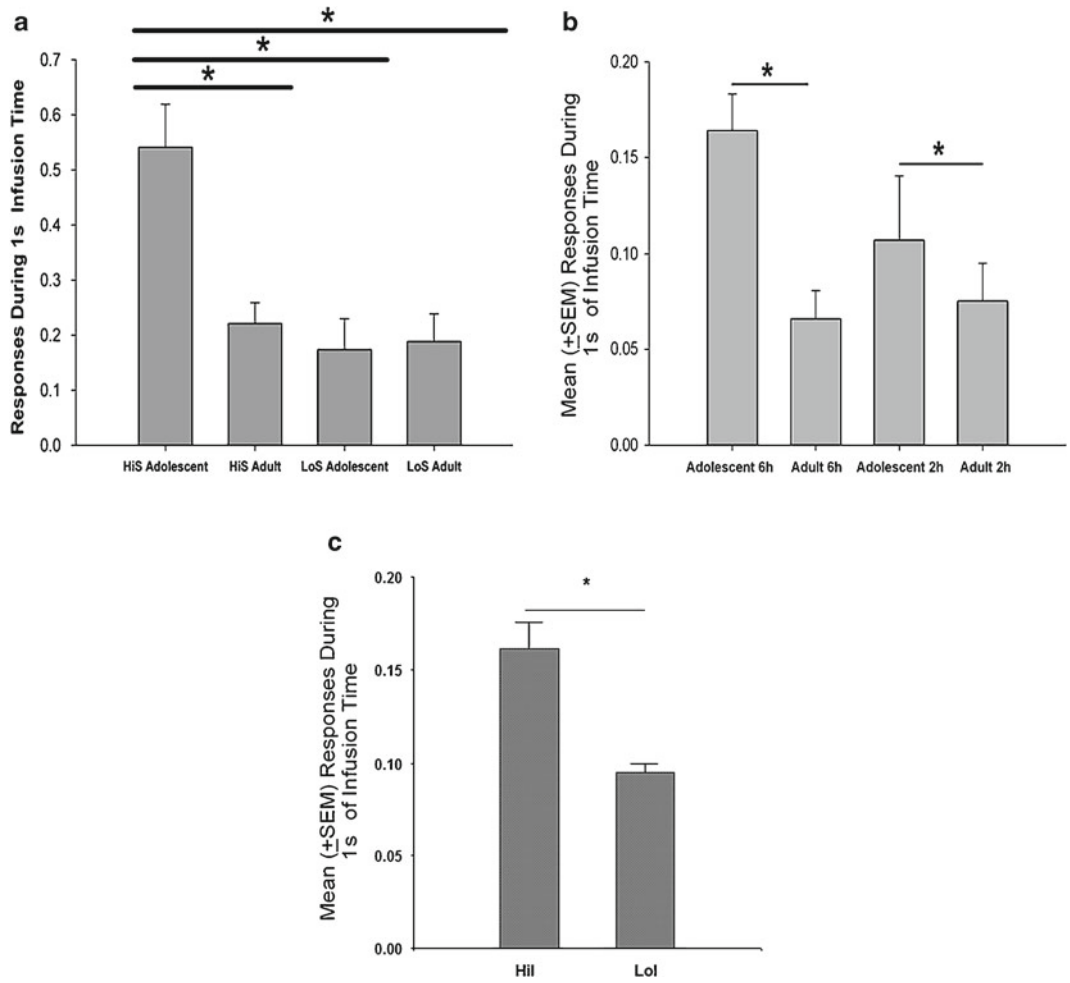

Fig. 2. Responses on the active lever expressed as infusions/per s of infusion time. $p = 0.05$. (**a**) and (**b**) are redrawn from (110), (**c**) is from Regier et al. (36).

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 (119) 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 highly-vulnerable rats, it is assumed that this behavior is directly related to the reinforcing effects of the drugs and stimulus properties of the manipulanda

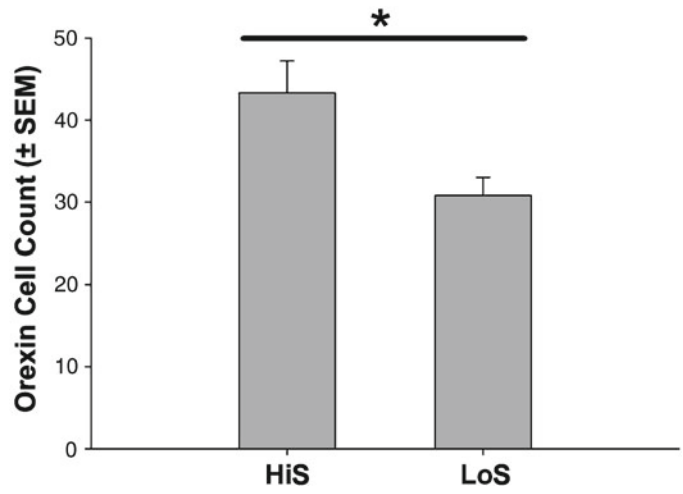

Fig. 3. Orexin cell counts in the lateral hypothalamus. $p = 0.05$. From Holtz et al. (109).

(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) (121). Other potential explanations suggested increased motivation because ineffective responding increases at lower drug doses (that may have a priming effect), under PR schedules (47, 119) that assess motivation, and during food restriction that increases motivation (119, 122, 123). Ineffective responding may also be an indicator of elevated impulsivity, as it is higher in rats selected for high (vs. low) impulsivity (Fig. 2d), and rats selected on other vulnerable features, such as HiS (21) or adolescents (111) 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 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 (124). Further, the orexin-1 antagonist blocks motivation for highly preferred rewards, such as high-fat chocolate in rats (125) and craving and reinstatement (126). 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. Assessing Responsiveness to Aversive Effects of Drugs in HiS vs. LoS Rats

Previous work has shown that LoS rats display greater negative reactivity, or emotionality, compared to HiS rats under stressful conditions (101, 127). For example, LoS rats show greater latency of emergence and increased defection in the novel open field, and more stress-induced anorexia (101) and analgesia than HiS rats (127). 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 (128, 129).

As stress is a powerful liability in many aspects of substance dependence (129), 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 of the interactions between genetic differences in drug abuse vulnerability (HiS vs. LoS) and several measures of stress reactivity.

3.2. Food Restriction-Induced Hyperactivity

One stressful condition, acute food restriction, increases wheel running more in LoS rats compared to HiS rats ((127); Zlebnik and Carroll, unpublished observations). In the rat's natural environment, this increased activity is considered paradoxical because 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. (131) 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 (136) and increases wheel running (137). 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 ((127), Zlebnik and Carroll, unpublished data; Fig. 4). This enhancement of the already elevated emotional reactivity in the LoS rats may be attributable to the augmentation of HPA functioning.

3.3. 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 (138). Startle can be modulated by environmental and intrinsic variables or exogenously administered (pharmacological) agents (139–141).

Table 5
Relationship between high and low responders for drug and nondrug rewards and response to aversive conditions (adapted from (17))

Groups	Positive effects	Negative effects	Reference
HiI vs. LoI (impulsivity)	HiI > LoI	LoI > HiI Cocaine extinction	(33)
		LoI = HiI	(36)
		Heroin withdrawal	(116)
		Cocaine withdrawal	(116)
HiS vs. LoS (saccharin intake)	HiS > LoS	LoS > HiS	(66, 130)
		Ethanol withdrawal	
		Glucose withdrawal	
		LoS > HiS	
		Food deprivation-induced wheel running	
		LoS > HiS Acoustic startle	(127)
		LoS > HiS	(131)
		Food-deprivation + methylphenidate-induced startle	
		LoS > HiS	(109)
		Punishment of cocaine intake	
HR vs. LR (novelty reactivity)	HR > LR	LR > HR Fear, anxiety and emotionality	(30, 132, 133)
HAC vs. LAC (ethanol intake)	HAC > LAC	LAC > HAC	(134)
		Ethanol withdrawal	
LEW vs. F344(inbred strains)	LEW > F344	F344 > LEW	(135)
		Fear, anxiety, emotionality, F344 > LEW	
		Taste aversion (morphine)	(18)

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

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. (127) 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 foot shock on startle amplitude between the phenotypes (142). Greater aversive effects in LoS (vs. HiS) rats were also indicated in another recent experiment from Mclaughlin et al. (131) 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. These studies illustrate

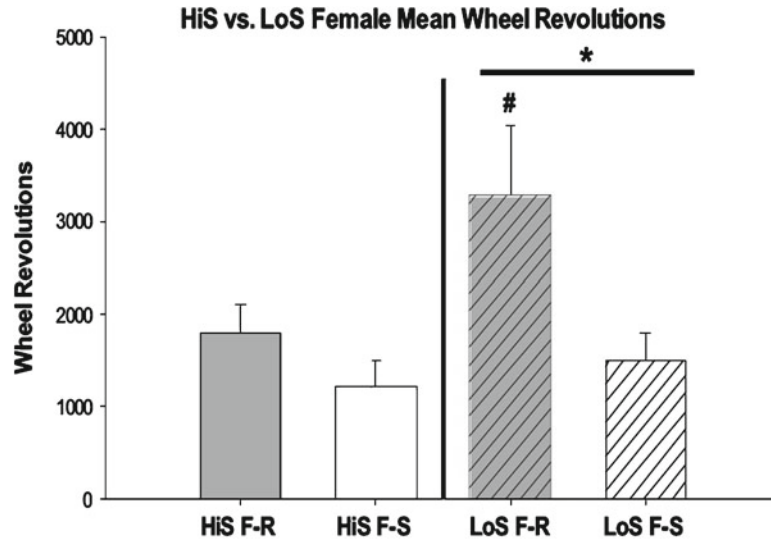

Fig. 4. Wheel revolutions in HiS and LoS rats that were food restricted (F-R) or food satiated (F-S). *$p < 0.05$, LoS F-R vs. LoS F-S and #$p < 0.05$, Los F-R vs. HiS F-R. From Zlebnik and Carroll, unpublished observation.

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 (143). Dess et al. (130) 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 (66). Because drug dependence, dysregulated food intake, and food addiction show strong parallels (144–146), 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 (binging) of ethanol and other drugs, while LoS rats showed more severe withdrawal effects and were more sensitive to the aversive aspects of chronic

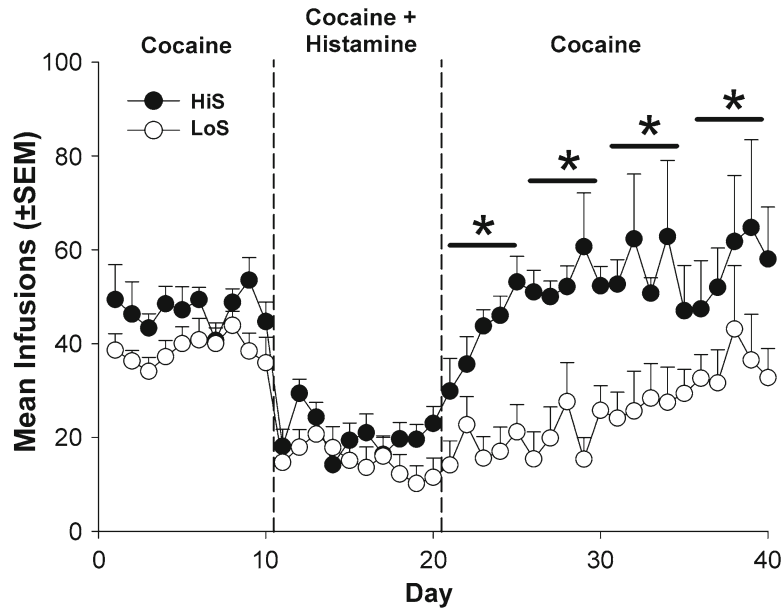

Fig. 5. Punishing effects of adding histamine to i.v. cocaine self-administration solution in HiS and LoS rats. *p< 0.05 HiS vs. LoS. From Holtz et al., unpublished observation.

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, then histamine was added directly into the cocaine solution. Systemic histamine administration is an effective punisher that reduces cocaine self-administration in monkeys (147, 148). 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. Results indicate that, compared to baseline, histamine reduced self-administered cocaine infusions 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 (Fig. 5a). These data suggest that the LoS rats are more sensitive to histamine's punishing effects than HiS rats.

3.4. 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

Table 6
Individual differences in sex and age and treatment effects

Behavioral model	Drug	Treatment	Treatment effect	Reference
Locomotor activity	Cocaine	Spiradoline	F > M	(149)
Acquisition	Cocaine	Baclofen	F > M	(150)
Self-administration	Heroin	Ketoconazole	F > M	(16)
	Cocaine	Exercise	F > M	(151)
	Phencyclidine	Bremazocine	F > M	(152)
	Phencyclidine	Saccharin	F > M	(153)
	Cocaine	Histamine	F = M	(109)
Escalation	Cocaine	Exercise	Adoles > adults	(154)
Reinstatement	Cocaine	Cocaine hydrolase	F = M	(110)

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

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 (149), and a similar drug, bremazocine, decreased phencyclidine self-administration under a FR schedule to a greater extent in female rhesus monkeys compared to males (152). 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 (150), and ketoconazole, a corticosterone synthesis inhibitor, suppressed heroin self-administration more in female than in male rats (155). Sex differences in responsiveness to 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 (151), and access to saccharin reduced phencyclidine intake to a greater extent in female compared to male monkeys (153).

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 (16, 156–158)). 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.

3.5. Treatment of 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. (159) showed that severe alcoholics 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 (24, 160), and saccharin intake, such as that displayed by the HiS vs. LoS rats (23), and impulsivity in selected HiI and LoI rats (22). 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 (143), 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 (e.g., (161, 162)), 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 et al. (108), chronic baclofen treatment was administered over a 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 (163), for review, see (164–166). Baclofen suppresses cocaine-induced dopamine increases in the nucleus accumbens (167). Animal models have generally supported baclofen's efficacy as a treatment for drug addiction (168–170); however, clinical studies have yielded equivocal results (171). 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 (Fig. 6; Table 7). The effect on LoS rats was similar to the

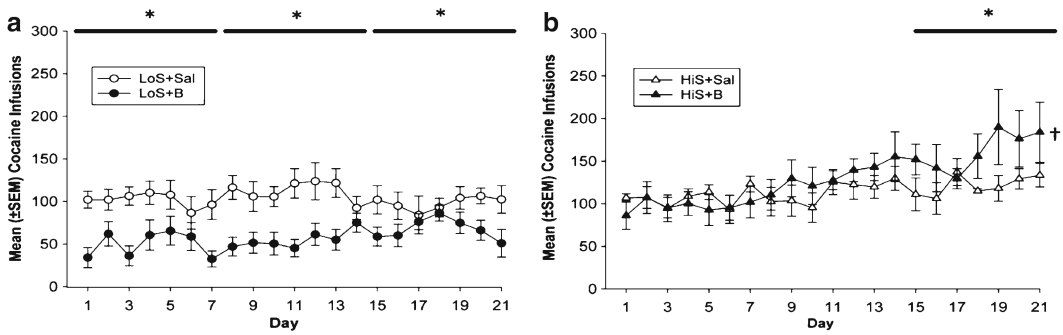

Fig. 6. Mean (±SEM) cocaine infusions are presented over a 21 day period when rats were allowed access to 0.4 m/mL cocaine for 6 h sessions 7 days per week. (**a**) baclofen- (*filled circles*) and saline -treated (*open circles*) rats selectively bred for low saccharin intake (LoS). (**b**) baclofen- (*filled triangles*) and saline-treated (*open triangles*) rats selectively bred for high saccharin intake (HiS). *Horizontal lines* and *asterisks* indicate blocks of 7 days when there were significant between group differences. Reproduced, with permission, from (108).

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 (Hil) and low (Lol) impulsivity or adolescents vs. adults

Behavioral model	Drug/reinforcer	Treatment	Treatment effect	Reference
Maintenance FR and PR	Sucrose	Naltrexone	HiS = LoS	(172)
	Cocaine	Histamine	LoS > HiS	(109)
	Cocaine	Histamine	HiI = LoI	(109)
Escalation	Cocaine	Baclofen	LoS > HiS	(108)
	Cocaine	Progesterone	LoS > HiS	(156)
	Cocaine	Exercise	Adoles > Adult	(154)
Reinstatement	Cocaine	Baclofen	HiS = LoS	(107)

HiS, high saccharin; *LoS*, low saccharin; *Hil*, high impulsive; *Lol*, low impulsive; *adoles*, adolescent

suppression of binge eating of a pure fat diet by baclofen in Sprague-Dawley rats (173).

In another recent study by Anker and coworkers (174), 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 (16, 174). Furthermore, exogenous progesterone administration has been investigated as a potential pharmacological treatment for substance abuse disorders (175, 176).

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 (Table 7). Together, these results suggest that some pharmacological treatments may be more 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.

3.6. Treatment for Operant Responding for Food Reward

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 binging was investigated in HiS and LoS rats by Gosnell et al. (172). 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 (177–179), 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 (66). 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 (180). 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).

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 (181), depression (182), attention-deficit/hyperactivity disorder (183), and substance abuse disorders (184). 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 (185, 186), as well as the reduction of positive subjective response to and self-administration of alcohol in individuals not diagnosed with alcoholism (187). 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 (188). In sum, our understanding of how to predict addiction severity and customize treatments based on individual vulnerability should lead to better management of drug abuse treatments.

4. Conclusion

As more studies on HiS and LoS rats accumulate, it becomes more apparent that sweet preference is a major drug-abuse predictor, and other major predictors such as sex, age, and impulsivity are additive to sweet preference. Initial studies suggest that vulnerability is not only determined by the positive rewarding effects of drugs and other stimuli, but also reactivity to the aversive effects of drugs. It is likely the balance between reactivity to positive and negative effects that determines the vulnerability for a given individual. It will be important to take these factors into account in evaluating vulnerability and how that will determine 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

further work on treatment for drug abuse. Several factors such as motivation and impulsivity for food and drugs are closely related determinants of addictive behavior (e.g., Fig. 1). Future research might take into account this interaction and target these behaviors in designing treatment strategies for drug abuse.

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Chapter 14

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 recently 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 that 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. However, to date, little evidence has been reported of continued food seeking and intake despite its harmful consequences in rats and mice.

Key words: Eating disorders, Food compulsion, Chocolate, Animal models, Prefrontal cortex, Norepinephrine

1. Introduction

1.1. Food Addiction

Studies in humans and laboratory animals show that, in addition to energy balance, eating is regulated by factors unrelated to metabolic control. Several studies suggest a link between stress, access to highly palatable food, and eating disorders (1–4). Eating in response to negative emotional states suggests the possibility that individuals overeat to self-medicate, such as with "comfort foods." However, data suggest that some individuals may develop addiction-like behaviors when consuming palatable foods (5, 6). It has been proposed that overeating of palatable food may produce long-term

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neuroadaptations in the reward and stress networks of the brain (7, 8), similar to those produced by long-term drug abuse (6).

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 (9, 10). These behavioral parameters have been reproduced in animal models of drug addiction (9, 10), and some of these parameters 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 (1, 6, 11, 12). Therefore, a critical question is the legitimacy of the term "food addiction."

The scientific definition of "food addiction" has emerged during recent years, and accumulating evidence in animal models suggests that, under certain circumstances, overeating can produce behavioral and central changes resembling an addiction-like state (7, 8). It has been suggested that the overconsumption of "refined" foods can be described as an addiction conforming to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), criteria for substance use disorders (13), and The Yale Food Addiction Scale (YFAS) was recently developed to operationalize the construct of palatable food dependence based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) (12), in humans. 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 (3, 7, 13–25). In addition, one study suggests that sugar-bingeing rats show cross-sensitization with some drugs of abuse (26).

1.2. Neuronal Circuits Underlying Food Addiction

In addition to the behavioral criteria described above, different brain studies also support the idea that overconsumption of certain foods has parallels with drug addiction (6). Several areas of the brain, as well as neurotransmitter systems, are involved in the reinforcement effects of both food and drugs, thus suggesting that natural and pharmacological stimuli activate some common neural systems (1, 27–33). Several recent review articles identified a connection between neural circuits activated in the seeking/intake of palatable food and drugs of abuse (6, 11, 12, 34, 35). 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 (6, 17, 19, 21, 27, 32, 36).

Like addictive drugs, certain palatable foods can activate brain circuitry involved in reward, motivation, and decision making (37). 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 (6, 38). 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 (38, 39).

The most clearly established mechanism common to food and drug intake is the activation of the dopamine (DA)-containing link in brain reward circuitry (1, 38, 39). 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" and lead to a loss of control over food and drug intake (38, 39). In addition, the degree of DA release seems to correlate with subjective reward from both drug and food use in humans (37, 39). 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 (6, 17, 32), and neuroimaging studies in obese subjects have revealed alterations in DA receptor expression that are similar to those found in drug-addicted subjects (1, 32, 39). 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 (38, 39). 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 (40, 41). 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 (PFC) to the striatum (41). Striatal D2 receptor availability in obese subjects correlates with glucose metabolism in some frontal cortical areas, such as the dorsolateral PFC, which is involved in inhibitory control (39). It is been suggested that reduced dopaminergic modulation from the striatum may lead to impaired inhibitory control over food intake and an increased risk of overeating (8, 38, 39). The same direct correlation between striatal D2 availability and glucose metabolism in the dorsolateral cortex has been reported in alcoholics (39). Several areas of the PFC are implicated in the motivation to eat (42), and a substantial body of evidence points to a critical role of PFC in motivated animal and human behavior related to food or drugs (1, 21, 30, 36, 43, 44). Thus, some prefrontal regions could reflect a neurobiological substrate common to the drive to eat or

take drugs. Abnormalities in these regions could enhance either drug-oriented or food-oriented behavior, depending on the established habits of the subject (1).

Abundant data suggest prefrontal cortical dysfunction in drug and food addicts, and are increasingly supported by experimental studies in both animals and humans (12, 38, 41, 45). Dysfunctional regions of the PFC involved with emotional processing (46) and inhibitory control (47) are particularly important to understanding addiction, as their disruption is linked to compulsive behaviors and poor impulse control.

In addition to DA, norepinephrine (NE) transmission in medial prefrontal cortex (mPFC) has been shown to be involved in the behavioral and central effects of drugs of abuse (48–52), and critical for food-related motivational behavior (30, 31). Finally, we recently showed that prefrontal NE transmission also plays a major role in aberrant motivation related to the seeking of palatable foods (53).

1.3. Eating Disorders, Palatable Foods, and Stress

Eating disorders are multifactorial conditions caused by environmental and genetic factors and the complex interactions among them (1, 54–56). Of the environmental factors that influence some eating disorders, such as binge eating disorder or bulimia nervosa, and obesity, the availability of palatable foods is the most obvious (1). It has been demonstrated that different foods induce different levels of compulsive behavior (1, 13). In particular, processed foods with high concentrations of sugar or other refined sweeteners, other refined carbohydrates, fat, salt, and caffeine have been suggested to be addictive substances (13). This hypothesis could explain why many people lose control of their ability to regulate intake of such palatable foods (13). Of all palatable foods, chocolate has been shown to have particularly rewarding properties in animals (30, 31, 36, 57), 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 (58).

Stress is another critical factor in the development and expression of eating disorders. Stress may play a role in eating disorders in both animals and humans (1, 4, 55, 56) because it is one of the strongest environmental influencers of psychopathology. Indeed, it affects the development, course, outcome, and recurrence or relapse after periods of remission of several psychiatric disorders (59–66).

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) (1), while chronic stress has been shown to increase the consumption of certain palatable foods, commonly referred to as “comfort food” (4, 67, 68), and

to precipitate binge eating (2, 25). 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 (8, 14, 15, 55, 56).

1.4. Food Restriction

Periods of food restriction, or dieting, are a very common finding among individuals with eating disorders (25), and theorists posit that diet exposure increases overall risk for onset and maintenance of eating disorders, such as binge eating disorder and bulimia nervosa (69–71). Indeed, recurrent periods of caloric restriction are consistently the strongest predictors of overeating in response to stress (72), whereas hunger alone appears not to be enough to induce such binge eating phenomena (70, 72).

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 (73), and many studies have shown that this manipulation alters levels of hormones, such as glucocorticoids, adrenocorticotrophic hormone, and corticotrophin-releasing factor, which are involved in the physiological response to stressful conditions (74–77). Finally, previous exposure to caloric restriction produces changes in stress neurocircuitry that increase stress sensitivity and the tendency to overeat high-fat foods (78).

Food restriction in rodents is a stressful condition that alters the sensitization of brain reward systems (79, 80), and greater sensitization of the reward systems can lead to excessive intake of highly palatable foods (55). In fact, repeated stimulation of reward pathways through highly palatable food consumption may lead to neural adaptations that make consumption more compulsive (1). 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 (14). 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 (7). 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, nondeprived state (7).

1.5. Animal Models

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 (8). Animal models can be used to

investigate molecular, cellular, and neuronal processes underlying normal or pathological behavioral patterns. Thus, 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. Inbred strains of mice are largely used to identify the genetic basis of normal and pathological behaviors, and strain-related differences in behavior appear to be highly dependent on genotype/environment interactions (73).

1.6. Food Seeking/Intake in Spite of Harmful Consequences

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 (9, 10, 16, 41); similar compulsion despite negative consequences is evident in some eating disorders, such as binge eating disorder, bulimia nervosa, and obesity (11). 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 (81). Although there is little evidence of continued food seeking/intake despite its possible harmful consequences (an index of compulsion) in rats (22, 81) and mice (53), animal models that have reproduced this behavior indicate that adaptive food seeking/intake can be transformed into maladaptive behaviors under specific experimental manipulations.

1.6.1. Food Seeking/Intake Despite Possible Harmful Consequences in Rats

The major models of compulsive eating of palatable foods in rats have been developed to study obesity and binge eating disorders (22, 81). In order to evaluate the compulsive nature of eating palatable food, these 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 the 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.

Johnson and Kenny (22) 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. 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 another study, Oswald and colleagues (81) 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 Chap. 2.

1.6.2. Food Seeking/Intake Despite Possible Harmful Consequences in Mice

Using a new paradigm of conditioned suppression, we investigated whether the ability of a foot-shock-paired conditioned stimulus to suppress chocolate-seeking behavior was reversed by previous exposure to a food restriction experience, thus modeling food seeking in spite of harmful consequences in mice (53).

The major sites of food-induced neuroadaptations are thought to be the DA mesolimbic and nigrostriatal circuits, and overconsumption of palatable food seems to downregulate dopaminergic reward circuitry through the same mechanisms observed in drug addiction (6, 38). However, several areas of PFC are implicated in influencing the motivation to feed (42), 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 (1, 21, 30, 36, 43, 44), thus suggesting that some prefrontal regions could contain a common neurobiological substrate (1). Finally, prefrontal NE transmission has been suggested to mediate behavioral and central effects of drugs of abuse (48–52) and to play a critical role in food-related motivational behavior (30, 31).

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 mPFC on a conditioned suppression test in stressed (calorie-restricted) mice. While control (non-food-deprived) animals showed a profound conditioned suppression of chocolate seeking during presentation of the conditioned stimulus, previously food-restricted animals showed food seeking/intake despite 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 behaviors and point to a "top-down" influence on eating disturbances, as well as new targets for treatment of aberrant eating behaviors.

2. Materials and Procedures

2.1. Animals

Male mice of the inbred C57BL/6JIco (C57) strain (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 0700–1900 hours). Each experimental group was comprised of eight animals (Fig. 1).

2.2. Drugs

Chloral hydrate, 6-hydroxydopamine (6-OHDA), and GBR 12909 (GBR) were used for these experiments. 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. 6-OHDA is a neutoxin, light sensitive, and has to be made fresh every day, dissolved in saline containing Na-metabisulphite (0.1 M) and stored at 4°C.

2.3. Food Restriction

We used food restriction as a stressful experience (73). In the chronic stressful condition the animals were placed on a moderate food-restriction schedule 5 days before conditioned suppression experiments started. Mice were assigned to a feeding regimen: they either received food ad libitum (ad lib) or were subjected to a food-restricted regimen (FR). In the food-restricted condition, food was

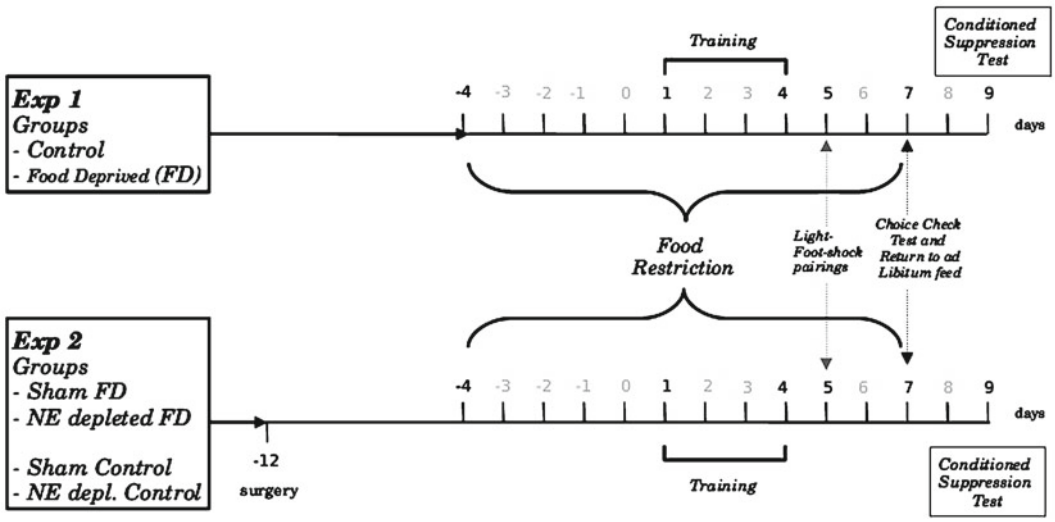

Fig. 1. Schematic timeline of the experimental procedure. See Box 1 for details.

delivered (provided) once daily (07:00 pm) in a quantity adjusted to induce a loss of 15% of the original body weight. In the control condition, food was given once daily (07:00 pm) in a quantity adjusted to exceed daily consumption (17 g). All testing sessions were carried out at least 48 h after food was again available ad libitum and animals reached their body weight before restriction.

2.4. Selective NE Depletion in Medial Prefrontal Cortex

Anesthesia and surgery were carried out as previously described (30, 50, 51). 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 mPFC (coordinates: +2.52 AP; ± 0.6 L; –2.0 V with respect to bregma (82)) 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 mPFC were assessed by high-performance liquid chromatography, with electrochemical detection analysis, as previously described (30, 50, 51), to evaluate the extent of depletion.

2.5. Apparatus for the Conditioned Suppression Test

The apparatus used for the conditioned suppression test was a modified version of the place conditioning apparatus (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)

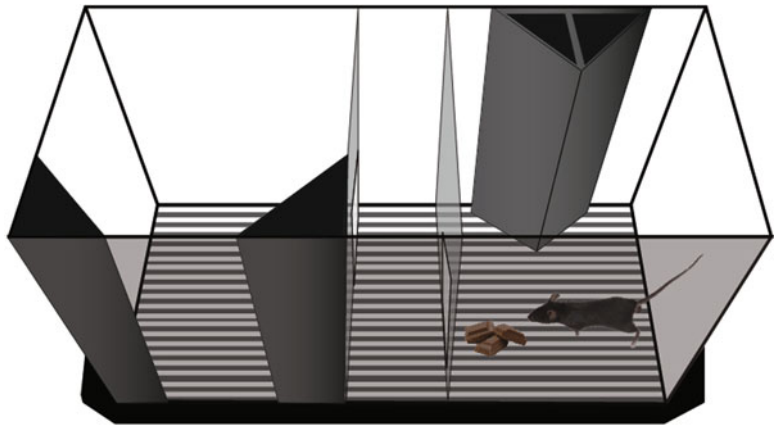

Fig. 2. Schematic apparatus used for training phase. The apparatus used for the conditioned suppression test was a modified version of a place conditioning apparatus (50, 51, 73); it consisted of two gray Plexiglas chambers (15 × 15 × 20 cm), a central alley (15 × 5 × 20 cm), and black spatial patterns, 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 pattern (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 day 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.

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 (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 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 (83). 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.

Box 1

Experiment 1: two groups of mice, control and food deprived (FD), were used.

Day –4 to 7: FD 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.

Day 1 to 4: Control (non-food-deprived) and FD mice were subjected to training phase.

Day 5: Animals were exposed to light-foot-shock pairing.

Day 6: Mice were left undisturbed in their home cage.

Day 7: Animals were subjected to the choice check test and then FD animals 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.

Experiment 2: two groups of FD mice, Sham (Sham FD) and norepinephrine (NE) depleted (NE-depleted FD), were used.

Day –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 (non-food-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 FD groups were subjected to surgery and after 7 days they were used for the behavioral test. From day –4 to 7 day, FD mice (Sham, NE depleted) were subjected to the food restriction procedure.

Day 1 to 4: Both control and FD groups were subjected to the training phase.

Day 5: Animals were exposed to light-foot-shock pairing.

Day 6: Mice were left undisturbed in their home cage.

Day 7: Animals were subjected to the choice check test and then FD 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.

2.6. Chocolate Consumption and Body Weight

Chocolate consumption was assessed during the conditioned suppression experiments. Intake was evaluated by weighing leftover chocolate at the end of each training phase session (on days 1–4), on day 7 (choice check test), and on day 9 (conditioned suppression test). Finally, mice were weighed daily throughout the experiments.

2.7. Procedures

The timeline of procedure is described in Box 1. The procedure was as follows: from day 1 to day 4 (training phase), individual

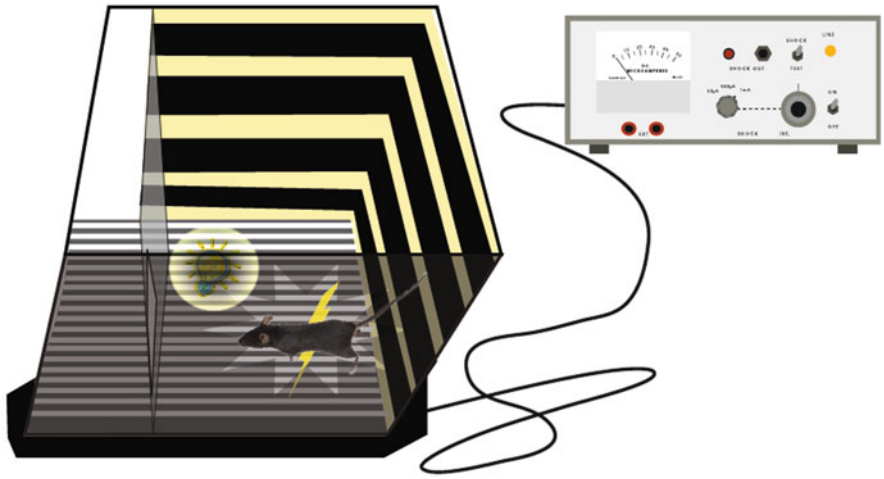

Fig. 3. Schematic apparatus used for light-foot-shock pairing. Acquisition of the conditioned stimulus CS (light)-shock association was established in a different apparatus comprised of a 15 × 15 × 20 cm Plexiglas chamber with a black and white striped pattern on two walls (to make it very different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

mice were 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 (Fig. 2). 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 (Fig. 3). 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 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). 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

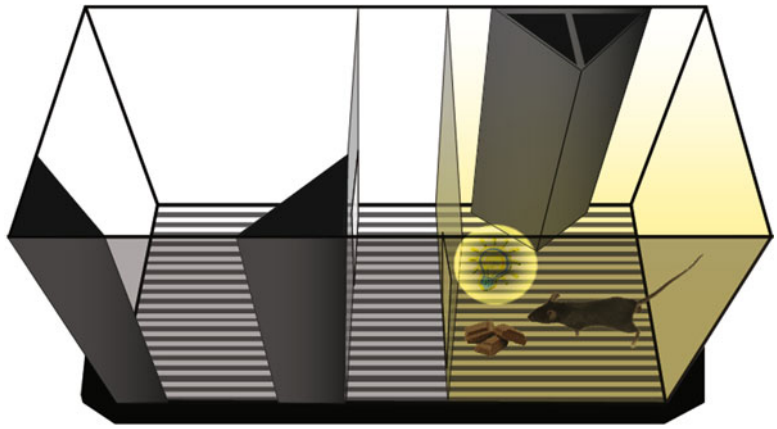

Fig. 4. Schematic apparatus used for conditioned suppression test. Conditioned suppression of chocolate seeking was assessed in a test session (day 9 of experimental procedure) that lasted 20 min in which the mice had access to chocolate in one of the two chambers in which chocolate had been placed during 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.

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 (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. Notes

3.1. Animals and Palatable Foods

While we used C57 mice, various inbred, outbred, and genetically modified mice may be used in this test. We are currently using another inbred strain (DBA/2J) to evaluate sensitivity of our procedure, possibility to generalize our results, and in order to

investigate how genetic and environmental factors interact to produce pathological eating behavior.

Moreover, while we used milk chocolate as palatable food, other different foods could be employed, based on preference of specific strain employed. Preliminary data suggest the possibility to use white chocolate in C57 mice, but different experiments are needed to verify it. Numerous behavioral test, like conditioned place preference test, may be used to obtain preliminary information regarding rewarding properties or palatability of new, different foods.

3.2. 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 previous exposure to a food restriction experience. However, 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 (9, 10), as well as compulsive eating emerges following extended access to a palatable diet (22). In agreement with these data, preliminary data from our laboratory suggest that extended access to chocolate is able to transform adaptive food-seeking/intake behavior into compulsive eating, depending on genotype (unpublished observation). Finally, our experimental procedure could be used to test relapse to compulsive-like behavior after different times from the end of the conditioned suppression test.

3.3. 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 medial 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.

3.4. 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 the 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 the cups, these have to be fixed on the floor to prevent mice from moving them.

3.5. 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 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 the feeding regimen that would lead to an increased value of the reinforcer 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.

3.6. Conditioned Avoidance Test

An important point is to rule out any unspecific effects of food restriction (or different stressful conditions) or different manipulations (e.g., prefrontal NE depletion) 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, the conditioned avoidance test was conducted (in one group of food-deprived mice and in Sham and NE depleted groups) like the conditioned suppression test (see Sect. 3), but there was no chocolate in either of two chambers.

3.7. Shock Sensitivity

Finally, it is needed to rule out differences in shock sensitivity between different strains or between different experimental groups subjected to environmental or pharmacological treatments.

To rule out alterations in sensitivity to foot-shock induced by NE depletion, Sham food-deprived, and NE-depleted food-deprived mice 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 μA, 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 hind foot left the grid floor. For each mouse, the mean value of shock thresholds recorded in each series was calculated.

4. 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. Indeed, such behavior conforms to the DSM-IV criteria for substance use disorders (13), and the YFAS was recently developed to operationalize the construct of palatable food dependence based on the DSM-IV-TR substance dependence criteria in humans (12). Although they are needed, studies of gene/environment interactions in human eating disorders are very rare; to date, only a few animals 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 (5, 22, 81) and mice (53). We are currently developing 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/6J and DBA/2J 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/6J mice (Fig. 5, unpublished data), which were previously shown to be more sensitive to drug effects than DBA/2J mice (73). Preliminary data confirm that compulsive eating emerges following extended access to a highly palatable diet (22), similar to how compulsive drug seeking emerges following an extended history of drug taking (9, 10), but only in genetically susceptible subjects.

Development of well-characterized animal models of compulsive overeating will be an essential tool to advance our understanding of etiological genetic and behavioral factors involved in eating disorders, to propose targeted pharmacological therapies, and to improve appropriate cognitive behavioral therapies.

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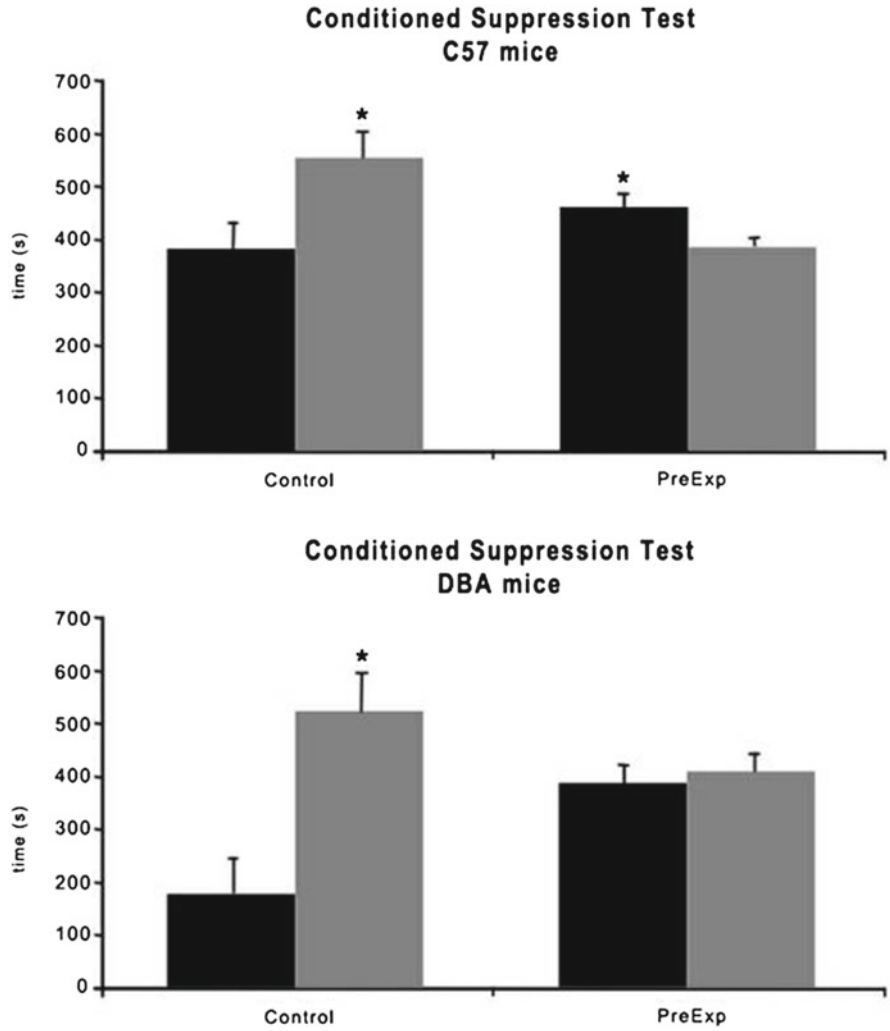

Fig. 5. Conditioned suppression test in control and preexposed (PreExp) mice from C57BL/6J (C57) and DBA/2J (DBA) strains. Mean (s ± SE) time spent in chamber containing chocolate (CC, black columns) and in empty-safe chamber (E-SC, grey columns) during the conditioned suppression test by C57 (*upper panel*) and DBA (*lower panel*) animals. PreExp animals were exposed to chocolate for 7 days before the experimental procedure started. $*p < 0.005$ in comparison with the other chamber.

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Part II

Anorexia and Undereating

Chapter 15

Introduction: Anorexia and Undereating

Guido K.W. Frank

Abstract

The eating disorder (ED) anorexia nervosa (AN) is a severe psychiatric disorder. Although the clinical diagnosis of AN has been recognized and included in the American Psychological Association's Diagnostic and Statistical Manual since the 1980s, the cause of AN remains largely unclear. A number of elements are suggested to be involved in the development of AN, including genetic and contextual. The use of animal models in research efforts to determine the causes of AN and expand upon the existing knowledge concerning the effects of this disorder may lead to significant progress in both understanding and treating this harmful illness.

Key words: Serotonin (5-HT), Dopamine, Brain reward system, Gonadal hormones, Neuropeptides

AN most commonly has its onset during adolescence (1). The AN phenotype presents with emaciation (body weight below 85% expected for height and age), intense fear of gaining weight, feeling fat despite being underweight, and amenorrhea for three or more months. Approximately 95% of affected individuals are female. A restricting type has been distinguished from a binge-eating/purging type, and many AN patients exercise excessively to burn calories and satisfy their drive for thinness (1).

The pathophysiology of AN is still unclear, but neurobiological studies have implicated neurotransmitters, such as serotonin (5-HT) and dopamine (DA), while gonadal hormones, genetic variations, or congenital insults could also be risk factors (2). Brain 5-HT is involved in behaviors such as mood, anxiety, feeding, and sleep (3). Symptomatic AN subjects show reductions of the cerebrospinal fluid (CSF) 5-HT metabolite 5-hydroxy-indole-acetic acid (5-HIAA) compared to healthy controls, but recovered AN individuals show elevated levels of CSF 5-HIAA (2). This suggests that abnormally high brain 5-HT levels could be a trait marker, and self-starvation might be a means to reduce 5-HT transmission. Brain DA plays a key role in the brain's reward systems (4). The DA pathways arise from the midbrain and project to three

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main regions—striatum, tuberoinfundibular area, and prefrontal cortex. Symptomatic and recovered restricting type AN subjects have reduced CSF homovanillic acid (HVA) concentrations, the major metabolite of DA, compared to controls (2). Whether individuals with AN have intrinsically lower DA remains uncertain, and this finding is in need of replication. However, if replicated, this might hold profound implications for the mechanism of reduced food reward in people with AN (4). Gonadal hormones are low during the symptomatic state of EDs, and multiple neuropeptides are also affected. Corticotropin-releasing hormone (CRH), opioids, neuropeptide-Y (NPY) and peptide YY (PYY), vasopressin and oxytocin, cholecystokinin (CCK), and leptin have all been reported to be altered, usually being low, but commonly remit with recovery (5). Whether the role of these abnormalities is causal or secondary remains to be clarified.

Food consumption is intimately connected to the brain reward system, and highly related to taste (6), driving the motivation to eat (7). The brain reward system is therefore an important research target for AN. Gustatory inputs from the tongue, immediately after food contact and prior to gut involvement, project via the brain stem and thalamus to the primary taste cortex comprised by insula and frontal operculum, and project from there to the ventral striatum and amygdala, and subsequently to the hypothalamus, midbrain, and frontal cortex (8). Hence, a highly complex network is involved in taste processing and food reward. In addition to the transmission of taste quality, there are learned associations between food and pleasurable experience, or fear of weight gain, that create an internal representation of food stimuli that gets activated when we see, smell, or think of food (6). This cephalic phase that includes desire and craving for food stimuli involves prefrontal and subcortical structures and activation of DA circuits that stimulate motivation to act and approach food rewards ("wanting"). The pleasurable experience of food ("liking") has been related to opioid release in the brain (9). Those pathways could be disturbed in altered eating behavior states.

Women recovered from AN showed increased DA D2/3 receptor availability in the ventral striatum in positron emission tomography (PET) studies (10). The presentation of body images during functional magnetic resonance imaging (fMRI) of the brain has revealed that AN individuals are more responsive in the striatum than controls to images of thin bodies (11). Recovered AN subjects show reduced functional brain response to a repeated application of taste in the insula and ventral and dorsal striatum (12), but increased response in the caudate, to randomly given monetary reward stimuli. AN subjects did not distinguish between win and loss in the ventral striatum (13). In summary, it appears that AN is associated with profound effects on brain DA and reward functioning, but the impact of those alterations is not yet clear.

Altered brain DA function could be related to abnormal food reward in AN (14) and subsequent undereating, but such hypotheses require further study (15).

The complex interplay between neurobiological, psychological, and environmental factors (16) 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, suggest strong psychological factors in conjunction with biological factors that perpetuate illness behavior. There have been recent advances with respect to describing brain neurocircuitry in AN (14), implicating the insular cortex, cingulate cortex, and basal ganglia and pointing toward involvement of reward-related brain pathways. However, how those alterations relate to the motivation for weight loss or brain pathway alterations that interfere with normal food reward processing is not yet known.

Animal models provide the ability to potentially model 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 (17) or the dynamics of neurotransmitter release (18) in those animals. Other studies subject animals to excessive exercising and can relate this behavior to neurotransmitter changes (19). Those studies can then inform potential mechanisms that underlie the AN phenotype and that could be targeted psychopharmacologically. A limitation of those studies is that they cannot model the intrinsic drive and motivation to starve that is seen in AN, which is a unique human characteristic. Yet animal research is crucial to model premorbid vulnerabilities or biologic changes that occur in the context of AN in order to systematically study this disorder.

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Chapter 16

Food Restriction and Reward in Rats

Kenneth D. Carr and Soledad Cabeza de Vaca

Abstract

Food restriction is a defining characteristic of anorexia nervosa and a risk factor for binge pathology. Basic research related to drug addiction indicates that food restriction increases drug reward magnitude, persistence of preference for a drug-paired environment, and relapse to drug seeking. These phenomena suggest that drugs of abuse subvert the adaptive mechanisms that normally facilitate foraging, learning, and ingestion when food is scarce. Similarly, if supranormally rewarding, energy-dense food is abundant but the physiological effects of underfeeding prevail due to restricted intake, the risk of developing maladaptive addiction-like eating behavior may increase. In this chapter, methods are described for assessing neurotransmitter receptor mechanisms and intracellular signaling pathways in the nucleus accumbens (NAc) that contribute to enhanced reward sensitivity in food-restricted rats. These methods combine intracerebral drug microinjection with the curve-shift rate-frequency protocol of intracranial self-stimulation testing. The addition of continuous intraventricular infusion of metabolic hormone or feeding-related neuropeptide receptor ligands is described as a means of assessing peripheral responses that may be antecedents to central nervous system changes of interest. When these studies are guided by biochemical findings in the NAc of food-restricted rats, the approach enables identification of neuroadaptations that increase reward sensitivity and suggests others that may increase synaptic plasticity and ingrain behavior. The goal is to generate a set of defined candidate mechanisms that can be evaluated for their involvement in the development and maintenance of disordered eating.

Key words: Reward, Nucleus accumbens, Self-stimulation, Food restriction, Addiction

1. Introduction

Food restriction is a defining characteristic of anorexia nervosa (1) and a risk factor for binge pathology (2–4). Among the functional mechanisms by which food restriction may contribute to binge eating are its effect as a stressor (5), a potentiator of the incentive effects of food and cues (6), and a modulator of synaptic plasticity (7). The pathogenic role of food restriction in the genesis of disordered eating is supported by animal models that combine alternating periods of food restriction/deprivation with access to palatable

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food, with or without an additional stress stimulus (8–13). Food deprivation and restriction also facilitate drug abuse, increasing drug reward magnitude (14), self-administration (15, 16), persistence of preference for a drug-paired context (17), and reinstatement of extinguished drug seeking (18). These observations are among many that are consonant with the hypothesized shared neural substrates of ingestive behavior and drug abuse (19–22). Moreover, addictive behavior, characterized by craving, compulsion, and persistence despite adverse consequences, is likely to include final common molecular mechanisms and neurocircuitry whether the object of addictive behavior is drug or food (23–25). Consequently, recent advances in understanding the neurobiological basis of drug addiction may provide insight into eating disorders that have addiction-like features, as well as illuminate the high comorbidity of disordered eating and drug abuse in adults (26–30) and adolescents (31–33).

The brain dopamine (DA) system is involved in normal and abnormal eating behavior (34–37) and mediates the rewarding and reinforcing effects of most drugs of abuse (38–40). The exact role of DA in normal feeding behavior has been debated, though current evidence indicates a role in the orosensory rewarding effect of sucrose (41–43), the acquisition of flavor preference based on postingestive consequences (44), incentive salience (45), cue-reward learning (46, 47), and the acquisition of new appetitive instrumental responses (48, 49). A method that has been useful to probe sensitivity of the DA-based brain reward circuitry, its modulation by diet, metabolic hormones, tastants, and drugs of abuse is electrical brain stimulation reward. Brain stimulation reward was discovered in 1954 (50), and there have been numerous reviews of methodology, neurophysiological underpinnings, and applications (51–55). Decades of research have been devoted to unraveling its neurobiological basis and using it in psychophysical studies to assess the effects of internal and external variables on reward sensitivity.

Stimulating electrodes are most commonly implanted in the lateral hypothalamic medial forebrain bundle, which supports more robust self-stimulation than electrodes implanted up- or downstream in the pathway. Lateral hypothalamic self-stimulation (LHSS) behavior is accompanied by DA release in the nucleus accumbens (NAc) (56, 57) and DA receptor-dependent activation of NAc neurons (58). Furthermore, LHSS is blocked by NAc microinjection of DA antagonists (59, 60). Nevertheless, pulse-pair stimulation studies that allow inferences to be drawn about refractory periods of neurons underlying elicited behavior, in conjunction with collision and anodal blockade studies that involve use of two electrodes implanted at different rostrocaudal levels of the stimulated pathway, have led to the conclusion that the reward signal is conducted by descending, rather than ascending, pathways (61, 62). This conclusion excludes mesoaccumbens DA elements from the population of

directly stimulated neurons. Subsequent studies suggest that activation of pontine cholinergic neurons, that in turn innervate and excite midbrain ventral tegmental area (VTA) DA neurons, link the LH to the ascending DA pathway (63, 64).

Addiction research indicates that persistent alterations in NAc neuronal circuitry play an important role in craving and compulsive drug use. For example, downregulation of D-2 DA receptors has been proposed to cause a reward deficiency syndrome which drives drug use aimed at restoring an optimal level of DA transmission (23, 65). A similar mechanism has been supported in an animal model of compulsive eating (66). Yet, hyperdopaminergia also drives excessive pursuit of rewards (67, 68), as would be expected from the role of NAc DA in incentive motivation (45). It is possible that excessive reward-directed behavior can result from either too much or too little DA transmission in NAc. In the case of drug addiction, the neuroplastic changes in NAc associated with persistent craving and vulnerability to relapse appear to involve glutamate more so than DA. Dedication of NAc neuronal ensembles to drug seeking (69–72) and loss of capacity for induction of new glutamate receptor-dependent synaptic plasticity (73–75) may ingrain drug-directed behavior and occlude acquisition of new, more productive alternatives. Increased neuronal membrane surface expression of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors (76) and disturbed mechanisms for regulating extracellular glutamate (77) appear to set up a "hair-trigger" for craving and relapse in response to environmental cues, stress, or a priming dose of drug via glutamatergic signals to NAc from prefrontal cortex (78–80). The extent to which similar mechanisms operate in eating disorders is unknown, although sucrose intake has been shown to increase NAc AMPA receptor function and membrane insertion with a greater effect in food-restricted relative to ad libitum fed rats (81). Convergent effects of DA and glutamate in NAc are important determinants of synaptic plasticity, and the role of synaptic plasticity in the genesis of disordered eating behavior is largely unexplored. It is also the case that NAc microcircuitry is complex, and multiple neurochemical players contribute to acute and enduring regulation of appetitive and consummatory behavior. Food seeking and/or ingestion are regulated by GABA, glutamate, DA, opioid, cholinergic, and other neurochemical mechanisms within NAc (82–86). The LHSS paradigm, in conjunction with microinjection cannulas implanted in NAc, offers a method by which one may assess changes in sensitivity of these multiple neurochemical mechanisms (87–89) as a function of a variety of independent variables, such as dietary history, body weight, hormonal status, and genotype.

The approach taken by our laboratory to investigate changes in reward induced by chronic food restriction involves the use of LHSS in combination with intracerebral drug administration and,

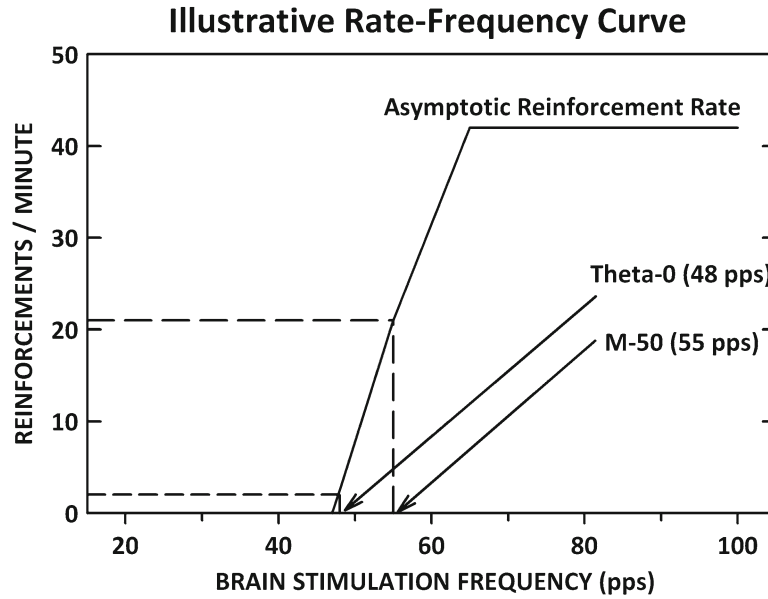

Fig. 1. Illustrative LHSS rate-frequency curve indicating the defining parameters: asymptotic or maximum reinforcement rate (reinforcements per minute), and the M-50 and theta-0 reward thresholds in pulses per second (pps).

in some cases, continuous intracerebroventricular infusion of metabolic hormones or ligands that interact with receptors for feeding-related neuropeptides. In recent years, our pharmacological studies have been guided by biochemical studies that have identified differences in NAc intracellular signaling, receptor phosphorylation, and trafficking following drug or sucrose challenge in food-restricted relative to ad libitum fed rats.

The LHSS protocol used is the rate-frequency curve-shift method, which generates a plot of the rate of reinforcement as a function of brain stimulation frequency for which the subject is responding (90, 91). The rate-frequency curve is analogous to the pharmacological dose–response curve and enables determination of the stimulation frequency that sustains half the maximum reinforcement rate (i.e., the M-50 threshold measure, which is analogous to the ED_{50} in pharmacology) and the minimum frequency at which stimulation becomes rewarding (i.e., the theta-0 threshold measure). Figure 1 depicts an idealized rate-frequency curve and identifies the M-50, theta-0, and maximum reinforcement rate. Treatments that increase or decrease reward effectiveness produce parallel leftward or rightward curve shifts, respectively, while treatments that preferentially affect performance alter the slope and/or asymptotic reinforcement rate. Empirical studies have indicated that the theta-0 measure is more resistant than the M-50 measure to treatments that alter performance capability (91–93). Drugs of abuse generally induce dose-related

leftward shifts (53), while DA receptor antagonists and withdrawal from chronic psychostimulant use produce rightward shifts (55). The interchangeability of food and drugs in this paradigm is indicated by findings that orosensory stimulation with sucrose also produces concentration-related lowering of the LHSS threshold (94, 95).

2. Materials and Procedures

2.1. Subjects

Adult male Sprague-Dawley rats are housed in a central animal facility in individual plastic cages with free access to water and laboratory chow, except when food restriction regimens are in force. The animal room is maintained on a 12:12 h light:dark cycle, with lights turned on at 0700 hours. Animals are transported to the laboratory where all behavioral testing is conducted during the light phase. All experimental procedures involving use of laboratory animals are approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine and are consistent with the *Principles of Laboratory Animal Care* (NIH Publication no. 85-23).

2.2. Food Restriction

All rats initially have ad libitum access to pelleted rat chow with a physiological fuel value of 3.36 kcal/g, consisting of 28.5% protein, 13.5% fat, and 58% carbohydrates (LabDiet #5001). Ad libitum fed rats consume 25–35 g of this diet per 24 h period. Half of the rats in each experiment are placed on a restricted feeding regimen in which daily intake is limited to 10–12 g of the laboratory diet. The daily meal is generally delivered at 1700 hours and meal size is held constant until a 20% loss of body weight is achieved; this takes approximately 2 weeks. Daily food allotment is then adjusted to stabilize body weight at this value for the remainder of the study. Generally, food-restricted rats consume their daily ration within 1 h of its delivery which results in approximately 23 h of daily food deprivation.

2.3. Surgical Procedures

All stereotaxic surgery is performed with animals under deep anesthesia using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally (i.p.). Atropine (0.4 mg/kg, i.p.) is also administered to prevent bradycardia associated with anesthesia. Before each surgery, forceps, hemostats, and other surgical instruments are washed and then sterilized in a dry glass bead sterilizer (Cole-Parmer, Vernon Hills, IL).

Once an animal is anesthetized, as confirmed by absence of corneal and pedal reflexes, the scalp is shaved and the animal is stabilized in an ear bar adaptor and positioned into the stereotaxic frame (#1404; Kopf Instruments, Tujunga CA). Initially, the incisor

bar is set at –3.5 mm to approximate the skull flat plane, later to be confirmed. Corneas are coated with ophthalmic ointment, and the scalp is prepared with three applications of Betadine and 70% isopropyl alcohol in alternation. A single longitudinal scalp incision is made exposing the skull suture landmarks, bregma, and lambda. Once the head position is adjusted to ensure that bregma and lambda lie in the same horizontal plane, stereotaxically guided holes for electrode(s) and/or cannula(s) are placed using a high speed stereotaxic drill. A hand drill is used to prepare holes for three stainless steel anchoring screws and a ground screw. Following placement of electrode(s) and/or cannula(s) plus screws, all are secured to the skull by application of dental acrylic. Upon completion of surgery, antibiotic ointment is applied topically to the margins of the scalp incision. Following removal from the stereotaxic frame, rats are placed in a clean cage with a warming pad beneath. Postsurgical analgesia is achieved by administration of the non-steroidal anti-inflammatory drug Banamine (2 mg/kg, s.c.).

2.3.1. Electrode Implantation

Commercially available (Plastics One, Roanoke, VA, USA) untwisted bipolar electrodes, 0.25 mm diameter and 12 mm length (part# MS303/1-A), are modified by removing 0.3 mm of insulation from the tip of one wire (the electrode) and all insulation from the other wire, which is bent at a 90° angle. One end of a 3.0 cm length of copper wire is soldered to the bent pole while the other is soldered to a stainless steel screw that will be placed in the anterior skull and serve as ground.

Rats are stereotaxically implanted with the monopolar stimulating electrode in the lateral hypothalamic medial forebrain bundle at coordinates: 3.0 mm posterior to bregma, 1.6 mm lateral to the sagittal suture, and 8.6 mm ventral to skull surface. Once dental acrylic is applied around the electrode cures, the ground screw is placed and additional acrylic is applied. The electrode socket is covered with a dust cap (Plastics One, part# 303DC/1) when not in use.

2.3.2. Cannula Implantation

In studies requiring acute intraventricular microinjections, rats are implanted with a single 26-gauge stainless steel guide cannula (Plastics One, part# C315G) placed 1.0 mm dorsal to the lateral ventricle contralateral to the stimulating electrode (coordinates: 1.0 mm posterior to bregma, 1.5 mm lateral, and 3.4 mm ventral to skull surface). During surgery a stainless steel wire is inserted into the guide to maintain patency. After surgery, patency is maintained using a commercially available stylet (Plastics One, part# C315DC).

For microinjections in the NAc shell, rats are implanted with two chronically indwelling 26-gauge stainless steel guide cannulas (Plastics One, part# C315G) placed bilaterally 2.0 mm dorsal to intended injection sites (1.6 mm anterior to bregma; 2.1 mm lateral with tips angled 8° toward the midline, 5.8 mm ventral). Guide cannula patency is maintained using stylets.

For continuous infusion of drug into the ventricular system, a 22-gauge guide cannula with a horizontal access port (Plastics One, part# C322OP) is implanted in the lateral ventricle (coordinates: 1.0 mm posterior, 1.5 mm lateral, and 4.5 mm ventral). The guide cannula is positioned so that the horizontal port faces rear to enable connection to an osmotic minipump. The horizontal port is covered with a short length of polyvinyl tubing melted at the tip as a temporary seal.

2.3.3. Minipump Installation and Removal

Following recovery from cannula/electrode surgery and initial behavioral training, a second surgery may be performed to install a subcutaneous "14-day" osmotic minipump (Alzet model 2002; Durect, Cupertino CA) and connect it to the horizontal port of the intraventricular cannula.

Minipumps and fill solutions are prepared the day before surgery using sterile procedures. In general it is necessary to prepare 0.3–0.4 mL of solution to fill each minipump. The filling of minipumps consists of (1) attaching a 4 cm length of gas-sterilized polyvinyl tubing to the minipump flow moderator; (2) loading a syringe with the prepared solution, connecting it to a Millex filter unit (Millipore, Billerica, MA), and attaching a sterile needle (23 g); (3) filling the tubing and flow moderator, removing the needle from the filter and attaching the filter to a filling tube; (4) inserting the filling tube into the minipump and delivering the solution slowly until overflow (at least 0.3 mL); (5) inserting the previously filled flow moderator into the filled minipump. Once a minipump is filled, it is placed in a sterile container in sterile 0.9% saline to incubate overnight at 36°C.

For surgery, rats are briefly anesthetized by 5-min exposure to isoflurane vapor and oxygen delivered at 1 L/min in an enclosed chamber, using a mobile laboratory animal anesthesia system (VetEquip Inc., Pleasanton, CA). Once rats are anesthetized, they are removed from the enclosed chamber but continue to receive the isoflurane mix through a tube connected to a nose cone. Rats are placed on a flat surface covered with a sterile drape. The temporary polyvinyl cap is removed from the intraventricular cannula port and the cannula is flushed with 10 µL sterile 0.9% saline. After shaving and disinfecting the surgical site, a longitudinal incision is made slightly posterior to the scapulae. A sterilized hemostat is inserted into the incision to create a subcutaneous pocket. The primed minipump is inserted into the subcutaneous channel and the attached polyvinyl tubing is connected to the intraventricular cannula port. The incision is closed with suture or surgical wound clips, the horizontal port and attached tubing are covered with dental acrylic, and the area around the incision is disinfected and swabbed with an antibiotic ointment.

Following the 2-week infusion period, rats are again briefly anesthetized with isoflurane. The minipumps are disconnected and

removed by cutting the polyvinyl tubing, extracting the minipump and flushing the subcutaneous channel with sterile 0.9% saline. The portion of the tubing attached to the cannula is sealed by melting and is then covered with dental acrylic. The incision is closed with a wound clip and the area is swabbed with Betadine followed by antibiotic ointment.

2.3.4. Histological Verification of Cannula and Electrode Placements

Upon completion of behavioral testing, rats are euthanized with CO_2 and decapitated. Brains are removed and placed in 10% buffered formalin for at least 48 h. Using a cryostat (Reichert Jung, Frigocut 2800), 40 μm frozen coronal sections are cut, thaw mounted on gelatin-coated glass slides, and stained with cresyl violet. Cannula and electrode placements are determined by visual inspection of sections under an Olympus (#343750) dissecting microscope.

2.4. Acute Microinjection Procedures

For microinjections, solutions are loaded into 30 cm lengths of polyethylene tubing (PE-50; Intramedic Clay Adams No 427411, Becton Dickinson, Sparks, MD) attached at one end to a glass microsyringe (Hamilton, Bonaduz, Switzerland) filled with distilled water, and at the other end to a 33-gauge injector needle, which extends 1.0 mm (ventricular) or 2.0 mm (NAc) beyond the implanted guide. For ventricular injections, a 250-mL syringe is mounted on a Harvard 2272 microliter syringe pump delivering the 5.0 μL injection volume over a period of 95 s. For intra-NAc injections, two 25-μL Hamilton are mounted on the twin holders of the syringe pump for simultaneous delivery of the 0.5 μL injection volumes over a period of 100 s. Alternatively, a smaller volume syringe may be used to slowly deliver the solution manually, through one (or both cannulas simultaneously) over a period of 2 min. One minute following the injections, the injector needle is removed, the stylet replaced, and the animal is placed in either a waiting cage or the test chamber depending on the interval to the postinjection test.

Accuracy of ventricular cannula placements is verified prior to drug testing by demonstrating an immediate (i.e., <60 s latency) and sustained (i.e., >30 s) drinking response to a 5 μL injection of 50 ng of angiotensin II.

2.5. LHSS Procedures

2.5.1. Apparatus

Brain stimulation training and testing are conducted in eight standard operant test chambers (26×26×21 cm; Med-Associates, Georgia, VT) placed within sound attenuating cubicles (Med-Associates, ENV 018 MD). Each chamber has a retractable lever mounted on one wall and a house light mounted on the opposite wall. Eight constant current stimulators (Med-Associates, PHM 152B/2) are used to deliver trains of 0.1-ms cathodal pulses, which are conducted to implanted electrodes by way of commutators and flexible cables. The eight chambers are separated into two groups

of four and each group is connected to a Dell computer and an interface (Med-Associates, DIG-700P2) that controls electrical stimulation, contingencies, and data recording. All stimulation parameters are monitored on two Tektronix (TAS 455) oscilloscopes.

2.5.2. LHSS Rate-Frequency Training

After 1 week of postsurgical recovery, rats are exposed to the operant chamber and trained to lever press for 0.5 s trains of electrical stimulation consisting of cathodal square wave pulses of 0.1-ms duration at a frequency of 100 pulses per second (pps). The stimulation is delivered to the implanted electrode via cable and commutator (Plastics One, part# SL2C), allowing the animal to move freely around the chamber. The initial stimulation intensity of 120 μA is systematically varied to locate, for each rat, the lowest intensity that will maintain a high rate of lever pressing without signs of motor impairment or aversive side effects. Once lever pressing is acquired, rate-frequency training starts with sessions consisting of 36 or 24 sixty-second trials. Extension of the response lever and a 2-s train of "priming" stimulation initiates each trial. Each trial is terminated by retraction of the lever and is followed by a 10-s intertrial interval. Each lever press produces a 1-s train of stimulation, except for those presses emitted during the stimulation train, which do not increase reinforcement density. The number of lever presses and stimulations delivered (reinforcements) is recorded for each trial. Rate-frequency curves are generated by presenting twelve trials in which the brain stimulation frequency decreases over successive trials (approximately 0.05 log units each trial) from an initial frequency of 100 pps to a terminal frequency of 28 pps. At least three such series are presented in each training session. The first series in any given session is always considered to be a "warm-up" and data are discarded. Training continues for approximately 2 weeks until rate-frequency curves stabilize. During the final few training sessions rats are habituated to the injection procedures to be used in future test sessions.

2.5.3. LHSS Test Sessions

Test sessions begin with a preinjection test. Rats are then removed from the chamber for acute intracerebral or systemic drug injection. Injection is followed by an interval that varies depending upon the drug injected and route of administration and is followed by the postinjection test. For each rate-frequency series, the number of reinforcements obtained at each brain stimulation frequency is recorded. Two series from each test are averaged to yield a single rate-frequency function per test, per rat.

2.5.4. Data Analysis

For each test, the rate-frequency function is used to derive four independent parameters. The asymptotic reinforcement rate or *maximum rate* (described by a line that parallels the *x*-axis) is defined as the mean of all consecutive values within 10% of the

highest rate for the curve. All remaining values comprise the descending portion of the curve, with the lowest point being at the highest frequency to produce fewer than 2.5 reinforcements per minute. Regression analysis of the descending portion of the curve is used to calculate three additional parameters: the stimulation frequency sustaining half the maximum reinforcement rate (*M-50 threshold*), the minimum stimulation frequency that maintains responding (*theta-0 threshold*), and the *slope* of the regression line. Antilog transformations are then applied and natural frequencies are used in subsequent calculations. Acute drug effects are expressed as the percentage change in a parameter in the postinjection test relative to the preinjection test. Treatment effects on each parameter are analyzed separately by two-way mixed design ANOVA with drug treatment as the within subjects factor and diet as the between subjects factor. If a study includes osmotic minipump-driven intraventricular infusions, results are analyzed by three-way mixed design ANOVA with osmotic minipump treatment as an additional between subjects factor.

3. Notes

3.1. Examples of Studies and Results Obtained

3.1.1. Acute Intracerebral Microinjections

Lateral ventricular injection of D-amphetamine or the D-1 DA receptor agonist, SKF-82958, activated neurons in NAc as indicated by immunostaining for the protein product of the immediate-early gene *c-fos*. Both drugs produced stronger activation in food-restricted than in ad libitum fed rats (96, 97). Corresponding increases in drug rewarding effects were predicted. These predictions were confirmed by demonstrations that microinjection of D-amphetamine and SKF-82958 into the lateral ventricle or NAc shell lowered the LHSS threshold and did so to a greater extent in food-restricted than ad libitum fed subjects (14, 89). Figure 2 displays actual rate-frequency curves for single ad libitum fed and food-restricted rats injected with D-amphetamine, and regression lines obtained from group parameters.

3.2. Investigations of Reward Modulation Based on a Biochemical Finding

3.2.1. Negative Case: ERK 1/2 Phosphorylation

Lateral ventricular injection of SKF-82958 produced a markedly greater increase in activation of ERK 1/2 /MAP kinase in NAc of food-restricted relative to ad libitum fed rats (98). Yet, inhibitors of the kinase upstream of ERK (i.e., MEK inhibitors: SL-327, U0126) injected systemically or directly into NAc shell did not alter the rewarding effect of D-amphetamine in food-restricted rats, despite the fact that MEK inhibition blocked activation of the downstream nuclear transcription factor, CREB, and the immediate-early gene, *c-fos* (99, 100). While this result will be instructive in pursuing altered D-1 DA receptor-mediated synaptic plasticity in food-restricted rats (101, 102), it did not illuminate the enhanced unconditioned rewarding effect of a DA-releasing psychostimulant drug.

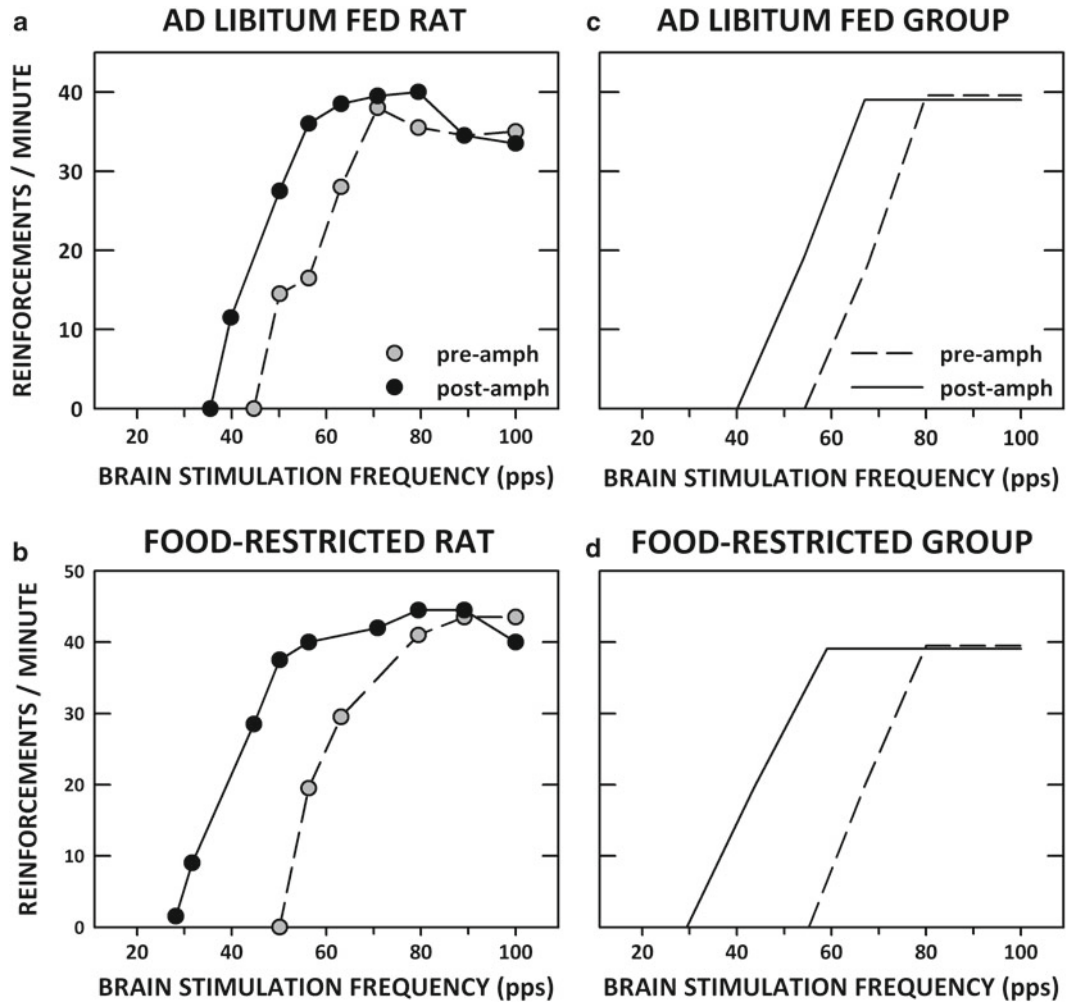

Fig. 2. LHSS rate-frequency curves before and after lateral ventricular microinjection of D-amphetamine (100 μg/5.0 μl). (**a**) and (**b**) display actual rate-frequency curves from one ad libitum fed (**a**) and one food-restricted rat (**b**) before (pre-amph) and after (post-amph) microinjection. (**c**) and (**d**) display rate-frequency regression lines obtained from the group parameters.

3.2.2. Positive Case: GluA1 Phosphorylation

SKF-82958 and cocaine were found to increase phosphorylation of the AMPA receptor GluA1 subunit on Serine-845 with a greater effect in food-restricted than ad libitum fed rats (103, 104). GluA1 phosphorylation on Ser845 increases channel open probability and mediates activity-dependent trafficking of cytoplasmic GluA1 to the extrasynaptic membrane as a first step in a two-step process that leads to synaptic insertion (105–111). Phosphorylation and trafficking of GluA1 are involved in synaptic strengthening and have been shown to produce both transient and enduring changes in neuronal excitability and behavior (112, 113). The relevance of this finding to ingestion of palatable food by food-restricted subjects was supported by the finding that brief intake of a 10% sucrose solution increased phosphorylation of GluA1 on Ser845 in

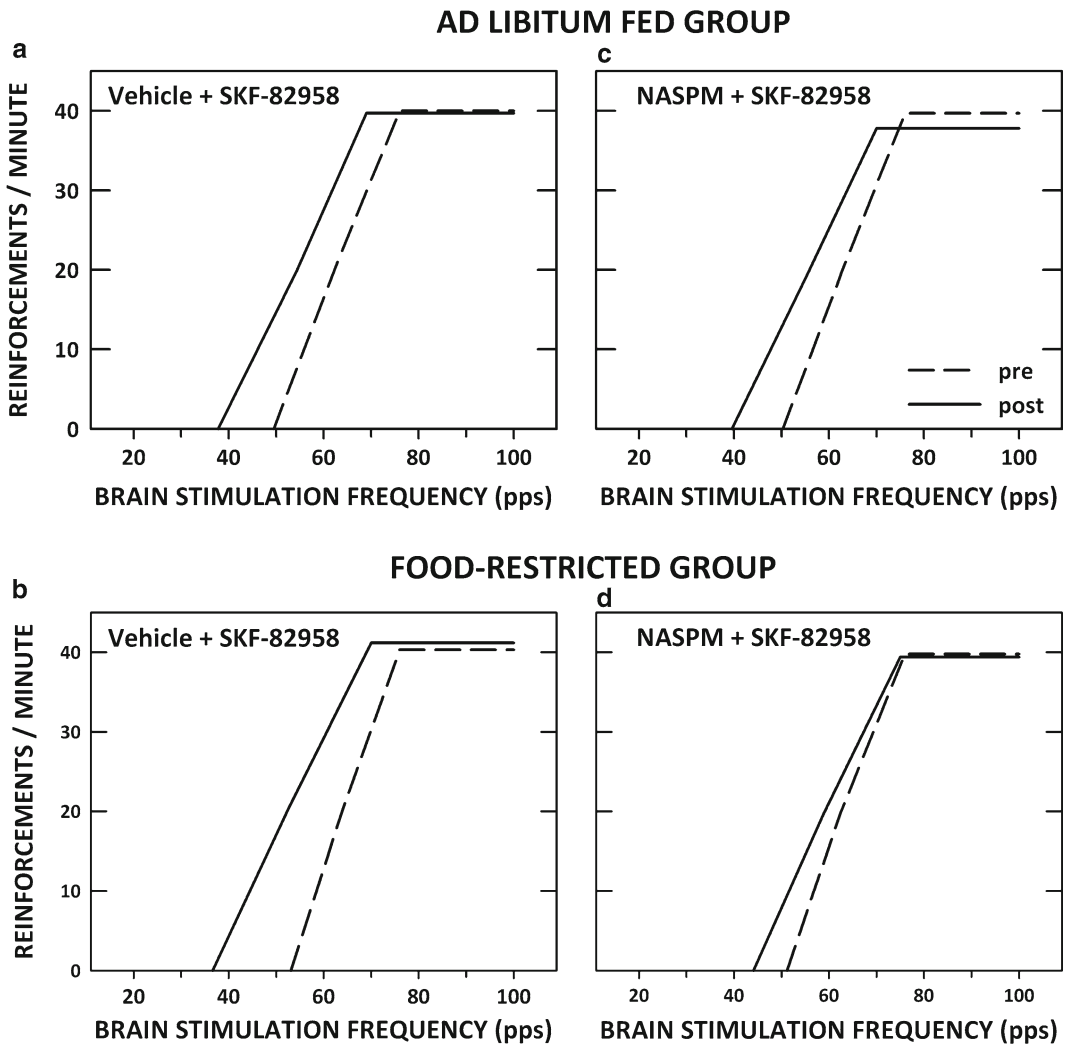

Fig. 3. LHSS rate-frequency regression lines derived from group parameters in ad libitum fed (**a**) and (**c**) and food-restricted (**b**) and (**d**) rats before (pre) and after (post) bilateral intra-NAc shell microinjection of the D-1 dopamine receptor agonist SKF-82958 (3.0 μg/0.5 μL/side) alone (**a** and **b**) or combined with 1-naphthyl acetyl spermine (25.0 μg; **c** and **d**).

food-restricted but not ad libitum fed rats (103) and increased AMPA receptor trafficking to the NAc postsynaptic density (81). To test whether D-1 DA receptor stimulation increases GluA1 involvement in acute rewarding effects, SKF-82958 microinjection in NAc was combined with 1-NA-spermine, a polyamine antagonist of Ca^{++}-permeable AMPA receptors (presumed to be GluA1 homomers). 1-NA-spermine selectively decreased the rewarding effect of SKF-82958 in food-restricted rats (103). This finding suggests that increased GluA1 function downstream of D-1 DA receptor stimulation accounts, at least partly, for the increased rewarding effect of NAc D-1 receptor stimulation in food-restricted rats. Figure 3 displays rate-frequency regression lines derived from

group parameters of ad libitum fed and food-restricted subjects tested in this study.

3.3. Assessment of Hypoleptinemia Involvement in the Modulation of Reward by Food Restriction

Leptin receptors are expressed by VTA DA neurons and modulate DA neuronal function (114–116). To assess whether hypoleptinemia underlies the enhancing effect of food restriction on drug reward, we continuously infused mouse recombinant leptin (0.5 μg/0.5 μL/h) via lateral ventricular cannulas for 12 days in ad libitum fed and food-restricted rats (117). Leptin infusion in ad libitum fed rats decreased food intake and body weight to levels comparable to those of vehicle-infused animals that were subjected to experimenter-imposed food restriction. Moreover, the leptin-infused subjects displayed enhanced rewarding effects of D-amphetamine, which reversed in tandem with body weight recovery during the 2 weeks following removal of the leptin-containing osmotic minipump. Thus, subjects that decreased intake and lost body weight as a result of chronic leptin infusion displayed a pattern of altered responsiveness to D-amphetamine similar to subjects undergoing and recovering from experimenter-imposed food restriction (118). Leptin infusions had no effect on intake, body weight, or the rewarding effect of D-amphetamine in food-restricted rats. The latter result suggests that hypoleptinemia is not involved in the enhancing effect of food restriction on drug reward and the former result suggests that decreased food intake and body weight can enhance drug reward in the absence of hunger and deprivation. Figure 4 displays rate-frequency regression lines derived from group parameters of ad libitum fed rats challenged with D-amphetamine before and during operation of vehicle- and leptin-filled osmotic minipumps.

3.4. Assessment of "Deprivation" Involvement in the Modulation of Reward by Food Restriction

To assess whether deprivation without weight loss alters the rewarding effect of D-amphetamine, ad libitum fed subjects were continuously infused in the lateral ventricle with the orexigenic melanocortin receptor antagonist SHU9119 (0.02 μg/0.5 μL/h) for 12 days (119). Half of the subjects had unlimited access to food and half were pair-fed to vehicle-infused controls. SHU9119 more than doubled daily food intake. Subjects that were pair-fed to vehicle-infused controls maintained body weights that were virtually identical to controls. Testing of D-amphetamine reward on days 3, 7, and 12 of the infusion revealed no alteration in SHU9119-infused rats that were pair-fed to controls. Thus, maintenance of baseline intake and body weight was associated with stable rewarding effects of D-amphetamine despite the fact that subjects were "deprived" relative to free-feeding SHU9119-infused subjects.

Although Sect. 3.3 casts doubt on the involvement of leptin in the modulation of reward by food restriction, there are numerous peripheral signals, known to interact with the brain DA system that

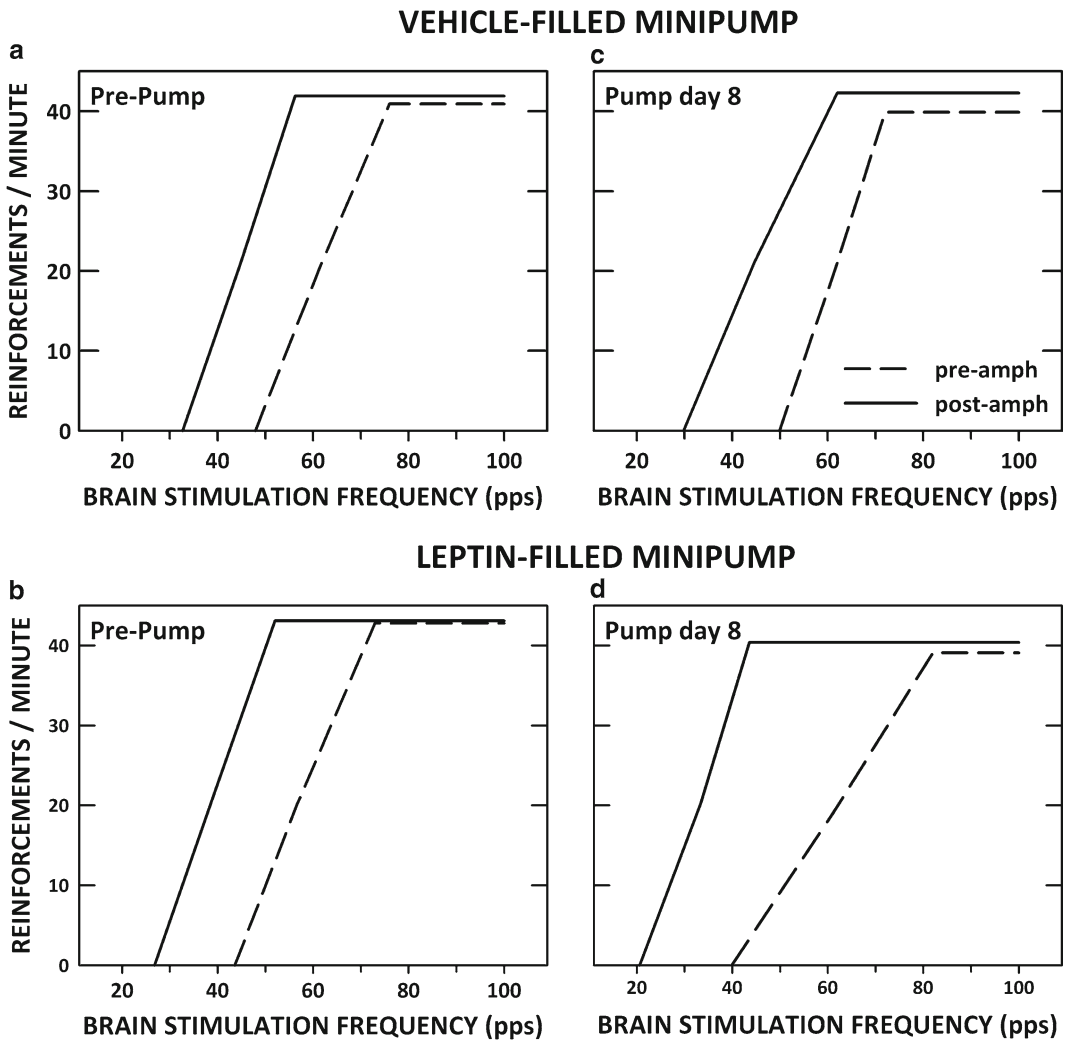

Fig. 4. LHSS rate-frequency regression lines for two groups of ad libitum fed rats before (pre-amph) and after (post-amph) systemic injection of D-amphetamine (0.5 mg/kg, i.p.). (**a**) and (**b**) display results from the test session that preceded insertion of subcutaneous osmotic minipumps (Pre-Pump), while (**c**) and (**d**) show results obtained on the eighth day (Pump day 8) of continuous lateral ventricular infusion of vehicle (0.5 μL/h; **a** and **c**) or leptin (12.5 μg/0.5 μL/h; **b** and **d**).

could plausibly play a role in the mesoaccumbens neuroadaptations and increased drug reward sensitivity observed in food-restricted subjects. For example, when blood is sampled from food-restricted rats on a day and time when brains are typically harvested for biochemical assay, and within the time frame when behavioral data are collected, plasma corticosterone levels are elevated 5–10-fold, activated ghrelin levels are elevated by 75%, IGF-1 levels are decreased by 50%, and insulin levels are decreased by 80%, relative to ad libitum fed rats (Zheng et al., in preparation). The relationship between these sustained alterations, the mesoaccumbens DA

system, and drug reward sensitivity, remain to be fully explored and are amenable to the strategies described for examining leptin and melanocortin involvement.

The representative findings outlined above suggest that a sustained decrease in food intake enhances the rewarding effects of psychostimulant drugs and D-1 DA receptor stimulation, and the effect may not require that the animal be hungry or deprived. In addition, downstream effects of NAc D-1 DA receptor stimulation on GluA1 phosphorylation and AMPA receptor trafficking may play a role in the enhanced rewarding effect with potential to mediate enduring changes in synaptic strength and behavior. Relevance of these findings to palatable food intake and the role of food restriction as a risk factor for binge pathology is suggested by findings that brief intake of sucrose also increases GluA1 phosphorylation and AMPA receptor abundance in the NAc postsynaptic density with a greater effect in food-restricted than ad libitum fed rats.

4. Conclusion

The experimental approaches described above are time consuming and labor intensive. However, they offer the possibility of identifying basal and receptor-stimulated molecular changes induced by food restriction that modulate reward. When used to illuminate results of biochemical studies, these behavioral assays may assist in the sorting of neuroadaptations that modulate responsiveness to acute reward stimuli versus those that may modulate synaptic plasticity and have the potential to ingrain maladaptive behavior, although the two categories are not mutually exclusive. To be clear, neuroadaptations induced by food restriction in the laboratory are assumed to be among those that facilitate adaptive behavior and survival when food is scarce in the wild. The potential for maladaptive outcome is hypothesized to be context dependent, as when severe dieting takes place in the presence of abundant, highly palatable, energy-dense food, when psychostimulant drugs are used as diet aids, or when drug abuse significantly displaces eating behavior. Mimicry or blockade of hormonal changes associated with chronic food restriction, using subcutaneous osmotic minipumps, may assist in the identification of physiological responses that are antecedent to CNS changes of interest. Of course, site-specific conditional gene modifications offer a range of powerful alternative approaches should mouse models be substituted for the rat model. The ultimate goals, on the basic research side, are to specify brain regional

mechanisms that facilitate reward and synaptic plasticity during chronic food restriction, assign strong candidacy to those that have known functional involvement in drug addiction, and investigate their role in the genesis and maintenance of disordered eating behavior in animal models.

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Chapter 17

Activity-Based Anorexia in the Rat

Nicole C. Barbarich-Marsteller

Abstract

Anorexia nervosa is a life-threatening psychiatric disorder characterized by unrelenting self-starvation, severe weight loss, and hyperactivity. Limited treatment efficacy and high rates of mortality provide strong justification for using animal models to study the biological mechanisms that promote the development and maintenance of these maladaptive behaviors. Activity-based anorexia is an animal model that combines restricted access to food with unlimited access to a running wheel. This programmed food restriction promotes hyperactivity that results in dramatic weight loss and increasingly greater levels of hyperactivity, thereby resembling a maladaptive behavioral pattern similar to some individuals with anorexia nervosa. This chapter describes the methodology for inducing activity-based anorexia in Sprague-Dawley rats, with a particular emphasis on adolescent female rats, given the predominately age- and sex-specific onset of anorexia nervosa in adolescent girls.

Key words: Anorexia nervosa, Activity-based anorexia, Eating disorders, Animal model, Food restriction, Self-starvation, Hyperactivity, Rat, Exercise

1. Introduction

Anorexia nervosa is a severe psychiatric disorder characterized by persistent self-starvation and life-threatening weight loss. Diagnostic criteria include a failure to maintain a minimal body weight, fear of gaining weight or becoming fat, disturbances in perception of shape or weight, and amenorrhea (1). Individuals with the restricting subtype of anorexia nervosa primarily engage in dietary restriction, whereas individuals with the binge eating/purging subtype engage in dietary restriction followed by periods of binge eating and/or the use of inappropriate compensatory behaviors (i.e., self-induced vomiting, laxative abuse, diuretic abuse). Although excessive exercise is not currently considered a formal diagnostic criterion for anorexia nervosa, physical activity has been termed a fundamental feature of the disorder in some individuals (2, 3), with up to 80% of individuals

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engaging in some form of excessive and/or strenuous physical activity (for review, see (4)). The onset of the disorder typically occurs during adolescence with 90–95% of cases occurring in women (1). The course of anorexia nervosa is variable, with some individuals recovering after the initial episode while others follow a chronic course characterized by episodes of recovery and relapse (5–8). There are few effective treatments for anorexia nervosa (9), resulting in one of the highest mortality rates among psychiatric disorders (10, 11).

Although dieting and drive for thinness are extremely prevalent among teenage girls, anorexia nervosa affects only 0.5–1.0% of women in the general population (1). The low prevalence of progression to anorexia nervosa in an environment with continuous pressures to diet and lose weight suggests that biological factors may increase the vulnerability of developing anorexia nervosa in certain individuals. Although a wide range of biological alterations during the course of anorexia nervosa have been found, it is unclear whether these alterations represent acute or chronic effects of starvation or whether they represent preexisting risk factors for the disorder. Thus, an inherent confound in the clinical research of this disease is the inability to identify at-risk individuals prior to the onset of illness. In contrast, the use of an animal model of anorexia nervosa enables the systematic study of biological factors involved in the development and maintenance of self-starvation in a controlled environmental setting. Clearly an animal model cannot represent every aspect of a clinical disorder. However, the use of an animal model of anorexia nervosa provides a rational approach for studying specific hypotheses related to the neurobiology underlying self-induced food restriction, weight loss, and hyperactivity.

The most widely utilized animal model of anorexia nervosa is activity-based anorexia. This paradigm combines unlimited access to a running wheel with limited access to food (typically 1 h per day) (12–14). This programmed food restriction promotes hyperactivity that results in dramatic weight loss and increasingly greater levels of hyperactivity, thereby resembling a maladaptive behavioral pattern similar to some individuals with anorexia nervosa (see Fig. 1 for a comparison between clinical and preclinical features). In early experiments using this model, some animals with activity-based anorexia would run themselves to death without experimental intervention, thereby mimicking the phenomenon of severe self-starvation seen in the clinical population of anorexia nervosa. This chapter provides an overview of the methodology for inducing the activity-based anorexia model in the rat, with specific considerations for utilizing adolescent female rats, given the age- and sex-specific onset of anorexia nervosa in adolescent girls.

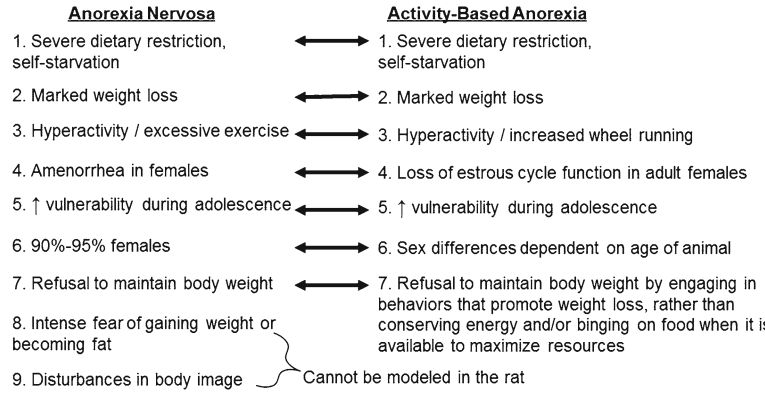

Fig. 1. Features of anorexia nervosa and activity-based anorexia. Reproduced, with permission, from (15).

2. Materials and Procedures

2.1. Materials

Induction of the activity-based anorexia model requires access to a running wheel. Although manual recording of wheel counts with a simple wheel running system is sufficient to induce the model and to measure activity levels, most investigators utilize more complex equipment that includes computer software specifically designed to measure wheel running activity on a second-by-second basis. This enables more accurate recording of wheel running activity as it relates to circadian rhythm, as well as the identification of food-anticipatory activity (i.e., increased wheel running activity in anticipation of food availability) in the hours leading up to food access. The standard activity wheel system in our laboratory measures 48.26 cm × 26.67 cm × 20.32 cm (Fig. 2; Med Associates, Inc., St. Albans, VT) and includes a standard home cage attached to an activity wheel with a 90° cam, microswitch, and LCD digital counter for standalone operation, as well as a computer interface for automated data collection and analysis with Med-PC-IV wheel counter software.

2.2. Procedures

Activity-based anorexia is induced through the combination of restricted access to food (typically 1 h per day) and unlimited access to a running wheel. Although these two factors are sufficient to induce the model, a number of methodological issues described below should be considered when designing an experimental paradigm (see Table 1 for an overview).

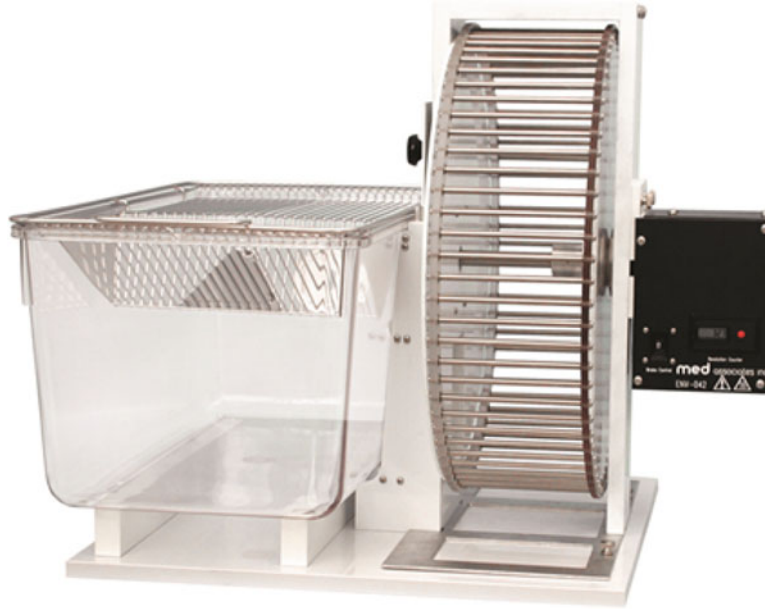

Fig. 2. An example of the experimental setup used to induce activity-based anorexia.

2.3. Acclimation and Housing

The activity-based anorexia model stresses the animal; thus, if animals are ordered from a vendor, it is important to allow time to acclimate to the animal facility prior to beginning restricted food access. As a general rule, animals in our lab arrive on postnatal day 21, the onset of adolescence; most animals are not put into experimental conditions until at least 14 days after arrival, although as little as 4 days is sufficient for acclimation in studies when timing is critical, such as when using early adolescent rats. In studies with age as a covariate or independent variable, all animal arrivals should occur on the same postnatal day of age. Animals should be individually housed upon arrival as this provides the most accurate method for measuring individual food intake and wheel running activity (see Note 1). Animals should be handled prior to the start of restricted food access in order to acclimate the animals and investigators to the process and to minimize stress from daily experimental manipulations.

2.4. Selection of Strain

Activity-based anorexia has been induced in multiple strains of rats, i.e., Sprague-Dawley, Long-Evans, and Wistar. To our knowledge, there has not been a systematic study comparing strain-dependent vulnerability to activity-based anorexia in rats. Our lab has utilized Sprague-Dawley rats to induce activity-based anorexia, thus the recommendations described below may be most pertinent to this strain.

Table 1
An overview of methodological issues to consider when inducing activity-based anorexia in the rat

Strain
• Typical strains used include Sprague-Dawley, Long-Evans, and Wistar, although there is a lack of data to definitively support differences in strain vulnerability
Age of animal
• Adolescent rats are more vulnerable to activity-based anorexia than adult rats and develop more severe weight loss and hyperactivity • Given the predominately age-specific onset of anorexia nervosa during adolescence, adolescent rats may provide important insight into this developmental vulnerability
Sex of animal
• Both males and females develop activity-based anorexia • Some sex differences exist in the rate of weight loss and level of hyperactivity • Given the predominately sex-specific onset of anorexia nervosa in women, female rats may provide important insight into this sex-specific vulnerability
Food access
• 1 h per day results in the most severe level of weight loss and hyperactivity • Up to 2 h per day can be used; however, the consequence is a less severe form of weight loss and hyperactivity with fewer animals developing the model
Wheel access
• Unlimited access for 24 h per day is most common • Some researchers restrict wheel access during the period of food access
Housing
• Individual housing should be used in order to accurately record food intake and wheel running activity
Experimental variables
• Daily changes in body weight, food intake, wheel running activity, and estrous cycle activity (when needed) should be measured • Variables should be recorded under the same conditions at the same time each day, in order to reduce animal stress and to obtain the most accurate assessment of data over the previous 24 h period • Baseline data (in the presence of ad libitum food) should be recorded for several days prior to the onset of activity-based anorexia, as this allows the investigator to identify any unusual differences in baseline activity levels, body weight, or food intake prior to experimental manipulation • Estrous cycle activity can only be measured in animals after vaginal opening occurs and involves stress to the animal; thus animals should be acclimated to the procedure and the procedure should be used only if the information that estrous cycle assessment will provide outweighs the potential effects of stress on the model
Determining day of experimental exit
• A predefined time point for removal of the animal from the study is typically used for collecting brain tissue for neurobiological studies (usually day 4 of restricted food access for adolescent rats in our lab) • An alternative approach for experimental endpoint is to allow animals to remain in the model until a certain level of weight loss has been met (i.e., up to 35% of baseline body weight) or predefined criteria for a decline in animal health have been reached

2.5. Selection of Age and Sex of Animal

Using animals that are the same age and sex helps reduce variability in the behavioral and physiological features of activity-based anorexia. Ideally, animals used in activity-based anorexia experiments should be bred at the institution so that monitoring of early life experiences can occur from the time of birth, in order to identify or control for potential stressors that might influence later development of activity-based anorexia. This is not possible at many institutions, so we recommend ordering animals at an exact day of age with the vendor, identifying specific littermates as part of the randomization procedure included in the experimental design.

Despite the predominately age- and sex-specific onset of anorexia nervosa in adolescent girls, the majority of early studies on activity-based anorexia utilized adult male rats. Existing literature comparing the vulnerability to activity-based anorexia across ages and sex is sparse; both males and females develop activity-based anorexia, but some sex differences may exist in levels of weight loss and hyperactivity (16, 17). The experience in our lab has been that using adolescent female rats provides unique challenges compared to using older rats, given that weight loss and hyperactivity appear to occur much more rapidly and severely during adolescence. To quantify this effect, our lab is currently conducting an NIH-funded study across three different ages in female and male Sprague-Dawley rats to determine the specific vulnerability to activity-based anorexia within this strain of rat. Our preliminary analysis of female data indicates an increased vulnerability to activity-based anorexia when the onset occurs during early and mid-adolescence compared to adulthood, with male data collection still ongoing (Barbarich-Marsteller, unpublished data).

2.6. Food/Wheel Access

In the traditional sense, the activity-based anorexia model combines unlimited access to a running wheel (24 h per day) with restricted access to food (unlimited access for 1 h per day; see Note 2). Timing of food access (i.e., light vs. dark cycle; at the beginning, middle, or end of the light or dark cycle) does not appear to affect the development of activity-based anorexia across studies, however, separating food access into four 15-min periods over a 24 h period, instead of a single discrete hour, prevents the development of activity-based anorexia in one study, suggesting that discrete food access is a necessary component for the development of activity-based anorexia (18).

2.7. Selection of Appropriate Control Groups

The inclusion of appropriate control groups when studying activity-based anorexia is an important and critical issue to consider. For studies examining the neurobiology of activity-based anorexia in our lab, four different groups are used (1) Control group—24 h per day access to food, no access to a running wheel—provides a comparison for changes induced by experimental manipulation; (2) Exercise control group—24 h per day access to food, 24 h per

day access to a running wheel—measures changes related to exercise; (3) Food-restricted control group—1 h per day access to food, no access to a running wheel—measures changes related to food restriction; (4) Activity-based anorexia group—1 h per day access to food, 24 h per day access to a running wheel—measures changes related to the combined effects of self-starvation and hyperactivity. While these control groups provide some basis for teasing apart the independent effects of exercise and food restriction/weight loss, an inherent difficulty that arises is the inability to control for the same level of severity of weight loss and hyperactivity found in the activity-based anorexia group. While the food-restricted control group (1 h per day access to food, no access to a running wheel) consumes a comparable amount of food to the activity-based anorexia group during the period of food access, the level of weight loss is more severe in the activity-based anorexia group, most likely due to the severe hyperactivity found in these animals (see Note 3). Moreover, while the exercise control group (24 h per day access to food, 24 h per day access to a running wheel) is used to control for changes induced by exercise, it is very rare for a control animal to run at the same level of hyperactivity as an activity-based anorexia animal. Therefore, it is important to consider differences in severity as well as the synergistic effects of combined weight loss and hyperactivity when attempting to independently analyze the effects of weight loss and activity levels in control groups.

2.8. Daily Measurement of Experimental Variables

Experimental variables, such as body weight, food intake, and wheel running activity, should be measured at the same time each day, in order to get an accurate representation of how behavior is changing over the previous 24 h. Handling animals at the same time each day also reduces animal stress. Our lab measures these variables approximately 15 min prior to the onset of the dark cycle when the 1 h per day period of food access begins for food-restricted and activity-based anorexia groups. Baseline variables, particularly wheel running activity in the presence of ad libitum food, should be measured for several days prior to restricting food access, as this allows the investigator to identify any unusual differences in baseline activity levels prior to experimental manipulation. In our laboratory, adolescent female rats typically run 2–4 km per day when given ad libitum access to food. Once restricted food access begins, a sharp increase in hyperactivity can be seen within 24–48 h, with animals in the activity-based anorexia group running on average 10 km per day by the fourth day of restricted food access, although some animals with activity-based anorexia will run in excess of 20 km per day within the same time frame.

2.9. Estrous Cycle Assessment in Female Rats

The stage of estrous cycle influences both food intake and activity levels in ad libitum fed female rats, and the predominately female nature of anorexia nervosa (90–95%) suggests that female

hormones may play a role in the development of the disorder. Interestingly, activity-based anorexia has been found to result in a disruption of estrous cycle activity (14), mimicking the diagnostic criteria of amenorrhea found in clinical anorexia nervosa. Depending on the experimental question, it may be important to measure estrous cycle activity in female rats that have reached puberty (see Note 4). This may be done via manual restraint of the animal and vaginal swabbing of fluid. Vaginal secretion should be collected at the same time each day and may be obtained with a plastic pipette filled with 10 μL of normal saline (NaCl 0.9%). Examination of the vaginal secretion should be visualized under a microscope and the ratios of leucocytes and cornified and nucleated epithelial cells can be used to determine the stage of the estrous cycle.

2.10. Study Endpoint

In a typical activity-based anorexia study, researchers should focus on analyzing changes in several key variables, including daily changes in body weight, food consumption, wheel running activity, and estrous cycle activity (in mature female rats, when needed). One approach for defining experimental endpoint is to utilize a predefined day for experimental exit in order to ensure that the characteristics of the model are present, yet animal health is still intact. For studies using adolescent female rats in our laboratory, this is typically day 4 of restricted food access. A second approach for defining experimental endpoint is to allow animals to remain in the model until a certain level of weight loss has been met (up to 35% of baseline body weight in our lab) or predefined criteria for a decline in animal health has been reached. This approach allows for more individual analysis of the response to restricted food access over time, but limits biological analyses collected postmortem, as the differences in the day of experimental exit are difficult to control for in analyses. This tradeoff should be considered in the experimental design.

3. Notes

Since activity-based anorexia is a model induced by stress, the following recommendations may help researchers to obtain more reliable and reproducible data.

3.1. Note 1

Individual housing is a stressor for animals; however, adaptation occurs more easily when the transition occurs at a young age. Sources of environmental enrichment, such as a nylon bone in the home cage, should also be added to minimize stress to the animals. Access to a running wheel, which is a key component of the activity-based anorexia model, also serves as a source of environmental enrichment.

3.2. Note 2

Although the activity-based anorexia model typically combines unlimited access to a running wheel (24 h per day) with restricted access to food (unlimited access for 1 h per day), this methodology varies slightly across studies, with some restricting wheel access during the period of food access and other studies increasing the period of food access to 2 h per day. The rationale for increasing the length of food access is often based on improving overall survival time in the model by allowing the animal more time to consume food. However, our experience would suggest this alteration in the paradigm often sacrifices the severity of activity-based anorexia behavior, resulting in lower levels of weight loss and hyperactivity and fewer animals developing the model. Again, the tradeoff should be part of the rationale in the experimental design.

3.3. Note 3

Given the limitations in understanding and interpreting comparable levels of weight loss in the food-restricted control group using a 1 h per day period of food access, a second approach is to use a control group that receives a predefined amount of food intake per day (typically 40–60% of baseline food intake) or to match intake to an experimental activity-based anorexia animal to induce weight loss. In our experience, this approach does not promote the more severe levels of weight loss seen in activity-based anorexia, given the inability to match the energy deficit state created by the excessive exercise typically found in the activity-based anorexia model. Moreover, this approach introduces variability in the experimental design without a significant effect on weight loss and therefore is not recommended.

3.4. Note 4

When considering the assessment of estrous cycle activity, it is important to consider the potential stress effects of the procedure. Typically, it is important to measure estrous cycle activity for at least several days prior to the onset of restricted food access in order to obtain an accurate baseline of estrous cycle activity as well as to acclimate the animal to the procedure. If an animal begins estrous cycle assessment at the same time as restricted food access, the stress of the new procedure may increase behavioral variability and therefore affect the outcome of the model. In adolescent rats, vaginal opening does not occur until approximately postnatal day 32–37, thus if restricted food access occurs around this age, the potential stress effects of beginning estrous cycle assessment without acclimation to the procedure need to be carefully weighed against the information that estrous cycle assessment will provide. Moreover, animals younger than puberty onset cannot undergo the vaginal swab procedure, thus if comparing activity-based anorexia across age groups, it is recommended that estrous cycle activity not be assessed in order to maintain consistency in experimental procedures across age groups.

4. Conclusion

This chapter has highlighted the major methodological issues that must be considered when designing an experiment aimed at utilizing an animal model of anorexia nervosa. Although it is clear that activity-based anorexia does not model the psychological components of anorexia nervosa, the model does provide a rational approach for studying the development and maintenance of self-starvation and hyperactivity induced by programmed food restriction. The activity-based anorexia model holds significant promise for identifying neurobiological pathways involved in these maladaptive behaviors and for testing novel pharmacological treatments.

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Chapter 18

Food-Anticipatory Activity: Rat Models and Underlying Mechanisms

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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, and includes increased locomotor activity and arousal prior to food access. These models can be used to shed more light on research questions, particularly "What happens in the brain when we are triggered to 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 are provided. In addition, the potential mechanisms underlying FAA, with a special focus on leptin, dopamine, and ghrelin signaling, are described.

Key words: Food-anticipatory activity, Activity-based anorexia, Ghrelin, Leptin, Dopamine

1. Introduction

1.1. Relevance for Studying Food-Anticipatory Activity

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 important 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

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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 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. 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 (≥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 ≤ 17.5 kg/m^2), (2) intense fear of gaining weight or becoming fat, (3) disturbances in body perception, and (4) amenorrhea in women. Although not mentioned in the DSM-IV 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 food-anticipatory activity (FAA) and its underlying mechanisms, as demonstrated through feeding models in rodents. 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 are 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 are also discussed.

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 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 focuses 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).

2.1.1. Experimental Set-up and Procedure

Female outbred Wistar WU rats weighing 160 g upon arrival 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 8,000–9,000 revolutions per day under ad libitum conditions. Their baseline chow intake is increased by 2–3 g compared to sedentary rats, probably to compensate for increased physical activity (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 baseline food intake. In addition, it will be reduced 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,

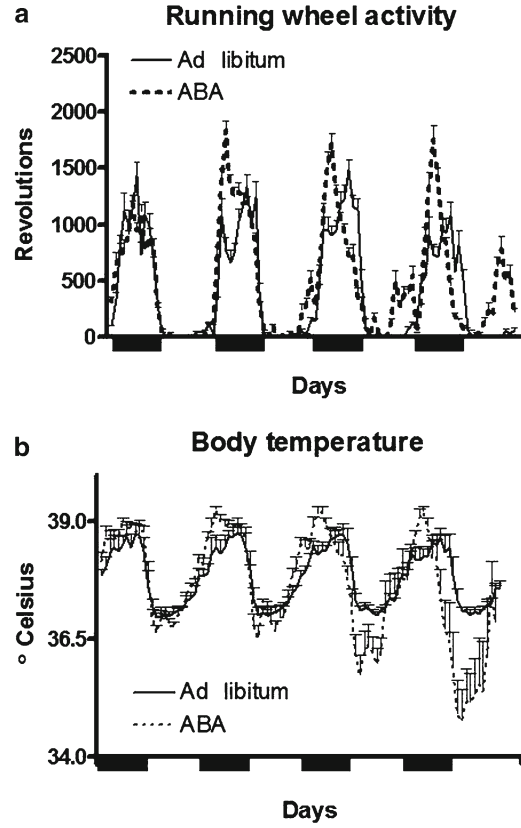

Fig. 1. Female Wistar rats were housed in running wheel cages and had ad libitum access to chow and water (*filled line*, $N=8$) or were subjected to an activity-based anorexia (ABA) model (*dotted line*, $N=8$) with 1-h access to food at the onset of the dark period. (**a**) depicts average running 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. In (**b**) represents the body temperature rhythms (averaged per hour per group) of rats during ad libitum feeding or ABA.

as depicted in Fig. 2a. 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 general, this threshold is reached within 4–6 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 the hours preceding food availability. The hyperactivity in the ABA model could reflect starvation, which induces 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.

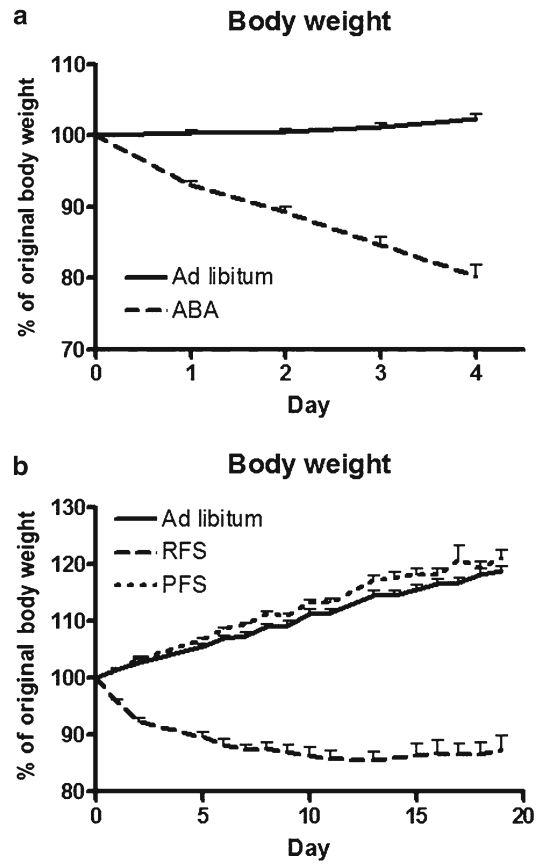

Fig. 2. Relative body weight gain, expressed as percentage of original body weight, of rats subjected to an activity-based anorexia model (ABA) (**a**), restricted feeding schedule (RFS) or palatable feeding schedule (PFS) (**b**) compared with ad libitum fed rats.

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 a 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 a RFS has also been reported in various other species, including mice, fish, birds, hamsters, and rabbits (33). Several parameters determine the course of a 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 Set-up 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 Sect. 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 RFS. On the first day, food is removed at zeitgeber time (ZT) 8, 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.

At first, rats on a 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 2–3 large meals of 5–6 g.

At the beginning of a 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 a 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, FAA can be observed within 3 days of the onset of a 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.

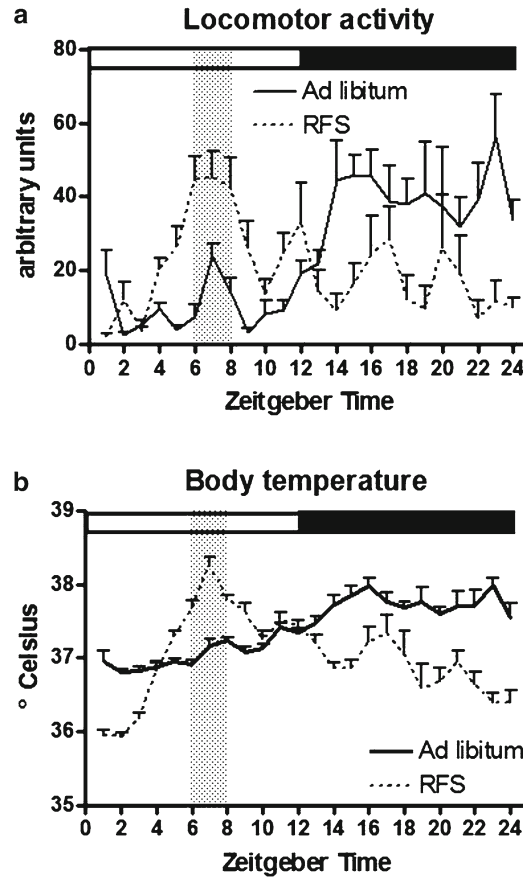

Fig. 3. Measures of locomotor activity (**a**) and body temperature (**b**) (averaged per hour) of male Wistar rats ($N=6$) were taken during ad libitum feeding (*filled line*) and when rats were subjected to a restricted feeding schedule (RFS, *dotted line*) with food available from ZT6–ZT8, as indicated by the *grey vertical bar.*

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

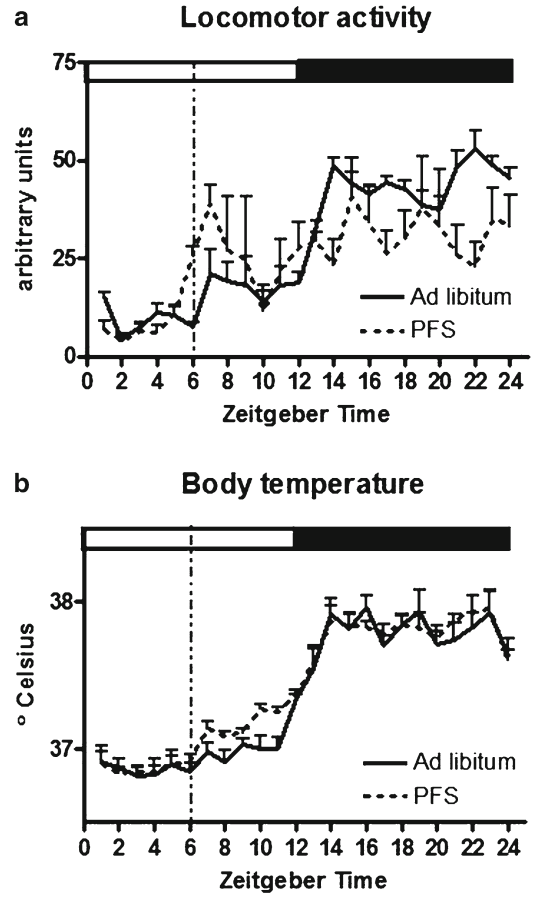

Fig. 4. Measures of locomotor activity (**a**) and body temperature (**b**) (averaged per hour) of male Wistar rats ($N = 6$) were taken during ad libitum feeding and when rats were subjected to a palatable feeding schedule (PFS, *dotted line*) with 5 g of chocolate available at ZT6 in addition to ad libitum chow access, as indicated by the *dotted vertical line*.

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).

2.3.1. Experimental Set-up 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 Sect. 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 a RFS, otherwise the running will become obsessive and rodents will run 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 down side, 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 do running wheels, since general locomotor activity is not exclusive to the dark phase in ad libitum fed rats. Furthermore, these devices not only record 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 are 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 min 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 interference has been applied to study FAA. Several knockout mice have been examined to determine whether certain genes play a role in FAA. Unfortunately, knock out models are scarce in rats. Another disadvantage of knock out models is that the gene is lacking from development onward, thereby enabling compensatory mechanisms. Conditional knock out 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.

The 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 are discussed in more detail in Sect. 3.3.3.

3.3.2. Involvement of Neural Circuits in FAA

Hindbrain

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).

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 a RFS. It has been demonstrated that dorsomedial hypothalamus (DMH), lateral hypothalamus (LH), tuberomammilary 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 a RFS. *Per2* rhythms shifted or changed in DMH, PVN, and VMH in rodents on a 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 vivid discussion takes place in the literature on 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, 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 a 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 a 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 brainstem 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 treatment 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 not only reduces hyperactivity 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 a 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 could also be considered leptin resistant because they possess a point mutation in the gene encoding the leptin receptor, still exhibited strong FAA to a 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 is 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, even 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 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). Posttranslational 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). Plasma ghrelin 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 in the 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, it has been found that ghrelin could play a role in FAA. Indeed, Plasma ghrelin 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 models involves hunger and motivational components, whereas FAA in the PFS model 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 a 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 models. 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

Anorexia and Drugs of Abuse Abnormally Suppress Appetite, the Result of a Shared Molecular Signal Foul-Up

Laetitia Laurent, Alexandra Jean, Christine Manrique, Mohamed Najimi, Fatiha Chigr, and Valérie Compan

Abstract

The brain serotonin (5-hydroxytryptamine, 5-HT) system is implicated in the neurobiological control of feeding and appears to be dysfunctional in patients suffering from feeding disorders, such as anorexia nervosa, bulimia nervosa, and obesity. Thanks to the identification and cloning of 5-HT receptors, the production of agonist and antagonist compounds, and the generation of 5-HT receptor knockout mice, our knowledge of the implications of different 5-HT receptor subtypes in feeding behavior has greatly increased. Studies have demonstrated an involvement of the hypothalamic 5-HT_{1B} and 5-HT_{2C} receptors in food intake and body weight control. In contrast, the connection between the brain 5-HT system and eating disorders has been less investigated. Little is known about the influence of 5-HT on the rewarding value of eating. Such value may not be linked to food consumption, but rather to voluntary reduction of food intake, as reported upon activation of the 5-HT_4 receptors in the nucleus accumbens, which induces downstream events (cAMP/PKA/CART). Here, we describe experimental procedures to study 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 treated with a recreational drug of abuse ("ecstasy"). We include the description of a micropunch technique, which allows for the analyses of specific downstream events (cAMP: FRET; pCREB: western blot), molecular biology (RQ-PCR), and radioautography. We conclude that abnormalities in the reward system, possibly disturbing the autonomic nervous system control of feeding behavior, might contribute to the anorexic behavior. Potential 5-HT receptor agonists/antagonists could be developed and used in association with psychological treatment to better cope with the stressors that trigger 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

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nervosa, can be found in a myriad of texts from the last six centuries. To the best of our knowledge, the most illustrious example is that of Catherine de Sienne (1347–1380) (1). However, only in the seventeenth century anorexia became the subject of a clinical description (Thomas Morton, 1689), and the first scientific accounts by Charles Lasègue in France and William Gull in England date from the beginning of the nineteenth century (2).

Eating disorders are now classified as mental disorders according to the Diagnostic and Statistical Manual of Mental Disorders (3). To simplify, bulimia nervosa or binge eating is characterized by impulsive and repeated phases of excessive food intake, whereas the main characteristic of anorexia nervosa is self-imposed food restriction despite energy requirements. The patients, usually women, may suffer from either anorexia nervosa or bulimia nervosa (DSM-IV-TR diagnostic codes 307.1 and 307.51, respectively), or from both disorders simultaneously. These disorders are complex dysfunctions of the central nervous system, as they often coexist with other mental diseases, such as pathological anxiety (4) and depression (5). Furthermore, a recent study has 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 of 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 requires deciphering the associations between nonvoluntary and voluntary brain controls, biological and environmental factors, and genetic and epigenetic influences, and thus the collaborative effort of psychologists, psychiatrists, and neurobiologists.

The central nervous system controls the organism's energy balance by regulating food intake and energy expenditure. The neurobiological abnormalities related to anorexia nervosa are poorly understood. The hypothalamus has a central influence on the regulation of feeding behaviors, but motivation disorders in which patients self-impose food restriction despite energy demand (anorexia) may also involve prominent disturbances in the nucleus accumbens (NAc), a key brain reward structure (6, 9). Among the numerous neural and hormonal messengers involved in feeding disorders, altered 5-HT transmission appears to be at the forefront of investigations. It is generally agreed that increased activity of 5-HT neurons and higher 5-HT expression in the brain trigger the reduction in food intake and body weight loss. Indeed, anorexia-like behavior (self-imposed food deprivation despite energy requirement) can be induced by treatments that increase 5-HT release in

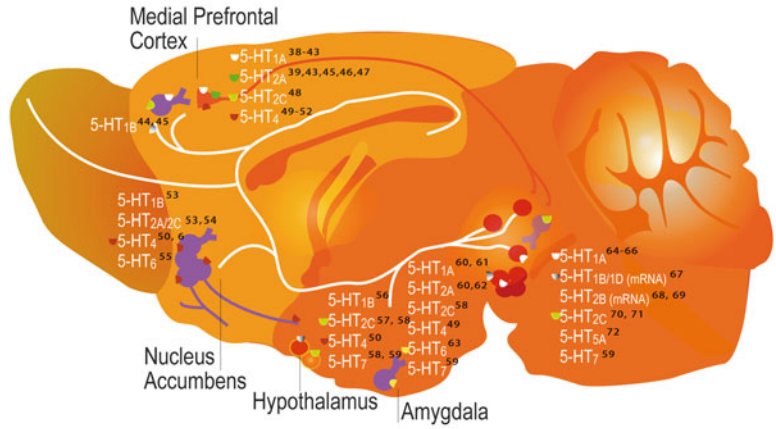

Fig.1. Schematic representation of 5-HT receptor subtypes present in brain regions involved in feeding behavior.

the brain (10). For instance, fenfluramine, which increases the extracellular 5-HT levels, lowers the consumption of food in humans and rodents (11, 12). Similarly, amphetamine and 3,4-*N*-methylenedioxymethamphetamine (MDMA, ecstasy) diminish food consumption in rats (13) and humans (14), and reduce deprivation-induced eating in mice (15).

The 5-HT neurons and receptors (5-HTR) are distributed throughout the brain, including all anatomical regions classically involved in feeding behavior (Fig. 1). Over the past three decades, seven families of 5-HTR have been identified with 18 5-HTR subtypes, without including mRNA editing and splice variant isoforms. The implication of these receptors in behavior has been widely described in studies using mice in which the genes encoding the subtypes 5-HT_{1A} (16), 5-HT_{1B} (17), 5-HT_{2A} (18), 5-HT_{2B} (19), 5-HT_{2C} (20), 5-HT_3 (21), 5-HT_4 (22), 5-HT_{5A} (23), 5-HT_6 (24), or 5-HT_7 (25) had been genetically invalidated. Both hypothalamic and NAc neurons express different 5-HTR. In the NAc, 5-HTR_4 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 motivation disorders to consume food, 5-HTR_4 could become targets for the pharmacological treatment of anorexia and likely bulimia. For now, both diseases remain untreated by pharmacological means.

Anorexia nervosa and bulimia nervosa in humans are extremely complex compared to the "simplicity" of animal models. However, animal models represent the first required step to propose molecular therapeutic targets and alternatives. Results obtained 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" (26).

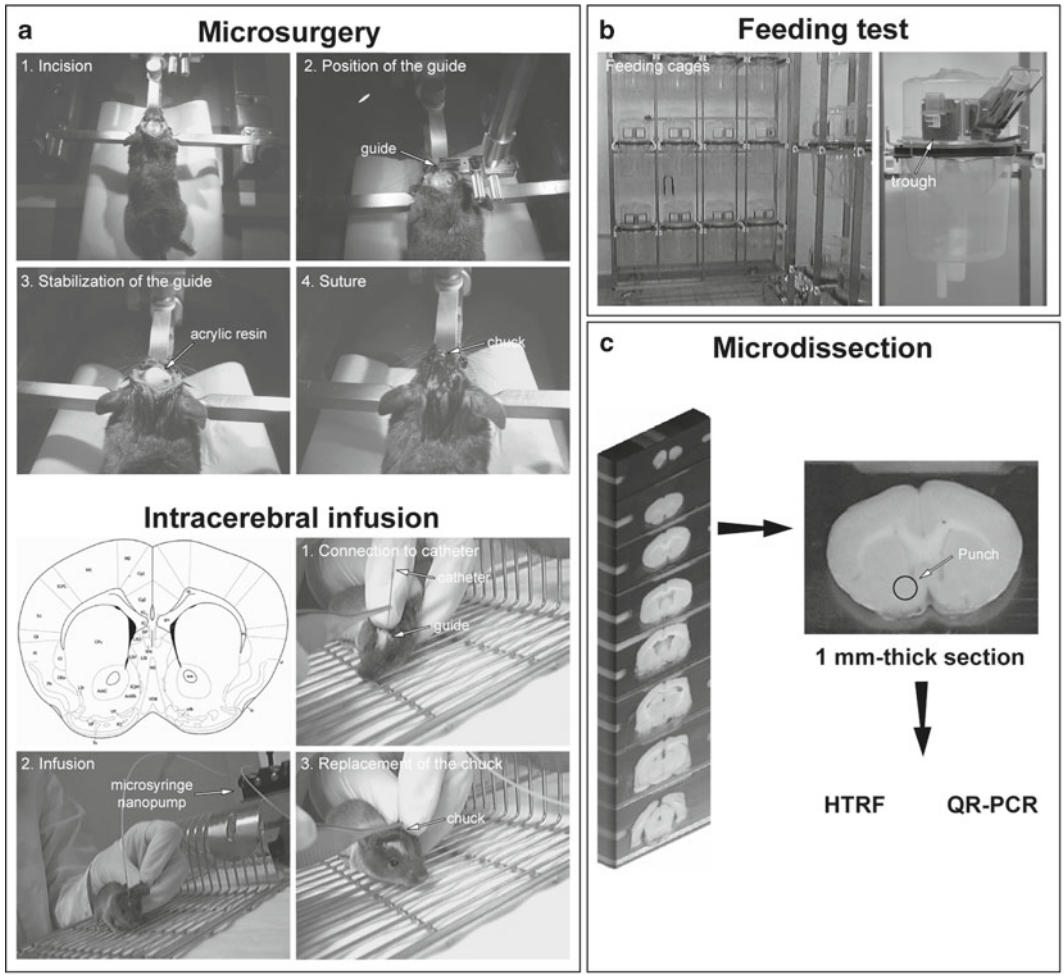

Fig. 2. Overview of the experimental strategy: from molecule to behavior analyses. (**a**) microsurgery for intracerebral infusion of compound (agonist, antagonist, inverse agonist, siRNA, transformed virus) in freely moving animals. Five days before any infusion of compounds into a specific brain area, a steel guide is unilaterally implanted at coordinates from the bregma according to the brain atlas (described in detail in Sect. 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, and are infused at 7 min intervals 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 are microdissected at −20°C. Tissue samples are then treated using the HTRF technique (described in detail in Sect. 2.2) or QR-PCR.

Using animal models, we postulated that anorexia might involve altered signaling events within the NAc (6). To address this possibility, we examined the effects on food intake of directly stimulating or inactivating 5-HTR_4 in the NAc (Fig. 2, microsurgery in freely moving animals). The 5-HTR_4 have been selected because we have previously observed that mice lacking these receptors displayed attenuated feeding responses to the restraint stress compared to wild-type mice (Fig. 2) (22). We also investigated whether these receptors are further involved 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-HTR_4 (si5-HTR_4) knockdown into the NAc (siRNA-mediated knockdown in adult mouse). Using 5-HTR_4 knockout (KO) mice, we further determined that 5-HTR_4 contributes to the appetite-suppressant effect of MDMA. In addition, the generation of 5-HT receptor knockout mouse required the use of different and specific complex methods previously and extensively reviewed (27–29). Additional experiments revealed that the activation of accumbal 5-HTR_4 increases the mRNA level of the satiety factor cocaine- and amphetamine-regulated transcript (CART) via a cAMP/PKA signaling pathway. Finally, we provide evidence that increased CART mRNA expression mediates the appetite-suppressant effects of both accumbal 5-HTR_4 stimulation and MDMA, the psychogenic compound of ecstasy. Using this set of combined methods, the 5-HTR_4 was observed to mediate upregulation of CART in the NAc, which triggers anorexia-like behavior and the appetite-suppressant effects of ecstasy.

2. Materials and Procedures

2.1. Microsurgery Materials, Equipment, and Setup

Intracerebral infusion of the compound into the NAc or any other brain area in freely moving mice requires the implantation of a permanent sterile stainless steel guide (internal diameter: 0.160 mm, external diameter: 0.405 mm). A chuck is required to temporarily fill the guide until injection. The lengths of the guide and chuck are strictly equal and have to be determined according to the coordinates of the brain atlas (30). The coordinates are adapted for mice weighing 30 g. 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-like state 15 s after mixing. The manipulation should be finished before 2 min after mixing when setting starts.

Mice are deeply anesthetized by intraperitoneal (i.p.) injection with ketamine (60 mg/kg) and xylazine (15 mg/kg). Ketamine is an anesthetic and xylazine has a double advantage of being sedative and analgesic. Lachrymal frost is systematically used in order to protect the eyes of each animal from light. This mixture of both ketamine and xylazine is 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 is stored and maintained systematically at 4°C until use and is dissolved in NaCl (9%) on the surgical day. The dissolved solution is maintained at 4°C for the entire 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 Materials, Equipment, and Setup

Animals are handled and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Mice are housed 3–6 per cage and allowed to acclimatize for at least 5 days after their arrival at the facility. All animals are then housed in individual cages and handled daily for 5–7 days before the experiment to minimize handling-associated stress; this period is thought to correspond to the complete adaptive process (31). Some mice were subjected to acute immobilization stress or restraint stress according to a well-established protocol (32, 33), as described in detail below. The animals are randomly assigned to stress procedure or control groups. Two methods can be used for the execution of immobilization stress: immobilization-induced stress with a restrainer device or immobilization-induced stress without a restrainer.

2.3. Micropunch Technique Materials, Equipment, and Setup

Animals are sacrificed after the various treatments and the brain area (e.g., NAc: 1.2 mm^3; hypothalamus: 2×3.9 mm^3) is 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) (30). Figure 3 illustrates the micropunch technique (6, 34). The 1-mm-thick brain sections are obtained using a brain slicer matrice, allowing the precise sectioning of the brain. From these tissue samples, total mRNA can be then isolated, for example, and the mRNA of interest can be analyzed (34, 35).

2.4. Microsurgery Procedures

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 on in a manner that allows them recover sufficiently before the active (dark) period.

Mice are anesthetized by i.p. injection of ketamine (60 mg/kg) and xylasine (15 mg/kg) and placed in a stereotaxic frame. The skin is precisely cut in only one movement from the top to the bottom of the skull 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. Small incisions are made at the surface of the bone in order to fix the resin in a later step. The bone is drilled in order

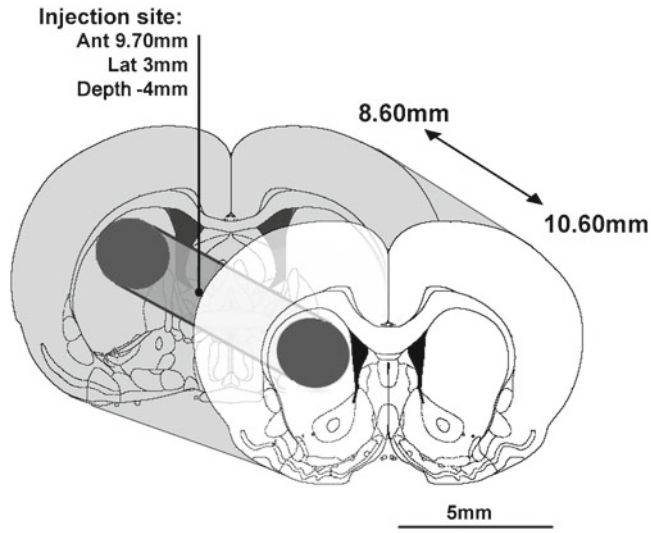

Fig. 3. Illustration of the punch technique. 2 mm striatal sections are obtained at –20°C using a specific slicer designed for the present investigation. Inside sections, a cylinder (2 mm diameter) was dissected with a micropunch allowing the experimenter to obtain cylindric striatal samples of 6.28 mm^3 according to the coordinates of the stereotaxic atlas (73): anterior: 8.60–10.60 mm from interaural line. Punched samples included the treatment injection site located at anterior: 9.70 mm from the interaural line.

to implant the guide. A saline solution is used to remove all bone dust. The guide is placed slowly according to the coordinates. The resin is then applied using a small spatula such that the mixture surrounds, but does not touch, the guide. The experimenter should wait 3 min for the resin to solidify and the guide to be permanently implanted. Using sterile surgical thread, the skin is stitched. The localizations of the injection sites are then systematically assessed for each mouse.

2.5. Restraint Stress and Forced Immobilization Procedures

Immobilization stress (IS) 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 human. IS, 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 (36) 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. For this, immobilization is considered as a complex stressor and includes physical as well as psychological dimensions.

In the restraint stress paradigm, the animal has to be immobilized individually in an adjustable, semicylindrical, acrylic restrainer

with air holes (Plexiglas restrainers) for 110 min. Other designs with device variations have been proposed. For example, to restrain the animal, a cylindrical wire mesh restrainer may be clamped at both openings and placed inside the animal's 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). This method ensures minimum movement, including that of the tail, and involves no pain. Control mice are left undisturbed in their home cages. The body weight of each animal is measured daily, beginning 1 week before stress and lasting until sacrifice.

In the immobilization paradigm, mice are placed on wooden boards in prone position by taping their limbs and shoulders to metal mounts at 0930 hours for 60 min, after which they are returned to their home cages. Head movements can be restricted with an appropriate loop around the neck. For the chronic stress, the experimental group of mice is subjected to immobilization daily for 1–10 days; control mice are handled each day for the same time period. All stress procedures begin at the same time of the day, between 0930 hours and 1030 hours, 3–4 hours after the start of the light cycle, to reduce the disruption to the circadian rhythm and associated variations on stress hormone levels as much as possible. The body weight and 24-hour-cumulative food intake of each animal are measured daily (at 0730 hours and 1500 hours) from 1 week before the chronic stress period, until sacrifice. Depending on the assay used, mice are sacrificed by decapitation after stress; controls are sacrificed at the same time.

2.6. Micropunch Technique Procedures

Following sacrifice, brains should be frozen in powdered dry ice to keep their original form. Blocks of dry ice would alter the form of the brain, and the tissue samples may not contain the target area. We commonly use the micropunch technique in mice, but it also can be used in rats. The brain matrice, micropunch, slides, pincer, and tubes for collecting tissue samples should be put into the cryostat and maintained at –20°C; otherwise, tissue samples could melt.

3. Anticipated Results and Notes

3.1. Anticipated Results

Using the restraint stress model in mice, to mimic anorexia-like behavior, we found that the 5-HTR_4 knockout mice displayed attenuated feeding responses to acute restraint stress (22). We also 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. Stimulating these receptors in mice reduced their drive to eat and increased production of the

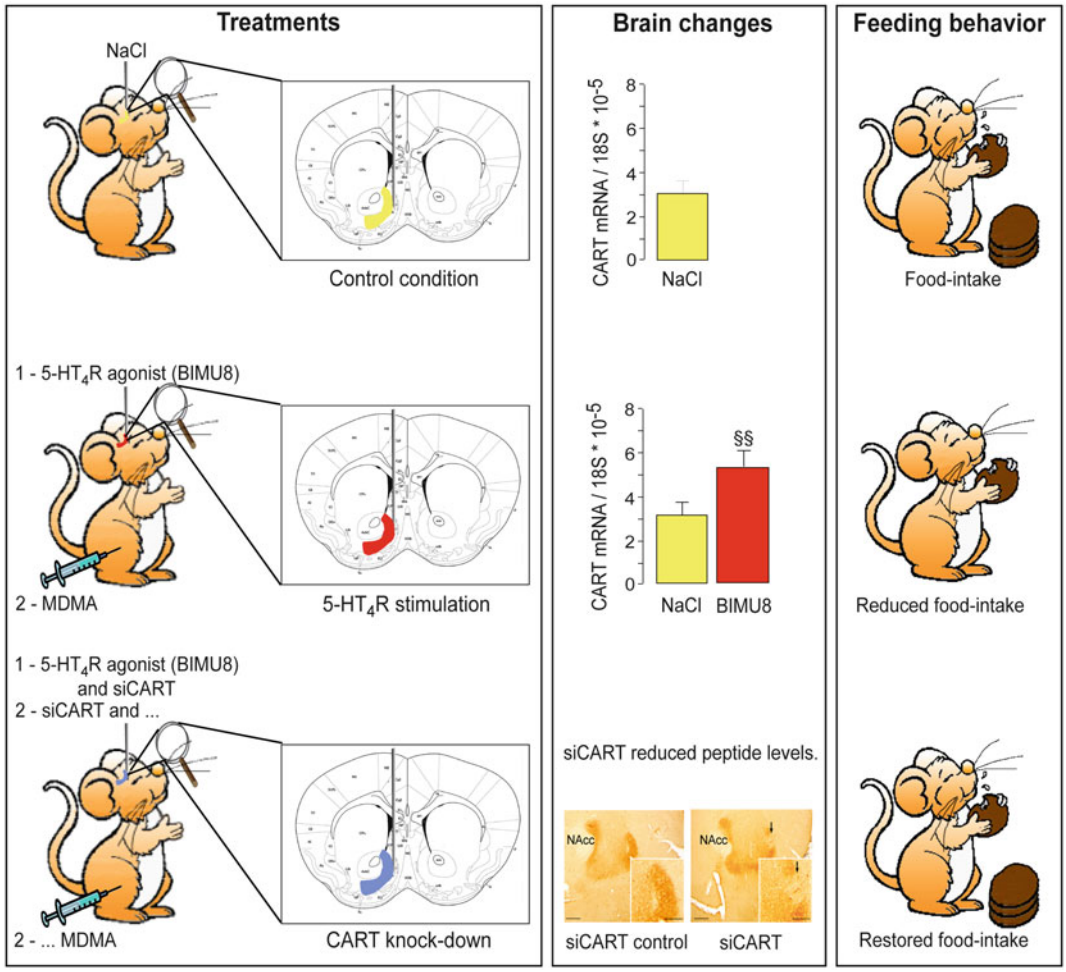

Fig. 4. Schematic representation of stimulating the 5-HT_4 receptors in mice reducing their drive to eat and increasing production of the same transcripts (CART) stimulated in response to MDMA (psychogenic compound of ecstasy).

same transcripts stimulated in response to cocaine and amphetamines (CART: Fig. 4). Blocking the receptor with RNA interference increased food intake. Mice lacking the 5-HTR_4 displayed attenuated feeding responses to the appetite-suppressant effects of ecstasy (Fig. 4).

3.2. Notes

1. 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 assessment before any period of treatments that feeding behavior is similar compared with a nonoperated group of mice.

2. Following surgery, we recommend placing the mice on a warm-water plastic pocket in order to preserve the life of the animals.
3. At the end of the surgery session, we recommend hydrating 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. The micropunch technique requires being extremely systematic to dissect the tissue samples at the same location. The samples should be maintained at –20°C. The cryostat chamber is then appropriate to perform this technique.
5. Prior to the restraint stress or immobilization and particularly before beginning handling, ensure that there are no animals presenting weight loss, abnormal locomotor activity, or wounds.
6. If several mice display abnormal weight loss during handling procedures, they should be excluded. Be sure that strain(s) used are not sensitive to handling.
7. It is important to minimize the impact of environmental conditions by keeping constant the dark/light cycle, temperature, humidity, water, and standard mouse chow (which should be available ad libitum), and the same experimenter for at least 1 week before and during the entire 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 the tape gently in order to avoid nociception.

4. Conclusion

There are clear benefits in combining different methodological approaches (stress, microsurgery, and molecular techniques) to analyze mechanisms underlying eating disorders, such as anorexia. Adaptive techniques allowing the visualization of a molecular cascade of events in the human brain are not yet available. The use of the stress paradigms proposed is appropriate and mimics the psychological stress and associated behavioral changes seen in anorexia.

The goal of detecting therapeutic targets using animal models can be realized (melanocortin 4 receptors and 5-HT_{2C} receptors for obesity). Our animal models are highly predictive in human clinical applications because, in agreement with our previous studies (6), recent findings of the G. Knudsen's team have observed that the 5-HTR_4 binding site density is modified in the NAc

in humans suffering from eating-related disorders (personal communication), but also from hyperphagia (37), which further encourages investigation of the role of 5-HTR_4 in eating disorders.

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Chapter 20

The Anorectic Phenotype of the *anx/anx* Mouse Is Related to Hypothalamic Dysfunction

Ida A.K. Nilsson, Charlotte Lindfors, Tomas Hökfelt, Martin Schalling, and Jeanette E. Johansen

Abstract

The anorectic *anx/anx* mouse, characterized by reduced food intake, is an interesting and useful model for studies of mechanisms involved in the regulation of food intake and anorexia. The anorexia (*anx*) mutation arose spontaneously at the Jackson laboratory in 1976 and has now 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*. 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.

Key words: Anorexia, Neuropeptides, Hypothalamus, Neuroinflammation, Neurodegeneration, Oxidative stress, *Ndufaf1*, Mitochondrial dysfunction

1. Introduction

The regulation of food intake is one of the most important tasks of the brain. Derangement of the systems involved in this process can have fatal 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 (1–19). When this sensitive system malfunctions, it can lead to disturbed eating behavior and/or appetite, which in turn impact an organism's well-being. In mice, the projections from neurons in the hypothalamic arcuate nucleus (Arc), involved in feeding behavior, primarily develop during the first 3 postnatal weeks (20–24). Thus, this period represents a time window when environmental factors can have long-term effects on eating behavior.

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Over the past 15 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 (25–36). Many of these models have been critical for the elucidation of pathways and molecules important for the regulation of food intake in humans, such as the leptin-signaling pathway identified in the obese *ob/ob* mouse (37). 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, useful for studies concerning regulation of food intake, in particular, anorexia and self-starvation. It is also relevant for other conditions associated with disturbed appetite, such as malnutrition, cachexia, and failure-to-thrive in infants (38–40). The *anx/anx* mouse is also a useful model for studies of hypothalamic neurodevelopment, -inflammation, and -degeneration (41–44).

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 (45). 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 (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 around 3–5 weeks of age, possibly due to the severe starvation. Histological analyses have neither revealed any abnormalities in the gastrointestinal system or blood parameters, e.g., total red blood cell count, hematocrit, or haemoglobin, nor have any abnormalities in other organs been found using routine staining techniques (45).

2.2. Genetics

The *anx* mutation arose spontaneously in the second filial generation (F2) of a cross between DW/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 (45). The *anx* interval was initially mapped to a region approximately 20 cM proximal to the agouti locus, on

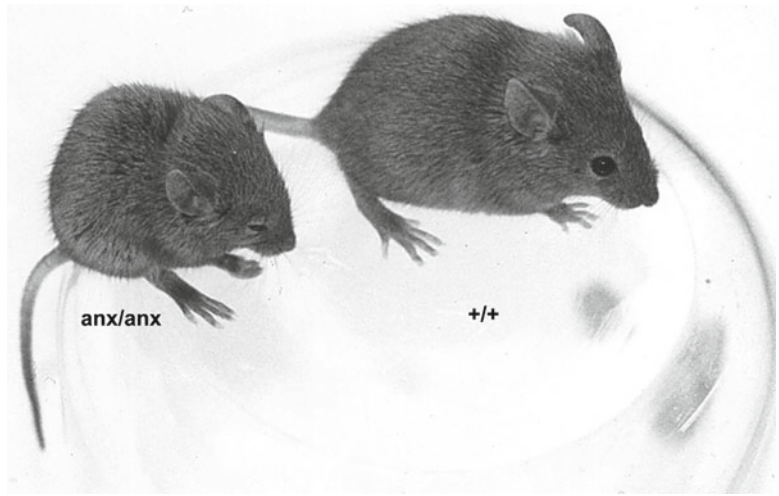

Fig. 1. An anorectic *anx/anx* mouse and a +/+ mouse, age 17 days.

chromosome 2 (Chr. 2) (45). By positional cloning, 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 cosegregate with markers *D2Mit104, D2Mit395,* and *Jojo8* (Fig. 2) (46). The interval includes approximately 40 identified genes.

2.3. Expression and Sequencing

Using an Affymetrix microarray analysis of the Arc, we identified a gene corresponding to one of the assembly factors for Complex I in the oxidative phosphorylation system (OXPHOS), *Ndufaf1*, to be downregulated in the *anx/anx* mouse (46). The downregulation was confirmed with both Western blot in the brain, and with real-time PCR in the brain, 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 have been identified. The mutation does, consequently, appear to be located in a regulatory element of the *Ndufaf1* gene.

3. Notes

3.1. 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) (14, 42, 47–54).

[1]Chr 2: bp 118,889,896-120,175,108; http://www.ensembl.org.

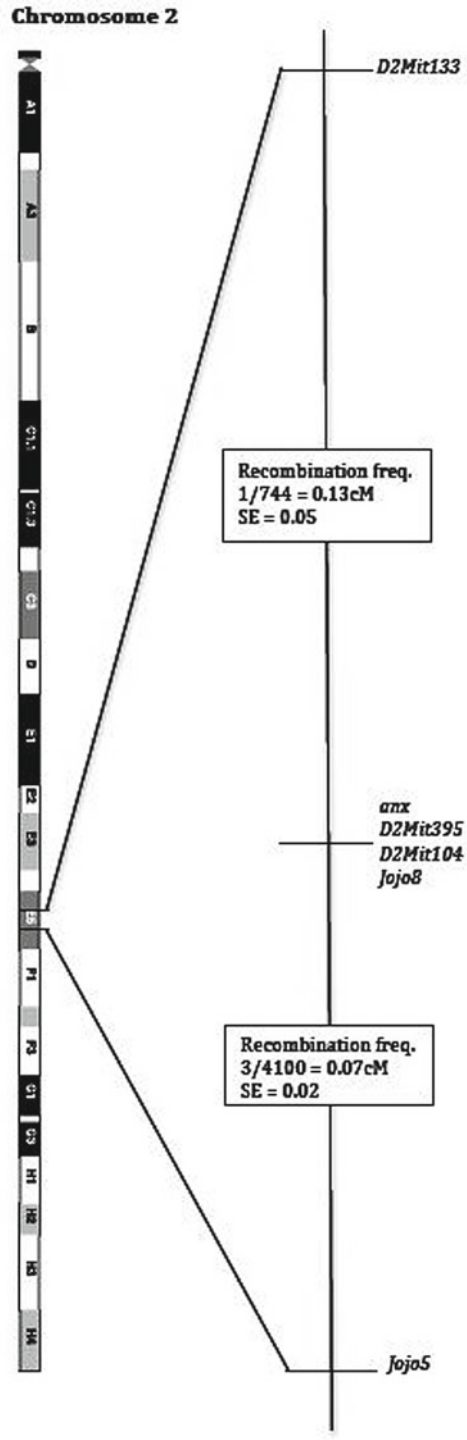

Fig. 2. Mapping of the *anx* mutation.

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

Abbreviations: *NPY* Neuropeptide Y, *AGRP* agouti-gene related transcript, *ACTH* adrenocorticotrophic hormone, *α-MSH* alpha-melanocyte stimulating hormone, *CART* cocaine- and amphetamine-regulated transcript, *POMC* proopiomelanocortin, *Y1, Y2, Y5* Neuropeptide Y receptors

The Arc is central in the energy homeostatic processes (1–12, 55–59). It harbors, in particular, two neuronal populations important for the regulation of food intake and energy metabolism (Fig. 3). One 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 expressed in multiple, widely distributed cell groups (50, 65–67). 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 alpha-melanocyte stimulating hormone (α-MSH). The AGRP/NPY neurons are known to partly be gamma-aminobutyric acid (GABA)-ergic (68), the POMC/CART cells glutamatergic (69). Both the AGRP/NPY and POMC/CART neurons are affected by 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 areas outside the hypothalamus (50). 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 (3, 5, 70, 71). It has also been established that the GABA/NPY/AGRP neurons innervate the POMC/CART neurons (72). Due to their roles

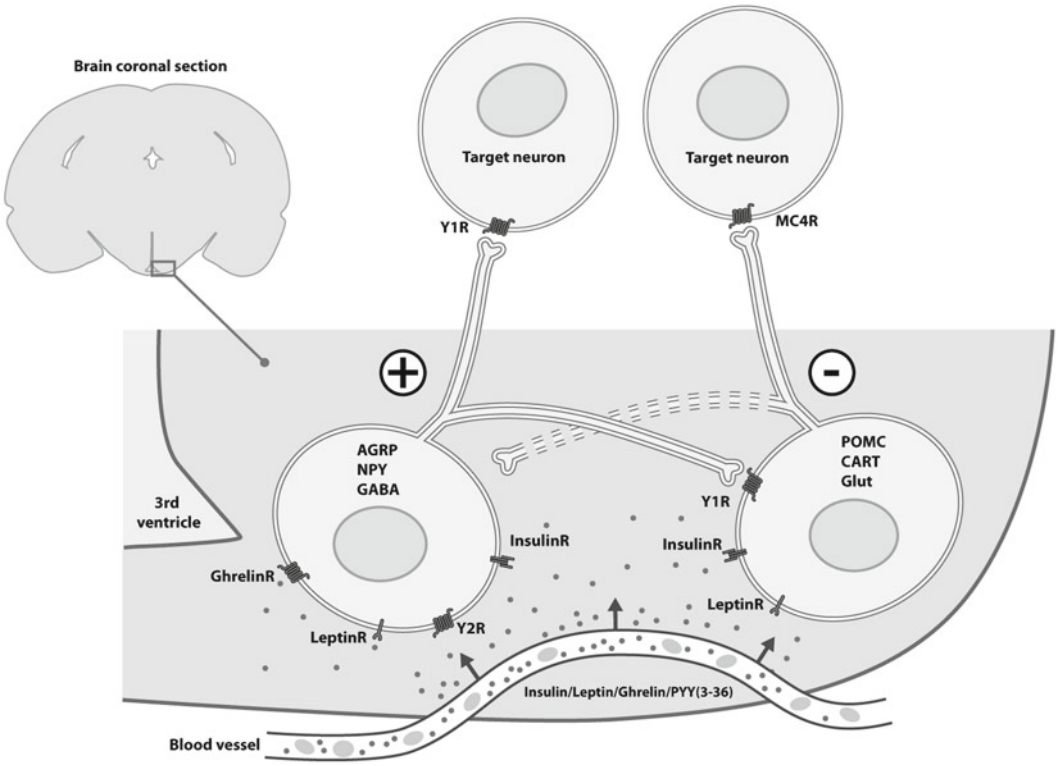

Fig. 3. The arcuate nucleus (Arc) is located in the most ventral, medial aspects of the hypothalamus, adjacent to the third ventricle and the median eminence, and ventral to the ventromedial nucleus. It harbors a large number of neuron populations characterized by different neurochemical markers, in many cases coexisting (60, 61). There are two prominent cell groups that have received much attention due to their involvement in control of food intake: the Neuropeptide Y (NPY)/agouti-gene-related peptide (AGRP) neurons (ns), in part gamma-aminobutyric acid (GABA)-ergic (NAGns), and the pro-opiomelanocortin (POMC)/*cocaine- and amphetamine*-regulated transcript (CART) neurons, in part glutamatergic (Glut; 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 (Y1R) on the PCGns. A reciprocal innervation, that is 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 (1–19, 62–64). *MC4R* melanocortin 4 receptor.

in the regulation of food intake, both the AGRP/NPY and POMC/CART neurons have been extensively studied in anorectic *anx/anx* mice (42, 44, 47, 49, 51, 54).

Immunohistochemistry analyses, using antibodies raised against NPY or AGRP, have revealed a reduced density of immunoreactive fibers in all projection areas studied, including the paraventricluar nucleus of hypothalamus (PVN), the lateral hypothalamic

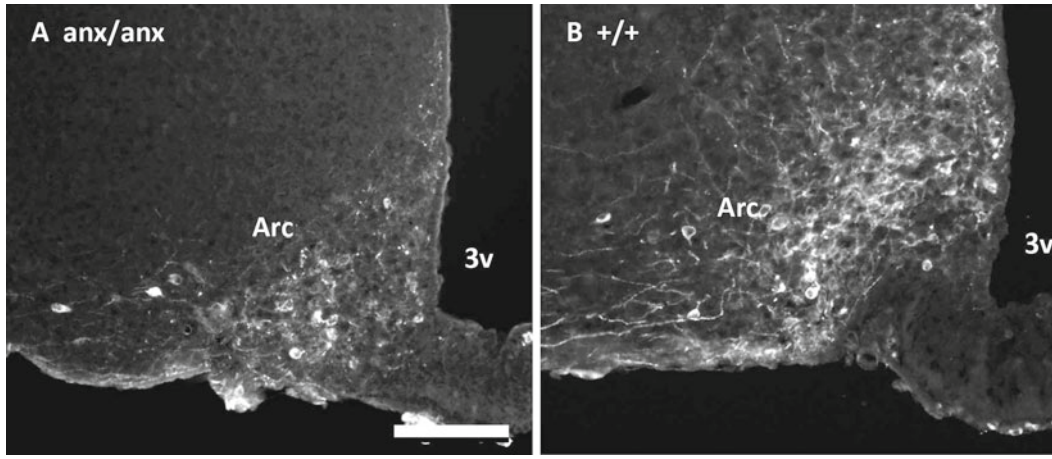

Fig. 4. Immunohistochemistry for Neuropeptide Y receptor 1 (Y1), a marker for POMC/CART neurons in Arc of an *anx/anx* (**a**) or +/+ mouse (**b**). Note the reduced density of Y1-immunoreactive cell bodies and dendrites in the *anx/anx* mouse. *3v* third ventricle; *Arc* arcuate nucleus. Scalebar = 10 μm.

area (LHA), the dorsomedial hypothalamic nucleus (DMH), and the Arc, when comparing *anx/anx* mice and +/+ mice at P21 (50, 54). 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 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 (73). In situ hybridization studies of NPY and AGRP mRNA levels have been conflicting (51, 53, 54). Broberger et al. (54) concluded 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. (53). However, a later study showed an increased expression of both AGRP and NPY mRNA in *anx/anx* mice of the same age as in the previous studies (51). 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 from the precursor protein POMC, show markedly attenuated immunoreactive fibers in *anx/anx* hypothalamus. This is, as expected, accompanied by reduced numbers of immunoreactive cell bodies for ACTH, another fragment generated from the POMC precursor. Furthermore, immunohistochemistry for the NPY receptor (Y1), which decorates the soma and dendrites of POMC/CART neurons (74), confirms these results by showing a reduced density of both immunoreactive dendrites and cell bodies in *anx/anx* hypothalamus, suggesting atrophy or degeneration (Fig. 4) (47).

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 (52, 53). 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. Maltais and colleagues report that 15-day-old normal mice that are treated with a serotonin precursor display these type of behaviors, and treating *anx/anx* mice with a serotonin antagonist has been shown to stabilize their motor abnormalities (45).

Abnormal dopaminergic neurotransmission has been demonstrated in the *anx/anx* mouse (75). 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 (75).

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 (76).

3.2. 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 (22, 23, 77). In *anx/anx* mice, this system initially appears to develop as in wild-type mice, but around P12-15 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. 5a, b, e, f) (42). Whether this represents increased release of AGRP and NPY or degeneration remains to be determined.

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. 5c, d, g, h) (44). In fact, the POMC neurons do not show signs of degeneration until P21, thus following the disappearance of AGRP/NPY fibers, which are already showing reductions at P12-15 (42).

From P8, *anx/anx* mice have significantly reduced serum leptin levels (49), 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 (20, 21), one can speculate that the low levels of leptin contribute to the aberrances, e.g., neurochemical, neurodevelopmental, and neurodegenerative processes, seen in these circuits in the *anx/anx* mouse.

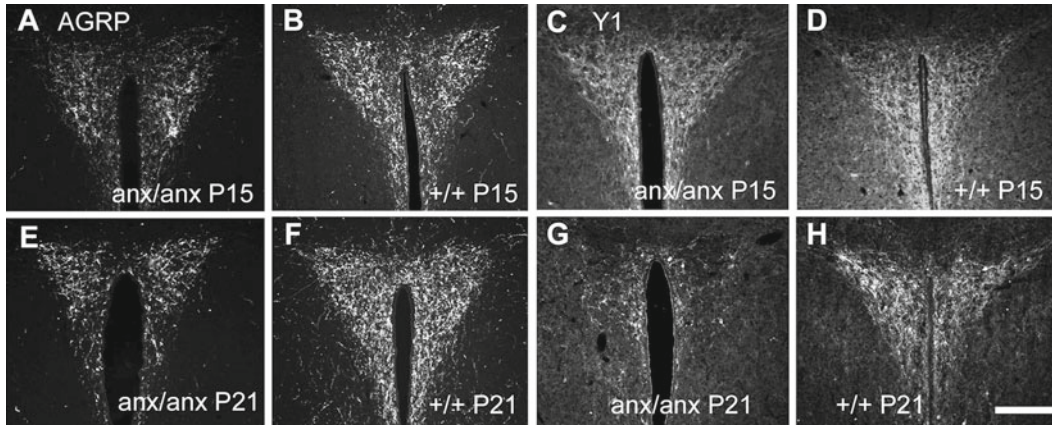

Fig. 5. Immunohistochemistry for agouti gene-related protein (AGRP; **a**, **b**, **e,** and **f**) and Neuropeptide Y receptor 1 (Y1) (**c**, **d**, **g,** and **h**) in paraventricular nucleus (PVN) of *anx/anx* (**a**, **c**, **e,** and **g**) and +/+ mice (**b**, **d**, **f,** and **h**) at P15 (**a**–**d**) and P21 (**e**–**h**). Note that a reduced density of AGRP-immunoreactive fibers in the *anx/anx* mice is visible already at P15, while such a reduction in Y1-immunoreactive processes is present first by P21. Scalebar = 200 μm.

Bouret et al. showed leptin's neurotrophic effect on the Arc neurons through studies on the obese leptin-deficient *ob/ob* mouse (20). The *ob/ob* mouse shows an abnormal development of Arc projections, resembling the pattern seen in *anx/anx* mice. By injecting *ob/ob* mice postnatally with leptin, they were able to normalize the development of these projections.

3.3. Hypothalamic Neuroinflammation

Several studies have indicated a relation 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 involved in the phenotype (41). In a second gene expression study of the *anx/anx* hypothalamus, Mercader and colleagues also concluded that the phenotype is related to changes in several inflammatory genes (43). In parallel, we have shown a strong activation of microglia cells specific for regions to which the food-intake-regulating AGRP neurons project, in particular hypothalamic regions, in *anx/anx* mice. Signs of activated microglia in these regions have been documented from P12, and progressively increase, reaching strong activation at P21, exemplified by LHA in Fig. 6 (42).

Microglia cells are normally activated in the central nervous system in response to neuroinflammation and/or neurodegeneration (78–80), and these 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 (44). 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 (81–85).

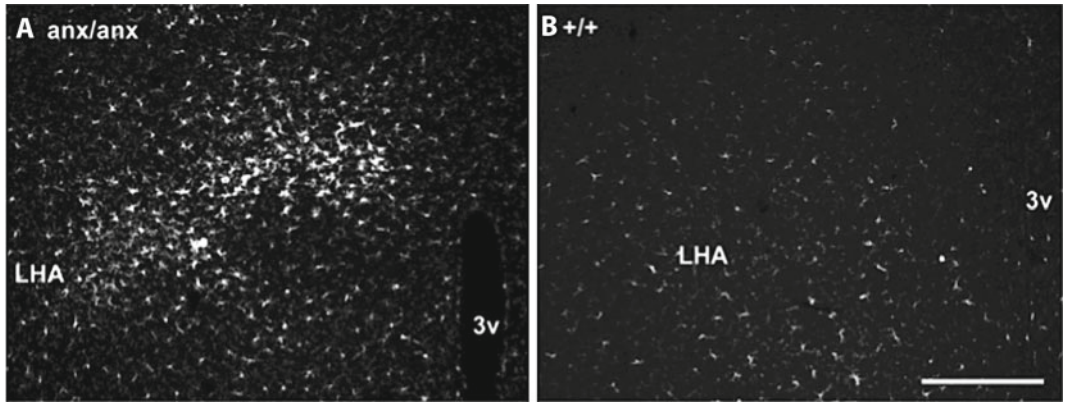

Fig. 6. Activated microglia cells, visualized by ionized calcium-binding adapter 1 (Iba1) immunohistochemistry, in hypothalamus of an *anx/anx* (**a**) or +/+ mouse (**b**). *LHA* lateral hypothalamic area; *3v* third ventricle. Scalebar = 200 μm.

Inflammatory mechanisms in the hypothalamus have been related to the impaired signaling seen after feeding with high-fat diet (86), obesity (87), and in cachexia (88). Thus, it seems that conditions of both excess and shortage of energy are coupled with hypothalamic mechanisms involving inflammation (89). This is paradoxical, and an extensive evaluation of the inflammatory progression in the different conditions is needed to understand this complex relationship between energy balance and hypothalamic inflammation. It is possible that studies of the *anx/anx* mouse may help resolve this paradox.

3.4. Hypothalamic Neurodegeneration

In addition to the signs of neuroinflammation and the loss of AGRP and NPY in nerve terminals, several observations have indicated 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 (47). 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, 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 fibers immunoreactive for AGRP (42), gave further support to the neurodegeneration hypothesis in the *anx/anx* Arc. These results are in agreement with results from Wu et al., showing that ablation of AGRP neurons results in gliosis in the target areas (90).

Moreover, the expression of MHC I by Arc neurons (44) may also support such a process being involved, since MHC class I has been shown to be expressed by degenerating (91, 92) or lesioned (93) neurons. We have detected an increased number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive neurons in *anx/anx* hypothalami as well as possible signs of what Ribak et al. call "microglia-associated cell death" (44, 94). This process of cell death appears different from both apoptosis and necrosis, whereby a 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 (44), a marker for axonal degeneration (95). 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 (96). A differential display analysis of the whole brain identified, among other genes, the apoptotic protease activating factor 1 as being regulated by the *anx* mutation (97). Last, our microarray analysis of the Arc region, mentioned previously, indicated several genes related to apoptosis/cell death/degeneration deregulated in the *anx/anx* mouse (Table 2).

3.5. Hypothalamic Mitochondrial Dysfunction

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 (Sect. 2.3), the microarray analysis also identified the Complex I assembly factor gene, *Ndufaf1*, to be downregulated in *anx/anx* mice (46). Blue native polyacrylamide gel electrophoresis revealed lower levels of fully assembled Complex I in *anx/anx* hypothalami 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 (46). We have also detected increased reactive oxygen species (ROS) and upregulation of antioxidative molecules in the hypothalami of these mice (46). Increased levels of ROS represent a common phenomenon accompanying dysfunction of Complex I. ROS is known to act as a signaling molecule affecting hypothalamic neurons, leading to decreased appetite (98–100), but is better known as a cause for oxidative stress and subsequently neuronal degeneration (101, 102). 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

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, 91kDa	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, 12kDa	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

(continued)

Table 2 (continued)

Symbol	Full gene name	Fold change
GJA1	Gap junction protein, alpha 1, 43kDa	1.5
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

of *anx/anx* mice. In conclusion, we suggest that the anorexia and premature death of the *anx/anx* mouse are related to a down regulation 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 (103). In addition, the mechanism of neurodegeneration related to Complex I dysfunction resembles events occurring in dopaminergic neurons in parkinsonism or Parkinson's disease (104–107). In fact, it has been shown that the degeneration of the dopaminergic neurons require a combination of Complex I dysfunction and disruption of microtubuli dynamics. It would thus be interesting to study microtubules in the Arc neurons in the *anx/anx* mice (108).

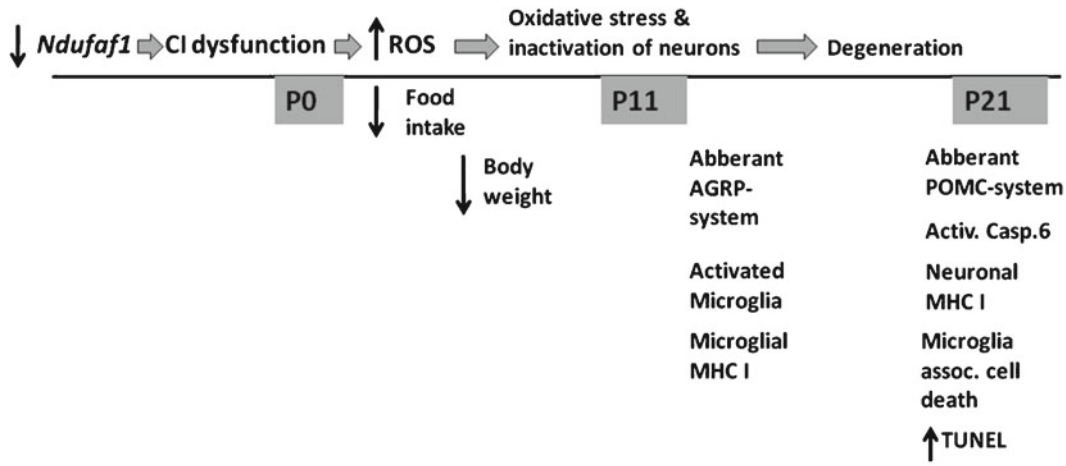

Fig. 7. Hypothetical illustration of the timeline and mechanisms underlying the anorectic phenotype of the *anx/anx* mouse. *CI* Complex I; *ROS* reactive oxygen species; *AGRP* agouti gene-related protein; *POMC* pro-opiomelanocortin; *MHC* major histocompatibility complex I.

4. Conclusion

The anorexia and premature death of the *anx/anx* mouse are related to aberrances in neuropeptidergic and neurotransmitter systems, neuroinflammation, neurodegeneration, oxidative stress, and mitochondrial dysfunction. A hypothetical scheme linking most of these phenomena is presented in Fig. 7. We suggest that the *anx* mutation leads to a downregulation of *Ndufaf1,* resulting in lower levels of fully assembled Complex I in OXPHOS, and subsequently accumulation of ROS. The increased levels of ROS may initially act as a signaling molecule via the food intake-regulating neurons in Arc, resulting in aberrant expression of neuropeptides and transmitters, and decreased food intake. Here a loss of the orexigenic NPY/AGRP signaling may be a contributory factor. In addition, ROS may give rise to increased oxidative stress and inflammation, and consequently, neurodegenerative processes in the hypothalamus of *anx/anx* mice.

The *anx/anx* mouse is thus a suitable model for studies on the neurobiology of regulation of food intake, particularly anorexia. The molecular mechanisms documented in the *anx/anx* mouse are involved in the anorectic phenotype in human conditions, such as anorexia nervosa, and other conditions involving disturbed food intake regulation, e.g., cachexia and failure to thrive. Future studies on these patient groups are needed to further test this hypothesis.

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Chapter 21

Functional Magnetic Resonance Imaging in Awake Rats: Studies Relevant to Addiction and the Reward Circuitry

Marcelo Febo

Abstract

Functional magnetic resonance imaging (fMRI) has been used to investigate human and laboratory animal brain reward function using a variety of experimental paradigms. The most popular functional imaging technique relies on the blood oxygen level-dependent (BOLD) contrast mechanism first reported in the anesthetized rat by Seiji Ogawa and coworkers in the early 1990s. A significant advantage of fMRI is that it allows a functional characterization of the awake rodent brain under different treatment and pharmacological conditions. We have performed fMRI of the neural actions of cocaine in awake male and female rats and the lactation stimulus in postpartum rats. Animal studies have the design flexibility to verify results with a multiplicity of invasive brain methods that can inform human work and aid in data interpretations. This review provides a summary of the methods used for fMRI experiments in rats with a special focus on awake imaging methods used in our laboratory, which can be applied to feeding behavior.

Key words: fMRI, Rat, Restraint, Acclimatization, BOLD signal, Addiction, Cocaine reward, Maternal brain, Lactation

1. Introduction

1.1. Functional MRI (fMRI), Perspective, and Mechanisms

Small animal fMRI originally led the way to what are now well-accepted methods in neuroscience that have added to our understanding of the human, nonhuman primate, and rodent brains. Although the applications of this technology to study the central nervous systems of smaller animals has trailed the development of human functional neuroimaging techniques, many of the methods can be applied to laboratory animal brain imaging, or first arose through direct experimentation in animals, such as rats. fMRI, which is a subset of methods within the broader nuclear magnetic resonance (NMR) imaging field, can be used in animals to track in vivo brain activation under a diversity of experimental manipulations. Moreover, the breadth of experimental designs in

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animal studies and the combinations with invasive tools and pharmacological testing make fMRI techniques an attractive and powerful strategy to use to understand the underpinnings of neural systems from a comparative viewpoint.

The methods presented here apply mostly to awake animal imaging experiments. We have used specialized methods in our laboratory to image animals while unanesthetized. It is obvious to those in behavioral neuroscience fields that this poses several challenges, not the least of which is how to deal with distress that arises during scanning of animals while awake and restrained. It is also challenging from a data processing standpoint, where the goal is to obtain high-quality data uncontaminated by severe movement artifact. As a comparison of the available methods and to present the broader range of tools available to researchers, I will also summarize some of the methods for imaging anesthetized animals. All experiments carried out using unanesthetized and restrained rats are done in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals (7th Edition, 1996) and adhere to the National Institutes of Health and the American Association for Laboratory Animal Science guidelines. The Institutional Animal Care and Use Committee at the University of Massachusetts, Northeastern University, and University of Florida approved the various protocols used for the cited studies.

1.2. The Blood Oxygenation Level-Dependent (BOLD) Signal

Contrast in magnetic resonance (MR) images depends on the behavior of hydrogen nuclei (protons) in water of major brain tissue compartments. Unpaired protons act as tiny magnetic dipoles that align along the length of the externally applied magnetic field (static field provided by the MR scanner). Precession, or "wobbling", along the longitudinal axis is manipulated during typical MR experiments. Radiofrequency (RF) excitation pulses and switching of magnetic field gradients ultimately result in the recovery of tissue RF signals from different areas of the brain. RF pulses excite protons away from their steady state position. Relaxation back to the steady state position is governed by two time constants, termed T_1 and T_2. T_1 and T_2 are associated with the intrinsic properties of specific tissue types (cerebrospinal fluid, white matter, grey matter) and allow for the generation of contrast through the experimenter-mediated adjustment of echo times (TE) and repetition times (TR), respectively. The excitation and relaxation processes result in the emission of RF signals from tissue compartments of the brain. These are detected with the aid of gradient and RF coils that localize signals from the tissue of interest. A variant of T_2, known as T_2* ("T-2-star"), is produced by inhomogeneities in the magnetic field that cause reductions in T_2 (faster transverse relaxation rate). The intravascular differences in magnetic properties between deoxyhemoglobin (dHb) and oxyhemoglobin (HbO_2) provide an endogenous contrast mechanism (1). Ogawa et al.

provided evidence that a decreased T_2* signal in blood vessels, particularly veins of the rat cortex, is due to blood oxygenation state (1). Darker veins in the cortex were distinguishable in rats inhaling low O_2 concentrations in inspired air (more dHb), while increased O_2 saturation (significantly less dHb) increased the brightness of images. The T_2* contrast was observed to be dependent on blood oxygenation. Therefore the BOLD signal arises from changes in the tissue concentrations of dHb. Seminal publications followed that provided support for task-dependent changes in the BOLD signal that occurs in T_2*-weighted images of the human somatosensory, motor, and visual cortices (2, 3).

1.3. Relation Between Cerebral Hemodynamics and Neuronal Activity

Neurometabolic coupling allows for inferences on changes in neuronal activity during most fMRI studies (4). Elevations in arterial blood flow supply glial cells and neurons with glucose and O_2, which serve as fuel to generate the cellular energy substrates ATP and lactate. The supply of oxygenated blood is associated with increased delivery to metabolically active regions of the brain. During conditions of high neuronal and metabolic activity, O_2 diffuses down a steep concentration gradient from plasma red blood cells across the capillary walls into the surrounding parenchymal tissue. This leads to dHb accumulation in the venous compartment. The paramagnetic effect of dHb is "felt" by local water protons in the intra- and extravascular compartments, and this increases the relaxation rate of protons, decreasing the signal intensity in T_2*-weighted MR images. This is a transient effect, however, as the BOLD signal increases (increased T_2*) within a few seconds of stimulus delivery. Fractional increases in plasma HbO_2 saturation from baseline levels therefore increase the T_2* signal. Indeed, visual and somatosensory evoked changes in O_2 metabolism were estimated to be 5% above baseline levels, but there is nearly a 30–50% increase in blood flow to the active cortical regions (5, 6). Therefore, increased cerebral blood flow (CBF) is several orders of magnitude above the O_2 demand of the tissue. The overcompensatory mechanism is instrumental in generating the BOLD response observed in many studies.

There have been thorough investigations of the possible neurovascular mechanisms contributing to the BOLD signal as well as the relation between the BOLD signal and neuronal activity. It appears that at the microscopic level (measured by intrinsic optical techniques) there is a tight coupling between single neuron activity and O_2 metabolism that contributes to generating the BOLD signal, but larger scale electrical oscillations at a macroscopic level may contribute to a larger extent. Using intrinsic optical imaging and laser Doppler flowmetry, Malonek and Grinvald (7, 8) investigated the dynamics of the hemodynamic response in the cat visual cortex. It was shown that an increase in HbO_2 and CBF response near single cortical columns occurs within several seconds (2–3 s)

of visual stimulus presentation (7, 8). At the single neuron level, it appears that there is an initial decrease in tissue O_2 content due to a greater O_2 extraction fraction immediately after increasing firing activity (9). This is followed by increases in O_2 that may be due to the elevated CBF (9). Simultaneous fMRI and neurophysiological recordings taken from the anesthetized rhesus macacque's visual cortex demonstrated a near linear relation between BOLD and local field potential (LFP), but this may not be the case for single unit activity (10). LFPs reflect larger scale electrical activity as a result of the cooperative interactions between populations of perhaps thousands of neurons, rather than spike input or output at the single neuron level (11). The steps between neuronal activity and BOLD involves coupling cellular metabolism and cerebrovascular reactivity (12–14).

2. Materials and Procedures

2.1. Magnetic Resonance Scanner

The high field MR scanner produces the external static magnetic field that is critical for MR studies. The proportionality between field strength and signal-to-noise ratio (SNR) has made it increasingly popular to carry out animal experiments, especially in small animals, in higher field systems. Typical systems are now actively shielded and some may contain cryogen recapture systems that minimize boil-off of helium. The room housing the system is also RF shielded from outside sources to prevent external noise sources from contaminating image data. These rooms are designed to maximize safety near the MR scanner environment. Figure 1 shows examples of Bruker and Agilent systems. The scanner also contains the spatial encoding gradient coils that are oriented along the longitudinal *z* and transverse *x*–*y* axes. Table 1 lists some popular vendors.

The use of 4.7 T and 7 T horizontal bore systems for rodent applications have been optimal for our experimental applications both because of the high SNR and good T_2/T_2* contrast for functional studies. There are higher field scanners now used frequently for fMRI studies in rats and mice, such as 9.4 T and 11.7 T systems. Scanners with high-quality spatial encoding gradients, automated or manual shimming (for correcting small field variations around the brain), and preinstalled pulse sequence routines that run on user-friendly console software are of choice for many applications-driven laboratories. Most animal imaging centers will use MRI methods in rodents of various sizes and small and large primates.

Among the important considerations upon purchasing a MRI for animal research are the clear bore size and different gradient sets with different inner bore diameters, which would allow applying the methods to small and larger animals. Typical clear bore sizes are 30–60 cm with internal gradient sizes of 12–20 cm.

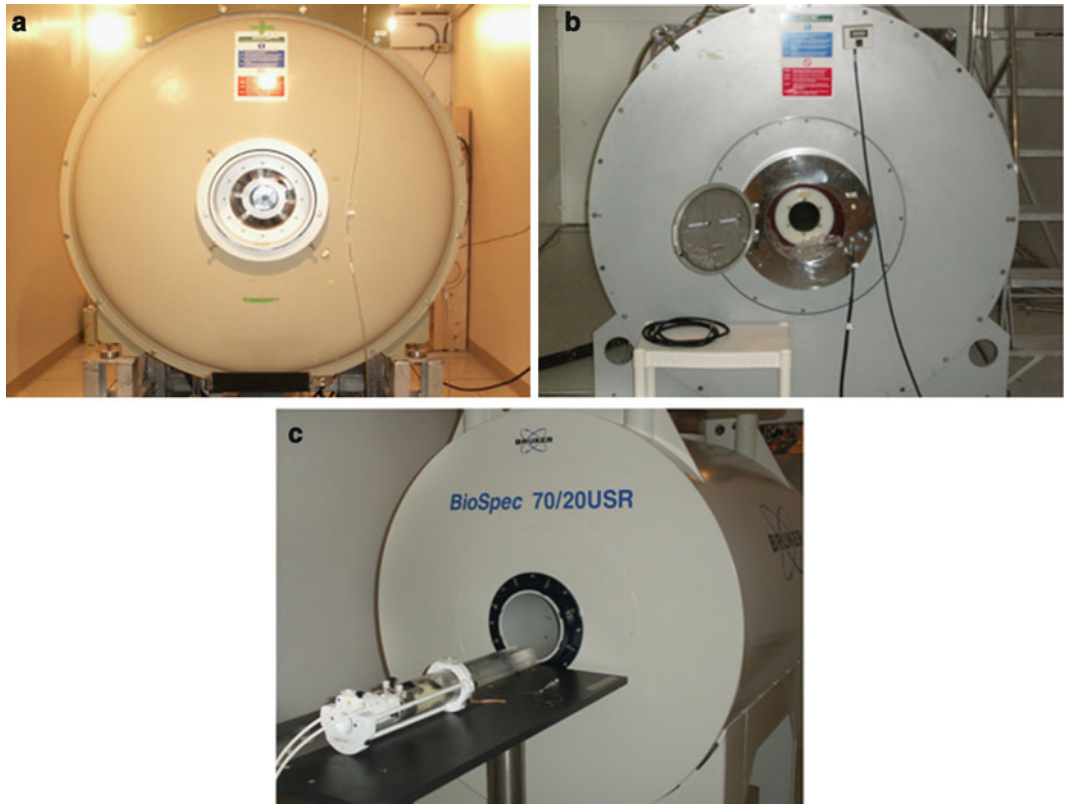

Fig. 1. Small animal MRI scanners available from various companies. (**a**) shows a refurbished 200 MHz 40 cm bore Oxford NMR system with passive shielding inside a Faraday cage. The scanner is lower in cost than actively shielded and new systems, but can be used for a variety of applications in rodents and small primates. (**b**) shows a 200 MHz 40 cm bore system from Magnex Scientific of similar dimensions. This system is actively shielded and contains a shielded front and back panel door to reduce MR field fluctuations. Both systems in (**a**) and (**b**) are filled with cryogens on a weekly basis. (**c**) shows an actively shielded 300 MHz Bruker USR 20 cm bore system with cryogen recapture system and coldhead for zero helium boil-off. This system also has a bed (not shown) that is used to move the RF coil along with the animal to adjust positioning inside the system.

Another factor to consider is bore length. Many 9.4 T and 11.7 T scanners have long bores that may present different challenges when planning to present stimuli. The length of the bore in the longitudinal *z*-direction may be quite large, reaching 160 cm to isocenter (center of the magnetic field of the scanner where the sample is placed for fMRI studies). This is more than arm's length, therefore setting up RF coils, centralizing animals, and ensuring that stimulus apparatuses are in place require a bit more ingenuity. It is important to work closely alongside vendors to ensure that everything needed to overcome these minor issues is included in the purchase of the scanner, especially if these are high field systems.

At high fields, it is possible to obtain an in-plane voxel resolution for functional scans of about 390–469 μm^2 with 12–20 coronal slices (1–1.2 mm slice thickness). This covers most of the rat brain from the olfactory bulb to the cerebellum using T_2-weighted

Table 1
Listing of equipment and analysis tools for small animal imaging studies. The purpose of the listing is to serve as a beginner's reference for researchers interested in small animal MRI methods

Essential item(s)	Vendor list and world wide web information
– Radiofrequency coils for animal MR experiments – Beds with manual or automated adjustment used to position RF systems inside the scanner – Mouse and rat cradles and head restraint devices, and options for adding on physiological monitoring and stimulation	Doty NMR (http://www.dotynmr.com/) M2M Imaging (http://www.m2mimaging.com/) Rapid MR International (http://www.rapidmri.com/) InsightMRI[a] (http://www.inslsystems.net/) Bruker BioSpin (http://www.bruker-biospin.com/) Agilent Technologies (http://www.agilent.com/)
– Physiological monitoring and stimulation equipment and accessories	BIOPAC Systems (http://www.biopac.com/) SA Instruments (http://www.i4sa.com/)
– Gradient Coils	Resonance Research Inc (RRI) (http://www.rricorp.com/) Bruker BioSpin Agilent Technologies
– Preclinical MRI Scanners	Bruker BioSpin Agilent Technologies
– Analysis software	FSL—Free Surfer Limited (http://www.fmrib.ox.ac.uk/fsl/index.html) SPM—Statistical Parametric Mapping (http://www.fil.ion.ucl.ac.uk/spm/) AFNI—Analysis of Functional NeuroImages (http://afni.nimh.nih.gov/afni) MIVA—Medical Image Visualization and Analysis (http://ccni.wpi.edu/)

[a]Only vendor selling equipment for awake animal functional MRI

fast spin echo sequences with minimal anatomical distortions. For localized rat brain studies with fewer slices, focusing on the coordinated activity of a few subsets of areas, the in-plane resolution can be increased to 100–250 μm^2. Many fMRI studies have been performed using gradient echo echo planar imaging (GE EPI) because of its

greater sensitivity to magnetic susceptibility and the BOLD effect. Gradient echo sequences use rapidly changing MR gradients to excite protons into the transverse plane (rather than using RF pulses). Because of the same magnetic susceptibility that contributes sensitivity to the BOLD effect, the GE EPI is highly vulnerable to signal loss at air–tissue interfaces in the temporal and paranasal regions. This leads to loss of data in important areas, such as the ventral hippocampus, amygdala, and medial and orbital regions of the prefrontal cortex (15, 16). GE EPI has a high sensitivity to physiological noise and shows anatomical distortions (spatial warping) that can produce alignment and registration errors. Most modern MR console software contains built-in algorithms that correct these distortions. For instance, Varian's VmrJ 3.1 "epip" sequence collects phase maps between repetitions that can be used to correct spatial warping in echo planar imaging (EPI). However, to correct the spatial warping, additional scan time must be added to acquire field maps that aid in unwarping image data upon reconstruction. Finally, GE EPI sequences are more sensitive to intravascular and extravascular large vein signals that are distant from the actual foci of activity (17). Spin echo EPI at high fields are more sensitive to intra- and extravascular compartments closer to the capillaries and therefore are commonly used for fMRI studies at higher field strengths (17). At high fields, T_2-weighted spin echo sequences appear to suffer less from the aforementioned issues (17–20). Single shot spin echo sequences (SE EPI) and multisegmented T_2-weighted fast spin echo can be used successfully with rats. The latter has been the sequence of choice for many of our experiments in awake rats. There is support in the literature for the use of fast spin echo and SE EPI sequences for BOLD imaging (17–20).

2.2. RF Coils

Studies of the rat brain using MR scanners require the use of RF coils that serve as the source of the B_1 field (the 90 and 180° pulses) that excite water protons in tissue to the transverse planes. The RF coils also serve to detect longitudinal and transverse signal relaxation. There are varieties of coils that are used for neuro-applications. Figure 2 shows examples of RF systems. Table 1 lists some current vendors.

Many laboratories design and construct their own RF coils (21–23). Some of the RF coils are as simple as single copper wire loops to more advanced actively decoupled surface receive/volume transmit dual coil systems tuned to the magnet frequency (4.7 T–7 T or 200–300 MHz range, respectively). The coil is aligned over or focused upon the area of interest, such as over the entire animal head or overlying the cortical area of interest. The loop coil configuration, however, has less spatial coverage and usually results in signal drop from dorsal to ventral areas of the brain that makes this type of configuration less favorable for developmental studies. The configuration prohibits coverage of signals

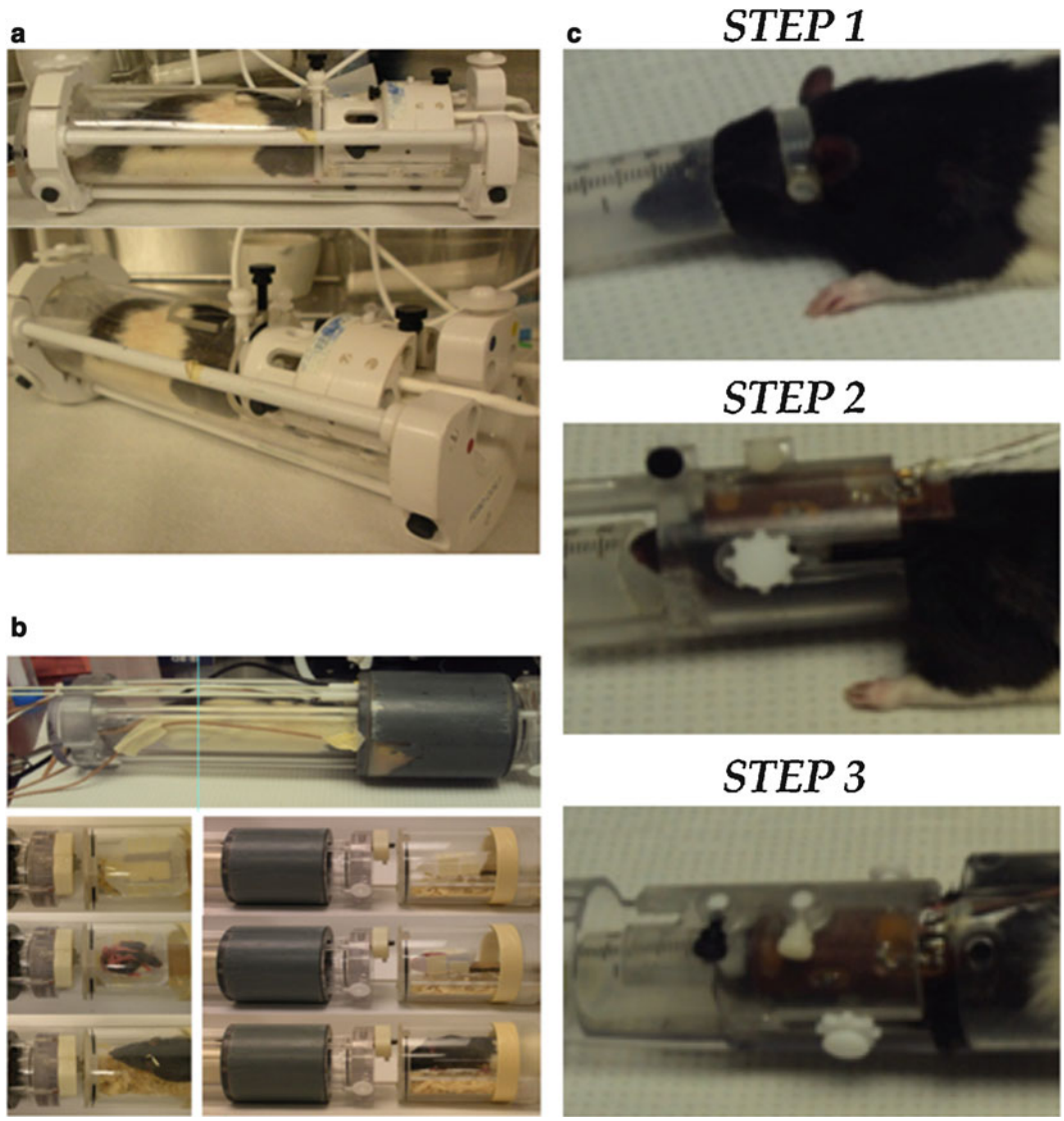

Fig. 2. Radiofrequency electronics for imaging awake rats. (**a**) shows a quadrature (volume) coil system capable of transmitting and receiving tissue RF signals. (**b**) shows a dual coil (volume transmit and surface coil receive) system. (**c**) shows the steps of a rat setup procedure. *Step 1* shows the placement of ear bars with lateral grooves for positioning inside the head restrainer. *Step 2* shows the animal positioned inside the head restrainder. Screws are used to affix the animal in place. These are guided into the lateral grooves of the ear bars. In *Step 3*, the animal is in its final position before being placed into the magnet. The nose bar and lateral ear bars are in place and the animal is positioned inside a body tube.

from brain structures such as the hypothalamus and midbrain that are farthest in distance from the coil. This can be overcome by using a dual RF coil system built into an MR compatible restrainer of the head and body (16), or a quadrature coil system with improved B_1 coverage of the brain (InsightMRI, Shrewsbury, MA) (Fig. 2; Table 1).

2.3. Accessory Equipment

In addition to the main electronics that are needed to run functional brain scanning in rats, there are other useful accessory devices (Table 1). For anesthetized preps, beds with integrated head and/or body holders are important to place animals correctly inside the bore of the magnet. Typically, these are necessary to align animals correctly within the isocenter of the MR-spectrometer prior to image acquisition. Physiological monitoring devices are also an essential part of the animal imaging setup. This includes MR compatible temperature probes, pulse oximeters, capnometers, electroencephalographic (EEG) and electromyographic (EMG) recorders, respiratory pillows and transducers, and other devices according to the needs of the investigator. The physiological measures are used to trigger the image acquisitions to remove respiratory and cardiac pulsations that appear as low-frequency oscillations (24–26). Stimulation devices may be needed when evoking sensory responses, such as for whisker and forepaw stimulations. The stimuli for sensory evoked responses can be timed to the functional image acquisitions for accurate correlations with BOLD signal responses during block design studies.

2.4. Anesthetized Preparation

Anesthetized preparations are used extensively for fMRI studies in rats. The methods used generally are not suitable for longitudinal studies in the same population of animals. In many applications, the femoral artery of the rat is catheterized to allow the sampling of arterial blood gases, and a close monitoring of arterial blood pressure and pH during scanning. Changes in the partial pressures of blood gases may be indicative of alterations of basal conditions that can alter the magnitude of the BOLD signal. This is important since hypoxia and hypercapnia modulate baseline BOLD signal in the rat, perhaps independently of basal neural activity and metabolism (27, 28). Controlling for movement is also important and some laboratories use chemical agents that can suppress muscle contractility during scanning. Marota et al. (29) used the paralyzing agent pancuronium to eliminate unwanted respiratory pulsations in the anesthetized rat. Others have used the muscle relaxant gallamine to paralyze animals during MR scanning (30). The animals in the cited studies were tracheostomized and mechanically ventilated during experiments (29). The experimenter-controlled activity facilitates removal of movement artifacts. The invasive procedure precludes long-term developmental studies in rats. Alternatively, arterial blood pressure and respiration rates can be measured noninvasively using a pulse oximetry over the tail of the rat and a respiratory pillow placed just underneath the animal's chest (Table 1).

2.5. Awake Setup

Our laboratory has utilized methods to image awake rats as an alternative to imaging under anesthesia. Before this is done, however, animals must be acclimated to the restraint conditions and MR pulse sequence noise. The procedures for acclimation are carried

out for 5 days prior to collection of imaging data. Both acclimation and actual imaging experimental setup procedures are done under similar conditions. Rats are first anesthetized under 2–4% isoflurane gas to enable placement into a head restrainer. There is evidence that isoflurane anesthetized animals regain motor function and coordination within minutes (31), and thus, volatile anesthetics such as isoflurane are useful when quick setup and awakening are desired. An important part of the restraint setup is the ear bars that allow the proper orientation of the head. A semicircular plastic headpiece containing blunted ear bars is first placed over the animal's head and fitted into the ear canals. These are noninvasive (requiring no surgery) and are not made of abrasive or harmful material. Their placement is the same as standard stereotaxic ear bars. Other laboratories permanently affix holders to the skull of the animals to ensure that the animals will not be able to move during scanning (32–34). The animal is guided through the center of the coil/head holder unit and the incisors placed over a bite bar. A plastic latch locks down over the nose with a screw. The lateral ear bars contain outer grooves that accommodate lateral screws that are used to align the animal in the holder and fix its position in the restrainer. The body is placed into a tube that has shoulder bars and an overlying square plastic peg that prevents up-and-down movement during scanning. The entire system is placed into a chassis that fits the bore of the magnet and can be fastened inside of it. In the experience of the author, the setup time is quite short (~10 min). The system has plenty of room to accommodate accessory equipment for stimulus delivery or physiological monitoring.

The above system allows the rodent to remain in a semicrouched position while being scanned (forelimb movement is more restricted). Most movement comes from the y direction (up and down movements). The rats seldom move in the back-and-forth (z) and side-to-side (x) if positioned correctly. The design of the newer coil system used in our laboratory (35) (Fig. 2) minimizes the y direction movement. Other groups have used positioning screws that are affixed to the skull and have obtained good results. For example, Desai and coworkers (32) carried out fMRI-optogenetic experiments in awake restrained mice. The mice had miniature plastic screws affixed to the skull. They used a short (3-day) acclimatization period and provided animals with "treats" after restraint sessions. It is possible that both approaches will yield good results, and using cranial fixtures to prevent movement may be preferable for methods that include additional invasive procedures during fMRI scanning.

2.6. Data Processing and Analysis

Motion must be minimized during MR scanning and residual motion should be corrected whenever possible. There are several preprocessing steps we have used over the years, some qualitative and others more stringent. These are done without a priori knowledge

of outcomes, which would bias the procedure. Both gross movements (for example, slow shifts in head position, sustained or transient leg motion, chewing, vocalizations) and physiological motion (pulsations due to cardiac and respiratory cycles) can significantly degrade multirepetition MR scans. They can also contribute to false activation patterns that correlate motion with stimulus presentations (36). One qualitative strategy is to generate movies of multi-repetition fMR scans and visually assess excess motion. We have done this in past work using Stimulate (http://www.cmrr.umn.edu/stimulate/) (37). Motion is detected across frames, and this provides a reasonable ability to detect permanent shifts in head position, and frequent movements during scanning. Recently, Ferris and coworkers generated artificial motion in MR images and assessed the amount of movement necessary to induce false activation patterns across the rat brain (38). Using the simulations, they were able to provide a numerical threshold for movement, above which motion is in excess and cannot be corrected. Scans above the threshold are discarded from studies. Animals included in the study are processed using standard Statistical Parametric Mapping software (SPM8; http://www.fil.ion.ucl.ac.uk/spm/). The latter corrects translational motions in x–y–z planes and rotational shifts. Signal drift also occurs in MRI scans and can contribute to false positive and negative signal changes. A simple voxel-wise or image-wise correction can be used. Drift correction uses square root minimization to fit time data to a straight line (using linear regression). This can be done in each voxel independently, which is more computationally intensive, or across the entire image volume. The reasoning behind the image volume correction is that drift due to electronics, temperature change, and physiological noise is distributed across the imaging volume; therefore the correction can be applied throughout the image plane.

Following prescreening and preprocessing procedures, individual scans are further processed using Medical Image Visualization and Analysis (MIVA; see Table 1 for other options). There are other software packages available, and some are more intuitive and user-friendly than others, depending on the knowledge of programming in UNIX language required. The program we have used does not require programming skills, although there are several standardized steps involved that are specific to our applications. Other programs are more "open-ended" and thus require programming skills to adjust the software to the specific MRI study at hand. Table 1 provides a short list of MRI software that has been used in small animal MRI studies. Many are freely available packages. Not all have electronic atlases of mouse and rat brains, and therefore this is researcher supplied. Most of the programs can import data in NIFTI (.nii) data format. See websites for specific details.

Using MIVA, each subject is registered to a fully segmented electronic rat brain atlas (39, 40) (Fig. 3). The program allows

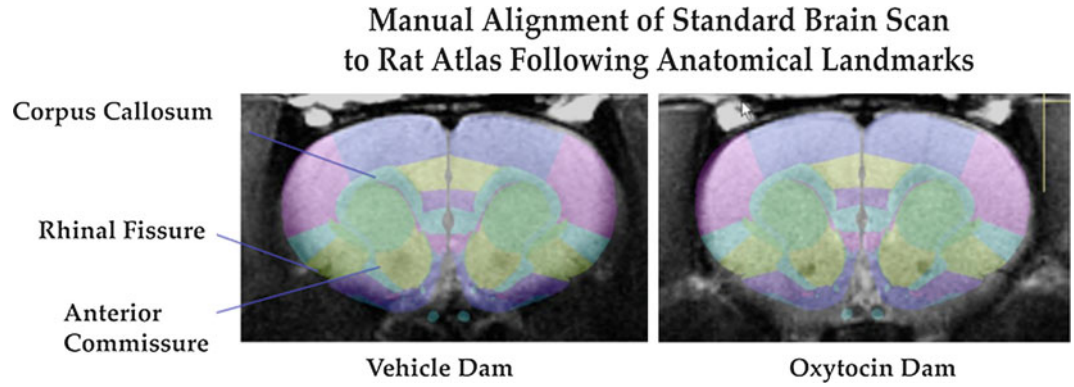

Fig. 3. Example anatomical reference image alignment to a digital atlas of the rat brain. Landmarks are used for optimal alignment. The rat atlas is part of the software package (Medical Image Visualization and Analysis, MIVA; ccni.wpi.edu).

alignment, segmentation and pixel-wise analysis for signal intensity changes in step-by-step modules that have been developed and incorporated into the main software (Fig. 3). Statistical *t* tests are performed on each subject within the original coordinate system. We determine the repetitions to be included as "baseline" where no stimulus is provided and a stimulus epoch of equal repetitions. Statistical t tests used a 95% confidence level, two-tailed distribution, and heteroscedastic variance assumptions. In order to provide a conservative estimate of significance, a false-positive detection-controlling algorithm is introduced into the analysis (41). This ensures that the false-positive detection rate is below our confidence level of 5% (42). Statistically significant pixels are assigned their percentage change values (stimulus mean minus control mean). Activated voxel numbers and percent signal changes are exported to statistical software to assess the presence of significant effects. For example, the number of voxels per region of interest (ROI) and their corresponding average percent change values can be statistically evaluated between 4 scan groups using Kruskall–Wallis analysis of variance (ANOVA $p < 0.05$). Independent variables will depend on the specific study design used.

3. Anticipated Results and Notes

3.1. Cocaine-Induced Changes in BOLD Signal

The work in our laboratory has partly focused on studies of the effects of psychoactive substances in rats. There has been significant previous work employing in vitro methods assessing cerebral glucose metabolism following acute and repeated cocaine exposure (43–46). Since the rewarding and psychomotor properties of cocaine are attributed to changes in neuronal and synaptic activity within mesocortical and mesolimbic systems (47, 48), we used fMRI to investigate the neural actions of cocaine in awake animals (15).

Prior to this experiment there were several human functional imaging experiments investigating changes in brain activation following intravenous cocaine administration (49–51); however, experiments seeking to understand the developmental events leading to an addicted state cannot be studied in humans. Animal studies carried out in anesthetized rats are hampered by the use of general anesthetics (29, 52) (see Sect. 3.4. below). We used spin EPI in conscious rats at 4.7 T following an intracerebroventricular injection of cocaine (20 μg) in artificial cerebrospinal fluid (10 μL). Within 5 min of injection, there was a significant increase in BOLD signal intensity in the substantia nigra, ventral tegmental area, nucleus accumbens, dorsal striatum, and prefrontal cortex, as compared to vehicle controls. Minimal negative BOLD signal changes were observed in response to cocaine, and there were no significant perturbations in normal cardiovascular and respiratory function. The findings demonstrated the technical feasibility of studying psychostimulant-induced brain activity using functional MRI in conscious rats.

The results using BOLD fMRI corroborate findings from previous animal studies. Metabolic mapping of radiolabelled deoxyglucose showed cocaine-induced, site-specific glucose utilization in the multiple areas of the brain (43, 53). In a follow-up study the repeated effects of cocaine were examined using the same methods. Seven days of pretreatment with cocaine significantly reduced the BOLD response to the drug. Although the lower BOLD response to cocaine appeared to be a generalized and nonspecific effect, several brain areas of acutely and repeatedly treated rats did not show differences in BOLD signal intensity (namely, the dorsal prefrontal cortex, cingulate, and somatosensory cortex). In addition, the lower BOLD response was not associated with differences in cerebrovascular reactivity between the two treatment groups, as measured by brief exposure to hypercapnia. One explanation proposed for the decreased BOLD response observed is that it might be associated with the previously observed decreases in glucose metabolism (46) and could also be related to reductions in basal and cocaine-stimulated synaptic monoamine concentrations (54–56). Alternatively, the reduced BOLD response could be due to differences in basal cerebrovascular reactivity.

To investigate changes in neural activity with repeated cocaine administration that may contribute to sex differences, we studied BOLD changes by fMRI in ovariectomized rats with and without circulating sex steroids (57). Our results indicate that cocaine-sensitized females having artificial treatments with estrogen show higher BOLD activity when reexposed to cocaine, particularly in frontal cortical areas (57). In contrast, rats without the steroid show a similar BOLD response to acute or repeated cocaine exposure. An exception was the hippocampus of OVX rats, which showed decreased neural activity compared to the response

recorded on the first day of exposure. These results contrast with those obtained in males. Male rats treated with a sensitizing regime of cocaine showed less positive BOLD activation as compared to drug-naïve rats receiving cocaine. This was observed for the volume of activation and percent change in BOLD. Interestingly, these findings suggest a tolerance-like effect in male rats that would otherwise show a sensitized behavioral response. It is possible that the sex differences observed may reflect temporal sex differences. Another potential explanation for the findings using fMRI is that the basal state for either CBF or baseline neuronal firing has been modified (58, 59). PET images from detoxified male cocaine addicts show decreased CBF in basal ganglia when presented with a cocaine video compared to their response to a non drug video (60). Interestingly, females addicted to cocaine show enhanced CBF in several brain areas when exposed to cocaine, compared to males (61). These results provide evidence of gender differences in the neural response to drugs of abuse, as measured using fMRI in awake female rats.

3.2. Imaging the Maternal Neural Response to a Natural Reward: Lactational Studies in Rats

A separate series of experiments in awake lactating rats has been carried out using the methods described above. Many of these have focused on the neural processing of the natural suckling stimulus from pups as a rewarding stimulus (42, 62–64). It has been reported that suckling stimulation from pups modulates the expression of maternal behaviors in rats by promoting arched back nursing postures (65, 66) and slow-wave sleep (67, 68). The fMRI technique was used to map the cortical pattern of activity during suckling, and the stimulus was compared to artificial suction in the absence of pups and mechanical stimulation on the ventrum skin (64). During the processing of somatosensory stimuli, information coming from the landscape of peripheral sensory receptors underlying body skin surface is relayed to the cortex through the spinothalamic pathway and topographically represented in the cerebrum. In the case of the mammillae, primary afferent fibers terminate in the ipsilateral dorsal root ganglia between spinal segments C5 and L6 (69), with afferent relays along the lateral cervical nucleus, the dorsal column nuclei, and the sensory and spinal portions of the trigeminal complex (66, 70). In contrast with studies using *c-fos* assays that provide exquisite cellular spatial detail (71–75), the detection of real-time brain activity during the actual act of nursing is limited by the very wide temporal window (usually taken 60–120 min post-stimulus). Findings from electrophysiological recordings taken from neurons in the somatosensory cortex of the anesthetized rat indicate that the receptive field for the ventrum skin surrounding the nipple area doubles in size during the lactation period (76). It was found in the fMRI study that wide areas of the postpartum rat cerebrum exhibit an increase in the fMRI BOLD signal during suckling stimulation, suggesting that neural

activity is modified over wide areas of the cerebral cortex in response to a rather specific stimulus (64). The artificial suckling stimulus caused a similar degree of cortical activation. Therefore, although auditory, olfactory, and nonsuckling tactile stimulation from pups may contribute to cortical activity, the suckling itself is fully capable of causing a widespread cortical response. There have been experiments examining the patterns of brain activity in mothers presented with infant sensory cues, but as of yet, none have investigated the effects of the lactational stimulus. Our rat studies suggest that there would be significant cortical activation not only in limbic cortical divisions, as reported previously (77), but also in areas that might correspond to long-term memory storage. These are cortical representations of the suckling stimulus that might be important for the maternal–infant bond during the early lactational period.

3.3. Comment on Interpretations and Comparisons with Existing Literature

One should be cognizant of the biological underpinnings contributing to the BOLD signal in order to interpret fMRI results. As in the original Ogawa et al. work, a greater O_2 tension resulted in increased local signal intensity, which is what is measured in most fMRI studies (1). In the cocaine BOLD activation experiment and the lactation study cited above, one assumes that the BOLD signal changes are predominantly related to changes in neuronal activity and not only due to changes in blood flow or volume alone. Differences in percent changes in BOLD between the different experimental conditions are expected to represent differences in neuronal activity of comparable magnitude.

There has been other significant research in awake animals that has primarily focused on investigating the neural actions of pharmacological agents (78), investigations of the differences in the hemodynamic response function in awake versus anesthetized rats (79), cerebellar-dependent motor learning though eye-blink conditioning in the rabbit (33), functional connectivity studies of the awake rat brain (80, 81), combined examination of sensory neural processing in specific circuits using fMRI, and optogenetics in awake mice (32), awake Rhesus macaques, and marmoset monkeys (82–84). The studies support the use of awake fMRI methods in neuroscience research and have been performed using a variety of elegant and creative custom procedures that will not be discussed here. In general, all involve head restraint and many used some form of animal training prior to studies. Miller et al. (33) used the rabbit model in their studies of eye-blink conditioning. There were changes in cerebellar BOLD signal responses during progressive conditioning trials that closely matched patterns of electrical activity during learning. Desai et al. recently used optogenetic methods that pair the virally mediated expression of photorhodopsin in glutamatergic neurons of the barrel field cortex to study light-stimulated increases in neuronal activity in the somatosensory cortex of the awake mouse during fMRI (32). Light-induced increases in

neuronal activity produced BOLD signal responses in the barrel field region that were comparable to activation evoked by whisker deflection. The combined use of fMRI and optongenetics is now a major approach in the investigation of the functional roles of specific neural circuits.

3.4. Potential Shortcomings and Methodological Challenges

3.4.1. Effects of Anesthetics on Basal Neuronal Firing and Hemodynamic Mechanisms

Agents typically used for anesthetizing animals can suppress certain forms of neuronal activity and modify specific patterns of neuronal activity and metabolism. In addition to these actions, anesthesia can influence global cerebrovascular reactivity. The choice of anesthetic and calibration of the depth of anesthesia are therefore important. There are differences in basal and stimulated brain glucose utilization (cerebral metabolic rate for glucose (CMR_{glu})) and CBF in awake versus anesthetized rats (85). Stimulation of the whisker-to-barrel cortex pathway, for example, resulted in differential CBF and CMR_{glu} across regions when rats were anesthetized with halothane (85). The results of the latter study suggest that although the barrel cortex is active in the anesthetized state, other regions along the pathway arising from the stimulation of peripheral sensory receptors to the cortex are suppressed and may thus require a conscious state (85). Evoked potentials in the barrel field cortex have been shown to vary between anesthetic conditions (79). The amplitudes of field potential responses to graded levels of repetitive electrical stimulation to the whisker pads are reduced to a greater degree in anesthetized vs. awake rats (79). The magnitude of neuronal activity and the specificity of activity of neural networks are hampered by anesthetics. Firing of action potentials over localized regions of the awake rat visual cortex showed higher frequencies and bursting, but lower pair-wise correlations between single units than ketamine-anesthetized rats (86). This suggests that the propagation of action potential in localized networks is modified by the induction of an anesthetized state. Halothane, isoflurane, and desflurane can differentially affect gamma band oscillations in the rat cortex (87, 88). This is important because field potential power has been correlated to BOLD signal changes (10). Graded levels of isoflurane (1.8–2.2%) also suppress EEG bursts measured in the primary sensory cortical area representing the forelimb of the rat and also reduced spontaneous variations in CBF (89). These levels of isoflurane are within the range that causes suppression of bursting in sensory cortical EEG patterns (90). Research on the role of anesthetic agents in modulating neuron activity raises concerns about the use of deep levels of anesthesia for rat brain imaging experiments (91–93). Variations in the pattern and magnitude of neuronal activity will vary according to anesthesia type and concentration.

The effects of volatile anesthetics on the BOLD signal, CBF, and CBV have also been investigated. Hypercapnia-induced BOLD signal changes, which occur in the absence of neuronal activity, are

of much greater magnitude in awake rats (94). This suggests that cerebrovascular reactivity is affected by anesthesia. It is also important to note that basal levels of O_2 metabolism and neuronal spiking frequency in the cortex are reduced by deep levels of alpha-chloralose (58). Larger magnitude changes in BOLD may reflect lower basal firing of neurons in animals that are deeply anesthetized whereas lighter levels of anesthesia allow for smaller magnitude changes in BOLD in the face of higher basal activity (58). Basal CBF levels in 2% isoflurane anesthetized rats were observed to be greater than in the awake state (95). Isoflurane anesthesia can act as a vasodilating agent that increases blood flow. The percent change in CBF and BOLD in response to CO_2, however, is lower in anesthetized rats (95). The lower magnitude response could be due to higher basal levels of blood flow (95). Thus, in isoflurane anesthetized animals there seems to be direct modulation of CBF that is independent of the effects on neuronal activity (93). Isoflurane reportedly increases CBF globally due to its vasodilating actions (89). Spontaneous CBF changes are suppressed by increasing levels of isoflurane from 1.8 to 2.2% (89). Light sedation with isoflurane (<1.8% in inspired air) might minimize the effects described above. It has also been reported that alpha-chloralose-specific parameters for forepaw-stimulated BOLD activity in the somatosensory cortex do not work under isoflurane anesthesia (93). The accumulating evidence again underscores the importance of considering the effects of anesthetics on both neuronal activity and cerebrovascular reactivity when designing fMRI studies and interpreting the data.

3.4.2. Restraint Stress

One of the concerns over restraint acclimation procedures is that it may produce effects similar to chronic stress exposure. Typical stress induction paradigms use a wire mesh that restricts total movement, while the imaging setup only involves restraint of the head as the limbs and torso are not fully restrained. The imaging setup seems to involve intermittent stress that may have transient, not chronic, effects. However, whether or not stress is present in restraint-acclimated rats is not a matter of debate. The aim in many experimental procedures using awake rat imaging is to minimize the impact of stress during data acquisition. King and coworkers (96) reported that various physiological variables (e.g., respiratory rates, blood pressure, corticosterone levels) of Sprague–Dawley rats are reduced following 5–8 days of restraint. Most measures are reduced near prestress baseline levels on days 4–5. An important outcome, however, was that there was a significant increase in contrast to noise in MR images (96). This signifies that the lower gross movement and physiological rhythms improve image quality. The data are consistent with past reports indicating that rats can habituate to repeated daily 1–2 h restraint for 4–9 days (97, 98). Rats show normal patterns of food intake and heart rate following habituation to restraint (99, 100). Importantly, habituation to

repeated daily sessions of restraint is not necessarily indicative of impaired hypothalamic-pituitary-adrenal (HPA) axis function, since rats acclimated to restraint stress still show increased *c-fos* activation and corticosterone levels to a novel stressor (97). Parry and McElligot (1993) devised a method for head immobilization in awake rats in order to study central regulation of cardiovascular function (101). They reported that side-by-side restraint acclimatization of rats reduced the stress of individual animals. We have used a similar acclimatization procedure in our studies in which groups of animals are simultaneously exposed to daily sessions of restraint. Heart rate and blood pressure normalized after initial exposure to restraint, indicating that the procedure was less stressful (101). Barnum et al. investigated the hyperthermia effects of chronic restraint stress and compared them to other forms of stress, such as the social defeat model and isolated cage confinement (102). They observed that corticosterone and stress-induced hyperthermic responses to restraint stress adapted after 5–6 days of intermittent exposure. However, this was not observed for the social defeat stress model, indicating that the two forms of stress have different behavioral and physiological outcomes.

Stress may still have a longitudinal impact. Interpreting data and considering distress as part of the shortcomings of studies employing awake rat imaging are therefore unavoidable. Naert et al. report a "depressed-like state" in rats chronically exposed to repeated restraint stress (103). This included behavioral changes in the elevated plus maze, indicative of higher anxiety levels, changes in hedonic state as measured by the sucrose preference test, depressed locomotion, and a reduction in body weight by 17% (103). The adaptation was accompanied by HPA-associated changes in brain derived neurotrophic factor (BNDF) expression, corticotropic releasing hormone (CRH), and arginine vasopressin (AVP) levels, suggesting several biological markers for plasticity within the stress axis (103). However, it is important to keep in mind that their study, as well as others focusing on stress, uses very intensive stress exposure schedules within the range of 90–120 min per day or more. Ferris et al. have had success with 60-min daily sessions over the course of 4–5 days (38). Animals are imaged the day after the last acclimatization session or several days later. Our laboratory has had success as well with incremental steps in restraint duration (20, 40, up to 60 min). The question remains whether immobility stress as classically studied has the same neurobiological and behavioral impact as the head restraint used in functional neuroimaging studies. There have been a host of other studies seeking to understand adaptations to restraint stress and whether the changes involved permanent modifications of the rodents brain (causing long-term changes in the behavioral and neural responses) (104–107). The latter effect may not be entirely avoidable. Indeed, even single exposure to immobility stress can have long-term effects on behavior, such as cross-sensitization of responses to stressors (108).

4. Conclusion

Awake rat imaging can be used to investigate reward brain function and the actions of drugs in the brain. We have studied reward from the perspective of the maternal rat brain, and with the administration of reinforcing drugs, such as cocaine. The fMRI method applications summarized here can be of potential use for studies seeking to understand a variety of neuropsychiatric conditions, among which are conditions involving eating disorders. These could involve the study of neural processing of rewarding stimuli such as tastes and smells that are associated with high-calorie and/or sugar- or fat-enriched foods. Studies along these experimental design lines have been carried out utilizing other methods, such as positron emission tomography, which provides direct links with dopamine receptor binding and obesity in inbred strains of rats (109, 110). Other MR imaging modalities involving administration of neuronal activity enhancing contrast agents, such as manganese chloride, have also been used (111, 112). There have also been several pharmacological MRI studies using BOLD-weighted MRI in anesthetized rats to examine the effects of compounds that could potentially control food intake through sire specific actions in the brain (113–115). There is a larger database in human fMRI work (116–121). However, as stated above, the ability to design fMRI studies in rats under well-controlled experimental conditions provides a strong case for using small animal fMRI methods to study eating disorders. It is important to keep in mind the underlying mechanisms of the BOLD fMRI technique when interpreting data. There are limitations to the use of the technique that mostly stem from the restriction of head movement in both anesthetized and awake preparations. This imposes an upper limit on the questions that can be asked regarding the relation of brain activity to behavior. The field of MR sees an ever-growing expansion of applications and technical advances that will surely impact the use of animal imaging methods. Many issues regarding anesthetized and awake animal imaging have been discussed here and continue to be investigated further.

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