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FEEDING BEHAVIORAL PHARMACOGENETICS OF A MURINE MODEL OF
THE *OPRM1* A118G POLYMORPHISM.

By

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ABSTRACT OF THE DISSERTATION

Feeding Behavioral Pharmacogenetics of a Murine Model of the *OPRM1 A118G*

Polymorphism.

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Binge eating disorder (BED) is the most prevalent eating disorder, and is characterized by a perceived “loss of control” over one's food intake, resulting in the consumption of large amounts of food in short periods of time. There is currently one FDA-approved drug for the treatment of BED, lisdexamfetamine dimesylate. A common single nucleotide polymorphism (SNP) of the mu-opioid receptor gene (*OPRM1 A118G*) results in an amino acid substitution (N40D) in the extracellular domain that is posited to alter receptor stability and ligand binding. The *OPRM1 A118G* SNP has been associated with alterations in nociception and analgesia, as well as altered susceptibility to substance abuse. GG allele status has also been associated with BED in an obese population, as well as increased preference and intake of highly-palatable foods. In this dissertation, an established rodent model of binge-like feeding was utilized to investigate the role of the homologous SNP in mice (*OPRM1 A112G; N38D*) in binge propensity, pharmacological efficacy, and taste and meal phenotyping in male and female mice. The 6-wk, intermittent 24-hr caloric restriction and/or 30-

min subsequent binge access feeding schedules (Restrict, R; Binge, B; Restrict-Binge, RB; Naïve, N) revealed no differences in binge intakes between AA and GG genotypes in male or female mice. Following the 6-wk protocol, female mice underwent acute or chronic dosing schedules: within-group, 1x/wk dosing of vehicle (Veh; water), lisdexamfetamine dimesylate (LDX; 0.15, 0.5, 1.5 mg/kg), and sibutramine hydrochloride (Sib; 0.3, 1.0, 3.0 mg/kg); or between-group, 2-wk daily dosing of either Veh, Sib (3.0 mg/kg), or LDX (1.5 mg/kg), respectively. There was no effect of AA or GG genotype on pharmacotherapeutic efficacy in reducing binge-like feeding. Two-bottle lipid preference tests in male mice previously exposed to the 6-wk feeding schedules revealed increased preference for Intralipid (IL) in R and RB groups compared to N, for 5% IL only, and N GG had lower preference than N AA mice. Genotype differences in brief-access taste responsivity in male and female mice were observed only in sweet, nutritive carbohydrate taste stimuli. There was no effect of genotype on meal microstructure in male mice, but female GG mice had larger average meal sizes and greater total caloric meal intakes. Although there may be some elements of taste and meal patterns mediated by *OPRM1* A112G SNP status, our data does not support a role for this common SNP in the predisposition to binge-like feeding nor the efficacy of pharmacotherapy. While the concept of “personalized medicine” remains intriguing, the current studies do not suggest a role for the involvement of the *OPRM1* A118G SNP in binge eating disorder.

Dedication

*For my dad, who taught me how to think like a scientist;
My mom, who convinced me I could achieve anything I put my mind to;
And for my husband, who reminds me I am more than my research.*

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Associated Publications

Chapters 2 & 3

Sachdeo BLY, Yu L, Giunta GM, Bello NT (2019). Binge-like eating is not influenced by the murine model of *OPRM1 A118G* polymorphism. *Front Psychol*, 10:246. Doi:10.3389/fpsyg.2019.00246

Additional Publications

Yeomans BL & Bello NT (2015). What's bugging you and your diet? *J Food Nutr Disor* 4:1. doi:10.4172/2324-9323.1000e110

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Gotthardt, J. D., Verpeut, J. L., **Yeomans, B. L.**, Yang, J. A., Yasrebi, A., Roepke, T. A., & Bello, N. T. (2016). Intermittent Fasting Promotes Fat Loss With Lean Mass Retention, Increased Hypothalamic Norepinephrine Content, and Increased Neuropeptide Y Gene Expression in Diet-Induced Obese Male Mice. *Endocrinology*, 157(2), 679-691. doi:10.1210/en.2015-1622

Chapter 1

Review of the Literature

Introduction

Binge Eating Disorder (BED) is the most prevalent eating disorder in the United States, with an estimated lifetime prevalence of 2.8%, and higher in women compared to men (3.5% and 2.0%, respectively)[1]. However, the underlying pathology of BED remains not well understood, and treatment options are not only limited, but relapse rates are high. There is currently only one pharmaceutical therapeutic that is approved by the Food and Drug Association (FDA) in the United States. Lisdexamfetamine dimesylate, commercially sold as VYVANSE® by Shire US Inc, was originally developed for the treatment of Attention Deficit Hyperactivity Disorder (ADHD) and FDA-approved for this use 2007. However, in 2015, VYVANSE® was FDA-approved for the treatment of moderate to severe BED in adults, becoming the only pharmacological treatment for this debilitating disease, and highlighting the neural overlap between these two psychological disorders that remains not well understood.

The occurrence of binge episodes is central to the BED diagnosis and involves the consumption of a larger-than-normal amount of food in a shorter-than-normal amount of time. Binges are often premeditated, occur in the absence of physiological hunger, and are defined by the experience of a “loss of control”

over ones eating behavior. Highly palatable, sweet-fat foods are typically consumed during binges, and the hedonic drivers of food intake involve classical reward processing by mesolimbic dopamine signaling. Opioidergic systems are salient in the consumption of high fat, highly rewarding foods, and dopaminergic signaling involves modulation by opioids [2].

Mu-opioid receptor (MOR) signaling is primarily responsible for the rewarding and analgesic effects of endogenous and exogenous opioids, including β -endorphin and morphine, respectively. The MOR gene (*OPRM1*) has numerous polymorphisms identified within the population, but the non-synonymous single nucleotide polymorphism (SNP), *OPRM1* A118G, is the most common functional SNP. With an allele frequency of ~11% in Caucasian populations, and as high as ~52% in some Asian populations [3-5], the role of the variant G allele in nociception, analgesia, and substance abuse/addiction has been investigated for decades. However, despite the demonstrated role of MOR signaling in hedonic feeding, there were few studies exploring *OPRM1* A118G status as a potential genetic risk factor aberrant feeding behaviors. In a pivotal clinical study exploring dopaminergic and opioidergic genetic contributors to obesity and/or BED, researchers found the a higher frequency of the G allele in obese BED subjects, as compared to those who were obese without BED[6].

Lisdexamfetamine dimesylate (LDX) is currently the only FDA-approved drug for the treatment of BED, and the *OPRM1* A118G SNP, observed to be enriched in

this clinical population, may affect its pharmacotherapy efficacy. Therefore, whether G allele status predisposes to greater risk for binge-like eating and/or alters the efficacy of the only approved drug for its treatment, remain particularly important questions. Furthermore, BED is more common in women, and there is a need for increased biomedical research in women and female animal models, so exploring the behavioral pharmacogenetic interactions in a sex-dependent manner is imperative.

The Regulation of Food Intake

The control of food intake to regulate body weight, or energy balance, is a multifaceted system involving physical, metabolic, endocrine, and neural mechanisms to facilitate communication between the gastrointestinal tract and the brain. Appetite is a function of the integration of these mechanisms and involves both homeostatic and hedonic regulation to modulate food intake. Whereas homeostatic mechanisms involve the stimulation or inhibition of appetite in response to metabolic need (i.e. tissue demand, energy stores), hedonic feeding involves stimulation of appetite due to the rewarding aspects of food (sensory, palatability). Appetite control involves the cross-talk between these two systems, and mediates food intake by determining aspects like meal size, duration, and frequency; the energy density and palatability of food selections; the variety of foods consumed and the variation between daily selections[7]. Some of these palatability variables increase the likelihood of over-consumption, and the cumulative nature of energy intake can result in positive

energy balance and body weight gain when numerous components of appetite consistently favor consumption that exceeds energy expenditure.

Homeostatic regulation

Peripheral satiation/satiety signals include gut peptides, hormones, nutrients, as well as mechanoreceptor activation in response to gastric distention. Whereas *satiation* signals refer to those that lead to cessation of a meal or bout of feeding, post-prandial *satiety* signals refer to those that delay initiation of subsequent intake[8]. These signals can be transmitted from the gastrointestinal (GI) tract to the brainstem by vagal afferents projecting to the nucleus of the tractus solitarius (NTS), and circulating hormones can also act on the CNS directly at regions with an incomplete blood-brain barrier: the median eminence (ME) at the hypothalamus, and the area postrema (AP) of the NTS in the brainstem[9]. These signaling molecules can exert short-term (or episodic) effects in response to the immediate fed-fasted state, or be long-term (or tonic) signals, reflecting tissue stores or energy status[7].

Enteroendocrine cells in the GI tract release signaling molecules including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide YY₃₋₃₆ (PYY), and the pancreatic hormone, amylin [8]. CCK is released from duodenal mucosal cells in response to dietary protein and fat, and activates vagal afferents to decrease food intake. GLP-1 is an incretin hormone released in response to ingested carbohydrate, which similarly reduces food intake, and stimulates

insulin release from pancreatic β -cells. PYY is released in the distal small intestine and colon in response to dietary fat and bile acids, and acts to increase satiety in the post-ingestive period [7]. The pancreatic hormone, amylin, acts as a satiation signal, reducing food intake and meal size. The release of these episodic signals terminates the eating episode thus playing an important role in controlling meal size. In contrast, the gastric peptide, ghrelin, peaks in circulation when fasted and initiates meal onset, and is posited to oppose satiety signals in an episodic and tonic fashion.

With regards to the long-term regulation of food intake and energy balance, the hormone leptin is secreted from white adipocytes in proportion to the bodily amount of adipose tissue, and provides negative feedback to regulate body fat mass and energy expenditure. Leptin receptors are located in several hypothalamic regions, including the ARC, paraventricular nuclei (PVN), ventromedial hypothalamus (VMH), lateral hypothalamic area (LHA), as well as the NTS and reward-related regions (mesolimbic dopamine pathway)[8]. The ARC is central to the integration of endocrine and nutrient signals, with two distinct populations of neurons that oppose in function. Neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) are orexigenic, stimulating food intake in response to ghrelin binding, or inhibited by leptin, insulin, CCK, PYY, or GLP-1. Opposing NPY/AgRP neuronal activity is a subpopulation of anorexigenic pro-opiomelanocortin (POMC) neurons, that decrease food intake in response to leptin or insulin, as well as NPY [10]. Both

AgRP (as an antagonist) and α -melanocortin stimulating hormone (α -MSH; as an agonist) act on melanocortin-4 receptors (MC4R) on post-synaptic neurons to affect energy balance.

Hedonic regulation

Hedonic drivers of food intake relate to reward processing, and motivate consumption regardless of physiological state. The mesolimbic dopamine system codes reward salience for behaviors, including palatable foods as well as drugs, and also interacts with homeostatic regulatory mechanisms. The primary area of DA production in the brain is the VTA, a region of the midbrain that projects to the nucleus accumbens (NA), or the ventral striatum, a limbic structure in the basal forebrain. In the VTA, mu-opioid receptor signaling modulates inhibitory GABAergic interneurons that synapse on dopaminergic neurons. Removal of the inhibition of GABA signaling activates dopaminergic neurons, and result in the release of large amounts of dopamine in the NA. This surge of NA DA marks behaviors as rewarding, to encourage future interactions, and thus motivates subsequent intake of highly palatable foods, or “wanting”. In coordination with DA “wanting” or reward-seeking, opioidergic signaling is responsible for the “liking” or positive affect experienced with the consumption of highly-palatable foods[6]. Whereas opioidergic “liking” is thought to establish a behavior, dopaminergic “wanting” sustains the behavior, whether “liking” remains present or not. In our current obesogenic environment, these hedonic cues override homeostatic inputs, driving over consumption and resulting in positive energy balance.

Dysregulation of Food Intake: Eating Disorders

Eating disorders are a set of psychiatric illnesses that include anorexia nervosa, bulimia nervosa, and binge eating disorder (BED). Although estimates of the global prevalence of eating disorders are often debated, a recent multinational survey approximated a lifetime prevalence of 0.6% for AN, 1.0% for BN, and 2.8% for BED [1]. Women are disproportionately affected by eating disorders, with a prevalence of 15.3% at midlife [11]. Eating disorders are often trivialized, and individuals face stigmatization as these illnesses are often viewed as a result of a lack of will power, or intentional behaviors due to vanity or selfishness [12, 13]. Furthermore, stigma internalization is correlated with greater symptom severity [14]. In addition to the stigma surrounding these illnesses, individuals with eating disorders present with a spectrum of symptoms and numerous comorbidities, which often results in underdiagnoses. The physiological alterations underlying these psychiatric disorders remains an active area of research, and the current behavioral and pharmacological therapeutic approaches are limited in number and efficacy, with respect to long-term management or remission.

Anorexia Nervosa & Bulimia Nervosa

Prior to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V)[15], anorexia and bulimia nervosa were the only recognized eating disorders. Anorexia primarily affects adolescent and young girls, and is

characterized by body dysmorphia leading to severe dietary restriction, accompanied by a pathological fear of weight gain. Individuals with anorexia often have symptoms of anxiety, depression, and/or obsessive-compulsive behaviors, and sometimes report the self-restriction behaviors help to cope with feelings of not being in control in other aspects of life. Severe and sustained caloric restriction can result in numerous gastrointestinal, cardiovascular, endocrine, neurological consequences. Anorexia has the highest mortality rate of any psychiatric disorder, estimated to be 10% [16]. Despite men representing 25% of individuals with AN, men have greater risk of mortality -- partly due to the assumption of some caregivers and clinicians that only women have EDs, and a lack of sex-specific approaches to treatment strategies.

Bulimia nervosa is characterized by cyclical binge-purge behaviors, where individuals engage in binge episodes, followed by a compensatory behavior. An episode of binge eating is defined as the consumption of a larger than normal amount of food in a shorter than normal amount of time, in addition to an experienced "loss of control." Further characteristics associated with binge episodes are described in greater detail in the following section. Compensatory behaviors occur in a cyclical fashion with binge episodes, and can include purging-type behaviors like self-induced vomiting or use of diuretics, or non-purge behaviors like excessive exercise, or fasting. Long-term outcomes remain poor, with one study finding 30% of participants continue to engage in binge/purge behaviors at a follow-up more than 10 years later[17].

Binge Eating Disorder

Binge eating disorder is the most common eating disorder in the US, but was not formally recognized as its own category of eating disorder until the DSM-V.

Under previous editions, individuals with disordered eating behaviors that did not fit a diagnosis of anorexia or bulimia would be categorized under the catch-all diagnosis, “eating disorder not otherwise specified,” in Appendix B: Criteria Sets and Axes Provided for Further Study. Binge eating disorder shares similarity to bulimia in that the presence of binge episodes is central to both diseases, but BED differs in that there is an absence of any compensatory or purge-type behavior. In a representative sample of US adults, lifetime BED prevalence estimates were higher using the updated, 2013 DSM-V criteria as compared to the former 2000 DSM-IV-TR criteria (2.03% and 1.52%, respectively) for recurrent binge episodes. Furthermore, only 3.2% of individuals meeting the DSM-V criteria for BED diagnosis had ever been formally clinically diagnosed[18]. These findings underscore the importance of the DSM-V updates to the criteria for BED, to improve diagnosis rates.

The primary DSM-V criteria for a diagnosis of BED is recurrent episodes of binge eating, at least twice per week for 6 months. A binge episode is not only defined as the consumption of a larger than normal amount of food in a shorter than normal amount of time, but also the feeling of a loss of control over ones eating behavior. Binges are often associated with a rapid rate of consumption, eating in

the absence of physiological hunger, and eating until feeling uncomfortably full. Binge episodes differ from nonpathological hyperphagia in the experienced subjective 'loss of control'. Individuals with BED often plan the binge in advance and hide the behavior from others due to embarrassment. They experience anxiety and distress in anticipation of a binge, accompanied by regret, shame, and disgust with oneself after overeating. Typical binge foods are high-caloric and highly-palatable; sweet-fat foods like cake, cookies, or ice cream. Some individuals do report binging on lower calorie food items like broccoli or plain popcorn, and although the caloric contribution of the behavior is less, the psychological distress associated with the behavior is the same.

There are numerous comorbidities associated with BED, including type-2 diabetes, anxiety, depression, bipolar disorder, and attention-deficit/hyperactivity disorder (ADHD), and BED carries a significant health care burden for individuals. On average, people with BED have elevated health care utilization and expenditure due to inpatient and hospital-based outpatient services, and prescription medication costs. This financial burden of BED is exacerbated by the high comorbidity rates in addition to under-diagnosis, resulting in the inverted-U-shaped data on health care use and costs [19]. These data emphasize the need to raise awareness of BED criteria and comorbidities, among patients and health care providers, in particular.

Neurobiological Alterations Associated with Binge Eating

The lifetime comorbidity of eating disorders with other mental disorders was estimated to be 56.2%, 94.5%, and 78.9% of individuals with AN, BN, and BED, respectively [20], suggesting shared neuropathologies. For BED specifically, lifetime comorbidities with anxiety disorders, mood disorders, impulse disorders, and substance use disorders were 65.1%, 46.4%, 43.3%, 23.3%, respectively [20]. Moreover, the only FDA-approved pharmacotherapy for the treatment of BED was first developed and approved for the treatment of ADHD. While the mechanisms underlying the self-sustaining nature of BED largely remain unknown, the utilization of animal models facilitates exploration of the associated neurobiological alterations.

Numerous studies by the Bello lab and colleagues have used rodent models of binge-like feeding to investigate the neural alterations associated with this debilitating eating disorder, largely focusing on dopaminergic and opioidergic mechanisms. An early study by Moran's group used a binge-access feeding schedule in male Sprague-Dawley (SD) rats to explore neural activation in hindbrain regions associated with feeding behavior, and circulating hormone levels [21]. Rats with a history of limited-access, but not continuous access, to highly palatable binge food, had elevated levels of the orexigenic hormone, ghrelin, and higher c-Fos-positive cells along the anterior-posterior nucleus of the solitary tract. C-Fos is an immediate early gene for which expression indicates neural activation. A review by Bello and Hajnal discussed human and animal

data supporting the role of binge-stimulated sustained dopamine signaling that perpetuates these dysregulated ingestive behaviors [22]. In a 2011 study in female SD rats, 6 weeks following diet-induced binge-like feeding schedules resulted in a reduction in MOR mRNA expression in the NTS in groups with intermittent and continuous-access to highly-palatable food [23]. Using a similar binge model in female rats, Bello *et al.* investigated the contribution of stress to binge-induced neural activation, and found elevated cFos staining in the medial prefrontal cortex (mPFC), a target region for pharmacotherapies for the treatment of both ADHD and BED [24]. A related study explored the involvement of central norepinephrine (NE) in binge-like feeding, and found that nisoxetine, a selective NE reuptake inhibitor, did not suppress palatable food intake in animals with caloric restriction preceding binge access, which also had reduced sensory-evoked response in locus coeruleus NE neurons [25].

Treatments for Binge Eating Disorder

Binge eating disorder is a psychiatric illness with a complex neuropathology, high degree of psychiatric comorbidities, and associated stigma that makes diagnosis and treatment challenging [26]. Cognitive behavioral therapy (CBT) and intrapersonal therapy (IPT) are considered effective approaches to treating BED [27]. However, a recent meta-analysis found that only 50.9% of individuals who completed treatment achieved abstinence from binge behavior, with IPT resulting in the highest rates [28]. Pharmacotherapies, alone or in combination with behavioral approaches, offer a treatment approach that may improve patient

compliance while also helping to reduce stigma [29]. Off-label use of several drug classes have been shown to decrease binge episodes, including selective serotonin reuptake inhibitors (SSRIs; i.e. fluoxetine) [30], antiepileptics (i.e. topiramate) [31], and appetite suppressants (i.e. sibutramine) [32]. However, there is currently only one FDA-approved drug for the treatment of moderate to severe BED in adults, lisdexamfetamine dimesylate, which is thus the first pharmacological approach.

Lisdexamfetamine dimesylate (VYVANSE®)

Lisdexamfetamine dimesylate is an orally administered central nervous system stimulant produced by Shire US Inc. under the name VYVANSE®. VYVANSE was originally developed for the treatment of ADHD and FDA approved for that use in 2007. In 2015, VYVANSE received FDA approval for the treatment of moderate to severe binge eating disorder in adults.

The chemical structure of lisdexamfetamine dimesylate is shown below (Fig. 1); its chemical designation is (2S)-2,6-diamino-*N*-[(1S)-1-methyl-2-phenylethyl] hexanamide dimethane sulfonate, and it has a molecular weight of 455.60 [33]. Lisdexamfetamine is a prodrug of amphetamine, containing a covalently bound lysine residue that requires enzymatic cleavage for activity. In the small intestine, the prodrug is posited to be taken up by the oligopeptide transporter, peptide transporter 1 (PEPT1) [34, 35]. In circulation, the lysine residue is then hydrolyzed by an unknown cytosolic aminopeptidase in erythrocytes [34-36]. The

mechanism of action for active amphetamine is via the stimulation of norepinephrine and dopamine release into the synaptic cleft and the inhibition of presynaptic reuptake of these monoamines [33]. However, the specific action responsible for the therapeutic effects of lisdexamfetamine is not known.

Following a single oral dose of lisdexamfetamine dimesylate (LDX), absorption under fasted conditions peaks after approximately 1 hr for LDX and 3.5 hr for dextroamphetamine [33]. When LDX was consumed with a high-fat meal or high-sugar beverage, the AUC for plasma concentrations of dextroamphetamine were not affected, however, food prolongs the time to reach max concentrations from 3.5 hr to 4.7 hr. In the blood, LDX is readily converted to dextroamphetamine and L-lysine via enzymatic cleavage due to the high capacity of red blood cells for this hydrolytic activity. In healthy adults, there is no accumulation of LDX or dextro; while LDX has a plasma elimination half-life of only 1 hr, dextro has a plasma elimination half-life of approximately 12 hr post-dose. Drug metabolites are almost entirely excreted in urine, the majority in the form of amphetamine-related compounds, and a smaller proportion as hippuric acid [33].

Clinical efficacy of lisdexamfetamine for the treatment of BED was demonstrated in one Phase II and two Phase III trials [37, 38], and was reviewed by our lab in a previous publication [26]. The Phase II trial was a multi-center, double-blind, placebo-controlled, randomized control trial (RCT) (n = 260) with an initial 3-week titration period followed by an 8-week maintenance dose period of either 30, 50,

or 70 mg/d or placebo [37]. Following the 11-week treatment, the number of binge episodes per week decreased with the 50 mg/d dose ($p < 0.01$), a decrease of 3.5 days per week, and with the 70 mg/d dose ($p < 0.001$), a decrease of 4.3 days per week, compared with placebo. In treatment groups, the incidence of treatment-emergent adverse events (TEAEs) was 84.7%, compared with 58.7% for placebo, and 1.5% of participants in treatment groups had severe TEAEs. Moreover, one participant in the 70 mg/d group died during the study, with postmortem toxicological analysis indicating methamphetamine overdose [37]. The two Phase III trials were also multi-center, double-blind, placebo-controlled, randomized control trials ($n = 383$; $n = 390$) with 3-week titration period followed by a 9-week maintenance dose period of either 50 or 70 mg/d or placebo [38]. Following the treatment period (weeks 11-12), binge frequency in treatment groups decreased by 3.87 and 3.92 days per week ($p < 0.001$ for both), compared to a decrease of 2.51 and 2.26 in placebo groups, in the two studies, respectively. In all groups in both studies, >50% of participants experienced TEAEs, with 10% of LDX-treated participants reporting dry mouth, headache, and/or insomnia [38]. In a study investigating long-term efficacy and prevention of relapse, participants that were first randomized to a 12-week open-label treatment phase ($n = 418$), after which, treatment responders (reduction in binge episodes to $\leq 1/\text{week}$; $n = 275$) were randomized to either placebo or continued LDX dosage for a 26-week double-blind, placebo-controlled relapse phase [39]. For continued LDX-treated participants, 3.7% met criteria for relapse (binge frequency of $\geq 2/\text{week}$), compared to 32.1% for placebo. Overall, these

studies support the long-term clinical efficacy and relative safety of lisdexamfetamine dimesylate for the treatment of BED.

The most common side effects of VYVANSE are dry mouth, constipation, trouble sleeping, feeling jittery, decreased appetite, increased heart rate, and feeling anxious [33]. Contraindications for VYVANSE include known hypersensitivity to amphetamines, or patients taking monoamine oxidase inhibitors (MAOIs) due to the risk of hypertension. As a CNS stimulant, VYVANSE has a high abuse potential, and the risk for cardiovascular complications, such as stroke and myocardial infarction, in addition to causing hypertension and tachycardia. CNS stimulants may also induce or exacerbate existing psychiatric conditions including manic episodes, hinder growth due to reduced food intake and a loss of body weight, as well as induce peripheral vasculopathy. Tolerance (physiological adaption resulting in an attenuated effect of the drug) and dependence (elicitation of withdrawal symptoms precipitated by abrupt cessation of drug treatment or administration of an antagonist) may occur in patients chronically treated with a CNS stimulant [33].

Lisdexamfetamine is the only FDA-approved drug for the treatment of BED, and is the experimental pharmacotherapy for the experiments outlined in Chapter 3 of this dissertation. Our positive control for these studies was sibutramine hydrochloride monohydrate, a drug once FDA-approved for the treatment of obesity, with demonstrated effectiveness in the reduction of binge-like feeding.

Sibutramine hydrochloride monohydrate (MERIDIA®)

Sibutramine hydrochloride monohydrate is an orally administered drug for the treatment of obesity that was formulated and originally manufactured by Knoll BV for Abbott Laboratories as MERIDIA®. MERIDIA was originally FDA approved in 1997 for weight loss and maintenance of weight loss in adults with a BMI >30 kg/m², but was withdrawn from the market in 2010 due to clinical data showing that long-term use was associated with a 16% increase in relative risk for major adverse non-fatal cardiovascular events.

The chemical formula for the active ingredient in sibutramine hydrochloride monohydrate is cyclobutanemethanamine, 1-(4-chlorophenyl)-*N,N*-dimethyl- α -(2-methylpropyl)-, hydrochloride, monohydrate, has a molecular weight of 334.33 [40]; its chemical structure and metabolites are shown below (Fig. 2). The mechanism of action of sibutramine and its secondary (M₁) and primary (M₂) amine metabolites is as norepinephrine and serotonin reuptake inhibition at the presynaptic terminal, and dopamine reuptake inhibition to a lesser extent, but not by additional stimulation of monoamine release into the synaptic cleft [40].

Sibutramine is readily absorbed following oral intake, with maximum uptake occurring 1.2 hr post-administration, and plasma levels of the pharmacologically active metabolites, M₁ and M₂, peaking at approximately 2-3 hr [40]. Sibutramine

undergoes hepatic metabolism by cytochrome P450 isoenzyme to M₁ and M₂, and further metabolized to M₅ and M₆, all which are protein-bound in circulation (relative concentrations, M₅>M₆>M₂>M₁>unaltered sibutramine). The elimination half-lives of M₁ and M₂ are 14 hr and 16 hr, respectively, primarily via hepatic metabolism [40]. Approximately 85% of a single dose is eliminated in urine (77%) and feces over a 15-day period, largely as M₅ and M₆ metabolites. When administered with a mixed meal, peak M₁ and M₂ plasma concentrations are delayed by approximately 3 hr, but there is no effect of food intake on plasma concentration AUCs [40].

Clinical data supporting the efficacy of the sibutramine hydrochloride for the treatment of binge eating disorder has been shown in two studies, in individuals with a BMI < 45 (no lower limit) [41] and between 30 and 45 (obese) [32]. In a 24-week multisite, double-blind, placebo-controlled RCT (n = 304), participants receiving sibutramine (15mg/d) treatment had reductions in binge frequency (2.7 d/week vs 2.0 d/week in placebo) and body weight (4.3 kg vs 0.8 kg in placebo), but greater incidence with side effects [41]. In patients with obesity and BED (n = 30), following a 2-week run-in, participants were randomized to sibutramine (15mg/d) or placebo in a 12-week double-site, double-blind RCT [32]. In sibutramine-treated participants, there were significant reductions in binge days (2.14 d; p < 0.05) and in body weight (7.4 kg; p < 0.001) compared to placebo.

Due to hepatic and renal clearance as the primary routes of excretion, sibutramine should not be used in individuals with severe renal or hepatic dysfunction, and concomitant use of drugs that interact with or inhibit cytochrome P450 enzymes should be avoided [40]. Sibutramine is contraindicated in patients taking MAOIs or CNS-acting antiobesity drugs, and in patients who have eating disorders. Sibutramine can cause hypertension and tachycardia, and should therefore not be used in people with cardiovascular disease. The most common side effects in placebo-controlled studies were dry mouth, constipation, headache, insomnia, and anorexia. Sibutramine is a Schedule IV drug as defined in the Controlled Substances Act (CSA), and thus has a high risk of abuse and dependence [40].

Binge Eating Disorders & Substance Abuse

Binge eating and substance abuse share a cyclical pattern of positive reinforcement (i.e. pleasure, reward) followed by negative reinforcement (i.e. craving, withdrawal), and comorbid anxiety behaviors. Food- and drug-seeking are both reward-driven, “intermittent excessive”[42] behaviors mediated by the mesolimbic dopaminergic system, and involve a common neuropathology. In participants with obesity and compulsive eating behaviors, functional magnetic resonance imaging (fMRI) revealed altered blood oxygen level-dependent (BOLD) response during exposure to highly-palatable, high-energy food cues, in brain areas associated with reward that are also implicated in drug addiction [43].

Anecdotal support for the concept of “food addiction” centers on the experience of intense craving and even withdrawal-like symptoms in the absence of commonly consumed highly-palatable foods. Although this term remains highly controversial due to the differences in food (energy and nutrient intake required for survival) vs drugs (exogenous compounds without biological requirement), there is greater agreement that “eating addiction” may be a suitable term.

Similarly, a recent review by Volkow *et al.* discusses where these two neurobehavioral phenotypes overlap and diverge, and the crosstalk between reward and homeostatic regulatory systems that mediate these two pathological conditions [44].

There are numerous animal studies designed to investigate the shared neural systems in binge-like feeding behaviors and substance abuse and addiction. Early studies by Avena, Hoebel, and colleagues address the concepts of bingeing, withdrawal, craving, and cross-sensitization in the context of sugar addiction in rats, finding alterations in opioid and dopamine signaling that support the concept of sugar dependence [45-50]. A critical distinction between binge-like or “addictive” feeding behaviors and drugs of abuse, is that the characteristics of the palatable food exposure (intermittency vs continuous) appears to determine the abuse potential, rather than solely the food composition [51].

Rodent Models of Binge-like Feeding

Eating disorders represent a family of complex psychiatric diseases involving cognitive and neurobiological perturbations, with clear social and cultural influences on body image and self-esteem. Eating disorders have a sustaining pathology that remains not well understood, and are accompanied by severe psychological distress. Animal models provide an important tool to investigate the biochemical basis for these aberrant feeding behaviors, to better understand the neurological causes or consequences, and elucidate mechanisms to target in the development of new pharmacotherapies. Although animal models are not able to recapitulate all aspects of the human experience, they are an essential complement to clinical research because they allow researchers to separate the neurobiological factors from the cultural and social components. There are a multitude of rodent models that have been used to study disordered feeding behaviors, and binge-like feeding, specifically. Depending on the research question, investigators have used variations of species, strain, phenotype-prone/-resistant selection protocols, feeding schedules, macronutrient compositions and food matrices, the presence, duration, or extent of caloric restriction, as well as the introduction and type of stressor. There have been several comprehensive reviews on this subject [42, 51-55]. Furthermore, because binge eating disorder presents with various subtypes, numerous behavioral models are used to study the respective set of symptoms they recapitulate.

Species, Strain, & Sex

Disordered eating behaviors are largely heritable, but the underlying genetics responsible for the disparity in vulnerability remains not well characterized. Identifying rodent strains that show differences in propensity for binge-like feeding provide the opportunity to explore genetic differences that may partially explain these behavioral differences. Hildebrandt and colleagues used female and male Sprague-Dawley and female Wistar rats to explore whether there were strain differences in vulnerability to binge behavior [56]. Using a binge eating-prone/-resistant paradigm (described in subsequent sections in further detail), investigators found that Sprague-Dawley females had the highest intakes of palatable binge food and were more frequently classified as binge-prone than Sprague-Dawley males and Wistar females. Thus, exploring the genetic or neurobiological differences between these strains could be an effective tool to identify factors that affect vulnerability to disordered eating.

Compared to rat models of binge-like eating behavior, mouse models are more limited due to a more pronounced effect of stress sensitivity on feeding behavior. Work by Teegarden and Bale support that mice display an altered macronutrient preference when exposed to chronic stressors, and stress sensitivity in mice is highly variable[57]. Furthermore, binge models in mice using stress (forced swim) and restriction-refeeding cycles (described in further detail below), produced only transient perturbed behavior[52]. The sustaining pathology of eating disorders is not only central to the clinical experience, but also required to reliably study the

neurobiological mechanisms involved. In response to this shortage of reliable mouse models, Czyzyk and colleagues developed an intermittent access mouse model of binge-like feeding, that notably does not require food restriction nor stressors [58]. This model also displayed clinical validity in that mice treated with fluoxetine, shown to decrease binge episodes in humans, dose-dependently reduced binge intake. Similar intermittent access models in mice have since been developed, and adaptations of rat models have been replicated in mice.

Binge-Prone/-Resistant Models

Binge behavior in humans is highly variable despite similar environmental conditions, where access to highly-palatable binge foods is ubiquitous. Innate differences in genetics or neurobiology may explain why some individuals are more vulnerable to binge eating pathologies than others. One technique to explore these innate differences in neurophysiology is the utilization of binge-prone/binge-resistant rodent models. Sisk and colleagues use a limited-access model to elicit binge-like feeding in adult female Sprague-Dawley rats [59]. The 3-week protocol involves a 4-hour access period to a purified high-fat diet, in addition to ad lib standard chow, during the dark cycle, occurring 3x/week. Rats are then grouped into tertiles based on the palatable food intakes during the 3-week protocol; the highest tertile of palatable food intake representing “binge-prone” animals, and the lowest tertile defined as “binge-resistant.” Neural response to a 1-hr palatable food exposure in binge-prone vs –resistant rats was assessed via quantification of Fos expression, the protein product of the c-Fos

gene that serves as a marker of early neural activation. In binge-prone rats exposed to the palatable food test, Fos expression was higher in the nucleus accumbens and limbic cortex than in binge-resistant rats, and in binge-prone rats not exposed to the high-fat diet test. These data support the hypothesis that there are neural alterations in mesolimbic reward systems associated with hedonic feeding, in animals with a high propensity for binge-like feeding behavior, and may partially explain why some individuals are more vulnerable to disordered eating behavior.

Composition of Binge Food: Sweet, Fat, Sweet/Fat, Chocolate, & Cafeteria

The typical foods individuals overconsume during binge episodes are highly palatable foods that are high in sugar and/or high fat. A variety of macronutrient profiles and formulations are used in animal models of binge-like feeding, and the associated neurobiological alterations observed vary somewhat with each model. Kreisler et al. use an intermittent access model with a chocolate-flavored sucrose-rich pelleted diet matched to standard chow in macronutrient composition and caloric density, but that is greatly preferred over chow in rats[60]. Avena and Hoebel's groups use a variety of sugar binging models, often using solutions of sucrose as the binge food [46, 48]. Yasoshima et al. used a sucrose solution limited-access model to explore binge-like consumption in mice, and the non-carbohydrate sweetener, saccharin, to explore whether consumption was a function of caloric need [61]. In contrast, Lardeux's intermittent access

model used a sweet-fat emulsion of corn oil, heavy cream, and sugar, to explore the effects of varying fat content and palatability on meal microstructure [62].

There are various high fat/high energy diets that also serve as effective binge foods in rodent studies. In their weekly, intermittent access model in mice, Czyzyk and colleagues used a nutritionally-complete high fat diet (40% kcal/g)[58]. Murphy et al., in a series of related, random-access binge experiments in mice, used a 60% high fat pelleted diet, a 10% sucrose solution, and a high-sugar, chocolate flavored complete liquid meal (Ensure™)[63]. Corwin's model of restricted access typically uses vegetable shortening as the binge food, which is a semi-solid and pure fat [53, 64-68]. Similarly, Bello's intermittent access rodent model used vegetable shortening plus 10% sucrose to mimic the sweet-fat binge foods often seen in clinical populations [21-25, 69].

Early binge models from Leigh used a "cafeteria diet" binge food, a varied selection of highly palatable human food items[70]. Hutson, Heal, and Vickers use chocolate in an intermittent access model, and chocolate-flavored pellets in a delayed-discounting task, in female rats to investigate opioidergic and dopaminergic alterations associated with binge-like feeding and impulsive behavior, and interactions with pharmacotherapies [71, 72]. Using human binge foods in animal models allows translation to clinical conditions, however, there are research limitations due to the inconsistencies in macronutrient composition and caloric content that create challenges in interpretation.

Caloric Restriction (presence, extent, duration)

In humans and in animal studies, cycles of food restriction and refeeding can elicit overconsumption during *ad libitum* fed periods, or “rebound hyperphagia.”

There are numerous animal models of binge-like feeding that utilize rebound hyperphagia to mimic binge episodes.

There are several research groups that were early contributors to the development of rodent models of eating disorders utilizing restrict-refeeding to precipitate binge-like feeding. Hoebel and colleagues, used repeated cycles of restriction/refeeding in rats, with both sweet and/or high-fat palatable foods [46, 73, 74]. The binge-like feeding patterns elicited were associated with altered dopamine and mu-opioid binding, and Hoebel's group explored the overlap in neural mechanisms of drug abuse and high-sugar or high-fat binge behavior. Similarly, Leigh's model of bulimia nervosa involved repeated cycles of alternating standard chow and a cafeteria diet to mimic restrict/refeed cycles [70]. Rodents exposed to this feeding paradigm develop reduced ovarian function while maintaining normal body weights. Hagan and colleagues developed a restrict-refeed model to explore the effect of a history of restricted eating behaviors on future feeding behaviors [75]. This model involved 12 restrict-refeed cycles, also providing access to cookies during the chow refeeding periods. After the 12 cycles, rats had a 30-day cessation from the feeding protocol, and then underwent a chow and cookies test without prior restriction. The neural

alterations associated with a history of restrict-refeed cycles resulted in greater non-rebound intake, despite the long period of restrict-refeed cycle absence. These animal studies support clinical findings that patterns of food restriction may have long-lasting effects on binge propensity.

Intermittency & Limited Access

In humans, self-imposed limitations on consumption of specific high-sugar/high-fat (palatable) foods are often the foods individuals binge on, a concept coined as the “forbidden foods” hypothesis [76]. In animal models, limited access to highly palatable foods is imposed by investigators (vs self-imposed), but results in increased consumption of those foods, even in the absence of homeostatic hunger.

Corwin and colleagues established a limited access model of binge-like feeding where rodents are provided with ad lib water and chow at all times, in addition to access to a high-fat food (i.e. vegetable shortening) that ranged from continuous choice to 2-hr access, three times per week [77]. Notably, there is no investigator-restricted caloric intake in this model, mimicking binge episodes in humans that frequently occur in the absence of physiological hunger. The more restricted the access to the high-fat shortening, the more was consumed during the access window, and overall as contribution to total kcal intake/day, with 70% of energy consumption coming from the highly palatable binge food. For this model, approximately four weeks of the limited access protocol is required to

establish binge-like eating behaviors, and subsequent binge eating is then easily maintained. Interestingly, some rats underconsume on non-binge days, developing a cycle of binge/compensation that mimics restrict-refeeding behavior without having investigator-restricted feeding. However, in subsequent studies, to parse the effects of prior caloric restriction vs limited access to a highly palatable food, Corwin's group showed that underconsumption on non-binge days is not required for overconsumption on binge days[64].

Sham feeding

Sham feeding is the technique by which esophageal or gastric fistulas are surgically introduced, allowing for consumed food or liquid to be eliminated from the gastrointestinal tract before entering the stomach or small intestine, respectively. Animal models fitted with chronic fistulas consistently display hyperphagia, with the drained food from the stomach mimicking the purge-type behavior associated with bulimia [47, 78-80]. Although the release of ingested food is controlled by the experimenter not the animal, the uncoupling of the orosensory drivers of hyperphagia, from the negative feedback of intestinal signaling, allows insight into the neurobiological underpinnings of the binge-like intake observed.

Stressors

The introduction of stressors has long been used in animal models to explore the neural control of feeding behaviors. Eating disorders have a large psychological

component involved in the development and maintenance of these maladaptive behaviors that is difficult to parse in human studies. Animal feeding studies using stressors allows researchers to look at the interaction between diet-induced binge-like feeding and the stress that accompanies these disorders. The specific stressor used is largely determined by the research question being asked, and can vary in type, severity, frequency or duration (acute vs. chronic), and effect span (immediate vs. historic)[42].

Two common methods used to study the effect of *immediate, acute* stress on food intake in rodents are tail pinch and foot shock. Both are brief in duration and considered mildly painful, and both techniques consistently yield effects on subsequent food intake. Hagan *et al.* explored the interaction between a history of restrict-refeed cycles and a one-time footshock, showing that as early as 2-hours post-stress exposure, intake of the highly-palatable food increased by 53% [81]. Moreover, Bello *et al.* found that a one-time, 1-hour restraint stress increased subsequent binge intake and neural activation, following a 6-week diet-induced binge-like feeding schedule [24].

In addition to immediate acute stressors, the extended restriction of physical space is an example of an *immediate chronic* stressor, due to its longer duration. Inoue and colleagues used a rat model of binge-like feeding involving an initial caloric restriction, followed by ad lib intake in either a small or normal sized cage. Investigators found that the stress induced by the restriction of physical space

during the refeeding period resulted in an increase in rebound hyperphagia through 24-hr intakes [82, 83].

In humans, severe stressors during development increase vulnerability to binge eating in adulthood [84, 85]. In rodent studies, intermittent maternal separation [86, 87], increase palatable food intake and rebound hyperphagia in adulthood. Maternal separation and social isolation are considered *historic chronic* stressors in that sustained or severe stress experienced in early life have enduring effects on food intake that persist well into adulthood [42].

Opioid signaling plays an integral role in mediating the rewarding aspects of both food and drugs. Mu-opioid receptor activity is primarily responsible for reward signaling, in addition to the function of opioids in nociception and analgesia.

OPRM1 A118G SNP

Characterization

The mu-opioid receptor (μ ; MOR; Fig. 3) is one of three classes of opioid receptors, in addition to delta- (δ ; DOR) and kappa- (κ ; KOR). Opioid receptors belong to the rhodopsin family of G-protein coupled receptors (GPCRs), comprised of seven transmembrane helices, three intracellular loops, three extracellular loops, one extracellular N-terminus, and one intracellular C-

terminus. While all three opioid receptor subtypes have high homology in the transmembrane regions, the extracellular loops have less homology. These residue differences in the ligand-binding domains facilitate ligand specificity and binding affinity that differs between μ -, δ -, and κ - classes, although many ligands bind multiple subtypes [88]. Further introducing complexity of opioid signaling and function is the ability of opioid receptors to act as single receptors or as heterodimers. The primary endogenous ligands for MOR are β -endorphin, a cleavage product of proopiomelanocortin (POMC), and endomorphins. The primary ligands for DOR are enkephalin & deltorphin, and for KOR, the dynorphins [88].

As GPCRs, opioid receptor signaling involves activation upon extracellular ligand binding, activating heterotrimeric G_i/G_o proteins and disassociation of α - and $\beta\gamma$ -subunits, which stimulate intracellular signaling pathways. Signaling results in the opening of postsynaptic G-protein-gated Inwardly Rectifying K^+ (GIRK) channels, the inhibition of presynaptic voltage-gated Ca^{2+} channels, reduction of adenylyl-cyclase activity and subsequent cyclic adenosine monophosphate (cAMP) concentrations, and activation of the mitogen-activated protein kinase (MAPK) pathway [88]. Collectively, these actions serve to decrease membrane potential, inhibit neuronal excitability and neurotransmitter release, and alter downstream signaling and gene expression.

Whereas KOR activation is largely responsible for feelings of dysphoria, MOR, and to a lesser extent DOR, activation results in analgesia and reward. Mu-opioid receptor distribution is vast, with expression in peripheral regions, particularly in the gastrointestinal tract, and dispersed throughout the CNS, and common analgesics such as morphine, fentanyl, codeine, and oxycodone are examples of exogenous mu-opioid ligands.

The gene that codes for μ -opioid receptors (*OPRM1*) is a 200 kb region of the long arm of chromosome 6. Of 3324 *OPRM1* polymorphisms identified, and 1395 variants with >1% global allele frequency, there are only two common non-synonymous variants[88]. The most common single nucleotide polymorphism (SNP) is the non-synonymous substitution in exon 1 resulting in an adenine (A) to guanine (G) substitution at position 118 (*A118G*; rs1799971). This SNP results in amino acid substitution in the extracellular region of the protein from asparagine (N) to aspartate (D) at position 40 (N40D; Fig. 4). The *OPRM1* *A118G* SNP has a relatively low prevalence in African American and Hispanic populations (1-3%), but is estimated to be much higher in individuals of Asian (40-50%) and European (15-30%) descent [89].

The *A118G* nucleotide substitution, and resulting N40D amino acid substitution, eliminates a putative N-glycosylation site in the extracellular region of the mu-opioid receptor [88]. This alteration in post-translational modification within the

extracellular N-terminal region is in close proximity to the ligand-binding domain, and therefore posited to affect receptor binding and/or cell signaling.

Biochemistry & Functional Outcomes

Early biochemical investigation of the *A118G* allele revealed that the G allele resulted in greater binding affinity for the 31-AA endogenous ligand, β -endorphin, whereas there were no allele differences in binding of small peptide ligands (4-5 AAs) [4]. These findings provided early evidence that the non-synonymous *A118G* SNP results in increased binding affinity for its primary endogenous ligand. However, subsequent studies by Mague and colleagues found no differences in MOR binding affinity for β -endorphin, morphine, and naloxone in mouse brain [90].

The effect of the *OPRM1 A118G* SNP on N-linked glycosylation and protein stability was investigated in mouse brain tissues and in stably transfected Chinese hamster ovarian (CHO) cells [91]. In a transgenic knock-in model of the equivalent SNP in mice (*A112G*; N38D), MOR protein molecular mass was measured in thalamic and striatal samples in AA and GG mice. In GG mice, MOR molecular mass was lower than in AA mice, but after PNGase F treatment to remove N-linked glycans, the molecular mass of GG and AA MOR proteins was equivalent. Furthermore, in CHO cells expressing human *OPRM1* homozygous for the major or minor alleles (GG or AA), similar differences in N-glycosylation were observed, and GG proteins had a shorter half-life (~12 hr)

than AA proteins (~28 hr). Together, these studies showed that the *A118G* SNP decreases the N-linked glycosylation and protein stability of mouse and human MOR.

Transgenic mouse models have also been used to study the effect of the *A112G* SNP on *OPRM1* mRNA and receptor expression in a brain region- and sex-specific manner. Mague et al. found that GG mice had lower *OPRM1* mRNA in the ventral tegmental area and nucleus accumbens, and lower whole-brain receptor expression levels [90]. Wang et al. used in vitro autoradiography to quantify and localize MOR expression in mice, and found reduced protein expression in GG mice in areas including the nucleus accumbens, thalamus, hypothalamus, periaqueductal grey, and ventral tegmental area. When exploring sex differences, they found that the trend of lower MOR expression in GG vs AA mice was more common in female vs. male mice [92]. In general, mice homozygous for the *A112G* SNP appear to have lower gene and protein expression of the mu-opioid receptor.

In addition to effects of the *A118G* SNP (N40D) on post-translational modification (i.e. N-glycosylation), this non-synonymous SNP may also affect epigenetic processes of gene expression. Recent evidence by Oertel and colleagues shows that the *A118G* SNP introduces a CpG site at position 117, thereby adding a DNA methylation site that can alter mRNA expression of the *OPRM1* [93]. This genetic-epigenetic interaction provides an additional mechanism that may explain

the functional outcomes of the *A118G* genetic polymorphism [93]. Previous studies from another group found that *OPRM1* methylation is elevated in former heroin addicts maintained on methadone [94]. In support of these findings, Oertel found that in postmortem human brain tissues from individuals with the *A118G* SNP, mu-opioid receptor expression was not upregulated, as is normally observed in conditions of chronic opioid exposure, and the lack of upregulation was associated with greater DNA methylation. This epigenetic regulation of mu-opioid signaling provides an additional mechanism by which the *A118G* SNP may affect neurobiological outcomes with long-term consequences on endogenous opioid signaling.

Several studies have also investigated the effects of the *OPRM1 A118G* polymorphism on downstream cell signaling pathways. Ishani Deb *et al.* found the *OPRM1 A118G* had a higher allele frequency in individuals who had a history of heroin or alcohol addiction or abuse, so they used stably-transfected Neuro 2A cells to investigate the potential signaling mechanisms that could help explain the genotypic association [89]. Morphine acutely inhibits cAMP and PKA signaling, and chronic opioid exposure results in the upregulation of this pathway. In neural cells expressing the human *A118G* SNP, chronic morphine exposure did not elicit the compensatory increase in PKA levels typically observed, despite finding equivalent levels of protein expression between prototype- and variant-expressing cells. Furthermore, basal ERK levels were reduced in *A118G* cells, but substantially increased in response to chronic morphine exposure [89].

Bond and colleagues also explored the effect of the SNP on downstream ion channel activation [4]. Activation of GIRK channels was higher in cells transfected with the A118G SNP receptor variant vs the prototype, suggesting altered potency of G-protein activated K⁺ channels to DAMGO activation of the variant mu-opioid receptor.

Associations with Drug Efficacy & Addiction

Clinical Studies

Although there is a genetic component of the susceptibility to substance abuse and addiction, identifying specific genetic components that contribute to disease development remains a challenge. Early work by Bond et al. found the variant (G) allele frequency not different between individuals with a history of opioid abuse/addiction vs no history of drug/alcohol abuse/addiction [4]. However, when evaluating allele frequencies by ethnic group of the participants, the minor allele was higher in Hispanic individuals without prior drug/alcohol abuse vs a history of drug abuse/addiction, suggesting a protective effect of the G allele in drug abuse susceptibility.

A recent review by RC Christ and WH Berrettini explored the association between *OPRM1* polymorphisms and nociception and rates of dependence for common drugs of abuse, as well as the pharmacogenetics of the respective treatments [88]. Overall, patients with the G allele of multiple ethnicities (Caucasian, Chinese, Japanese, Taiwanese), required higher doses of numerous

types of analgesics (morphine, fentanyl, sulfentanil) to achieve the same pain relief as AA patients following various surgical procedures (abdominal, oral, cesarean section, knee, gynecological) or due to cancer pain. Although several studies found no association between *A118G* allele status and analgesia, there is a significant body of literature supporting the finding that GG genotype incurs the need for a higher dose of analgesic treatment to provide the same degree of pain relief, as compared to individuals with the AA prototype. In patients receiving naltrexone treatment for alcohol abuse or transdermal patch for nicotine addiction, individuals with the *A118G* SNP had higher abstinence rates and lower relapse rates. Although the literature supports pharmacogenetic differences in dosage and efficacy in the treatment of pain and/or substance abuse, there has not been consistent data to support an association between *OPRM1* polymorphisms and opioid, nicotine, or alcohol dependence.

Mouse Models

There have been many transgenic mouse models developed to study the functional effects of the *OPRM1 A118G* SNP and the underlying biological mechanisms involved. Many models, including our own, incorporate the equivalent murine SNP (*OPRM1 A112G*; N38D) [69, 90-92, 95-100], whereas others use a “humanized” model involving the introduction of the *A118G* SNP of the human *OPRM1* gene [101-110].

Associations with Obesity & BED

In addition to a posited association with drug action and abuse, the *OPRM1* A118G polymorphism has also been investigated in the context of feeding behavior, and the rewarding aspects of palatable food consumption. Early work from Davis *et al* found the G allele to be enriched in individuals with obesity and BED, compared to obese individuals without BED [6]. Subsequent studies from their group examined this relationship further, and found that, in healthy adults, the G allele was associated with higher reported preference for sweet and fatty foods, which correlated to measures of overeating and which accounted for variance in body mass index [111].

Although mouse models of the *OPRM1* A118G/A112G SNP have been extensively utilized in the context of nociception, analgesia, and substance abuse, there is currently no known literature using this model to study potential genotypic differences in feeding behavior, or binge-like feeding behavior. Moreover, the concept of “personalized medicine” to improve treatment options and outcomes garners attention from the biomedical community. The experiments in this dissertation seek to address questions centering on the role of *OPRM1* A118G allele status in feeding behavior, binge propensity, and pharmacological efficacy for the treatment of BED.

General Hypothesis

In the outlined experiments, a transgenic knock-in mouse model of the homologous murine SNP, A112G/N38D, and an established intermittent access

model of binge-like feeding were used to investigate these interactions of diet, genes, drugs, and sex.

The general hypothesis is that the *OPRM1* polymorphism increases feeding behavior to promote overeating phenotype in the murine model of *A118G* variant.

Through the subsequent chapters of this dissertation, the following experimental questions are addressed:

- 1) Does *OPRM1 A112G* allele status affect vulnerability to binge eating behaviors in male and female mice? Does the SNP have sex-dependent effects on binge propensity and/or body weight change?
- 2) What is the pharmacogenetics of the *OPRM1 A112G* SNP in lisdexamfetamine efficacy for the treatment of binge-like feeding in female mice, during acute and chronic dosing?
- 3) Does the *OPRM1 A112G* allele status affect lipid preference in male mice, naïve or previously exposed to an intermittent access model of binge-like feeding? Does allele status affect taste responsivity or meal patterns, in male and female mice?

Figures

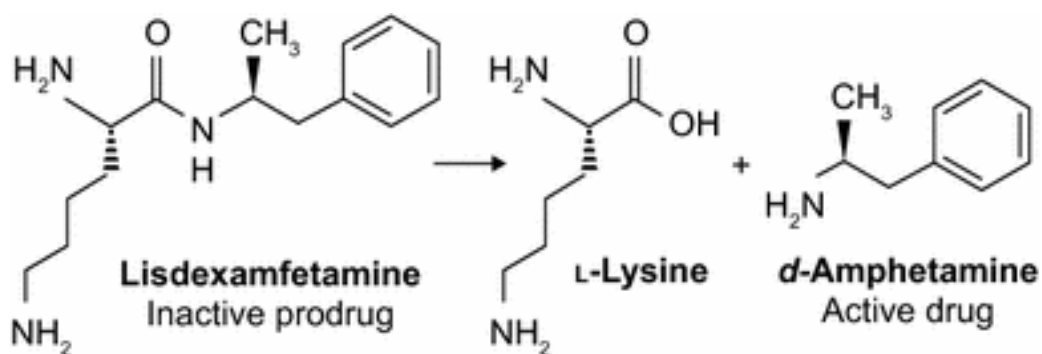
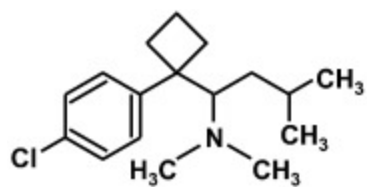
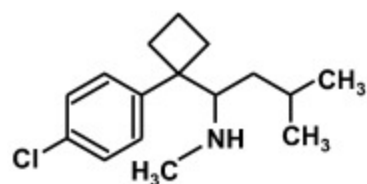


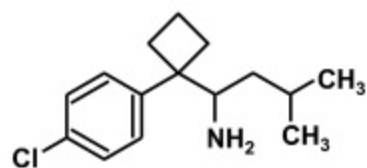
Figure 1: Chemical structure of lisdexamfetamine. Inactive prodrug lisdexamfetamine and its cleavage to active d-amphetamine. Image from Ermer JC et al, *Clin Drug Investig*, 2016.



Sibutramine



N-Desmethylsibutramine



N-Didesmethylsibutramine

Figure 2: Chemical structure of sibutramine hydrochloride and metabolites.
Image from Morikawa Y *et al.*, *Toxicol Appl Pharm*, 2017.

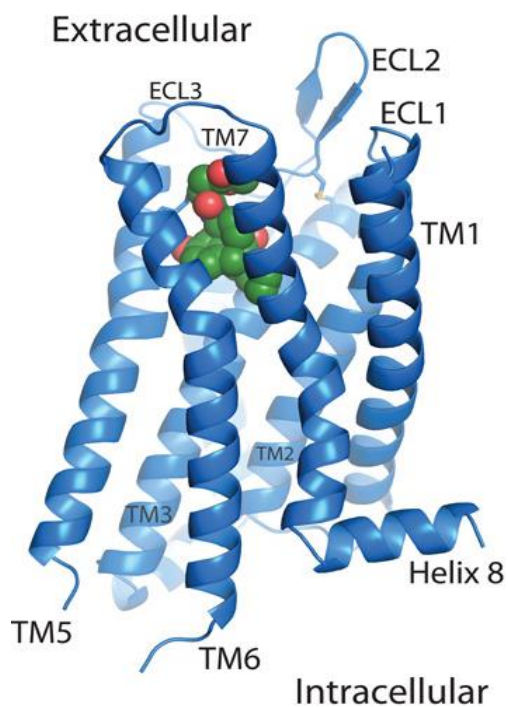


Figure 3: Crystal structure of the mu-opioid receptor (MOR) bound to morphanin, a semi-synthetic, irreversible MOR antagonist derived from morphine. Image adapted from Manglik A *et al.*, *Nature*, 2012.

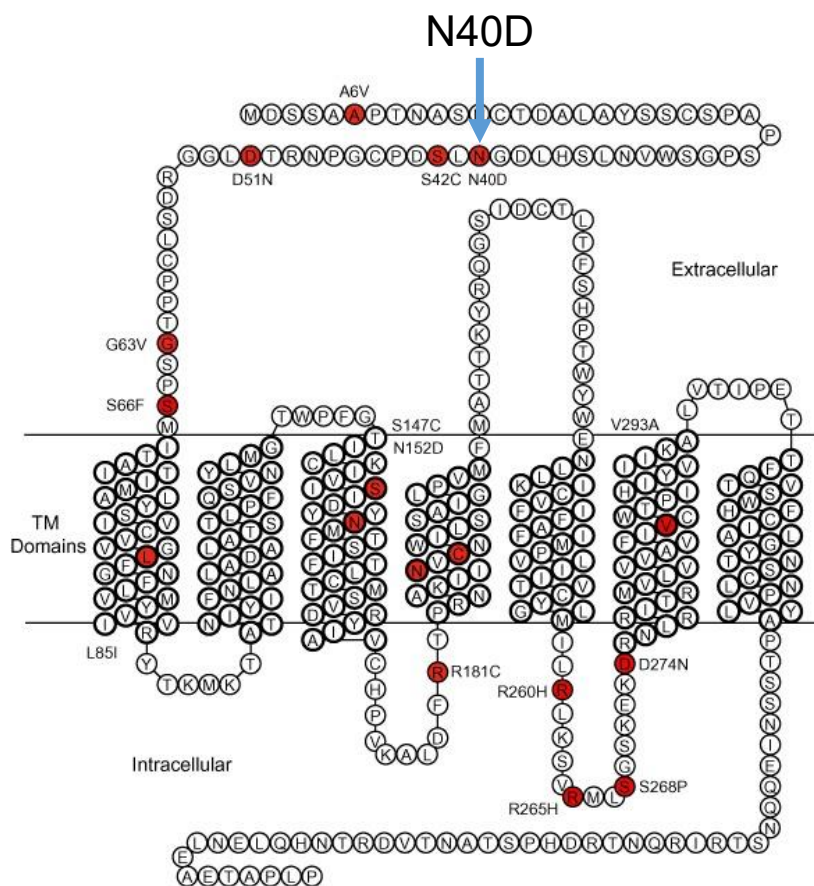


Figure 4: Illustration showing asparagine (N) to aspartate (D) substitution within the extracellular region of the mu-opioid receptor, due to the *OPRM1* A118G single nucleotide polymorphism. Image adapted from Knapman A *et al.*, *Br J Pharmacol*, 2014.

Chapter 2

Binge-like Feeding Behavior in Male and Female Mice Homozygous for *OPRM1* A112G Prototype (A) and Variant (G) Alleles

Introduction

Binge eating disorder (BED) is the most common eating disorder, with a lifetime prevalence of 2.8%, and is more commonly diagnosed in women vs men (1.6% and 0.8%, respectively) [1]. BED is characterized in the DSM-V as the frequent or repeated occurrence of *binge episodes*, defined as the consumption of a larger than normal amount of food in a shorter than normal amount of time, and a marked experience of a loss of control. The neuropathology of BED remains not well understood, and there are few treatment options available with long-term success.

Opioids, and mu-opioid receptor (MOR) signaling specifically, play a salient role in the hedonic regulation of food intake, and consumption of highly palatable, sweet-fat foods, in particular. There are few functional polymorphisms of the MOR gene (*OPRM1*) that are common within the population. One such single nucleotide polymorphism (SNP) is *OPRM1* A118G, which results in an amino acid substitution (N40D) in the extracellular N-terminal region of the protein. *OPRM1* A118G is the most common functional SNP, shown to affect nociception and analgesia, and with a posited interaction with substance abuse and addiction of opioids and non-opioid drugs. Furthermore, there is population-based data

supporting enrichment of the GG allele in people with obesity and BED, compared to obese individuals without BED [6]. Despite this important potential genetic predisposition to binge-like feeding behavior, there are few studies utilizing mouse models to explore this interaction. This study used a transgenic knock-in mouse expressing the homologous murine SNP (*OPRM1* A112G) and an established intermittent access model of binge-like feeding behavior to investigate whether *OPRM1* A112G allele status affects binge-like feeding behavior in male and female mice.

Materials and Methods

Animals

The animal care protocol was approved by the Institutional Animal Care and Use Committee of Rutgers University (OLAW #A3262-01).

OPRM1 A112G Mice

The common non-synonymous SNP of the human mu-opioid receptor gene (*OPRM1 A118G*) involves the substitution of adenine (A) to guanine (G) as position 118 within exon 1. This single nucleotide substitution results in a functional amino acid substitution of asparagine (N) to aspartate (D) at position 40, eliminating a putative N-glycosylation site within the extracellular region of the mu-opioid receptor (MOR). The mouse homolog of this SNP, *OPRM1 A112G*, involves the same A to G substitution, at position 112 in exon 1, resulting in the same N to D substitution, at amino acid 38. The homologous mouse SNP eliminates the N-glycosylation site in the extracellular region, as in humans.

Transgenic *OPRM1 A112G* conditional knock-in mice were generated on a C57BL6 background by Lei Yu (Department of Genetics, Rutgers University, New Brunswick, NJ) with Caliper Discovery Alliances & Services (Caliper Life Sciences Inc., Hanover, MD). As described in the project report provided by Caliper, the conditional knock-in was created using homologous recombination in mouse embryonic stem (ES) cells, followed by blastocyst injection with the

targeted ES cells. The mouse chromosome 10 sequence (n.t.# 3,510,000,000~3,590,000; Ensemble database) was used as a reference, and the RP23-263A7 bacterial artificial chromosome (BAC) clone was used as template to generate the homology arms, conditional knock-out (KO) region, and probes for southern blot screening tests. The 5' homology arm (5.3 kb), 3' homology arm (3.0 kb), and conditional KO region (2.2 kb) were generated using Taq DNA polymerase, and fragments cloned in the bacterial plasmid, pCR4.0™ (Invitrogen, Thermo Fisher Scientific, Waltham, MA), were confirmed by restriction digestion and end-sequencing. The A to G mutation at position 112 in exon 1 was introduced into the conditional KO region by PCR-based site-directed mutagenesis using the QuickChange II™ kit (Stratagene, Agilent Technologies, Santa Clara, CA). In addition to the homology arms, the final cloned vector contained LoxP sequences flanking the conditional KO region, Frt sequences flanking the neomycin (Neo) expression cassette (positive selection of ES cells), and diphtheria toxin A (DTA) cassette (negative selection of ES cells). The final vector (Fig. 1) was confirmed by restriction digestion and end-sequencing, and the restriction enzyme, NotI, was used for linearization of the plasmid before electroporation into ES cells. ES clones were expanded, and the A to G mutation was confirmed. Blastocyst (C57BL/6 Tyr) injection was performed on confirmed clones, and heterozygous mice were obtained from male chimera breeding to C57BL/6 wildtype females.

Mice were originally maintained by Taconic Laboratories (Rensselaer, NY). Two male and two female heterozygous *OPRM1 A112G* transgenic mice were purchased from Taconic Laboratories, and paired as two sets of breeders to establish a colony at the Rutgers University Cook Campus vivarium located in Bartlett Hall (New Brunswick, NJ).

Breeding

OPRM1 A112G heterozygous mice were bred for three generations. In order to excise the Frt-flanked Neo cassette, four male ACTBFLPe mice (C57BL/6J background) were purchased from The Jackson Laboratory (cat #005703, Bar Harbor, ME) and bred with heterozygous *OPRM1 A112G* females. Heterozygous offspring from several generations (>3) were then used as founder mice for the *OPRM1 A112G* colony.

Twelve-week-old (PND84) male and female transgenic mice heterozygous for the SNP (AG) were bred, one pair per cage, and provided ad libitum water and standard chow (LabDiet[®] Mouse Diet 5015, 19.75% protein, 26.1% fat, 54.15 CHO, 3.8Kcal/g), and a plastic shelter. Cages were checked daily, and new litters were recorded. Pups were ear-notched for identification between PND 14 and 21, and the tissue was used for genotyping (described in detail in subsequent sections). Pups were weaned at PND 21 and separated by sex. All pups were group housed with ad libitum water and standard chow (LabDiet[®] Laboratory Rodent Diet 5001, 3.36 Kcal/g; 28.67% protein, 13.38% fat, 57.94% CHO), and

provided a plastic shelter and wooden block. Heterozygous (AG) mice were paired as new breeders at maturity, PND 84, one male and one female per cage. Male and female mice homozygous for the prototype (AA) or variant (GG) alleles were single-housed and transported to a garage at PND 38.

Housing

Six-week-old (PND42) male and female transgenic mice (*OPRM1* A112G) homozygous for the prototype or variant alleles (AA or GG, respectively) were single-housed in standard mouse 'shoebox' cages (7.5" x 11.75" x 5"). Each cage bottom was lined with paper-enriched corn cob bedding, and cages were given a plastic shelter, a paper Nestlets®, and a wooden block to promote gnawing behaviors. Rooms were maintained on a 12/12h light/dark cycle (automatic lights off at 1900 h), and garages were temperature and humidity controlled (18-25°C and 30-70%, respectively), with twice-daily documentation by vivarium staff. Mice were provided ad libitum water through the duration of the protocol, and clean water bottles were provided by vivarium staff weekly. Cage bottoms were changed by Bello lab trainees once per week.

Diet

All mice were maintained on a standard chow (LabDiet® Laboratory Rodent Diet 5001, 3.36 Kcal/g; 28.67% protein, 13.38% fat, 57.94% CHO), provided ad libitum in custom, stainless steel chow hoppers, except when otherwise noted during Binge protocol, and described in detail in subsequent sections. Mice in

restrict groups have biweekly, 24-hr periods of food deprivation, and mice in binge groups receive intermittent access to a “sweetened fat” binge food, consisting of commercially-available, food grade vegetable shortening (Crisco™) with 10% sucrose (w/w) added; 8.6 Kcal/g.

Genotyping

As described in previous sections, between PND 14 and 21, mice were ear-notched for identification, and tissue punches were collected for genotype analysis by standard polymerase reaction (PCR) of genomic DNA (gDNA).

The ReliaPrep™ gDNA Tissue Miniprep System (Promega, Madison, WI) was used to digest tissue samples, and isolate gDNA, using the standardized supplier protocol.

For PCR, target gDNA (3 ul/sample) was amplified using GoTaq® Green Master Mix, 2X (10 uL/sample) and nuclease free water (7 uL/sample) (Promega, Madison, WI), and forward and reverse primers: 5'-

GCACACAAAAGAGCAATAGAACGGAAATA-3' (0.5 uL/sample) and 5'-

GATCCCCTCAGAAGAACTCGT-3' (0.5 uL/sample). Reaction mixtures (21 uL

total/sample) were run on a thermocycler (2 ABI GENEAMP 2700, Applied

Biosystems, Thermo Fisher Scientific) with the following reaction conditions: Hold

(95C/F? for 2 min) for one cycle; annealing (95C for 30 s, 55C for 30 s, 72C for

1.5 m) for 35 cycles; holds (72C for 5 m, 4C until run is complete).

For characterization of genotypes by electrophoresis, PCR products and a 100bp ladder were run on a 2% agarose gel (130V constant, 40 min), and bands were visualized using Gel Doc™ EZ System (Bio-Rad Laboratories, Inc, Hercules, CA). The prototype allele (A) amplicon is ~0.26 kb, and the variant allele (G) is ~0.4 kb (Fig. 2).

Body Weight, Chow Intake

Mouse body weight and chow weight were measured twice weekly, days 2 and 5, between 0900 and 1600 hours. To measure body weight, a plastic cup was weighed and tared (CQT 202 portable compact balance, Adam Equipment, Oxford, CT), and mice were transferred to the cup. Body weights (g; to the hundredth place) were recorded. Chow weights were measured (Scout® portable topload balance, CA# 10805-278, OHAUS®, Parsippany, NJ) as hopper weight plus chow (g; to the thousandth place), and intakes were calculated from recorded values.

Dietary-induced Binge Eating

The diet-induced binge eating protocol used for these experiments involves intermittent access to a highly-palatable “sweetened-fat” (vegetable shortening plus 10% sucrose; 8.6 Kcal/g) that mimics the macronutrient composition of common binge foods in humans. At 6-weeks of age (PND 42), one week before starting the binge access protocol, all mice underwent a 24-hr pre-exposure to

the sweetened-fat binge food, to minimize novelty-induced hypophagia and to control for innate food preferences when assigning experimental groups. Mice were then randomly assigned to one of four feeding conditions (Table 1), controlling for pre-exposure body weight and sweetened-fat intake: *Naïve* (N), *Restrict* (R; 24-hr chow restriction), *Binge* (B; 30-min access to sweetened-fat), or *Restrict-Binge* (RB; 24-hr chow restriction, followed by 30-min access to sweetened-fat).

At 1630 hours, on Days 2 and 5, restrict (R/RB) mice had chow hoppers removed from the home cage, and bedding was searched for loose chow pellets which were removed. On days 3 and 6, the sweetened fat was prepared directly preceding the binge period. Chow pellets and sweetened-fat were portioned into labeled, small, glass jars, and weighed (as the glass jar plus food). At 1630 hours, chow and binge food (B/RB mice) were given to mice in their home cage, the glass jars positioned away from the water bottle spout. All mice were allowed to eat freely during the 30-minute binge access (1630-1700 hours). At 1700 hours, all chow and binge jars were retrieved from the cages, final weights were recorded, and all regular chow hoppers were returned to the home cages. Chow and sweetened-fat intake during the thirty-minute binge window was calculated. The binge-like feeding protocol was repeated for 6 consecutive weeks (12 binges) in all male ($n = 8/\text{Genotype/Feeding Group}$, $n = 6$ AA RB, $n = 6$ GG RB, $n = 7$ AA R; total $n = 59$); and female mice ($n = 32/\text{Genotype/Feeding Group}$; $n = 31$ AA RB; total $n = 255$).

Vaginal Cytology

Vaginal cytology was performed on female mice twice-weekly, days 3 and 6, between 0800 and 1000 hours (~8 h before the scheduled binge), to determine the stage of estrous on binge days. Disposable, fine-tip transfer pipettes were used to collect vaginal cells via lavage with sterile saline (0.9%). Fluid samples were placed on glass slides and allowed to dry at room temperature overnight. Once dry, vaginal smears were treated using a cytology aerosol fixative (SlideRite™, Fisher Scientific). In addition, sham cytology was performed on male mice. Fine-tip transfer pipettes were used to gently poke male mice in the lower abdomen.

Fixed cytology slides were stained using toluidine blue, under a fume hood. Slides were gradually rehydrated using descending concentrations of ethanol (100%, 95%, 70%), stained for two minutes in foil-wrapped toluidine blue (0.5 mg/mL), rinsed in dH₂O, and rinsed then kept in xylenes until coverslipped. Samples were coverslipped using slide mounting medium (Permount™, Fisher Scientific) and glass coverslips.

Once dry, stained cytology slides were examined by light microscopy to determine the relative ratio of the cell types present. Proestrus was classified by the predominance of round, nucleated epithelial cells; Estrus was classified by the presence of cornified squamous epithelial cells; Metestrus was indicated by

the predominance of polymorphonuclear leukocytes (immune cells), but also some cornified epithelial cells; and diestrus is characterized by the dominance of leukocytes, with a minimal number of nucleated and/or cornified epithelial cells. All classification of estrous stage was done by blinded, trained research assistants, and repeated in duplicate.

Statistical Analysis

All statistical analyses were performed with Statistica 7.1 software (StatSoft Inc.). Significance was set at $\alpha=0.05$, and Neuman-Keuls post-hoc comparisons were made when appropriate. Total caloric intake for 30-min binge access, cumulative intakes, and body weights were analyzed using repeated measures analysis of variance (ANOVA). Two-way ANOVAs with repeated measures was used to analyze the contribution of genotype, feeding groups, and the genotype-diet interaction.

Results

I. Males:

Pre-exposure body weights and sweetened-fat intakes

One week before beginning the intermittent access feeding protocol, all mice underwent a 24-hour Pre-exposure to the binge access food (sweetened-fat, provided ad libitum), in addition to ad libitum standard chow. Two-way ANOVAs revealed no genotype or feeding group differences in animal body weights, sweetened-fat intakes, or chow intakes (data not shown).

Caloric intakes during 30-minute refeeding/binge access periods

Repeated-measures two-way ANOVA revealed overall effects of Group [$F(3, 49)=109.29$, $p=0.0000$]; Time [$F(11, 539)=7.2864$, $p=.000$]; and a Time x Group interaction [$F(33, 539)=3.6370$, $p=.000$] in male mice (Fig. 3A). Neuman-Keuls post hoc analysis showed total caloric intakes during intermittent access periods in all feeding groups were different from each other ($p < 0.001$ for all), intakes increased over time ($p < 0.05$). There was no effect of genotype on the escalation of palatable food intake observed in either group that received binge access.

Contribution of sweetened-fat and chow to caloric intakes during first vs last 30-minute binge access period

In Binge and Restrict-Binge feeding groups, an escalation of caloric intake during access periods was observed from the first to last (#12) binges, and the increase over time was due to increased intake of the highly-palatable sweetened-fat binge food (Fig. 3B). In Binge groups, individual repeated-measures ANOVAs revealed an overall effect of Time on total caloric intake [$F(1, 14)=8.0926$, $p=.013$] and sweetened fat intake [$F(1, 14)=7.8890$, $p=.014$]. In Restrict-Binge groups, there was also an effect of Time on total [$F(1, 10)=17.649$, $p=.002$] and sweetened fat [$F(1, 10)=15.918$, $p=.003$] intake (kcal).

Cumulative caloric intakes during 6-week feeding schedules

Two-way repeated-measures ANOVA on twice-weekly intakes of intermittent access periods and ad libitum chow revealed an overall effect of Group [$F(3, 51)=3.8067$, $p=.015$], Time [$F(11, 561)=2643.8$, $p=0.000$], Time x Group [$F(33, 561)=4.0460$, $p=.000$], and the Time x Genotype x Group interaction [$F(33, 561)=1.4523$, $p=.052$] approached significance (Fig. 3C). Neuman-Keuls post-hoc tests revealed that Restrict-Binge mice had lower cumulative caloric intakes than Naïve animals ($p < 0.05$), and Restrict animals followed that trend with a reduction compared to Naïve ($p < 0.06$) that approached significance. At the final time point (end of week 6), Restrict-Binge intakes were less than Naïve ($p < 0.001$) and Binge ($p < 0.01$) mice, and caloric intakes of Restrict animals was less than Naïve ($p < 0.01$) and Binge ($p < 0.05$) mice.

Final body weights

Repeated-measures two-way ANOVA of twice-weekly body weights for the 6-week feeding protocol revealed overall effects of Time [$F(11, 561)=174.91$, $p=0.000$], a Genotype x Group interaction [$F(3, 51)=3.2276$, $p=.029$], a Time x Genotype interaction [$F(11, 561)=2.1633$, $p=.015$], and an overall effect of Genotype [$F(1, 51)=3.4419$, $p=.069$] that approached significance (Fig. 3D).

Neuman-keuls post-hoc analyses showed that body weights increased over the 6 weeks of feeding protocols ($p < 0.001$), and Restrict-Binge AA body weights were lower than Restrict-Binge GG mice ($p < 0.05$). Two-way ANOVA of final body weights revealed Restrict-Binge AA body weights were lower than Restrict-Binge GG mice, a trend that approached significance ($p = 0.05$).

II. Females:

Pre-exposure body weights and sweetened-fat intakes

One week before beginning the intermittent access feeding protocol, all mice underwent a 24-hour Pre-exposure to the binge access food (sweetened-fat, provided ad libitum), in addition to ad libitum standard chow. Female body weights (mean \pm SEM) were 17.64 ± 0.12 g in AA mice and 17.83 ± 0.15 g GG mice. Sweetened-fat intakes were 14.56 ± 0.29 kcal in AA mice and 15.36 ± 0.30 kcal in GG mice, and chow intakes were 2.30 ± 0.55 kcal in AA mice and 2.09 ± 0.39 kcal in GG mice. Individual t-tests revealed no genotype differences in animal body weights, sweetened-fat intakes, or chow intakes.

Caloric intakes during 30-minute refeeding/binge access periods

Repeated-measures two-way ANOVA revealed overall Group [$F(3, 233) = 526.7$, $p = 0.000$], Time [$F(11, 2563) = 41.3$, $p = 0.000$], and Group x Time. [$F(33, 2563) = 12.5$, $p = 0.000$] effects (Fig. 4A). Neuman-Keuls post-hoc analysis showed an increase in caloric intake over time in Restrict and Restrict-Binge groups ($p < 0.0005$ for both). There were no genotype effects on caloric intakes during 30-min access periods in any group.

Contribution of sweetened-fat and chow to caloric intakes during first vs last 30-minute binge access period

Repeated-measures ANOVA revealed an overall effect of Time [$F(1,60) = 64.0$, $p = 0.000$] in Restrict-Binge groups and [$F(1,61) = 25.9$, $p = 0.000$] in Binge animals (Fig. 4B). Neuman-Keuls post-hoc shows an increase in sweetened-fat intake over the 6-week intermittent access protocol in both groups ($p < 0.005$ for both). There was no effect of genotype on the escalation of palatable food intake observed in either group that received binge access.

Cumulative caloric intakes during 6-week feeding schedules

There was an overall effect of feeding Group [$F(3, 247) = 43.9$, $p < 0.001$] on 6-week total caloric intake (Fig. 4C). Post-hoc testing revealed a lower cumulative food intake in Restrict and Restrict-Binge groups ($p < 0.005$ for both) compared to Naïve mice. There was no effect of genotype on total caloric intake over the 6-week feeding protocol.

Final body weights

Female mice body weights after 6 weeks of the feeding paradigm, 12 bouts of 30-minute intermittent access, were analyzed by repeated-measures, two-way ANOVA, revealing an overall Genotype x Group [$F(3, 247) = 4.69, p < 0.005$] effect (Fig. 4D). Neuman-Keuls post-hoc showed changes in body weights of GG Restrict mice were greater than AA Restrict mice ($p < 0.05$), and a similar but non-significant trend was seen in Restrict-Binge mice, where GG mice had a greater change in body weights than AA mice ($p = 0.06$). An overall effect of Time [$F(11, 2717) = 939.6, p = 0.000$] was also seen, whereas body weights in all groups increased over time. Final body weights were analyzed by two-way ANOVA and revealed an overall effect of Group [$F(3, 247) = 4.8, p < 0.005$], and the interaction of Group x Genotype [$F(3, 247) = 2.6, p = 0.05$] approached significance. Post-hoc testing showed GG Restrict mice had higher final body weights than GG Binge and GG Naïve mice ($p < 0.05$ for both).

Frequency of Estrous Cycle Stage on Binge Access/Refeeding Days

There were no apparent differences in cycling between genotypes nor feeding groups; mice were most frequently in metestrus or diestrus (Fig. 5).

Discussion

The *OPRM1* A112G polymorphism, in this diet-induced intermittent access murine model of binge-like feeding, did not appear to incur a predisposition to, nor protection from, binge-like feeding on highly palatable, sweet-fat food. In male and female mice in Binge and Restrict-Binge feeding groups, hyperphagia during 30-min access periods and an escalation of intake during those binge windows was observed over the 6-week protocol. Even when provided continuous *ad libitum* chow (i.e. in the absence of physiological hunger), Binge mice consumed more calories during the access periods, supporting the use of this intermittent access model to mimic the hyperphagia observed in individuals with binge eating disorder. There were no differences in binge-like feeding between mice homozygous for the gene prototype (AA) or variant allele (GG).

In male and female mice in Restrict and Restrict-Binge feeding groups, overconsumption during 30-min refeeding/binge access periods was not sufficient to compensate for twice-weekly 24-hour chow restriction. Chow-restricted groups (R and RB) had lower cumulative caloric intakes after 6 weeks of the feeding paradigm, regardless of sweetened-fat “binge” access status, compared to Naïve and Binge groups. Furthermore, Binge mice showed compensation for hyperphagia during sweet-fat binges, reducing *ad lib* chow intake during regular feeding periods resulting in cumulative caloric intakes not different from Naïve mice. Whether this underconsumption in Binge mice was

self-restriction preceding binge access or prolonged satiety following sweet-fat access, is not known. Although there were no genotype differences in binge consumption nor cumulative caloric intakes, in male and female chow-restricted mice, there were genotypic differences in final body weights. For male Restrict-Binge mice, GG weighed more than AA mice after 6 weeks of the feeding protocol. Similarly, for female Restrict mice, GG weighed more than AA mice. This suggests reduced energy expenditure in GG vs AA mice, under conditions of caloric restriction.

Although population data suggests greater allelic frequency of the *A118G* variant in obese individuals with binge eating vs. without binge eating, these studies do not support the hypothesis that the *OPRM1 A118G* results in an increased propensity for binge-like eating behavior. However, higher final bodyweights in GG (vs. AA) mice under conditions of intermittent chow restriction, suggests a gene-diet interaction that may result in attenuated physical activity, or other metabolic perturbation. Whether this finding has clinical implications for individuals homozygous for the *A118G* SNP should be explored further, as there are currently various iterations of intermittent fasting that are popular diet trends, despite sufficient supporting research.

Figures

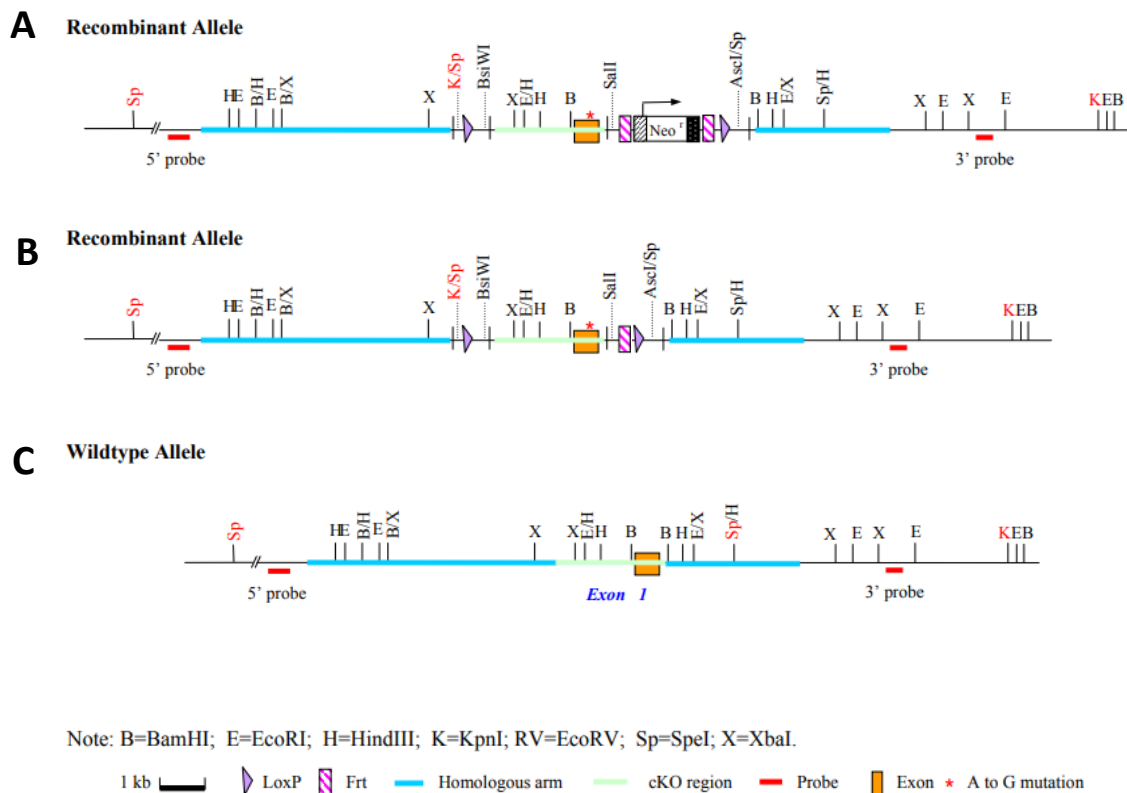


Figure 1: Schematic of murine *OPRM1* A112G transgene construct (A); Excision of Neomycin cassette following breeding with male ACTBFLPe mice via FLP-FRT homologous recombination (B); Prototype allele (C). Prepared for Dr. Lei Yu by Caliper Discovery Alliances and Services; image adapted from progress report. * indicates A to G mutation in exon 1 of the *OPRM1* gene.

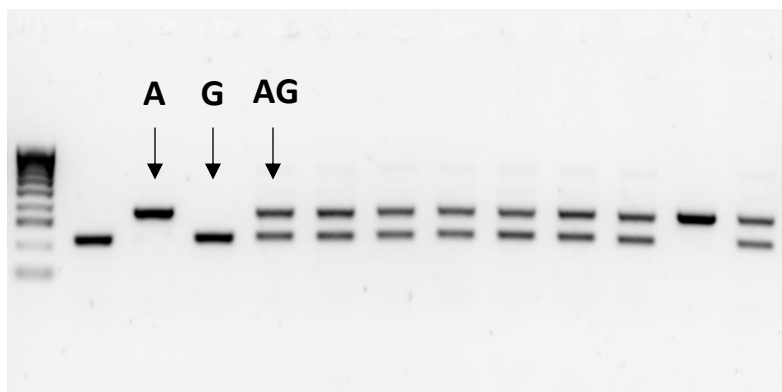


Figure 2: Representative genotyping gel image (2% agarose gel; 100bp ladder); OMPR1 A112G prototype allele (A) amplicon is ~260 bp, variant allele (G) amplicon is ~400 bp, heterozygous samples show as double bands.

Table 1: Feeding groups for the dietary-induced binge eating protocol. Adapted from Sachdeo BLY et al, *Front Psychol*, 2019.

Groups	Calorie restriction (days 2 and 5)	Sweetened fat access (days 3 and 6)
Restrict binge	Intermittent (24 h, twice a week)	Intermittent (30 min, twice a week)
Binge	None	Intermittent (30 min, twice a week)
Restrict	Intermittent (24 h, twice a week)	None
Naïve	None	None

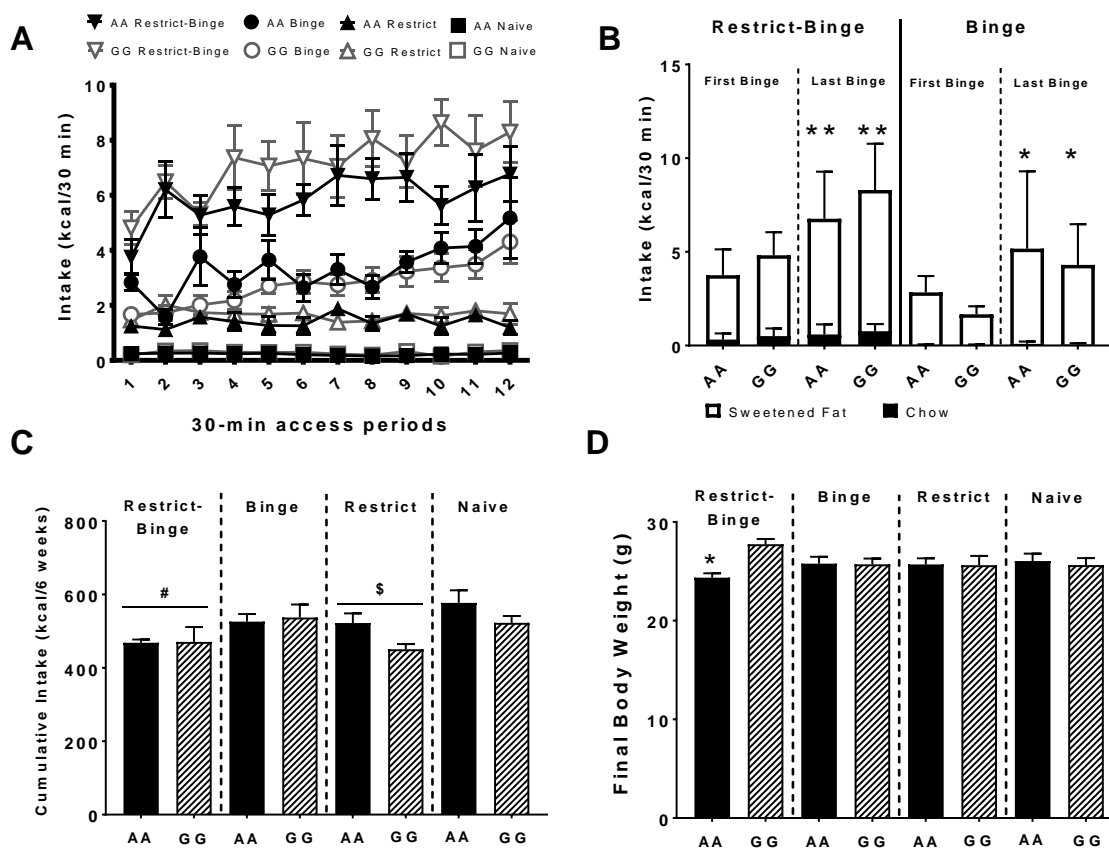


Figure 3: Dietary-induced binge eating protocol over 6 weeks, in male mice homozygous for the major (AA) and minor (GG) allele of *OPRM1*. **(A)** Caloric intakes during twice-weekly (days 3 and 6) 30-min access periods. **(B)** Comparison of 30-min caloric intakes during first vs last binge, and contribution from chow (black bars) vs sweetened-fat (white bars); ** indicates $p < 0.005$ and * indicates $p < 0.05$ compared to first binge. **(C)** Total cumulative caloric intake (ad lib chow plus binge access) during 6-week feeding protocol; # indicates $p < 0.001$ from Naïve, and $p < 0.01$ from Binge; \$ indicates $p < 0.01$ from Naïve, and $p < 0.05$ from binge. **(D)** Final body weights following the 6-week feeding protocol; * indicates $p = 0.05$ from GG Restrict-Binge.

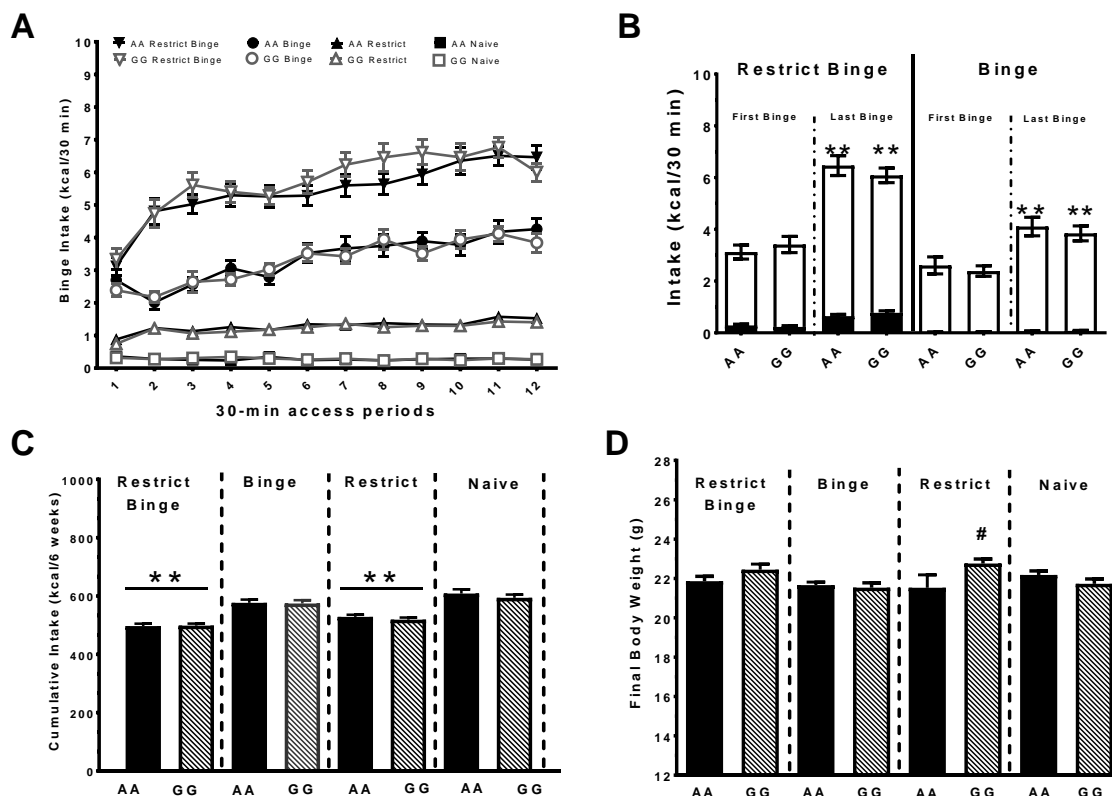


Figure 4: Dietary-induced binge eating protocol over 6 weeks, in female mice homozygous for the major (AA) and minor (GG) allele of *OPRM1*. **(A)** Caloric intakes during twice-weekly (days 3 and 6) 30-min access periods. **(B)** Comparison of 30-min caloric intakes during first vs last binge, and contribution from chow (black bars) vs sweetened-fat (white bars); ** indicates $p < 0.005$ compared to first binge. **(C)** Total cumulative caloric intake (ad lib chow plus binge access) during 6-week feeding protocol; ** indicates $p < 0.005$ from Naïve group. **(D)** Final body weights following the 6-week feeding protocol; # indicates $p < 0.05$ from GG Binge and GG Naïve groups.

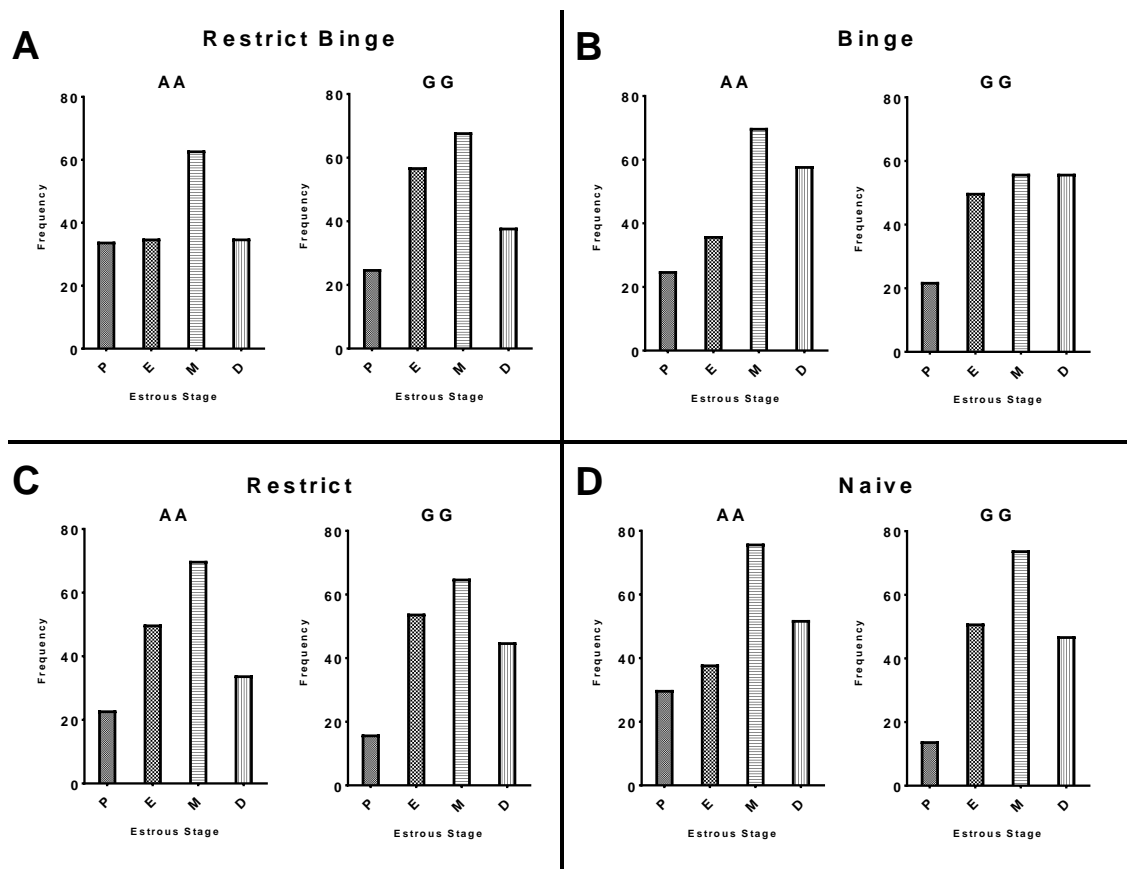


Figure 5: Estrous cycle frequency during the dietary-induced binge-like feeding protocol. Vaginal cytology was used to determine stage of estrous cycle. Data are from binge access days (days 3 and 6) expressed as the number of occurrences during the 6-week protocol, in Restrict-Binge (**A**), Binge (**B**), Restrict (**C**), Naïve (**D**) feeding groups. P, Proestrus; E, Estrus; M, Metestrus; D, Diestrus.

Chapter 3

Effects of Acute and Chronic Dosing of Lisdexamfetamine, and Sibutramine control, on Binge Behavior in *A112G* Female Mice

Introduction

The most common eating disorder, binge eating disorder (BED), with a lifetime prevalence of 2.8%, and a disproportionate number of women [1]. As classified in the DSM-V, BED is defined by the presence of binge episodes, or the consumption of an extremely large amount of food in an extremely short amount of time, and a marked experience of a loss of control over one's consumption. The neuropathology of BED remains not well understood, as such, therapeutic strategies are limited, and relapse rates remain high. There is currently one FDA-approved pharmacotherapy for the treatment of moderate to severe BED, Lisdexamfetamine dimesylate (LDX), commercially available as VYVANSE®, produced by Shire pharmaceuticals. LDX is an amphetamine pro-drug, with a covalently-bound lysine residue that requires cleavage in circulation, providing a “time release” effect. LDX first developed and FDA-approved for the treatment of attention deficit hyperactive disorder (ADHD), but was later discovered to be effective in reducing binge episodes.

Opioids, and mu-opioid receptor (MOR) signaling in specific, play a salient role in the hedonic regulation of food intake. The most common functional SNP of the MOR gene, *OPRM1* A118G, has an allele frequency of ~11% in Caucasian populations, but as high as ~52% in some Asian populations [3-5]. The *OPRM1* A118G SNP results in an amino acid substitution (N40D) in the extracellular N-terminal region of the receptor, and results in alterations of nociception and analgesia, in addition to posited interactions with substance abuse and addiction. Furthermore, population data suggests the G allele is enriched in obese individuals with BED vs those who are obese but not binge eaters.

The *OPRM1* A118G SNP is common in the global population and perhaps further enriched in BED populations, and may affect not only reward-processing involved in palatable food intake, but also drug efficacy. Therefore, the potential gene-diet-drug interactions may modulate pharmacotherapy efficacy in individuals with BED and the A118G variant allele. Transgenic knock-in mouse models of the murine homologous SNP, *OPRM1* A112G, have yet to be used to explore this pharmacogenetic interplay. In these studies, an established diet-induced intermittent access model of binge-like feeding was used to investigate whether *OPRM1* A118G allele status affects LDX efficacy to reduce binge-like feeding in female mice, under acute and chronic dosing conditions.

Materials and Methods

Animals

The animal care protocol was approved by the Institutional Animal Care and Use Committee of Rutgers University (OLAW #A3262-01).

OPRM1 A112G Mice

The common non-synonymous SNP of the human mu-opioid receptor gene (*OPRM1 A118G*) involves the substitution of adenine (A) to guanine (G) as position 118 within exon 1. This single nucleotide substitution results in a functional amino acid substitution of asparagine (N) to aspartate (D) at position 40, eliminating a putative N-glycosylation site within the extracellular region of the mu-opioid receptor (MOR). The mouse homolog of this SNP, *OPRM1 A112G*, involves the same A to G substitution, at position 112 in exon 1, resulting in the same N to D substitution, at amino acid 38. The homologous mouse SNP eliminates the N-glycosylation site in the extracellular region, as in humans.

Transgenic *OPRM1 A112G* conditional knock-in mice were generated on a C57BL6 background by Lei Yu (Department of Genetics, Rutgers University, New Brunswick, NJ) with Caliper Discovery Alliances & Services (Caliper Life Sciences Inc., Hanover, MD). As described in the project report provided by Caliper, the conditional knock-in was created using homologous recombination in mouse embryonic stem (ES) cells, followed by blastocyst injection with the

targeted ES cells. The mouse chromosome 10 sequence (n.t.# 3,510,000,000~3,590,000; Ensemble database) was used as a reference, and the RP23-263A7 bacterial artificial chromosome (BAC) clone was used as template to generate the homology arms, conditional knock-out (KO) region, and probes for southern blot screening tests. The 5' homology arm (5.3 kb), 3' homology arm (3.0 kb), and conditional KO region (2.2 kb) were generated using Taq DNA polymerase, and fragments cloned in the bacterial plasmid, pCR4.0™ (Invitrogen, Thermo Fisher Scientific, Waltham, MA), were confirmed by restriction digestion and end-sequencing. The A to G mutation at position 112 in exon 1 was introduced into the conditional KO region by PCR-based site-directed mutagenesis using the QuickChange II™ kit (Stratagene, Agilent Technologies, Santa Clara, CA). In addition to the homology arms, the final cloned vector contained LoxP sequences flanking the conditional KO region, Frt sequences flanking the neomycin (Neo) expression cassette (positive selection of ES cells), and diphtheria toxin A (DTA) cassette (negative selection of ES cells). The final vector (Fig. 1) was confirmed by restriction digestion and end-sequencing, and the restriction enzyme, NotI, was used for linearization of the plasmid before electroporation into ES cells. ES clones were expanded, and the A to G mutation was confirmed. Blastocyst (C57BL/6 Tyr) injection was performed on confirmed clones, and heterozygous mice were obtained from male chimera breeding to C57BL/6 wildtype females.

Mice were originally maintained by Taconic Laboratories (Rensselaer, NY). Two male and two female heterozygous *OPRM1 A112G* transgenic mice were purchased from Taconic Laboratories, and paired as two sets of breeders to establish a colony at the Rutgers University Cook Campus vivarium located in Bartlett Hall (New Brunswick, NJ).

Breeding

OPRM1 A112G heterozygous mice were bred for three generations. In order to excise the Frt-flanked Neo cassette, four male ACTBFLPe mice were purchased from The Jackson Laboratory (cat #005703, Bar Harbor, ME) and bred with heterozygous *OPRM1 A112G* females. Heterozygous offspring from several generations (>3) were then used as founder mice for the *OPRM1 A112G* colony.

Twelve-week-old (PND84) male and female transgenic mice heterozygous for the SNP (AG) were bred, one pair per cage, and provided ad libitum water and standard chow (LabDiet® Mouse Diet 5015, 19.75% protein, 26.1% fat, 54.15 CHO, 3.8Kcal/g), and a plastic shelter. Cages were checked daily, and new litters were recorded. Pups were ear-notched for identification between PND 14 and 21, and the tissue was used for genotyping (described in detail in subsequent sections). Pups were weaned at PND 21 and separated by sex. All pups were group housed with ad libitum water and standard chow (LabDiet® Laboratory Rodent Diet 5001, 3.36 Kcal/g; 28.67% protein, 13.38% fat, 57.94% CHO), and provided a plastic shelter and wooden block. Heterozygous (AG) mice were

paired as new breeders at maturity, PND 84, one male and one female per cage. Female mice homozygous for the prototype (AA) or variant (GG) alleles were single-housed and transported to a garage at PND 38.

Housing

Six-week-old (PND42) female transgenic mice (*OPRM1 A112G*) homozygous for the prototype or variant alleles (AA or GG, respectively) were single-housed in standard mouse 'shoebox' cages (7.5" x 11.75" x 5"). Each cage bottom was lined with paper-enriched corn cob bedding, and cages were given a plastic shelter, a paper Nestlets®, and a wooden block to promote gnawing behaviors. Rooms were maintained on a 12/12h light/dark cycle (automatic lights off at 1900 h), and garages were temperature and humidity controlled (18-25°C and 30-70%, respectively), with twice-daily documentation by vivarium staff. Mice were provided ad libitum water through the duration of the protocol, and clean water bottles were provided by vivarium staff weekly. Cage bottoms were changed by Bello lab trainees once per week.

Diet

All mice were maintained on a standard chow (LabDiet® Laboratory Rodent Diet 5001, 3.36 Kcal/g; 28.67% protein, 13.38% fat, 57.94% CHO), provided ad libitum in custom, stainless steel chow hoppers, except when otherwise noted during Binge protocol, and described in detail in subsequent sections. Mice in restrict groups have biweekly, 24-hr periods of food deprivation, and mice in

binge groups receive intermittent access to a “sweetened fat” binge food, consisting of commercially-available, food grade vegetable shortening and sucrose (plus 10% sucrose; 8.6 Kcal/g).

Genotyping

As described in previous sections, between PND 14 and 21, mice were ear-notched for identification, and tissue punches were collected for genotype analysis by standard polymerase reaction (PCR) of genomic DNA (gDNA).

The ReliaPrep™ gDNA Tissue Miniprep System (Promega, Madison, WI) was used to digest tissue samples, and isolate gDNA, using the standardized supplier protocol.

For PCR, target gDNA (3 ul/sample) was amplified using GoTaq® Green Master Mix, 2X (10 uL/sample) and nuclease free water (7 uL/sample) (Promega, Madison, WI), and forward and reverse primers: 5'-GCACACAAAAGAGCAATAGAACGGAAATA-3' (0.5 uL/sample) and 5'-GATCCCCTCAGAAGAACTCGT-3' (0.5 uL/sample). Reaction mixtures (21 uL total/sample) were run on a thermocycler (2 ABI GENEAMP 2700, Applied Biosystems, Thermo Fisher Scientific) with the following reaction conditions: Hold (95C/F? for 2 min) for one cycle; annealing (95C for 30 s, 55C for 30 s, 72C for 1.5 m) for 35 cycles; holds (72C for 5 m, 4C until run is complete).

For characterization of genotypes by electrophoresis, PCR products and a 100bp ladder were run on a 2% agarose gel (130V constant, 40 min), and bands were visualized using Gel Doc™ EZ System (Bio-Rad Laboratories, Inc, Hercules, CA). The prototype allele (A) amplicon is ~0.26 kb, and the variant allele (G) is ~0.4 kb (Fig. 2).

Body Weight, Chow Intake

Mouse body weight and chow weight were measured twice weekly, days 2 and 5, between 0900 and 1600 hours. To measure body weight, a plastic cup was weighed and tared (CQT 202 portable compact balance, Adam Equipment, Oxford, CT), and mice were transferred to the cup. Body weights (g; to the hundredth place) were recorded. Chow weights were measured (Scout® portable topload balance, CA# 10805-278, OHAUS®, Parsippany, NJ) as hopper weight plus chow (g; to the thousandth place), and intakes were calculated from recorded values.

Dietary-induced Binge Eating

The diet-induced binge eating protocol used for these experiments involves intermittent access to a highly-palatable “sweetened-fat” (vegetable shortening plus 10% sucrose; 8.6 Kcal/g) that mimics the macronutrient composition of common binge foods in humans. At 6-weeks of age (PND 42), one week before starting the binge access protocol, all mice underwent a 24-hr pre-exposure to the sweetened-fat binge food, to minimize novelty-induced hypophagia and to

control for innate food preferences when assigning experimental groups. Mice were then randomly assigned to one of four feeding conditions (Table 1), controlling for pre-exposure body weight and sweetened-fat intake: *Naïve* (N), *Restrict* (R; 24-hr chow restriction), *Binge* (B; 30-min access to sweetened-fat), or *Restrict-Binge* (RB; 24-hr chow restriction, followed by 30-min access to sweetened-fat).

At 1630 hours, on Days 2 and 5, restrict (R/RB) mice had chow hoppers removed from the home cage, and bedding was searched for loose chow pellets which were removed. On days 3 and 6, the sweetened fat was prepared directly preceding the binge period. Chow pellets and sweetened-fat were portioned into labeled, small, glass jars, and weighed (as the glass jar plus food). At 1630 hours, chow and binge food (B/RB mice) were given to mice in their home cage, the glass jars positioned away from the water bottle spout. All mice were allowed to eat freely during the 30-minute binge access (1630-1700 hours). At 1700 hours, all chow and binge jars were retrieved from the cages, final weights were recorded, and all regular chow hoppers were returned to the home cages. Chow and sweetened-fat intake during the thirty-minute binge window was calculated. The binge-like feeding protocol was repeated for 6 consecutive weeks (12 binges) in all female mice (n = 32/Genotype/Feeding group; n = 31 AA RB; total n = 255).

After 6 weeks of the respective feeding protocols, mice were assigned to either an *acute dosing* (within group design; n = 8/Genotype/Feeding Group; n = 7 AA RB total n = 63) or *chronic dosing* (between group design; n = 8/Genotype/Feeding Group/Treatment Group; total n = 192) schedule.

Acute Dosing

For the acute dosing experiment, female mice continued the binge feeding protocol for an additional 7 weeks (13 weeks, 26 binges). For weeks 7-13, mice were orally dosed with vehicle and 3 concentrations of two drugs, lisdexamfetamine dimesylate (0.15, 0.5, and 1.5 mg/kg) and sibutramine hydrochloride monohydrate (0.3, 1.0, and 3.0 mg/kg), once-weekly on schedule Day 6, 1 hour before the binge/refeeding period.

Chronic Dosing

For the chronic dosing experiment, in a separate cohort of female mice and following 6 weeks of the binge feeding schedule, mice underwent daily oral dosing with either vehicle, lisdexamfetamine (1.5 mg/kg), or sibutramine (3.0 mg/kg) for two additional weeks (8 weeks total of binge feeding protocol).

Drug Preparation and Dosing Schedule

Acute Dosing

After 6 weeks of the binge feeding protocol (12 binges), mice (n=64) underwent once-weekly acute dosing, in a within-subjects crossover design, for 7 additional weeks (14 additional binges). Mice were orally gavaged using single-use, sterile plastic feeding tubes (20 ga x 30 mm; cat # FTP-20-30, Instech Laboratories, Plymouth Meeting, PA) at 1530 h on day 6 of feeding schedule (1 h prior to scheduled binge/refeeding period). Each mouse was orally dosed with vehicle (ultrapurified deionized water), 0.15, 0.5, and 1.5 mg/kg lisdexamfetamine dimesylate (VYVANSE®, lot # AF7299B; Shire Pharmaceuticals, Lexington, MA), and 0.3, 1.0, and 3.0 mg/kg of sibutramine hydrochloride monohydrate (cat # S9944; Sigma-Aldrich, St. Louis, MO). Each mouse received all doses once, in ascending concentrations of each pharmacological treatment, and the drug treatment order was switched for half in order to control for potential order effects.

Chronic Dosing

After 6 weeks of the binge feeding protocol (12 binges), mice (n=192) underwent 14 days of daily dosing, in a between-subjects design, with two additional weeks of their respective feeding protocol (8 weeks total, 16 binge/refeeding periods total). Mice were orally gavaged using single-use, sterile plastic feeding tubes (20 ga x 30 mm; cat # FTP-20-30, Instech Laboratories, Plymouth Meeting, PA) daily between 0900 and 1100 h. Mice were orally dosed with either vehicle (ultrapurified deionized water), 1.5 mg/kg lisdexamfetamine dimesylate (VYVANSE®, lot # AF7299B; Shire Pharmaceuticals, Lexington, MA), or 3.0

mg/kg of sibutramine hydrochloride monohydrate (cat # S9944; Sigma-Aldrich, St. Louis, MO). Following the 16-week binge protocol, including 2 weeks of daily oral dosing, mice were sacrificed and tissues collected (detailed in subsequent sections).

Vaginal Cytology

Vaginal cytology was performed on female mice twice-weekly, days 3 and 6, between 0800 and 1000 hours (~8 h before the scheduled binge), to determine the stage of estrous on binge days. Disposable, fine-tip transfer pipettes were used to collect vaginal cells via lavage with sterile saline (0.9%). Fluid samples were placed on glass slides and allowed to dry at room temperature overnight. Once dry, vaginal smears were treated using a cytology aerosol fixative (SlideRite™, Fisher Scientific).

Fixed cytology slides were stained using toluidine blue, under a fume hood. Slides were gradually rehydrated using descending concentrations of ethanol (100%, 95%, 70%), stained for two minutes in foil-wrapped toluidine blue (0.5 mg/mL), rinsed in dH₂O, and rinsed then kept in xylenes until coverslipped. Samples were coverslipped using slide mounting medium (Permount™, Fisher Scientific) and glass coverslips.

Once dry, stained cytology slides were examined by light microscopy to determine the relative ratio of the cell types present. Proestrus was classified by

the predominance of round, nucleated epithelial cells; Estrus was classified by the presence of cornified squamous epithelial cells; Metestrus was indicated by the predominance of polymorphonuclear leukocytes (immune cells), but also some cornified epithelial cells; and diestrus is characterized by the dominance of leukocytes, with a minimal number of nucleated and/or cornified epithelial cells. All classification of estrous stage was done by blinded, trained research assistants, and repeated in duplicate.

Sacrifice and Tissue Collection

Following the final binge/refeeding session of the chronic dosing experiments (binge # 16), mice were sacrificed and tissues collected for analysis. Between 0700 and 0900 h, 5 h prior to the sac, mice were food restricted, body weight was measured, vaginal cytology was performed, and mice were switched to clean, empty cages with wire bottoms. Following the 5-h restriction (1200-1400 h), mice were sacrificed; blood, feces, and brain were collected; and blood glucose was measured. Mice were decapitated and a glucometer was used to measure blood glucose (AlphaTrak, Abbott Laboratories, Inc, Alameda, CA; AlphaTrak2 test strips, Zoetis, Inc, Kalamazoo, MI). Trunk blood was collected into EDTA tubes, protease inhibitor (1 uL/100 uL blood; AEBSF, item # 61132709, Worldwide Medical Products, Inc., Bristol, PA) was immediately added, and the tube was gently inverted and then placed on ice. Blood was centrifuged for 10 min (3.0 rcf, 4C), and plasma was pipetted into nuclease-free microtubes and stored in a -80C freezer for future hormone analysis. Brains were

then dissected out and transferred to a beaker of chilled Sorenson's phosphate (0.1 M). A stainless steel mouse brain matrix and double-edged razor blades (cat # 121-6, Ted Pella, Inc) were then used to slice the brain tissue into 1mm coronal slices containing the hypothalamus and ventral striatum, and slices were transferred to a nuclease-free 6-well plate containing chilled 50% RNA Later (ddH₂O). The 6-well plate containing tissue samples was stored overnight in a 4°C refrigerator until microdissection the following day. Using forceps, fresh fecal pellets were collected from cage bottoms and placed in nuclease-free microtubes. Microtubes were then stored in a -80°C freezer for future microbial composition analysis.

The following day, microdissection of coronal brain slices was done to isolate the arcuate nucleus (ARC) and paraventricular nucleus (PVN) of the hypothalamus, and the nucleus accumbens (NA) within the ventral striatum of the basal ganglia. Microdissected nuclei were transferred to nuclease-free microtubes and placed on dry ice until stored in a -80°C freezer for future gene expression analysis by real-time PCR.

Statistical Analysis

All statistical analyses were performed with Statistica 7.1 software (StatSoft Inc.). Significance was set at $\alpha=0.05$, and Neuman-Keuls post-hoc comparisons were made when appropriate. Individual ANOVAs with repeated measures were used to analyze the effect of genotype on drug treatment for each feeding group.

Results

I. Acute Dosing

Caloric intakes during 30-minute binge access/refeeding periods following acute dosing

Following 6 weeks of the intermittent access binge-like feeding paradigm, female mice were dosed once weekly with one of seven drug treatments (Vehicle (water, Veh); Lisdexamfetamine-low (Lis L), -medium (Lis M), -high (Lis H); or sibutramine-low (Sib L), -medium (Sib M), or -high (Sib H)) for 7 additional weeks of the protocol. Oral doses were administered 1 hr preceding the 30-min refeeding/access periods, and food intake was quantified. Repeated-measures two-way ANOVA revealed overall effects of Group [$F(3, 54) = 105.1, p = 0.000$], with post-hoc testing showing all groups were different from each other ($p = 0.000$); and Treatment [$F(6, 324) = 5.8, p = 0.000$], with post-hoc testing showing an overall reduction in intake following high-dose sibutramine (3.0 mg/kg, Sib H) compared to vehicle ($p < 0.001$) & all doses of lisdexamfetamine ($p < 0.01$ for all) (Fig.1). Individual repeated-measures ANOVAs for each feeding group revealed an effect of Treatment [$F(6, 84) = 3.16, p < 0.01$] in the Binge group, with intakes following med- & high-dose sibutramine (1.0 mg/kg, Sib M; 3.0 mg/kg, Sib H) were lower than the lisdexamfetamine low-dose (0.15 mg/kg, Lis L; $p < 0.05$ for both); and an effect of Treatment [$F(6, 84) = 2.8; p < 0.01$] in Restrict mice, with lower intakes following the sibutramine high-dose (3.0 mg/kg; Sib H) than the

lisdexamfetamine low-dose (0.15 mg/kg; Lis L; $p < 0.05$). There were no significant differences in Naïve or Restrict-Binge groups with respect to caloric intake during access periods following acute dosing, and there were no genotype differences in treatment effect within feeding groups.

II. Chronic Dosing

Caloric intakes during 30-minute binge access/refeeding periods during two weeks of chronic dosing

In a second set of female mice ($n = 8/\text{Genotype/Feeding Group/Treatment Group}$; total $n = 128$), following 6 weeks of the intermittent access binge-like feeding paradigm, mice underwent two weeks of daily oral dosing with one of three drug treatments (Vehicle (water, Veh); sibutramine (3.0 mg/kg, Sib); or lisdexamfetamine (1.5 mg/kg, Lis)) while continuing the feeding protocol.

Repeated-measures multivariate ANOVA revealed an overall effect of Feeding Group [$F(3, 165) = 300.5$, $p = 0.000$], and post-hoc testing showed all feeding groups were different from each other (Fig. 2). Within each feeding group, individual two-way ANOVAs with repeated-measured did not reveal any differences in treatment effects, nor effect of genotype.

Cumulative food intake during 2-week chronic dosing period

Repeated-measures multivariate ANOVA revealed an overall effect of Feeding Group [$F(3, 168) = 8.3$, $p < 0.001$] on cumulative intake (intermittent access

periods plus *ad libitum* chow) during two weeks of daily dosing, with Neuman-Keuls post-hoc showing lower cumulative intake in Restrict-Binge & Restrict feeding groups ($p < 0.005$ for both) compared with Naïve mice (Fig. 3). Within each feeding group, individual two-way ANOVAs did not reveal differences in treatment effect, nor between genotypes.

Body weights during 2-week chronic dosing

Repeated-measures multivariate ANOVA revealed overall effects of Feeding Group [$F(3, 168) = 2.75, p < 0.05$] with Neuman-Keuls post-hoc showing Binge mice weighed less than Restrict mice ($p < 0.05$); an interaction of Genotype x Feeding Group [$F(3, 168) = 3.7, p < 0.05$], with post-hoc revealing a higher body weight in GG vs AA Restrict mice ($p < 0.05$) (Fig. 4C); Time [$F(3, 504) = 75.0, p < 0.005$], with all body weights increasing over time; Time x Treatment [$F(6, 504) = 4.2, p < 0.005$], and a Time x Genotype interaction [$F(3, 504) = 3.1, p < 0.05$], with post-hoc revealing an increase in body weights in both genotypes over the two-week chronic dosing period ($p < 0.005$) (Fig. 4).

Data and Tissues Collected at Sacrifice Following 8-week Feeding & Chronic Dosing Schedule

At sacrifice, blood glucose was measured, plasma was collected for analysis of circulating hormone levels, brain nuclei (Arc, PVN, NA) were microdissected for analysis of relative gene expression, and fecal samples were collected for

analysis of gut microbiota composition. Due to negative results pertaining to hypothesized genotype differences, samples were stored and not analyzed.

Discussion

In these acute and chronic dosing experiments in female mice, *OPRM1 A112G* allele status did not affect caloric intakes during 30-min access periods, nor cumulative caloric intakes during the respective dosing schedules. Following 6 weeks of the respective feeding paradigms, and subsequent oral administration of vehicle and ascending doses of lisdexamfetamine and sibutramine, caloric intakes during 30-min access periods remained different between all feeding groups, and the high dose of sibutramine reduced intakes compared to vehicle and all doses of lisdexamfetamine. Within Binge feeding groups, medium and high doses of sibutramine resulted in a reduction in 30-min binge intakes compared to the low dose of lisdexamfetamine. Similarly, in Restrict feeding groups, the high dose of sibutramine attenuated 30-min caloric intakes compared to low dose lisdexamfetamine.

Furthermore, following 6 weeks of feeding paradigms, during the 2-week daily dosing schedule and continued respective feeding protocols, caloric intakes during 30-min access periods was different between all feeding groups, but there no differences in treatment efficacy within feeding groups, nor between genotypes. Similar to cumulative caloric intakes during the first 6 weeks of the feeding protocols (Chapter 2), cumulative intakes during the 2-week chronic dosing period were lower in Restrict and Restrict-Binge groups, compared to Naïve groups. Regardless of sweet-fat binge food access status or

overconsumption during 30-min access periods, time-restricted feeding groups (R and RB) could not compensate for twice-weekly 24-hour chow restriction preceding access periods. There were no differences in treatment efficacy on cumulative intakes within feeding groups, nor between genotypes.

Despite a lack of differences between *OPRM1* prototype (AA) vs variant (GG) allele on caloric intakes during 30-min access periods and total cumulative intakes, there was a small but significant effect of genotype on body weights during the 2-week chronic dosing schedule. Both genotypes had body weights that increased over time, and Binge mice weighed less than Restrict mice. However, similar to body weight differences observed during the first six weeks of the feeding paradigm, GG Restrict mice had higher body weights than AA Restrict mice, despite 30-min refeeding intakes and cumulative intakes that were not different. Again, this suggests the *A112G* SNP may predispose to protection of a higher body weight, although the underlying mechanisms has not yet been explored.

Together, these studies suggest that there is not a functional association between the common *OPRM1 A112G* polymorphism and an altered propensity for binge eating nor efficacy of pharmacotherapy for its treatment.

Figures

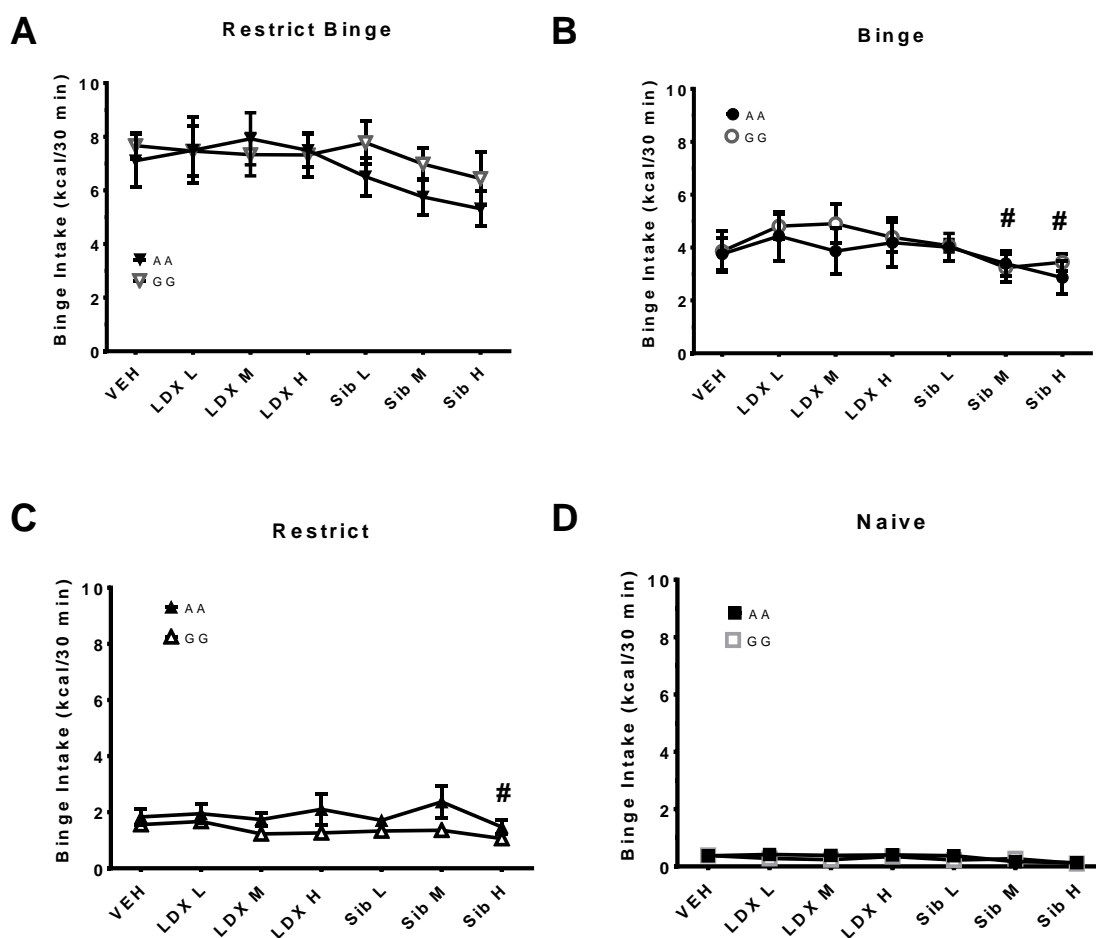


Figure 1: Total caloric intakes (kcal) during 30-min access periods following 6-week binge-like feeding protocol and during once-weekly (day 6) acute dosing, in female mice homozygous for the *OPRM1* A112G prototype (AA) or variant (GG) allele. Data are expressed as mean \pm SEM. Feeding groups were Restrict-Binge (A), Binge (B), Restrict (C), and Naïve (D). Each mouse was orally dosed with vehicle (VEH; water), lisdexafetamine low (LDX L; 0.15 mg/kg), medium (LDX M; 0.5 mg/kg), and high (LDX H; 1.5 mg/kg), and sibutramine low (Sib L; 0.3 mg/kg), medium (Sib M; 1.0 mg/kg), and high (Sib H; 3.0 mg/kg), with 1-week washouts, during 7 subsequent weeks of the respective feeding protocol. # indicates $p < 0.05$ from LDX L dose.

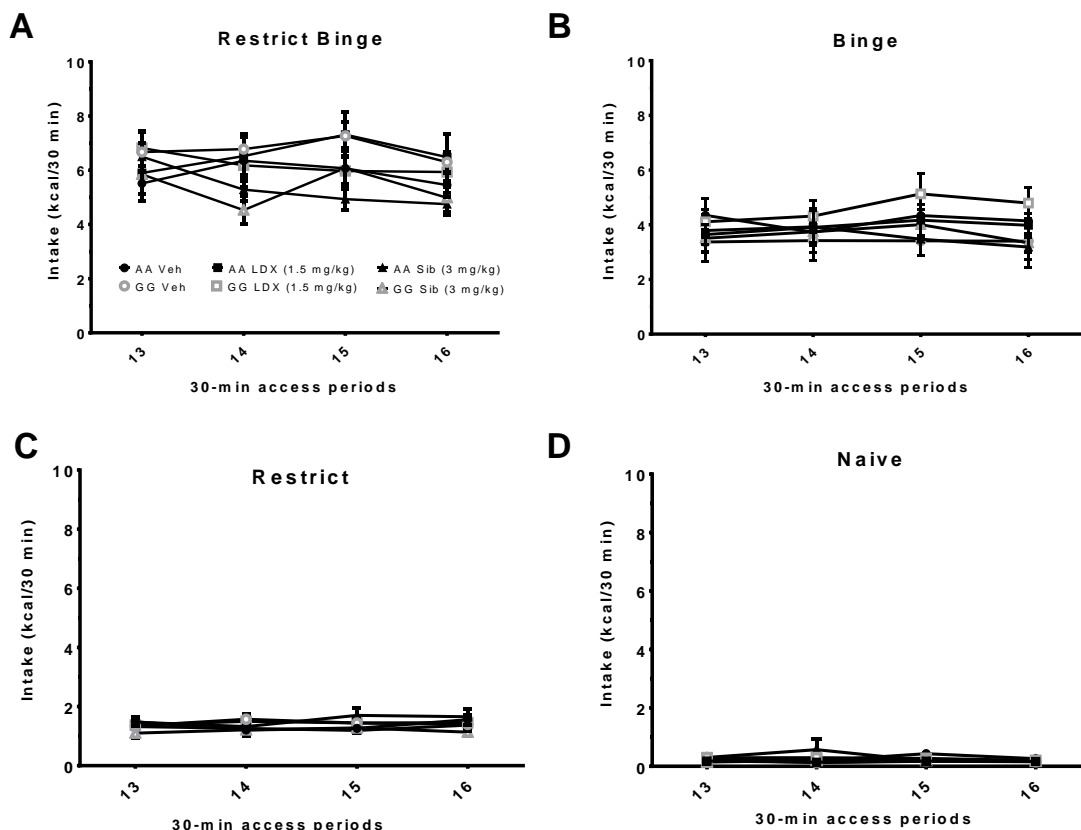


Figure 2: Total caloric intakes (kcal) during 30-min access periods following 6-week binge-like feeding protocol during daily chronic (14-day) dosing, in female mice homozygous for the *OPRM1* A112G prototype (AA) or variant (GG) allele. Data are expressed as mean \pm SEM. Feeding groups were Restrict-Binge (**A**), Binge (**B**), Restrict (**C**), and Naïve (**D**). Mice ($n=8$ /genotype/feeding group/treatment) were orally dosed with either vehicle (Veh), lisdexamphetamine (LDX; 1.5 mg/kg), or sibutramine (Sib; 3.0 mg/kg), daily, during two subsequent weeks of the respective feeding protocol.

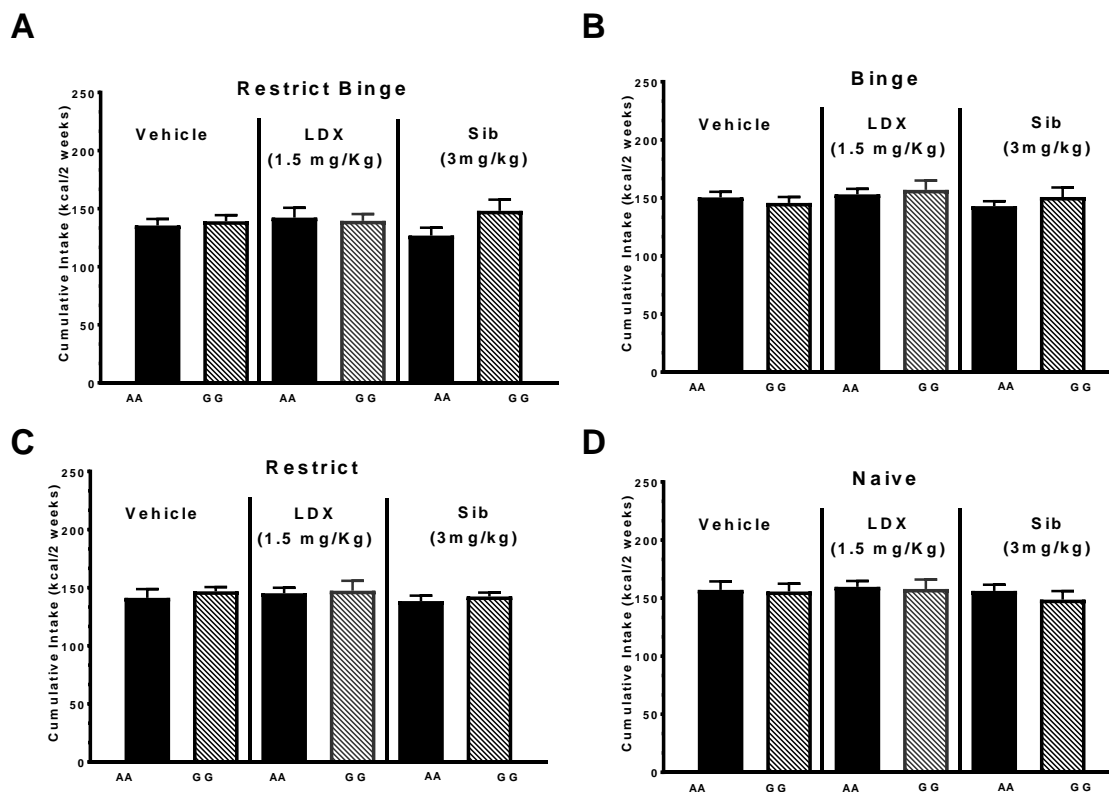


Figure 3: Cumulative food intakes (kcal) during daily chronic (14-day) dosing. Data are expressed as mean \pm SEM. Feeding groups were Restrict-Binge (A), Binge (B), Restrict (C), and Naïve (D). Mice (n=8/genotype/feeding group/treatment) were orally dosed with either vehicle (Veh), lisdexamfetamine (LDX; 1.5 mg/kg), or sibutramine (Sib; 3.0 mg/kg), daily, during two subsequent weeks of the respective feeding protocol.

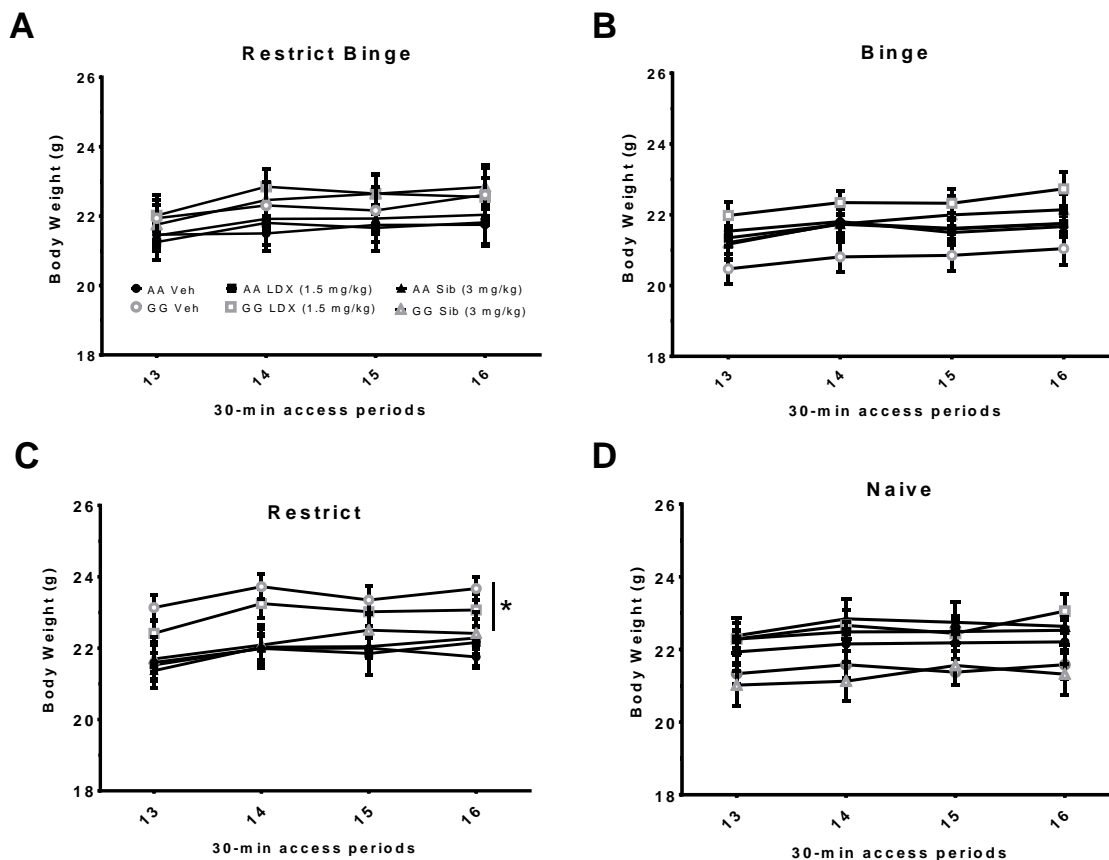


Figure 4: Body weights during daily chronic (14-day) dosing. Data are expressed as mean \pm SEM. Feeding groups were Restrict-Binge (**A**), Binge (**B**), Restrict (**C**), and Naïve (**D**). Mice ($n=8$ /genotype/feeding group/treatment) were orally dosed with either vehicle (Veh), lisdexamfetamine (LDX; 1.5 mg/kg), or sibutramine (Sib; 3.0 mg/kg), daily, during two subsequent weeks of the respective feeding protocol, and body weights were measured on protocol days 2 and 5. *Indicates $p < 0.05$ higher body weight in GG compared to AA mice.

Chapter 4

***OPRM1* A112G SNP Phenotyping: Lipid Preference, Taste Responsivity, & Meal Patterns**

Introduction

Endogenous opiates, and mu-opioid receptor (MOR) signaling, in particular, has a salient role in the hedonic regulation of food intake. In addition to MOR expression in the ventral tegmental area (VTA) involved in mesolimbic dopamine reward pathways, MORs are highly distributed throughout the central nervous system and in the periphery. The gastrointestinal tract, in specific, is enriched with MORs, and thus they play an interesting role in sensing and signaling the rewarding characteristics of highly palatable, sweet and fat foods.

A common functional single nucleotide polymorphism (SNP) of the gene that codes for human MOR (*OPRM1* A118G), has been shown to have an effect on nociception and analgesia, and a posited interaction with vulnerability to substance abuse and addiction. In addition, there is population data supporting enrichment of the GG allele in individuals with obesity and binge eating disorder (BED), compared to obese individuals without BED [6]. Binge foods are typically highly-palatable, sweet-fat items [112], and follow-up studies in healthy adults revealed GG status was associated with higher reported preference for sweet

and high fat foods, which correlated with measures of overeating and explained higher body mass indexes (BMIs) [111]. Furthermore, a history of caloric restriction and/or intermittent access to highly-palatable foods can result in binge-like feeding behavior that persists long after cessation of cyclic restrict-refeed schedules in female rats [75], and 2-weeks but not 4-weeks after cessation in male rats [21]. However, a recent study from our lab found that a history of 24-hr/24-hr restrict/refeed cycles on both high- and low-fat diets had only transient effects on subsequent feeding patterns in diet-induced obese male mice [113].

Transgenic mouse models expressing the murine homologue of the *OPRM1* A118G SNP (*OPRM1* A112G) have not yet been used to explore potential phenotypic differences in taste and meal microstructure that may be driving these observed differences in feeding behavior. In the following experiments, an automated gustometer was used to measure taste responsivity, two-bottle testing was performed to assess lipid preference, and meal microstructure was analyzed using an automated instrument for biological data acquisition (BioDAQ). Whereas taste responsivity measures utilize brief taste exposure to exclude the role of post-ingestive signaling, two-bottle testing measures 48-hour lipid intakes, thus inclusive of central reward processing, and BioDAQ quantification of meal pattern represents the integration of peripheral and central signaling to regulate food intake. We investigated whether *OPRM1* A112G allele status affected taste responsivity in male and female mice, meal patterns in female mice, and whether

a history of binge and/or caloric restriction interacts with genotype to affect lipid preference in male mice.

Materials and Methods

Two-Bottle Taste Preference

For the two-bottle lipid preference tests, Intralipid® 20%, a sterile lipid emulsion formulated for intravenous provision of essential fatty acids, was diluted to the required concentrations for oral consumption. Intralipid® 20% is manufactured by Fresenius Kabi (Uppsala, Sweden) for Baxter Healthcare Corporation (Deerfield, IL 60015). The lipid fraction of Intralipid® is primarily in the form of soybean oil (20%), containing triglycerides comprised of a mixture of saturated fatty acids (palmitic acid, 7-14%; stearic acid, 1.4-5.5%), monounsaturated fatty acids (oleic acid, 19-30%), and polyunsaturated fatty acids (linoleic acid, 44-62%; α -linolenic acid, 4-11%). Also included in the formulation are egg yolk phospholipids (phosphatidylcholine and phosphatidylethanolamine; 1.2%) and glycerin (2.5%) which function as emulsifying agents. Sodium hydroxide is also added. The Intralipid® 20% emulsion provides 2.0 kcal/mL total caloric value, from triglycerides, phospholipids, and glycerin. (Fresenius Kabi, Manufacturer Medical Information and Instruction, revised 1/20/2006)

Following 6 weeks of the binge feeding protocol (12 total binge/refeeding periods), male mice (n=64) were exposed to a series of 48-h 2-bottle preference tests with a lipid emulsion (Intralipid® 20%, Fresenius Kabi, Sweden) diluted to 2.5%, 5%, and 10% concentrations. Bottles were fabricated from plastic 10-mL serological pipette tips with stainless steel sipper-tube feeders attached to the

pipette tip with rubber tubing. Once pipette tip “bottles” were filled with their respective fluid, rubber bulbs were fixed onto the upper, open pipette end to seal the tube, and any air bubbles were tapped out before measuring fluid volumes. The volumetric bottles were positioned with bottle tips extending down into the cage at an angle, held in place by the metal bars spanning the stainless steel cage lid (Figure 1).

For each trial, a 24-h acclimation period preceded the test period, where mice were presented with two volumetric bottles filled with water only. At time, $T=0$, each mouse was provided one bottle of the lipid solution (with trial concentrations presented in a random order between cohorts), and one bottle of water, and initial volumes were recorded. At $T=24\text{-h}$, volumes were measured and 24-h intakes calculated. The lipid solution was replaced with a fresh preparation, and the bottle positions in the cage lid were switched to account for potential side-preference. At $T=48\text{-h}$, final volumes were recorded, and the standard water bottles were returned to the cages.

Intake volumes were calculated for each liquid, during each 24- and 48-h period. Preference scores (%) for each 48-h 2-bottle test, at 2.5%, 5%, and 10% lipid concentrations, were calculated by dividing lipid intake (mL) by water intake (mL), then multiplying by 100.

Taste Responsivity

In two separate cohorts of adult homozygous male (n=9 AA, n=11 GG) and female (n=7 AA, n=7 GG) mice, a brief access taste assessment was performed using an automated gustometer (Davis Rig, Dilog Instruments, Tallahassee, FL; Fig. 2). Each animal was presented with a range of concentrations of different taste stimuli, and lick number was quantified. During the light cycle, mice were placed into the plexiglass testing chamber (6"W x 11"H x 10"D) with a stainless steel wire grid bottom, and access to a single spout of water. The test chamber was furnished with a computer-driven automated bottle rack with a capacity of 16 tubes. For this experiment, a maximum of 7 tubes was used for any given taste stimuli. Prior to each test, mice were acclimated to the chamber for 5 min, and then the session was initiated by a shutter opening to reveal the first tube spout. Each concentration was presented for 5 s with an inter-trial interval (ITI) of 8 s, and each tube was presented 15 times, with a maximum of 105 trials occurring over a 25-min test session. Before the tasting sessions commenced, all mice underwent a 24-hour water restriction followed by 4 consecutive days of behavioral water training during which water was provided only during training sessions. On days 1 and 2 of water training in the gustometer apparatus, mice were allowed 15 min of ad libitum access to 2 water bottles. On days 3 and 4, mice were allowed 25 min of access to 7 water bottles, with a presentation duration of 5 sec and an ITI of 8 sec, for 105 total trials. Following water training, mice underwent taste responsivity testing for the following taste stimuli (Table 1): sucrose (sweet disaccharide; 0, 0.01, 0.03, 0.1, 0.3, 1, 1.5M), fructose (sweet monosaccharide; 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6M), alanine (sweet amino acid; 0,

0.01, 0.03, 0.06, 0.1, 0.3, 1M), sodium chloride (NaCl, salty; 0, 0.01, 0.03, 0.06, 0.1, 0.3, 1M), monosodium glutamate (MSG, umami; 0, 0.01, 0.03, 0.06, 0.1, 0.3, 1M), citric acid (sour; 0, 0.001, 0.003, 0.006, 0.01, 0.03, 0.1M), quinine hydrochloride (quinine-HCl; bitter; 0, 10^{-5} , 3×10^{-5} , 10^{-4} , 3×10^{-4} , 10^{-3} , 3×10^{-3} M). Concentrations were presented in a standardized design to minimize contrast and order effects (i.e., non-ascending/-descending), and each test was followed by one day of ad libitum water and chow before the next test day. All mice were exposed to each taste stimuli twice, and only gustometer data for the second trial was used for analysis. A total of 1 female and 3 male mice were excluded following water training because they failed to lick during training sessions. However, mice that failed to lick only at a certain taste stimuli concentration received a lick response of "0."

Meal Pattern Analysis

Meal microstructure in homozygous male (AA n = 8 Naïve, 6 Binge, 6 Restrict, 6 Restrict-Binge; GG n = 13 Naïve, 8 Binge, 8 Restrict, 7 Restrict-Binge) and homozygous naive female (n= 10 AA; n= 15 GG) mice was measured using the Biological Data Acquisition System (BioDAQ; Research Diets Inc., New Brunswick, NJ). This meal pattern analysis system uses standard shoe-box style cages fashioned with gated, front-mounted food hoppers. The food hoppers rest on scales that detect changes in food hopper weight. Bouts of food consumption were clustered into meals, defined as a minimum of 0.02 g eaten, with an inter-meal interval of 300 sec, per supplier recommendation. Mice were acclimated to

test cages for at least 3 days preceding a meal pattern measurement period of 24 hours. Recorded chow intake patterns were used to calculate the average meal number, size (kcal), duration (sec), and rate of consumption (kcal/min).

Statistical Analysis

All statistical analyses were performed with Statistica 7.1 software (StatSoft Inc.). Significance was set at $\alpha=0.05$, and Neuman-Keuls post-hoc comparisons were made when appropriate.

For 2-bottle preference tests, 48-hour fluid intakes and preference scores were calculated. Preference scores (%) were calculated by dividing 48-h Intralipid intake by 48-h water intake and multiplying by 100. Repeated measures ANOVA was used to determine effect of genotype, prior history of binge exposure, or genotype-binge history interaction.

For taste responsivity tests, mean lick number across trials was calculated, and concentration-response curves were generated, for each taste stimulus.

Repeated measures ANOVA was used to determine genotype differences across concentrations for each taste stimuli.

Results

Lipid Taste Preference

Following 6 weeks of the intermittent access binge-like feeding paradigm, male mice underwent 2-bottle lipid preference testing for three concentrations of Intralipid® (2.5% (n = 4-8/group), 5% (n = 8/group), 10% (n = 6-8/group) of the premade lipid emulsion). Preference scores were analyzed using two-way repeated-measures ANOVA, and revealed an overall effect of Concentration [$F(2, 64)=3.4730$, $p=.037$], with a Group effect approaching significance [$F(3, 32)=2.8499$, $p=.053$] (Fig. 3). Neuman-keuls post-hoc testing showed that male mice had higher preference scores for IL 10% compared to IL 2.5% & 5% ($p < 0.05$ for both), and Restrict and Restrict-Binge mice trended towards higher preference scores than Naïve animals ($p = 0.05$ and 0.06 , respectively). There was no effect of genotype on lipid preference. Based on prior literature showing highest taste preference at a 5% concentration of IL [114], two-way ANOVA of preference scores at the 5% concentration were performed, revealing overall effects of Group [$F(3, 56)=4.2804$, $p=.009$], and a Genotype x Group interaction [$F(3, 56)=3.3725$, $p=.025$]. Newman-Keuls post-hoc revealed higher fat preference scores in Restrict ($p < 0.05$) and Restrict-Binge ($p < 0.01$) mice compared to Naïve, and GG Naïve mice had lower scores than GG Binge, Restrict, or Restrict-Binge, and all AA groups ($p < 0.01$ for all).

Taste Responsivity

Males

Potential genotype effects on concentration-response data for each taste stimuli was analyzed using repeated-measures ANOVA and LSD post-hoc testing, and revealed a lower average lick number at the 0.3 M concentration in AA male (n = 9) mice compared to GG males (n = 11) [$F(6, 108) = 2.7036$, $p = 0.017$] in sucrose only (Fig. 4).

Females

Potential genotype effects on concentration-response data for each taste stimuli was analyzed using repeated-measures ANOVA and LSD post-hoc, and revealed lower mean lick numbers in GG (n = 7) vs AA (n = 8) females at 1.5 M concentration of sucrose [$F(6, 78) = 2.3639$, $p = 0.038$] and at 0.3 M and 1.0 M concentrations of fructose [$F(6, 78) = 2.6660$, $p = 0.021$] (Fig. 5).

Meal Pattern Analysis

Males

In male mice, regardless of feeding schedule history (Naïve, Restrict, Binge, or Restrict-Binge), individual two-way ANCOVAs using body weight as a covariate revealed no effect of genotype on average meal number (15.73 ± 0.72 for AA; 14.86 ± 0.67 for GG), average meal size (g; 0.213 ± 0.011 for AA; 0.233 ± 0.013 for GG), or average meal duration (sec; 1091.895 ± 106.634 for AA; $1114.126 \pm$

96.814 for GG), nor consumption rate (mg/sec; 0.223 ± 0.014 for AA; 0.232 ± 0.012 for GG) or cumulative meal intake (g; 3.256 ± 0.142 for AA; 3.246 ± 0.091 for GG) during the 24-hour assessment period (Fig. 6). Male mean body weights per genotype were $27.5\text{g} \pm 0.05$ for AA mice and 27.8 ± 0.42 for GG mice.

Females

In homozygous naïve female mice ($n = 10$ AA; $n = 15$ GG), individual one-way ANOVAs revealed an effect of genotype on meal size (g) [$F(1, 23) = 4.4692$, $p = 0.046$] and cumulative meal intake (g) [$F(1, 23) = 7.4698$, $p = 0.012$], with GG female mice having larger meals and greater cumulative intakes during the 24-hour assessment period (Fig. 7). There was no effect of genotype on meal number (14.6 ± 1.94 for AA; 13.87 ± 0.82 for GG) or average meal duration (sec; 1602.3 ± 302.52 for AA; 1540.4 ± 193.41 for GG), or consumption rate (mg/sec; 0.143 ± 0.013 for AA; 0.179 ± 0.014 for GG). The observed effects of genotype persisted when individual ANCOVAs were run using body weight as a covariate, with GG female mice having a larger average meal size (g) [$F(1, 22) = 6.9379$, $p = 0.015$] and greater cumulative meal intakes (g) [$F(1, 22) = 4.3165$, $p < 0.05$]. Female mean body weights per genotype were $21.8\text{g} \pm 0.72$ for AA mice and $23.17\text{g} \pm 0.05$ for GG mice.

Discussion

These experiments utilized a murine model of the *OPRM1* A118G SNP (A112G) to explore taste and meal pattern phenotype in male and female mice. Two-bottle preference testing and brief-exposure gustometry were used to parse orosensory properties from post-ingestive consequences, and quantification of meal microstructure parameters reflected the sum total of peripheral and central appetitive signals that regulate food intake.

Male mice that had a history of intermittent calorie restriction/binge access had greater lipid preference scores than naïve animals at the 5% concentration, and an overall concentration effect that approached significance. In addition, and contrary to previous literature [114], preference for the 10% lipid solution was higher than the lower concentrations. However, similar to literature showing strain differences only at low concentrations of nutritive lipids [115], the only differences in lipid preferences associated with *OPRM1* A112G allele status was at the 5% concentration, where GG naïve mice had lower preference scores than all other groups.

Taste responsivity was evaluated in naïve male and female mice homozygous for the prototype (AA) or variant (GG) allele of the *OPRM1* gene. Genotype differences in lick response were only observed in nutritive sweeteners, with AA males having lower lick numbers than GG males at the 0.3 M concentration of

sucrose, but GG females licking fewer times at 1.5 M sucrose and 0.3 and 1.0 M fructose. Sexual dimorphism in lick response, as a function of *A112G* allele status, only reached statistical significance for sweet-tasting carbohydrates.

Lastly, due to the additive effect of food intake, small differences in meal microstructure can result in substantial differences in cumulative caloric intake and energy balance. Furthermore, a history of caloric restriction and/or intermittent access to highly-palatable, sweet-fat foods can alter subsequent feeding behavior [75, 113]. However, in this study, quantification of meal patterns in male mice with a history of intermittent caloric restriction and/or binge access revealed no effect of genotype or prior feeding schedule. In naïve females, GG mice had a larger meal size and cumulative meal intake than AA mice, an effect which persisted with body weights included as a covariate in the analysis.

Figures



Figure 1: Two-bottle taste preference testing apparatus. Standard home cage wire lid is adapted to fit two volumetric bottles, in addition to ad libitum chow. Left bottle contains lipid emulsion, right bottle contains water.

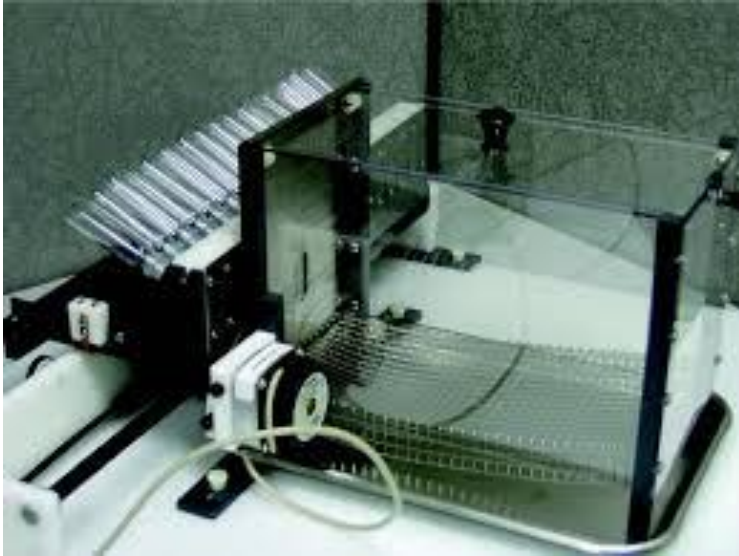


Figure 2: “Davis Rig” automated gustometer apparatus to measure very brief access taste responsivity in mice. Image from pubs.rsc.org.

Table 1: Taste stimuli and respective concentrations for taste responsivity analysis using an automated gustometer (Davis rig).

Taste Stimuli	Concentration (M)						
Sucrose	0	0.01	0.03	0.1	0.3	1.0	1.5
Fructose	0	0.05	0.1	0.2	0.4	0.8	1.6
Alanine	0	0.01	0.03	0.06	0.1	0.3	1.0
Sodium Chloride	0	0.01	0.03	0.06	0.1	0.3	1.0
Monosodium Glutamate	0	0.01	0.03	0.06	0.1	0.3	1.0
Citric Acid	0	0.001	0.003	0.006	0.01	0.03	0.1
Quinine Hydrochloride	0	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}

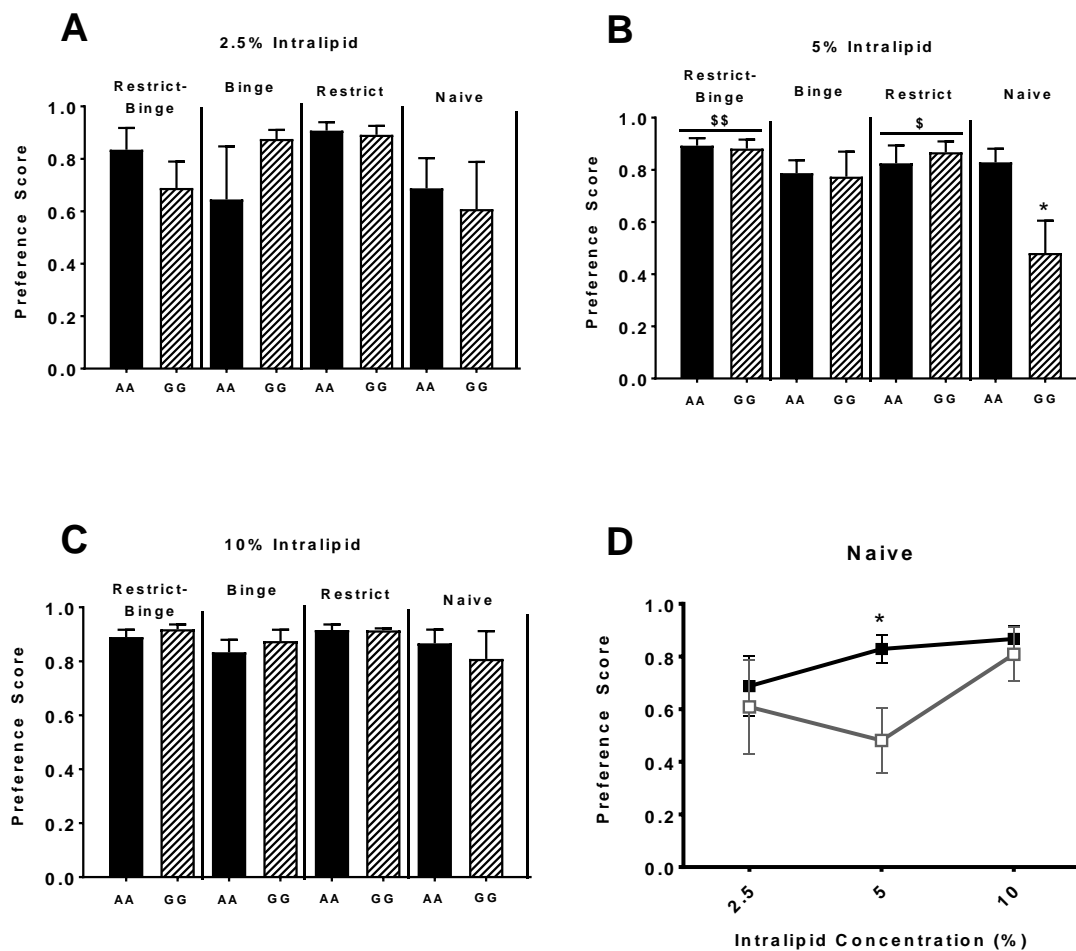


Figure 3: Lipid preference in male *OPRM1* A118G (AA/GG) mice following 6-week binge-access feeding protocol, using Intralipid® solution at 2.5% (**A**), 5% (**B**), 10% (**C**) concentrations, and in Naïve animals at all concentrations (**D**). Values indicate Mean \pm SEM. \$ indicates $p < 0.05$ from Naïve; \$\$ indicates $p < 0.01$ from Naïve; * indicates $p < 0.05$ between genotypes.

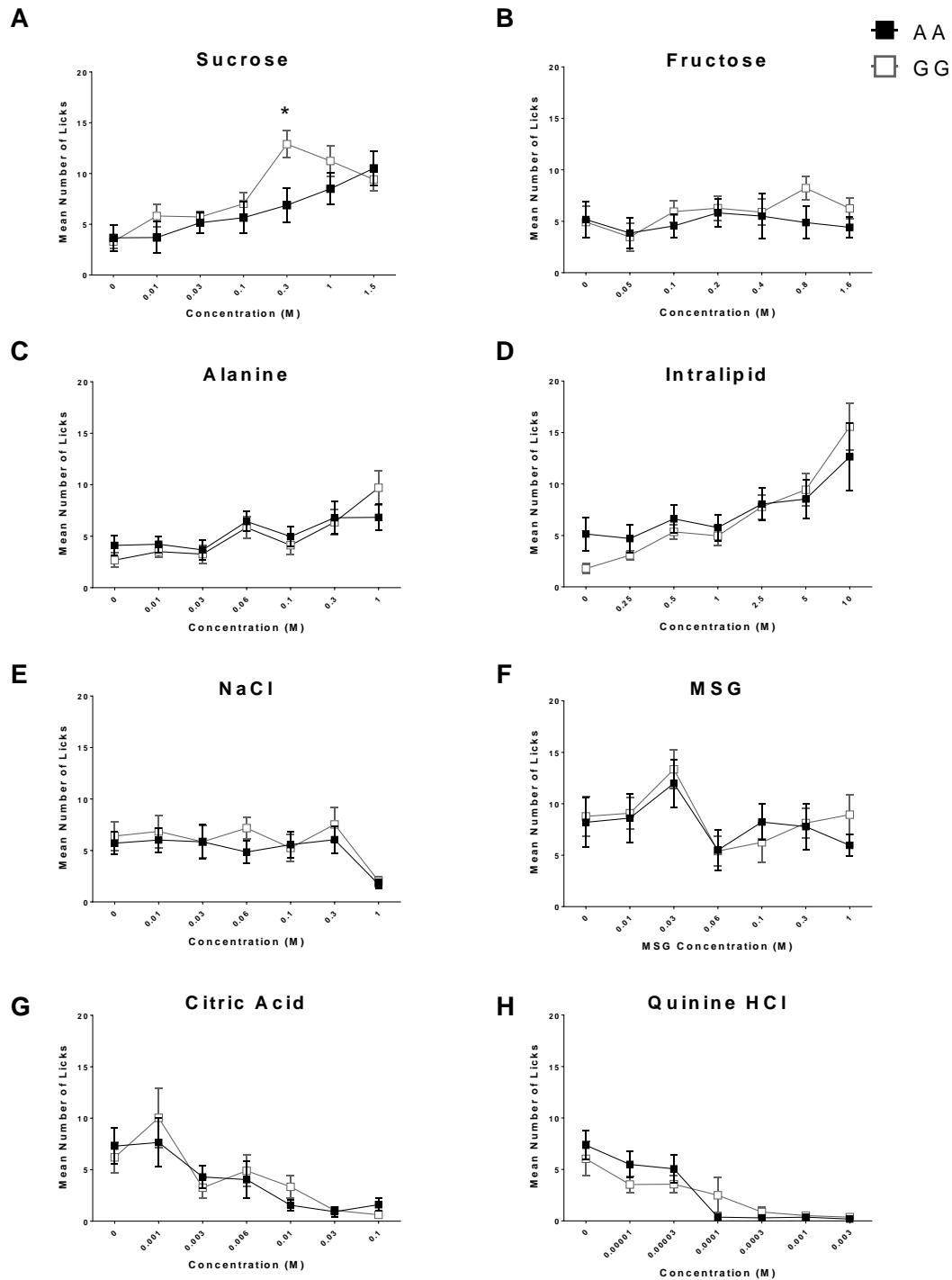


Figure 4: Taste responsivity concentration response curves in male *OPRM1* A112G AA (n = 9) and GG (n = 11) mice for sucrose (**A**), fructose (**B**), alanine (**C**), Intralipid® (**D**), sodium chloride (NaCl) (**D**), monosodium glutamate (MSG) (**E**), citric acid (**G**), and quinine hydrochloride (HCl) (**H**). Values indicate Mean \pm SEM. * indicates $p < 0.05$.

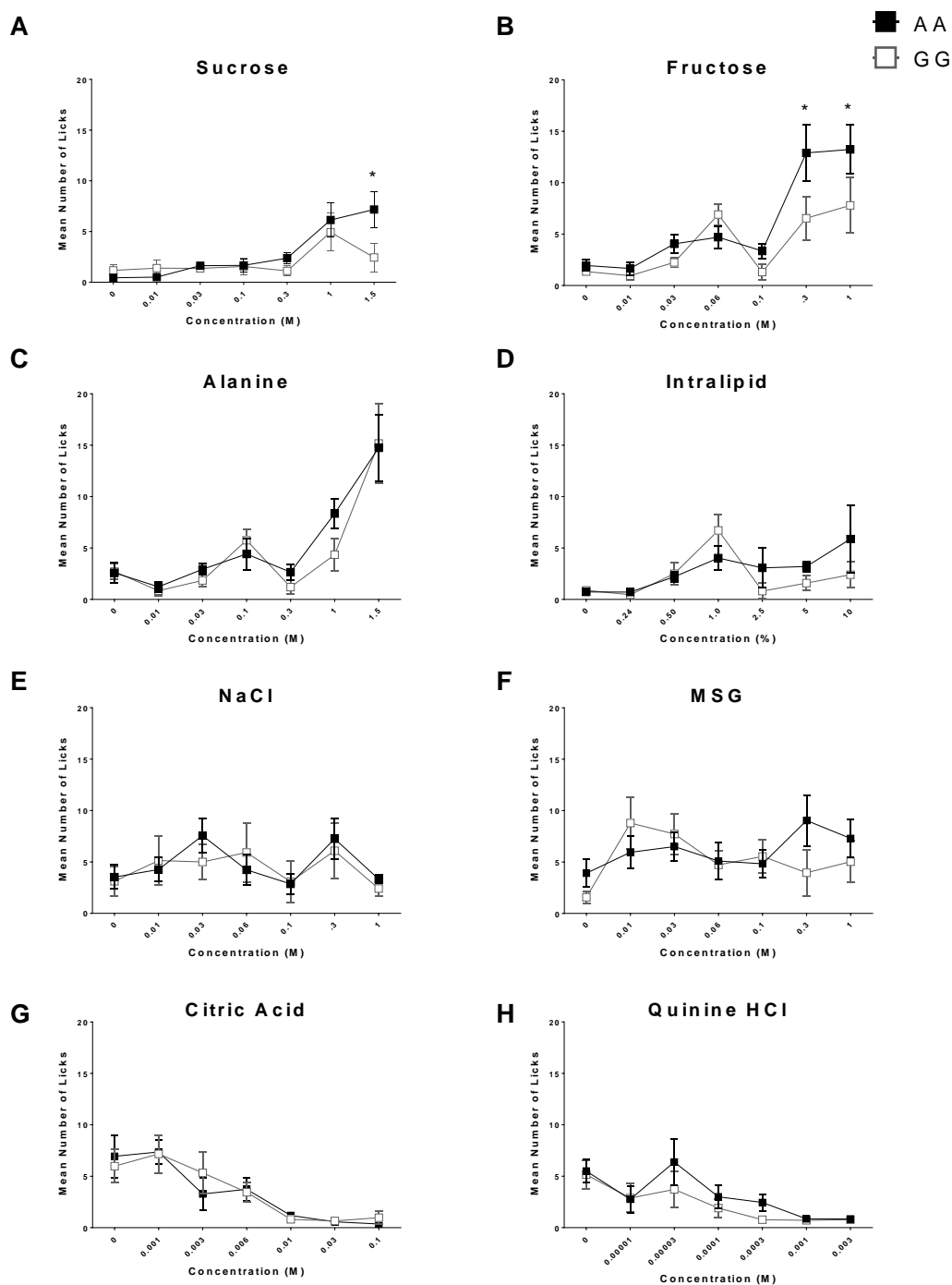


Figure 5: Taste responsivity concentration response curves in female *OPRM1* A112G AA (n= 7) and GG (n= 7) mice for sucrose (**A**), fructose (**B**), alanine (**C**), Intralipid® (**D**), sodium chloride (NaCl) (**D**), monosodium glutamate (MSG) (**E**), citric acid (**G**), and quinine hydrochloride (HCl) (**H**). Values indicate Mean \pm SEM. * indicates $p < 0.05$.

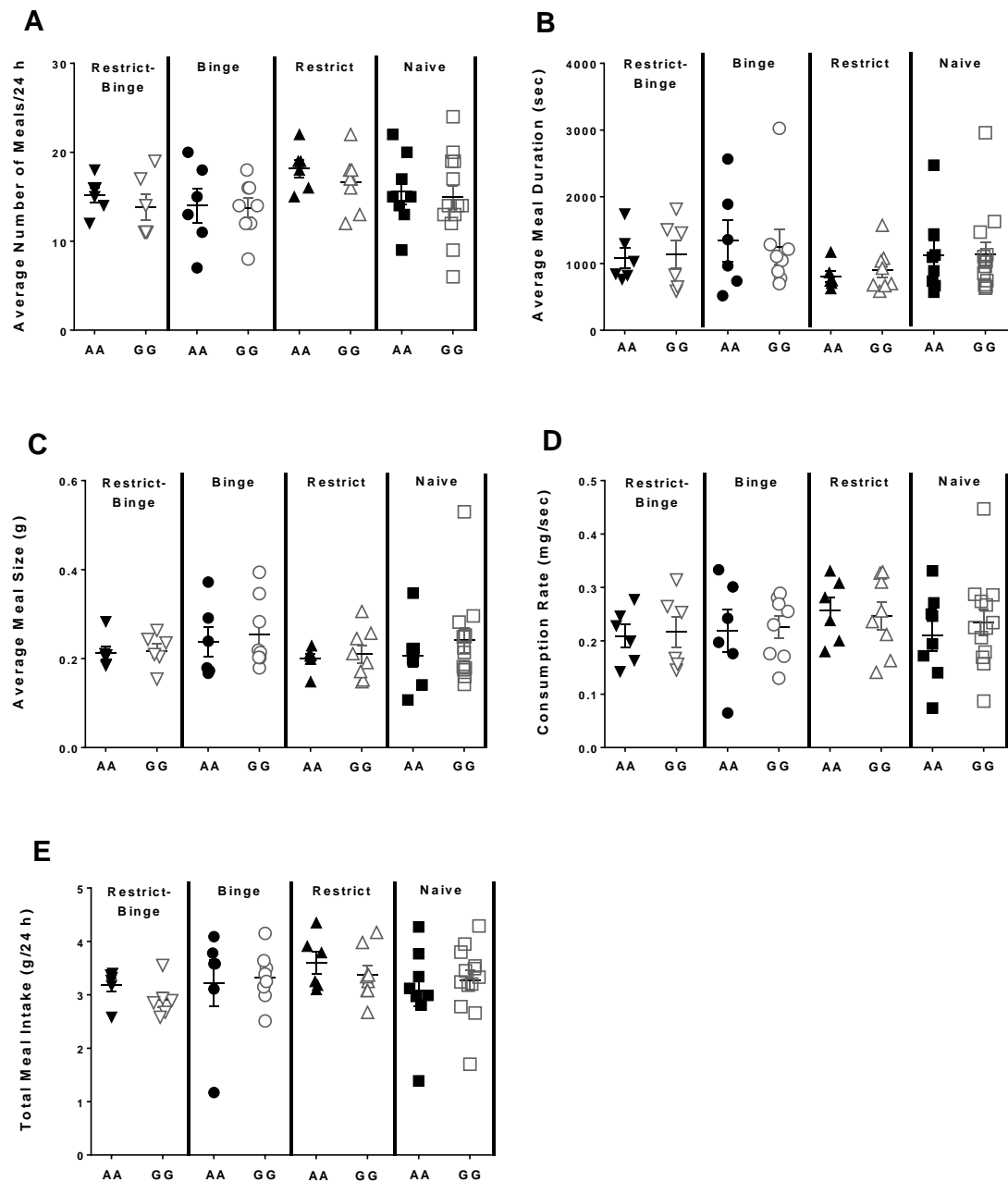


Figure 6: Meal microstructure during 24-hour measurement period in male *OPRM1* A112G AA (n = 6-8) and GG (n = 7-13) mice following the 6-week binge-like feeding paradigm. Data are represented as Mean \pm SEM. **(A)** Average number of meals. **(B)** Average meal duration (sec). **(C)** Average meal size (g). **(D)** Consumption rate (mg/sec). **(E)** Total meal intake (g).

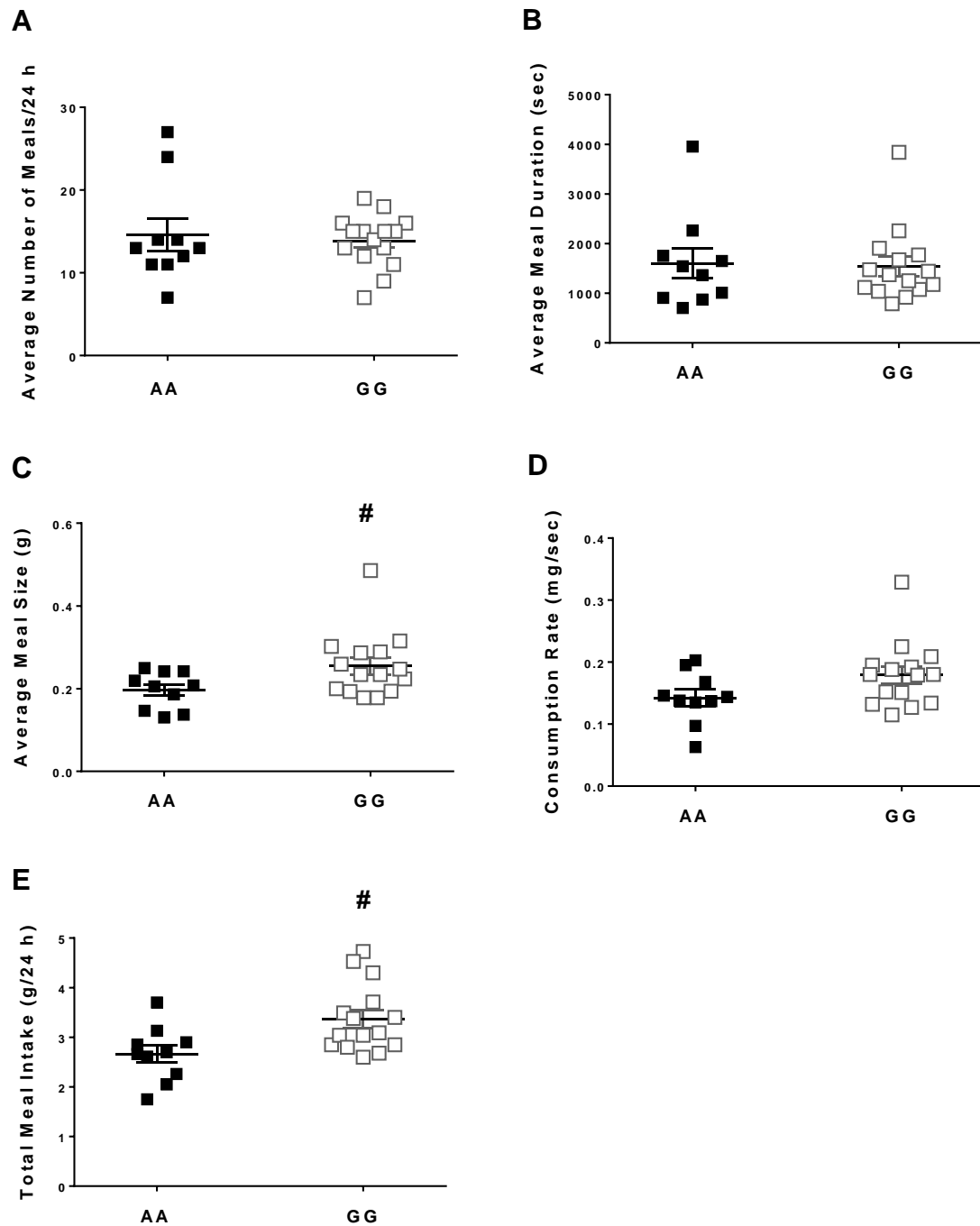


Figure 7: Meal microstructure during 24-hour measurement period in naïve female *OPRM1* A112G AA (n = 10) and GG (n = 15) mice. Data are represented as Mean \pm SEM. **(A)** Average number of meals. **(B)** Average meal duration (sec). **(C)** Average meal size (g); # indicates $p < 0.05$ compared to AA. **(D)** Consumption rate (mg/sec). **(E)** Total meal intake (g); # indicates $p < 0.05$ compared to AA.

Chapter 5:

Discussion and Conclusions

The experiments described in this dissertation were designed to explore the role of the common SNP of the mu-opioid receptor (*OPRM1* A118G; A112G in mice) in three main areas of interest: 1) propensity for binge-like feeding of a sweet-fat food; 2) pharmacological efficacy of lisdexamfetamine for the treatment of binge eating behavior; and 3) altered hedonic sensitivity to taste stimuli and fat preference that may shift meal patterns and drive overconsumption.

OPRM1 A118G allele status effects nociception and analgesic effectiveness, with variant allele carriers (GG) requiring greater doses of medications to reach similar levels of pain relief. In prior literature, GG status has also been observed to have an association with binge eating disorder (BED), in addition to higher sweet-fat food preference and correlating BMIs. With the current attention on “personalized medicine” in the biomedical community, whether A118G status may necessitate altered pharmacological dosage in the context of BED treatment, is an important question. Furthermore, psychiatric disorders including eating disorders, Alzheimer’s, and dementia, display sexual dimorphism in diagnosis, efficacy of pharmacotherapy, and prognosis. Female preclinical animal data, as well as clinical studies in women, remain underrepresented, despite recent pressures from the NIH to encourage this research. Therefore, a

major strength of this dissertation is the inclusion of female mice to identify potential sex differences in these behavioral pharmacogenetic studies.

In Chapter 2, we investigated whether the *OPRM1 A112G* SNP mediated binge-like feeding in a 6-week intermittent access model involving twice weekly exposure to 24-hr caloric restriction and/or 30-min sweet-fat “binge” food choice. Our operational definition of binge-like feeding was met, with 30-min palatable food consumption escalating over the study duration, and calorie intakes during access periods exceeding their restriction-matched controls; measures supported as reliable based on the larger body of rodent binge research [116]. Whereas Binge groups represent binge-like intake in the *absence* of physiological hunger, as they have access to continuous *ad libitum* chow, the Restrict-Binge feeding schedule mimics binge-like intake in the *presence* of physiological hunger, due to their food restricted condition. An interesting observation is the difference in standard chow consumption during binge-access periods between Binge and Restrict-Binge groups. Whereas Binge mice consume only the sweet-fat food, Restrict-Binge mice consume standard chow in addition to the highly palatable binge food, suggesting that physiological hunger drives chow consumption. This may be due to the nutritionally complete nature of the pelleted chow diet, or perhaps textural differences between the firm pelleted chow and the malleable semi-solid binge food.

Interestingly, our established rodent binge model uses partially-hydrogenated vegetable shortening (i.e. Crisco) plus 10% sucrose as the highly-palatable food [21-25, 69], but the formulation of this commercially-available food product has changed over time due to federal regulation pertaining to the use of *trans*-fats in the food supply. However, a study comparing binge-like intake of *trans*-fat vs non-*trans*-fat vegetable shortening in rodents found no difference resulting from the shift in fatty acid composition [67].

The Restrict-Binge schedule could be interpreted as a model of bulimia nervosa (BN), with caloric restriction mimicking a “purge” behavior. Furthermore, Restrict groups serve as a control for the Restrict-Binge schedule, but both groups could also be considered a variation of intermittent fasting (IMF), with twice-weekly 24-hr food restriction that was not compensated for when comparing 6-week cumulative intakes. If considering the Restrict feeding schedule as IMF, a common dietary strategy for weight reduction and maintenance, it is notable that female GG Restrict and male GG Restrict-Binge mice had higher final body weights despite having lower cumulative caloric intakes. Because these mice did not have an obese phenotype, it may be appropriate to analyze body composition using Echo Magnetic Resonance Imaging (MRI) to determine if elevated body weights were due to body fatness or lean muscle. The primary ligand for MOR is β -endorphin and is associated with both exercise and fasting, but whether *OPRM1 A118G* allele status alters activity or energy expenditure is unknown. Follow-up studies could use the Oxymax Comprehensive Lab Animal

Monitoring System (CLAMS) system to measure spontaneous activity, as well as fuel utilization via quantification of macronutrient oxidation (respiratory exchange). Our findings of elevated body weights in GG mice supports previous literature showing an association between the *A118G* variant and BMI in adults [111]. However, in a study of morbidly obese patients undergoing bariatric surgery [117], and a recent large, population-based study in mothers and children, no association between *OPRM1* genotypes and BMI was observed [118]. Along similar lines as personalized medicine, “personalized nutrition” based on genetic screenings is gaining momentum in the entrepreneurial space, utilizing wearable tech, algorithms, and machine learning strategies [119]. Personalized nutrition generally refers to the use of personal data pertaining to genetics, physiology, metabolism, diet, behavior, and/or medical history, to develop an individualized diet strategy to maximize one’s health [120]. Conceptually, this strategy is based on data showing a high degree of interindividual variability in physiological responses to specific diets and nutrients (i.e. Postprandial blood glucose; blood cholesterol in response to chronic high-fat feeding; autoimmune response to gluten in celiac disease, etc.) and that personalized advice may be more effective in producing sustained behavioral changes than general population recommendations. Despite considerable growth of this sector of business, the breadth of claims is scarcely supported by the current body of scientific evidence. However, this finding suggests the *A118G* SNP may warrant further exploration when considering the metabolic consequences of intermittent fasting techniques.

Chapter 3 centered on potential genotypic differences in the pharmacological efficacy of lisdexamfetamine dimesylate to reduce binge-like feeding in female mice in acute and chronic dosing schemes. Following 6 weeks of the intermittent-access feeding schedules, female AA and GG mice underwent once weekly acute dosing via oral gavage, one hour preceding the 30-min binge/refeed period. Acute dosing followed a within-group design, so each mouse received a one-time dose of vehicle (water), and three ascending doses of both lisdexamfetamine dimesylate (LDX; 0.15, 0.5, 1.5 mg/kg) and sibutramine hydrochloride (Sib; 0.3, 1.0, 3.0 mg/kg), with the initial pharmacotherapy presentation switched for half of the trials to reduce potential order effects. There were no genotype differences in intakes during 30-min access periods following any of the acute doses. However, a one-time acute dose of 3.0 mg/kg Sib reduced binge-like feeding compared to vehicle and LDX doses. Sibutramine is a monoamine reuptake inhibitor originally formulated for the treatment of obesity, and FDA-approved for that use in 1997. Although subsequently withdrawn from the market due to adverse cardiovascular outcomes, Sib has been demonstrated to be effective in reducing binge episodes in people with BED [41].

In another set of mice, after the 6-week binge-feeding paradigm, mice underwent a 2-week daily chronic dosing schedule. Mice received oral gavage of either vehicle, or high dose of either LDX (1.5 mg/kg) or Sib (3.0 mg/kg), in a between-group design. There were no observed effects of genotype on pharmacological

attenuation of binge intakes or cumulative food intake during chronic dosing. Although an acute dose of Sib had reduced binge intake, chronic exposure did not have an effect on food intakes nor body weights. This discrepancy in Sib efficacy at the 3.0 mg/kg dose may be explained by data showing that chronic Sib treatment is more effective in obese vs lean rodents, and particularly ineffective under low-fat fed conditions [69, 121, 122]. Body weight differences between Restrict groups suggest that GG status may predispose to body weight gain during intermittent fasting conditions, similar to observations in Chapter 2. Together, these studies do not support a functional association between *OPRM1* A112G allele status and binge propensity nor efficacy for pharmacologic treatments.

There is a large body of evidence supporting an association between *OPRM1* A118G genotype and nociception, analgesia, and drug abuse and treatment, with outcomes specific to the type of pain and/or drug, and patient ethnicity [88]. The strongest data supports an association between the G allele and the treatments for pain and alcohol dependence [88]. Furthermore, studies in a similar mouse model of *OPRM1* A112G found that in female mice with the G allele, buprenorphine analgesia was blunted and the buprenorphine-induced reduction of neohypophagia was blocked [99]. Thus, our findings that the efficacy of lisdexamfetamine and sibutramine were not mediated by *OPRM1* A112G genotype, were somewhat surprising. However, the mechanism of action of common opioid treatments for pain, including morphine, codeine, oxycodone,

fentanyl, and hydrocodone, is via direct activation of MORs. Similarly, the pharmacotherapy for alcohol dependence showing differential efficacy due to *A118G* genotype was naltrexone, a high-affinity MOR antagonist that precipitates withdrawal symptoms. In our studies, we investigated the pharmacogenetics of LDX, an amphetamine pro-drug that acts as a CNS stimulant by stimulating release of norepinephrine and dopamine, and by monoamine reuptake inhibition. Similarly, the mechanism of action of Sib is as a monoamine reuptake inhibitor, and as such, neither of our drugs of interest act directly through MOR signaling.

A limitation to this study was that circulating levels of LDX during acute and chronic dosing paradigms were not verified via plasma analysis. Literature containing pharmacokinetic data for LDX in rodents was limited to rat models, and mouse studies of LDX efficacy remain scarce. As such, it is possible that peptidase cleavage of the lysine residue of pro-drug LXD in circulation, to its active form, is not occurring. Previous studies using an intermittent-access binge model in female rats, showed LDX (0.1-1.5 mg/kg) reduced chocolate consumption a dose-dependent manner [123]. Additional behavioral pharmacological studies revealed LDX (0.8 mg/kg) reduced binge-induced impulsivity in a delay-discounting task in female rats [72], and chocolate binging as well as compulsive and perseverative behaviors in female Wistar rats [124]. In a recent study in mice investigating the potential for LDX dependence, an oral dose of 2.5 mg/kg, but not 1.0 mg/kg, increased conditioned place preference and self-administration, suggesting that high doses may be producing off-target

effects [125]. The lower doses used in our studies were intended to parse genotype differences without producing a ceiling effect on reduction of binge intake [69]. Because BED is often accompanied by obesity, it would be useful to explore LDX and Sib efficacy on the reduction of binge intake in a model of high-fat induced obesity in mice.

In Chapter 4, we characterized the taste and meal pattern phenotype in male and female mice homozygous for the *OPRM1* A112G prototype (AA) or variant (GG) allele. Lipid taste preference was measured in male mice that had previously been exposed to the 6-week binge-like feeding paradigm described in Chapters 2 and 3. Although overall lipid preference scores were higher at the 10% Intralipid® (IL) concentration, feeding group history and genotype differences were only observed in the 5% IL exposure. Male mice previously exposed to Restrict and Restrict-Binge feeding schedules had higher lipid preference scores compared to Naïve mice, and genotype differences were only seen in Naïve animals, where GG mice had lower lipid preference than AA mice. Although prior literature found the highest IL preference and intake at the 5% concentration [114], our findings are similar to work by Sclafani *et al.* showing that strain differences in IL preference in mice was observed at low (0.313-5%) but not high (10-20%) concentrations [115]. Furthermore, by repeating each preference test, they found that experience induced preference for IL at low concentrations (0.313-0.625%), particularly in strains that were relatively indifferent in the first test, increasing the lipid preference score to >90% in the second test, but eliminating

strain differences [115]. This draws attention to a limitation in our lipid preference studies, due to inconsistencies in the sequence of exposures to different IL concentrations. The first 92 male mice underwent 2-bottle lipid preference testing at each of the three concentrations (2.5%, 5%, 10% IL), in a random order. However, due to limited funding to maintain cage *per diems* for this project, the remaining 37 of the final cohorts were prioritized to complete the 5% IL test, due to the literature suggesting this concentration yields the highest preference [114]. Moreover, due to variation in the delay between conclusion of the 6-week feeding paradigm and the preference tests (1 week- 2.5 months), mice varied in age of exposure from 13 to 22 weeks. Although in humans, taste perception decreases with age [126], a study in C57BL/6J mice that repeated 2-bottle testing in mice aged 4-125 weeks found little effect of age on taste preference in males [127]. Despite these limitations in study execution, this data suggests that a history of intermittent caloric restriction, regardless of prior access to a high fat-sweet “binge food,” may predispose to heightened lipid preference in the future, and a history of repeated intermittent restriction and/or binge access may eliminate potential differences in lipid preference due to *OPRM1* A112G allele status.

In two additional groups of naïve male and female mice homozygous for the prototype (AA) or variant (GG) allele of the *OPRM1* A112G SNP, concentration-response curves were generated using an automated gustometer to quantify lick number in response to eight taste stimuli. Genotype differences in lick response were only observed in nutritive sweeteners, with AA males having lower lick

numbers than GG males at the 0.3 M concentration of sucrose, but GG females licking fewer times at 1.5 M sucrose and 0.3 and 1.0 M fructose. Whereas Sclafani and colleagues found that mouse strain differences in preference for nutritive (i.e. Intralipid) vs non-nutritive (i.e. Olestra) lipid were similar to that of nutritive (sucrose) vs non-nutritive (saccharin) sweeteners [115], similar patterns in taste responsivity were not observed in our experiments. Moreover, lick response nor taste preference of a non-nutritive lipid was explored in the context of *OPRM1 A112G* genotype differences, providing an interesting area for future study. Sexual dimorphism in lick response, as a function of *A112G* allele status, only reached statistical significance for sweet tasting carbohydrates, with female AA mice with higher lick response at the highest concentrations of sucrose and fructose. Our findings support decades of prior research highlighting sex differences in taste responsivity and preference in rodents, as well as humans. As reviewed by Martin *et al.*, several studies show female rodents prefer caloric and non-caloric sweet stimuli, and sex differences in brief-access studies are stimuli- and concentration-specific [128]. In a recent study in healthy adults, women perceived taste intensity for five stimuli at five concentrations to be higher overall [126]. A study exploring changes in taste perception associated with weight gain, found that adult men perceived sweet and salty tastants as less intense after modest weight gain (3.9%), whereas females perceived an increase in sour taste intensity with a similar amount of weight gain [129]. A similar sex-adiposity interaction was observed in children, where overweight/obese males

had lower perceived intensity of sweetness, but this was not observed in females [130].

Lastly, the BioDAQ system for quantifying meal microstructure was used to explore meal patterns in AA vs GG naïve female mice, and male mice previously exposed to the 6-week intermittent access feeding paradigm, in addition to 2-bottle lipid preference testing. No prior feeding group nor genotype differences in meal microstructure were observed in male mice. However, in naïve females, GG mice had larger average meal sizes and greater total meal caloric intakes, an effect sustained when body weights were included in covariate analysis. Meal pattern analysis was done with *ad libitum* standard chow to explore any baseline differences. However, due to the role of endogenous opioids in high-fat feeding, providing *ad libitum* high fat diet may better tease-out genotype-related differences due to the hedonic aspects of the high energy diet.

Together, these taste and meal pattern experiments provide information regarding orosensory vs post-ingestive signaling mechanisms in this murine model of the *OPRM1* A118G SNP. Our brief-access, taste response experiments revealed great lick number in GG male mice for sucrose, suggesting heightened orosensory stimulation as a result of the MOR polymorphism. Interestingly, whereas female GG mice had a reduced lick response at high concentrations of sucrose and fructose, this could be interpreted as a decrease in response due to heightened sensitivity to the nutritive sweet tastants. A study by Ostlund *et al.*

quantified lick microstructure for 30-min bouts in female MOR knockout mice, compared to wildtype, found considerable evidence in support of mu-opioid mediation of palatability [131]. MOR knockout mice had lower levels of licking behavior in response to sucrose and sucralose, suggesting an attenuated hedonic response to the sweet stimuli, encompassing the sum of orosensory drive and post-ingestive inhibition. Interestingly, Ostlund and colleagues found that although wildtype mice had a two-fold increase in licking bursts of sucrose solutions under caloric deprivation, MOR knockout mice licking response was not sensitive to fasting conditions [131], suggesting a role for MOR signaling in mediating the palatability-enhancing effect of food restriction [132].

In contrast to sweet stimuli, we observed no differences between AA and GG mean lick scores for Intralipid in male or female mice. However, our 48-hour, two-bottle preference studies revealed a higher lipid preference in mice with a history of intermittent caloric restriction, but with no differences between AA and GG mice. The only genotype difference in lipid preference was observed in naïve male mice at a 5% concentration, with a lower preference in GG mice compared to AA. This suggests that genotype did not alter orosensory characteristics, but that post-ingestive reinforcing effects may be attenuated in GG mice, or that post-ingestive inhibitory mechanisms may be elevated. In a study by Sakamoto *et al.*, administration of the opioid antagonist, naltrexone, attenuated the intake and reinforcing effects of IL only at high IL concentrations, whereas olfactory and glossopharyngeal nerve transections attenuated the effects only at low IL

concentrations [114]. This suggests that opioid signaling may be more important for regulating lipid intake at high concentration, whereas olfactory and glossopharyngeal nerve signaling may be more important for lipid intake of low concentrations. Furthermore, larger average meal size and total meal intake were higher in female GG mice compared with AA females, but no differences in meal microstructure was observed. Regulation of food intake incorporates input from orosensory and post-ingestive signals. Despite a higher sweet taste responsivity and lower lipid preference in GG males, no differences in meal patterns was observed under standard chow-fed conditions. In females, lower sweet taste responsivity in GG mice compared to AA females may have partially mediated higher meal size and intake under standard chow-fed conditions.

Despite the numerous strengths of the described studies, there are several limitations that require mention. During colony establishment, after breeding three generations of the *OPRM1 A112G* transgenic “knock-in” mice, we were advised to cross heterozygous females with FLPase males. Due to the transgene structure, FLPase breeding would result in the excision of the Neo cassette, an artifact of gene construction process. However, sequencing of gDNA from mice before- and after- FLPase breeding was not performed, so we cannot be certain that the Neo cassette is no longer included. In addition, mice were single-housed cages at the initiation of the experimental period. As social animals, this may be considered a limitation, as its effect on behavior cannot be ruled out. However, for accurate measurement of food and liquid intakes, the primary measures of

this work, removing the influence of cage-mates is necessary, and strengthens the study by improving precision of intake measures. Furthermore, fecal samples were collected throughout the female binge and chronic dosing paradigm intended for microbial analysis, and coprophagy would transfer microbiome profiles between cage-mates eliminate potential community differences. Ideally, perforated dividers can be used to create a physical barrier between two mice that allows for social interaction while limiting interferences.

To add to this body of work, there are several experimental avenues that are of interest. In female mice exposed to the 6-week binge paradigm followed by a 2-week daily dosing period, brain tissue was collected at sacrifice for mRNA expression analysis by qPCR. The arcuate nucleus (ARC) and paraventricular nucleus (PVN) of the hypothalamus, in addition to the nucleus accumbens (NA) of the ventral striatum were microdissected and stored in a -80°C freezer.

Because no difference in binge behaviors were observed between *OPRM1* A112G AA and GG mice, gene expression was not explored. Similarly, colonic fecal samples were collected at T=0, 6 weeks (effect of feeding schedules, preceding chronic dosing), and 8 weeks (sacrifice). Gut microbial communities reflect host diet and genes, metabolize ingested drugs, and alter neurotransmitters directly via production or indirectly via its constituents, and MOR is highly expressed throughout the intestine. Thus, we intended to sequence the gut microbiome to explore potential effects of host genotype, diet, and pharmacological treatment. However, as with brain gene expression, no

genotypic behavioral differences were observed that warranted further investigation. Another area to potentially explore is stress-like behavior in the *OPRM1 A112G* mice. A “loss of control” over one’s eating is central to the experience of BED, and endogenous opioids modulate neuroendocrine stress pathways. Although our binge model addresses the binge-like feeding aspect of BED, it does not address the comorbid anxiety symptoms. There is a battery of behavioral tests designed to explore stress-like behaviors in rodents, including the elevated-plus maze, open-field test, and light-dark box, that could be used to explore the behavioral outcomes of the *A112G* SNP in the context of stress behavior in binge-like feeding.

Moreover, although our studies used a diet-induced binge-like feeding model to explore the potential effects of *OPRM1 A112G* genotype on palatable food consumption, it is important to readdress the comparison between food or eating “addiction” and drug addiction. In addiction, the highly pleasurable effects of drugs are mediated by the mesolimbic dopamine system, and positive reinforcement drives initial drug consumption. As use continues, neural adaptations result in a blunted reward response, and tolerance encourages abuse of the substance. In addition to the heightened memory of drug cues and experience, avoidance of withdrawal symptoms (i.e. negative reinforcement) drives further drug-seeking behavior [133]. Addiction involves this cyclical experience of the compulsion to seek and take drugs, a loss of control to limit intake, and the emergence of a negative emotional state reflecting withdrawal

[134]. Anecdotally, and clinically as seen in individuals with BED, it is easy to draw a parallel between the cyclical pattern of drug addiction (a fixation with and compulsion to consume, driven by both the pleasure of consumption and the expectation of alleviation from negative affect), and the excessive consumption of highly palatable foods. As such, there are numerous studies exploring the neural and physiological systems underlying the similarities between these two pathological behaviors [43, 44, 49, 50, 74, 112, 135-141]. However, food addiction remains a particularly controversial topic. Despite powerful hedonic drivers of foods high in fat and/or sugar, food, and the nutrients it contains, is required for life and under extensive homeostatic regulation [44]. Drugs, however, have no biological necessity, thus hedonic drivers of intake and inhibitory self-control mediate substance use behavior. Furthermore, despite binge-like intakes of highly-palatable foods as seen in intermittent-access models, providing continuous-access of the same foods does not elicit binge feeding behaviors, suggesting there is not an innate, addictive quality to the foods [116]. This observation is supported by the difference between the pharmacological actions of palatable foods vs. drugs of abuse. Palatable foods have non-specific neural action mediated by several redundant systems, whereas drugs are ligands for receptors that facilitate specific mechanisms of action.

The studies in this dissertation contribute to the larger body of scientific work in a few salient ways. First, this was the first study to use a mouse model of the

OPRM1 A118G SNP to investigate clinical findings that GG allele status may influence binge eating behaviors and high-fat food preference. Second, twice as many women vs men are diagnosed with eating disorders, so this study primarily included female mice, and also male mice for most experiments. By addressing sex as a biological variable, these studies aim to improve the health disparity resulting from a historical deficit of biomedical research in women and female animal models. Third, these studies illustrate the necessity to publish and share negative data, particularly in the context of one-gene hypotheses for psychiatric diseases. A very recent publication from Border *et al.* provides a critical analysis, by means of an extensive GWAS data set, of the body of evidence (or lack-there-of) supporting candidate genes that predispose individuals to depression [142]. Although there have been vocal critiques of these optimistic, overly simplistic theories resulting from less sensitive investigative techniques of the time, they generated a momentum that created a research environment resistant to a paradigm shift. It is for this reason that the publication of negative data is so important to the scientific process. Lastly, although these studies did not support the hypothesis that the *OPRM1 A118G* polymorphism influences binge eating and pharmacotherapy efficacy, the goal of pharmacogenetics to tailor treatments to patient genetics for improved clinical outcomes, deserves continued attention from the scientific community.

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