SEX AND PHENOTYPIC DIFFERENCES OF OBESITY-INDUCED GHSR VENTRAL HIPPOCAMPAL DISRUPTIONS IN THE CONTROL OF APPETITE

By

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ABSTRACT

The gastric hunger signal, ghrelin, influences feeding behavior via activation of the growth hormone secretagogue receptor (GHSR). GHSR's are abundantly expressed in cells in the ventral hippocampus (VHPC) where they function to regulate food intake. In this study, we used a GHSR-IRES-Cre mouse to examine whether feeding behaviors driven by GHSR cells in the VHPC are influenced by vulnerability to dietary obesity. Both males and females were exposed to 8 weeks of a high fat diet (HFD)—the top and bottom quartiles of weight gainers from each respective sex were designated as diet-induced obese (DIO) and diet resistant (DR), respectively.

These mice received targeted injections of an excitatory (hM3Dq) or inhibitory DREADD (hM4Di) virus. This enabled chemogenetic control of GHSR-expressing cells in the VHPC as mice engaged in consumption of lab chow or HFD. Only DR female mice displayed the expected increase in food intake when tested with lab chow following DREADD stimulation, indicating that female mice that are resistant to dietary obesity maintain typical function for GHSR's in VHPC. Surprisingly, in males, DREADD stimulation decreased meal intake, which for chow occurred in DIO mice, whereas for HFD testing this was observed in DR mice. On the other hand, DREADD inhibition attenuated chow intake only in female mice.

In the final series of studies, I used licking microstructure to examine the pre-ingestive (e.g., orosensory, palatability) and post-ingestive (e.g., gastrointestinal negative feedback) variables that regulate GHSR-dependent food intake. Multiple findings were revealed, including that the increased consumption of food in female mice following GHSR stimulation reflects a reduction in gastrointestinal negative feedback. Overall, my findings stress the need to implement a rigorous examination of a host of variables with refined analyses of meal intake to determine a role for how feeding signals in the brain impact ingestive behavior.

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LIST OF SYMBOLS

<	Less Than
>	Greater Than
=	Equal to
♀	Female
♂	Male

LIST OF ABBREVIATIONS

AAV	Adeno-associated Virus
AgRP	Agouti-related Protein
AHA	American Heart Association
ANOVA	Analysis of Variance
ARC	Arcuate Nucleus of the Hypothalamus
BBB	Blood-brain Barrier
BMI	Body Mass Index
CDC	Center for Disease Control
CNO	Clozapine-N-Oxide
CNS	Central Nervous System
COVID	Coronavirus SARS Cov-2
Cre	Causes Recombination
DASH	Dietary Approaches to Stop Hypertension
DHPC	Dorsal Hippocampus
DI	De-ionized
DIO	Diet-Induced Obesity
DR	Diet-Resistant Obesity
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
E2	Estradiol
FDA	Food and Drug Administration
FN	Feature Negative
GABA	γ-Aminobutyric acid

GHSR	Growth Hormone Secretagogue Receptor
GOAT	Ghrelin-o-Acetyltransferase
HFD	High Fat Diet
HE	High-energy
HM	Henry Molaison
IACUC	Institutional Animal Care and Use Committee
ICU	Intensive Care Unit
ICV	Intracerebrovascular
IP	Intraperitoneal
IRES	Internal Ribosome Entry Site
Kg	Kilogram
KO	Knockout
LHA	Lateral Hypothalamic Area
LTD	Long-term Depression
LTP	Long-term Potentiation
NAc	Nucleus Accumbens
NHANES	National Health and Nutrition Examination Survey
NO	Nitric Oxide
NPY	Neuropeptide Y
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
TD-NMR	Time Domain Nuclear Magnetic Resonance
US	United States

VEH	Vehicle Solution
VTA	Ventral Tegmental Area
vHPC	Ventral Hippocampus
WD	Western Diet
WT	Wild Type

CHAPTER 1: INTRODUCTION

Significance: Obesity Prevalence and Diet

Prevalence

Obesity is a highly prevalent issue domestically and worldwide. From a global perspective, the World Health Organization indicates that obesity rates have tripled since 1975, with a now estimated 650 million adults worldwide considered obese, as determined by a body mass index (BMI = kg/m^2) greater than 30 (Abarca-Gómez et al., 2017; Blüher, 2019; Ezzati, 2016). In the US, the most recent statistics from the CDC indicate that as of 2020, 42.4% of all adults aged 20 and over qualify as obese. Additionally, 9.2% of the adult US population is determined to have "severe obesity" which is defined by a BMI greater than 40 (Bryan et al., 2021; Hales, 2020). Both values are significantly increased from prior measurements in 1999 where obesity prevalence was calculated at 30.5% and severe obesity at 4.7% (Bryan et al., 2021). More recent figures from the National Health and Nutrition Examination Survey (NHANES) were unavailable due to restrictions of field operations in 2020 as a result of COVID-19, however it is estimated the rates of obesity and severe obesity have continuously increased since the start of the pandemic (Di Renzo et al., 2020).

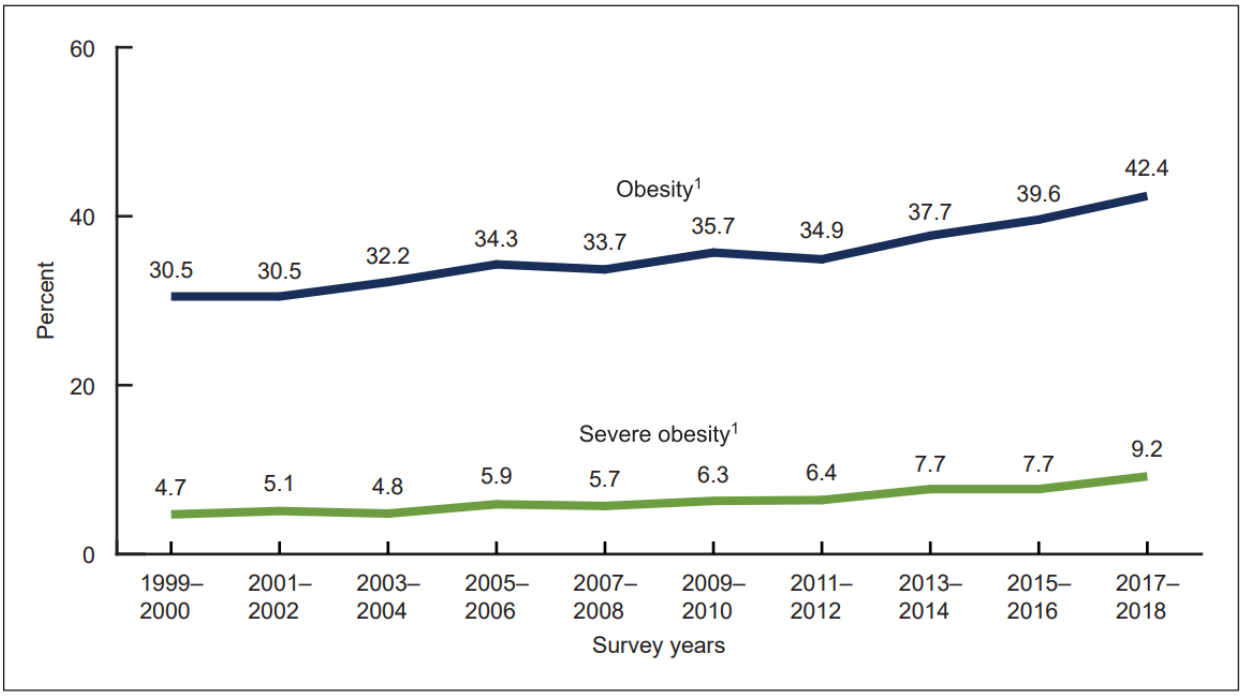


Figure 1. Trends in age-adjusted obesity and severe obesity prevalence in adults aged 20 and over. Rates of obesity increased from 30.5% and severe obesity from 4.7% in 1999 to 42.4% and 9.2% in 2018 respectively. Image adapted from Hales et al. 2020.

Cost and Societal Impact

The resultant increase in obesity rates has a significant societal impact as well as associated healthcare costs. In one of the most comprehensive overviews of nationally represented data from Finkelstein et al., it was found that obesity-related costs accounted for 9.1% of the US medical expenditures, with an obese patient paying on average $732 more in annual medical premiums than non-obese individuals (Finkelstein et al., 2003, 2005; Peterson & Mahmoudi, 2015). However, these data were calculated in 1998 and do not consider the vast increases in the rates of obesity and severe obesity in the last 25 years. Indeed, more recent data indicates that there has been a jump in associated medical costs in relation to obesity, which in 1998 were estimated to be $78.5 billion and as of 2008 rose to $147 billion (Finkelstein et al., 2009). It is estimated by 2030 that healthcare costs attributable to obesity may reach over $860 billion by 2030, and account for 16-18% of all US related healthcare costs (Revels et al., 2017; Wang et al., 2008). These increased healthcare costs are found to be a result of increased in-patient, out-patient, and emergent

medical visits, rehabilitation services, surgical care and complications, and prescription medications (Bamgbade et al., 2007; Dzien et al., 2003; Kim & Basu, 2016; Thompson & Wolf, 2001; Tremmel et al., 2017; Withrow & Alter, 2011).

Comorbidities

With the prevalence of obesity steadily increasing, special notice must be made regarding various comorbidities, one of the most widely studied being Type II Diabetes Mellitus. Higher BMI levels are positively correlated with increased rates of diabetes and associated decreases in quality of life as well as subsequent increases in morbidity and mortality (Carnethon et al., 2012; Kahn et al., 2006; Lazar, 2005). This is primarily due to the increases in consumption of simple carbohydrates and fats in a caloric surplus that results in insulin resistance as the body contends with persistently increased blood glucose levels along with circulating lipids and cytokines which can progress to a diabetic phenotype (Astrup & Finer, 2000; Corkey, 2012; Malone & Hansen, 2019; Yaturu, 2011). As a result, these patients are at high risk for developing cardiovascular disease, retinopathy, nephropathy, neuropathy, and venous insufficiency, ultimately decreasing life expectancy in this patient population (Abdelaal et al., 2017; Hollenberg, 2006; Mbata et al., 2017; Smith & Singleton, 2013).

Similar trends hold true for cardiovascular disease without associated diabetes which continues to show an increased prevalence with increasing BMI as well as increased rates of morbidity and mortality (Khan et al., 2018; Koliaki et al., 2019; Krauss et al., 1998; Parto & Lavie, 2017; Pi-Sunyer, 2009). With a caloric surplus, there is an excess of lipids available which can deposit in the vasculature as atherosclerotic plaques. This deposition occurs more easily in obese populations due to endothelial dysfunction because of inflammation leading to a reduction in nitric oxide (NO) bioavailability and thus a pro-atherogenic state. It is the decreased patency and flexibility as a result of these atherosclerotic plaque depositions,

particularly in the coronary arteries that can readily progress to myocardial infarcts and associated sequelae including death.

Additionally, obesity is associated with increased risks for developing certain types of cancer including those of the colon, breast, endometrium, kidney, and esophagus (Basen-Engquist & Chang, 2011; Calle et al., 2003; Calle & Thun, 2004; Pi-Sunyer, 2009). Using BMI as an outcome, a dose-response relationship for obesity can be extrapolated for all cancers for both men and women. Compared to a BMI below 25, female patients with a BMI over 30 are at an 18% higher risk of developing cancer, while patients with a BMI over 40 have a 62% increased risk (Calle & Thun, 2004; Zhang et al., 2008). In comparison, males with a BMI over 30 have an elevated cancer risk of 9% and a with a BMI over 40 have a 52% risk of developing cancer (Basen-Engquist & Chang, 2011; Calle & Thun, 2004).

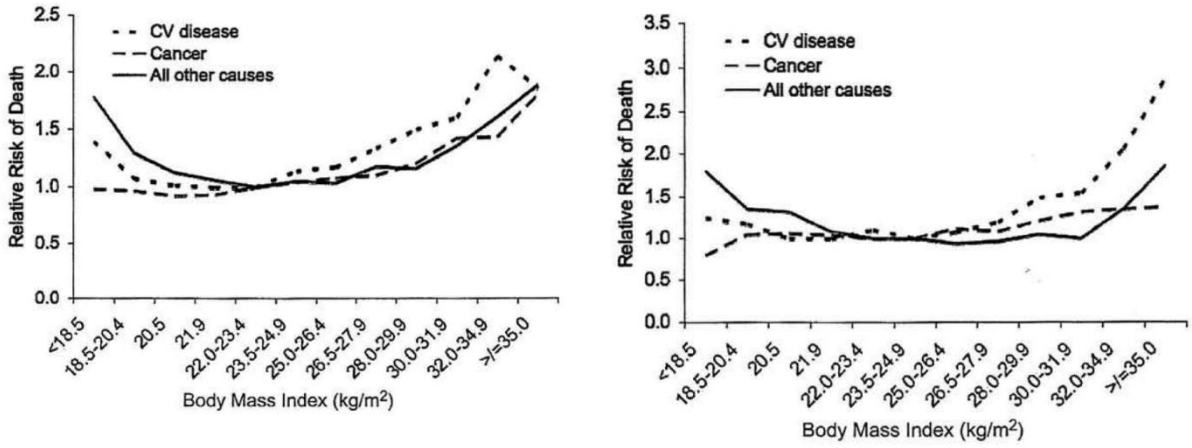


Figure 2. Relative risk of death from cardiovascular disease, cancer, and all other causes in relation to BMI. Regardless of sex, both female (left) and male (right) show increased relative risk of death from cardiovascular disease, cancer, and all other causes as BMI approaches and surpasses the current metric for obesity (BMI > 30). Image adapted from Pi-Sunyer et al., 2020, as modified from the Massachusetts Medical Society.

In light of more recent events, obesity has also played a detrimental role in the progression of the COVID-19 pandemic. Obese patients were found to have overall greater rates of hospitalization, ICU admittance,

mechanical ventilation requirements, long-term complications, and death compared to lower BMI patients when controlling for co-morbidities and vaccination (Denova-Gutiérrez et al., 2020; Popkin et al., 2020; Simonnet et al., 2020; Townsend et al., 2020, 2021). The issue—especially at high BMI levels—is that obesity is associated with decreased expiratory reserve volume, functional capacity, general respiratory compliance, and difficulties with diaphragmatic excursion (Dietz & Santos-Burgoa, 2020; Kwok et al., 2020; Yu et al., 2021). Considering coronaviruses primarily target the pulmonary system, this constellation of factors along with general increases in inflammatory markers such as C-reactive protein and D-Dimer in obese patients drives a worsening prognosis and has resulted in millions of additional COVID-associated deaths worldwide (Alberca et al., 2021; Burn et al., 2021; Petrilli et al., 2020; Popkin et al., 2020; Westheim et al., 2021).

The Western Diet

One of the major factors implicated in the obesity epidemic is the prevalence of the modern Western Diet (Bortolin et al., 2018; Kopp, 2019). In comparison to healthier diets recommended by the CDC, FDA, and AHA such as the DASH or Mediterranean diet for example—which place an emphasis on consumption of fresh vegetables, fruit, whole grains and legumes while limiting saturated fats and red meat—the Western Diet is defined by calorically dense sources of highly processed sugar, salt, and saturated fat (Cena & Calder, 2020; Kanoski & Davidson, 2011; Rakhra et al., 2020; Sandouk & Lansang, 2017; Wartella et al., 2010). These food sources are found to be nutritionally poor and in many cases lack basic and essential vitamins and minerals (Bortolin et al., 2018; Kaidar-Person et al., 2008).

Despite these nutritional shortcomings, the Western Diet remains incredibly prevalent and popular due to its high level of palatability which is purposely engineered to draw individuals to these highly-processed foods and to eschew more nutritious options in the process and to eat beyond one's points of metabolic need (A. W. Johnson, 2013, 2018; F. Johnson & Wardle, 2014). This is due to the high-insulinemic nature

of the Western Diet which is inherently less satiating yet appetite inducing, as the high sugar and fat content in these highly-processed foods allows for easier consumption of a large amount of calories in a relatively short period of time (Holt & Miller, 1995; Kopp, 2019; Rakhra et al., 2020; Rodin et al., 1985). This blunted satiation response is also largely driven by additives such as high fructose corn syrup which is found to disrupt satiation signals of the gut-brain axis and promote addictive-like feeding behavior (Holt & Miller, 1995; López-Taboada et al., 2020). As a result, this perpetual cycle of overconsumption of calorically dense food more readily promotes a state of calorie storage via increasing adiposity in comparison to healthier, filling foods such as fiber-filled vegetables, legumes and lean proteins (Rakhra et al., 2020). Within the context of the 3 major macronutrient components, it is estimated that the average Western Diet consists of 51.8% carbohydrates, 32.8% fat, and 15.4% protein in comparison to the healthier diets which suggest dropping fat consumption to less than 30% while increasing carbohydrates and even more so—protein due to increasing bodies of evidence indicating that elevated protein consumption can improve blood lipid profiles (Cordain et al., 2005; O'dea, 1984; O'Dea et al., 1989; Wolfe & Giovannetti, 1991).

Current Treatments and Lack of Efficacy

Current treatment options for obesity are limited in scope and long-term success. The gold-standard recommended treatment for obesity is regular daily, sustained exercise and a lower fat, lower carbohydrate, higher protein diet such as the DASH or Mediterranean diets as previously discussed (Anton et al., 2017; Farhadnejad et al., 2019; Park et al., 2017). Unfortunately, long term success is severely lacking with some meta-analyses indicating that as little as 15% of behavioral interventions via diet and exercise leads to long-term sustained weight loss (Ayyad & Andersen, 2000; Curioni & Lourenço, 2005; Mann et al., 2007; Volek et al., 2005). Finding an ideal combination of diet and type of exercise should

theoretically work on an individual basis but access to consistent multidisciplinary treatment is limited thus leading to generalizing treatment options based on population data and expecting an individual to maintain a consistent, yet restricted dietary regimen on their own volition (Raynor & Champagne, 2016; Volek et al., 2005).

From the pharmacological standpoint, current first-line therapies primarily seek to control symptoms of obesity by lowering cholesterol to decreasing levels of low-density lipoprotein in the blood, as well as controlling highly comorbid hypertension as examples (Gaudette et al., 2015; Modan et al., 1991; Muls et al., 1997; Seravalle & Grassi, 2017). The issue is these therapeutics do not address the primary cause of obesity which is the increased adiposity due to excess calorie consumption—and as a result—increased weight gain (Sharma & Padwal, 2010). To directly treat these root causes of obesity, a line of appetite suppressant drugs has been developed in the last couple decades, the most recently FDA-approved form being the injectable semaglutide in 2021 (Kalyanasundar et al., 2016; Moon et al., 2021). These drugs can include synthesized analogs of glucagon-like peptide 1(GLP-1) or amphetamine as examples, which target various anorexigenic pathways in order to decrease hunger and increase satiety (Halford, 2001; Kalyanasundar et al., 2016; Knudsen & Lau, 2019; Nammi et al., 2004; Rebello & Greenway, 2020; Rothman & Baumann, 2009). Current efficacy data is promising however there is a lack of long-term data to support whether these drugs are able to support a sustained form of weight loss for patients over years and decades (Bray, 1993; Greenway et al., 1999; Tak & Lee, 2021).

If diet and exercise or pharmacotherapy interventions fail, the next step in management involves more invasive interventions—typically involving some form of bariatric surgery including Roux-en-Y gastric bypass or laparoscopic adjustable gastric banding (Franco et al., 2011; Tice et al., 2008). The goal of these interventions is to reduce the amount of macronutrients that can be consumed and absorbed during a meal, thus reducing overall calorie intake leading to a more sustainable form of weight loss management. While initial weight loss metrics show promise following bariatric surgery, as many as 50% of patients will

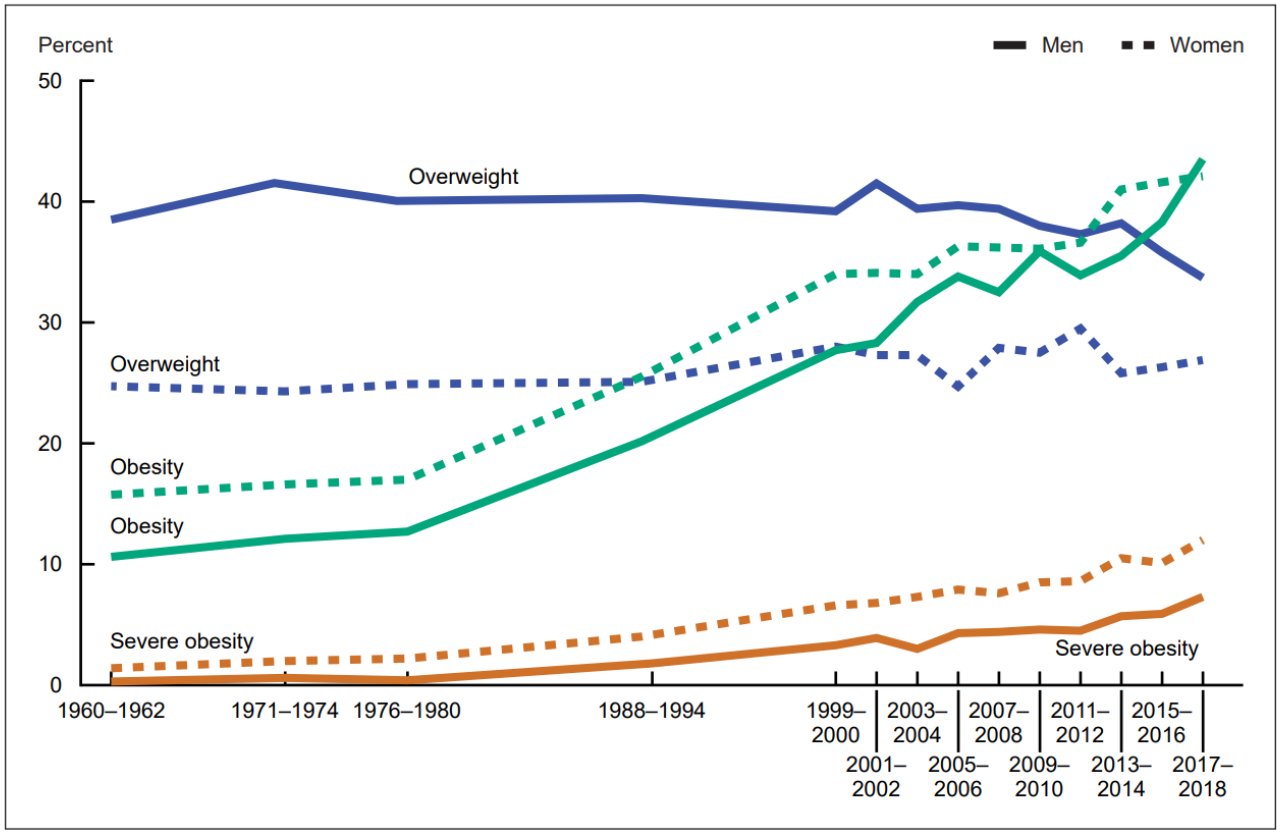
regain or surpass their pre-surgical weight by 24 months post-operation (Bastos et al., 2013; Cooper et al., 2015; Kushner & Sorensen, 2015; Magro et al., 2008; Velapati et al., 2018). Considering the health risks for long term complications following these surgeries and the variable success rate, it is important to note that even the most stringent interventions for weight loss management can lack sufficient efficacy in the long term.

Obesity Resistance

Despite all the issues regarding the prevalence and availability of highly processed foods in our obesogenic environment, not all individuals become obese. In fact, despite similar diet and nutrition access, these individuals are resistant to developing an obese phenotype (Farooqi & O'Rahilly, 2006; Madsen et al., 2010). Development of these phenotypes is partially driven by genetics, as twins raised in completely separate environments report similar BMI's, regardless of the variances in the environment they were individually raised in (Price et al., 1987; Stunkard et al., 1986). Comparatively, within-sets of twins exposed to chronic overfeeding show relatively little variance in their weight gain. Comparatively, between twin-sets show drastic differences where over the course of an 84 day overfeeding study the range in weight gain was between 4.3 and 13.3 kg depending on the twins. This supports the idea again that there are inherent genetic factors at play that predispose certain individuals to becoming more obese than others given the same dietary access (Bouchard et al., 1990). Rodent models support these findings; when given ad-libitum access to a high fat diet meant to approximate the macronutrient proportions found in the previously discussed Western diet, some animals will rapidly cultivate mass and develop a diet-induced obese phenotype while the diet resistant animals will increase in weight, though not in proportions as seen in the obese group (Farley et al., 2003; Huang et al., 2004; Levin et al., 1989, 1997; Reuter, 2007).

Additionally, there are sex differences noted in dietary obesity. Human males are found to have higher rates of overweight classification (BMI > 25) compared to females while females have a higher incidence of severe obesity (BMI > 40) compared to males (Fryar et al., 2020). These trends have remained consistent until about 2020 where it is now estimated that there are more obese males and females than there are overweight individuals within each sex (Figure 3). Trends for severe obesity follow similar trajectories where the rates for both males and females have increased significantly since 1960. This indicates there may be factors beyond the obesogenic environment itself that are driving differences in the proportion of these obese-prone and obese-resistant phenotypes between either sex. This is further supported by the difference in proportion of males and females who qualify as a healthy BMI between 18.5-24.9 which now constitutes 35.5% of males and 27.9% of females (Bryan et al., 2021).

Figure. Age-adjusted trends in overweight, obesity, and severe obesity among men and women aged 20–74: United States, 1960–1962 through 2017–2018



NOTES: Data are age adjusted by the direct method to U.S. Census 2000 estimates using age groups 20–39, 40–59, and 60–74. Overweight is body mass index (BMI) of 25.0–29.9 kg/m^2. Obesity is BMI at or above 30.0 kg/m^2. Severe obesity is BMI at or above 40.0 kg/m^2. Pregnant women are excluded from the analysis.
SOURCES: National Center for Health Statistics, National Health Examination Survey and National Health and Nutrition Examination Surveys.

Figure 3. Age-adjusted trends for overweight, obese, and severely obese males and females since 1960. Males (solid lines) have consistently higher rates of overweight classification compared to females (dotted lines) since 1960, however the rates of obesity for both sexes has increased significantly where the rate of obesity for both males and females (green lines) in 2018 is now higher than incidences of overweight classification for either sex (blue lines). Figure adapted from Fryar et al., 2020.

Ghrelin: Gastric Feeding Signal That Contributes to Eating Behavior and Obesity

Ghrelin and the Ghrelin System

One of the primary factors implicated in driving ingestive behavior is the peripheral gut hormone ghrelin. Ghrelin is a 28-amino acid peptide secreted from the oxyntic glands of the stomach and to a lesser extent, the small intestine (Cui et al., 2017; Kojima et al., 1999; Müller et al., 2015). Once acylated by ghrelin O-acyl transferase (GOAT) at the serine-3 position, ghrelin with its octanoic acid side chain is capable of

crossing the blood-brain barrier (BBB) to act on its target growth hormone secretagogue receptor (GHSR1a) also known as the ghrelin receptor (Banks et al., 2002; Perello et al., 2019; Rhea et al., 2017, 2018). This fatty acid modification is unique to ghrelin and explains how this peptide is able to exert effects peripherally as well as in the CNS. However, the truncated isoform GHSR1b—derived from the same gene—is non-functioning (Callaghan & Furness, 2014).

Ghrelin is released in a diurnal fashion, with systemic levels peaking pre-prandially, and reaching a nadir post-prandially (Alvarez-Castro et al., 2013; Cui et al., 2017; Zigman et al., 2016). This diurnal secretion pattern is not as readily apparent in obese individuals, where ghrelin levels do not peak as high pre-prandially and at a basal level are lower than in lean individuals (Ariyasu et al., 2002; English et al., 2002; Shiiya et al., 2002). This attenuated ghrelin secretion in a dietary obese phenotype drives a hypothesis for central ghrelin resistance wherein alterations in homeostatic ghrelin secretion in obese-prone individuals dysregulates GHSR-mediated feeding circuits (Zigman et al., 2016). This has been shown in obese mice which have reduced ghrelin receptor mRNA in the nodose ganglion and hypothalamus along with resistance to ghrelin-evoked feeding (Naznin et al., 2015). Thus, the diminished activity of ghrelin signaling in the vagal afferent pathway impairs metabolic regulation in the obese state via the disrupted transmission of gastrointestinal sensory information to the CNS (Naznin et al., 2015).

When coupled with increased levels of inflammatory markers in the obese state which are found to decrease BBB permeability, this ultimately drives a negative feedback loop that potentiates a persistent state of obesity as hunger and satiety mechanisms fail to regulate when and how much to eat for a given meal (Cui et al., 2017). This is supported by studies showing that neither peripheral nor central administration of ghrelin induces feeding behavior in mice on a high fat diet—in part due to blunted downstream agouti-related peptide (AgRP) and neuropeptide Y (NPY) expression from the arcuate nucleus of the hypothalamus, a region critical in feeding control (Briggs et al., 2010; Mondal et al., 2005).

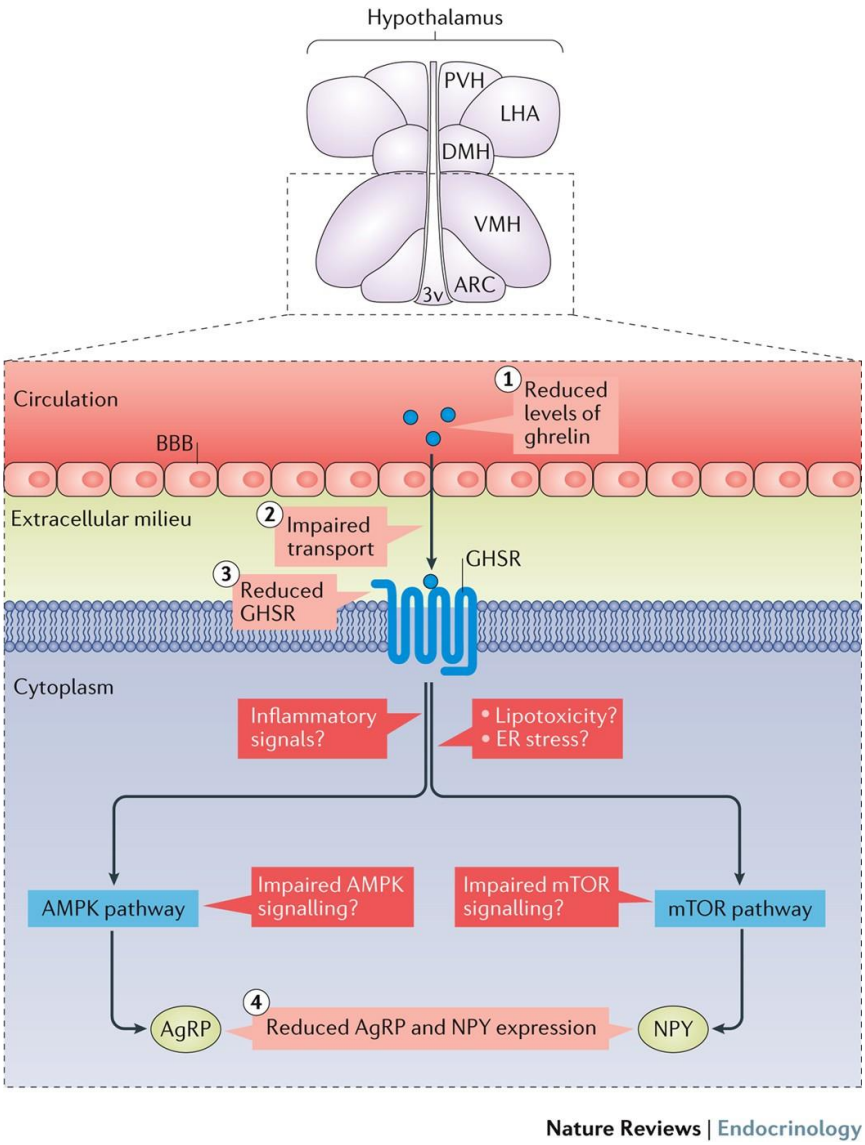


Figure 4. Proposed mechanism of obesity associated ghrelin resistance. Reduced diurnal rhythmicity of ghrelin secretion (1) is impaired in crossing the BBB due to increases in inflammatory markers in the obese state (2). As a result, there are less ghrelin molecules able to bind GHSR in the CNS (3) which further decreases intracellular signaling and expression of downstream factors involved in feeding regulation including AgRP and NPY (4). This leads to dysregulation in hunger and satiety, ultimately driving a state of overeating due to the abundance of highly-processed, calorically dense foods available in the obesogenic environment. Figure adopted from Cui et al., 2017.

Energy Regulation and Intake

Various rodent models have been developed to further elucidate the central role of ghrelin in driving feeding intake and energy regulation via GHSR and how sensitivity of this interaction is affected by obesity.

It is well characterized that ghrelin binding to GHSR drives neuronal activation via a Gq-coupled protein signaling cascade, which exerts various downstream appetite stimulatory and feeding effects depending on the brain region. In the ARC for example, this leads to initiation of feeding, while in the ventral hippocampus this leads to an increase in overall meal size as well as frequency (Briggs et al., 2010; Cui et al., 2017; Kanoski et al., 2013; Kojima & Kangawa, 2005; Rediger et al., 2009). GHSR knockout mice show no increase in feeding on a chow diet with ghrelin administration compared to wild-type (WT) mice indicating that the receptor is indeed necessary to induce feeding behavior (Sun et al., 2004). Interestingly, these mice maintain similar appetite and bodyweight patterns to their WT counterparts. However, when a GHSR-null mouse is given a high-fat diet instead of chow, these mice eat less food and have overall lower levels of adiposity than WT counterparts in both sexes, with GHSR-KO females accumulating less weight and adiposity than GHSR-KO males (Zigman et al., 2005). On a chow diet similar results are seen in exclusively females indicating a potential sex difference in utilization of calories depending on the diet driven by activity of the GHSR receptor or the lack thereof.

Additional findings consider ghrelin's role in energy utilization and regulation. Ghrelin-KO mice maintained on a HFD that are switched to a calorie-restricted diet gain less rebound weight compared to WT mice when the calorie-restricted chow diet is later altered to ad libitum access (Briggs et al., 2013). This may serve as a potential explanation for the high rates of rebound weight gain following bariatric surgery given that calorie restriction is a necessary component of the post-operative treatment and plan (Magro et al., 2008). This is further supported by studies showing that intracerebroventricular (ICV) infusion of ghrelin upregulates the gene expression of lipogenic enzymes involved in formation of white adipose tissue with HFD despite of a lack of associated hyperphagia compared to chow, implicating a role for ghrelin in energy balance and homeostasis regardless of dietary access (Perez-Tilve et al., 2011). This is especially poignant considering the modern obesogenic environment in which decreases in energy expenditure and increases

in energy consumption have been noted since the 1960s, when processed sugars and fats became a more integral part of the Western Diet (Blüher, 2019).

GHSR and the Hippocampus

GHSR is expressed in multiple regions of the CNS including the midbrain, hypothalamus, and hippocampus to name a few (Zigman et al., 2006). The hippocampus is well known for its integral role in learning and memory. For example, in the amnesic patient HM, the resection of his medial temporal led to an incapacity to form new declarative memories; thus, implicating the hippocampus as an important hub controlling long term memory (Corkin, 2002; Gabrieli et al., 1988; Tulving & Markowitsch, 1998). However, one of the other drastic changes HM experienced was the inability to identify a state of hunger or satiation. HM was asked to rate his overall level of satiety before and after a meal, where he would consistently rate his overall level of hunger after the meal equivalent to what it was before the meal, indicating he lacked the ability to interpret any internal hunger cues—which in this case was also made more difficult as his anterograde amnesia prevented him from remembering he had even consumed a meal in the first place (Hebben et al., 1985). This was further validated in an animal model where rats given selective hippocampal lesions were trained on a discrimination task in a food-deprived and non-food deprived state compared to control rats and counterbalanced to assess performance with a shock contingency. Hippocampal-lesioned rats failed to discriminate regardless of hunger or satiety state indicating these rats were impaired in their ability of utilizing hunger cues to discriminate. However, these results were not a result of general deficits in learning and memory as these rats were still able to utilize and interpret auditory cues, indicating these deficits in performance were directly related to their inability to process interoceptive hunger signals (Davidson & Jarrard, 1993).

Hippocampal-dependent changes in introspective learning and memory towards feeding are also supported by a study by Davidson et al., where a deprivation intensity discrimination paradigm was

utilized to assess interoceptive sensory properties of ghrelin in a rodent model (Davidson et al., 2005). In this model, a group of rats were trained to anticipate a sucrose pellet reward after 24 hours of food deprivation, while another group were trained to anticipate the reward after 1 hour of food deprivation. When all rats were given exogenous ghrelin mimicking the period of 24-hours of food deprivation, only the rats that were rewarded with sucrose under the 24-hour deprivation paradigm increased their activity. This indicates the process of introspective learning of food cues is indeed ghrelin-dependent and lends credence to the hypothesis that the main receptor for ghrelin—GHSR—in driving this process (Davis, 2018).

The Ventral Hippocampus as a Conduit for Regulating Feeding Behavior

Function as a Region Important in Food Intake and Energy Homeostasis

With this uncovered role of the hippocampus in regard to interpreting states of hunger and satiation, it was then pertinent to determine what structures within the hippocampus carry out these functions and further elucidate the level of involvement the hippocampus has in regulation of ingestive behavior. Anatomically speaking, the hippocampus can be split into dorsal and ventral sections. The dorsal hippocampus (DHPC) has not been shown to be a key component in feeding (Fanselow & Dong, 2010). This idea is supported by the finding that ghrelin delivery to the DHPC was not found to affect feeding through either meal size or meal frequency (Hock & Bunsey, 1998; Kanoski et al., 2013). However, GHSR-expressing neurons that are sensitive to ghrelin have been isolated to the ventral hippocampus (vHPC), and moreover, ghrelin injections to the vHPC promote meal frequency and size (Johnson & Leinninger, 2020; Kanoski et al., 2013; Mason et al., 2014; Zigman et al., 2006)—driving the hypothesis that the vHPC plays an important role in regulating feeding behavior. Findings of ventral hippocampal-mediated meal frequency are also supported by studies in rats showing that hippocampal lesions increase meal frequency

with no effect on overall food intake (Clifton et al., 1998). A suspected reason for this phenomenon plays into the role of the hippocampus in memory formation and consolidation. Temporary pharmacological inactivation of the vHPC following a sucrose meal was found to reduce latency until the next meal. Additionally, the amount of sucrose consumed in the subsequent meal was increased. Overall, the number of meals and intake of sucrose increased as well indicating the vHPC indeed plays a vital role in memory consolidation and inhibition of the vHPC affects postprandial energy intake (Hannapel et al., 2017).

Orexigenic Feeding Behavior and GHSR Expression

One of the ghrelin receptor's main roles in the ventral hippocampus is to drive orexigenic behaviors. In a rat model, ghrelin delivery to the vHPC was found to increase food intake particularly through an increase in meal frequency, yet higher doses of ghrelin administration were also found to increase meal size (Kanoski et al., 2013). This increase in meal size is found to occur via downstream orexin receptor signaling to the lateral hypothalamic area which projects to the laterodorsal tegmental nucleus of the hindbrain, indicating a potential circuit by which the vHPC can modulate feeding behavior (Hsu et al., 2015; Suarez et al., 2019).

From a systemic viewpoint, ghrelin signaling to the vHPC was also found to counteract food-intake reducing signals from the gut including cholecystokinin, a glucagon-like peptide-1 receptor agonist, amylin, and mechanical stomach distension, which all serve to reduce consumption as a feeling of satiation is achieved (Suarez et al., 2019). Additionally, vHPC ghrelin signaling in this model is found to produce interoceptive cues which cognitively serves to create a perceived state of negative energy balance which may explain this noticeable attenuation of satiation processing. This is further supported by Suarez et al., as they showed that rats in a food-deprived state spent more time seeking out a food magazine awaiting a sucrose reward compared to rats in a sated state. When ghrelin is administered to

the hippocampus of sated rats who associated the hunger state with anticipation of a food reward, the time spent in the magazine for the sucrose reward was increased as if they were in the food-deprived condition. Conversely, in rats trained to predict no reward in the presence of food deprivation, when given ghrelin under sated conditions responded by decreasing the time spent in the magazine as if they were in the food-deprived condition. This indicates that ghrelin administration to the ventral hippocampus not only drives food intake, but at subthreshold doses still plays a role in interoceptive cue processing to affect how a state of hunger or satiety will affect how future meals are approached.

Effects of Western Diet on Hippocampal-Dependent Learning & Memory

To explore how the WD can directly affect processes of hippocampal-dependent learning and memory, Davidson et al., trained rats on both a hippocampal-dependent discrimination task while on a standard lab chow diet. Rats were then split such that one group stayed on the chow diet, while the other group was given access to a high-energy (HE) diet. The highest weight gaining rats in the HE-diet group showed impairment to the hippocampal-dependent paradigm (Davidson et al., 2012). In comparison, the HE-diet resistant rats performed like the chow controls. Interestingly, all rats from all the groups performed the same on the hippocampal-independent discrimination task indicating the main learning impairment for the HE-diet prone group which gained the most weight was hippocampal-dependent in nature. This phenomenon was also found to be specific to the WD in particular and not just high fat diets in general as a follow-up study in rats showed that on a ketogenic diet—which is also high in fat and low in carbohydrates by design in order to induce ketosis—did not show the same level of hippocampal-dependent impairment in the FN discrimination task compared to the WD rats, specifically those that also developed obesity as a result of their WD exposure (Davidson et al., 2013). This indicates that not only does the WD directly impair hippocampal-dependent learning, but the severity of that impairment is

determined by one's level of obesity, with diet-resistant animals performing similarly to chow-fed controls in these studies. This impairment leads to a decrease in interoceptive cue processing such that obese animals show diminished ability in learning to utilize internal satiation cues, driving chronic overconsumption as a result and further worsening their obese state as the animal struggles to process when they are full (Davidson et al., 2005; Davidson & Jarrard, 1993; Kanoski & Davidson, 2011). The caveat here though is that the experiments described above were only performed in male rodents and thus there remains a distinct lack of understanding on the role sex plays in these hippocampal-dependent learning processes with WD exposure.

Summary and Aims of Dissertation

The growth hormone secretagogue receptor plays an integral role in ingestive behavior. In the ventral hippocampus, GHSR activation is found to modulate meal size and frequency as well as processing interoceptive cues regarding hunger and satiation. However, there is a lack of understanding as to how these processes are affected by obesity, particularly considering the role obesity plays in disrupting ghrelin secretion and ghrelin receptor sensitivity. Additionally, there is a distinct lack of investigation in the role of sex noted in studies regarding the ventral hippocampus, as most studies tend to skew towards a male rodent population. There is critical need to understand how these factors relate as both sex and vulnerability to obesity interact to influence ghrelin and GHSR signaling (Andrews, 2011; Briggs et al., 2010; Briggs & Andrews, 2011; Lockie et al., 2015; Naznin et al., 2015; Zigman et al., 2016). Chapter 2 describes the role sex and obesity proneness play in consumption, specifically testing the sufficiency and necessity of GHSR-expressing cells in invoking feeding behavior for chow or a high fat diet.

In addition, there is a lack of understanding as to the pre- (e.g., orosensory) and post-ingestive (e.g., gastrointestinal negative feedback) variables underlying GHSR vHPC modulation of ingestive behavior.

Accordingly, through a refined analysis of meal intake, I will examine whether GHSR-expressing vHPC cells control meal intake through their dissociable variables and the extent to which key macronutrient components contained within the HFD alter GHSR vHPC-dependent feeding behavior. I predict due to the perceived insensitivity of the ghrelin receptor as described in the aforementioned studies, that chemogenetic stimulation of GHSR-expressing cells in the vHPC will only modulate feeding in obese-resistant mice across all tests compared to obese-prone mice. Additionally, I expect chemogenetic inhibition will only reveal effects in obese-resistant mice—again due to sensitivity of GHSR in this group that is not present in obese-prone mice. Together, this dissertation serves to highlight sex differences and the role of dietary phenotype on the sufficiency and necessity of GHSR-expression in the ventral hippocampus for invoking feeding behavior.

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CHAPTER 2: SUFFICIENCY AND NECESSITY OF GHSR CELL-EXPRESSION IN HOME CAGE CONSUMPTION OF CHOW AND HIGH FAT DIET

Abstract

The gastric hunger signal, ghrelin, influences feeding behavior via activation of the growth hormone secretagogue receptor (GHSR). GHSR's are expressed in multiple brain regions including the ventral hippocampus (VHPC). This region plays a critical role in initiation and overall consumption during bouts of feeding. In this study, we used a GHSR-IRES-Cre mouse line in which the IRES-Cre cassette was inserted 3 bp downstream of the GHSR stop codon (Mani et al., 2017). Both male and female heterozygous GHSR-IRES-Cre mice were exposed to 8 weeks of high fat diet exposure (HFD) to discern phenotypes of diet-induced (DIO) or diet resistant (DR) obesity based off a sex-specific quartile split. The top and bottom quartiles were designated either DIO or DR respectively while the middle two quartiles were removed from further study. Mice then received either a Cre-dependent excitatory DREADD, inhibitory DREADD, or control mCherry virus into the VHPC to examine whether chemogenetic stimulation or inhibition of GHSR-expressing cells within this region would influence consumption of lab chow or a palatable high fat diet (HFD). On test days, mice were injected with clozapine-N-oxide (CNO) or vehicle solution prior to the onset of the dark cycle and given portioned access to either lab chow or HFD. CNO-evoked DREADD stimulation of VHPC GHSR-expressing cells significantly enhanced lab chow in DR females and decreased HFD consumption in DR males. Surprisingly, GHSR-expressing cell stimulation attenuated chow consumption in DIO males. CNO evoked inhibition of GHSR-expressing cells in the VHPC significantly decreased chow consumption in DR males and females and increased HFD consumption in DIO females exclusively. CNO evoked inhibition was also found to decrease chow consumption in DIO females. These data provide insight into the differential role of central nervous system GHSR-expressing cells on ingestive behavior that is in part sex-dependent as well as differences in obesity status that may affect the sensitivity of these cell populations to external manipulations.

Introduction

The hippocampus is an area traditionally associated with learning and memory, though more recently it has also emerged as an important region for the regulation of ingestive behavior. Anatomically, the hippocampus is split into two functionally distinct regions; the dorsal (DHPC) and ventral hippocampus (vHPC). The dorsal hippocampus is seen as an area important for cognitive functions related to the exogenous environment, such as spatial memory (Jung et al., 1994; Moser et al., 1995). Meanwhile, the ventral hippocampus has been depicted as the hippocampal region critical for interoceptive and endogenous signaling that includes meal regulation. Inhibition of vHPC neurons with the GABA-receptor agonist muscimol infused intra-vHPC postprandially was found to decrease latency and increased the amount of food consumed in the subsequent meal. (Hannapel et al., 2017). Additional support for the vHPC's role in meal regulation involves central administration of ghrelin to the ventral hippocampus which was found to increase meal intake via an increase in meal frequency as well as meal size (Dess et al., 2018; Johnson & Leinninger, 2020; Kanoski et al., 2013; Suarez et al., 2019). This is in contrast to the dorsal hippocampus in which ghrelin administration shows no effect on meal consumption (Kanoski et al., 2013).

The ability to regulate meal intake at the level of the vHPC is made possible—in part—by high expression levels of the target receptor for ghrelin; the growth hormone secretagogue receptor (GHSR). (Zigman et al., 2006). GHSR-expressing cells in the ventral hippocampus project to downstream hypothalamic feeding centers including orexin neurons in the lateral hypothalamic area (LHA), through which vHPC GHSR-expressing cells in modulate orexigenic feeding behavior (Suarez et al., 2019).

The magnitude of the effect of GHSR activity on ingestive behavior is modulated by diet. Mice that are more prone to becoming obese when exposed to a high fat diet (HFD) show a decreased sensitivity to ghrelin administration in the ARC. In fact, neither peripheral nor central administration of ghrelin is found to induce feeding in this population (Briggs et al., 2010; Mondal et al., 2005). This GHSR-specific resistance

is supported by studies in which activation of GHSR via ghrelin fails to induce NPY/AgRP expression in the ARC; however, central administration of NPY stimulates food intake. This suggests that certain brain structures (e.g., the ARC) are more vulnerable to the effects of HFD on disrupting ghrelin-evoked signaling, whereas as other downstream targets of NPY/AgRP are unaffected by dietary obesity (Zigman et al., 2016b). Accordingly, additional studies are required to determine whether upstream targets such as the vHPC are vulnerable to HFD exposure, and moreover whether the resistance seen with ghrelin generalizes to cells that express its receptor, GHSR.

The influences of HFD over the ghrelin system and vHPC are also modulated by biological sex (Sample & Davidson, 2018). Typically, ghrelin receptor binding in the hippocampus promotes dendritic synaptic spine synapse formation as well as generation of long-term potentiation (LTP) (Diano et al., 2006). However, DIO males show overall lower magnitudes of synaptic plasticity including LTP and long-term depression (LTD) compared to DIO females and diet resistant controls, particularly in Schaffer collateral-CA1 synapses. While the mechanism underlying these differences is unclear, given that the CA1 region displays high GHSR expression in the vHPC (Guan et al., 1997; Suarez et al., 2019; Zigman et al., 2006) these sex-dependent differences in plasticity could reflect GHSR-dependent changes. Additionally, DIO males also show poorer performance in hippocampal-dependent learning that is not present in DIO females nor diet resistant mice of either sex, indicating the effect of sex is potentially also dependent on how prone one is to developing dietary obesity (Hwang et al., 2010). Therefore, DIO male mice may be particularly susceptible to the effects of HFD exposure on disrupting vHPC GHSR-dependent feeding behavior.

Given the aforementioned findings, I chose to examine if GHSR-expressing cells in the vHPC—a region critical in regulation of meal intake and size—have differential effects on feeding depending on sex as well as proneness to obesity. To achieve this, I utilized a designer receptor exclusively activated by designer drugs (DREADD)-based chemogenetic approach in a GHSR-IRES-cre knock-in mouse model to determine the sufficiency (Experiment 1A/2A) and necessity (Experiment 1B/2B) of GHSR expression in the ventral

hippocampus in modulating consumption of either chow (Experiment 1) or a palatable high-fat diet (Experiment 2). I hypothesize that mice with a DIO phenotype will show decreased sensitivity to DREADD-evoked manipulations compared to DR mice. Additionally, I hypothesize that female DIO mice will be more sensitive to DREADD-evoked feeding effects compared to DIO males who I expect to show the greatest resistance to these manipulations.

Methods

Animals

An in-house GHSR-IRES-cre knock-in breeding colony was established in the vivarium of the Interdisciplinary Science and Technology Building at Michigan State University. The original colony mice were graciously donated by Dr. Jeffrey Zigman from the University of Texas-Southwestern. Heterozygous male and female GHSR-IRES-cre mice between the age of 3-9 months were bred and offspring were weaned between postnatal day 21-28. Heterozygous genotypes of offspring were confirmed using PCR where a mouse was heterozygous-positive if containing one copy of the WT GHSR allele and one copy of the GHSR-IRES-cre allele (Transnetyx Corp, Cordova, TN). Prior to HFD exposure, all mice were maintained on a standard lab chow diet 8940 (Envigo Corp., Indianapolis, IN). Mice were run in six separate cohorts, within each cohort of 32 mice—16 males and 16 females were placed on a high fat diet beginning at 6-8 weeks of age (HFD; see dietary manipulation) until a total of 6 cohorts, totaling 192 mice were bred and run through the HFD feeding paradigm. Mice were maintained under a 12:12 dark-light cycle for the duration of all studies with lights off corresponding to 6:00 PM EST and lights on at 6:00 AM EST.

Body composition analysis

All 192 animals were run through the Bruker Body Composition Analyzer, which utilizes time domain nuclear magnetic resonance (TD-NMR) to analyze signals from all protons throughout the sample volume to provide three values of interest: adipose, muscle, and free water composition of each mouse. Mice were placed in individual plastic cylinders and restrained with a rubber plunger before being placed into the sample chamber. This process involved no anesthesia and each mouse was restrained and scanned within 10 seconds, minimizing stress to the animals (Bruker Corp., Billerica, MA). All mice were analyzed on day 1 of the study—the first day of HFD exposure. Mice were then re-weighed at week 8 at which point only the mice that were designated DIO and DR had their body composition re-analyzed. This allowed for retroactive analysis to examine the nature of any weight differences between the DIO and DR phenotypes prior to and following HFD exposure.

Dietary manipulations.

Immediately after body composition analysis at 6-8 weeks of age, all mice in each cohort were randomly assigned to group housing of 4 mice per cage based on sex, with the exception of the first cohort in which all mice were single housed for the duration of study following the introduction of the HFD. Once in the new housing condition, laboratory chow was removed and replaced with *ad-libitum* access to a 4.73 kcal/g D12451 formula HFD (45% fat, 35% carbohydrates, and 20% protein; Research Diets, New Brunswick, NJ, USA). This rodent diet was chosen as the macronutrient composition closely resembles that of the human-consumed WD (Gajda, 2008; Hariri & Thibault, 2010; Hintze et al., 2012). Mice were maintained on this diet for 8 weeks with free access to water and the HFD in order to promote discernible differences in dietary obesity. Mice were weighed weekly to determine the percent change in body weight in relation to their baseline weight which was measured immediately prior to the first day of HFD access. A quartile

split was then performed at 8 weeks of HFD exposure with the top and bottom 25% weight gainers for each sex designated as diet induced obese (DIO) and diet resistant (DR), respectively. At this point, the middle 50th percentile of mice for each sex were excluded from further study and were immediately euthanized following IACUC-approved procedures for humane CO2-induced euthanasia techniques.

Stereotaxic Surgery

Following dietary cohort placement and body composition analysis, stereotaxic surgery was performed 8 weeks after initial HFD exposure. Mice from each cohort were randomly assigned to one of three viral manipulations such that there would be an even distribution of virus within each cohort and sex utilizing a chemogenetic approach via designer receptors exclusively activated by designer drugs (DREADD). 8 mice from each of the respective conditions (i.e., female DIO, DR; male DIO, DR) received bilateral injections of the excitatory AAV8-hSyn-DIO-hM3Dq(Gq)-mCherry virus to test the sufficiency of GHSR-expressing cells in the vHPC for evoking feeding behavior [henceforth referred as hM3Dq; Addgene]. A similar approach was adopted to examine whether inhibition of GHSR-expressing cells would disrupt feeding behavior such that 8 mice from each condition received an AAV8-hSyn-DIO-hM3Dq(Gq)-mCherry inhibitory DREADD virus [henceforth referred as hM4Di; Addgene]. Finally, in order to rule out effects of stereotaxic surgery or the chemogenetic manipulations on consumption and activity, a cre-dependent mCherry virus with no G-coupled receptor activity [henceforth referred as mCherry; Addgene] was given to the final group of 8 mice within each condition.

Viral injections were performed under isoflurane anesthesia with induction at 5% saturation and maintenance at 4.0% saturation which was dropped by ~1% every 10 minutes until 1.0% maintenance was reached for the remainder of each procedure, mainly to reduce complications of over-exposure to the lipid-binding capacity of isoflurane in the DIO mice (Meinwald et al., 1991; Turkyilmaz et al., 2011). Mice

were restrained using a Stoelting Brand stereotax (Stoelting Co., Wood Dale, IL). The coordinates for viral injection into the vHPC (-3.20 mm from Bregma, -5.00 mm from the dorsal surface, +/- 3.40 mm lateral) were chosen based on a mouse brain atlas and confirmation from prior injections performed by the Robison Research Group at Michigan State University. Under aseptic conditions, a dental drill was utilized to drill through the skull at the A.P. and M.L. coordinates before a Hamilton syringe (Hamilton Corp., Reno, NV) could be inserted through the drill site to reach the D.V. coordinates at which point 0.5 µl of the virus could be infused unilaterally into the vHPC and retained in that position for 5 minutes, after which the syringe was slowly retracted and the process repeated on the contralateral side. The incision site was closed using surgical staples and protected with a layer of triple antibiotic ointment.

All mice were also given buprenorphine 1 mg/kg s.c once per 24-hour period, and not exceeding more than one total injection per mouse except in cases of hunched posture or slow movements > 24 hours post-operation. Mice were postoperatively kept single-housed with free access to additional HFD and in certain cases solid chow and liquid chow supplementation in the event a mouse lost >20% of bodyweight from pre-operative weight. Post-operative weigh-ins and protocols were maintained daily for 10 days at which point staples were removed for all mice and were placed back in group housing conditions for 11 more days to reach 21 days of total post-operative recovery prior to experimentation, with the exception of the first cohort in which all mice were single-housed throughout the 21-day recovery period. This recovery period was also chosen to allow sufficient time for the virus to transfect the cell population of interest (Roth, 2016; Smith et al., 2016).

Feeding Tests

All mice first received home-cage feeding tests with laboratory chow. To prepare for these testing conditions and to reintroduce the laboratory chow, mice were housed individually in home cages and

were continually maintained on the HFD. However, they were also provided with 24-hour access to an ID-labeled plastic tray that would be exclusive to each mouse and remain in the cage for all test preparation days as well as on testing days. One pellet of laboratory chow 8940 comprised of 17% fat, 54% carbohydrate, and 29% protein generating 3 kcal/g (Envigo Corp., Indianapolis, IN) was placed on the tray. On each test preparation day mice were also handled for 5 minutes to acclimate to human touch for injections under the testing conditions. On the second day of preparation, chow pellets that were entirely consumed were replaced with a fresh pellet that was placed in the exact same plastic boat for another 24 hours in order minimize risk of neophobia. Uneaten or minimally eaten pellets were kept in the cage and were not replenished. At this time mice were also given a sham injection using a 30-gauge insulin syringe inserted intraperitoneally. On the third day of pre-test preparation, chow pellets that were entirely consumed were replaced with a fresh pellet as in day 2 conditions. Additionally, sham injections were repeated as in day 2 with the exception that mice were now injected with 5ml/kg volume of 0.1M phosphate buffer solution (PBS) which would also serve as the vehicle condition for test days.

Experiment 1A/B: Sufficiency and Necessity of GHSR Activity for Consumption of lab chow

To test the sufficiency and necessity of GHSR activity in the vHPC for modulating consumption, all access to the HFD and trays containing the laboratory chow were removed for mice given either the hM3Dq excitatory DREADD (Experiment 1A) or inhibitory DREADD hM4Di (Experiment 1B) or the control mCherry virus 1 hour prior to the study start time. Each test began at the start of the dark cycle with injections of either clozapine-N-oxide (CNO) (0.3 mg/kg) or vehicle (0.1M PBS). Injections were counterbalanced so that half of all virally-transfected mice tested on that particular day would receive CNO, while the other half would receive vehicle. These conditions were reversed for the alternate test day which would occur

after a 72-hour washout from the prior injection time, thus regardless of phenotype or sex, all mice received both a CNO and vehicle injection, the only difference being the order of delivery.

Additionally, for the control experiments, the same paradigm was run through a third time using mice transfected with an mCherry virus that has no associated G-protein coupled activity (supplementary figure 6). This was to determine if there were potential confounding variables of the drug treatments themselves aside from their DREADD-associated activity, as prior studies have described a risk of the pharmacologically inert CNO reverse metabolizing to the parent compound clozapine and exerting clozapine-like behavioral effects in rodents (MacLaren et al., 2016; Manvich et al., 2018).

Consumption began 20 minutes after the first injection to allow for CNO to cross the BBB and bind the mutant human muscarinic receptor to exert the pharmacological effects on GHSR-expressing cells (Alexander et al., 2009). At this time, the mouse was weighed and the plastic tray containing ~10 g of chow (between 2-3 pellets) was reintroduced into the home cage. Subsequently, the amount of lab chow consumed was weighed at 1hr, 2hr, 4hr and 12hr timepoints. At the 12-hour timepoint, the chow pellets were weighed and discarded, plastic trays removed from the cage until the next testing period, and the mice were all weighed to determine changes in weight over the duration of testing under both vehicle and CNO conditions. All cages were replenished with *ad libitum* access to HFD between testing and protocols for the second testing day were performed in the exact manner as outlined above, with the exception that the alternative injection was given on this day.

Experiment 2A/B: Sufficiency and Necessity of GHSR Activity for Consumption of HFD

HFD testing took place 72-hours after the start of the last day of chow testing to account for washout of CNO prior to this new testing condition. During that washout period, mice were reacclimated to the plastic tray 48 hours prior to testing, only this time a single HFD pellet was placed in the tray so the mouse could

routinely access the diet from the tray instead of the food hopper, however the mice were still maintained on *ad libitum* access to HFD during this acclimatization and washout period.

On test days, protocols followed the exact steps as outlined in the chow consumption study, with the exception that the plastic food tray used for the 12 hours of testing would contain ~10g of pre-weighed HFD. Mice and the HFD were weighed at the time of injection and were both re-weighed at the conclusion of the study at the 12-hour timepoint. The HFD was also weighed at the 1, 2, and 4-hour timepoints as described in Experiment 1 for mice transfected the excitatory hM3Dq (Experiment 2A), inhibitory hM4Di (Experiment 2B), or the control mCherry virus.

Statistical Analyses

To examine differences in weekly body weight (in grams) a repeated measures 3-way ANOVA was applied with week (1-8) X sex (male, female) X phenotype (DIO, DR) as factors. Follow-up phenotype X week ANOVAs were examined for each sex separately to determine the time point (in weeks) at which weight changes manifested between groups of animals. For the body composition analysis, phenotype X diet exposure (pre-, post HFD) was applied for fluid, lean and fat mass, with each sex analyzed separately.

To understand the effect of GHSR inhibition (hM4Di cohort) or activation (hM3Dq cohort) when exposed to either a chow diet or HFD, an analysis of variance (ANOVA) was performed to compare between-subject variables of phenotype and sex as well as drug and hour interactions.

For each viral condition, a drug (Veh, CNO) X hour (1,2,4,12) X phenotype (DR, DIO) 3-way ANOVA was applied to examine the overall effects of phenotype. Follow-up two-way ANOVAs were also applied. To examine whether baseline or drug differences in intake were observed between the phenotypes, an hour X phenotype ANOVA was applied for each drug. In addition, to examine within-subject sensitivity of the chemogenetic manipulations for within each phenotype, a hour X drug ANOVA was applied.

Overall sex differences between the phenotypes were explored via a drug X hour X sex (male, female) three-way ANOVA. Follow-up two-way ANOVAs were implemented to examine hour X sex for each drug, and hour X drug for each sex.

Finally, each of the effects of hM3Dq stimulation or hM4Di inhibition was examined separately for sex and each phenotype using two-way drug X hour ANOVAs. Significant interactions were followed up by tests of simple main effects and a planned comparison to determine at which time point of testing the effect of drug was significant. The α level for significance was .05 and all analyses were performed using Statistica software (Statsoft). Analysis of viral transfection is described in Chapter 3. This analysis was used to determine the exclusion criteria, where mice with no or minimal transfection would be excluded from analysis as they could not exhibit an effect of DREADD-mediated stimulation or inhibition. For the excitatory DREADD, the total included mice were n=4 DIO females, n=7 DR females, n=8 DIO males, and n=7 DR males. For the inhibitory DREADD, this included n=6 DIO females, n=6 DR females, n=7 DIO males, and n=6 DR males.

Results

Body Composition Changes with HFD Exposure

Figure 5 outlines differences in overall body weight and composition in DIO and DR male and female mice. In general, DIO mice gained weight at a greater rate than DR mice, with females (Fig. 5b) displaying weight differences between the phenotypes earlier following HFD administration than males (Fig. 5a). The week x sex x phenotype 3-way ANOVA revealed a significant 3-way interaction ($F[6,552] = 2.37$, $p<0.05$), which reflected significant differences in weight gain in females from week 2 (smallest $F[1,46] = 4.39$, $p<0.05$), whereas in males DR and DIO groups differed from week 4 onwards (smallest $F[1,45] = 5.15$, $p<0.05$). Additionally, housing status did not attribute to any significant variation in weight gain. Follow-up ANOVAs

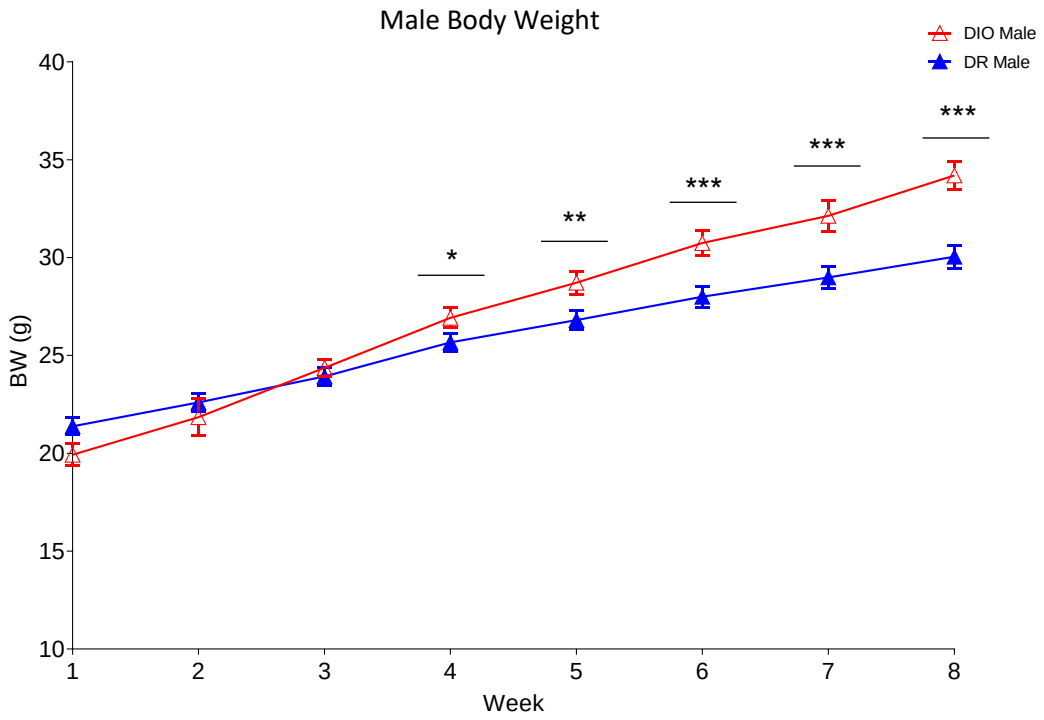
for weight gain by housing status revealed single compared to group housing had no major effect on resultant body composition data or weight gain (p's>.2), thus the individual group data could be collapsed without factoring in housing condition for the statistical analyses.

When body composition was examined in males, differences in fat composition both prior to and following the introduction of the HFD were revealed (Fig. 5c; Fat). A diet X phenotype ANOVA revealed in males a significant interaction between the two variables, with a follow-up planned comparison revealing a main effect of phenotype both pre-HFD exposure ($F[1,45] = 6.83$ $p<0.05$) as well as post-HFD exposure ($F[1,45] = 10.41$, $p<0.01$). In females, no baseline differences in fat composition were noted, though following the introduction of the HFD, DIO females displayed significantly greater fat composition compared to DR females (Fig. 5d; Fat). The drug x phenotype 2-way ANOVA for body fat revealed an interaction ($F[1,46] = 42.51$, $p<0.001$). A planned comparison revealed a main effect of phenotype post-HFD exposure ($F[1,46] = 39.29$, $p<0.001$) but not pre-HFD exposure ($F[1,46] = 1.95$, $p=0.17$).

For lean mass, prior to the introduction of the HFD, both DIO and DR males displayed equivalent lean mass composition, which was reduced significantly for DIO males following maintenance on the HFD (Fig. 5c; Lean). At this stage, DR male mice displayed elevated lean mass compared to DIO males. The diet X phenotype ANOVA revealed a significant two-way interaction ($F[1,45] = 12.36$, $p<0.01$), with follow-up planned comparison revealing a main effect of phenotype for lean mass post-HFD exposure ($F[1,45] = 15.05$, $p<0.001$) but not pre-HFD exposure ($F[1,45] = 3.04$, $p=0.09$). In females, similarly no baseline differences were noted in lean mass composition, but after HFD-exposure DR females were found to have higher lean mass composition (Fig. 5d; Lean). A diet x phenotype ANOVA revealed a significant interaction between the variables ($F[1,46] = 32.20$, $p<0.001$) due to a main effect of phenotype post-HFD exposure ($F[1,46] = 47.90$, $p<0.001$) but not pre-HFD exposure ($F[1,46] = 0.23$, $p=0.63$).

Finally, for fluid neither male (Fig. 5c; Fluid) nor female (Fig. 5d; Fluid) mice displayed any baseline differences between the phenotypes; however, following HFD exposure DR groups in both sexes displayed elevated fluid distribution. In males, a diet x phenotype ANOVA for fluid revealed a trend towards interaction ($F[1,45] = 3.95$, $p=0.05$) due to post-HFD exposure ($F[1,45] = 9.71$, $p<0.01$) but not pre-HFD exposure differences ($F[1,45] = .34$, $p=0.56$). In females, a similar pattern emerged, where a diet x phenotype ANOVA for fluid composition revealed a significant interaction ($F[1,46] = 11.26$, $p<0.01$). Follow-up planned comparisons revealed a main effect of phenotype post-HFD exposure ($F[1,46] = 27.43$, $p<0.001$) but not pre-HFD exposure ($F[1,46] = 0.10$, $p=0.75$). Overall, these results indicate that apart from a subtle baseline difference in fat composition for male DIO mice, all mice displayed similar weight and body composition prior to the introduction of HFD. However, following maintenance on this diet for 8 weeks, DIO mice displayed significant weight increase compared to DR mice due to an increase in fat body composition.

(a)



(b)

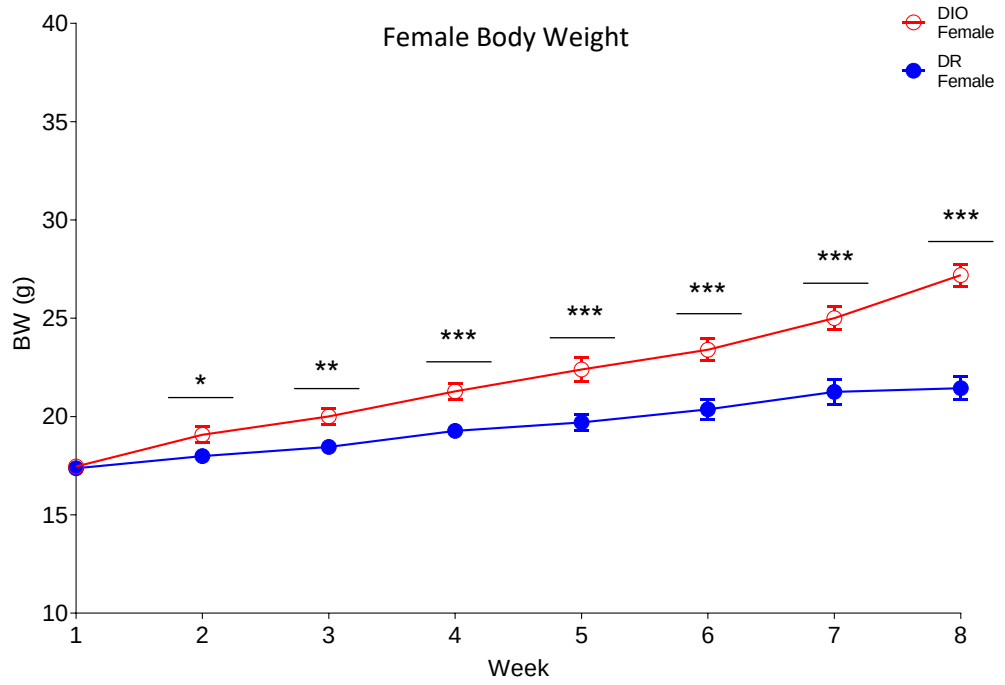
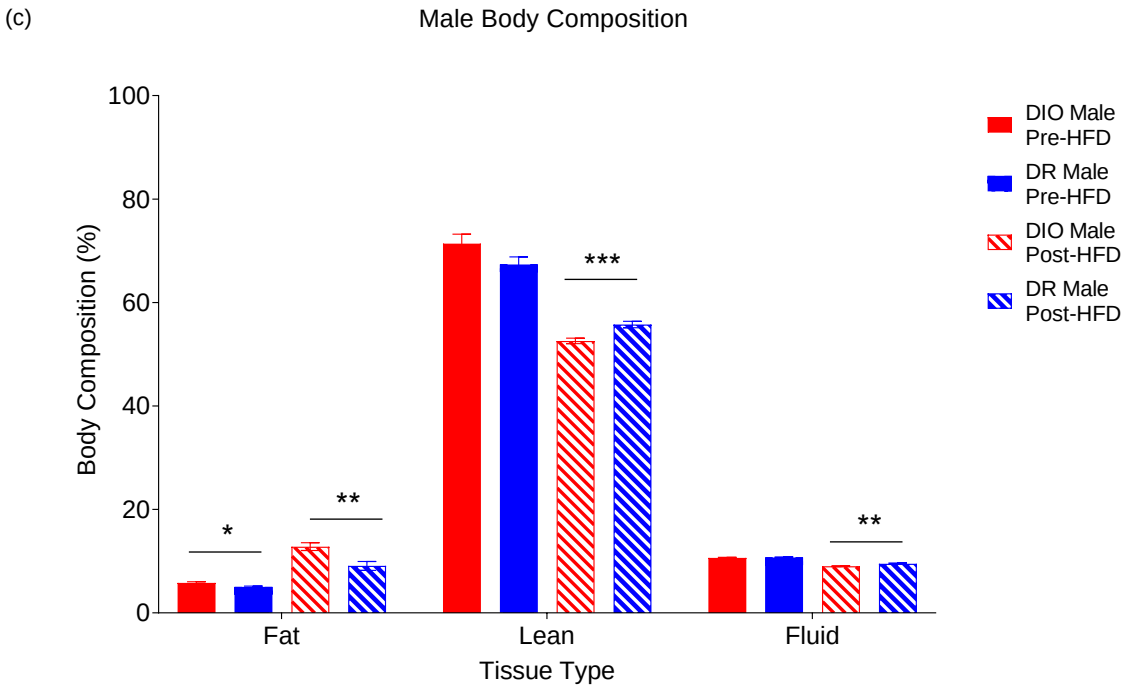


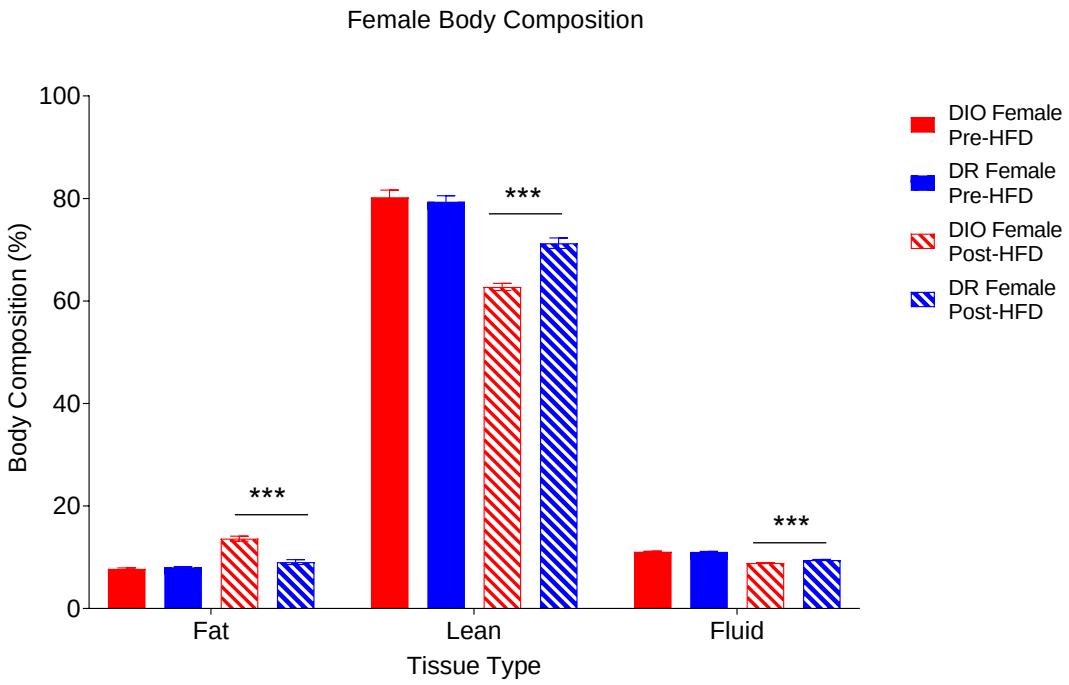
Figure 5. Phenotypic differences in weight gain for (a) male body weight and (b) female body weight after exposure to high fat diet. Body composition analysis before and after high fat diet exposure reported as percent of total body mass in (c) males and (d) females including metrics of fat mass, lean mass, and fluid mass. Main effects of diet indicated with *p<0.05, **p<.01, ***p<.001.

Figure 5 (cont'd)

(c)



(d)



Experiment 1A: Stimulation of GHSR-Expressing Cells in Ventral Hippocampus Increases Chow Consumption in DR Females

To begin, CNO administration to the mCherry group revealed no effect of drug under chow or HFD for any of the sex and phenotype combinations, revealing that CNO nor its metabolite clozapine induced any extraneous changes in behavior (F's<2.81, p's>.14), thus their data will not be included in the analyses but is available in supplementary figure 6.

Chemogenetic activation of vHPC GHSR-expressing cells led to differential effects on lab chow consumption dependent on sex and dietary phenotype (Figure 6). A sex x drug x hour 3-way ANOVA revealed a main effect of hour ($F[3,66] = 189.47$, $p<0.0001$), with a tendency towards effect of sex ($F[1,24] = 4.15$, $p=0.05$). A significant two-way drug X sex interaction ($F[1,24] = 5.63$, $p<0.05$) was also revealed. To better investigate the role of sex in regard to drug, sex x hour analyses for each drug were performed where an effect of hour ($F[1,72] = 127.75$, $p<0.0001$) and sex ($F[1,24] = 6.80$, $p<0.05$) was revealed for vehicle while only an effect of hour was revealed for CNO ($F[3,72] = 179.09$, $p<0.0001$), indicating that there were baseline differences in consumption with vehicle dependent on sex that ceased with administration of CNO (supplementary figure 1a). Thus, under vehicle conditions, male mice consumed more lab chow than females, whereas following CNO treatment, intake in males (but not females) was surprisingly reduced, resulting in equivalent chow intake for both sexes under these conditions. Finally, to confirm whether—within each sex—CNO had effects over chow intake, a drug X hour ANOVA was performed separately for males and females. This revealed in males a tendency for CNO to attenuate chow intake ($F[1,14] = 4.43$, $p=0.05$), whereas females were unaffected by this manipulation (F's<2.38; p's>.15).

To compare consumption differences based on phenotype, a drug x phenotype x hour 3-way ANOVA revealed a drug x phenotype interaction ($F[1,24] = 6.15$, $p<0.05$) and a main effect of hour ($F[3,72] =$

202.38, $p<0.001$). Hour x phenotype analyses for each drug revealed a main effect of phenotype ($F[1,24] = 6.00$, $p<0.05$) and hour ($F[1,72] = 139.76$, $p<0.001$) with vehicle while only a main effect of hour was revealed with CNO ($F[3,72] = 185.65$, $p<0.001$) indicating that CNO ameliorated baseline differences in consumption between phenotypes. In other words, CNO reduced chow intake in DIO mice such that intake was comparable to that of DR mice under both vehicle and CNO conditions. This was further confirmed by a drug X hour ANOVA for each phenotype. This revealed a main effect of drug for DIO ($F[1,11] = 6.51$, $p<0.05$) but not DR mice ($F<1$) (supplementary figure 1b).

Finally, the data of significant interest from this study, the individual group comparisons revealed in female DR mice, an expected increase in chow intake (Figure 6b), with a main effect of both drug ($F[1,6] = 24.01$, $p<0.01$) and hour ($F[3,18] = 139.05$, $p<0.001$). By contrast, male DR mice were insensitive to the chemogenetic manipulations (Figure 6a), with the ANOVA revealing a main effect of hour only ($F[3,18] = 27.29$, $p<0.001$). Interestingly, an opposite pattern was revealed in DIO groups, such that DIO females were insensitive to drug administration (Figure 6d; $F[1,3] = 2.2$, $p=0.23$), whereas males displayed a tendency for a reduction in chow intake following drug administration (Figure 6c; $F[1,7] = 5.29$, $p=0.05$).

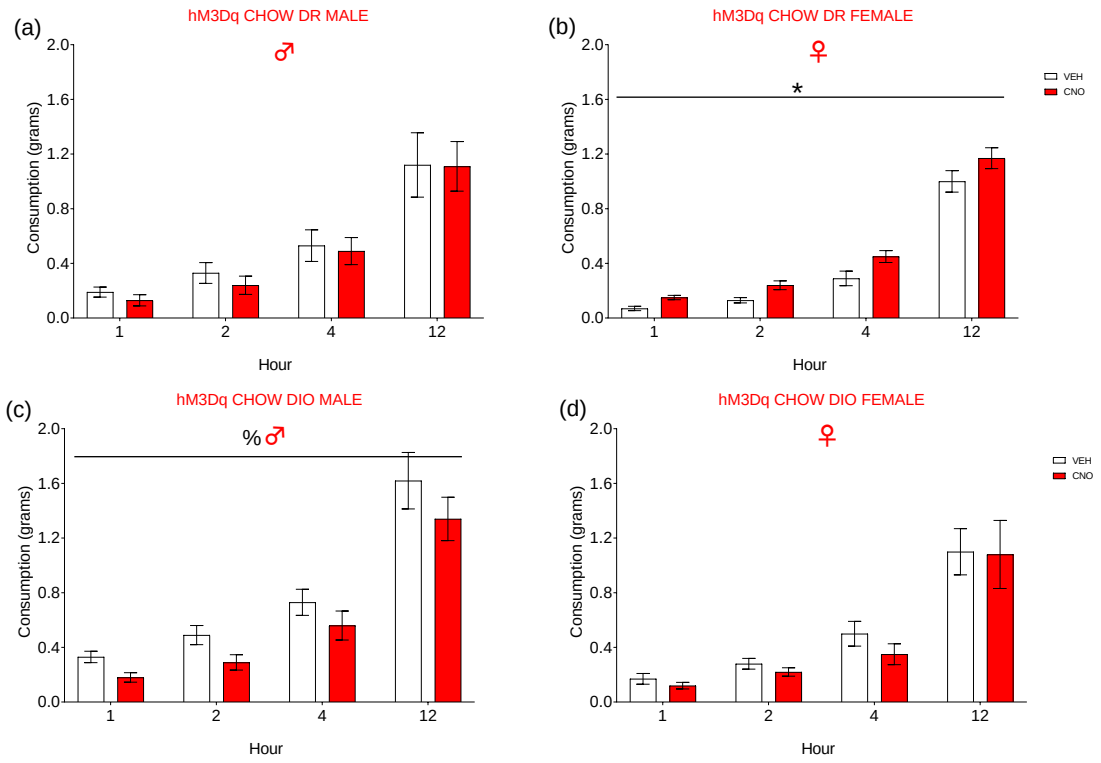


Figure 6. Experiment 1A. (a) Cumulative chow intake with Excitatory vHPC GHSR DREADD stimulation compared to vehicle. Individual group differences of chow consumption in (a) DR males and (b) females as well as (c) DIO males and (c) females. Main effects of drug indicated with *p<0.05, **p<.01, ***p<.001, # indicates main effect of drug at each time point, p≤0.05. % Indicates tendency towards effect of drug, p=0.05.

Experiment 1B: GHSR-Expressing Cell Inhibition in the Ventral Hippocampus Attenuates Chow Consumption in DIO Females

Chemogenetic inhibition of vHPC GHSR-expressing cells led to differential effects on lab chow consumption dependent on sex and dietary phenotype (Figure 7). A drug x hour x sex 3-way ANOVA revealed an effect of sex ($F[1,23] = 5.16$, $p<0.05$), drug ($F[1,23] = 7.84$, $p<0.01$), and hour ($F[3,69] = 157.97$, $p<0.0001$), but no interaction between the variables (F's<2.44, p's>.13). Follow-up hour x sex two-way ANOVA revealed a main effect of sex ($F[1,23] = 7.17$, $p<0.05$) and hour ($F[3,69] = 111.10$, $p<0.0001$) for CNO and an effect of hour for vehicle ($F[3,69] = 139.90$, $p<0.0001$). Thus, there were no baseline differences in chow consumption between males and females, but with administration of CNO females

were found to attenuate chow consumption (supplementary fig. 1c). To confirm whether—within each sex—CNO had effects over chow intake, a drug X hour ANOVA was performed separately for males and females. This revealed in females a main effect of CNO to attenuate chow intake ($F[1,11] = 11.02$, $p<0.01$), whereas males were unaffected by this manipulation ($F's<1$; $p's>.42$).

To compare consumption differences based on phenotype, a drug x hour x phenotype 3-way ANOVA revealed a main effect of hour ($F[3,69] = 152.22$, $p<0.0001$) and drug ($F[1,23] = 6.75$, $p<0.05$) indicating there was a general effect of drug, reflected by an attenuation of chow intake with CNO compared to vehicle (supplementary figure 1d). To confirm within each phenotype that CNO influenced chow intake, a drug x hour ANOVA was run separately for the DIO and DR groups. This revealed in the DR group a main effect of drug ($F[1,11] = 8.56$, $p<0.05$), whereas the DIO group was unaffected by CNO administration ($F[1,12] = 2.44$, $p=0.14$). Thus, DR mice in general were more sensitive to chemogenetic inhibition as reflected by a decrease in chow consumption compared to vehicle.

Individual group comparisons revealed no major changes with consumption in the DR males or females following CNO administration. Separate drug X hour ANOVAs confirmed that neither DR male (fig. 7a) or DR female (fig. 7b) mice displayed any changes in lab chow intake following CNO administration, with the analysis revealing a main effect of hour only for female ($F[3,15] = 16.73$, $p<0.0001$) and male ($F[3,15] = 74.82$, $p<0.0001$) DR animals and no effect of drug ($F's<4.12$, $p's>.1$).

Alternatively, in DIO mice, female (fig. 7d) but not male (fig. 7c) DIO mice displayed an attenuation of chow intake following DREADD-mediated inhibition of vHPC GHSR-expressing cells. Separate drug X hour ANOVAs revealed a main effect of hour only in DIO males ($F[3,18] = 61.07$, $p<0.001$), whereas in female DIO mice a significant drug X hour interaction was noted ($F[3,15] = 3.622$, $p<0.05$) due in part to significant reduction in lab chow at each time point (smallest F-value; hour 12, $F[1,5] = 6.85$, $p<0.05$). Overall, these

findings suggest that in both male and female DR mice, DREADD inhibition had little impact over chow consumption, whereas in DIO mice, females but not males showed a marked reduction in chow intake.

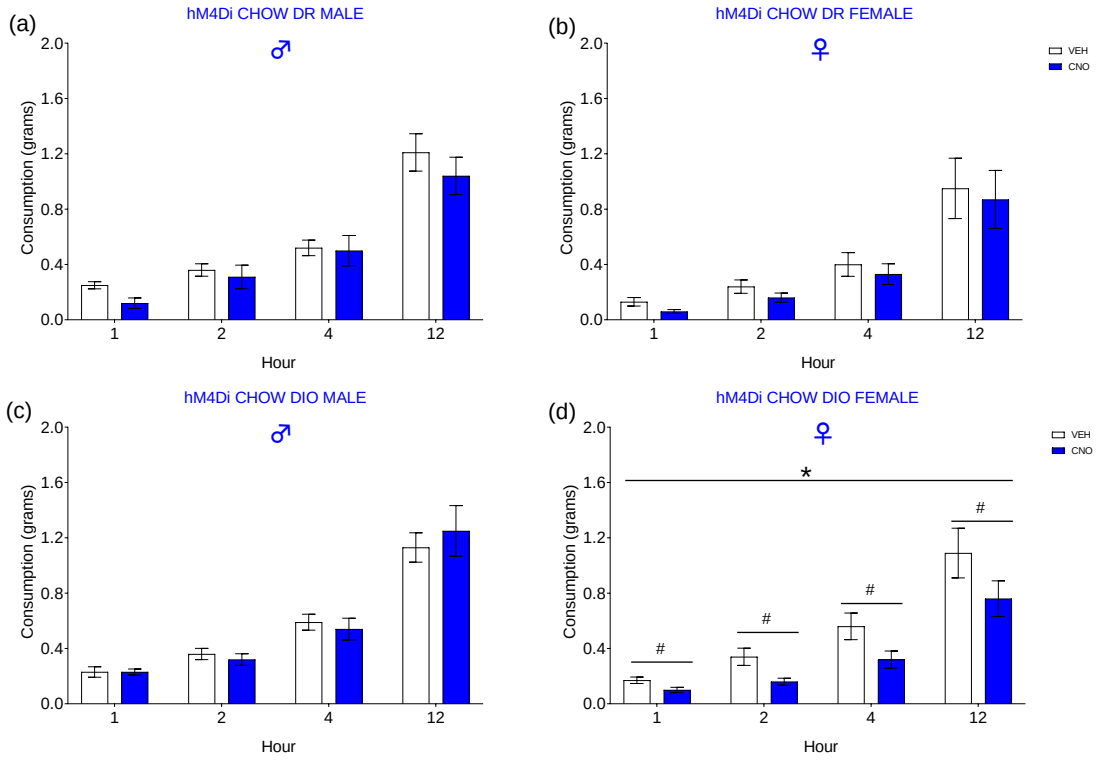


Figure 7. Experiment 1B. Cumulative chow intake with CNO-evoked inhibition of vHPC GHSR DREADD compared to vehicle. Individual group differences of chow consumption in (a) DR males and (b) females as well as (c) DIO males and (c) females. Main effects of drug indicated with *p<0.05, **p<.01, ***p<.001, # indicates main effect of drug at each time point, p≤0.05.

Experiment 2A; *GHSR-Expressing Cell Stimulation in the Ventral Hippocampus Attenuates HFD Consumption in DR Mice*

Chemogenetic activation of vHPC GHSR-expressing cells led to differential effects on HFD consumption dependent on sex and dietary phenotype (Figure 8). A sex x drug x hour 3-way ANOVA revealed a main effect of hour ($F[3,72] = 214.97$, $p<0.0001$) as well as drug ($F[1,24] = 4.75$, $p<0.05$). Hour x sex analysis for each drug revealed a main effect of hour for vehicle ($F[3,72] = 165$, $p<0.001$) and CNO ($F[3,72] = 137.46$,

p<0.001) indicating the absence of any sex differences at either baseline or following CNO (supplementary figure 2a). This was followed with a drug x hour analysis for each sex which only revealed a main effect of hour for males ($F[3,42] = 90.22$, $p<0.001$) and females ($F[3,30] = 269.9$, $p<0.001$).

To compare consumption differences based on phenotype, a drug x phenotype x hour 3-way ANOVA revealed a tendency towards interaction between the variables ($F[3,72] = 2.56$, $p=0.06$) along with significant main effects of drug ($F[1,24] = 4.57$, $p<0.05$) and hour ($F[3,72] = 231.51$, $p<0.001$). Hour x phenotype analyses for each drug revealed an interaction for the CNO condition ($F[3,72] = 3.18$, $p<0.05$) while for vehicle only a main effect of hour was revealed ($F[3,72] = 167.34$, $p<0.0001$) indicating CNO administration altered baseline differences in HFD consumption between the phenotypes. A planned comparison did not reveal a main effect of phenotype at any time point (F's<1.9, p's>0.18). In addition, a drug X hour ANOVA for each phenotype revealed an interaction for DR mice ($F[3,39] = 4.31$, $p<0.01$) but not DIO mice (F's<1, p's>.73) (supplementary figure 2b). Follow-up planned comparisons for the DR group revealed a significant main effect of drug at all tested timepoints (smallest F-value; hour 12, $F[1,3] = 7.43$, $p<0.05$). This indicates that stimulation of vHPC GHSR-expressing cells attenuated HFD consumption in DR mice, whereas in DIO mice these chemogenetic manipulations had no effect on intake.

The individual group comparisons revealed in male DR mice (fig. 8a), but not DR females (fig. 8b), a surprising attenuation in HFD consumption with CNO administration. This was confirmed with separate drug x hour 2-way ANOVAs which revealed a trend towards interaction in DR males ($F[3,18] = 3.07$, $p=0.05$) as well as a main effect of drug ($F[1,6] = 11.77$, $p<0.05$) and hour ($F[1,3] = 24.81$, $p<0.0001$). Follow-up tests of simple main effects revealed the attenuation was present at all timepoints (smallest F-value; hour 12, $F[1,6] = 6$, $p<0.05$). With DR females, only a main effect of hour was revealed ($F[3,18] = 128.97$, $p<0.0001$) indicating an insensitivity to the DREADD manipulations in this cohort of mice. In addition, DIO conditions were similarly insensitive to the effects of CNO on DREADD-mediated intake as the analysis revealed only a main effect of hour for male ($F[3,21] = 79.51$, $p<0.0001$) and female ($F[3,9] = 245.58$,

p<0.0001) DIO animals (fig. 8c,d). Overall, these findings suggest that in DR males, DREADD excitation led to a surprising decrease in HFD consumption, with all other groups of animals displaying an insensitivity to the excitatory DREADD manipulations.

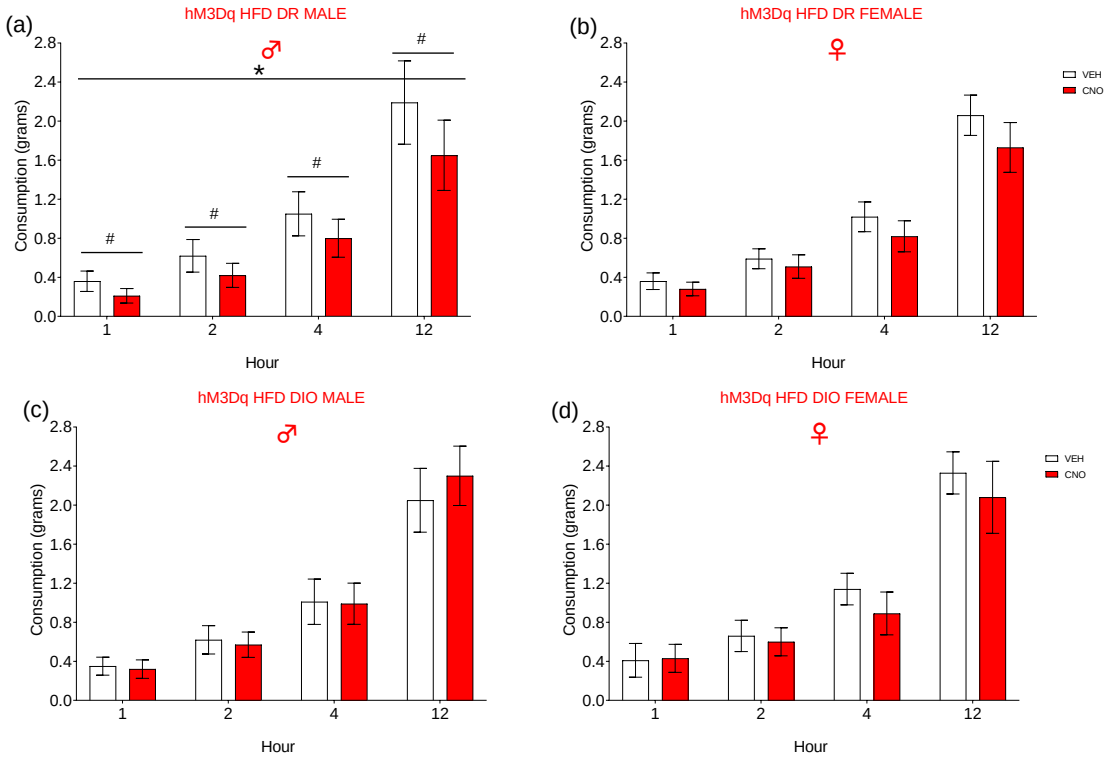


Figure 8. Experiment 2A. (a) Cumulative HFD intake with Excitatory vHPC GHSR DREADD stimulation compared to vehicle. Individual group differences of HFD consumption in (a) DR males and (b) females as well as (c) DIO males and (c) females. Main effects of drug indicated with *p<0.05, **p<.01, ***p<.001, # indicates main effect of drug at each time point, p≤0.05.

Experiment 2B: *GHSR-Expressing Cell Inhibition in the Ventral Hippocampus Does Not Influence HFD Consumption*

Chemogenetic inhibition of vHPC GHSR-expressing cells led to no changes in HFD consumption regardless of sex or phenotype (Figure 9). A sex x drug x hour 3-way ANOVA revealed a main effect of hour only ($F[3,69] = 316.52$, $p<0.0001$) (supplementary figure 2c). To compare consumption differences based on phenotype, a phenotype x drug x hour 3-way ANOVA revealed a main effect of hour ($F[3,69] = 348.90$,

p<0.001) as well as a tendency for a phenotype x hour interaction (F[3,69] = 2.7, p=0.05) (supplementary Figure 2d). Follow-up tests did not reveal an effect of phenotype at any time point (F's<3.17, p's>0.09).

Individual comparisons of the groups revealed no major differences in consumption. Separate drug X hour ANOVAs showed that neither male (fig. 9a) or female (fig. 9b) DR mice displayed any changes in HFD intake following CNO administration (F's>2.76, p's>0.08), with the analysis revealing a main effect of hour only for female (F[3,15] = 54.77, p<0.0001) and male (F[3,15] = 94.06, p<0.0001) DR animals. Similarly, drug x hour ANOVAs revealed only a main effect of hour in DIO males (F[3,18] = 59.75, p<0.0001) (fig. 9c) and DIO females (F[3,15] = 511.35, p<0.0001) (fig. 9d). Overall, these findings suggest that DREADD inhibition does not alter consumption of HFD regardless of phenotype or sex.

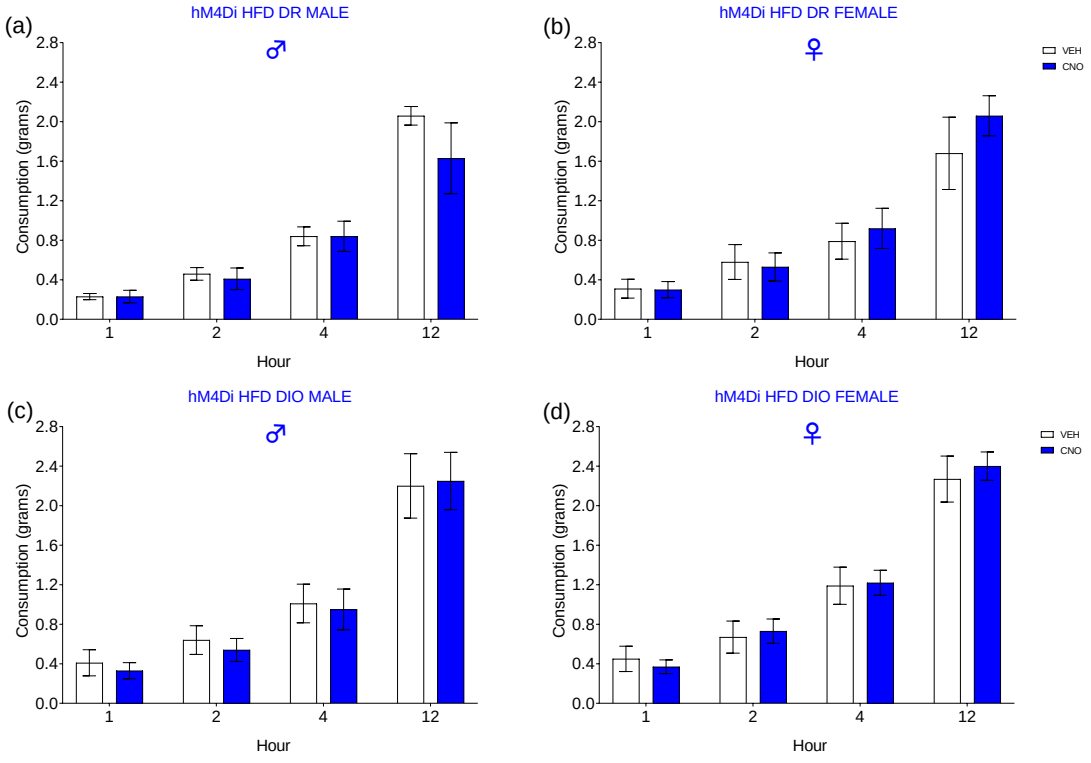


Figure 9. Experiment 2B. Experiment 2B. (a) Cumulative HFD intake with CNO-evoked inhibition of vHPC GHSR DREADD compared to vehicle. Individual group differences of HFD consumption in (a) DR males and (b) females as well as (c) DIO males and (c) females. Main effects of drug indicated with *p<0.05, **p<.01, ***p<.001, # indicates main effect of drug at each time point, p≤0.05.

Table 1. Summary of chemogenetic stimulation and inhibition of GHSR-expressing cells in vHPC on chow and HFD intake

CHOW Intake	DR Male	DR Female	DIO Male	DIO Female
Stimulation	-	↑	↓	-
Inhibition	-	-	-	↓
HFD Intake	**DR Male**	**DR Female**	**DIO Male**	**DIO Female**
Stimulation	↓	-	-	-
Inhibition	-	-	-	-

Discussion

Males in general were found to have increased baseline consumption of chow compared to females which has been validated in multiple studies prior; however, these differences were ameliorated with CNO administration as demonstrated by feeding suppression with GHSR-expressing cell stimulation in males; this is a novel finding considering GHSR activity typically increases meal intake (Giles et al., 2016; Kanoski et al., 2013) (supplementary figure 1). Surprisingly, baseline differences between the sexes with chow consumption were more apparent in the hM3Dq cohort than the hM4Di cohort, potentially reflective of increased variability due to the number of exclusions made for mice that did not meet criteria for sufficient viral transfection, with the greatest amount of exclusions made in the female DIOs given hM3Dq (n=4 exclusions). Regardless of sex, males and females consumed an equal amount of HFD at baseline, and both were found to attenuate HFD consumption with CNO-evoked stimulation (supplementary figure 2). When comparing phenotypes, DIO mice expectedly consumed more chow than DR animals at baseline. Comparatively, no baseline differences in HFD consumption were noted, however GHSR-expressing cell activation was found to attenuate HFD consumption in the DR group, revealing important novel differences in sex and phenotype on consumption of CHOW with CNO-evoked activation or inhibition of GHSR-expressing cells in the vHPC.

DIO mice provided *ad-libitum* access to HFD gained significantly more weight than their DR counterparts. Interestingly, the timing of when these phenotypes manifested differed based on sex, where females

established phenotypic differences by week 2 of HFD exposure while males exhibited these differences by week 4. This is in contrast to other studies that indicate females develop DIO at a slower rate than males (Giles et al., 2016). However, the majority of these studies investigating sex differences in obesity phenotyping use a rat model whereas phenotyping in the GHSR-IRES-cre mouse model is poorly characterized, particularly with females. (Mani et al., 2017; Stoltenborg et al., 2022). In fact, a literature search revealed no such phenotyping has ever been performed in females of this mouse strain with *ad libitum* access to HFD. Our results reveal a novel finding of weight gain based on obesity proneness in both male and female GHSR-IRES-cre mice.

Body weight of females has been shown to vary with levels of lean and fat distribution such that higher weight females may be heavier due to higher lean composition while smaller females can have higher fat composition and less lean mass; again with the caveat that most of these phenotypic effects were revealed in rats (Chang et al., 1990; Giles et al., 2010, 2012). This was not found to be the case in our study as the DIO females were all significantly heavier than DR females by the 8-week point, with the highest proportion of that mass derived from adipose tissue and not lean muscle for the DIO group. This demonstrates another novel finding in the GHSR-IRES-cre model that female DIOs have consistently higher fat mass and lower lean mass which can be reflected by the average weight where in our case—regardless of sex—the highest weight mice also had the highest proportion of fat mass to lean mass, contrasting the aforementioned studies that indicate higher weight in and of itself may not be indicative of diet-induced obesity in rats.

Our body composition results demonstrated in both sexes that DIO's have higher fat and lower lean composition compared to their DR counterparts which is consistent with the aforementioned studies that reflect similar body composition differences with exposure to HFD (Giles et al., 2016; Le May et al., 2021). Surprisingly, DIO males had a modestly higher fat composition than DR males prior to the HFD; the magnitude of which was increased after HFD exposure. Comparatively, DIO and DR females showed no

body composition differences pre-HFD exposure. This is a novel finding in our GHSR-IRES-cre mouse model that may reflect a potential predictor of dietary-induced obesity and warrants replication to confirm that higher adiposity under a chow diet predicts DIO status with HFD exposure in males. Replication is also warranted to confirm this isn't a result of the mouse model itself, as heterozygous GHSR-IRES-cre knock-in mice—which we used in our study—were reported to have inconsistencies in metabolic characteristics compared to WT including a lack of fasting-induced growth hormone release and decreased insulin and blood glucose levels compared to WT. (Peris-Sampedro et al., 2021).

With regard to chow feeding tests, effects of CNO administration were dependent on the combination of sex and phenotype. DR females showed an expected increase in chow consumption with CNO-evoked stimulation of GHSR-expressing cells in the vHPC while DR males showed an insensitivity to the manipulations. Considering this is the first known study exploring the effects of vHPC GHSR-expressing cell excitation in females, these findings in the DR females are consistent with prior studies in males of ghrelin activity evoking a hyperphagic response by binding to its target receptor GHSR (Faulconbridge et al., 2003; Swartz et al., 2014). In the vHPC this has been depicted as an increase in meal frequency and meal size (Kanoski et al., 2013; Suarez et al., 2019). Interestingly, this effect was not replicated in our DR male model. We must make note that our experiments also explored phenotypic differences in GHSR activity which have not been thoroughly explored in the vHPC before. Other studies describe diet-induced obesity leading to a form of ghrelin resistance in males, particularly in areas of the hypothalamus such as the ARC whereas our model shows the opposite effect and an insensitivity to GHSR stimulation in the vHPC is revealed in the DR males (Briggs et al., 2010; Lockie et al., 2015; Zigman et al., 2016a). Additionally, our study utilized a quartile split and eliminated the middle 50th percentile of weight gain so we could better investigate the most extreme examples of DIO and DR to elucidate phenotypic differences more readily. The aforementioned studies in the vHPC could not address phenotypic differences as the animals in said studies were not chronically exposed to HFD. A follow-up study could explore the differences in

the non-extremes of weight gain or resistance in that middle 50th percentile. This should indicate if the GHSR-IRES-cre mouse line does indeed replicate results previously shown in WT-mice or rat models, or if there are inherent differences in behavior with our model regardless of obesity phenotype.

Conversely, DIO males showed a surprising attenuation of chow consumption with stimulation while DIO females showed an insensitivity to the manipulations. These findings contrast with multiple studies in which males are expected to show a hyperphagic response with neuronal excitation, particularly in the vHPC (Kanoski et al., 2013; Suarez et al., 2019). These differences may be due to experimental design as the other studies examining GHSR activity tend to use ICV or direct ghrelin administration whereas we utilized a chemogenetic approach in our GHSR-IRES-cre model. Although this is the first study to utilize chemogenetics in assessment of GHSR-expressing cell activity in the vHPC, chemogenetics has been successfully used in the GHSR-IRES-cre model to elucidate GHSR's role in the mediobasal hypothalamus, where CNO-evoked inhibition of this orexigenic feeding center led to an expected attenuation in feeding (Peris-Sampedro et al., 2021). Our study may demonstrate a more robust understanding of GHSR's role in the vHPC that is indeed dependent on sex and phenotype, such that DIO males responding atypically to GHSR-evoked stimulation may reflect a rescuing of the mechanism for regulation of energy balance through interoceptive cue processing resulting in the decrease in chow consumption, as GHSR activation at the level of the vHPC is found to be a critical component of satiety processing (Davidson & Jarrard, 1993; Kanoski, 2012; Suarez et al., 2019). Considering DIO animals have lower plasma ghrelin and blunted pre-prandial ghrelin spikes—and therefore less bioavailability for GHSR binding—we hypothesize that this leads to a decrease in interoceptive cue processing for DIO animals represented as an insensitivity to satiety cues as described by Davidson et al., (Briggs et al., 2010; Davidson et al., 2012; Levin et al., 2003; Li et al., 2011; Sample et al., 2015; Zigman et al., 2016a). With our mouse model, GHSR stimulation can rescue the response to those interoceptive cues such that DIO males can re-learn meal intake patterns to resemble those of DR animals and reduce consumption accordingly.

GHSR-expressing cell activity had minimal impact on HFD consumption, with the surprising exception of attenuation in feeding with stimulation in DR males. This is mostly in agreement with multiple studies that demonstrate central ghrelin administration—and therefore activation of the target GHSR—does not result in hyperphagia of HFD as it does with chow (Perez-Tilve et al., 2011). In fact, ghrelin administration is found to increase preference for chow (Bake et al., 2017). These results have also been demonstrated with endogenous ghrelin, as fasting-induced ghrelin secretion is found to increase preference for chow as well. This is believed to be due to ghrelin driving food-seeking towards more nutritious foods (Bake et al., 2017). This may also explain the novel result in our DR males where GHSR activation was found to attenuate HFD consumption, potentially due to a preference for chow that was not available during the HFD feeding tests. Additionally, GHSR activation is found to increase novelty seeking in rodents as well as human males (Hansson et al., 2012). Considering the mice were exposed to HFD for over 12 weeks by the point of testing, the chow would have been considered novel compared to the HFD and thus potentially further increased preference for the more highly nutritive diet.

Conclusion

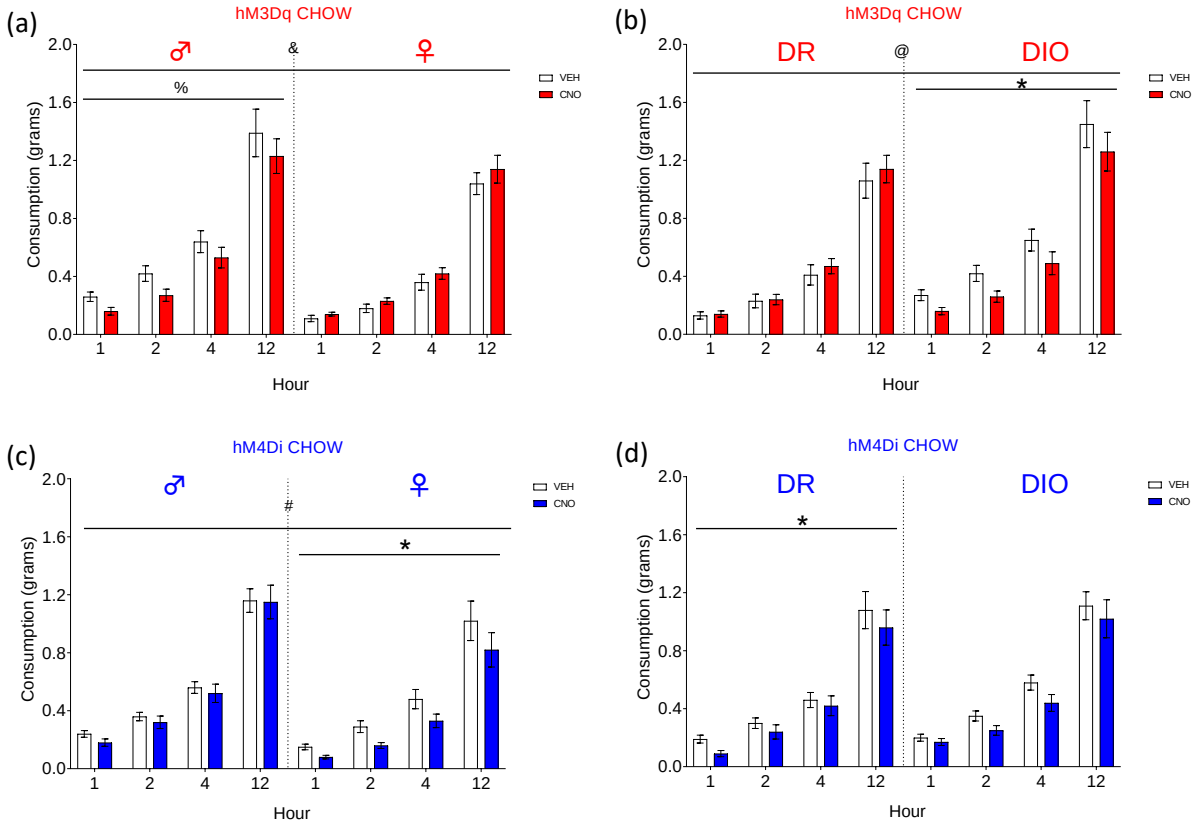
For the first time in the vHPC, effects of sex were noted with GHSR activity as demonstrated by differences in both chow and high fat diet consumption with GHSR activation and inhibition. These sex differences in dietary consumption highlight the importance of including both biological sexes in experimental design. We also showed that susceptibility to dietary obesity affects overall consumption of both chow and HFD, indicating differences in feeding are potentially also affected by one's proneness to developing dietary obesity.

Finally, we showed that the combination of phenotype and sex plays a differential role in consumption where stimulation of vHPC GHSR was found to increase chow consumption in DR females while DIO males

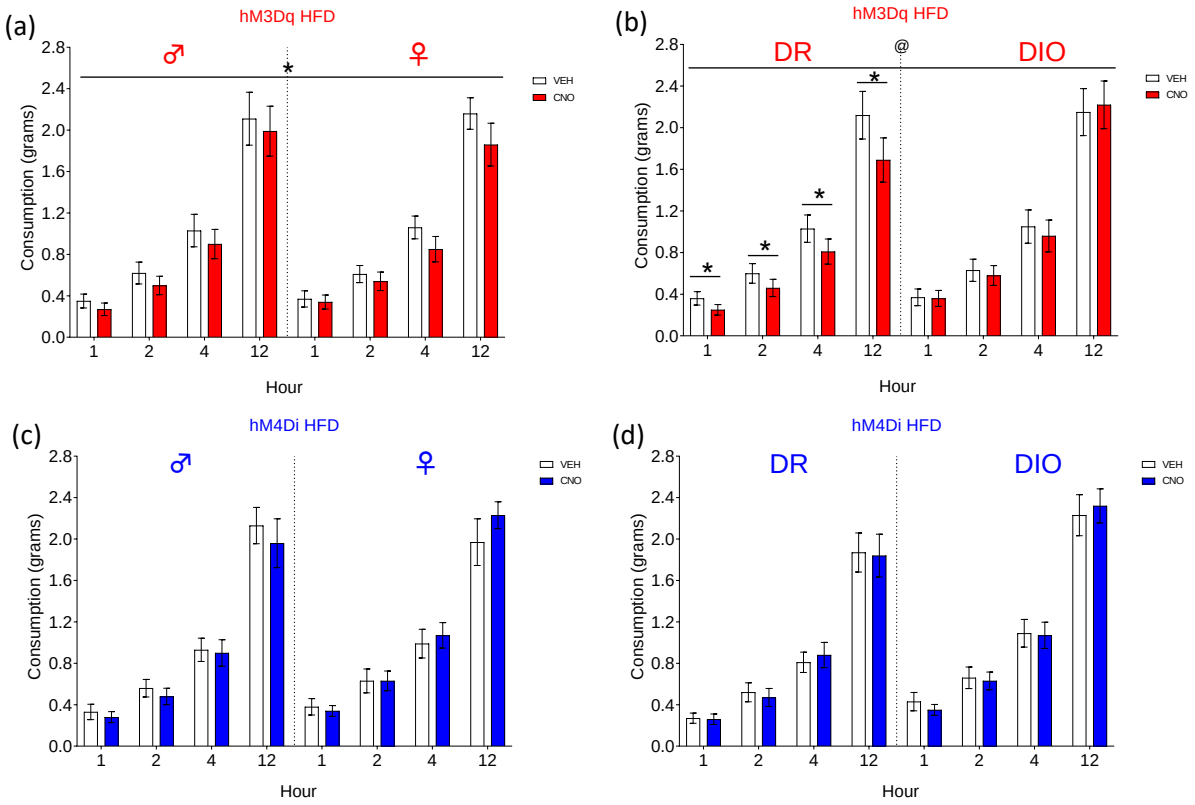
were found to attenuate chow consumption. Comparatively, CNO-evoked inhibition of GHSR-expressing cells was found to decrease chow consumption in DIO females exclusively. Surprisingly, activation of GHSR-expressing cells was found to attenuate HFD consumption in DR males. Overall, these results build on the established role of the vHPC as an important site for the regulation of meal intake, the ability of which is affected both by sex and dietary obesity.

APPENDIX

Supplementary Figures



Supplementary Figure 1. Cumulative chow intake with Excitatory vHPC GHSR DREADD stimulation comparing (a) sex as well as (b) phenotype. Cumulative chow intake with Inhibitory vHPC GHSR DREADD comparing (c) sex and (d) phenotype. Main effects of drug indicated with *p<0.05. & Indicates main effect of sex for vehicle condition p<0.05. # Indicates main effect of sex for CNO condition p<0.05. @ Indicates main effect of phenotype for vehicle p<0.05. % Indicates tendency for main effect of drug p=0.05.



Supplementary Figure 2. Cumulative HFD intake with Excitatory vHPC GHSR DREADD stimulation comparing (a) sex as well as (b) phenotype. Cumulative HFD intake with Inhibitory vHPC GHSR DREADD comparing (c) sex and (d) phenotype. Main effects of drug indicated with *p<0.05. @ Indicates main effect of phenotype for CNO condition p<0.05.

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CHAPTER 3: SUFFICIENCY AND NECESSITY OF GHSR-EXPRESSING CELL ACTIVITY FOR ASSESSMENT OF PALATABILITY AND POST-INGESTIVE FEEDBACK INHIBITION FOR MACRONUTRIENT COMPONENTS OF HIGH FAT DIET

Abstract

The Western Diet is characterized by calorically dense and nutritionally poor foods containing excess amounts of processed sugar and fat. These macronutrients increase the palatability of foods while also decreasing satiety signaling and postingestive negative feedback from the gut to the CNS leading to overconsumption beyond metabolic need. The vHPC is implicated in this processing of postingestive satiety cues via GHSR cell activity in this region. Stimulation of this cell population has been previously shown to counteract the food intake-reducing effects of peripheral satiety signaling.

To examine intake of specific macronutrients present in the Western Diet and the nature of any changes in ingestive behavior via GHSR-expressing cell activity at the level of the vHPC, we separately investigated fluid intake of lipid (45% corn oil), and sugar (20% sucrose) through an analysis of licking microstructure. This quantitative approach is capable of dissociating overall intake into individual components of pre-ingestive (e.g., orosensory, palatability; via burst size) and post-ingestive (e.g., negative feedback; via burst number) variables that regulate meal intake. To interrogate this cell population, we utilized a chemogenetic approach to stimulate or inhibit GHSR-expressing cells of the vHPC.

The chemogenetic manipulations mainly influenced short-term sucrose solution intake in female mice, such that excitation of GHSR-expressing cells in this region increased lick rate of sucrose for DR females and decreased post-ingestive negative feedback for females in general. Meanwhile, inhibition was found to decrease palatability for sucrose in DR females exclusively. Conversely, neither DREADD activation or inhibition of GHSR-expressing cells significantly altered the burst size or burst number with corn oil for either males or females. These data provide insight into the differential role of central nervous system GHSR-expressing cells on ingestive behavior that is in part dependent on the neuronal loci in which they are expressed and the nature of the tastant being consumed.

Introduction

Consumption of food involves the intricate coordination of CNS and sensory taste signals as food travels through the oral cavity to the periphery, where gastrointestinal post-ingestive feedback helps guide meal intake. (Davis & Campbell, 1973; Davis & Levine, 1977; Poothullil, 1995; Sclafani, 2013; Treesukosol et al., 2011). These processes work in tandem to maintain a state of energy homeostasis (Hopkins & Blundell, 2016; Lovejoy et al., 2009). However, with increasing prevalence of the Western Diet (WD), a state of persistent caloric excess and a misbalance of caloric intake to caloric expenditure can manifest, leading to increased adiposity, obesity and a host of associated sequelae (Kopp, 2019; Rajala & Scherer, 2003; Rakhra et al., 2020). Because overconsumption is so critical to the development of obesity, we sought to explore what in particular drives a mouse to consume. Specifically, we chose to investigate differences in pre-and post-ingestive measures of feeding behavior. Given the difficulties of assessing chow consumption through a liquid medium, I selected to investigate two of the more palatable components of the HFD—sucrose and fat. These macronutrients were chosen given their high palatability more easily drives excess consumption beyond metabolic need and increases the risk of developing obesity (Hallfrisch et al., 1981; Malafaia et al., 2013; Rasool et al., 2018; Surwit et al., 1995).

Additionally, prior studies show sex and obesity proneness significantly impact the consumption of these tastants. Females are shown to have higher preference for sucrose over water by body weight compared to males and consume proportionally more calories from sucrose over a 24-hour period (Grimm et al., 2022; Sclafani et al., 1987). Females are also shown to have a lower licking rate at low concentrations of sucrose solutions compared to males (Curtis et al., 2004). Additionally, DIO phenotypes are found to consume less sucrose in a consumption test compared to control animals reflecting a potential interaction of phenotype that warrants further exploration as well (Johnson, 2012). Corn oil is regularly used as a tastant for assessing fat preference, and findings show there is minimal difference in preference for corn oil between sexes in rodent models (J. C. Smith et al., 2000; Stratford et al., 2008). Interestingly for corn

oil, a more pronounced effect on consumption is seen between obesity proneness and resistance instead of sex, where obese prone rats are found to prefer fat consumption whereas obese resistant rats prefer consumption of carbohydrates (Gilbertson et al., 2005; Okada et al., 1992)

These macronutrient components of the HFD—mainly sugar and fat—can be investigated through a refined analysis of meal intake known as licking microstructure (Davis, 1973; Johnson, 2018). Licking microstructure is capable of calculating temporal distributions between pauses of licking behavior as well as individual licks to distinguish between stimulatory and inhibitory influences of ingestive behavior. Licks with an interlick interval—periods between licks—for a mouse of < 250 ms are grouped together to determine the burst size (Davis & Smith, 1992). The average size of all these bursts of licking serves as a measure of how palatable a mouse finds a particular tastant. This has been validated by studies showing that as one increases the concentration of a sucrose solution, a monotonic relationship forms with the rate of licking such that the average burst size increases as a result of the increased sucrose concentration (Davis & Perez, 1993; Davis & Smith, 1992; Spector et al., 1998). Comparatively, overall intake is depicted as an inverted-U-shape (rather than monotonic) function, where as one increases the sucrose concentration, a peak is reached at intermediate concentrations and decreases at higher concentrations due to increased caloric density and thus earlier satiation (Johnson, 2018).

Additionally, a separate factor controlling meal intake—post-ingestive negative feedback—can also be measured through licking microstructure analyses via the total number of bursts of licking behavior. With this measure, a pause in licking behavior is defined by intervals > 1000 ms (Johnson et al., 2010). Burst number reflects post-ingestive feedback, whereby the caloric and colligative properties of food accumulate in the gastrointestinal tract and diminish the likelihood of further engaging in meal intake. Thus, burst number diminishes greatly when consuming high energy foods (Davis & Smith, 1992; Spector et al., 1998), whereas it remains persistently high regardless of caloric load, when rats are tested under

conditions that prevent post-ingestive feedback (i.e., via gastric fistula) (Johnson, 2018; Johnson et al., 2010; Spector et al., 1998).

Because of its role in ingestive behavior as well as learning and memory, the hippocampus—particularly the ventral hippocampus (vHPC)—is an optimal candidate for licking microstructure analysis. The reason being is that most animals including rodents and humans have habitual feeding patterns in which meals are consumed based off learned and environmental factors which are processed—in part—by the vHPC (Hsu et al., 2016, 2018; Kanoski et al., 2013). A large proportion of cells in the vHPC express the growth hormone secretagogue receptor 1a (GHSR), the target for the peripheral feeding signal ghrelin (Zigman et al., 2006). GHSR-specific signaling in the vHPC is found to produce interoceptive cues that emulate a perceived state of energy deficit, thereby attenuating the response of reciprocal satiation signals (Suarez et al., 2019).

However, quantifiable differences in licking microstructure via GHSR activity in the vHPC have yet to be elucidated, nor have sex differences or phenotypic differences between DIO and DR animals been explored in this region. These factors are particularly important as not only do rates of obesity differ between males and females, but prior studies indicate that obese-prone animals are found to develop a resistance to ghrelin-mediated feeding effects in other brain regions (Briggs et al., 2010; Link & Reue, 2017; Lovejoy et al., 2009; Mauvais-Jarvis, 2015).

To examine the variabilities in licking microstructure between DIO and DR phenotypes as well as between sexes, we utilized a GHSR-IRES-cre knock-in mouse model. Mice were given local infusions of one of three DREADD vectors: the excitatory Gq-coupled hM3Dq, a Gi-coupled inhibitory hM4Di, or a control mCherry virus as described in Chapter 2. This allowed for chemogenetic activation or inhibition of GHSR-expressing cells in test subjects when the designer receptor is bound by its highly selective exogenous ligand CNO. Mice were given separate 20-minute consumption tests with a highly palatable 20% sucrose solution as

well as a 45% corn oil solution—best approximating the proportions found in the HFD. A third test was run with DI water to serve as a control using a non-nutritive substance (Johnson et al., 2010). The average licks per minute, as well as average burst size and total burst number were measured to determine whether CNO-evoked excitation or inhibition of GHSR-expressing cells in the vHPC differentially affected licking behavior of mice based on sex and dietary phenotype.

Methods

Animals

The in-house GHSR-IRES-cre knock-in breeding colony as described in Chapter 2 was established in the vivarium of the Interdisciplinary Science and Technology Building at Michigan State University.

Apparatus

The licking microstructure paradigm began immediately after conclusion of the home-cage consumption studies as outlined in Chapter 2. Here, all experiments were performed in consummatory chambers consisting of clear polycarbonate sides and ceiling, aluminum front and back walls, and a floor comprised of parallel, stainless-steel rods, all housed in sound-attenuating shells (Med Associates, St. Albans, VT). The chamber was outfitted with a custom-built food cup into which 50 µl of a liquid reward could be delivered. Any unconsumed liquid could be removed via a programmable vacuum connected to the food cup. The food cups contain a lickometer with a fiber optic cable on either end to introduce a light beam through the fluid-air interface of the fluid bolus (Schoenbaum et al., 2001). Licks could be registered through disturbances in the beam as the mice licked and consumed the bolus. This allowed for time stamped intervals of individual licks recorded through MedPC software (Med Associates, Inc., Fairfax, VT).

The liquid solutions used in this apparatus included a 20% sucrose solution dissolved in deionized (DI) water, a 45% corn oil solution emulsified in DI water, or DI water exclusively to serve as a control.

Experiment 3/4: Brief-Access Licking Microstructure Testing of Sucrose and Corn Oil

In order to habituate the mice to the consummatory chambers, mice were given two sessions of food cup magazine training over two days prior to testing. Each session involved 16 trials wherein 50 µl of the 20% sucrose reward solution was distributed under a random-time 120s schedule with each session taking approximately 20-45 minutes to complete.

Following food cup training, mice from each cohort were randomized for testing so that of the 16 mice in a single cohort, 4 mice would be run each day taking 4 days to complete one round of testing for all the mice in one cohort. Each day, 1 DIO female, 1 DIO male, 1 DR female, and 1 DR male mouse were run (Experiment 3). During this phase of testing, half the mice in each running group received CNO, and the other half received vehicle. On completion, testing was repeated, however the alternate drug injection was provided. The running order was randomized through a cyclic permutation so that no phenotype/sex combination was run first or last more than once for each cohort in order to mitigate variances in timing in relation to the onset of the testing period.

During these short-access licking microstructure tests, 20 minutes prior to testing and the onset of the dark cycle, the first mouse received either CNO or vehicle injection. During testing, mice were placed into the consummatory chamber. The consumption tests consisted of 20-minutes of free access to the food cup, with the food cup automatically refilling as tastant volume depleted. This occurred every 10 licks, where ~10-12 µl of solution would automatically be replenished into the food cup. While one mouse was running, the next mouse in the running order was immediately injected to synchronize with the end of the prior run after the 20-minute post-injection period. At the conclusion of the 20-minute testing period,

mice were immediately removed from the consummatory chamber and replaced with the next mouse in the running order and the process was repeated until 4 mice were run per test day. This testing protocol was repeated for corn oil (Experiment 4), as well as a third test with just DI water to test the effects of drug on licking behavior with a non-nutritive substance. Due to the viscosity of the corn oil solution and to better approximate the volume as present in the HFD but in liquid form, corn oil was diluted to 45% by volume by mixing with DI water and emulsified with 0.2g/100mL of sodium-stearoyl lactylate, a food-grade emulsifier capable of stabilizing hydrophobic lipids within a hydrophilic medium. This reduced viscosity also allowed the solution to flow through the liquid delivery lines to the consummatory chambers and did not disrupt the infrared beam within the food cup allowing accurate tracking of time-stamped licking for each mouse during testing. All licks were time-stamped with the temporal distribution in pauses of licking recorded via MedPC software for additional analyses of the licking microstructure. Two licking measures were implemented. (1) To examine tastant palatability, burst size was defined as the average number of licks that occurred prior to each pause in licking of >1000 ms. (2) To examine post-ingestive negative feedback, burst number was defined as the total number of bouts of licking behavior that were initiated after a >1000 ms pause in licking behavior. (Johnson, 2018; Johnson et al., 2010).

Viral Confirmation and Analysis

At the conclusion of behavioral testing, all mice were euthanized via 0.15 cc of sodium pentobarbital prior to intracardiac perfusion of 0.9% saline, followed by a 4% paraformaldehyde solution to fix the brain tissue. Brains were immersed in individually labeled jars of 12% sucrose 4% paraformaldehyde solution for 24 hours before being transferred to aluminum foil containers in cold storage at -80° C. Whole brains were then coronally sectioned on a microtome for the first 3 cohorts, or cryostat for the latter 3 cohorts with all slices measuring 30 µm in thickness. Sections were stored in cryoprotectant solutions in -20° C

freezers until further processing. Sections were then mounted onto subbed SuperFrost microscope slides before addition of Prolong Gold antifade mountant to highlight DAPI before cover slipping and microscope analysis.

Mounted brains were viewed with an Olympus Immunofluorescent Microscope (Olympus Corp., Center Valley, PA) targeting a red fluorescent signal as all 3 viruses utilized expressed an mCherry reporter which is optimally viewed at 550-650 nm (Shu et al., 2006). Photomicrographs were captured with a Qimaging Retiga 2000R digital camera attached to a fluorescent microscope (Olympus Corporation, Shinjuku City, Tokyo, Japan) using Stereo Investigator software (MBF Bioscience, Willington, VT). Adobe Photoshop was used to optimize the image quality for visualization and counting (Adobe Inc., San Jose, CA).

To isolate analyses of behavioral manipulations to ventral hippocampal transfection of either hM3Dq or hM4Di viruses, visualized slices were anatomically oriented to brain atlas images as a reference and labeled as "hit" in cases of bilateral transfection of the vHPC and a "miss" in the cases of unilateral or no visualizable transfection in the target region. Mice with insufficient unilateral transfection or low cell counts in hippocampal regions even if transfected bilaterally were also excluded from statistical behavior analyses. Mice included for analyses were grouped by phenotype and sex to generate heat maps to localize transfection based off bregma coordinates and anatomical location (supplementary figure 5). All confirmed hits were then followed up with additional analyses to determine cell count of viral transfection as well as anatomical regions of transfection coverage using the cell counter plug-in of ImageJ Software (Ahmadzai et al., 2022) (fig. 10).

Statistical Analyses

General statistical analyses utilized a repeated-measures analysis of variance (ANOVA) in order to investigate average lick rate (in licks/min), as well as licking microstructure measures associated with

tastant palatability (burst size) and post-ingestive feedback (burst number). These measures were examined to understand the effects of GHSR-expressing cell inhibition (hm4Di) or excitation (hM3Dq) on licking behavior when exposed to sucrose or corn oil. Along with the above-mentioned exclusions for viral transfection which were described for Chapter 2, mice that did not meet licking criteria—which required a minimum of 30 licks over the course of vehicle testing with sucrose—were also excluded from this study given these insufficient baseline responses in the behavioral paradigm. For the excitatory DREADD, the total included mice were n=4 DIO females, n=5 DR females, n=4 DIO males, and n=6 DR males. For the inhibitory DREADD, this included n=4 DIO females, n=5 DR females, n=5 DIO males, and n=6 DR males.

To examine rate of consumption, licks per minute were calculated by totaling all licks during the consumption session divided by the duration of the tests (20 minutes). To examine whether there are any changes in the palatability of the tastants, we utilized burst size which is quantified as the average number of licks occurring within a burst of licking which is defined by a separation from the next licking burst by a period of > 1000 ms. To calculate post-ingestive negative feedback measures, we utilized the burst number which is quantified as the total number of bursts initiated following a pause of licking > 1000 ms. Two-way phenotype (DIO, DR) x drug (CNO, VEH) ANOVAs were performed to compare between subject variables of phenotype as well as drug. Two-way sex (male, female) x drug (CNO) ANOVAs were run to examine sex differences regarding sex. Significant interactions were followed up by tests of main effects to determine the nature of said interactions for the dependent measures. The α level for significance was .05 and all analyses were performed using Statistica software (Statsoft).

Results

Viral Quantification

Viral quantification via mCherry fluorescence was performed to determine the extent of virus transfection in the vHPC, providing an estimate as to the number of cre-expressing cells present in the vHPC of each individual group. This also allowed us to analyze which mice to exclude from the study, as mice with no or minimal infection of DREADD virus would be unresponsive to our chemogenetic manipulations. Additionally, we excluded all mice that did not respond with at least 30 licks under vehicle conditions for the sucrose tests, as this indicated the mice were unable to learn to use the apparatus needed for the licking microstructure tests. This led to final n's for analysis for each virus as listed below (Table 2).

Overall, there were a trend towards interaction between the individual groups noted in total cell expression, revealed by a one-way ANOVA ($F[3,40] = 2.81$, $p=0.05$) (fig. 10e). To compare effects of sex on total cell count, a one-way ANOVA was performed which revealed no effect ($F<1$). Comparisons between phenotypes also revealed no effect ($F<1$). One-way ANOVAs of total cell count were then run between DIO and DR females with a separate analysis performed between DIO and DR males to see if phenotype affected within sex expression of GHSR. In general, DIO females were found to have significantly higher mCherry expression in the vHPC than DR females. A one-way ANOVA revealed an effect of phenotype for females ($F[1,40] = 6.37$, $p<0.05$) (fig. 10f). An effect of phenotype was not revealed between DIO and DR males ($F[1,40] = 2.06$, $p=0.16$) indicating the DIO phenotype led to higher viral count in DIO females compared to DR. A follow-up one-way ANOVA was performed to determine if differences were due to variabilities in expression in rostral (bregma -2.8 to -3.16) or caudal (bregma -3.28 to -3.64) regions of the vHPC which revealed no effects ($F's<2.4$, $p's>.08$). This indicates that in general there was a trend in variation of total cell count between the groups, driven by a higher cell count in DIO females compared to DR females.

Table 2. Summary of exclusions for individual groups under each viral condition. All groups had an initial n = 8 mice for use in the study. Mice with insufficient viral expression or targeting that was not in the region of the vHPC were excluded from further analyses.

hM3Dq	DR Male	DR Female	DIO Male	DIO Female
Inclusions	**7**	**7**	**8**	**4**
Exclusions	**1**	**1**	**0**	**4**
hM4Di	**DR Male**	**DR Female**	**DIO Male**	**DIO Female**
Inclusions	**6**	**5**	**7**	**6**
Exclusions	**2**	**3**	**1**	**2**

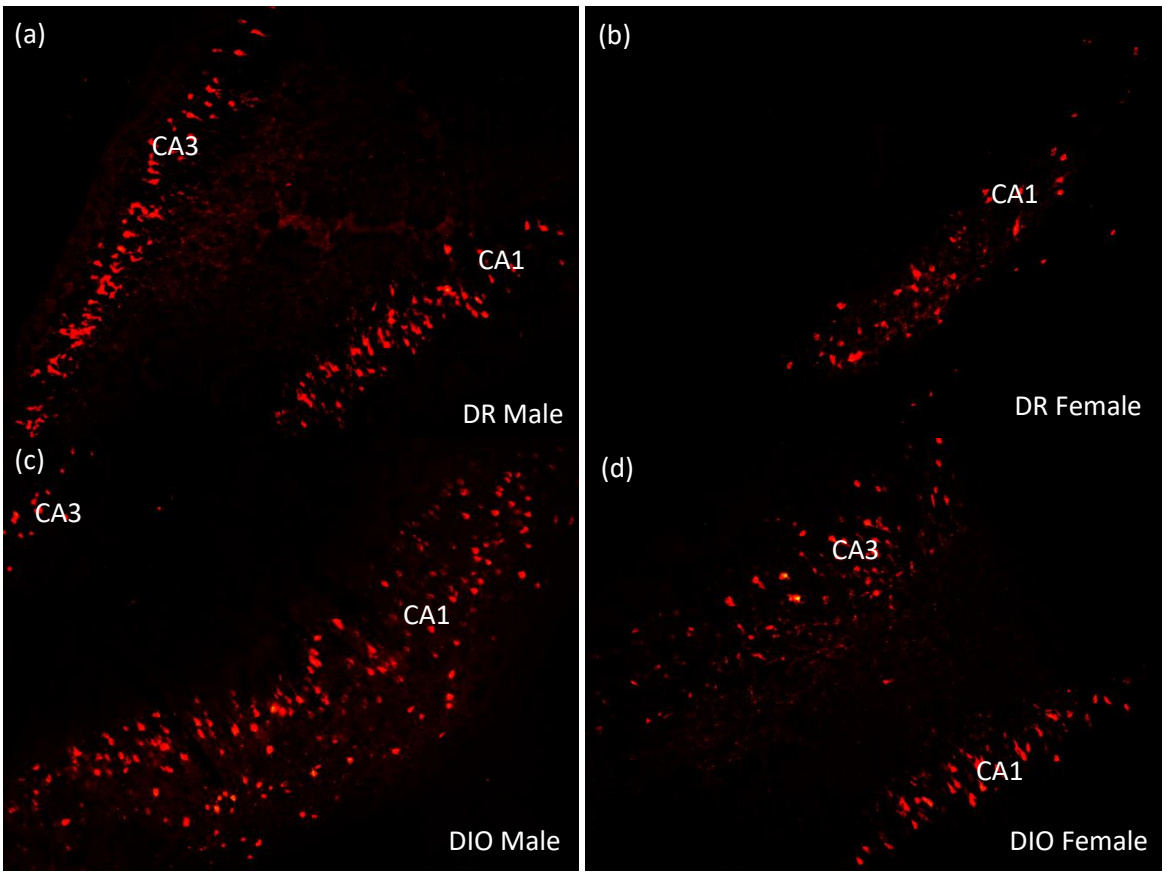
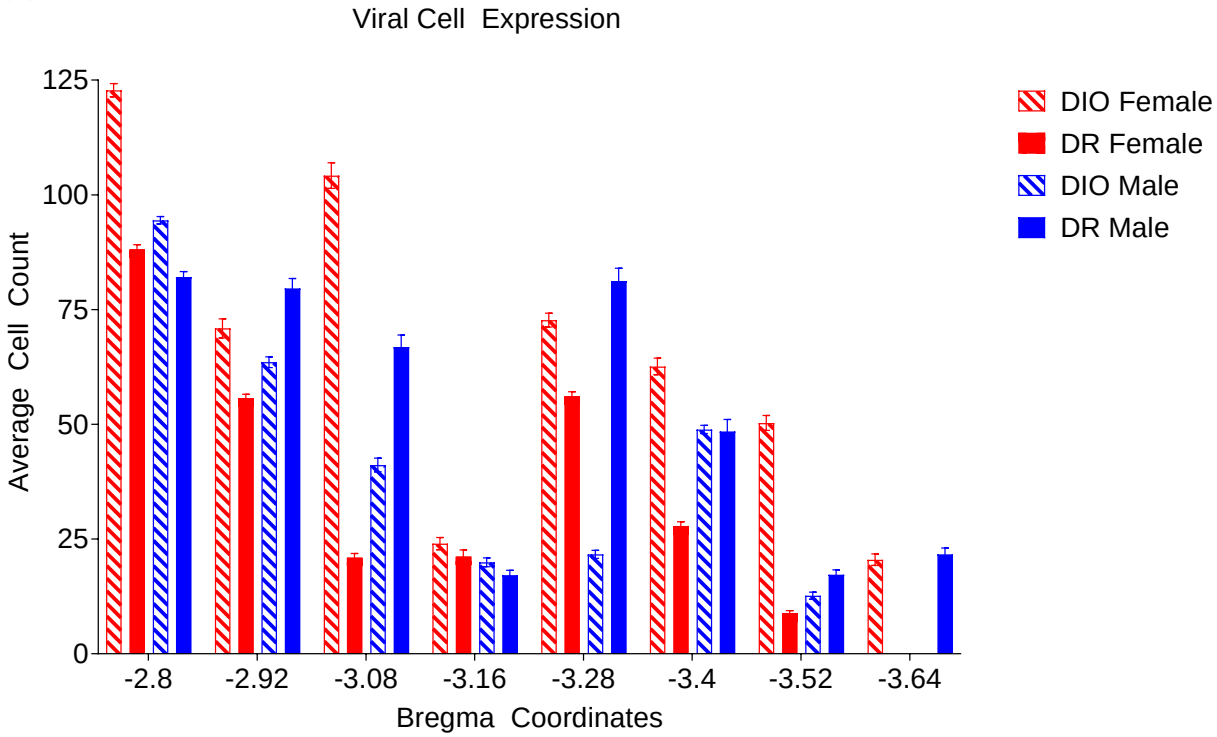


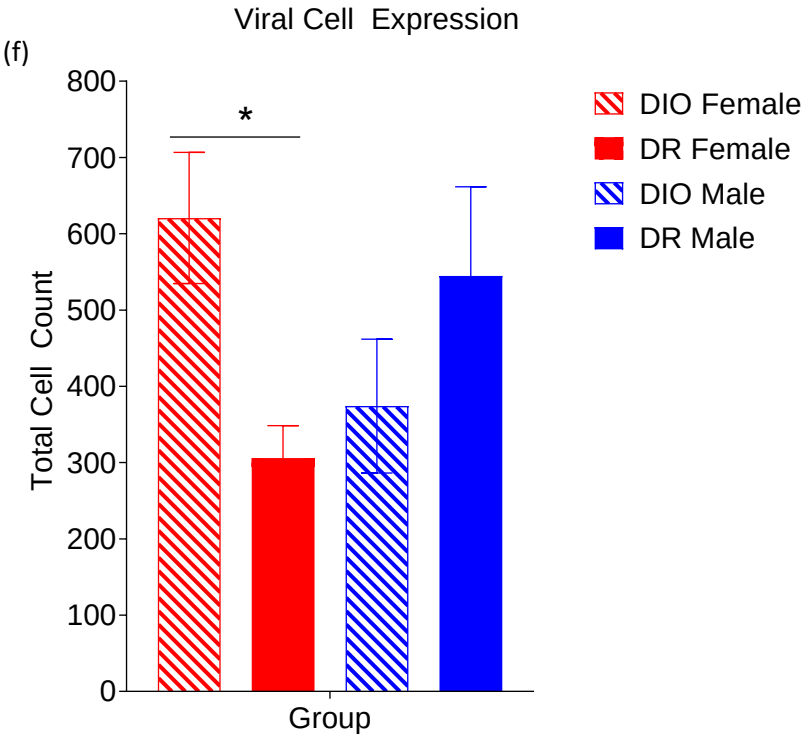
Figure 10. Representative photomicrographs of cre expression in vHPC regions. Representative photomicrographs of cre expression in vHPC regions at 10x magnification for (a) DR males and (b) females and (c) DIO males and (d) females. (e) Average cell count per mouse of cre-positive virally-infected cells including hM3Dq and hM4Di in ventral hippocampal regions for individual groups across all measured bregma coordinates from -2.8 to -3.64. (f) Total cre-mediated expression in vHPC for all groups. * Indicates main effect of phenotype for females $p<0.05$.

Figure 10 (cont'd)

(e)



(f)



Licking Tests with DI Water and mCherry Control Conditions

CNO administration to any group during the DI water licking tests revealed no changes in lick rate, measures of palatability, or post-ingestive feedback indicating CNO was not indiscriminately stimulating licking behavior or other locomotor functions of ingestive behavior beyond baseline (F's<1) (supplementary figure 8). CNO administration to the mCherry group revealed no effect of drug with exposure to sucrose or corn oil as well, revealing that CNO nor its metabolite clozapine induced any changes in behavior reflected in the licking microstructure (F's<1.7) (supplementary figure 7). Thus, licking microstructure analysis for water and mCherry control data in general will not be included in the analyses but is available in the supplementary figures section.

Experiment 3A: Effects of Excitation in GHSR-Expressing Cells of the Ventral Hippocampus on Lick Rate, Palatability, and Post-ingestive Negative Feedback of Sucrose

Chemogenetic activation of vHPC GHSR-expressing cells led to differential effects on lick rate dependent on sex and dietary phenotype (Figure 11; licks/min). When examining sex differences, CNO tended to increase lick rate for sucrose in females but not male mice (supplementary figure 3a). A sex x drug ANOVA for lick rate revealed a main effect of drug only ($F[1,17] = 6.42$, $p<0.05$). In addition, no sex differences were noted under either CNO or vehicle administration (F's<2.46, p's>.14). However, when each sex was examined separately, a main effect of drug was revealed in females ($F[1,8] = 5.83$, $p<0.05$) but not males ($F[1,9] = 0.73$, $p=0.42$). Thus, in female but not male mice, CNO led to a modest increase in lick rate for sucrose. To compare lick rate based on phenotype, a drug x phenotype 2-way ANOVA revealed an effect of drug ($F[1,17] = 5.02$, $p<0.05$) only indicating a general increase in lick rate following CNO administration (supplementary figure 3b). The within-subject investigation of the individual phenotypes further revealed a significant increase in lick rate following CNO in DR ($F[1,10] = 6.79$, $p<0.05$) but not DIO mice ($F[1,7] =$

1.09, p=0.33). The individual group comparisons also revealed an increase in lick rate in DR mice, however this was specific to females only (fig. 11b) ($F[1,4] = 9.62$, $p<0.05$), where a main effect of drug was revealed. In comparison, no effects of drug on lick rate were revealed in DR males, DIO males, or DIO females ($F's<5.18$, $p's>0.11$) indicating CNO-evoked stimulation of lick rate was specific to DR females.

For measures of burst size, which reflect the palatability of the tastant, CNO was not found to have an effect regardless of sex ($F's<1$, $p's>.55$) or phenotype ($F's<2.45$, $p's>.14$). Similarly, individual group comparisons revealed no effects of drug in any of the groups of mice ($F's<3.79$, $p's>.5$). This indicates that DREADD stimulation of GHSR-expressing cells in the vHPC had no effect on the palatability of sucrose as reflected by no changes in burst size (fig. 11).

Finally, for post-ingestive measures of negative feedback (i.e., burst number), a sex effect was revealed where CNO stimulation led to an increase in burst number in females (supplementary figure 3a). A sex x drug ANOVA revealed a sex x drug interaction ($F[1,17] = 5.42$, $p<0.05$). Follow-up analysis of drug revealed a main effect of sex for CNO ($F[1,17] = 5.07$, $p<0.05$) but not vehicle ($F[1,17] = 0.33$, $p=0.57$) indicating there were no differences in baseline measures of burst number, but with CNO administration females were found to show increased burst number indicative of reduced post-ingestive negative feedback. To confirm within each sex whether CNO influenced post-ingestive feedback, a main effect of drug was noted in females ($F[1,8] = 6.78$, $p<0.05$) but not males ($F<1$). Thus, female mice in general displayed an attenuation of post-ingestive feedback following CNO treatment. The individual group comparisons revealed no major effects of drug in DIO males or females, or DR males or females ($F's<3.64$, $p's>0.15$) (fig. 11) confirming CNO's effect of reducing post-ingestive negative feedback was generally seen in females and not males, and it was not specific to any phenotype.

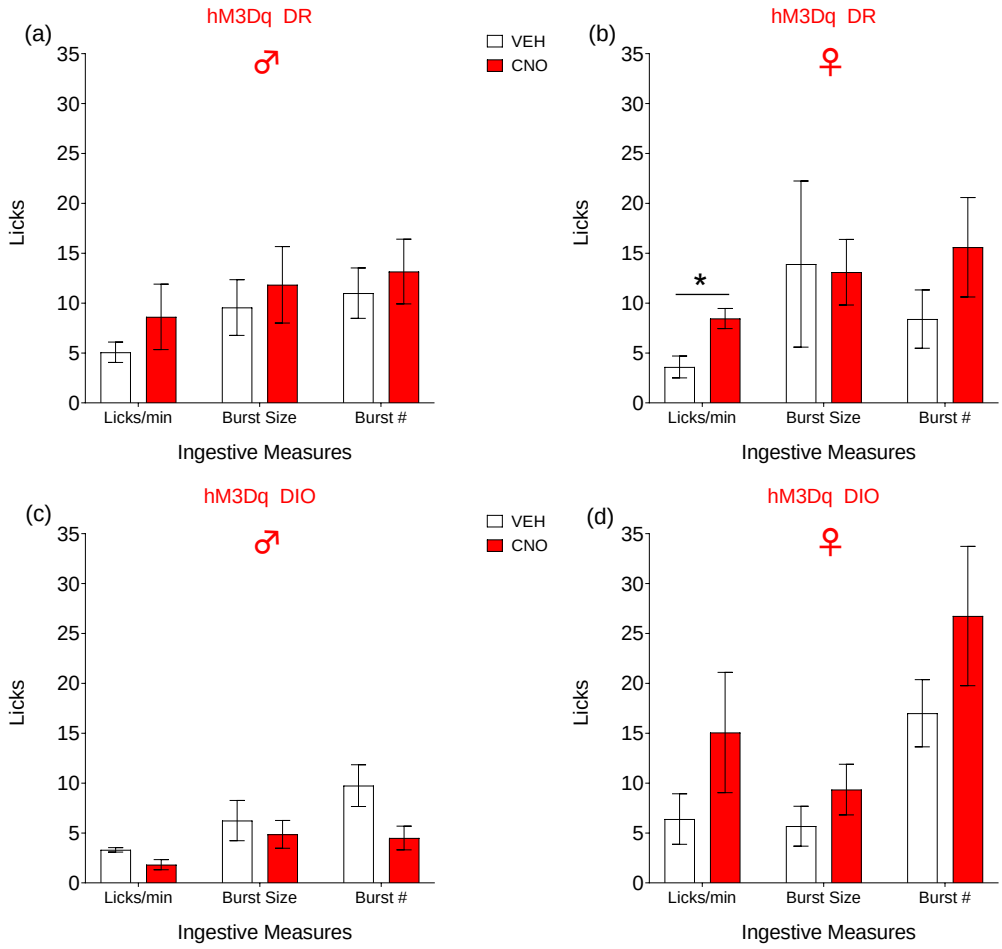


Figure 11. Experiment 3A. Cumulative measures of licking microstructure for sucrose including licks per minute, burst size, and burst number with Excitatory vHPC GHSR-expressing cell DREADD stimulation. Individual group differences of licking behavior between (a) DR males and (b) females and (c) DIO males and (d) females. Main effects of drug indicated with *p<0.05.

Experiment 3B: Effects of Inhibiting GHSR-Expressing Cells in the Ventral Hippocampus on Lick Rate, Palatability, and Post-ingestive Negative Feedback of Sucrose

Chemogenetic inhibition of vHPC GHSR-expressing cells was not found to influence lick rate of sucrose regardless of sex or phenotype (supplementary figures 3c,d). The sex x drug ANOVA revealed no main effects or interactions (F's<1, p's>.38). Similarly, the drug x phenotype ANOVA also revealed no main effects or interaction (F's<1, p's>.48). Finally, individual group comparisons (fig. 12) revealed no effects of

drug on lick rate in any group of mice (F's<2.59, p's>.21). This indicates that DREADD inhibition of GHSR-expressing cells in the vHPC had no effect on the lick rate regardless of sex or phenotype.

Similarly, for burst size, CNO was not found to have any general effects based on sex or phenotype (supplementary figures 3c,d). The sex x drug (F's<1, p's>.55) and drug x phenotype (F's<2.45, p's>.14) ANOVAs revealed no main effects of drug nor interaction between the variables. Nevertheless, the individual groups analyses (fig. 12) did reveal that DR females were found to attenuate burst size when administered CNO (fig. 12b) ($F[1,3] = 18.29$, $p<0.05$). The analysis for all other groups failed to reveal any effects of drug (F's<2.66, p's>.18), indicating the attenuation in burst size with CNO was specific to DR females.

For post-ingestive negative feedback, CNO was not found to have an effect on burst number for any group (supplementary figures 3c,d). Neither the sex x drug (F's<1.82, p's>.19) or drug x phenotype ANOVA (F's<1.33, p's>.26) revealed main effects or interactions. Finally, individual group comparisons revealed no effects of drug in DR males or females, or DIO males or females (F's<3.08, p's>.18). This indicates that DREADD inhibition of GHSR-expressing cells in the vHPC had no effect on post-ingestive negative feedback as reflected by a lack of differences in burst number for any group (fig. 12).

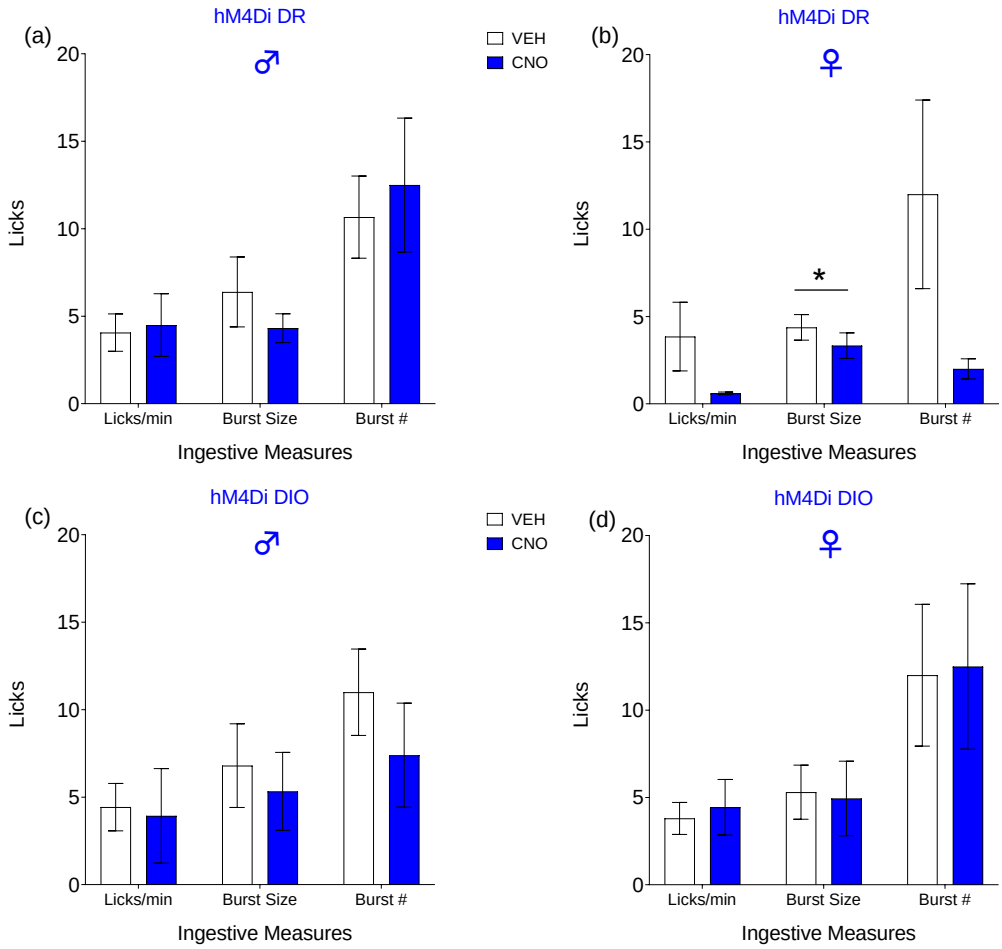


Figure 12. Experiment 3B. Cumulative measures of licking microstructure for sucrose including licks per minute, burst size, and burst number with inhibitory DREADD hM4Di. Individual group differences of licking behavior between (a) DR males and (b) females and (c) DIO males and (d) females. Main effects of drug indicated with *p<0.05.

Experiment 4A: Activation of GHSR-Expressing Cells in the Ventral Hippocampus Does Not Affect Lick Rate or Palatability of Corn Oil, But Does Decrease Burst Number in Females Compared to Males

Chemogenetic excitation of vHPC GHSR-expressing cells was not found to alter lick rate for corn oil regardless of sex or phenotype (supplementary figures 4a,b). In terms of sex, a sex x drug ANOVA revealed no interactions (F's<2.6, p's>.12). Similarly, a drug x phenotype ANOVA revealed no interaction (F's<1, p's>0.34). Finally, individual group comparisons revealed no effects of drug in DR males or females, or DIO

males or females (F's<1.58, p's>.28). This indicates that DREADD stimulation of GHSR-expressing cells in the vHPC had no effect on the lick rate of corn oil regardless of sex or phenotype (fig. 13).

For the burst size, CNO was not found to induce any general effects based on sex or phenotype (supplementary figures 4a,b). In terms of sex, a sex x drug ANOVA revealed no interactions (F's<1, p's>.37). Similarly, a drug x phenotype ANOVA revealed no interaction (F's<2.33, p's>.15). Individual group comparisons followed a similar pattern for DR males and females, and DIO males and females which revealed no effects of drug (F's<1, p's>.52), indicating CNO had no effect on burst size for any group when exposed to corn oil (fig. 13).

For measurements of post-ingestive negative feedback, an effect of sex was seen in males depicted as a lower burst number compared to females (supplementary figures 4a,b). A drug x sex ANOVA revealed a main effect of sex ($F[1,17] = 6.61$, $p<0.05$). Follow-up analysis of each drug revealed a main effect of sex for vehicle ($F[1,17] = 6.32$, $p<0.05$) with a tendency towards effect of drug for CNO ($F[1,17] = 3.85$, $p=0.06$). However, the analysis within each sex revealed no main effect of drug in either males or females (F's<2.53, p's>.14). Thus, in general males displayed an increased sensitivity to post-ingestive negative feedback, an effect that was modestly increased following CNO treatment. Finally, individual group comparisons (fig. 8) revealed no effects of drug in DR males or females, or DIO males or females (F's<2.87, p's>.17).

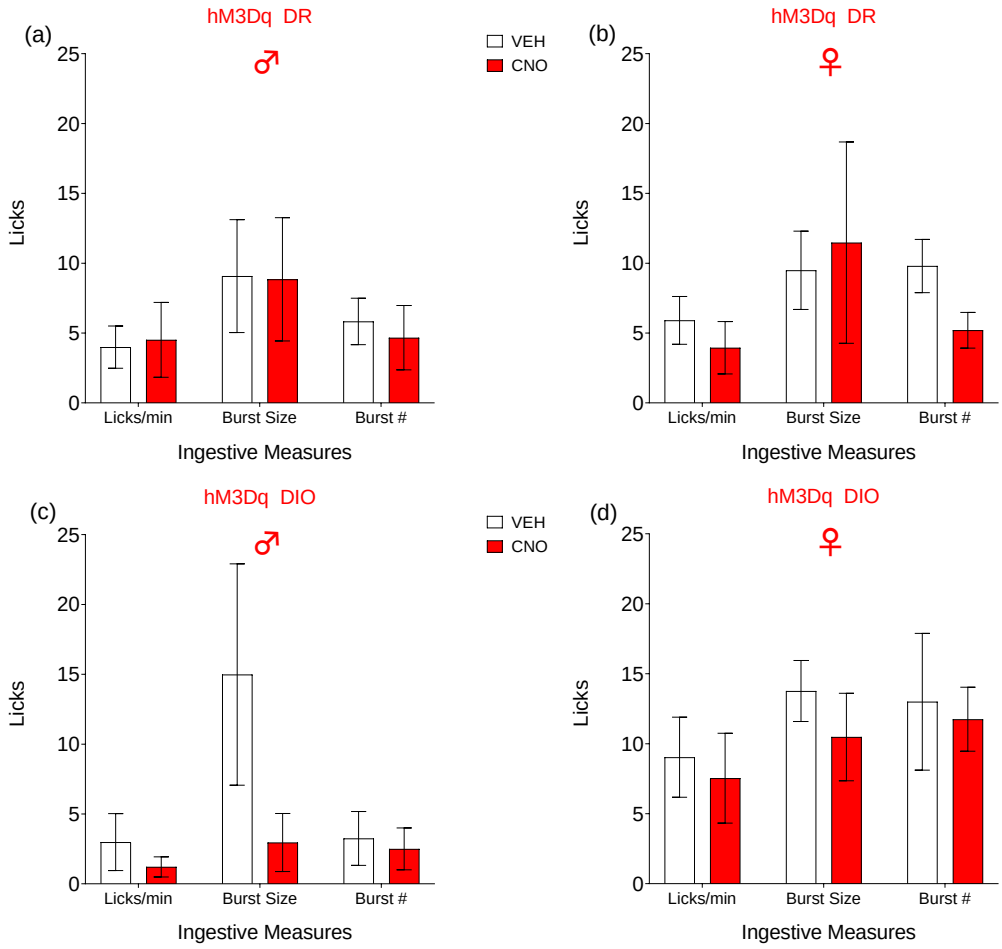


Figure 13. Experiment 4A. Cumulative measures of licking microstructure for corn oil including licks per minute, burst size, and burst number with excitatory vHPC DREADD stimulation compared to vehicle. Individual group differences of licking behavior between (a) DR males and (b) females and (c) DIO males and (d) females. Main effects of drug indicated with *p<0.05.

Experiment 4B: Inhibition of GHSR-Expressing Cells in the Ventral Hippocampus Ameliorates Sex Differences in Lick Rate of Corn Oil Does Not Affect Palatability or Post-ingestive Negative Feedback

Chemogenetic inhibition of vHPC GHSR-expressing cells was found to affect lick rate of corn oil dependent on sex and not phenotype (supplementary figures 4c,d). A sex x drug ANOVA revealed a trend towards interaction of sex ($F[1,16] = 4.29$, $p=0.05$). Females were found to have a higher baseline lick rate compared to males which disappeared upon CNO administration. Under vehicle conditions, a main effect

of sex was revealed ($F[1,16] = 6.11$, $p<0.05$) but not under CNO conditions ($F[1,16] = 0.8$, $p=0.38$) indicating that CNO tended to ameliorate the baseline differences in lick rate of corn oil between males and females (supplemental figure 4c,d). To examine within sex effects of CNO on lick rate, an effect of drug was assessed which revealed no effect in either males or females ($F's<1$, $p's>.61$). Comparatively, a drug x phenotype ANOVA revealed no effect of drug or interaction ($F's<1$, $p's>.34$). Finally, individual group comparisons revealed no effects of drug in any of the groups of mice ($F's<2.42$, $p's>.19$). This indicates that DREADD inhibition of GHSR-expressing cells in the vHPC modestly eliminated baseline differences in lick rate of corn oil between males and females (fig. 14).

For the burst size, CNO was not found to have any general effects based on sex or phenotype (supplementary figures 4c,d). A sex x drug ($F's<2.22$, $p's>0.16$) or drug x phenotype ($F's<2.2$, $p's>.16$) ANOVAs revealed no main effects or interactions. Individual group comparisons followed a similar pattern, with no effects noted for any group of mice ($F's<5.25$, $p's>.07$), indicating CNO had no effect on burst size for any group when exposed to corn oil (fig. 14).

Finally, for post-ingestive negative feedback, CNO was not found to have an effect on burst number for any group (supplementary figures 4c,d). In terms of sex, a sex x drug ANOVA revealed no interactions ($F's<3.09$, $p's>0.1$). Similarly, a drug x phenotype ANOVA revealed no interaction ($F's<3.17$, $p's>.09$). Finally, individual group comparisons revealed no effects of drug in DR males or females, or DIO males or females ($F's<4.01$, $p's>.12$). This indicates that DREADD inhibition of GHSR-expressing cells in the vHPC had no effect on post-ingestive negative feedback (fig. 14).

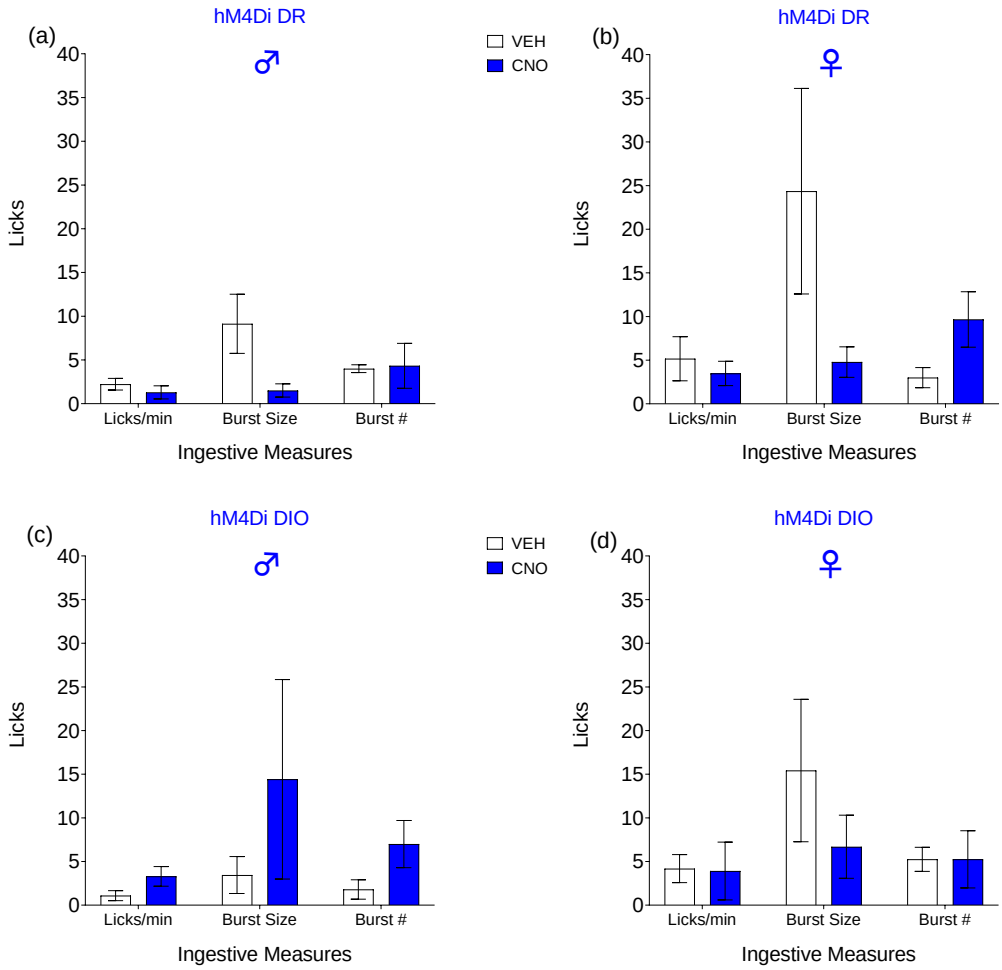


Figure 14. Experiment 4B. Cumulative measures of licking microstructure for corn oil licks per minute, burst size, and burst number with inhibitory DREADD hM4Di. Individual group differences of licking behavior between (a) DR males and (b) females and (c) DIO males and (d) females. Main effects of drug indicated with *p<0.05.

Discussion

In this study, we examined the role of the GHSR-expressing cells in the vHPC in modulating licking behavior using macronutrient components of the HFD as tastants. In the case of our sucrose tests, prior studies indicated that females are less sensitive to licking lower concentrations of sucrose (Curtis et al., 2004). Considering our mice would also be tested under non-deprived conditions, we utilized a higher concentration 20% sucrose solution to promote licking behavior as DIO animals are found to consume less sucrose while on HFD putting the results at risk for floor effects as the DIO animals may not respond with

CNO-evoked inhibition if they are already sated on the palatable HFD (Johnson, 2012). High concentration sucrose also shows no sex differences in lick rate at baseline and our results are consistent with these findings (Curtis et al., 2004).

Our study is the first to show that GHSR-expressing cell stimulation in the vHPC increases lick rate and burst number for sucrose in females and not males, indicating females consume more sucrose mainly through reduced post-ingestive negative feedback. This is a novel effect of sex that has not been reported previously. These data do partially support the idea that GHSR-expressing cells of the vHPC are important for regulation of sucrose consumption, but in our case was specific to females. Peripheral ghrelin has been shown to enhance consumption of sweet solutions in males, indicating GHSR cell activity may drive sucrose consumption in males, but not at the level of the vHPC (Disse et al., 2010). Instead—in males—it is suggested to occur via GHSR activity at the level of the VTA where ghrelin administration is found to increase dopaminergic signaling in the VTA and an increase in operant level presses for sucrose (Overduin et al., 2012; Perello & Dickson, 2015; Skibicka et al., 2011). The ARC is another region important in mediating sucrose consumption in male rodents, where GHSR acts in an indirect manner by engaging NPY/AgRP neuronal activity to increase sucrose consumption through activation of downstream NPY-receptor expressing cells in the VTA (Skibicka et al., 2012). Comparatively, our data suggests that vHPC GHSR activity does directly increase sucrose consumption in females reflected by an increased lick rate and burst number, indicating there may be a unique female-specific signaling pathway for driving consumption of sucrose.

This difference in burst number may also be due to a sex-specific insensitivity to the chemogenetic manipulations in males, as male mice maintained on HFD show a reduced burst number to sucrose compared to chow-fed controls (Johnson, 2012). This indicates a lack of motivation to consume sucrose once satiated as the mice also had *ad libitum* access to a more palatable HFD. Indeed, reverting HFD-fed mice back to chow rescues the drive to consume sucrose beyond satiety reflected by an increase in burst

number that resembles that of the control mice (Johnson, 2012). This demonstrates that the HFD diet may have reduced the incentive motivation to consume the sucrose in the male cohorts regardless of stimulation as they were already satiated, while females were still sensitive to GHSR stimulation and were more willing to consume the sucrose solution even though they also had *ad libitum* access to HFD.

Additionally, phenotype differences were essential to unraveling the drug interactions with the individual groups, as we revealed the effect of CNO on increasing lick rate was specific to DR females with the excitatory DREADD, and not DR females with the inhibitory DREADD or in any other group with either viral manipulation. These results are in contrast to prior studies indicating that females rodents will consume proportionally more sucrose than males over a 24-hour period (Grimm et al., 2022; Sclafani et al., 1987). In our study the increase in sucrose consumption was driven by GHSR stimulation in the vHPC of DR females exclusively. These differences may be due to the animal model, where we used mice while the aforementioned studies used rats. Additionally, our study maintained animals on HFD which is shown to reduce sucrose consumption in DIO animals through decreases in burst size and burst number, potentially blunting chemogenetic effects on consumption (Johnson, 2012).

This may explain the general insensitivity to chemogenetic activation or inhibition on sucrose consumption we saw in our DIO animals. As we noted in chapter 2, our DIO animals were responsive to DREADD manipulation with chow. This indicates that DIO animals may just have a lower preference for sucrose that is resistant to chemogenetic manipulation while maintained on a palatable HFD, and that sensitivity of GHSR can be rescued with transition to a more nutritious chow diet (Johnson, 2012). Indeed, the Johnson study shows HFD-maintained DIO mice switched back to chow show comparable licking of sucrose to chow-maintained controls indicating the *ad libitum* access to the HFD may be a confounding variable diminishing the effects of GHSR activity on sucrose consumption for DIO animals.

Our investigation of burst size revealed no main effects between sex or phenotype. We do find a sensitivity to GHSR inhibition in the DR female group specifically as depicted by a decrease in burst size. This indicates in DR females—when GHSR-expressing cell activity in the vHPC was inhibited—found the sucrose solution less palatable, again reflecting the findings above that there is a sensitivity to GHSR-expressing cell activity in DR females towards sucrose consumption that is not prevalent in the other groups. This further emphasizes the importance of examining phenotypes as subtle phenotypic differences in behavior can be obscured when broader heterogenous sample of subjects are used instead. A potential explanation for this reduction in palatability may also be due to the ad libitum access to the HFD itself. Prior studies indicate that maintenance on HFD affects the licking behavior between sexes. Male mice maintained on HFD are found to have a decreased burst size of a glucose solution compared to females (Carr & Weiner, 2022). Other studies exploring phenotypes show that DIO animals find sucrose less palatable than DR animals as depicted by a lower burst size (Johnson, 2012).

Our results somewhat contrast with these data as there were no effects of sex noted with burst size at baseline, indicating males and females found the sucrose solutions equally palatable even with all groups maintained on HFD without chemogenetic manipulation. The difference with our results may be partially due to lower n's as a result of mice being excluded from analysis due to lack of viral transfection in cre-expressing cells and inadequate licking responses, decreasing the statistical power. Additionally, the aforementioned studies explored either one variable of sex (in rats) or phenotype (in male mice), in the latter study with one group maintained on HFD to develop an obese phenotype and compared to chow-fed controls. Our study developed the phenotypes by determining weight differences with HFD exposure. Additionally, our study assessed both variables of sex along with phenotype concurrently, highlighting the importance of examining sex along with dietary phenotype in ingestive behavior as aggregating data to analyze singular variables (i.e., either sex or phenotype) can ameliorate some of the key differences revealed by the individual groups.

In fact, with examination of the individual groups we uncover the novel finding that DR females are particularly sensitive to the drug manipulations, indicating palatability in DR females is partially driven by GHSR activity in the vHPC—inhibition of which decreases palatability for this group specifically. This indicates that preserving palatability for sucrose in DR females requires intact GHSR signaling in the vHPC. The novelty of these findings warrant further investigation as the mechanism of GHSR activity in females is not fully understood. The increase in lick rate with stimulation and decrease in burst size with inhibition indicate that DR females are particularly sensitive to manipulation of GHSR-expressing cell activity in the vHPC when exposed to sucrose specifically. The question remains if that activity is driven by the ventral hippocampus exclusively or if the effects include downstream signaling pathways to orexigenic feeding centers of the vHPC such as the lateral hypothalamic area (LHA) and laterodorsal tegmental nucleus (LDTg). These regions in particular are found to increase orexin receptor signaling in response to GHSR stimulation at the level of the vHPC (Lee et al., 2021; Noble et al., 2019; Novelle & Diéguez, 2018).

With the sex specific effects of GHSR-expressing cell stimulation increasing lick rate and burst number noted in females, investigation as to the role of estrogen will be critical in future studies. Estrogen is found to induce tonic inhibitory effects on meal size and intake as well as increase GHSR expression which has been well characterized in the ARC (Asarian & Geary, 2006; Clegg et al., 2007; Conde & Roepke, 2020; Drewett, 1973; Geary, 2004; Yasrebi et al., 2016). DIO and DR females are not found to have a difference in circulating estradiol (E2) (Balasubramanian et al., 2012; Giles et al., 2016). However, DIO females are found to have reduced sensitivity to the effects of estrogen, indicating a potential mechanism as to the profound behavioral differences between our DIO and DR female mice (Giles et al., 2016). To address these differences, it will be important to validate the level of GHSR expression through in situ hybridization of GHSR mRNA between males and females and investigating how those expression patterns are affected by dietary phenotype. This will provide better insight as to why DR females are particularly sensitive to GHSR-expressing cell manipulations compared to any other group. Our current analyses in this study

reveal that between DIO and DR females, there is a difference in total cre-mediated expression, wherein DIO females are found to have nearly double the amount of fluorescent cre-positive cells compared to DR females. Comparatively, DIO and DR males showed no significant differences in overall expression. Considering cre in the GHSR-IRES-cre model is expressed downstream of the GHSR stop codon, immunofluorescence of the attached mCherry fluorophore in our viral constructs serves as a functional metric to estimate the number of GHSR-expressing cells in the vHPC (Mani et al., 2014, 2017; Zigman et al., 2006). This indicates in our model at least, that DIO females have higher cre-mediated expression than DR females in the vHPC, potentially reflective of a compensatory mechanism in DIO females due to a lack of receptor activity from endogenously impaired and blunted ghrelin secretion (Cui et al., 2017; Zigman et al., 2016).

These are again novel findings as GHSR-expressing cells have not been quantified between DIO and DR females before, nor between DIO and DR males in the vHPC. In the ARC, GHSR expression has been quantified such that DIO males are found to have lower GHSR expression than control animals (Yasrebi et al., 2016). In our case DIO males indeed had a lower average cell count than DR males, however the values did not approach significance. This may do with the issue for both sexes in these studies is that the DIO animals referenced were compared to low-fat diet controls, whereas our DR mice are fed the exact same high fat diet as the DIO cohorts, they are just resistant to the diet's effect of promoting obesity. Regarding our female cohorts, our findings are somewhat in agreement with Yasrebi et al., as they indicate DIO does not suppress GHSR expression in the ARC compared to controls (Yasrebi et al., 2016). In our case, although we find that the DIO females have the highest overall cre-mediated expression in the vHPC, a follow-up study utilizing in-situ hybridization will be critical to determining if there are sex or phenotypic specific effects on GHSR expression in the vHPC.

This was not only the first study to examine licking microstructure at the level of the vHPC, but given most studies utilize male rodents exclusively, our findings are the first to reveal sex differences with regard to

the consumption of corn oil (Davis, 1996; Davis et al., 1995; B. K. Smith et al., 2001). We show that at baseline, males consume less oil than females as depicted by a lower lick rate per minute. A literature search revealed that no such comparable test has been performed between GHSR-IRES-cre males and females before, indicating a novel finding. A study by Glendinning et al., assessed oil consumption at different concentrations in male and female mice across eight different strains, however they did not assess sex differences and instead aggregated mouse data solely by strain, thus data regarding effects of sex on oil consumption are inconclusive (Glendinning et al., 2008). We also show that at baseline, males exhibit a lower burst number with corn oil than females. This indicates that male mice experience greater levels of satiation and post-ingestive negative feedback while consuming oil than females. This also reflects a general effect of sex that was not seen with phenotype as no differences in licking behavior were noted between DIO and DR animals. Additionally, we found that GHSR-expressing cell activity in the vHPC does not affect licking microstructure for corn oil in the individual groups, as neither stimulation nor inhibition of GHSR-expressing cells was found to affect individual licking behaviors. Instead, we demonstrate that females in general will consume more corn oil due to experiencing less satiety and post-ingestive negative feedback. One must then question if other brain regions and/or neuropeptide systems may instead be involved in driving consumption of oil and fat.

It is also worthwhile considering that in addition to reflecting post-ingestive negative feedback, more recent studies suggest that burst number may also indicate the capacity of a food to override post-ingestive satiation through the incentive motivational properties of food (Naneix et al., 2020). Although additional studies are required to decipher the relationship between incentive value and burst number, it is notable that the ventral tegmental area (VTA) dopamine system that controls the incentive value of foods (Berridge, 2009; Morales & Berridge, 2020) also contains GHSR receptors (Abizaid, 2019; Abizaid et al., 2006; Zigman et al., 2006). Moreover, GHSR activity in the VTA is required for ghrelin-evoked feeding behavior (Abizaid et al., 2006; Perelló & Zigman, 2012; Zessen et al., 2012). In addition, ghrelin-dependent

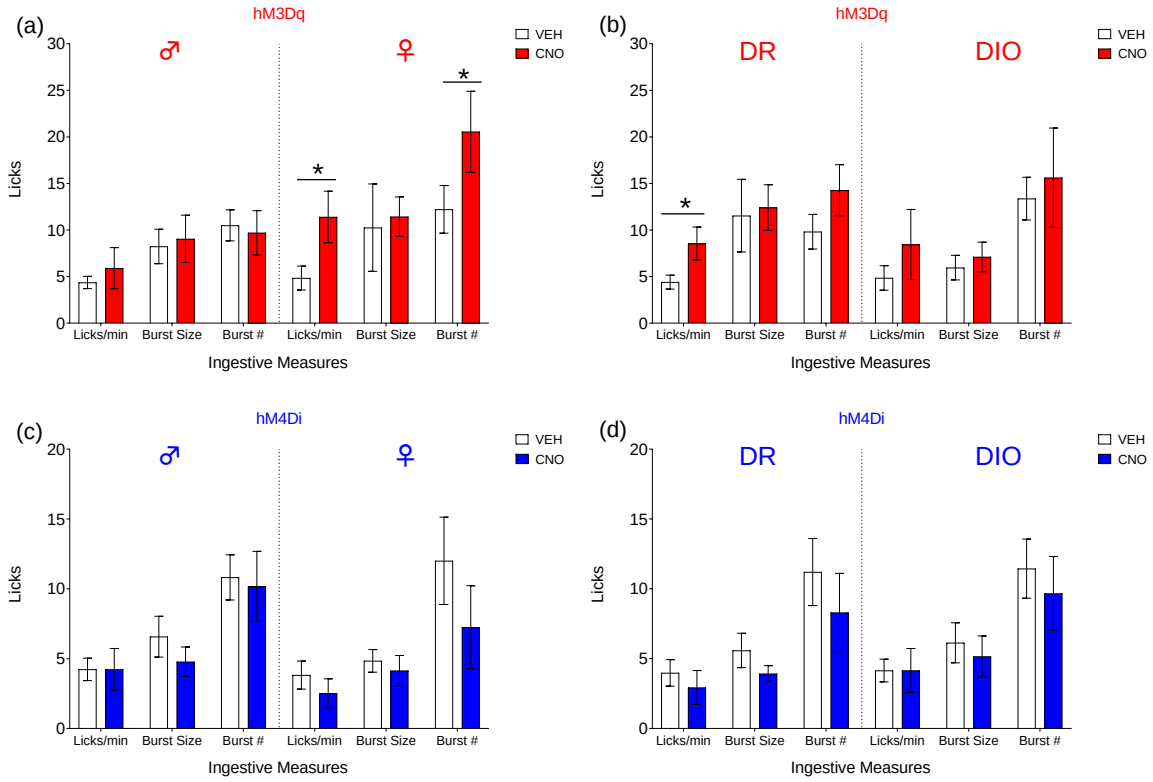
feeding also requires intact GHSR activity in the VTA and intra-VTA ghrelin administration increases dopamine (DA) turnover and dopaminergic neuron activity in a downstream target of the VTA—the nucleus accumbens (NAc) (Abizaid et al., 2006; Skibicka et al., 2011; van Zessen et al., 2012). These findings suggest a critical role for ghrelin and GHSR's in the mesostriatal reward system. Notably, the NAc is also found to be responsive to corn oil—where corn oil administration in rats leads to an increase in NAc dopamine (DA) release (Liang et al., 2006). Therefore, this outlines a potential route of GHSR signaling from the VTA to the NAc that may influence the consumption of fats such as corn oil that were not as clearly demonstrated in GHSR-expressing cells of the vHPC.

Conclusion

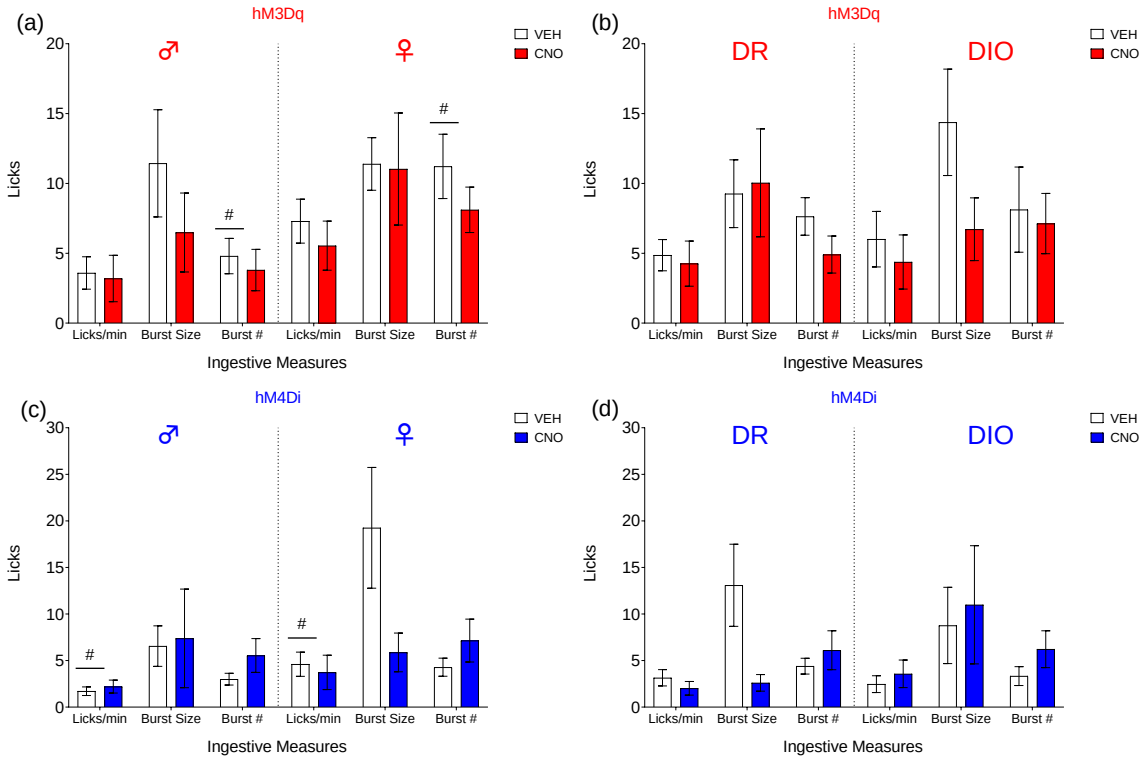
Our study suggests that GHSR-expressing cell stimulation in the vHPC differentially affects males and females as assessed by measures of licking microstructure with sucrose consumption, where females are found to increase licking and consume more due to being less satiated compared to males. Comparatively, GHSR cell inhibition is found to decrease the palatability of sucrose for DR females specifically. Finally—with corn oil—although we saw no effects of GHSR cell activation or inhibition on licking behavior, for the first time we have demonstrated that baseline sex differences exist where females will consume more corn oil and do so due to feeling less satiated than males. These results demonstrate the need to further explore sex and phenotypic differences in ingestive behavior to determine potential causes of dietary obesity.

APPENDIX

Supplementary Figures

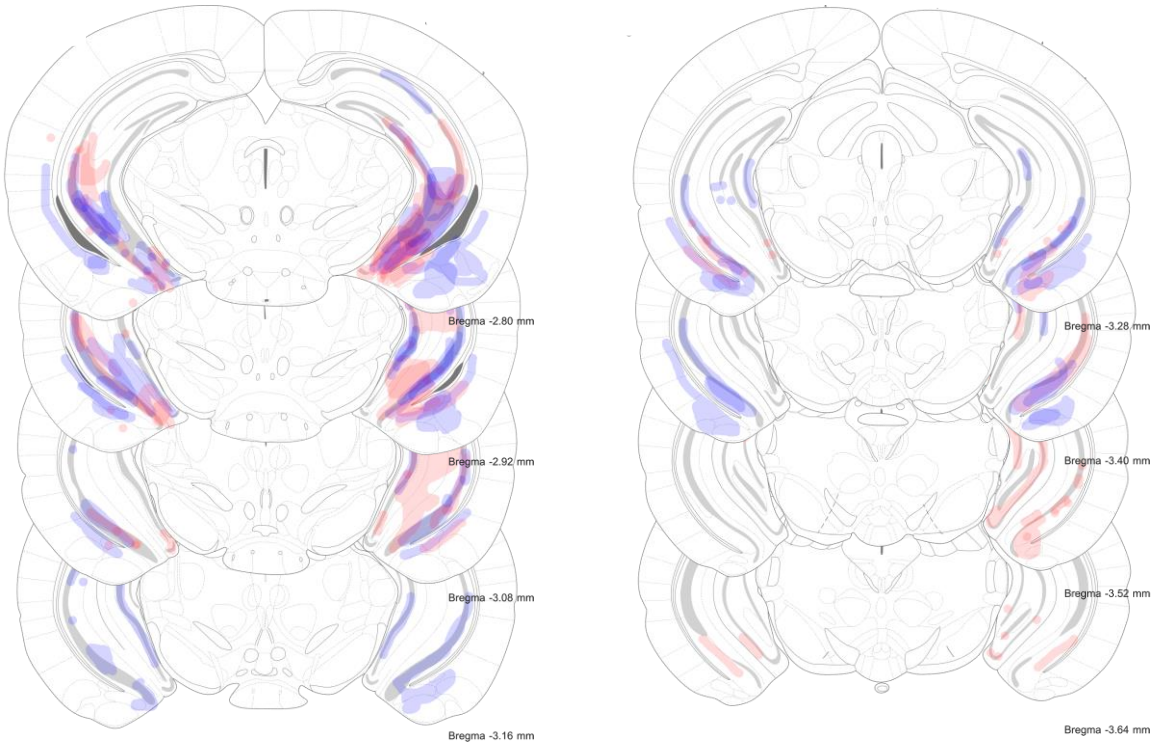


Supplementary Figure 3. Cumulative measures of licking microstructure for sucrose with Excitatory vHPC GHSR DREADD stimulation comparing (a) sex as well as (b) phenotype. Cumulative measures of licking microstructure for sucrose with Inhibitory vHPC GHSR DREADD comparing (c) sex and (d) phenotype. Main effects of drug indicated with *p<0.05.



Supplementary Figure 4. Cumulative measures of licking microstructure for corn oil with Excitatory vHPC GHSR DREADD stimulation comparing (a) sex as well as (b) phenotype. Cumulative measures of licking microstructure for corn oil with Inhibitory vHPC GHSR DREADD comparing (c) sex and (d) phenotype. Main effects of drug indicated with *p<0.05. # Indicates main effect of sex for vehicle p<0.05.

(a)

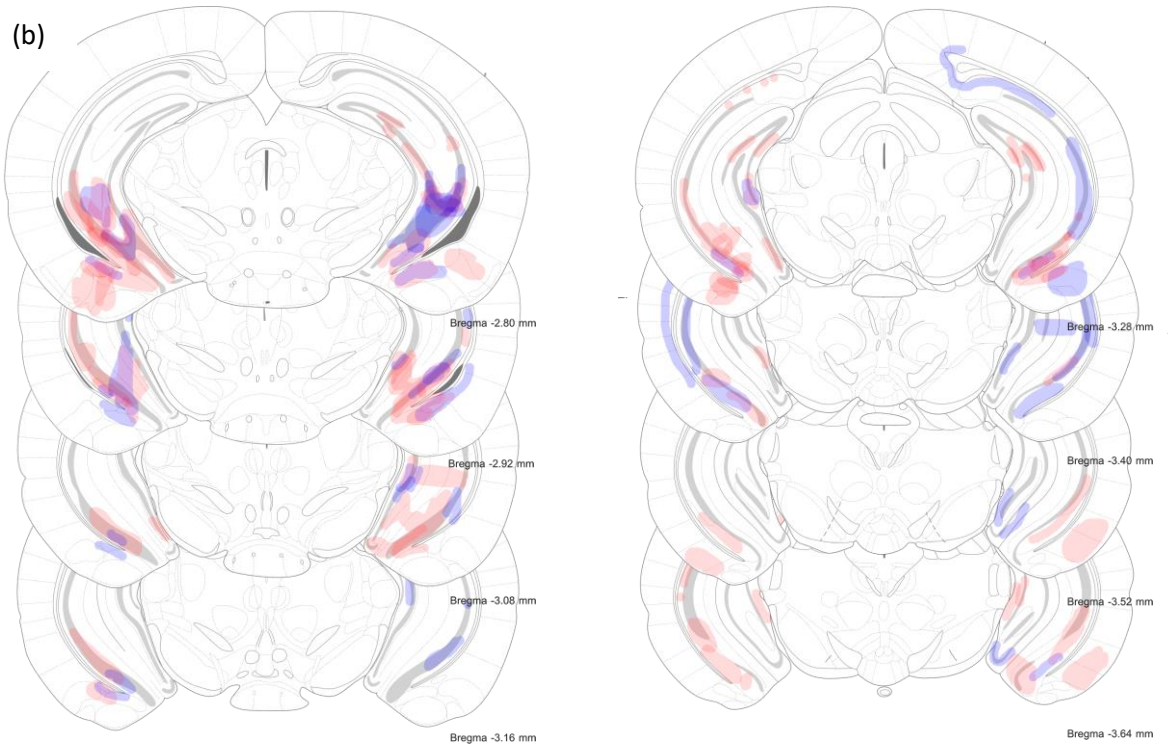


Supplementary Figure 5. Heat maps of viral expression for individual groups combining hM3Dq (red) and hM4Di (blue). Expression patterns from Bregma -2.80 to -3.64 in (a) DR males and (b) females and (c) DIO males and (d) females.

Supplementary Figure 10 (cont'd)

DR FEMALE

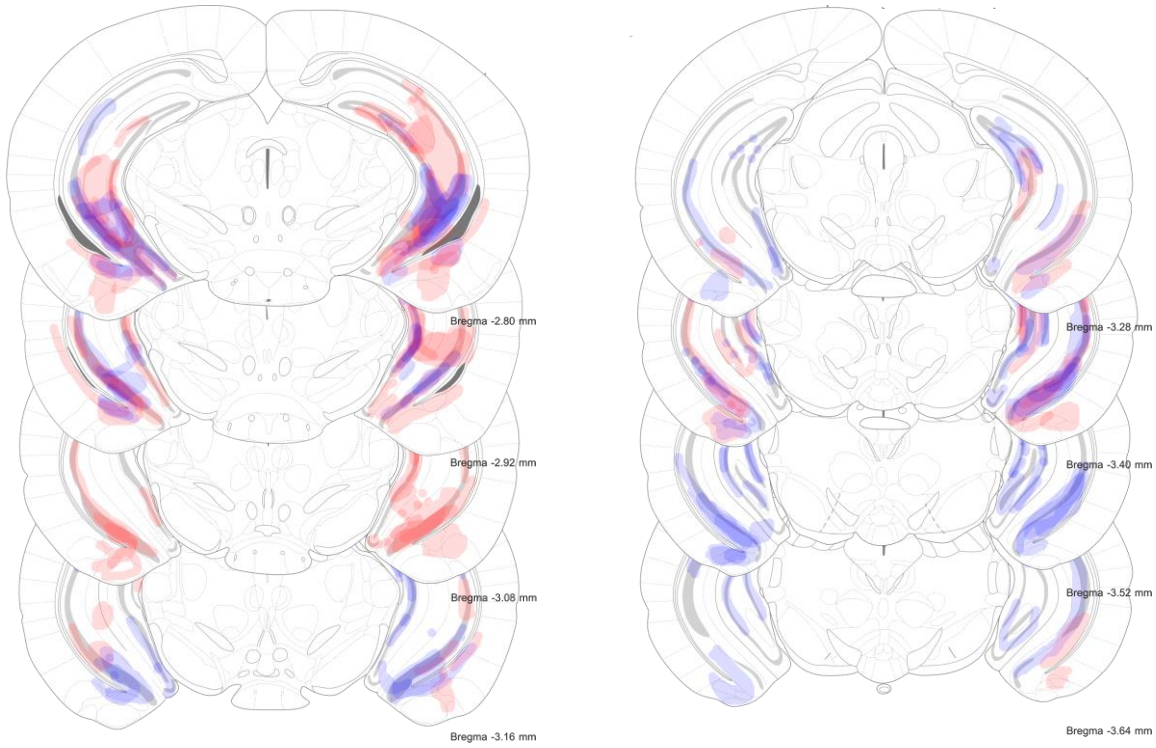
(b)



Supplementary Figure 10 (cont'd)

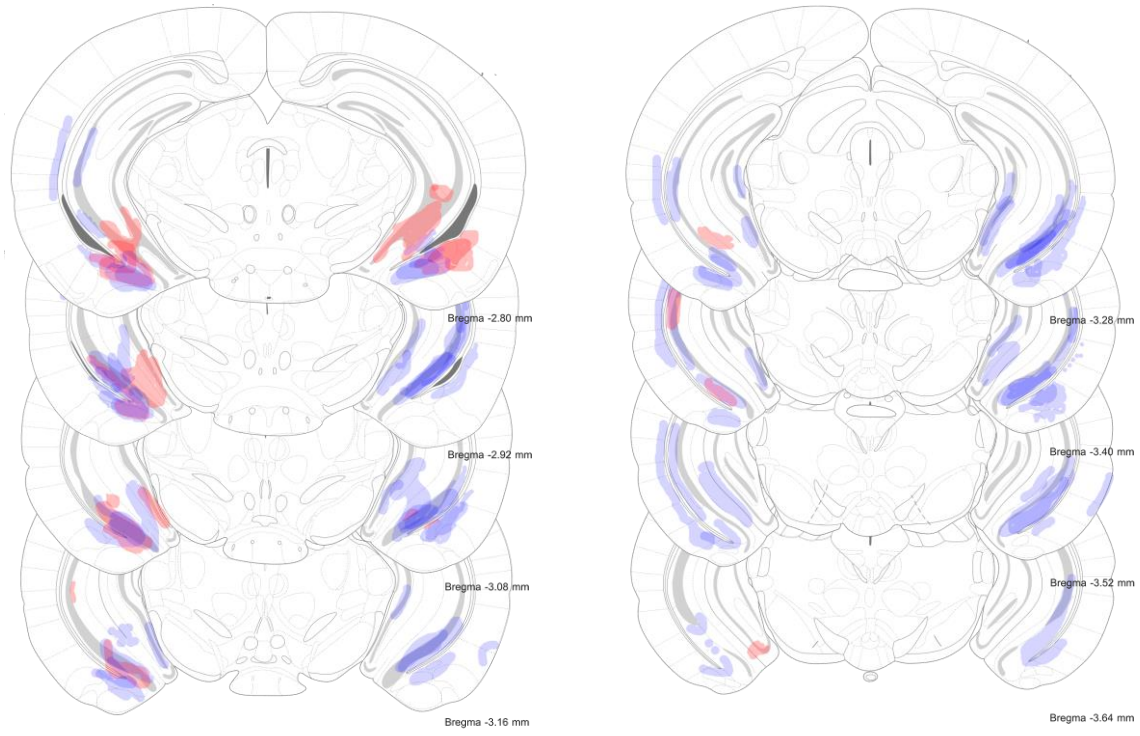
(c)

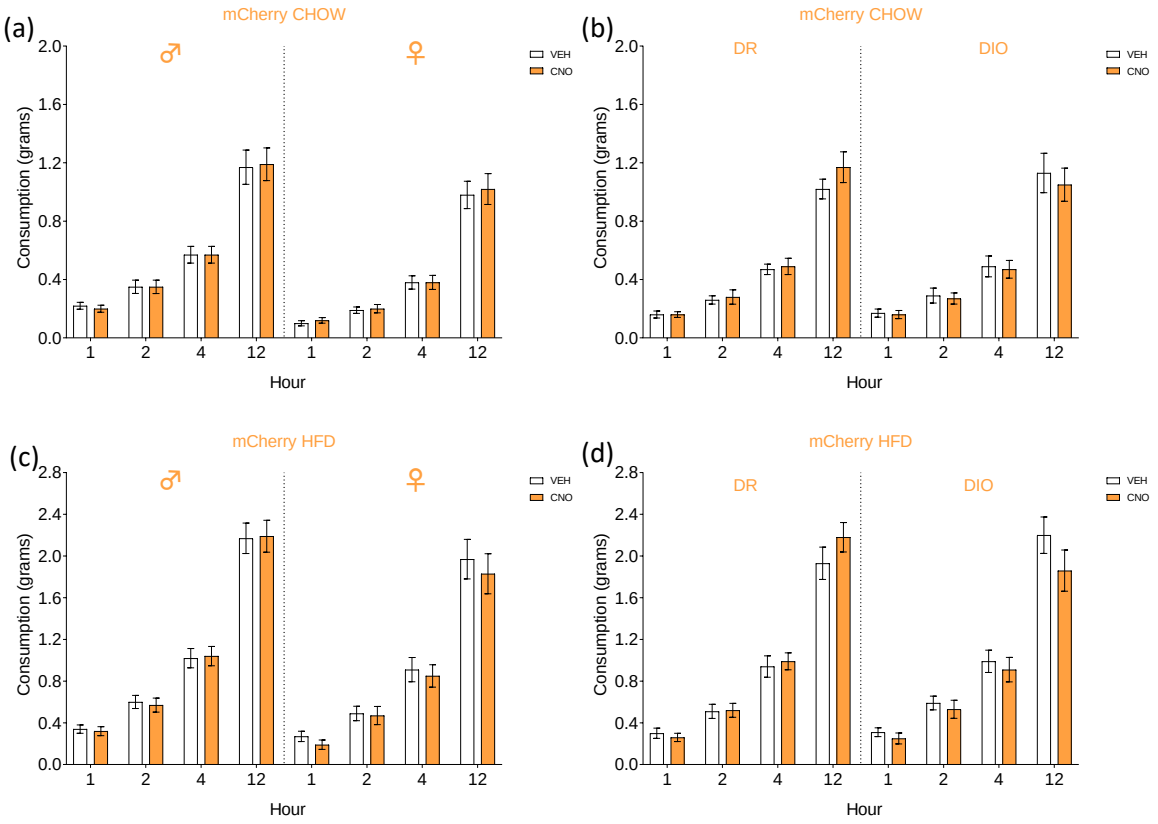
DIO MALE



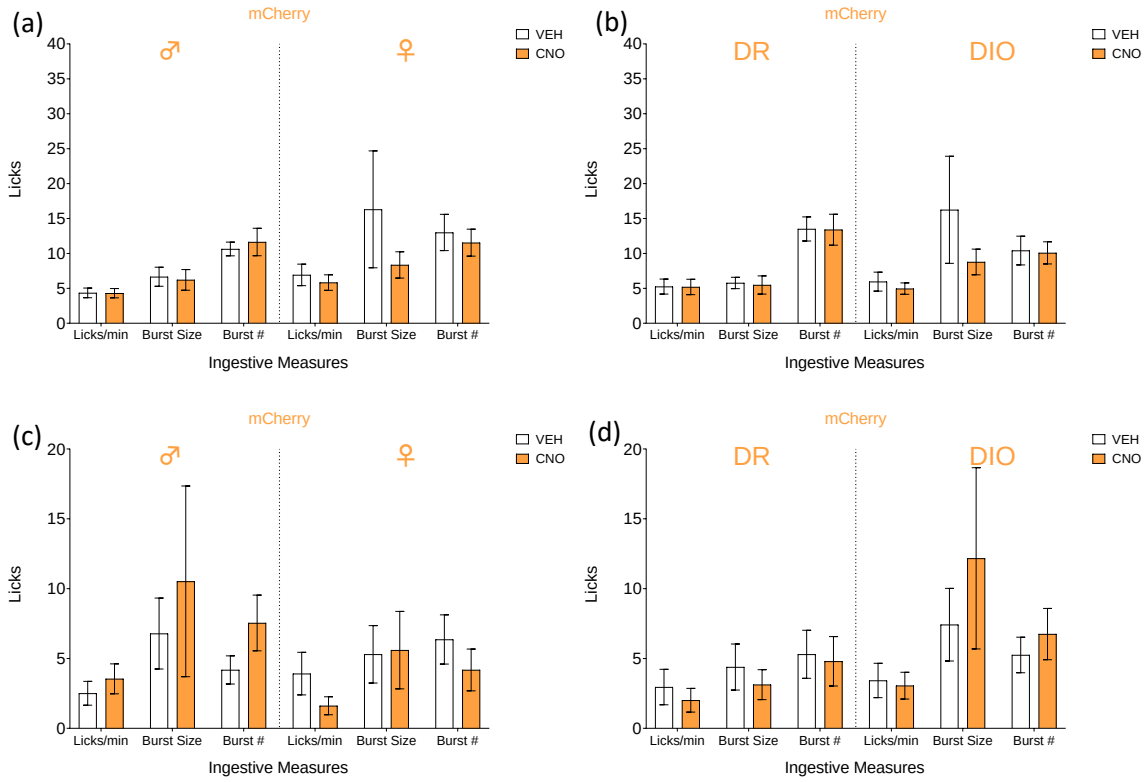
(d)

DIO FEMALE

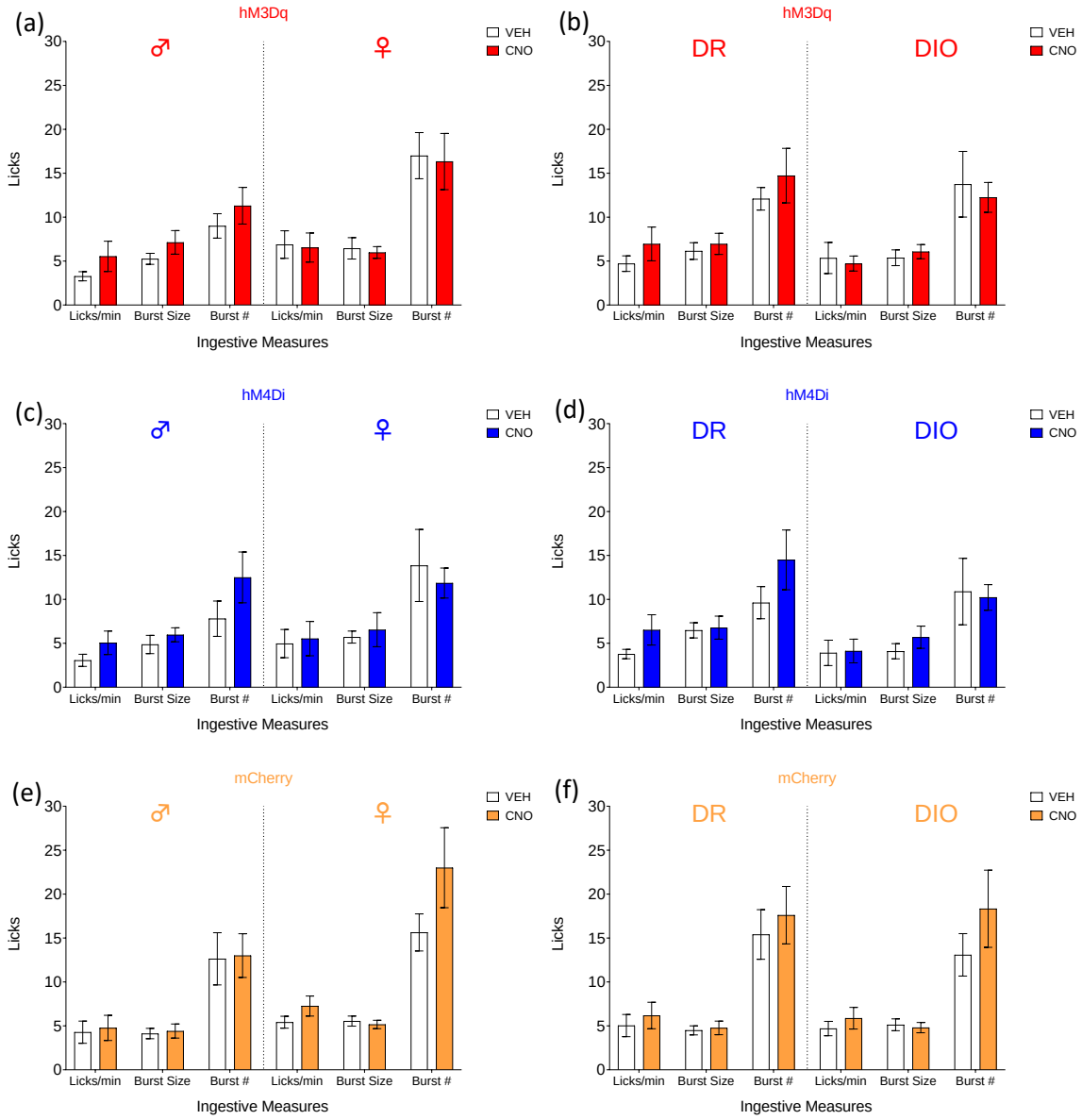




Supplementary Figure 6. Cumulative Chow intake with control mCherry virus comparing (a) sex as well as (b) phenotype. Cumulative HFD intake with control mCherry virus comparing (c) sex and (d) phenotype. Main effects of drug indicated with *p<0.05.



Supplementary Figure 7. Cumulative measures of licking microstructure for sucrose with control mCherry virus comparing (a) sex as well as (b) phenotype. Cumulative measures of licking microstructure for corn oil with control mCherry virus comparing (c) sex and (d) phenotype. Main effects of drug indicated with *p<0.05.



Supplementary Figure 8. Cumulative measures of licking microstructure for DI water comparing sex as well as phenotype for excitatory hM3Dq (a,b), inhibitory hM4Di (c,d) or control mCherry virus (e,f). Main effects of drug indicated with *p<0.05.

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CHAPTER 4: KEY FINDINGS AND FUTURE DIRECTIONS

Key Findings

The ventral hippocampus is increasingly recognized for its role in regulation of meal intake, via activity of the growth hormone secretagogue receptor (GHSR)—the target of the peripheral hunger hormone ghrelin. This interaction of ghrelin with GHSR has garnered particular attention to understanding the obesity epidemic considering the role ghrelin plays in driving consumption; an issue worsened by the pervasive presence of the Western Diet which increases the risk of developing obesity (Kanoski & Davidson, 2011; Kopp, 2019; Rakhra et al., 2020). Critically, ghrelin binding to GHSR in the vHPC is found to drive overconsumption through increases in meal size as well as meal frequency (Kanoski et al., 2013). Although in the early stages, studies are beginning to explore sex differences in GHSR expression across various regions of the CNS and their implications for driving overeating behavior in males compared to females. These investigations of sex on ingestive behavior are warranted as obesity rates vary between males and females, and prior studies indicate that GHSR plays a differential role in consumption depending on sex and the region in which it is expressed (Lovejoy et al., 2009). GHSR blockade in the VTA is found to decrease consumption of palatable sucrose in males, while in females acute GHSR blockade in the LHA is found to decrease sucrose consumption, but not vice versa (Hansson et al., 2012; López-Ferreras et al., 2017; Perello & Dickson, 2015). This indicates that the neuronal pathways utilized in ingestive behavior differ between males and females, however prior to this dissertation, the role of GHSR in the vHPC of female rodents had yet to be explored.

Our decision to examine phenotypic differences as well was driven by a lack of understanding as to why certain individuals are more prone to becoming obese than others given equivalent dietary access (Chang et al., 1990; Levin et al., 1986, 1989a, 1989b). Our chemogenetic approach allowed us to address variables of sex and phenotype via the ability to regulate activity of the ghrelin receptor at the level of the vHPC to

determine what drives a mouse to consume and how those patterns of consumption can lead to a state of obesity.

Chapter 2 Findings

In Chapter 2, we demonstrated that sex and phenotypic differences in consumption are present with both standard lab chow and highly palatable HFD exposure. While others have examined sex differences with regard to obesity—including at the level of the hippocampus—our study is the first to show sex differences in consumption occur both with and without chemogenetically modifying GHSR-expressing cell activity in the vHPC (Dorfman et al., 2017; Hwang et al., 2010). At baseline, males were found to consume more chow than females. However, we found that GHSR-expressing cell activation via the excitatory DREADD hM3Dq ameliorated those differences via attenuation of chow consumption in males, specifically DIO males. This is in contrast to the prior seminal work in males that showed vHPC GHSR activity was found to increase consumption through increases in meal size and frequency (Kanoski et al., 2013). In our study, that effect was only seen in DR females. The surprising attenuation in DIO males may be a novel finding specific to GHSR activity in the vHPC with chronic HFD exposure. In the mediobasal hypothalamus, CNO-evoked inhibition of this orexigenic feeding center led to an expected attenuation in feeding (Peris-Sampedro et al., 2021). Our study indicates that DIO males responding to activation of GHSR-expressing cells by attenuating consumption may be due to regulation of energy balance through interoceptive cue processing that was not present with vehicle. This is because GHSR activation in the vHPC plays a vital role in satiation, and a lack of satiety processing leads to overconsumption as the mouse cannot utilize interoceptive cues to realize when they are full and thus stop consuming (Davidson et al., 2012; Davidson & Jarrard, 1993; Kanoski, 2012; Suarez et al., 2019). Part of this may be due to the arhythmic nature of ghrelin secretion in DIO animals. These animals have lower plasma ghrelin—including

at the onset of the dark cycle—and blunted pre-prandial ghrelin spikes in general, indicating there is less bioavailability of ghrelin for GHSR binding, especially at scheduled times of feeding (Briggs et al., 2011; Li et al., 2011). This is in contrast to non-DIO animals who experience preprandial spikes in ghrelin and a diurnal rhythmicity to ghrelin secretion (Bodosi et al., 2004; Sanchez et al., 2002). This lack of rhythmic ghrelin secretion may decrease in interoceptive cue processing for DIO animals as there is less ghrelin available to bind GHSR in the vHPC during meal time, of which activation is necessary for satiation processing (Briggs et al., 2010; Davidson et al., 2012; Levin et al., 2003; Li et al., 2011; Sample et al., 2015; Zigman et al., 2016). As a result, DIO mice are unable to integrate those satiety cues to terminating consumption, leading to a chronic state of overeating. GHSR stimulation in the vHPC can potentially rescue the response to those interoceptive cues such that DIO males can re-learn meal intake patterns and limit overconsumption. This is supported by our HFD feeding tests, where DR animals—and DR males in particular—attenuated HFD consumption with CNO-evoked stimulation of GHSR-expressing cells. This indicates that satiety processing can potentially be rescued even with chronic HFD exposure. The question is if a follow-up study can replicate these results in DIO animals, and if meal pattern timed stimulation of GHSR-expressing cells in the vHPC can serve as a long-term treatment option to limiting overconsumption.

Regarding the effect of inhibiting GHSR-expressing cells on chow feeding, sex-specific effects were noted where females—but not males—were sensitive to chemogenetic inhibition. The effect of drug on attenuating chow consumption was specific to DIO females. This striking contrast with GHSR cell activation in DIO males and GHSR cell inhibition in DIO females both leading to an attenuation in chow consumption reflects a potential difference in GHSR cell activity between sexes that warrants additional study, especially considering the contradictory nature of the prior described role of the ghrelin receptor in males and the results of our study (Hsu et al., 2015; Kanoski et al., 2013; Suarez et al., 2019). This may be due to the higher proportion of GHSR-expressing cells in the vHPC of DIO females compared to any other group as measured through our viral quantification, This inhibitory effect does reflect other findings where

inhibition of GHSR reduces food intake though this was previously shown in male mice (Ge et al., 2018). It will be important to validate these novel findings of cell count, particularly in our female samples, by utilizing in-situ hybridization to quantify the amount of GHSR mRNA expression to confirm that cre activity mirrors the GHSR expression pattern of the vHPC (Mani et al., 2017).

This increased cell count was not unexpected, as females were found to express more GHSR in the LHA than males, another key orexigenic feeding center, and indeed an orexigenic vHPC to LHA to hindbrain pathway has been recently revealed (Suarez et al., 2019). This pathway may more potently drive feeding in females compared to males, inhibition of which could explain the female-specific decrease in chow consumption. Comparatively, males may have more prevalent compensatory regions for regulating feeding with sufficient GHSR expression in the VTA or ARC, though most of that speculation being specific to males is due to said regions not being sufficiently explored in females whatsoever. These regions may potentially compensate for the decreased activity of the vHPC to stabilize consumption patterns accordingly.

With HFD, we saw that sex did not play a role in consumption. However, GHSR cell activation led to a general attenuation in HFD consumption for all animals, while cell inhibition played no role. Here, the phenotypes played a more important role where DR animals were more sensitive to the manipulations, as depicted by a significant attenuation in HFD consumption with stimulation at all time points; however this effect was mainly driven by the DR males. This introduces a very unique possibility that GHSR agonism—at the level of the vHPC, may actually decrease consumption of HFD in animals by increasing interoceptive cue processing, i.e., the mouse can learn to know when it's full even when exposed to a highly palatable diet. It has been shown that DIO mice have deficits in this cue processing and overeat due to not accurately processing when they are satiated. Part of this may be due to blunted plasma ghrelin levels leading to insufficient stimulation of GHSR-expressing cells of the vHPC, leading to insufficient satiation processing (Davidson et al., 2012; Davidson & Jarrard, 1993; Sample et al., 2015). This could lead

to a vicious cycle of further blunted ghrelin secretion leading to less stimulation of GHSR in the vHPC, driving additional overconsumption due to even lower satiation processing, leading to a progressively worsening obese state. Our data suggests DR males may have intact postingestive negative feedback that is sensitive to stimulation even with chronic HFD exposure, leading to this group reaching satiety with less HFD than at baseline. This reflects a potential mechanism to address overfeeding by learning to process those interoceptive satiety cues in order to stop eating before excess calories are consumed.

When discussing variabilities of sex, one cannot overstate the potential role of the gonadal sex hormones, particularly estrogen. Estrogen is found to induce tonic inhibitory effects on meal size and intake as well as increase GHSR expression which has been well characterized in the ARC (Asarian & Geary, 2006; Clegg et al., 2007; Conde & Roepke, 2020; Drewett, 1973; Geary, 2004; Yasrebi et al., 2016). However, DIO females are found to have reduced sensitivity to the effects of estrogen, indicating a potential mechanism as to the profound behavioral differences between our DIO and DR female mice (Giles et al., 2016). In our case, it would be helpful to confirm estrogen levels are similar between DIO and DR females in the GHSR-IRES-cre model as fasting induced growth hormone release and glucose homeostasis have been found to be impaired in this model compared to WT controls (Peris-Sampedro et al., 2021). A difference in E2 signaling may reflect the difference in viral expression in the vHPC, but would contrast prior results, as we find DIO females have nearly double the total number of fluorescent cells in the vHPC compared to DR females, indicating estrogen modulation of GHSR expression may vary depending on anatomical location in the brain. Additionally, estrogen levels fluctuate with the estrous cycle. In rodents, the rise in estrogen during the proestrus phase of the cycle is associated with a reduction in food intake; however this response is found to be delayed in DIO females (Giles et al., 2016). Thus, it may be beneficial in future studies to determine the phase of the cycle for female subjects prior to testing to determine if estrous cycle phase is a potential confounding variable leading to inconsistencies in chemogenetic manipulation of GHSR-expressing cells in the vHPC.

Chapter 3 Findings

In Chapter 3 we provided new evidence as to the nature of the interaction between sex and dietary phenotype in the consumption of individual macronutrient components of the HFD—specifically sucrose and corn oil. We demonstrated sex effects with vHPC GHSR-cell stimulation, where females will consume more sucrose compared to males as also reflected by an increase in lick rate and burst number, with the finding of increased lick rate specifically attributed to DR females.

Peripheral ghrelin has been shown to enhance consumption of sweet solutions in males, indicating GHSR activity may be an important factor in modulating sucrose consumption in males, but not at the level of the vHPC (Disse et al., 2010). Instead—in males—it may potentially be driven by GHSR activity at the level of the VTA where ghrelin administration is found to increase dopaminergic signaling as well as increase in operant level presses for sucrose (Overduin et al., 2012; Perello & Dickson, 2015; Skibicka et al., 2011). The ARC is another region important in mediating sucrose consumption in male rodents, where GHSR acts in an indirect manner by engaging NPY/AgRP neuronal activity to increase sucrose consumption through activation of downstream NPY-receptor expressing cells in the VTA (Skibicka et al., 2012). It will also be important in future studies to see if these aforementioned regions affect sucrose consumption in females, as the VTA in particular is poorly characterized in regard to sex differences.

vHPC GHSR cell stimulation did not affect burst size in any group during the sucrose tests, indicating that at least for our GHSR-IRES-cre mouse model, GHSR cell stimulation does not affect palatability of sucrose for any individual group. Comparatively, a reduction in burst size was seen with GHSR cell inhibition in DR females, demonstrating that inhibition of GHSR-expressing cells of the vHPC reduced palatability of sucrose for this group. This indicates there is a sensitivity to GHSR in DR females towards sucrose palatability that is not prevalent in the other groups. This reduction in palatability may have been due to the ad libitum access to the HFD. Prior studies indicate that maintenance on HFD affects the licking

behavior between sexes. Male mice maintained on HFD are found to have a decreased burst size of a glucose solution compared to females (Carr & Weiner, 2022). Other studies exploring phenotypes show that DIO animals find sucrose less palatable than DR animals as depicted by a lower burst size (A. W. Johnson, 2012). Here, the combination of sex with phenotype in DR females revealed the greatest sensitivity to the effects of the HFD, revealed with GHSR inhibition decreasing burst size. This also further exemplifies the importance of investigating behavior beyond sex as a variable, as females in general did not show any change in palatability with CNO-evoked inhibition. It was the females were resistant to dietary obesity which revealed a sensitivity of GHSR inhibition to decreases in palatability of sucrose.

Our study also revealed unique effects of sex when consuming corn oil. We found that at baseline, females had a higher burst number and lick rate when consuming corn oil than males. This suggests that females are more willing to continue consuming corn oil as they become satiated and consume more oil overall during the testing period. This indicates that GHSR cell activity in the vHPC is not necessary for driving corn oil consumption. Instead, other brain regions be involved in driving consumption of oil and fat and should be investigated accordingly.

The VTA is one such target that holds promise for investigating differences in licking microstructure when exposed to corn oil. GHSR activity in the VTA is required for ghrelin-evoked feeding behavior (Abizaid et al., 2006; Perelló & Zigman, 2012; Zessen et al., 2012). In addition, ghrelin-dependent feeding also requires intact GHSR activity in the VTA and intra-VTA ghrelin administration increases dopamine (DA) turnover and dopaminergic neuron activity at a downstream target of the VTA—the nucleus accumbens (NAc) (Abizaid et al., 2006; Skibicka et al., 2011; van Zessen et al., 2012). These findings suggest a critical role for ghrelin and GHSR in the mesostriatal reward system. Notably, the NAc is also found to be responsive to corn oil—where corn oil administration in rats leads to an increase in NAc dopamine (DA) release (Liang et al., 2006). This suggests a potential VTA to NAc signaling pathway that may be influencing consumption of corn oil. To investigate this, we would seek to use a different model from the GHSR-IRES-cre mice as

preliminary studies we performed revealed a variable levels of cre-expressing cells in the VTA, compared to the vHPC which contained more abundant and consistent expression. This would also support the notion that consumption is a complex process that involves multiple signaling pathways of the CNS, disruptions or alterations of which can drastically affect the palatability and satiety of highly palatable foods promoting a state of obesity.

This dissertation highlighted one such region—the ventral hippocampus—and its various effects on consumption depending on sex as well as phenotype. DR males reduced HFD intake with GHSR cell stimulation. DR females increased chow intake and lick rate of sucrose with GHSR cell stimulation. Comparatively, DR females found sucrose less palatable when GHSR cells were inhibited in the vHPC. DIO males were found to increase chow intake with GHSR cell stimulation. Finally, DIO females decreased chow intake when GHSR cells were inhibited.

Future Directions

The results of our studies open up multiple possibilities to further explore the interplay of sex and dietary phenotype in ingestive behavior. From chapter 2, our striking result of chemogenetic activation in DIO males and chemogenetic inhibition in DR females both leading to attenuation of chow intake ponders the question of what are the downstream signaling pathways that could be modulating such opposite effects to drug treatment. Suarez et. al have established a pathway in male rats from the vHPC to lateral hypothalamic area (LHA) to laterodorsal tegmental nucleus (LDTg) in the hindbrain and this pathway is found to regulate meal size in males (Suarez et al., 2019). The question is if that same pathway regulates meal size in females as well or if there is another pathway that could potentially explain our noted differences in consumption as vHPC neurons also project to the nucleus accumbens and ventromedial prefrontal cortex for example (Bakogiannis, 2021; Sweeney & Yang, 2017).

Our results also demonstrate a need for further investigation into the role of GHSR in the context of licking microstructure given sex and phenotypic differences in lick rate, palatability, and post-ingestive feedback were seen with both chemogenetic activation and inhibition. In particular, it will be important to investigate why DR females were the most sensitive to these manipulations with the sucrose solution, given their increase in lick rate with CNO stimulation and decrease in burst size with inhibition. This reflects a general issue in the literature where effects in males or diet-induced obesity tend to be emphasized, while females continue to be underrepresented, which is problematic considering a better understanding of how dietary resistance occurs between the sexes can help inform future treatment plans to combatting the obesity epidemic.

Given this is the first known study to assess the licking microstructure of a fat- as opposed to sucrose-laden solution in both males and females, it will be important to validate these findings both in regard to sex and phenotype, but also to establish if the concentration of oil used in this study elicits an appropriate response in licking behavior. The concentration we chose best approximates the proportion seen in the HFD, however in an isolated medium may not be as palatable, particularly in liquid form. Thus, it will be important to establish if different concentrations of corn oil demonstrate a similar monotonic relationship in males and females for measures of palatability or even an inverted U-shape in measures of post-ingestive feedback and overall consumption as one either increases or decreases the concentration of oil. Davis et al., have demonstrated that male rats find corn oil highly palatable at up to 64% concentrations and patterns of post-ingestive follow a similar inverted-U shaped function as demonstrated with increasing sucrose concentrations, indicating that our 45% corn oil concentration was well within reason for assessing licking microstructure (Davis et al., 1995). However, the question remains if these measures of burst size and burst number are equally replicable with different concentrations of corn oil solution, particularly in females. Considering the baseline differences in multiple of our measures between males

and females, this marks an important distinction that a highly palatable solution for males may not be as palatable for females and vice versa.

Additionally, given that palatability is largely driven in humans by the combination of fat with sugar (cookies, cake, ice cream, and chocolate as examples) (Drewnowski & Greenwood, 1983; F. Johnson & Wardle, 2014), it will be important to determine how a combination of fat and sugar affects palatability and post-ingestive feedback. In a preliminary study, we utilized a modified form of an emulsified cream/oil/sugar mixture developed by Lardeux et al., that was found to be highly palatable to rodents, and our data confirm those findings as our mouse model readily consumed the solution (Lardeux et al., 2013). Unfortunately, due to the opacity of the solution we were unable to acquire meaningful licking microstructure data, however the question remains if modification of the solution—potentially through removal of the highly opaque cream—can provide a sugar and fat laden mixture that can draw a more accurate assessment as to what drives consumption of the HFD beyond the individual macronutrient components (B. K. Smith et al., 2001; J. C. Smith et al., 2000).

The next step in my career aspirations following these studies is to translate some of these key findings to address the human obesity epidemic and perform studies in a human population to potentially develop screening tools to determining proneness to obesity through measurable differences in consumption.

One such possibility for screening involves the noted differences in satiation processing between our DIO and DR females. In our licking microstructure analysis, we found females were particularly sensitive to fats—in our case corn oil—as reflected by an increased burst number in females compared to males. This indicates that in our mice, the females were more willing to consume oil even as they became satiated whereas the males were satiated with less oil and consumed less oil accordingly. This poses an opportunity to assess licking microstructure in humans in a way that is reflective of rodent microstructure analysis, though not quite literally examining licking behaviors as outside of ice cream and certain sweets, humans

are not found to exclusively lick many foods. Instead, microstructure has been analyzed in humans using metrics of eating rate—including deceleration rate as one approaches satiety, chewing frequency, and total bouts of feeding during a meal as examples (Berridge, 2000; Gero, 2020).

Thus, continued examination of microstructure analysis in human consumption can better elucidate what comprises postingestive negative feedback in humans, such that we can potentially take younger individuals and test how they respond to certain foods. In females for example, decreased satiety and increased eating bouts with exposure to palatable fatty foods may indicate these individuals will be at higher risk of developing obesity due to diminished postingestive negative feedback as our mouse data demonstrates. This allows behavioral modifications and interventions to be performed before obesity manifests such that these individuals can be taught how to better manage and process satiety cues when they are exposed to fattier foods. This reflects a potential route for behavioral intervention that may provide relief beyond pharmacological options and may address the discrepancy in obesity prevalence between biological sexes. This also can provide clinicians with a primary preventative screening tool combined with a form of personalized medicine that can address obesity risk early on and minimize risk of obesity-associated sequelae from developing.

For those that have already become obese and are looking to manage their weight, one could develop a treatment plan to address and regulate meal intake such that patients can learn to process when they are full and restricting variables to overconsuming highly palatable processed foods that are prevalent throughout the environment. Again, this is reflected by our mouse data as *ad libitum* access to HFD led to discernible weight gain regardless of diet; with the DIO group gaining the most weight overall. One way this could be addressed Is through how food itself is served. Humans are found to scale consumption based on the size of their food plate, and will eat more solely because there's more real estate to put food onto (Kosīte et al., 2019; Ravandi & Jovanovic, 2019; Wansink & van Ittersum, 2013). Teaching individuals how to portion control and calorie restrict can be cumbersome even with the advent of weight loss apps.

However, a recent meta-analysis indicates that portion control through sizing of the serving medium (i.e., plates) on its own shows marginal effects at best at the moment (Robinson et al., 2014). Thus, potentially teaching patients to manage portions regardless of plate size along with learning how to better process postingestive satiation cues, may lead to more consistent calorie intake patterns, leading to a lower total daily energy intake.

Combining potential behavioral interventions above—some of which already exist in the nutritional and dietetic sciences—with the expanding and ever-advancing catalogue of anti-obesity treatments—particularly those regarding the ghrelin system—may lead to more promising clinical outcomes. Currently, selective pharmacological blockade of GHSR is not well-established and the current FDA-approved drugs targeting ghrelin or GHSR have generated mixed results in reducing weight, mainly due to the complexity of interactions with the ghrelin receptor and compensatory mechanisms to regulating appetite if and when GHSR activity is interrupted.

This was potentially reflected in our own results as males across all studies were resistant to chemogenetic inhibition, indicating even if GHSR cells are inhibited in the vHPC, other regions involved in ingestive behavior may be compensating including dopaminergic signaling in the VTA/NAc, or NPY/AgRP neuronal activity in the ARC as examples, regardless of GHSR activity (Briggs et al., 2010; Perello & Dickson, 2015).

This highlights a key point that obesity is a multi-faceted and complex issue and combining medication with behavioral therapy may provide the best case of clinical efficacy for addressing and potentially reversing the weight gain trends of the last 70 years. My goal is to provide my future patients with treatment options to prevent or reverse obesity in a sustainable way that does not require lifelong pharmacologic interventions or invasive surgery, but instead investigates the relationship the individual has with highly palatable foods and learning to process palatability and interoceptive satiation cues to

prevent overconsumption. The results of this dissertation will inform my future studies and set a translational framework to begin unraveling these complex interactions in humans.

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