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Hypothalamic Circuits Mediating Consumption And Anxiety

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HYPOTHALAMIC CIRCUITS MEDIATING CONSUMPTION AND ANXIETY

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A

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Ryan Michael Cassidy

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Dedication

To my grandparents, who had the adventure of a lifetime moving to Alaska in its earliest years.

This work represents a different kind of adventure, but driven by the same spirit.

&

To my patients.

Acknowledgments

Patience is a virtue, and if there is one virtue the manifold people who have made this project possible all share, it is their saintly patience with me!

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Abstract

HYPOTHALAMIC CIRCUITS MEDIATING CONSUMPTION AND ANXIETY

Ryan Michael Cassidy

Advisory Professor. Qingchun Tong, PhD

From 1960-2016, U.S. obesity prevalence increased 13-40% and diabetes increased from 3-15%. There is an epidemic of *metabesity*, the aggregate metabolic disorders produced by chronic overeating. Drugs for metabesity were developed in the 1930s with limited effectiveness; agents today rely on the same principles and are similarly ineffective. Particularly surprising has been the failure of satiety enhancers; this indicates it may not be that physiologic hunger drives chronic overeating. Although hunger and satiety affect traditional reward circuitry (Cassidy & Tong 2017), evidence for the primacy of this effect is mixed. Being hungry reduces anxiety-like behavior in mice; whether the act of eating also reduces anxiety is not well-known. If true, this represents a different avenue for the beneficial effects of eating than just reward manipulation or satiation. The aim of this dissertation is to discover hypothalamic neurocircuits involved in this relationship.

This body of work is the result of two projects in mice. In the first project, I demonstrate that GABAergic eating neurons in the lateral hypothalamus (LH) inhibit GABAergic anxiety neurons in the basal forebrain (BF). Activating this circuit causes feeding and reduces anxiety; fiber photometry shows LH neurons are active both during

food approach and food consumption, but BF neurons only drop in activity during eating. Conversely, BF neurons activate in response to environmental anxiogenic stimuli. It appears that the act of eating can, through the LH-BF circuit, directly reduce sensitivity to threatening stimuli The second project is in collaboration with Yuanzhong Xu and Yungang Lu on a parallel circuit from the paraventricular hypothalamus (PVH) glutamatergic projections to the ventral lateral septum (LSv) GABA neurons. My work with fiber photometry shows that both the PVH and LS respond to anxiety-provoking stimuli and are silenced by food, but with divergent activation/deactivation dynamics.

This data shows at the circuit level that the act of eating itself, not hunger *per se*, reduces activity of anxiety regions in the brain via hypothalamic neurocircuits. It may be that some metabesity arises from chronic overeating of food eaten for anxiolytic manipulation of these circuits, a concept popularly called "stress eating". Until this behavior is addressed by new psychotherapy or pharmacology, these patients will likely struggle to recover.

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Abbreviations

ANATOMY:

3V: third ventricle

aca: anterior commissure anterior limb

AHP = anterior hypothalamic area, posterior part

BF: basal forebrain

BMA = basomedial amygdala

CeA = central nucleus of the amygdala

DBB: diagonal band of Broca

DMH: dorsomedial hypothalamic nucleus

fx: fornix

HDB: horizontal limb of the diagonal band of Broca

ic: internal capsule

mt: mammillothalamic tract

MS: medial septum

LS: lateral septum (LSv = ventral lateral septum)

LH = lateral hypothalamus

NAc: nucleus accumbens (NAcSh nucleus accumbens shell)

PVH: paraventricular hypothalamic nucleus

VDB: ventral limb of the diagonal band of Broca

VMH: ventromedial hypothalamic nucleus

VTA: ventral tegmental area

ZI: zona incerta

OTHER:

2-DG: 2-deoxy-glucose

4-AP: 4-aminopyridine

5-HT#(letter): serotonin receptor (number) (family)

Beta-#: adrenergic receptor beta (number)

D#R: dopaminergic receptor (number)

AAV: adeno-associated virus

ACO: acute-on-chronic overeating

AgRP: Agouti-related protein

BMI: body mass index (kg/m^2)

CART: Cocaine- and amphetamine-related transcript

Cb1: cannabinoid receptor 1

CCK1R: cholecystokinin receptor 1

CDC: Centers for Disease Control

ChAT: Choline acetyltransferase

ChR2: channelrhodopsin-2

CNO: clozapine-N-oxide

CPP: conditioned place preference

CRH: corticotropin releasing hormone

DIO: double-inverse orientation

Ef1a: elongation factor 1 alpha

EGFP: enhanced green fluorescent protein

EPM: elevated plus maze

ER: endoplasmic reticulum

EYFP: enhanced yellow fluorescent protein

FDA: Food and Drug Administration

FGF#: fibroblast growth factor (number)

GABA: gamma-aminobutyric acid

GCaMP6m: GFP-calmodulin Protein 6 (medium-term)

GCGR: glucagon receptor

GIPR: gastric inhibitory peptide receptor

GLP-1: Glucagon-like peptide 1 (receptor)

H2B: histone H2B

HbA1C: hemoglobin A1C (glycosylated hemoglobin)

HDL: high density lipoprotein

LAGB: Laparoscopic gastric banding

LDL: low density lipoprotein

LMWCr: low-molecular weight protein chromium (binding substance)

MC4R: melanocortin receptor 4

MCH: melanin-concentrating hormone

MCH1R: MCH receptor 1

MetS: metabolic syndrome

MHO: metabolically healthy obesity

MSE: Minnesota Starvation Study

NAD+:NADH: nicotinamine (+): nicotinamide adenine dinucleotide (+ charge or hydrogen)

NHANES: National Health Assessment and Nutritional Examination Survey

NHES: National Health Examination Survey

oEPSC: observed excitatory post-synaptic current

oIPSC: optically-invoked inhibitory post-synaptic current

OFT: open-field test

OxyR: Oxytocin receptor

PAM: positive allosteric modulator

PDE#: Phosphodiesterase (number)

PDx1: pancreatic-duodenal homeobox protein 1

PDyn: predynorphin

PYY: peptide YY

REE: resting energy expenditure

RTPP: real-time place preference test

RYGB: roux-en-Y gastric bypass

SAD: standard American diet

SBP: systolic blood pressure

SGLT2: sodium-glucose transporter 2

SOM: somatostatin

Sim1: single-minded 1

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

TC: total cholesterol

TDEE: total daily energy expenditure

TGs: Triglycerides

TRH: thyrotropin-releasing hormone

TTX: tetrodotoxin

VAT: visceral adipose tissue

VLCD: very low calorie diet

WAT: white adipose tissue

WL: weight loss

Y#R: Neuropeptide Y receptor (number)

Chapter 1. The untreated etiology of metabesity: acute-on-chronic overeating

This chapter represents original review work produced for this dissertation

1.1. The Lost War on Obesity.

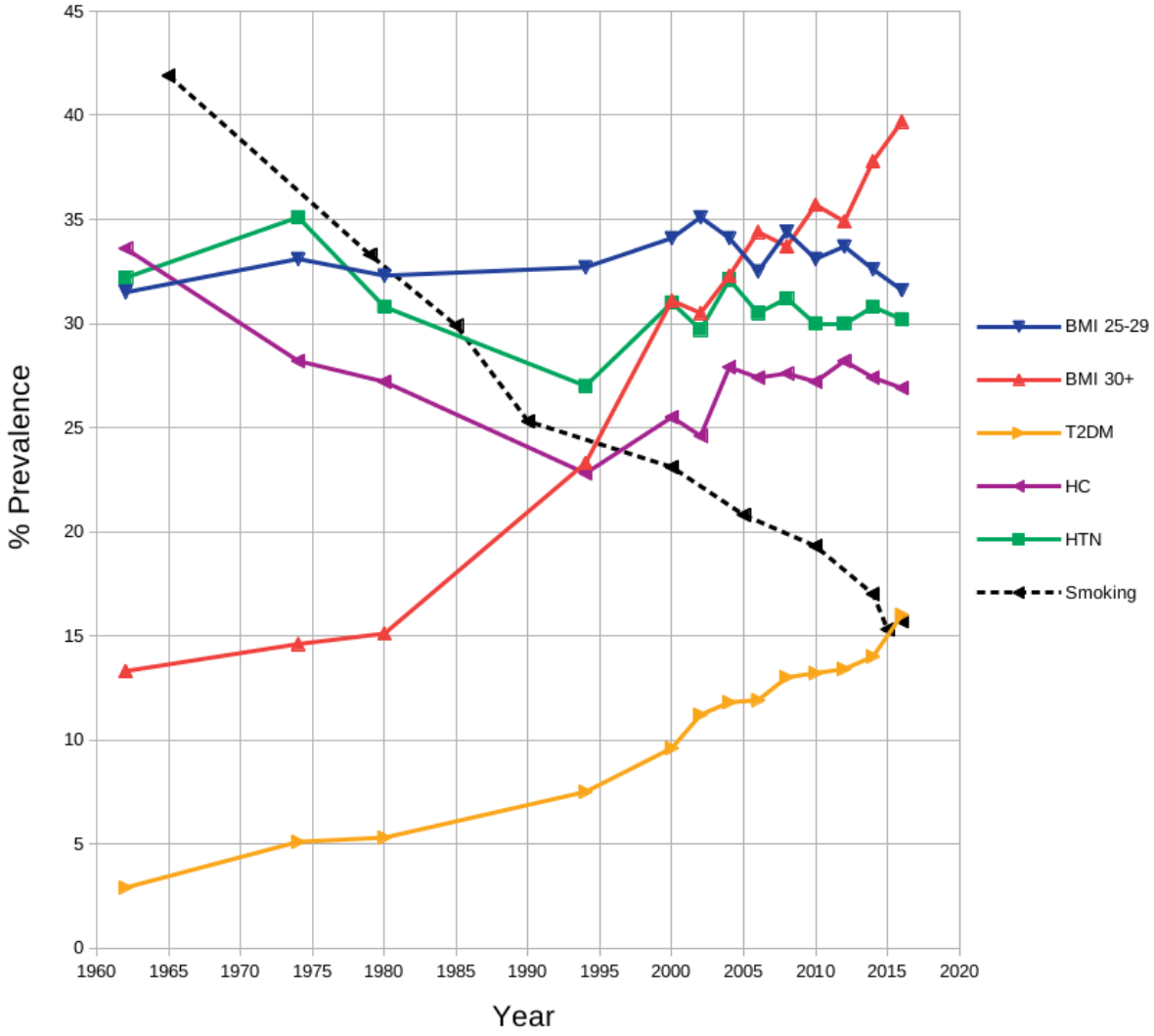
> *Corpulence. Of all the parasites that affect humanity I do not know of, nor can I imagine, any more distressing that of Obesity [...] Obesity seems to me very little understood or properly appreciated by the faculty and the public generally, or the former would long ere this have hit upon the cause for so lamentable a disease, and applied effective remedies, whilst the latter would have spared their injudicious indulgence in remarks and sneers [...]*
>
> *Oh! that the faculty would look deeper into and make themselves better acquainted with the crying evil of obesity-that dreadful tormenting parasite on health and comfort. Their fellow men might not descent into early premature graves [...] from what is termed apoplexy, and certainly would not, during their sojourn on earth, endure so much bodily and consequently mental infirmity.*
>
> *-- William Banting, 1863 "A Letter on Corpulence, Addressed to the Public"*

Mr. William Banting, an English undertaker, published the first widely popular and successful dieting book of the modern era [1]. He was 66 years old, 5'5 and 202lbs. After visiting many physicians who recommended exercise or ignored his complaints, he finally found one Dr. William Harvey (who got the idea from Claude Bernard and bears no relation to the famous William Harvey of the 17th century who described the circulation of blood). Dr. Harvey suggested he 1) abstain from beer (but not liquor) and 2) practice what is recognizable now as a low carbohydrate diet. Banting lost 46lbs over a year and stayed at his ideal weight afterwards. Because of his struggle to find effective dieting advice and to find physicians who took his struggles seriously, Banting published this pamphlet in 1863 and distributed several thousand copies, for free, to the general public. The pamphlet was so popular that “Banting” became a synonym for dieting for many years afterwards.

Low carbohydrate and ketogenic diets like Banting's are the popular recommendation du jour for weight loss. Like most popular diets and exercise programs, they have demonstrable efficacy for weight loss [2]. Banting's desire to spread information has been far exceeded, at least in the U.S., by various governmental agency initiatives such as: the U.S. Department of Agriculture's Dietary Guidelines for Americans and ChooseMyPlate.gov; the Centers for Disease Control (CDC) "Healthy Weight" publication series; the U.S. Public Health Service's Dietitian Professional Advisory Committee's Weight Management Modules; the National Institute of Diabetes and Digestive and Kidney Diseases Weight-Control Information Network; the Department of Health and Human Service's Physical Activity Guidelines for Americans and it's "Healthy People 2020" initiative; etc [3]. These are not new. Such public health initiatives specifically for weight control have been ongoing since the 1970s and have been termed, in keeping with the rhetoric of many policy initiatives from that era, "*The War on Obesity*" [4–9]. However, such initiatives have been ineffective: the prevalence of obesity and type 2 diabetes mellitus (T2DM) have tripled since such statistics were first recorded on a national level. These trends are depicted in **Figure 1** [10–15].

Figure 1. Metabesity prevalence in the U.S. over time

Data integrated from multiple different Centers for Disease Control (CDC), National Health Examination Survey (NHES) and National Health and Nutrition Examination Survey (NHANES), reports on population prevalence estimates of each disease [10–15]. Includes adults over the age of 18, both sexes. Current guideline recommendations for threshold values for hypercholesterolemia, hypertension, and hyperglycemia/%HbA1C were used for historical values, as discussed in the references.



The sobering reality of these data is not meant as a repudiation of these initiatives. The great triumph of behavioral interventions in public health is the concurrent dramatic reduction in smoking also seen in **Figure 1**. Beyond the deleterious effects of tar and particle inhalation on the lung causing inflammation, chronic obstructive pulmonary disease, and lung cancer, smoking also directly contributes to metabolic disease because chronic nicotine ingestion independently induces hypertension, hypercholesterolemia, and insulin resistance [16–19]. Yet, despite halving the prevalence of smoking, these chronic diseases are relentlessly increasing in prevalence. At the surface level, it appears that the intervening 150 years since Banting's publication have not produced any meaningfully more efficacious advice or information on how to control weight. Indeed, although they all can produce dramatic changes in weight, the factors that determine whether any particular individual will respond remain unknown. It seems that knowledge of effective diets is necessary, but not sufficient to stem the epidemic of obesity.

In order to progress beyond Banting, it is thus necessary to revisit the pathophysiology leading to weight gain and metabolic disease. As will be described below in detail, obesity and metabolic disease are multi-factorial and thus many different therapeutic approaches have been implemented. One crucial driver of obesity and metabolic disease is overeating. Although satiety agents have been developed to curb hunger and prevent overeating, their success has been limited. The possibility that some eating behavior is not driven by physiologic hunger has not been examined in detail. One reason that this may have been relatively understudied is the diverse nature of research

on eating behavior, obesity, and metabolic diseases. Thus, the goal of this chapter is also to integrate findings from each of these fields into a unified story and demonstrate the key role of non-physiologically driven eating behavior within it.

1.1.1. Metabesity and weight loss: useful short-hand

Before discussing the potential causes of obesity and metabolic diseases, the disease state itself must be defined. There are a variety of terms used to describe the cluster of chronic diseases believed to be of metabolic origin such as metabolic syndrome, metabolic disease, diabesity. For the purposes of simplicity, I will use the neologism ***metabesity,*** discussed in greater detail in **section 1.2**. It is a broad term, first coined by the endocrinologist Alexander Fleming in the early 2010s, meant to link a diverse set of chronic diseases that share a common metabolic etiology driven at least in part by the environment, rather than pure genetic concerns [20,21]. The biomarkers of obesity, hypertension, hypercholesterolemia, hypertriglyceridemia, hyperglycemia and T2DM and chronic inflammation are all included within metabesity, as are their diverse sequelae such as atherosclerosis and microvascular complications of advanced glycation end-products [21]. Given that obesity and other metabolic illnesses are interrelated and tightly associated with weight gain and that weight loss - or at least reduction in calories - improves all of them (**section 1.2**), I will also use the short-hand of ***weight loss*** to refer to recovery from metabesity.

The increase in the prevalence of metabesity cannot be primarily attributed to an aging population nor to older adults who quit smoking and thus lost the hunger-

suppression mediated by nicotine [22,23]. The percentage of children aged 6-11 years old with metabesity has increased at the same rate, if not faster. For example, in 1965, an estimated 4.2% of this group was obese; in 2016, 18.4% were obese [11,24]. Around the 1990s, endocrinologists stopped referring to T2DM as adult-onset diabetes, because so many children were being diagnosed with it that childhood onset of hyperglycemia was no longer pathognomonic for autoimmune diabetes (type 1). Now, nearly half of all children (<18 years old) diagnosed with diabetes have T2DM, rather than T1DM [25]. Such trends are worldwide [5].

Nor can the lack of progress in treating metabesity be attributed to a lack of advancement in medical therapy. A person with an acute myocardial infarction was twice as likely to die in the hospital or 1 month after discharge in 1960 than in 1990 [26]. The pharmacotherapy available for management of metabesity has similarly expanded considerably. The majority of drugs approved by Food and Drug Administration (FDA) target metabolic illness are, in fact, either 'me too' drugs with pharmacokinetics optimized for certain patient subsets, or drugs targeting the same disease via a different mechanism [27]. There has even been substantial legislation-driven intervention in the pharmaceutical market attempting to promote development of new and less costly drugs to try and maximize access [28,29].

Even if the rise in biomarkers of metabesity was inevitable, many experts predicted that the development of newer and better treatments will reduce morbidity, mortality, and ultimately cost of care for the manifestations of metabesity [30]. This has not been the

case; morbidity and costs have only increased [31]. The burden of metabesity may even have stymied the expected growth in life expectancy in the early 21st century. It is disappointing to compare historical estimations of what the average lifespan of a 65 year old woman would be in 2000 to what they actually became [32]: in 1981, 1984, 1995, and 2000, the estimated remaining years of life were 21, 20.5, 19, and 18.5, respectively. Although much attention has been paid in recent years to the decline in life span attributable to drug overdoses and suicide in people aged 55-64y since the 2010s, it is also true that metabesity-linked deaths are also increasing. In fact, the increase in deaths attributed to heart disease has risen at the nearly same rate as drug overdose since 2011 [33]. In general, the majority of the top ten causes of death in 2017 as reported by the CDC share some or all origin in metabesity [34].

The diverse set of diseases encapsulated by metabesity have all risen in prevalence, indicating a common connecting etiological factor. In my view, as I will elaborate in **section 1.2.1** and **1.2.2,** metabesity is driven by overeating and subsequent weight gain. However, because the concept of obesity has intersected with social forces interested in reducing guilt and shame for body type, and that obesity is usually the only disease readily connected to overeating, this connection has often been neglected or misinterpreted. For example, a short missive from Medscape in 2006 titled *"End the War on Obesity: Make Peace with Your Patients"* makes the following claims [35]: weight has been greatly exaggerated as a health risk; sustained weight loss is not practical nor well-established to improve health; health-improvements arise from health behaviors

regardless of loss of fat mass. While I agree with this to some extent, as I will argue in **1.2.2**, sustaining healthy behaviors that improve health should inherently result in weight loss. This article is emblematic of some of the confusion that must be resolved on the pathophysiology and etiology of metabesity.The sobering reality of these data is not meant as a repudiation of these initiatives. The great triumph of behavioral interventions in public health is the concurrent dramatic reduction in smoking also seen in **Figure 1**. Beyond the deleterious effects of tar and particle inhalation on the lung causing inflammation, chronic obstructive pulmonary disease, and lung cancer, smoking also directly contributes to metabolic disease because chronic nicotine ingestion independently induces hypertension, hypercholesterolemia, and insulin resistance [16–19]. Yet, despite halving the prevalence of smoking, these chronic diseases are relentlessly increasing in prevalence. At the surface level, it appears that the intervening 150 years since Banting's publication have not produced any meaningfully more efficacious advice or information on how to control weight. Indeed, although they all can produce dramatic changes in weight, the factors that determine whether any particular individual will respond remain unknown. It seems that knowledge of effective diets is necessary, but not sufficient to stem the epidemic of obesity.

1.2. Overeating produces metabesity

To determine the necessary etiologies of metabesity, it is important to first set the definition. I specifically have used metabesity (section 1.1.1) to emphasize the common origin of multiple chronic diseases; this term, though a bit clunky, is an encompassing categorizer that has at least some nomenclature distinctness from traditional descriptors of metabolic disease. This concept owes its origin to metabolic syndrome (MetS). MetS , described below, refers to the finding that there is a greater-than-chance clustering of elevated biomarkers for several chronic diseases in a given individual – this has subsequently been attributed to insulin resistance. MetS has been well-studied clinically, and the influence of short-term changes in behavior has a surprisingly profound effect on it's constituent biomarkers. In particular, there have been a series of rigorous studies on the effects of short-term fasting on biomarkers of MetS. In their totality, these studies demonstrate both the utility of the term metabesity and of the behaviors that lead to its manifestation – overeating.

1.2.1. The metabolic syndrome and "metabolic healthy" obesity

The metabolic syndrome (MetS) otherwise known as syndrome X, insulin resistance syndrome, cardiometabolic syndrome, or Reaven's syndrome, is an anchoring concept created to understand the correlation of various metabolic diseases together [36–38]. Specifically, it refers to a cluster of clinical findings - central adiposity (usually measured by waist circumference, though imaging now is more specific and can identify visceral adipose tissue instead), hypertension, insulin resistance, and atherosclerotic biomarkers

like dyslipidemia with elevated low-density lipoprotein to high density lipoprotein ratio LDL/HDL. The 2016 NHANES survey estimates that up to 35% of adults in the U.S. have MetS [14]. The fact that these diseases cluster together indicates that they share a common etiology.

1.2.1.1. Healthy at every size?

While insulin resistance is often the proximal culprit of these findings, the origin of such insulin resistance is murky and often not attributed to obesity. Indeed, a 2014 publication in the *American Journal of Public Health* titled *"Obesity, health at every size, and public health policy"* made the following statement [9]:

> *Obesity is associated with chronic diseases that may negatively affect individuals' health and the sustainability of the health care system. Despite increasing emphasis on obesity as a major health care issue, little progress has been made in its treatment or prevention [...] Evidence is accumulating that a weight-neutral, nutrition- and physical activity–based, Health at Every Size (HAES) approach may be a promising chronic disease-prevention strategy*

There is some truth to this; obesity itself (having a high total body weight) independent of endocrine abnormalities likely only drives accelerated osteoarthritis [39]. Instead, it is the metabesity associated with excess adiposity that causes chronic disease; the differentiation is relevant for making decisions about intensiveness of medical intervention [40]. However, it seems incorrect that "nutrition and physical activity" can correct MetS without also inherently correcting for the factors leading to the development of excess adiposity, hence leading to long-term weight loss. This implies that process

leading to obesity is not an ongoing process that continues to accumulate metabolic damage.

Fortunately, some rigorous prospective cohort studies have been conducted to answer questions like this; the Framingham Heart Study is a classic example, but there are several others. The specific question as to the temporal relationship between excess adiposity and future metabesity was evaluated by the CARDIA study [41]. This 30-year prospective cohort study demonstrated that metabolically healthy obesity (MHO; i.e. excess adiposity without metabolic disease) is, for most people, a transient stage. After several years of MHO, most individuals then develop metabesity (defined here as hypertension, dyslipidemia, or hyperglycemia) [41]. Further, few individuals developed metabesity without passing through the MHO stage, indicating that obesity may be an early marker of the metabesity disease process. These findings have been replicated in several other large scale cohort studies; the more time is spent living with MHO, the higher the probability of developing metabesity [42–45]. Some public health researchers question the utility of MHO as a concept entirely [46].

1.2.1.2. Adiposopathy versus overeating on top of adiposopathy

This raises the next logical question: if obesity almost always precedes metabesity, is it the excess adiposity itself that drives the elevation of biomarkers? This is sometimes called adiposopathy or the adiposocentric paradigm [47,48]. The identification of excess visceral fat as far more predictive of metabesity as compared to subcutaneous fat is an

example of the utility of this model. Put another way, the question is this: which of the following is a more useful generalization of the transition from MHO to metabesity?

Chronic overeating --> adiposopathy --> sustained elevated biomarkers

OR

Chronic overeating --> adiposopathy + acute overeating --> elevated biomarkers

The short-term fasting literature on healthy humans and humans with metabesity can help differentiate these two conditions. If adiposopathy is the primary driver of metabesity, a certain amount of weight loss should precede and predict the degree of recovery in any given biomarker of metabolic illness. Conversely, if acute overeating on top of these resistance mechanisms causes the elevated biomarkers, short-term fasting without substantial fat-mass loss should result in meaningful recovery of biomarkers. This latter behavior can be referred to as acute-on-chronic overeating (ACO). The significance of this distinction will be discussed after reviewing this evidence.

1.2.2. Short-term fasting dramatically reduces metabesity biomarkers

Short term fasting studies in humans with metabesity are powerful experimental tools to differentiate whether it is excess adiposopathy or ACO that is the primary driver of the perpetuation of the biomarkers of metabesity. **NOTE:** *the following is not demonstrating that fasting is "curative" of the known resistance mechanisms that are related to adiposopathy. Instead, it is a demonstration that eating excess energy acutely, on top of the adiposopathy, is what drives the elevated biomarkers known to cause the sequelae of*

metabesity like coronary artery disease from cholesterol and hyperglycemia. **Table 1** is a non-exhaustive summary of the effects of short- and medium-term fasting on relevant biomarkers of metabesity [49–57]. Further work on the subject of fasting, very low calorie diets (VLCD) and their effect on metabesity and longevity can be found here [58–66].

Table 1. Short-term negative energy balance reduces metabesity biomarkers

Data consolidated from sources indicated in the first column. Each value is the reported mean change from baseline. - means no value, n.s. means change was not significant. All lab values were taken in the morning. Units were standardized. Most studies include men and women. See Abbreviations for acronyms.

Citation	Duration (days)	Method	Disease	N	Weight (kg)	SBP (mmHg)	Glucose (mg/dL)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TGs (mg/dL)	Insulin (uU/mL)	Leptin (ng/mL)
Andersson 1988	**2**	VLCD	Obese & HTN	11	-2	-17	-	-	-	-	-	-	-
Browning 2012	**2**	Water	Healthy (F)	9	-	-	-11	31	-	-	-35	-13	-15.5
			Healthy (M)	9	-	-	-15	11	-	-	-40	-9	-4.1
Chan 2003	**3**	Water	Healthy	8	-1.8	-	-	-	-	-	-	-4.3	-1.97
Felber 1981	**3**	VLCD	Obese & T2DM	6	-1.5	-	-100	-	-	-	-50	-12	-
Watts 1990	**3**	Water	Obese & T2DM	10	-3.5	-	-138	-	-	-	-	n.s.	-
Dessi-Fulgheri 1999	**4**	VLCD (+7g NaCl)	Obese & HTN	8	-2.9	-10.9	-	-	-	-	-	-	-
Hall 2015	**6**	-30% carb	Obese	19	-1.8	-	n.s.	-8	n.s.	-3	-17	-2.8	-3.89
		-30% fat			-1.3	-	-7.1	-19	-11	-7	-4	-2	-2.89
Jackson 1971	**7**	Water	Obese	7	-5.1	-	-15	n.s.	-	-	-40	-3	-
			Obese & T2DM	7	-3.6	-	-105	n.s.	-	-	-110	-15	-
Li 2013	**7**	VLCD	Obese	18	-5.3	-14	-	-34	-23	-7	n.s.	-15.3	-15.3
			Obese & MetS		-5.6	-20	-	-47	-42	0.7	n.s.	-8.1	-19.9
Stange 2013	**7**	VLCD	Obese & T2DM	16	-2 (BMI)	-	-32	-	-	-	-	-2.3	-
			Obese & MetS	9	-2.3 (BMI)	-	-11	-	-	-	-	-2.5	-
Lingvay 2013	**10**	RYGB-diet	Obese & T2DM	10	-7.3	-	-36	-	-	-	-	-	-
		RYGB			-4	-	-28	-	-	-	-	-	-
Goldhamer 2001	**10**	Water	Overweight & HTN	174	-6.3	-31.7	-	-	-	-	-	-	-
Goldhamer 2002	**14**	Water	Overweight & pre-HTN	68	-7.7	-17	-	-	-	-	-	-	-
Umphonsathien 2019	**14**	VLCD	Obese & T2DM	19	-9.5	-	-108	n.s.	-	n.s.	-38	-6.1	-
Leiter 1984	**14**	VLCD	Obese	12	-7.8	-6	-	-	-	-	-	-	-
Morel 2011	**30**	VLCD	Obese	24	-6	-6	-11	-54	-70	-12	-12	-3	-25.2
Henry 1986	**36**	VLCD	Obese & T2DM	10	-11.1	-	-190	-45	-	-6	-58	-18	-
			Obese	5	-16.2	-	-20	-121	-	-7	-295	-26	-
Marliss 1970	**42**	Water (+NaCl/KCl)	Obese	14	-21.3	-	-15	-	-	-	-	-25	-
Steven 2015	**60**	VLCD	Obese & new T2DM	15	-14.5	-19	-68	-40	-38	n.s.	-46	-	-
			Obese & chronic T2DM	14	-13.9	-27	-90	-42	-38	n.s.	-19	-	-

The data in **Table 1** demonstrate that changes in the biomarkers of metabolic illness are acute, largely independent of changes in body weight, and meet or exceed the expectations for guideline-recommended chronic dietary interventions. *Hypertension:* a mere 2 days of VLCD resulted in a drop in systolic blood pressure of 17 mmHg (reaching below hypertension cutoffs) [49]. By comparison, a recent network meta-analysis of dietary interventions for blood pressure found that the DASH diet results in between 2.3 to 8.7mmHg drop in systole [67]. *Hyperglycemia*: 3 days of VLCD resulted in a drop of fasting glucose by an average of 100mg/dL (to a below-diabetic level in this group). By comparison, a recent meta-analysis of low glycemic index diets for T2DM found a standard mean reduction by ~2mg/dL [68]. *Dyslipidemia:* 7 days of VLCD in patients with MetS resulted in a reduction of TC by 47 mg/dL, LDL by 42mg/dL [53]. By comparison, the low glycemic index diet for T2DM resulted in a standard mean reduction of TC by 6.4mg/dL and in LDL by 8mg/dL [68]. *Resistance:* Insulin and leptin levels also both drop substantially during the first few days of VLCD/fasting. Further, there was a reduction in markers of insulin resistance (e.g. glucose tolerance test [69] and insulin clamp [66]) and leptin resistance (soluble leptin receptor levels (Lep-R) [53]) after a week of VLCD/fasting.

The rapidity of these results in these small, highly controlled experiments is equivalent or superior to the aggregated changes observed in large scale, randomized control trials of dietary interventions run over several months. Interestingly, it appears that as the length of the VLCD/fasting intervention increases, weight loss continues linearly but the biomarkers begin to stabilize. The level of analysis offered in these studies

did not examine whether the leveling off occurred once pre-disease levels were reached; however, VLCD and short-term fasting with supplementation are well-established to be safe 70,71 (see section 1.2.4). This is unlike the drugs that forcibly reduce the blood levels of these biomarkers; these medications, such as insulin for hyperglycemia or certain alpha-receptor 2 agonists like clonidine for hypertension, can induce pathologically low levels that harm human health [70,71].

Short term negative energy balance is also sufficient to reduce serum concentration of hormones that are normally chronically elevated in MetS - insulin [72–76] and leptin [50,76–78] among others. These findings are largely independent of substantial fat-mass loss (see citations in **Table 1** for more information - the methods for fat mass determination are too heterogeneous to report in a systematized way). However, as discussed above, excessive adiposity appears to be a prerequisite for the development of leptin and insulin resistance and then metabesity.

1.2.3. The acute-on-chronic overeating model of metabesity

It seems that it is **neither** the case that people are "healthy at every size" **nor** that possession of certain level of adiposity is sufficient to drive the expression of various metabolic diseases. Instead, I believe it will be useful to introduce the concept of **acute-on-chronic overeating (**ACO) as the best descriptor of the etiology and driver of metabesity.

ACO is a concise summary of the following concept, introduced in **Section 1.2.1.2**. Over a protracted period of time, overeating (i.e. eating beyond caloric need) results in weight gain and eventually, adisopopathy Eventually, the adisopopathy is significant enough that the excess energy cannot be stored inside adipocytes, and instead circulates in blood. That is ACO, and it drives the elevation of biomarkers known to be causal in the development of metabolic disease. **Figure 2** depicts this concept graphically where, for known quantity of calories in excess of total daily energy expenditure (TDEE) and dependent upon the level of adiposity, excess energy is stored in white adipose tissue (WAT), visceral adipose tissue (VAT), or ultimately manifests itself in the blood for a period of time (such as hyperglycemia or dyslipidemia). The molecular evidence for these dynamics has been established [47,48,79–81]. Each point represents a different day for a given individual across several years.

A) At point A, the patient has relatively low adiposity and is consuming only as many calories as is needed to meet their TDEE.

B) Either because of increased energy consumption or the same amount of energy consumed as in point A but with reduced TDEE, the patient now consumes excess energy; it all gets stored into WAT.

C) Between point B and C, the patient continued to eat above the TDEE and gained significant adiposity. This results in leptin and insulin resistance and cellular mechanisms preventing further incorporation into adipocytes [82]. Thus, some of the

excess energy is incorporated into VAT or deposited into extracellular locations. Notably, it is hypothesized that the failure for WAT and VAT tissue to undergo further adipogenesis to accept the excess energy may instead cause the release cytokines driving the chronic inflammation phenotype so commonly seen in MetS [81,83,84].

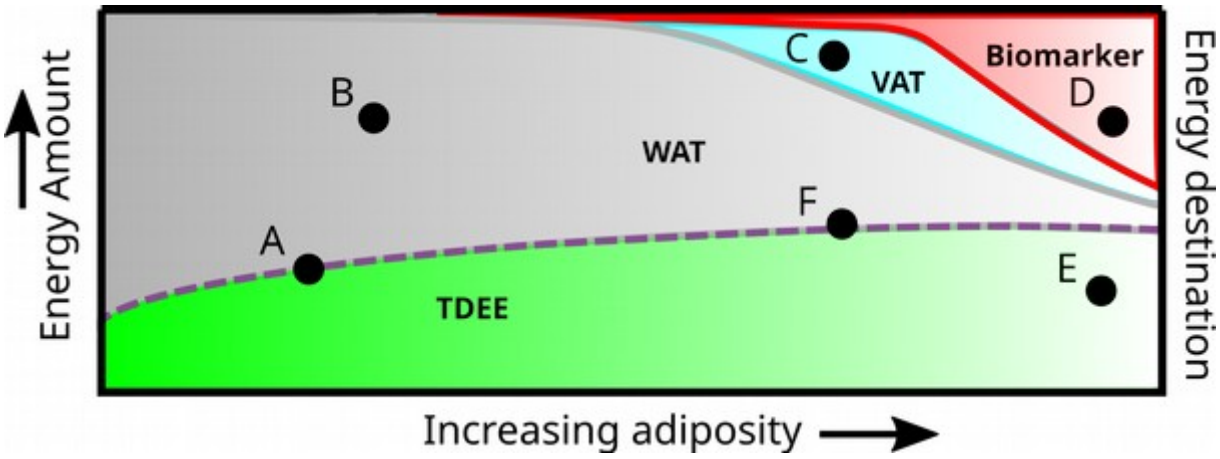
D) Between point C and D, the patient gains even more weight, despite consuming less energy. It is still above TDEE and neither the WAT nor VAT can functionally accept any further energy; this instead is spilled into tissue-deposited extracellular fat (such as seen in non-alcoholic fatty liver disease), or into the blood as hyperglycemia, dyslipidemia, etc.

E) An acute reduction in caloric intake below TDEE, such as seen in **Table 1,** means no further excess energy is spilled into the blood; after several days allows dissipation of some resistance mechanisms, biomarkers of metabesity reduce substantially.

F) Over time, patients unconsciously increase calorie consumption despite supposedly maintaining a diet (see **section 1.3**), and arrive at a heaver weight with intake matching TDEE.

Figure 2. A schematic of how metabesity is derived from acute-on-chronic overeating

A schematic demonstrating the development of metabesity by continuous and chronic overeating. Across a given patient's life time (points A-F), as they consume energy above TDEE, they (B) first incorporate the excess energy into WAT (C), then VAT (D), then dump the remainder into the blood as biomarkers of metabesity, causing further disease (e). Dramatic reduction in energy intake results in no further excess energy being spilled into the blood and reduced risk, without requiring sustained weight loss (F). TDEE= total daily energy expenditure; WAT = white adipose tissue; VAT = visceral adipose tissue. See section 1.2.3 for citations and further details. NOTE: this is not meant to be predictive of all aspects of metabesity, but instead to demonstrate that, in true negative energy balance (which in some people may require eating only VLCD), these biomarkers remit quickly.



NOTE: Hypertension (HTN) deserves some special discussion, as it is less directly related to overeating compared to the other biomarkers of MetS and metabesity [85]. First, excess energy intake causes hyperinsulinemia with increased sodium-water retention - this causes acutely readily-reversible HTN [86]. Second, obesity-linked leptin resistance appears to drive elevated sympathetic activity, resulting in increased renal response with release of renin - this is reversible across several days to weeks as described above. However, physical compression of the kidneys by fat and damage secondary to both HTN and glomerular hyperfiltration lead to early and permanent kidney damage. Since chronic kidney disease itself causes HTN, but HTN is an etiological factor in so many other chronic diseases, it can be considered both a biomarker and a sequela of metabesity.

1.2.3.1. Interpreting the ACO model and its caveats

There is extensive evidence that chronic exposure to hyperglycemia, dyslipidemia, hypertension, and chronic inflammation are the etiological agents that cause end organ damage [37,70,87]. Understanding the origin and preventing chronic exposure is the major challenge of medicine today. The ACO model presented in **Figure 2** provides a unique explanation of the origin and persistence of these biomarkers while incorporating a wide body of evidence. *First*, it provides some validity for the "Health at Every Size" public health approach, wherein meaningful reductions in exposure to risk factors for serious disease can be accomplished just by short-term reduction in feeding behavior. For example, a recent randomized control trial of a 7 day VLCD for patients with T2DM provides some evidence for this; it found that 4 months after the VLCD ended, the fasting

group sustained reduced BMI, SBP, serum glucose, and had an increased subjective quality of life [88]. *Second,* it incorporates the concepts of metabolically healthy obesity, unhealthy obesity, and adiposopathy. As adiposity increases, the same amount of excess energy consumption is spilled into VAT, then the blood, rather than inducing adipogenesis/ hypertrophy and incorporation into newer/bigger cells. *Third,* it anchors eating behavior as a fundamental determiner of metabesity as a whole, both in it's etiology and continual manifestation.

Of course, much of the difficulty in studying the origins of metabesity arises from how many knobs can be tuned to move a threshold seen in **Figure 2**. For example, when three individuals are exposed to the same food environment, the fact that one remains healthy throughout life, one develops type 2 diabetes mellitus, and one develops hypercholesterolemia is a manifestation of genetic susceptibility. Indeed, there are hundreds of metabolic knobs that can be tuned to alter basal metabolic rate and energy expenditure. For example, some individuals with hypothyroidism have reduction in basal metabolic rate (and hence reduced TDEE) and consequently gain substantial amounts of weight in relatively short periods of time, despite eating the same or fewer calories [89–91]. Further, it is well-evidenced that metabolic factors such as excess cellular aging [92–94] and sarcopenia [95–97] directly dysregulate normal levels of biomarkers of metabesity.

Exercise induces similar effects as short-term fasting on most metabolic factors within a short period of time - likely representing increased TDEE. For example, 7 days of high intensity interval workout [98] produced similar improvements in insulin resistance in

individuals with metabolic illness. Mechanisms driving this are currently under research, such as how mitochondrial exposure to excess fatty acids leads to incomplete fatty acid oxidation, contributing to skeletal muscle insulin resistance [83,99] and are reviewed in detail here [74].

Dietary composition is also a major factor driving metabolic response to foods. Macronutrient and micronutrient composition and the cellular and hormonal responses to their ingestion substantially affect whether excess energy is stored into muscle, WAT, VAT, or not incorporated. For example, high carbohydrate foods produce a higher peak glucose concentration in the serum, provoke more insulin release, and cause more storage of calories into fat or hyperglycemia in T2DM-susceptible individuals [100]. Comparatively, extremely low carbohydrate diets (ketogenic diets) seem to produce relatively less weight gain, are less likely to alter adaptive thermogenesis, and mimic fasting's effects on circulating hormones for the same amount for calories [101–104]. Even time-restricted eating, where the same amount of calories are consumed on a daily basis, but only during a few hours per day, seems to reduce the amount of energy incorporated into fat and hence is less "obesogenic" [105,106]. Heavy alcohol consumption is also a unique driver of metabolic illness beyond the pure calorie content that is probably under-assessed in the general population [107–110]. And of course, certain diets, particularly the "Standard American Diet" (SAD) seem to induce over-consumption [75,111–113].

Nevertheless, ACO is an important and necessary etiological driver of not only obesity, but most metabolic illness. It seems to be the primary common causal

factor; targeting overeating should be the first and most successful tool used by clinicians for weight loss. Yet, it is rare for real-world medical practitioners to see their patients successfully alter their lifestyle, at least in the United States. A synthesis of NHANES data from 2011-2016 found that 55% of patients with T2DM report receiving any lifestyle advice from their doctor; only 45% of obese patients, 30% of patients with high blood pressure, and 27% with high cholesterol reported receiving lifestyle advice [114].

Why is this the case? This question will be explored in **section 1.3** where standard practice has not succeeded in resulting in sustained weight loss. However, it is first necessary to discuss several concerns about the deleterious nature of weight loss, calorie reduction, and fasting.

1.2.4. Commentary on the safety and efficacy of prolonged fasting and calorie restriction when treating metabesity

It is worth noting that, to this day, the shadow of the Minnesota Starvation Experiment (MSE) has an outsized negative influence on popular conceptions of fasting and calorie restriction. An article published by Kelsey Miller July 11, 2016 on news.yahoo.com is titled *"The Starvation Study That Changed The World"* and cites the ~1600 calorie amounts the men consumed (see below) and their subsequent deterioration as incontrovertible proof that even mild reductions in calorie intake are dangerous and harmful, which is typically extended to view fasting as very dangerous. Indeed, studies that recapitulate the MSE in recent decades further use this to support both the conclusion that even 50% calorie restriction leads to rapid starvation, post-

dieting binging behavior, and irreversible compensatory adaptive thermogenesis [115]. This is commonly invoked to explain the extremely high relapse of contestants in the television show *Biggest Loser*, where a followup study found that they reported eating fewer calories per day but that their estimated metabolic rate was reduced, causing them to regain all of the weight they lost [116]. As discussed below and in the longer-term fasting studies in **Table 1**, however, more rigorous analyses do not actually support these conclusions and often show the opposite. Why is there such discrepancy? It may be due to a misinterpretation of the MSE itself. Since these ideas owe their conceptual origin to the MSE and are extraordinarily common in the clinical world as a counter to recommendations of food restriction, it is worth discussing the MSE in some detail.

1.2.4.1. The Minnesota Starvation Experiment

The MSE was conducted by Josef Brozek and Ancel Keys, developed initially to simulate wartime starvation in Western Europe [117]. Early observations were published in 1948 and later incorporated into a book titled *The Biology of Human Starvation* (1950, University of Minnesota Press). In this study, 36 young adult men were fed on a diet of ~3500 calories for 3 months, then on ~1600 calories for 6 months, then refed with slowly incremented increased in calories/macronutrient content for another 3 months, then finally given unrestricted food access for a final 2 months. Perhaps surprisingly given the data in Table 1, by 3 months the subjects had all of the symptoms of starvation such as muscle wasting, edema, micronutrient deficiency manifestations like paresthesia, slow

wound healing, hypokeratosis, etc. After the rehabilitation period, they consumed heroic amounts of food. It is illustrative to quote this section of the article directly:

> *[…] on the weekend of October 20, the whole group was at long last free to satisfy their personal food cravings and, most importantly, to eat as much as they wanted. The men gorged prodigious quantities of food, which approximated six to seven thousand calories per day. Many ate more or less continuously throughout the two-day period. Some reported eating as many as three consecutive lunches. […] The free choice of ingredients, moreover, stimulated "creative" and "experimental" messing with food. Licking of plates and neglect of table manners persisted. Attempts to avoid wasting even a particle continued in the face of unlimited supplies of immediately available food. An irrational fear that food would not be available or that the opportunity to eat would somehow be taken away from them was present in some of the men. This may have motivated their eating as much as they could hold at any given time […] Follow-up studies made at thirty-three and fifty-five weeks after the end of semi-starvation showed that the men had returned, by and large, to their pre-experimental "normal" status except that a number of men exceeded their pre-starvation weight.*

The technical aspects of the Minnesota Starvation Experiment clearly showed these men had indeed undergone starvation. Dr. Key's work on the techniques of safe re-feeding and avoiding re-feeding syndrome provided critical evidence that has saved thousands upon thousands of lives. However, he was not a psychologist; further, there is some controversy surrounding the selection of data used to demonstrate an association of increased dietary fat with cardiovascular disease mortality across countries [118–120]. There are several major confounders that limit the applicability of the MSE to all forms of calorie restriction:

1. The subjects were volunteers, but were selected from a pool of draftees who were conscientious objectors, usually for strong religious beliefs such as being a Quaker, and in later interviews they discuss their desire to be doing "something" for the war

effort, since they could not deploy. Thus, some of the intensity of emotions reported may be a reflection of the personality type. Further adding to the behavioral consideration, they were kept isolated for an entire year and asked questions incessantly about hunger.

2. Their diet was extremely high carbohydrate - meant to reflect wartime food supplies in Europe, according to a recent estimate, it only provided 55g/d of protein [115]. While debate is ongoing, evidence has emerged that meeting a minimum threshold of protein consumption per day is much more important than calories for preserving muscle mass and maintaining growth hormone during calorie restriction [121,122]. Hence, some of the starvation was exacerbated by specific nutrient deficiencies rather than caloric restriction.

3. The subjects were also required to exercise and walked 20+ miles per week and had non-specified "work" to do around the camp. Thus, energy expenditure was much higher than would be expected for the average 21st century American adult.

1.2.4.2. Extended therapeutic fasts show high tolerability, minimal reduction in basal metabolic rate beyond expected for weight

These factors likely reduce the relevance of the MSE to understanding therapeutic fasting and calorie restriction to the average healthy or metabese individual, though certainly do not affect the importance of this study for understanding starvation. As alluded to in **Table 1,** numerous prolonged fasting studies (>1 month without calorie consumption) for treatment of obesity, conducted in the wake of the MSE, did not

replicate these findings [123–127]. In discussion section of *Drenick et al. 1964*, for example, the authors make the following observations on 11 patients with metabesity treated by prolonged fasting and a supplement containing 5000U vitamin A, 400U vitamin D, 2mg of thiamine, 3mg of riboflavin, 75mg of ascorbic acid, and 20mg of nicotinamide [123]:

> *The most astonishing aspect of this study, to the patient and to the physician, was the ease with which prolonged starvation was tolerated. This experience contrasted most dramatically with the hunger and suffering described by individuals who, over a prolonged period, consume a calorically inadequate diet. It is remarkable that the fast seemed easier for the patient the longer it lasted. […] most subjects will be able to continue long enough to reach the point when appetite or hunger is no longer felt. From then on, he fast can be safely continued, provided close clinical supervision is maintained. It is not clear why the sensation of hunger subsides, but the disappearance is apparently not related to ketosis. It is also interesting that severe, prolonged hypoglycemia does not produce the hunger sensation or other symptoms associated with abnormally low blood-sugar levels. […] Those who attained an normal body weight were enthusiastic and delighted to resume living as normal-weight human beings. The eating habits and appetites of these individuals seemed to have undergone a decisive change; and, during a limited follow-up period, these patients seemed to have little difficulty in maintaining their weight.*

The fact that every descriptor here runs counters to those seen in the MSE indicates, again, that it may not be representative of all persons experiences with fasting. There are certainly complications associated with long-term fasting and the attendant rapid weight loss such as choledocholithiasis [128] or the possibility of electrolyte depletion in a small subset of susceptible individuals especially during the early natriuresis phase [86,129]. The question as to whether binging behavior occurs after dieting, compared to being an unacknowledged cause of the original weight gain, is not so easy to answer. The inherent act of dieting will make subjects more attentive to their eating behavior and hence a positive confirmation bias if they feel they are struggling with maintaining the

diet in general.[130,131] On the whole, subsequent research errs far more on the side of Drenick than Keys when it comes to understanding the effects of voluntary, protein-sparing and micro-nutrient-repleted fasting [65,105,132].

1.2.4.3. Metabolic fasting versus "overnight fasting" in medical research

Before moving on to discussing modern day successes and failures with dietary, medical, and bariatric interventions for metabesity, two questions related to this data remain:

1) If fasting reverse metabesity biomarkers, why has so much importance been placed on "fasting glucose" and "fasting cholesterol" as markers of metabesity?

The answer is that in the medical literature, "fasting" almost uniformly refers to "morning before breakfast" samples. The questionable utility of this metric can inferred from the results of an elegant study by Shubhroz Gill and Satchidananda Panda [106]. In this study, they deployed a cellphone app to 47 subjects; the subjects took pictures of everything they ate that were then calculated for caloric content by the experimenters. They also sent several push notifications per day asking if the subject had eaten anything. This was combined with an activity tracker (wrist actigraphy) that allowed assessment of baseline eating habits compared to wake-sleep times over 3 weeks.

The results of this study were striking and best put in Dr. Panda's own words: "if your eyes are open, your mouth is open". Subjects ate food within the first hour and a half of waking up (median 1hr 18 mins) and two and a half hours before bed (median 2hrs 22

mins). Amount of time slept per night varied widely, but generally was between 6-8 hours. Further, the data revealed the subjects did not really consume 3 meals per day, but instead distributed calories fairly evenly across the day.

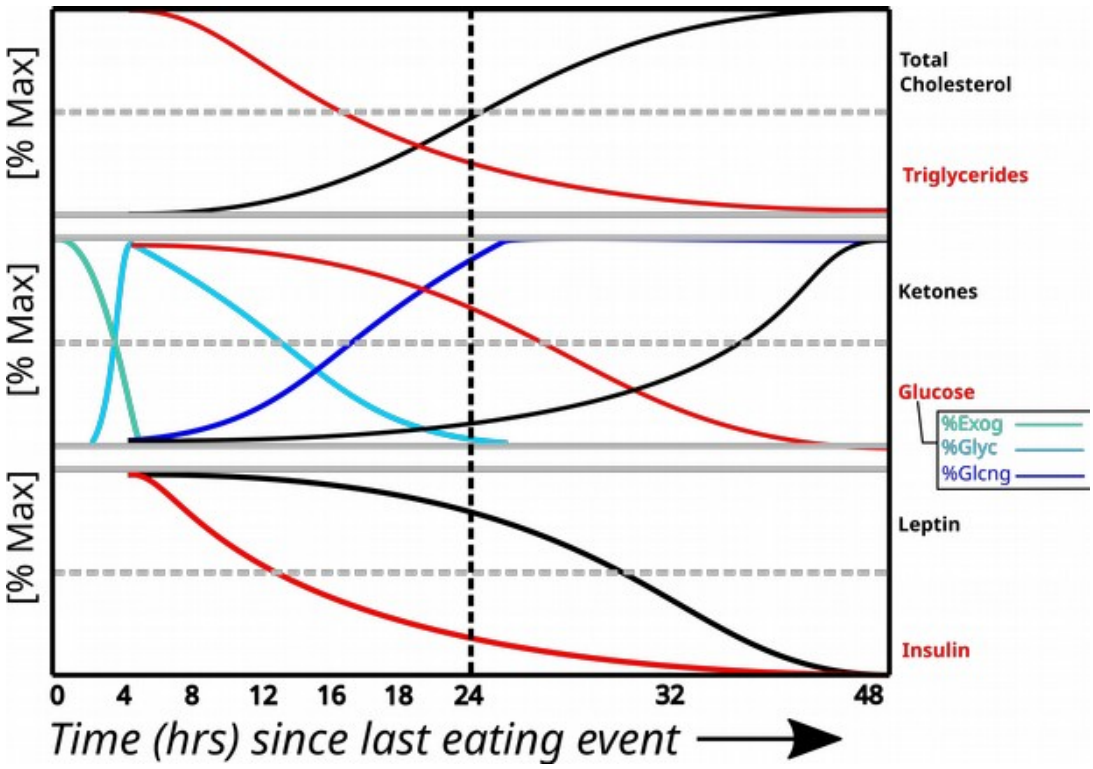
While not the focus of Gill and Panda's study, it reveals that a "morning fasting glucose level" or "overnight fasting cholesterol level" probably represents between 8-14 hours after the last eating event. From the perspective of whole body metabolism, this is not very much time and sits in the middle of a transition period between the metabolic postprandial period and true fasted state. While a detailed review of the transition from fed to fasted state is beyond the scope of this dissertation (see here for such an overview [133,134]) it is clear that a “true” fasting physiology, predicated upon the cessation of glycolysis and initiation of ketosis, does not fully engage until at least 24 hours after the last eating event. Figure 3 shows the dynamics of glucose, ketones, leptin, insulin, total cholesterol, and triglycerides during the transition from the early post-prandial period (4hrs after last meal) to the fully fasted, ketosis state (48hrs after last meal), derived from data reported here 135 and reviewed more extensively here 63,127,136. The massive variations in these biomarkers seen between ‘fed’ and ‘fasting’ days seen in alternate-day fasting studies demonstrates the importance of this nuance [135].

Notably, the literature often refers to the 48-72hr period when ketone bodies become the primary source of fuel as "starvation". It is important to differentiate this from the medical conditions of starvation; these are the diseases of chronic malnutrition, either from total energy restriction or insufficient intake of proteins or lipids with

isocaloric intake (predominated instead by carbohydrates) [136]. Manifestation of these diseases include loss of subcutaneous fat (e.g. 'sunken eyes'), muscle loss in a use-independent manner (e.g. wasting of the temples and reduced grip strength), and pitting edema due to reduced activity of ATP-dependent ion pumps [136,137]. It appears that the negative consequences of protracted fasting/starvation are due to depletion of nitrogen (technically, loss of electrolytes may be the first negative effect). The timeline for the onset of significant nitrogen-loss induced muscle wasting is likely weeks after the beginning of fasting [138] and is due primarily to the need to balance urinary excretion of anion ketone bodies with ammonium [139]. Providing exogenous sodium bicarbonate and potassium chloride is sufficient to substantially reduce ammonium excretion and preserve muscle mass [140].

Figure 3. Serum metabolite changes during the fed-fasted transition

The data presented are derived from Browning et al 2012 [134] and concepts elaborated extensively in the literature [63,125,133,134,138,141–144]. The bottom of each graph segment represents the lowest recorded average value in that condition and the top represents the highest recorded average value: glucose, 73-90 mg/dL; ketones, 0.05-1.94 mmol/L; insulin 3-16 µU/mL; leptin 1.8-19.3 ng/mL; triglycerides 75-115 mg/dL; total cholesterol 145-176 mg/d. The percent change from 4hrs to 24hrs compared to 48hrs was used to determine the location of the 24hr inflection point; a sigmoid line was used based off of aforementioned references. For glucose, %Exog means percent derived from exogenous sources, %Glyc means % derived from glucagon, and %Glcng means % derived from gluconeogenesis



Compared to the detrimental effects of starvation, glycogen depletion and reliance on ketone bodies is not inherently physically impairing. It has long been reported that long-distance runners and hikers can enter into ketosis after several hours of physical exertion [145], that non-energy-restricted ketogenic diets do not meaningfully alter exercise tolerance [102,103], and that calorie-restricted ketogenic diets may actually improve endurance exercise performance [146].

My conclusions from this data are twofold. First, "overnight fasting" does not represent a true "fasted" value that might reflect the baseline metabolic dysregulation stemming from sarcopenia, excess adiposity, genetic variation, etc. Second, there is a significant difference in the metabolic effects of fasting and ketosis versus starvation. The first two are much safer than may initially be believed; however, this leads to the second question.

2) In healthy individuals, fasting decreases insulin sensitivity: isn't it paradoxical that it would improve insulin sensitivity in T2DM and metabesity generally?

This delves into the topic of tissue-specific insulin resistance and sensitivity and the progressive sparing of glucose for tissues that require glucose to function - namely the brain (potentially only glia), red blood cells, and the renal medulla [63]. In a healthy individual, we would expect that, as glycogen stores are depleted and ketone bodies are produced alongside liver gluconeogenesis of glucose, it is adaptive to prevent the liver and muscle from utilizing the gluconeogenesis-originated glucose since they can use

alternate fuels (see Figure 3) 134,148. Thus, those tissues develop insulin resistance. However notably, this only occurs in relatively low levels of serum glucose. In individuals with metabesity, excess serum glucose has induced insulin resistance in liver, muscle, *and adipose tissue* with concomitant high levels of leptin and leptin resistance. Once the adipocytes begin liberating free fatty acids for conversion into ketones in the liver, they regain relative insulin sensitivity as they are no longer at what can be referred to as storage capacity [74,141,147–149]. Similar statements can be made about total cholesterol. While fasting induces an increase in serum cholesterol and free fatty acids in order to supply ketone bodies in healthy individuals, this increase is likely much smaller in magnitude as is seen in hypercholesterolemia – instead, hypercholesterolemic individuals see a relative reduction in serum cholesterol over time, because more is being utilized [149].

1.3. Medical treatment of overeating is ineffective.

The best evidence indicates that dietary and exercise interventions only delay the onset of metabesity for a few years, though this still is a highly cost-effective way to reduce morbidity and mortality [150,151]. Most physicians report not seeing much utility in providing such advice [152–154]. Further, there is a major gap in the patient education guidelines currently available for treatment of obesity, and the difficulty of implementing, in practice, lifestyle modification [155,156]. For example, few studies even report physician adherence to psychotherapeutic interventions like motivational interviewing across time [157].

1.3.1. Dietary changes and lifestyle intervention fail primarily because of lack of adherence

Most studies that evaluate weight loss find a plateau effect around 6 months. This has traditionally been attributed to reductions in resting energy expenditure (ERE) that are disproportionate for an individual's body weight [116,158–162]. However, accounting for differences in ERE does not appear to predict the amount of weight regained 16[163] It may even reflect a discrepancy in the way ERE is measured. There is wide variability in estimations of TDEE and fat-free mass-adjusted ERE (which determines true metabolic need). Further, in more tightly controlled studies, such as in a month-long alternate day fasting regimen [135], or a 6 day metabolic ward trial [52], no reduction in TDEE was detected. Interestingly enough, it appears that these findings are strongest in studies of long-term weight loss with reduced monitoring of patient activity, and generally a recommendation for "continuous energy restriction" (i.e. cutting calories) either in proteins or in fats [164]. Fasting generally preserves TDEE. It seems to be the case that diets promoting growth hormone expression (fasting, low carbohydrate) are least likely to have this effect. Thus there is a possibility that the reduction in TDEE may be appropriate for the amount of weight lost or attributed to one specific dietary intervention [165–169].

The lack of patient monitoring is important, because it is also clear that dietary adherence is often not sustained for lengthy periods of time. Relapses in behavioral interventions often begin to occur 6 months after a change in habit, which times quite closely with the weight loss plateau observed above [163].

This occurs even after highly controlled intensive lifestyle therapy and training, as was tested by the Look AHEAD Research group comparing this intervention to conventional diabetes support and education in over 5000 patients [170]. As soon as the intensity of intervention ended one year after the trial began (with substantial improvements compared to conventional treatment), weight began to increase again and glycemic control diminish. The study's methodology makes it clear this was primarily due to failure to continue adherence to the intervention - ultimately, 10 years after the study was started, there was no significant difference in rates of cardiovascular morbidity and mortality. The failure of this intensive behavioral intervention to produce weight loss has been replicated in many other studies [171]. Given that weight loss per se is not necessarily the only factor that is going to cause dietary intervention to reduce morbidity and mortality from metabesity (Figure 2), these findings could indicate that the patients may no longer successfully diet at any meaningful rate. A recent review of literature on lifestyle modification for weight management has shown that factors such as depression, stress, unemployment, strong body shape concern, and a history of previous failed weight loss attempts all predict poor adherence to change [172].

1.3.1.1. Evidence from medication adherence that long-term behavioral change is a serious barrier to treatment

However, it is not necessarily easy to determine what a person's real dietary habits are. The Gill & Panda 2015 study using cellphone push notifications, discussed in **section 1.2.4.3** provided valuable insight into 'standard' human eating behavior, which does not

really reflect the perceived cultural norms of eating 3 meals a day, for example [106]. However, even the app still relies on accurate patient reporting.

Adherence to medication regiments has been more extensively studied - examining this field may provide insight into dietary adherence. Certainly, there are differences in motivation for a patient to adhere to lifestyle intervention compared to a medication, since weight loss and fitness are positively reinforcing whereas medication side effects are almost uniformly negatively reinforcing. However, medication regimens are far simpler to follow compared to a lifestyle intervention and require less intentionality throughout the day - intuitively, it would appear that adhering to medications is logistically simpler than to a lifestyle intervention.

Unfortunately, it appears medication adherence rates are generally poor and get worse the more rigorously patient behavior is examined or the more complex the regimen becomes. A retrospective analysis of 5,500 hypertensive medication refills over one year at two community pharmacies in Washington state found that, in total, ~75% of all prescriptions were filled in compliance with the regimen [173]; however, when narrowed down to the first three months after initiating a prescription, 35% of patients were undercompliant (did not refill enough times). Complexity of regimen, such as taking several medications at once, reduces adherence, as does the type of drug (lipid lowering drugs have a nearly 20% drop in adherence compared to anti-hypertensives) [174].

1.3.1.2. More intensive observation of medication adherence reveals challenges with delivering effective, patient-controlled therapy

This does not address the question as to whether the patients are actually taking all of the medication received at each refill or following instructions to take the medicine in an efficacious way (for example, thyroid hormone should not be taken with food as it has dramatically reduced absorption). To answer this question, objective biomarkers of drug presence or efficacy can be analyzed.

Biomarkers: A recent analysis on the subject of medication adherence notes that the Vaginal and Oral Interventions to Control the Epidemic (VOICE) trial for HIV per-exposure prophylaxis (Pr-P) was a surprising failure to differentiate, despite enrolling over 5000 patients [175]. In this study, 90% of patients reported taking the medication 100% of the time and pill counts suggested 86% of doses were taken: however, only 30% of blood and cervicovaginal fluid samples had detectable drug. The drugs themselves were well-established and effective clinically, indicating it was not a formulation issue.

Drug efficacy: Curing tuberculosis requires a 4-medication regimen for many months; to increase cure rate and reduce the hazard to the public health, governments have implemented directly observed therapy (DOT) across the world. In most cases, the medication is free to the patient whether self-administered or through DOT; thus, a comparison of cure rates in a given time period can elucidate the degree of influence that habitual non-adherence (rather than economic factors) has on real-world drug efficacy. A 2015 Cochrane Review of six trials from Africa, Thailand, Taiwan, Pakistan, and Australia

interestingly showed that DOT was *not* more effective than self-administration (RR 1.08) in resulting in cure (between 41-67% of the time) [176]. However, this was actually modified by the control condition where patients still went and picked up medication very frequently; if the patient only refilled their prescription once per month, DOT was more effective (RR 1.15). Thus, frequent contact with healthcare providers provided an external reinforcer for adhering to medication to a great enough degree to result in a cure. This is in keeping with the the Look AHEAD study's result, where the beneficial effect of intervention was maintained so long as there was the frequent contact with a provider.

1.3.2. Bariatric surgery - forcible adherence to dietary intervention

Given that patients struggle with adhering to behavioral changes long term without extensive, frequent support, medicine has long sought tools to increase assistance; namely, bariatric surgery and anoretic agents. Bariatric surgery is an aggressively simple solution to the problem of low adherence rates - shrink the stomach so overeating is extremely uncomfortable, or remove enough intestine so excess calories cannot be absorbed. The first well-documented bariatric surgeries for weight loss occurred in the 1950s in Germany, with major resection of the small bowel [177]. This extremely effective, but those patients suffered from severe malnutrition and no way to reverse the operation.

In response to this, bariatric surgeons have rerouted and restricted the stomach and intestines in a wide variety of ways. These less drastic surgeries, such as the laparoscopic gastric banding (LAGB) produces fairly unimpressive results in the prolonged

remission of metabesity and require frequent revisions because of band slippage [178]. The surgeries with substantial intestinal rerouting like the Roux-en-Y gastric bypass (RYGB) appear to sustain weight loss and remission of metabolic illness; for example, in a randomized control trial comparing RYGB to LAGB in T2DM, the mean reduction in percent body weight was 25% and 15% respectively [178,179]. If we assume these patients were at the minimum BMI with T2DM to be included (35), that means the RYGB resulted in an average reduction of 9 points (to 26) whereas the LAGB reduced by 5 points (to 30). The RYGB has also been shown to substantially reduce blood pressure and other cardiometabolic risk factors - likely, the number of indications for RYGB will increase in the future [180].

Bariatric surgery may be a necessary intervention in some subset of people and appears to successfully induce sustained remission in certain cases. It has the benefit of, in the most invasive types, having very high long-term success rate, at least in the field of remission of metabesity. However, it is expensive, time and labor intensive, and requires close outpatient follow-up with substantial vitamin and mineral supplementation, and has an estimated 4.4% readmission rate within 30 days of the operation [181]. The less-invasive surgeries, moreover, usually require at least one surgical revision [178]. All of this means bariatric surgery will likely not become a primary go-to approach to treating metabesity.

1.3.3. Pharmacotherapy for weight loss has limited efficacy

Because drugs are far easier to reliably administer and adhere to as compared to lifestyle interventions, and generally much safer and more reversible than

bariatric surgery, there has been a long history of pharmacotherapy targeted towards weight loss. Perhaps not surprisingly, given the data in **Figure 1**, none of the available drugs are all that efficacious; otherwise, most Americans would be on them.

I have consolidated the most clinically well-established and/or unique pharmacotherapy for weight loss and reversal of metabolic illness into **Table 2.** Each drug listed has either previously been marketed in the U.S. or Europe for weight loss and since removed (strike-through), is currently FDA approved for weight loss (highlight), has been used off-label to assist with weight loss, has been used in off-label clinical trials, or is currently undergoing research. Phase is indicated with year of last known result published either in an academic paper or on cinicaltrials.gov. Date of FDA approval, if not widely known, was taken from accessdata.fda.gov.

Table 2. Pharmacotherapy for weight-loss

*Consolidated from multiple sources [23,182–223]. Highlighted and bold drug names currently have FDA approval for weight loss. Italicized and underlined drug names are only investigational; for these, I have only listed the most successful (has reached Phase 1 clinical trials or has been studied in humans). Strike-through drug names were used clinically but have since been removed from the market. Dates indicate FDA or European approval for any use, not just weight loss; if not approved, latest research status indicated. * indicates commercially available without prescription.*

Monoamine Release		Monoamine Receptor	
Dopamine reuptake inhibitor	Methylphenidate 1955	**5-HT1A agonist / 5HT2A antagonist**	Flibaserin 2015
Norepinephrine-dopamine reuptake inhibitor	Bupropion 1985	**5-HT2C agonist**	Lorcaserin 2012
	~~Fenbutrazate~~ 1957	**5-HT receptor family agonist**	~~Meta-chlorophenylpiperazine~~ Preclinical (1970s+)
	Modafinil 1998	**Beta-3 agonist**	Mirabegron 2012
	~~Phenylpropanolamine~~ 1947	**D2R agonist**	Bromocriptine 1975
	Pipradol 1953	**D3R antagonist**	Gsk598809 Phase I (2010)
Norepinephrine releasing agent	Diethylpropion 1950	**H1R agonist, H3R antagonist**	Betahistine 1972
	Phendimetrazine 1960		Ly377604 Phase II (2010)
	~~Phenmetrazine~~ 1954	**Nicotinic receptor agonist**	Nicotine 1980s (gum)*
	~~Phenylpropanolamine~~ 1938	**Endocrine/Immune**	
Serotonin-norepinephrine reuptake inhibitor	Duloxetine 2004	**Amylin analog**	Pramlintide 2005
	~~Sibutramine~~ 1997	**ATP-sensing K channel PAM (reduces insulin release)**	Diazoxide 1973
Serotonin-norepinephrine-dopamine reuptake inhibitor	~~Aminorex~~ 1962	**LMWCr-binding substance agonist (insulin receptor PAM)**	Chromium picolinate 1989*
	Amphetamine 1935	**CCK1R agonist**	GSKI181771X Phase II (2009)
	~~Benzphetamine~~ 1960	**FGF21 analogue**	Pegbelfermin Phase II (ongoing)
	~~Clobenzorex~~ 1966	**FGF4 receptor antagonist**	ISIS-FGFR4RX Phase II (2016)
	Dextroamphetamine 1996	**GHRH analogue**	Tesamorelin 2010
	~~Fenproporex~~ 1966	**PDE4 inhibitor**	Roflumilast 2010
	~~Levamphetamine~~ 1944	**PDE5 inhibitor**	Sildenafil 1998
	~~Mazindol~~ 1973		Tadalafil 2003
	~~Mefenorex~~ 1966	**Somatostatin mimetic**	Recombinant HGH 1985
	Methamphetamine 1944		Octreotide 1998
	Phentermine 1959	**TBK:IκB kinase inhibitor (reduces downstream NF-κB activation)**	Amlexanox 1996
	Tesofensine Phase III (ongoing)	**Thyroid hormone mimetics and metabolites**	~~Sheep's thyroid extract~~ 1890s
Serotonin releasing agent	~~Chlorphentermine~~ 1962		~~Iodinated casein strophanthin~~ 1944
	~~Clortermine~~ 1973		3,5-diiodothyropropionic acid Phase II (2007)
	~~Dexfenfluramine~~ 1995		Eprotirome Phase II (2008)
	~~Fenfluramine~~ 1973		Sobetirome Phase II (2018)
Serotonin reuptake inhibitor	~~Benfluorex~~ 1973	**Cellular Metabolism**	
	Sertraline 1991	**Pleotropic inhibitor of ER stress (insulin sensitization)**	Tauroursodeoxycholic acid Preclinical (ongoing)*
	Fluoxetine 1986		Sodium phenylbutyrate 1996
Neuropeptide/Neurohormonal Modulation		**Increases intracellular nad/nadh ratio**	Metformin 1957 (EU) 1995 (US)
AgRP inhibitor	TTP435 Phase II (2009)		~~Phenformin~~ 1958
Dual GLP1-GCGR agonist	Cotadutide Phase I (ongoing)	**Intestinal diacylglycerol acyltransferase 1 inhibitor**	Pf-04620110 Phase I (2011)
Dual GLP1-GIPR agonist	Tirzepatide Phase III (ongoing)	**Matrix metalloproteinase inhibitor**	Als-l1023 Phase III (2015)
GLP1 agonist	Efpeglenatide Phase III (ongoing)	**Methionine aminopeptidase 2 inhibitor**	Beloranib Phase III (205)
	Exenatide 2005	**Microsomal triglyceride transfer protein inhibitor**	Lomitapide 2012
	Liraglutide 2014	**Oxidative phosphorylation decoupler**	~~2,4-dinitrophenol~~ 1930s
	Semaglutide Phase I (ongoing)	**White→Brown adipocyte differentiator**	RZL-012 Phase II (ongoing)
Leptin receptor agonist	Metreleptin 2014	**Sirt1 allosteric activator**	Resveratrol Phase I-III (ongoing, many)
MC4R agonist	Setmelanotide Phase III (ongoing)	**Nutrient re/uptake**	
MCH1R inhibitor	Bms-830216 Phase II (2015)	**Intestinal glycoside hydrolase (alpha-glucosidase) inhibitor**	Acarbose 1995
Mu opioid receptor competitive antagonist	Naltrexone 1984		Miglitol 1996
	Naloxone 1971	**Exocrine (Pancreatic/ Intestinal) lipase inhibitor**	Orlistat 1999
Y2R agonist	PYY3-36 Phase II (2008)		Cetilistat Phase III (ongoing)
Y2R/Y4R agonist	Obinepitide Phase II (2007)	**Sglt2 inhibitor**	Dapagliflozin 2014
Y5R antagonist	Velneperit Phase II (2011)		Canagliflozin 2013
OxyR agonist	Oxytocin Phase II (ongoing)	**Combination therapy**	
Neurotransmitter Release		**Combination of other medications**	~~Fenfluramine/phentermine~~ 1992 (never official)
Cb1 inverse agonist	~~Rimonabant~~ 2006		Bupropion-naltrexone 2014
	Taranabant Phase IIII (2008)		Dapagliflozin-exenatide Phase III (ongoing)
Pleotropic ion channel modulator	Topiramate 1996		Phentermine-topiramate 2012
	Zonisamide 2000		Pramlintide-metreleptin Phase II (2009)

1.3.3.1. Five FDA-approved drugs that result in ~5-10kg weight loss.

Despite the extraordinary number of drugs that have been developed for weight loss, only **5** drugs currently have FDA approval for long-term weight loss. They are liraglutide, lorcaserin, bupropion/naltrexone, phentermine/topiramate, and orlistat. While metformin is not FDA-approved for uncomplicated weight loss, it receives an honorable mention in this list as it is commonly used as adjunctive therapy for antipsychotic-induced weight gain and off-label for weight loss [215,224]. Diethylpropion, phendimetrazine, and phentermine are only approved for assisting weight loss for the first few weeks. **Note:** at the time of this dissertation's submission to the graduate school, the FDA had requested a voluntary recall for Belviq, the branded version of lorcaserin, because of a 0.6% increase in cancer incidence (7.1% placebo (n=324) to 7.7% lorcaserin (n=462)) in a 12,000 person randomized controlled trial. Whether this is a permanent recall is not yet known.

The first four FDA drugs all act on satiety signaling and the latter two reduce energy uptake/utilization. They do not work well: the average weight lost on these drugs over 8-12wks is between *6-10kg* [204]. For a 170cm male, this represents a reduction by 1-2 points of BMI. With reference to **Table 1**, a similar amount of weight loss can be achieved in 1-2 weeks via a VLCD or water fast. This is not surprising. The history and present state of the search for weight loss medication is replete with examples of drugs that were minimally effective and unpleasant such as the over-the-counter non-absorbable fat substitute Olestra [225]; mildly effective but addicting (such as amphetamines) [186]; and mildly effective but with serious side effects. Such side effects include cardiac valve fibrosis

associated with serotonin-releasing agents,cardiovascular disease associated with norepinephrine releasing agents like sibutramine, and depression and suicidality associated rimonabant [191,196,226]. To my knowledge, no medication that has historically been used for weight loss/reversal of metabolic illness via weight loss has ever surpassed the 6-10kg mark in long-term efficacy. This is despite almost every imaginable molecular target having been tested preclinically and often in clinical trials. At the time of this writing, there continues to be breathless coverage of the many drugs currently undergoing clinical trials for potential FDA approval for either weight loss or reversal of metabolic illness via reduced eating.

Many of the drugs in **Table 2** are stuck in phase 1 or phase 2 and do not move on to phase 3 or clinical use. In most cases, this is not because of issues with safety; an analysis of 2013-2015 clinical trials found that safety concerns sank ~25% of all drugs undergoing clinical trials. [227,228] Instead the drugs never reach market either because of a lack of demonstrable efficacy or a lack of a viable commercial market for the effects of the drug.

1.3.3.2. A historical perspective on weight loss treatment reveals insights applicable to today

How is it possible that 100+ drugs, many with totally distinct mechanisms of action, have been tested for weight loss, with no real advances? An examination of the first attempts at medical therapy for weight loss may be useful to understand the inherent problems with the approaches tried so far. Serious study into pharmacological

treatment of obesity can probably be said to have started in the late 1930s, with high protein diets (i.e. low carb, low fat), amphetamines, thyroid extract, and 2,4-dinitrophenol all understood as potential weight loss agent and under intense scrutiny for their efficacy [229–231]. Much of the research conducted on these drugs at that time has meaningful implications for the study of weight loss today.

One example, a retrospective cohort study published in 1949, is illustrative. This study evaluated 299 patients who attended an outpatient weight loss clinic and divided them into three treatment groups. The first was dietary intervention alone (1200 kcal/day), the second was diet + thyroid, and the third was diet + amphetamine sulfate. The average amount of weight lost by group at the end of two months was 3.3, 2.3, and 3.1lbs respectively. At the end of treatment, the greatest weight loss was in the most obese individuals who lost on average 9.1(over an average of 9 months), 10.4 (over an average of 6.3 months), and 19.6lbs (over an average of 12 months) on average. This is about the same amount of weight loss seen with the FDA approved drugs of today [222]. Further, the clinical data for these agents show a similar exponential decay in the amount of weight lost over time (i.e. they observed the same plateau as noted in dietary interventions today, see **section 1.3.1**). Researchers at the time noted each of these weight loss strategies manipulated distinct aspects of the physiology regulating weight [232]. These included the following:

Increased basal energy requirement:

1. Decreased energy use efficiency: dinitrophenol (an oxidative phosphorylation de-coupler discovered when ammunition manufacturers during WWI lost weight by chronic inhalation of the chemical) [230,231,233]

2. Increased thermogenesis: thyroid extract and dinitrophenol.

3. Reduced energy absorption: amphetamine (minor increase in gut motility)[232]

Increased voluntary energy expenditure:

1. amphetamine

2. exercise regimens

Decreased food intake:

1. Reduced sensation of hunger: amphetamine

2. Voluntary reduction in eating excess energy: diet and psychotherapy

The drugs in **Table 2** can easily be fit into each of these categories. In particular, many satiety agents targeting hypothalamic neuropeptide systems have been tested and failed, which is particularly relevance to my work in Chapter 2 and Chapter 3 that specifically does not emphasize this aspect of hypothalamic neuroscience. Because of the current deluge of basic science publications on leptin-mediated satiety and increased metabolic rate via hypothalamic nuclei, it is worth examining why leptin therapy fails to control weight [234].

1.3.3.3. Satiety agents fail because of "satiety resistance" - generalizing the case of metreleptin

Metreleptin is a synthetic analogue of human leptin that did not pass phase 2 trials for treatment of obesity after several attempts. It also failed to meaningful alter long-term efficacy of glucose control (when paired with insulin) for treatment of T1DM [235,236]. Instead, it was FDA-approved in 2014 for treatment of several forms of lipodystrophy associated with leptin deficiency [236].

Obesity and metabolic illness are generally considered to be diseases of insulin and leptin resistance; even in T1DM [235] patients develop higher-than-population levels of insulin resistance because of the exogenous insulin. Adding more ligand to these receptors will only perpetuate the resistance mechanism in the long term. The natural response to this problem is to develop some sort of sensitizing agent; multiple agents that could potentially accomplish this were proposed in a recent review titled *"Renaissance of leptin for obesity therapy"* [237]. Many of them can be found in **Table 2,** either as failed/stalled investigational drugs or ones with only modest efficacy in the clinical setting such as liraglutide and metformin reviewed previously [237]. This parallels the fact that these agents have already been used as insulin sensitizing agents in T2DM, as have drugs like the thiazolidinediones and SGLT2 inhibitors [87]. The former has fallen out of favor clinically for lack of efficacy and side effects and the latter seems to work by forcible reduction in serum glucose rather than targeting the problem of insulin sensitivity directly.

This concept can be broadened: the failure of the myriad satiety-targeting (a.k.a. anorectics) drugs in Table 2 should indicate the metabese person suffers from "satiety resistance". Put another way, the primary driver of the eating habits that lead to obesity and metabolic illness is *not* lack of satiety signaling. Instead, the overeating leads to feelings of hunger. As described in section 1.2.4.2, much of the fasting research in the 1960s and 70s reported that patients who ate no food for months at a time often stopped feeling hungry all together, until they reached substantial levels of protein catabolism.

A recent rigorous study of appetite in weight loss put individuals put on a VLCD ketogenic diet for 8 weeks [104]. They had an average weight loss of 18kg (over 10% of body weight loss, 10%WL) over 8 weeks (nearly double an FDA-approved drug's efficacy). They had the worst level of hunger at day 3 of the diet, but which nearly returned to baseline by week 9. However, during the next 4 weeks, they were introduced to a normal diet, and *hunger levels at the end of 4 weeks resembled the day 3 hunger.* Notably, serum GLP-1 was highest at baseline, second highest at week 13 (the very end), and lowest at 10%WL. Active ghrelin was suppressed throughout until week 13, when it nearly doubled. PYY was also at its lowest at 10%WL, as was insulin (which paralleled GLP-1).

From the failure of satiety enhancers and evidence that many peripheral satiety hormones do not necessarily correlate with reported appetite during rigorous monitoring of patients undergoing weight loss (except for leptin), it can be concluded that it may not be a deficit in satiety signaling that prevents people from maintaining diets. Instead, it

may be the case they are eating foods for other reasons besides hunger, i.e. "satiety resistance".

1.3.3.4. Metabolic rate modulators and energy wasting drugs are effective but too dangerous to give at sufficiently high doses

Metformin comes up repeatedly in the discussion of pharmacotherapy for metabesity. It is unique in that it may be the best cellular mimetic of the fasting condition available today. Perhaps ironically, it may also be an agent that most closely recapitulates the effects of dinitrophenol (minus thermogenesis) without it's more deleterious side effects. Metformin's predecessor, phenformin, suffered similar side effects as dinitrophenol related to mitochondrial toxicity, even though it acts on a different aspect of mitochondrial ATP production and in the acute experimental setting has opposing effects on the respiratory chain [233,238,239]. All three agents have been noted to be insulin sensitizing agents, [240,241] and it even has been postulated that the issue with dinitrophenol was that the doses used were too high and a reevaluation with smaller doses could meet acceptable safety standards [233]. It has recently resurfaced as an illegal weight loss agent in the body-building community for this reason [233,242].

Metformin's effects are pleotropic and a single mechanism of action is not clear [243]; however, a net effect of increased NAD+:NADH ratio appears to predict the effects. This mimics cellular depletion of ATP and negative energy balance i.e. fasting [244]. This has been borne out by many clinical studies that show, except for subjective feelings of fatigue, muscle weakness, nausea, and diarrhea, metformin has few common side effects [245–250].

Probably because of the reduced efficiency of ATP utilization, there is concomitant reduction in peak aerobic capacity and reduced adaptation to aerobic exercise training 25[250–252] Nevertheless, its pleotropic effects on metabolic illness clearly resemble those seen in fasting as described in Table 1. Metformin is broadly effective for metabolic illness beyond reducing glucose levels; it induces weight loss [193,253,254], corrects dyslipidemia and hypertriglyceridemia [255–257], and even some evidence for blood pressure reduction [256,258,259]. However, likely because it has very low potency (most drugs are dosed in the milligram range whereas metformin is dosed in grams), metformin is rarely sufficiently effective to be used as monotherapy.

Although thermionic compounds are experiencing a similar degree of excitement as neuropeptide satiety modulators lately, they too have had predecessors that have largely failed and were similarly plagued by patient toxicity [211].

In conclusion, it seems that current pharmacotherapy for weight loss and subsequent treatment of metabesity is at an impasse. Satiety agents, no matter how novel the mechanism, seem to have limited efficacy long term due to habituation and resistance. Drugs that prevent nutrient re/uptake, reduce the efficiency of energy utilization, or induce thermogenesis have upper limits to their dosage before toxicity or malnutrition occurs, and this upper limit does not appear to produce substantial weight loss.

1.4. To treat the metabesity epidemic, target the cause - acute-on-chronic overeating driven by non-physiological factors

1.4.1. Early observations of non-physiologic drivers of hunger may still hold promise today

Several times while preparing this dissertation, I have noticed the prescience of early researchers and clinicians in the field of weight loss. When it comes to understanding non-hunger and non-endocrine related eating behavior, the same has held true. An influential primer by an gynecologist and endocrinologist, Dr. Charles Freed, in 1947 describes what he believes are the "psychic factors" that drive overeating (emphasis mine) [260]:

> *There are a variety of psychologic factors which intensify the appetite for food. Many of these are unconscious, while others are recognized by the patients and can be controlled by them [...] Five hundred consecutive patients who requested treatment for their overweight were asked the question "When you are nervous or worried do you eat more or less?" [370 eat more, 130 eat when bored instead, and the last eat all the time...] The following conditions, many of which are capable of being treated by the general physician, have been found to result in overeating.*
>
> *1. **Environment** [growing up in families which eat excessively, businessmen who eat rich foods commonly during meetings]*
>
> *2. **Economics** [growing up poor makes food relatively insecure and hence one more likely to overeat when given access, as well as how cheap food is high calorie]*
>
> *3. **Monotony** [lack of interest or distractions or even mere inactivity, such as staying at home at the weekend, leads to relative overeating]*
>
> *4. **Occupation** [working in kitchens affords greater access to food]*
>
> *5. **Organic diseases** [being bedridden does not alter the typical amount of calories eaten per meal, despite reduced daily expenditure]*
>
> *6. **Nervousness**.- That which makes a patient worried or anxious, such as a social upset, domestic difficulty, or illness in the the family, will result in overeating [...] it is not uncommon that after the death of a member of the family the others will find*

themselves getting heavier, much to their consternation and shame because loss of weight is considered a classic response to such an occurrence.

7. ***Glandular imbalance*** *[endocrine causes, typically rare]*

8. ***Subconscious factors*** *[Psychoanalytic-Freudian assessment …]*

Childhood and adult food environment and poverty map directly onto current discussions of food deserts and the obesogenic American diet; for an article written in 1947, this is prescient [261–263]. Some of the other observations are prosaic, but Dr. Freed is unique for describing, so clearly and explicitly, how people may use eating as a damper on their emotions (anxiety, nervousness, and boredom alike). These drivers of eating behavior remain poorly recorded in an experimental or systematic way until very recently and almost exclusively come from psychology [264–266]. But, I believe the data in the other chapters shows that such a focus on non-hunger-alleviating benefits of eating may be fruitful for really targeting patient behavior.

Dr. Freed's psychoanalytic discussion, however, focuses on fairly esoteric concepts of maturity and oral fixation. Perhaps because of this, a prominent psychiatrist sent a letter to the editor of *JAMA* in response to Dr. Freed and stated the following [267]:

> *The fundamental problem is whether emotional problems per se, even as "inciting stimuli," cause obesity unless there is already an inherent, constitutional disposition. The term "constitution" which clinicians consider primarily essential in the problem of obesity, is never mentioned in Dr. Freed's article. His article conveys the impression that a psychiatric approach to the emotional problems of the obese individual [is the only important therapy]. This assumption, I feel, is not justified, because the constitutional potential limits a priori psycho-therapeutic efforts.*

These are questions discussed to this day, with many researchers adhering tightly to genetic/epigenetic or alterations in basal metabolism "constitutional" factors [237,268]. Or,

they may believe people who overeat do so because of irresistible hunger like that described by the men in the MSE that resembles a reflex more than a complex behavior.

1.4.2. The state of emergency: a metabesity epidemic with no known way to alter ACO in the long-term

The medical field has developed and is currently testing over 100 drugs (**Table 2**) with mechanisms of action hitting nearly every known constitutional factor and nearly every known satiety center, as well as all conceivable forms of dietary macro- and micro-nutrient intake modifications. We have only seen relentless increase in the prevalence of metabolic illness (**Figure 1**). While the terminology and conceptual models used by Dr. Freed are a bit outdated, his is the only therapeutic approach that has not been tested with the full might of either basic science or clinical research. The "psychic" origins of overeating are worth exploring in more detail.

In this chapter, I have endeavored to demonstrate the fundamental importance of studying eating behavior and the non-homeostatic factors that may affect it. From the data presented, I believe the following conclusions can be made:

1. Metabolic illness is an epidemic that is the causal origin of most chronic diseases (1.1. and Figure 1)

2. Acute-on-chronic overeating – that is, the act of eating excess calories on top of the obese state produced by overconsumption – is a necessary causal factor in the expression of metabolic illness , as demonstrated by short-term fasting (*1.2.*, Table 1,

and Figure 2). Thus, changing eating behavior is crucial not only for obesity, but for most metabolic diseases,

3. Diet and lifestyle interventions for weight loss are successful, but fail in the long term primarily because of patient relapse, rather than adaptive thermogenesis or metabolic changes not proportional to lost weight. Further, bariatric surgery is effective primarily because it forces diet adherence or simple calorie malabsorption (1.3).

4. No long-term, meaningfully effective weight loss drug has been developed, despite intensive targeting of satiety, thermogenesis, energy utilization efficiency, and nutrient absorption. Further, these concepts are not new and have been targeted since the 1940s, without any increase in success (1.3.3, Table 2)

5. The common thread uniting these findings and more than 70 years of research on eating behavior is this: there are untreated behavioral drivers of overeating that are not related to the physiology of energy balance. Discovering these drivers and a mechanistic basis for them (rather than the psychoanalytic model as done by Dr. Freed) may finally give credibility to this concept and provide truly new avenues for treatment.

1.5. Conclusion

1.5.1. A new neuroscience of overeating in the model of the neuroscience of addiction

It is in light of the above observations that I became intensely interested in understanding the basic neurocircuitry regulating the interaction between hunger and other internal states. It appears that the fields of behavioral and pharmacological therapies for sustained and effective weight-loss, and by proxy, metabesity, need to target novel drivers of eating behavior. Further, it appears that some of these drivers are derived from affective (i.e. emotional) experiences like anxiety, stress, and depression. For example, in the 1947 review cited earlier, Dr. Freed expressed a conundrum that strikes us as odd even today "*after the death of a member of the family the others will find themselves getting heavier, much to their consternation and shame because loss of weight is considered a classic response.*"

Dr. Freed's report indicates the possibility that eating is used as a method to suppress the negative aspects of other affective experiences, even though eating itself has consequences. Certainly, one repeated observation in **Chapter 1** has been that human beings are not behaving the way we should over time. We continue to override internal satiety signals, mental plans, and external social pressures to continue to consume food. In psychiatry, "stress eating" and "food addiction" are controversial concepts; however, the use of food as a way to reward oneself and reduce anxiety is commonly observed. Clinically, patients with binge eating disorder uniformly report stress as a trigger for eating [269]. Even more intriguingly, most drugs that reduce clinical levels of

anxiety, such as atypical antipsychotics, tricyclic antidepressants, beta-blockers, benzodiazepines, marijuana, and alcohol, generally increase eating behavior and chronically can lead to weight gain [270].

The concept of stress eating seems quite similar in expression to the concept of "self-medication", wherein people suffering from mental illness, typically depression, begin using drugs like cocaine or alcohol specifically as a way to feel better [271]. The common pathway for clinically-identifiable addictive behaviors is the heightened increase in activity of opioid receptors, most easily accessed by increased ventral tegmental area (VTA) release of dopamine in the nucleus accumbens (NAc) in such a way as to bias for D1R activation/expression [272]. However, in this case, there was a conscious choice to use the drug for the amelioration of psychiatric symptomatology (depression may represent an abnormal extended blunting of this neurocircuits activity), rather than purely for euphoria.

It is reasonable to ask whether these hypotheses of non-physiologic drivers of eating behavior have been targeted directly by behavior therapy. Currently, there are many diets of every macro- and micronutrient composition and calorie content. Further, there are also some behavioral therapies for overeating that are derivatives of addiction therapy - these target reward-seeking behavior or employ behavior change techniques like motivational interviewing. These all have some degree of efficacy, and produce significant effects in certain subsets of patients [171,220]. Targeting mindless eating through portion control has also shown some efficacy [273]. However, to my knowledge, no clinically

common weight loss program has targeted replacing the unique suppressive role eating may have on the experience and expression of other affective states like anxiety or depression. Yet, stress, depression, and unemployment are predictors for poor adherence to weight loss regimens of all kinds [172].

The reason that this correlation has not been targeted directly for improvement of adherence to weight loss regimens may be because of a lack of mechanistic evidence that this correlation is relevant. Indeed, the non-hunger drivers of eating behavior described above - stress eating and anxiety reduction – do not yet have an expansive mechanistic literature demonstrating how they might operate at the neurocircuit level. It is worth noting that this approach has already paid dividends in the excellent work that produced the neurocircuit theory of addiction. For example, it is not intuitive that opioid receptor antagonists like naltrexone would be effective at reducing alcohol-consuming behavior. However, this treatment was tested and is effective because of the demonstration that addictive behaviors require the interaction of GABA, dopamine and opioid release in the nucleus accumbens [274].

Thus, it is my hope that by leveraging the neurocircuit approach pioneered to understand and treat addictive behavior, a similar approach will be fruitful in the new neuroscience of eating and provide avenues for new therapy, both pharmacologically and behaviorally.

1.5.2. Example neurocircuit evidence underpinning the possibility that hunger and eating may alter the expression of other affective states

A neurocircuit basis for the interaction between hunger and other affective states such as anxiety will expand the field of metabesity therapy. Amidst many publications on the primary neurocircuitry of hunger, some interesting data on how hunger alters the expression of other affective states has also been revealed. However, interpreting much of this data has been challenging because of the confounding effects of the typical experimental manipulations used to elicit hunger behavior (i.e. fasting).

For example, it is well established that hunger and eating behavior reduce mouse expression of anxiety [275]. However, this finding is derived from observations of the fasted state and is difficult to distinguish from a negative drive to find food. Further, it has so far been difficult to determine if, when comparing between physiological hunger (often called homeostatic) and non-physiological eating (often called hedonic), the latter is only driven by traditional reward circuitry [276,277] or has unique pathways that may underpin it. This is a crucial question for separating whether all non-physiologically driven eating behavior is driven by 'food addiction', or whether hunger and eating can directly alter the experience and expression of affective states.

One way to demonstrate the difference is to test a relatively reward-neutral behavior, like maternal care, and examine whether neurocircuits exist that connect the circuits driving eating behavior and those driving maternal care. In a recent *Journal of Neuroscience* publication (Li et al. 2019), the investigators demonstrated this point [278]. In

this study, the investigators used optogenetics to activate hypothalamic arcuate AgRP-expressing neurons that project to the hypothalamic median preoptic area (MPOA) in mouse dams (mothers with pups). They demonstrated the following: 1) protracted fasting reduces maternal nest building and increases the latency to scattered pup retrieval 2) the AgRP-->MPOA projections are GABAergic 3) activating this projection prevents maternal nest building, but not pup retrieval 4) a different set of MPOA neurons underlie pup retrieval than do nest building. This data provides a unique glimpse into how eating behavior and care behavior interact with each other, disrupting complex expressions of affective state. Further, it demonstrates this occurs directly, without relying upon interaction with traditional drivers of reward behavior like the ventral tegmental area or nucleus accumbens.

Given this fascinating neurocircuit finding that two very distinct behaviors appear to 'compete' in real time, with electrophysiological evidence that this occurs via monosynaptic connection, it is natural to wonder if such direct competition can be observed and tested in other circuits. Indeed, research in the Tong laboratory, which will be discussed more extensively in Chapters 2 and 3, also has demonstrated the competition between hunger and aversion driven purely by subcortical circuits, where neuroanatomcial areas known to generate these aversive behaviors such as self-grooming directly synapse and act on hunger areas – and vice versa 281,282.

If it is true that 1) the act of eating elicits activation of certain brain regions associated with hunger more generally and 2) direct projections from those brain regions

activate or inhibit other brain regions that are associated with anxiety, these data would provide a mechanistic platform for the context of "stress-eating" and have all the implications described in **Section 1.5.1**. My work in Chapters 2 and 3 discusses my small addition to the suite of evidence showing that eating and anxiety-linked brain regions do, in fact, directly interact with each other, independent of physiologic hunger.

Chapter 2. A lateral hypothalamus to basal forebrain neurocircuit promotes feeding by suppressing responses to anxiogenic environmental cues

This chapter is based upon: Cassidy RM, Lu Y, Jere M, Tian JB, Xu L, Mangieri LR, Felix-Okoroji B, Selever J, Xu Y, Arenkiel B, Tong Q. A lateral hypothalamus to basal forebrain neurocircuit promotes feeding by suppressing responses to anxiogenic environmental cues. Science Advances 2019 5:eaav1640

2.1. Rationale

Given the information presented in Chapter 1 it becomes clear that identifying hunger and eating neurocircuits driving or suppressing non-hunger affects will yield new behavioral approaches to treating acute-on-chronic overeating. There are an extraordinary number of subcortical targets to examine that may bear fruit, and it is clear that the mammalian brain is possessed with an abundance of redundancy such that any single anatomical nucleus is not the sole arbitrator of most functions. However, the hypothalamus and lateral hypothalamus (LH) in particular may prove to be important nodes in the neurocircuits that produce these functions.

Hypothalamic neurocircuits are developmentally programmed and highly distinct in their ontogeny; disruption of individual hypothalamic neurocircuits during development can cause lasting changes in eating behavior [279–281]. There is mounting evidence that some of the known genetic disorders that lead to overeating such as Prader-Willi syndrome, have prominent disruption of hypothalamic neurocircuit development – further, treatment with neuropeptides produced in the hypothalamus like oxytocin has been effective for regulating eating behavior in that condition [200,282]. The LH sits at the center of a large number of functionally integrated affective systems that have been identified in a diverse set of experimental conditions, receiving input from the ventral pallidum, central amygdala and basal nucleus of the striatal tract (BNST), and prefrontal cortex [283,284]. The LH has many diverse functions and projections, as will be discussed below; however, few of its outputs regulate motor output directly. Instead, it

alters the activity of *other* nuclei that integrate this input with other input before generating motor output [283–285]. This is consistent with the hypothesis that its primary role is to generate an affective state and alter the expression of other affective states.

Thus, hypothalamic neurocircuits in general, and LH neurocircuits in particular, are promising targets for investigating major nodes connecting the hunger and eating to expression or suppression of other affective states. Previous work in the Tong laboratory has indeed discovered such neurocircuits, defined by fast neurotransmitters and neuropeptides both, which mediate the intersection between peripheral signals of energy balance and the central nervous system [286–294]. In particular the Tong laboratory has demonstrated that the lateral hypothalamus (LH) sends both GABAergic and glutamatergic projections to the paraventricular hypothalamic nucleus (PVH). Activation of the former cause feeding and the latter cause self-grooming, a common rodent marker of anxiety [286,294]. In addition to this projection and the LH-basal forebrain projection I describe below, at the present date the LH has been demonstrated to send **GABAergic** projections to the ventral tegmental area (VTA) [295–297], periaqueductal gray (PAG) [298], ventrolateral preoptic area [299,300], dorsal raphe nucleus (DRN) [301], bed nucleus of the stria terminalis (BNST) [302,303], locus coeruleus (LC) [299], and tuberomammillary nucleus (TMN) [299]. Crucially, it appears that GABAergic outflow is responsible for eating behaviors, whereas glutamatergic outflow is anoretic [304,305]. While not all of these **GABAergic** circuits have been tested extensively, activation of the LH-VTA, LH-PVH, and LH-BNST circuits induces

robust feed behavior, activation of the LH-PAG drives attack, and those to the LH-VLPO induce awakening from sleep.

This diversity of functions demonstrates well that the LH is highly heterogeneous, even within the subset of LH neurons that release GABA. There is also abundant evidence for functionally distinct subsets of LH neurons that synthesize glutamate, dopamine, galanin, hypocretin/orexin (orexin), melanin concentrating hormone (MCH), dynorphin, enkephalin, neurotensin, cocaine- and amphetamine regulated transcript (CART), parvalbumin, thyrotropin releasing hormone (TRH), corticotropin releasing hormone (CRH), nociceptin/orphanin FQ, and unocortin-3 [306,307]. Further, many of these neurotransmitters and neuropeptides are co-expressed and co-released, and it is unclear to what extent their projection patterns diverge. Some progress has been made in identifying anatomical subdivisions within the LH (i.e. some studies refer to the perifornical region, the posterior LH, and the anterior LH) that may have distinct functions, but it generally remains difficult to conclude whether the region determines the projection or cellular type determines the projection [284]. This may explain why there has been relatively little success in studying this region using genetic markers as there has been for other brain regions. The primary exception to this, of course, is work on the sleep-wake cycle driven by orexin and MCH neurons [308]; these are regionally segregated within the LH and mostly do not co-localize with other neurotransmitters. By comparison, calcium imaging of LH GABAergic neurons has revealed that adjacent GABAergic cell bodies have distinct activity patterns during the feeding process; some are active only

during food approach, some are active only during food consumption, some are active during both, and some are not activated in either condition [305,309]. Do these adjacent neurons target distinct regions? It is not yet known and will require intensive study to do so.

Studying specific projections instead remains one of the most effective ways to examine what behaviors each subset of LH^{GABA} neurons mediates. Of particular relevance to the question of how eating interacts with anxiety is the projection of the LH to the medial septum (MS) and diagonal band of Broca (DBB), which for the purposes of brevity will be referred to as the DBB or basal forebrain (BF). The DBB shares strong reciprocal connections with the primary sensory cortex, the thalamus, and activation causes behavioral inhibition and enhanced attention to the environment [299,310–313]. Conversely, destruction of the DBB or BF more generally appears to cause generalized behavioral *dis*inhibiton, including excess eating and drinking [314–317].

The existence of an LH-DBB projection has been controversial, as it has variously been reported as present and absent within the older tracing literature [318–321]. This likely reflects the underlying heterogeneity of the LH discussed earlier. Orexin activity in the BF drives wakefulness and LH^{orexin} neurons are documented to project to the BF [322,323]. Viral retrograde tracing from the MS/DBB portions of the BF has shown that $LH^{neurotensin}$ [324] project to this area, and that the LH sends input to cholinergic neurons [313]. However, no connection related to feeding behavior has yet been identified prior to this work and the putative LH^{GABA}-BF circuit has not been discussed in reviews of this aspect of LH

neuroscience [304,306]. A major joint publication with our collaborators wherein it was demonstrated that destroying cholinergic neurons within the DBB induces profound obesity and chronic activation causes starvation and death [325], lead to this hypothesis: LH projections to the basal forebrain drive feeding behavior. Further, I hypothesized that this likely related to some aspect of reducing responsiveness to the environment as activation of the BF broadly increases alertness and attentional modulation of sensory processing [322]. To test these hypotheses, I used the materials and methods in **section 2.2**.

2.2. Materials and Methods

The following tools were selected as they were best suited to answer the questions posed in **Section 2.1.** They provide the ability to both manipulate (via optogenetics) and observe the activity of (via fiber photometry) specific, molecularly defined neurocircuits in real-time and in freely moving mice. Since the mice are tested in real-time and are freely moving, they can be tested using standard behavioral neuroscience techniques such as the open field test or elevated plus maze. The acuity of effect also allows pre- and post-intervention comparisons, as well as paired comparisons across two different interventions (e.g. a refeeding assay paired with or without laser stimulation, described below), all in the same animal. This reduced the effect of inter-subject variation. The relatively large effect sizes produced using these techniques increased the power of these tests. This made it possible to minimize the number of mice used, in keeping with IACUC goals to minimize potential pain and suffering of experimental animals.

Given that many of these experimental techniques are used and reused across several different experiments and questions, the **Results** section has been written to integrate the use of these techniques into a logical and cohesive story.

2.2.1. Mouse care and strains

All experiments were performed with genetically modified mice. These mice were housed inside the semi-barrier animal facility located inside the Institute of Molecular Medicine, part of the University of Texas Health Science Center at Houston (UTH). After weaning at 3 weeks of age, between 2 to 5 littermates were group-housed in cages with

corncob bedding. They were fed *ad libitum* with access to standard pelleted laboratory mouse diet (referred to here as chow) and water. The vivarium was kept between 21-22℃ and has a 12-hour light/dark cycle. All animal care and procedures were approved by the UTH Institutional Animal Care and Use Committee (IACUC) and all guidelines for care and use were followed.

Preliminary analysis demonstrated no meaningful difference in elicited behaviors between males and females, so these were both used in roughly equal numbers. Multiple litters of mice were used for testing and mice were at least 6 weeks old prior to any behavioral testing. The following genetically modified strains of mice were used: Pdx1-Cre [292], Vgat-Cre [326], and Vglut2-Cre [326]. The details of how these mice were generated may be found within the references; they were derived from C57BL/6 and 129 mouse strain crosses. In order to reduce the possibility of a strain effect, nearly all experiments discussed in the **Results** section were performed with internal comparison of an acute gain-of-function intervention (such as activating a neurocircuit, discussed below).

Pdx1-Cre is a transgenic mouse line where expression of Cre is dependent upon expression of the pancreatic-duodenal homeobox 1 (Pdx1); Cre is expressed in the pancreas, and in the medial preoptic area, arcuate nucleus, dorsomedial hypothalamic nucleus, DRN, inferior olivary nucleus, and in the LH [292]. This limited expression of Cre in the brain makes it easier to reduce off-target expression of cre-dependent proteins after viral infection. Vgat-Cre is a knock-in mouse line where ires-Cre has been inserted just downstream of the SLC32A1 gene (which encodes for vesicular GABA transporter/Vgat)

[326]. The internal ribosome entry site (IRES) gene sequence allows translation initiation at that site on the mRNA [327]. The net effect is that Cre is expressed at the same level and in the same tissue as Vgat, allowing targeting of GABAergic neurons. Vglut2-Cre is also a knock-in mouse line, with ires-Cre inserted downstream of SLC17A6 (encodes for vesicular glutamate transporter 2/Vglut2) such that only tissue that expresses vglut2 (a subset of glutamatergic neurons that are the predominant type in the hypothalamus and basal forebrain [326,328].

2.2.2. Neurosurgery

Stereotaxic surgery for the delivery of viral vectors to localized brain regions and for optical fiber implantation was performed as previously described in work from the Tong Lab, with 0.1-mm precision in the anterior-posterior (AP), mediolateral (ML), and dorsal- ventral (DV) axes [286,294]. Mice were anesthetized with a ketamine, xylazine, acepromazine cocktail (80, 8, and 2.5 mg/kg); after confirmation of the absence of the pedal reflex, mice were affixed into the stereotaxic frame, and skin over the cranium was incised. Viral vectors were delivered with a 0.5-μL syringe (Neuros Model 7000.5 KH, point style 3;Hamilton, Reno, NV, USA) with the infusion rate controlled by a micro-injector motor (Quintessential Stereotaxic Injector; Stoelting, Wood Dale, IL, USA) between 25 and 50 nl/min. Viral preparations were titered at ~1012 particles/ml. Coordinates for injection were as follows: AP −1.5, ML −0.9, and DV −5.1 (for LH); AP +1.1, ML −0.1, and DV −5 (for DBB). For optogenetic experiments, an uncleaved fiber optic cannula [Ø1.25-mm stainless ferrule, Ø200-μm core, 0.39 numerical aperture (NA); Thorlabs, Newton, NJ, USA] was cut

to 4.8 to 5.0 mm in length for implant over the BF. For fiber photometry, a precleaved wide-aperture fiber optic cannula (Ø1.25-mm stainless ferrule, Ø400-µm core, 0.66 NA; Doric Lenses, Quebec, QC, Canada) were implanted over the LH or BF. For GABAzine and saline *in vivo* micro-infusions, a guide cannula was placed over the BF, which permitted preinjection of drug, before placing a removable optic fiber. Fast-drying acrylic glue and then dental cement were used to secure implantation before suture. Two injections of carprofen (5 mg/kg) were administered after surgical analgesia, 24 hours apart. All LH injections were unilateral since preliminary data showed no difference between right and left activation; BF injections were centrally located (with 0.1-mm right shift to avoid penetration of the superior sagittal sinus) and thus bilateral.

2.2.3. Viral vectors, plasmids, and Cre-dependent protein expression

A core aspect of these experiments is the use of viruses that deliver plasmids to neurons. Those plasmids encode for the machinery necessary to initially package the virus, a mammalian promoter sequence, in this case, elongation factor 1 alpha (Ef1α), and a protein of interest. In most cases, there is also a genetic construct that makes expression of the gene Cre-dependent.

Cre (Causes Recombination) is a bacteriophage enzyme used to excise integrated viral DNA through recombination [329]. It does this by finding two recombinase recognition sites that match a specific DNA sequence template like loxP1 (locus of excision P1), then recombine them so that the 3' end of the first site and 5' end of the second site are connected in a circular piece of excised DNA. However, if one of the loxP1 sequences is

inverted, Cre will actually invert the gene instead of excise it. Further, if two different loxp sequence types are used, Cre recombines by the matched sequences (this study uses vectors with loxP1, loxp2272, and fas sequences) . A specific combination of two sets of loxp sites (loxP1 and loxp2272) in the inverse orientation, called the flip-excision (FLEX) switch or double inverse orientation (DIO) cassette causes the following effects [330]: Cre binds one set and flips the gene into the sense position; this also causes the internal loxp site on the pair not used to be uninverted and in the forward position. Then, Cre binds the forward position loxp sites, which flanks one of the original paired loxp sites, and cuts that sequence out. Now, the gene is stable in the sense position. This results in Cre-dependent expression of the protein. It is also possible to use the opposite approach with a simple loxp-site flanked protein of interest in the sense position - allowing a mixture of Cre-Off and Cre-On protein expression [331].

Using the mouse strains and neurosurgical techniques described above, this allows both anatomical and molecular specificity in the introduction of new proteins to the mouse genome. The primary proteins of interest used in this study are: channelrhodopsin 2 (ChR2) [332], which is a blue-light sensitive non-selective cation channel that depolarizes neuronal membranes to cause action potentials; hm3dq (or GqDREADD) [333], which is a muscarinic acetylcholine Gq protein-coupled receptor derivative only activated by a synthetic drug, clozapine-N-oxide (CNO); and GFP-calmodulin protein 6 (GCaMP6m), a fusion of the Green Fluorescent Protein (GFP) and Calmodulin that increases in fluorescence intensity at higher intracellular calcium levels [334,335]. For synaptic

tracing, synaptophysin fused to GFP allows localization of synapses with GFP and largely avoids the issue of mistaking fibers of passage for synaptic input [336]. For retrograde tracing, I used two methods. First, I used a mutated version of the adeno-associated virus (AAV) viral vector, called rAAV2-retro, which is presumed to enter into neurons only at the presynaptic terminal and be transported to the nucleus [337]. rAAV2 allows retrograde tracing; the version I used has a plasmid containing a histone protein fused fluorescent protein (H2B::Venus) such that only the nucleus of the presynaptic neuron expresses GFP. It also contained an enhanced version of Cre, called iCre. Finally, I also used a viral vector containing FLEX-TVA-mRuby and another one containing FLEX-EGFP to use for Cre-dependent fluorescent labeling of neurons for electrophysiology.

The following plasmids were packaged in viral vectors by either the Baylor College of Medicine Neuroconnectivity Core (with special credit to Dr. Benjamin Arenkiel for assisting us in procuring them) or by commercial sources with the catalog number as indicated. The viral capsid serotype was either mixed AAV 2/9 or DJ/8 unless otherwise indicated.

- AAV-Ef1α-FLEX-hChR2(H134R)-EYFP-WPRE-hGHpA (Addgene, 20298)
- AAV-Ef1α-FLEX-hChR2(H134R)-EYFP-WPRE-pA (Univ.of North Carolina Vector Core)
- AAV-Ef1α-FLEX-EGFP-WPRE-pA (Baylor Neuroconnectivity Core)
- AAV-Ef1α-FLEX-Syn::EGFP-WPRE-hGHpA (Baylor Neuroconnectivity Core)
- AAV-Ef1α-FAS-ChR2(H134R)-mCherry-WPRE-pA (Addgene, 37090)

- AAV-Ef1α-FLEX-TVA-mRuby (Baylor Neuroconnectivity Core)
- AAV-EF1α-DIO-GCaMP6m-WPRE-pA (Baylor Neuroconnectivity Core)
- AAV-Ef1α-DIO-hM3Dq-pA-mCherry (Baylor Neuroconnectivity Core)
- (capsid AAV2/1) AAV-Ef1a-EGFP-2A-iCre (Baylor Neuro- connectivity Core)
- (capsid rAAV2-retro)AAV-Ef1a-iCre-P2A-H2B::Venus (Baylor Neuroconnectivity Core)

2.2.4. Ex vivo brain slice electrophysiology - ChR2 assisted circuit mapping

NOTE: while I was present during the majority of electrophysiological recordings, patched a few neurons myself, and performed all surgeries and post-hoc statistical analysis, the large majority of recordings were performed by a post-doctoral fellow in the Tong Lab, **Yungang Lu, PhD.**

Coronal brain slices (250 to 300 μm) containing the DBB (centered around the ventral portion) were harvested from Cre+ mice at least 3 weeks after unilateral LH injection of AAV-FLEX-ChR2-EYFP or AAV-FAS-ChR2-mCherry. After inhalational anesthesia of isoflurane, mice were decapitated and brains were quickly harvested and preserved in ice-cold cutting solution. After slicing, the brains were incubated in 32°C artificial cerebrospinal fluid (aCSF) for an hour and then maintained at room temperature until used for recording. aCSF composition was as follows: 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 1.25 mM NaH2PO4, 25 mM NaHCO3, and 10 mM d-glucose, bubbled with 95% O2/5% CO2. During recording, slices were superfused with aCSF (2 ml/min) warmed at 32°C (feedback-controlled in-line heater TC-324B, Warner Instruments). Cells

were identified through a 40× water-immersion objective with differential interference contrast and infrared illumination. DBB neurons were selected for recording if found medial to the islets of Cajal and below the horizontal midline and recording for the presence or absence of cre-dependent fluorescence in the pertinent experiments. Whole-cell voltage-clamp recordings were made using patch pipettes (3 to 5 megohms) filled with Cs+-based low internal Cl- solution, containing 135 mM CsMeSO3, 10 mM Hepes, 1 mM EGTA, 3.3 mM QX-314, 4 mM Mg–adenosine triphosphate, 0.3 mM Na2–guanosine triphosphate, and 8 mM Na2-phosphocreatine (pH 7.3 adjusted by CsOH; 295 mOsm). For ChR2-assisted circuit mapping, 473-nm laser light (Opto Engine LLC, Midvale, UT, USA) was pair-pulsed onto the DBB at 1 to 5 ms). When an inhibitory current (IPSC) was identified (no excitatory currents were identified), GABAzine (10 μM) was bath-perfused to block GABA-A receptors and eliminate the current. After recovery, tetrodotoxin (0.5 μM; Alomone Labs, Jerusalem, Israel) then 4-aminopyridine (100 μM; Acros Organics, Fisher Scientific, Pittsburgh, PA, USA) was bath applied. The tetrodotoxin blocks conduction of action potentials in local neurons, preventing non-light induced release of neurotransmitter at the synaptic cleft. The 4-aminopyridine blocks K+ channels that normally respond to depolarization within the presynaptic neuron. The goal is to block polysynaptic effects while enhancing optogenetically-mediated neurotransmitter release to demonstrate a monosynaptic connection.

2.2.5. Behavioral Testing

All tests were conducted during the light cycle after at least 3 weeks of recovery from surgery in clean empty cages without bedding. For optogenetic feeding experiments, an integrated rotary joint patch cable connected the ferrule of the implanted optic fiber to the 473-nm diode-pumped solid-state laser (Opto Engine LLC, Midvale, UT, USA). Light pulse protocol (10 Hz with 50-ms pulses at ~10 to 15 mW/mm2 was selected empirically on the basis of feeding data) was generated by a Master-8 pulse stimulator (A.M.P.I., Jerusalem, Israel). For the RTPP test, OFT, and competing choice feeding as- says, the laser activity was routed through a Noldus EthoVision XT (Noldus Information Technology, Wageningen, Netherlands) behavioral chamber camera; EthoVision XT 11.5 software was used to control laser activity dependent on mouse position

2.2.5.1. Feeding Behavior Assays A: discussion of "normal" laboratory mouse behavior

Often discussions of overeating and undereating, particularly in mice, are difficult to interpret without some familiarity as to what typical feeding behavior actually looks like. Thus, this section is dedicated to understanding what ‘normal’ is for a lab mouse with regards to eating.

The following represents a brief overview of considerations when evaluating mouse eating behavior and the typical diurnal pattern of a laboratory (lab) mouse eating. Lab mice come in a wide variety of strains, some of which have much greater potential to

develop metabolic disease or to starve on a 'normal' rodent diet than others [338]. The mice used in our experiment are typical for lab mice (derivatives of C57BL/6 and 129) Further, lab mice are typically fed only a single food that has complete nutrition. Further, they usually have minimal enrichment of their cage besides nesting material and several other cage mates. Finally, the environment is tightly controlled - there is minimal variation in the temperature, humidity, or light cycle duration [339]. Thus, most detailed analyses of feeding behavior across multiple days in lab mice reflect extremely basic, entrained patterns. This has a benefit for reductive simplicity, but also does not allow expression of compensatory strategies that could alter eating behavior. For example, most lab mice do not have an easy way to engage in voluntary energy expenditure that, if it were available, may cause them to increase meal size or feed more frequently.

With this caveat, lab mice demonstrate the following diurnal pattern of behavior [340]. Mice are nocturnal. When entrained on a 12 hour light cycle, mice typically 'wake up' (have no more protracted bouts of immobility, they move around even at night) around 15-30 minutes prior to lights off. The first feeding bout begins immediately and lasts around 3 minutes and is followed by a water drinking bout the large majority of the time [340]. A study of food intake across three diets of high carbohydrate, high fat, or high protein provides some useful bounding parameters [341]. First, the number of feeding bouts per day was 38, 33, and 48 respectively. The amount of kilojoules (kJ) consumed per bout was at 1.2, 1.4, and 1.01 respectively. The average inter-bout period was 30, 40, and 25.9 minutes respectively. And finally, the total daily energy intake was 46, 43, and 48kJ

respectively, with no statistical difference across dietary conditions. This is consistent across methods for analyzing mouse behavior [342].

Work on the role of circadian clock genes in feeding behavior has demonstrated that feeding bouts are largely restricted to the dark period, with a high likelihood to concentrate around the time immediately after lights off and right before the lights on period [343] . Notably, in the all-dark condition, feeding retains its diurnal rhythmicity; conversely, restricted access to food to short periods of time during the day can sustain molecular clock expression rhythmicity or the downstream rhythmicity of activity normally attributed to the clock [344]. In summary, mice tend to eat several times per hour throughout the entire lights off period, while only consuming a small amount of food per bout. Having established the general parameters of feeding, we can next explore the behaviors preceding, during, and following a single feeding bout.

2.2.5.2. Feeding Behavior Assays B: the ethological action map of a single feeding bout in the mouse

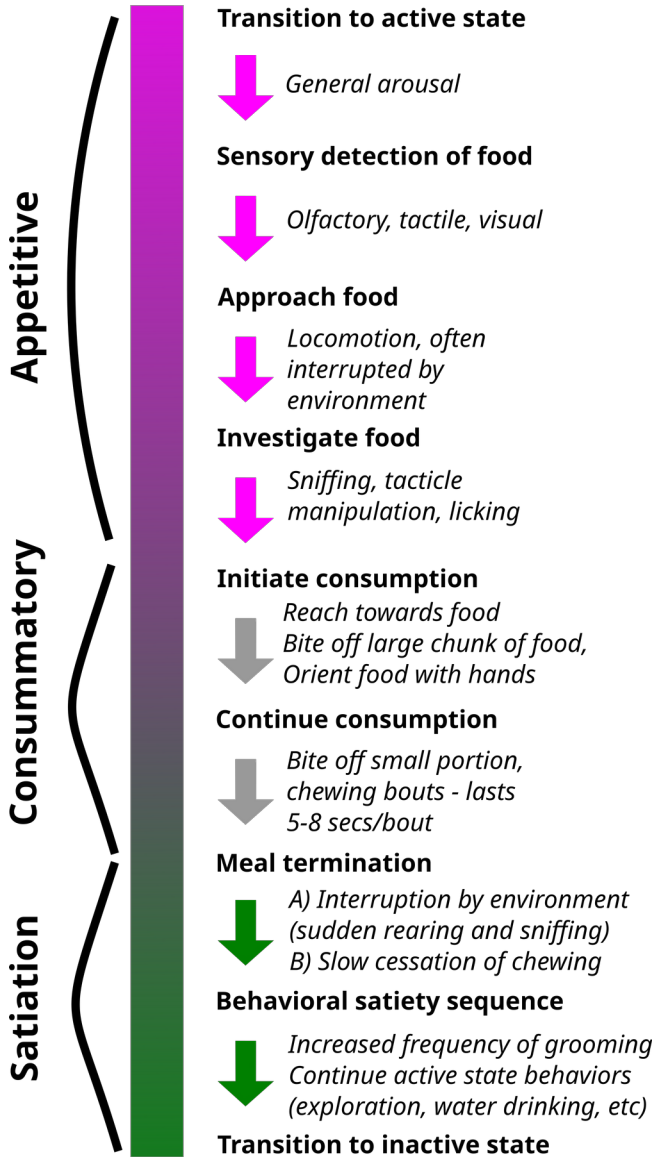
Unfortunately, the word 'micro-structure' as it relates to feeding behavior has largely been used in reference to the diurnal pattern of eating behavior (number of bouts, size of bouts), and not the structure of feeding. So, instead, I have elected to incorporate the phrase "ethological action map", derived from the set of motor movements that typically appear together in coordinated complex action to accomplish ethologically relevant tasks [345]. Here, I will describe the ethological action map of mouse free feeding [346]. Currently, automated analysis of complex mouse behavior - computational ethology -

is only just starting to become standardized and widely implemented [347]. Thus, quantitative analysis of such behavior is lacking. However, from the detailed action maps provided by Whishaw 349,351–353, my own unpublished video recordings of free-eating behavior in mice without experimental intervention, the Behavioral Satiety Sequence (BSS) cluster of behaviors commonly used in pharmacological research, 354,355 and grooming behavior analysis, 356 I propose the behavioral modules engaged during feeding are as depicted in Figure 4.

Delineation of this sequence is useful, because it reveals how many different behavioral tasks are accomplished before feeding behavior is successfully completed. Interruption of any of these tasks can alter feeding behavior - and in truth, this is probably why nearly every intervention made on the behavior of experimental models produces some alteration in feeding behavior and overall weight gain.

Figure 4. The anatomy of a single feeding bout - an ethological feeding action plan for the mouse.

Demonstration of the series of behaviors and their triggers that a mouse engages in before, during, and after a feeding bout. This is an ethological action plan for feeding at the behavioral state level rather than locomotor level. Its utility is understanding the drivers and inhibitors of feeding behavior in a systematic way. This is the uninterrupted eating bout: ***novel stimuli or intermittent threat scanning can interrupt any phase of the eating bout.***



2.2.5.3. Feeding Behavior Assays C: experimental techniques

Unless otherwise specified, mice were fed ad libitum in their home cages with standard pellet chow in their home cage before testing. All mice were previously exposed to HFD (Research Diets D12492; 20% protein, 60% fat, 20% carbohydrate, 5.21 kcal/g) in their home cage several days before tests with HFD. For Pdx1-cre mice, the amount of food consumed over a 15-min laser stimulation period was compared between FLEX-ChR2–transfected and FLEX-GFP–transfected mice. For Vgat-cre, amounts of food consumed per mouse over a 10-min prestimulation period, 10-min laser stimulation period, and 10-min post–laser stimulation period were compared. To determine food preference, mice were given free access to both regular chow and HFD during a 10-min laser stimulation period. To test competing preference for laser versus food, mice were habituated to the Noldus chamber for 10 min with laser paired to one side of the chamber and then, one of two paradigms was used: (i) regular chow was placed in laser on side, and HFD was placed in the laser off side; and (ii) regular chow was placed in the laser off side, and no food was placed in the laser on side. Latency to food consumption was calculated as the time spanning from the beginning of food exposure after a 24-hour fast or onset of laser stimulation and the first time the mouse bit the pellet.

2.2.5.4. Pharmacology

GABAzine or saline (~200 nl) was infused into the DBB via a guide cannula. The dose is comparable to other microinjection techniques targeting the basal forebrain in mice [316]. After 15 min, an optic fiber connected to a cap was fixed into place into the guide

cannula, and laser stimulation pulsed. For diazepam experiments, mice were intraperitoneally injected with either saline or diazepam (3 mg/kg) dissolved in 0.1% carboxymethyl cellulose at 1:10 dilution of stock dissolved in dimethyl sulfoxide (DMSO) and then placed into testing cage; timing began immediately after injection, with regular chow (ad libitum fed state) or regular chow and HFD (fasted state) placed into the testing cage with water. Food consumption was measured after 2 hours.

Two mouse models were used for the clozapine-N-oxide (CNO) experiments. 1) Vgat-cre with $LH^{FLEX\text{-}ChR2}$ and $DBB^{DIO\text{-}GqDREADD}$, and Vglut2-cre with $LH^{Fas\text{-}ChR2}$ and $DBB^{DIO\text{-}GqDREADD}$. In both cases non-glutamatergic (i.e. GABAergic et al) neurons in the LH express ChR2, and either GABAergic or glutamatergic neurons in the DBB express GqDREADD. Both had an optic fiber implanted over the DBB. These mice then received intraperitoneal injection of either saline or CNO (3 mg/kg at 1:15 stock solution in DMSO then dissolved in normal saline) and left in a testing chamber for 30 minutes prior to onset of assay. Food intake during this time was measured, then mice were exposed to 10 minutes of laser stimulation - food intake was measured again. Finally, mice were given another 10 minutes post-laser in the testing chamber, and food intake was measured again.

2.2.5.5. Locomotion and anxiety assays

Four mouse models used for these assays - real time place preference test (RTPP), open field test (OFT), conditioned place preference test (CPP), and elevated plus maze (EPM). Either Pdx1-Cre or Vgat-Cre with $LH^{FLEX\text{-}ChR2}$ or $LH^{FLEX\text{-}EGFP}$. Mice were internally compared with and without laser stimulation, as well as between ChR2 and EGFP groups.

RTPP: mice were placed into the Noldus behavior chamber and had activity monitored for 20 min, with laser stimulation paired to one side of the chamber. Testing was initiated when mouse was in the laser off zone. Half of the mice in each group had the left side of the chamber paired with laser stimulation, and half with the right side, to counterbalance possible testing differences. Mouse movement was tracked and analyzed using EthoVision XT software (version 11.5; Noldus Information Technology, Wageningen, Netherlands).

OFT: the periphery of the chamber was determined as ~2 mouse (10 cm) widths; the patch remaining in the center is the "center." Mice were tested for baseline levels of exploration into the center with laser on or off.

CPP: the center area was paired with laser activation over 7 days. The first day, the laser was off; then at the same time every day for the next 7 days, mice were exposed to the laser on-paired with center condition. On the last day, the pairing was extinguished and mouse preference for the center after 7 days was compared to the first day.

EPM: mice fasted overnight were then placed on the center of the EPM and habituated to the apparatus for 10 min. Then, regular chow was placed on either the open or closed arm. The mouse was then placed on the open arm with the food to allow discovery. Latency to food consumption was determined as in the feeding behavioral assay; the mice were allowed 10 min to consume food after first bite.

2.2.6. Fiber photometry

2.2.6.1. Equipment

All fiber photometry was conducted using a Doric Lenses setup, with a light-emitting diode (LED) driver controlling two connectorized LEDs (405 and 465 nm) routed through a five-port Fluorescence MiniCube [order code: FMC5_AE(405)_AF(420-450)_E1(460-490)_F1(500-550)_S] to deliver constant illumination of excitation light for calcium-independent and calcium-dependent signals to the implanted optic fiber simultaneously. Emitted light was received through the MiniCube and split into two bands —420 to 450 nm (autofluorescence: calcium-independent signal) and 500 to 550 nm (GCaMP6 signal: calcium-dependent signal). Each band was collected by a Newport 2151 Visible Femtowatt Photoreceiver module (photometer) with an add-on fiberoptic adapter. Output analog signal was converted to digital signal through the fiber photometry console and recorded using the “Analog-In” function on Doric Studios (V4.1.5.2).

2.2.6.2. Behavioral tests

Looming threat. Mice are acutely sensitive to potential predatory presence and approach, and this stimulus is anxiogenic i.e. induces behavioral inhibition and potentially a fight-or-flight response. While laboratory environment in general has a major influence on mouse behavior, evidence suggests that experimenters themselves can dramatically increase rodent stress in ways that seem ethologically indistinguishable from their response to the presence of actual predators likes cats [348–353]. Given this, rather than work with complicated mimickers of natural predators for laboratory mice such as a pigeon-sized

object flying over the cage to mimic a flying predator or an expanding, looming disk [354], I used a well-established and simple predatory stimulus to induce anxiety in the animal - the looming threat of an experimenter's hand. Mice were tested in their home cage with the roof removed; after 3 min of acclimation to the connected cable (recording the entire time) and while standing over the cage, I placed one hand 2 cage heights above the mouse for 3 s; and the onset of hand movement was set as time 0. I was the only experimenter to perform any behavioral assay and responses are only compared pre-stimulus, during stimulus, and post-stimulus for a given an animal, thus eliminating the potential influence of inter-experimenter or inter-mouse variation.

Loud sound. Similarly, mice are wary of sudden loud sounds indicating potential danger. Mice were tested in their home cage with the roof removed; after 3 min of acclimation to the connected cable, I (positioned across the room out of sight of the mouse and immobile) clapped hands five times in rapid succession. The onset of clapping was set as time 0.

Novel object. Mice were placed in a testing chamber and acclimated for 10 min. Then, recording was initiated; 30 s after recording onset, a novel object (15-ml Falcon tube cap) was placed in a corner of the cage. The novel object interaction onset was determined by the moment when the mouse's nose touched the novel object.

As an aside, I also performed some preliminary tests where the mouse was exposed to a cotton swab (and later petri dish with a small amount of fluid) soaked in

commercially available bobcat urine from two different brands, as well as 2-phenylethylamine (2-PEA), which is a volatile chemical found in cat urine and is known to activate the vomeronasal system in mice [355]. However 2-PEA did not induce any sustained response distinguishable from when I first placed the dish in the cage. Further, from my subjective perspective, the mice interacted with the swab exactly the same as any other novel object, generally approaching it within about 5 minutes and showing a short spike in activity upon interaction with the novel object (see **Figure 22**) . Although predator urine has long been used in risk-assessment tests and measures of anxiety with strong circuit evidence of interaction with the ventromedial hypothalamus [356,357], it apparently does not have substantial influence on this system. Alternatively, the laboratory strains of mice that have been inbred for generations for selection of Cre and flox genes, may have reduction in sensitivity to these chemicals; knockout of a single trace amine-associated receptor has been shown to reduce sensitivity to predator odor [358,359]. Finally, there is a possibility that this may represent fiber implant-related brain damage effects; the midline placement of the fiber, which must inevitably destroy some midline tissue, could conceivably have disrupted this sensory pathway [360]. Since I did not pursue this line of experimentation further, no conclusions can be drawn.

Fasted-refeeding. After a 24-hour fast, mice were placed in a testing chamber and acclimated for 10 min. Then, recording was initiated; 30 s after recording onset, HFD and regular chow were placed into two different corners of the testing chamber. Food consumption bout onset was determined by the moment when the mouse bit the pellet

(fecal consumption was excluded); food consumption bout offset was set when the mouse ceased chewing and began investigatory sniffing behaviors/rearing. Two experimenters (myself and a summer undergraduate researcher, Madhavi Jere) independently viewed the videos to determine the offset, and the averaged onset/offset time between the two was used.

Diazepam. Mice were habituated for 2 to 3 min with the cable attached in their home cage, and then, recording was started. After 2 min, mice were given an intraperitoneal injection of either saline or diazepam (3 mg/kg; Sigma-Aldrich) and were monitored for the next 20 min. No food or other stimulus was present during testing.

2.2.6.3. Data analysis

Fluorescence data were acquired through Doric Studios V4.1.5.2. and saved as comma-separated files (header was deleted) at a sampling rate of either 1 or 2.5 kS/s. I wrote a custom analysis script in the R programming language (V3.4.4. “Someone to Lean On,” packages ggplot2, reshape, zoo, plyr, viridis, and scales) to calculate the baseline fluorescence (F0) using linear regression across a chosen period of recording. The change in fluorescence (dF) was then determined from the residuals and multiplied by 100 to arrive at percentage of dF/F0. A sliding median with a window 51 data points in width was used to reduce noise. For looming threat, loud sound, and novel object, the baseline was calculated from 10 s be- fore stimulus onset to 20 s after stimulus onset. For fasted-refeeding, because mice typically engage in a series of quick successive feeding bouts, the F0 was calculated across a 50-s period with the feeding bout in the center. The

average percentage of dF/F0 across the whole period was compared to the average percentage of dF/F0 during the feeding bout. For diazepam, 1.5 min before intraperitoneal injection was used to calculate the baseline, and this period was averaged and compared to a 1.5-min time segment occurring 5 min after intraperitoneal injection.

Stimulation frequency–dependent inhibition. For this test, there was leakage of laser-emitted light into the fiber photometer. Two filtering methods were applied: (i) a raw intensity cutoff slightly higher than the highest natural peak observed in the fluorescence trace and (ii) a sliding median window 1800 sample points in width (~0.7 s) applied to eliminate remaining laser-induced artificial light increases.

Fiber photometry heat map. Individual traces were aligned to zero at stimulus onset and averaged to produce an average trace. These averaged traces were then normalized from 0 to 1 using the R scales package; each mouse's scaled average trace was combined into a heat map. The viridis package was used to generate a colorblind-friendly color scheme for heat map.

NOTE: The calcium-independent fluorescence signal was also recorded during all tests. No significant alteration in signal level was observed compared to calcium-dependent signal (indicating steady LED excitation), so I do not display these data for simplicity's sake.

2.2.7. Tissue section imaging and immunohistochemistry

To harvest brain tissue, mice were given a lethal injection of ketamine/ xylazine/ acepromazine. After loss of the pedal reflex, mice were transcardially perfused with 15 ml

of saline and 15 ml of 10% buffered formalin. The brain was then extracted and stored in 10% buffered formalin for at least 1 day, and then, the brain was switched to 30% sucrose solution and stored for at least 1 more day. Brains were frozen with dry ice on a microtome and sectioned into 30 μM slices. The slices were mounted onto microscope slides and coverslipped with Fluoromount (Diagnostic BioSystems Inc., Sigma-Aldrich). A confocal microscope was used to image the slices (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany).

For immunohistochemistry, brains were washed and placed in a blocking solution for 1 hour (10% donkey serum, 0.3% Triton X-100 dissolved in phosphate- buffered saline), and then incubated overnight at 4°C with the appropriate primary antibody. Then, after washing, they were incubated overnight at 4°C with the appropriate secondary antibody. Purple and green, rather than red and green, were used for ease of visual discrimination. The following antibodies were used:

Primary:

- c-Fos rabbit monoclonal antibody (mAb) (9F6) (#2250, Cell Signaling Technology)
- Orexin A rabbit mAb (#H-003-30, Phoenix Pharmaceuticals)
- Melanin-concentrating hormone rabbit mAb (#H-070-47, Phoenix Pharmaceuticals)

Secondary:

- Alexa Fluor 647–conjugated AffiniPure Donkey (H+L) anti- rabbit immunoglobulin G (Jackson ImmunoResearch)

Distance from bregma (the intersection of the sagittal and coronal sutures in the cranium) in millimeters is written next to the scale bar in each image; these are approximated with reference to Franklin and Paxinos [361].

2.2.8. Statistical analysis

Data were collected into organized Excel (2016) sheets and exported into tab-delimited format. All statistical tests were run using R (V3.4.4. “Someone to Lean On,” packages ggplot2, reshape, zoo, plyr, viridis, and scales). Mice exposed to two different conditions were compared using two-tailed paired t test. When two different groups of mice are tested, they are compared using two-tailed Welch’s unequal variance t test. For the CNO-blocked feeding test, a one-way ANOVA was used for the three conditions, and Tukey’s honestly statistical difference (HSD) test was used for post-hoc analysis. Means and SE are displayed as gray bar graphs behind individual data points. Refer to *** for analysis of fiber photometry data. *Note: in all figures, the following notation is used for representation of the p value; n.s., not significant. *P < 0.05; **P < 0.01; ***P < 0.001.*

2.3. Results

2.3.1. LH^{GABA} neurons project to and inhibit DBB neurons to drive feeding

In order to establish the existence of an LH-BF connection, I performed a unilateral injection of a viral vector encoding FLEX-ChR2-EYFP into the lateral hypothalamus ($LH^{FLEX\text{-}ChR2\text{-}EYFP}$) of Pdx1-Cre mice. After several weeks to allow full expression, I sacrificed the mouse to look at the projection targets of these neurons. Previous work in the Tong laboratory has shown the specificity of this type of injection for the LH [286,294]. $LH^{Pdx1\text{-}ChR2\text{-}EYFP}$ neurons showed prominent projections to the DBB; implantation of an optic fiber also successfully targeted those projections in the DBB (**Figure 5**).

Notably, the dorsomedial hypothalamic nucleus (DMH) is adjacent to the LH and expresses Pdx1-cre. Thus, it is possible it was also exposed to sufficient level of the viral vector to also express the fluorescent reporter. Notably, the stereotaxic coordinates and small volumes of virus used to target the LH generally result in minimal expression in this region and have been reported in our previous work cited above. **Figure 5** shows a representative image of typical LH^{Pdx1} infection with ChR2. However, some small contribution of DMH infection cannot be excluded from any of these findings and is a necessary caveat in the interpretation of the following data.

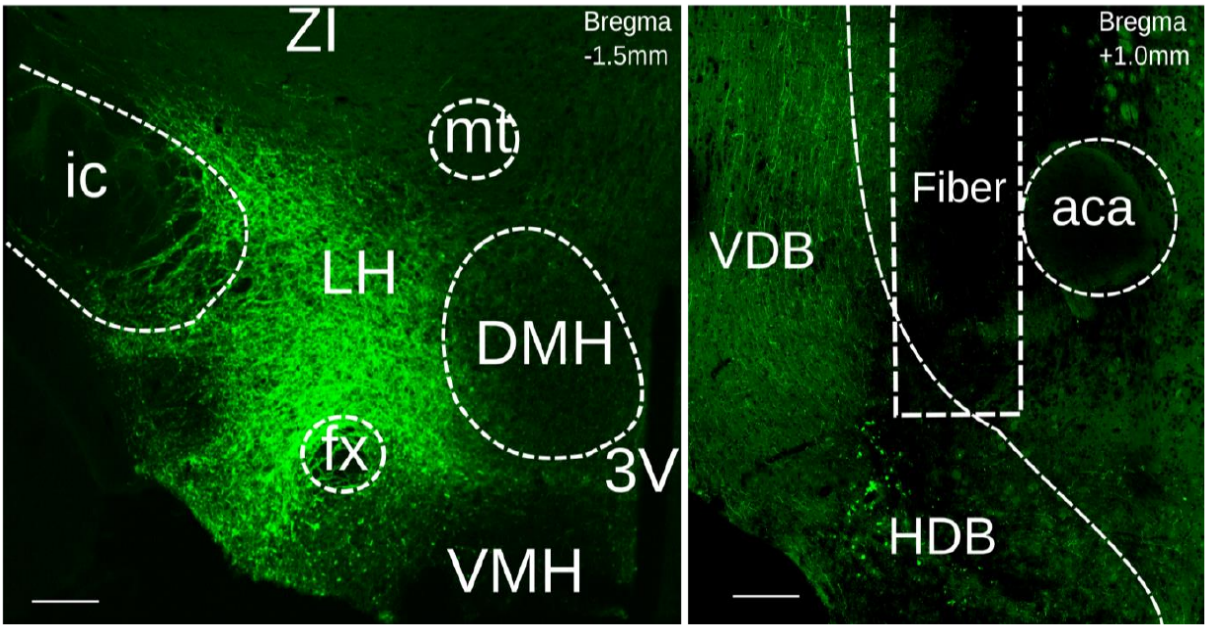
Photostimulation of those $LH^{Pdx1\text{-}Chr2}$-DBB^{Fiber} approximated projections resulted in robust feeding, but photostimulation of $LH^{Pdx1\text{-}GFP}$-DBB^{Fiber} did not resulted in any feeding, as seen in **Figure 6A.** Since LH^{Pdx1} neurons are a mix of GABAergic and glutamatergic neurons, Dr. Yungang Lu and I used brain slice electrophysiology of postsynaptic DBB

neurons to determine whether the photostimulation-linked current was excitatory or inhibitory. Since only optically evoked inhibitory postsynaptic currents (oIPSCs) were observed, we concluded the $LH^{PDx1\text{-}DBB}$ projections were GABAergic, as seen in **Figure 6B,C**.

I also used two trans-synaptic tracing strategies to further confirm the existence and molecular identity of the LH→DBB projection. The first strategy used a single-synapse anterograde virus, AAV1, carrying a plasmid encoding Cre [362]. Injection into the LH of Ai9+ animals revealed Cre activity in the DBB (**Figure 7A,B,C**) and antibody staining against choline acetyltransferase revealed that most of the downstream neurons were cholinergic (**Figure 7D-I**).

Figure 5. Optogenetic targeting projections from LH neurons to neurons in the DBB

Coronal brain slices of Pdx1-cre mouse with LH injection of FLEX-ChR2-EYFP (left) and placement of optic fiber over the DBB (right). Scale bar- 250μM. aca= anterior commissure anterior limb; DMH = dorsomedial hypothalamus; fx = fornix; HDB = horizontal limb of the diagonal band of Broca; ic = internal capsule; LH = lateral hypothalamus; mt =



mammillothalamic tract; VMH = ventromedial hypothalamus; VDB = ventral limb of the diagonal band of Broca; ZI = zona incerta; 3V = third ventricle

Figure 6. Photostimulation-induced feeding behavior in LH$^{Pdx1\text{-}ChR2}$:DBB^{Fiber} vs. LH$^{Pdx1\text{-}GFP}$:DBB^{Fiber} mice and electrophysiological recordings of DBB post-synaptic neurons receiving monosynaptic input

(A) Activation of LH Pdx1→DBB neurons induces feeding behavior. (B) Brain slice electrophysiology of downstream neurons in the DBB (n=4 animals) identifies 16 neurons with inhibitory post-synaptic current (oIPSC), 14 with no response, and 0 with excitatory postsynaptic current (oEPSC). (C) oIPSC is monosynaptic as it was not blocked by tetrodotoxin (TTX) + 4-aminopyridine (4-AP). Further, it was also dependent on local GABA-A receptors as it is blocked by GABAzine (bottom). Photopulse is 2ms. Details for the electrophysiological experimental setup can be found in ***Section 2.2.4***

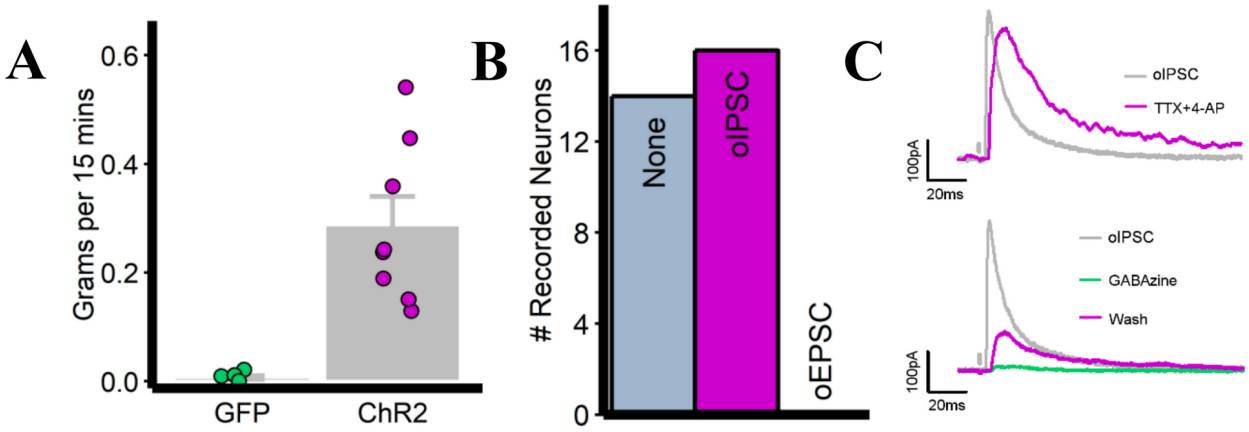
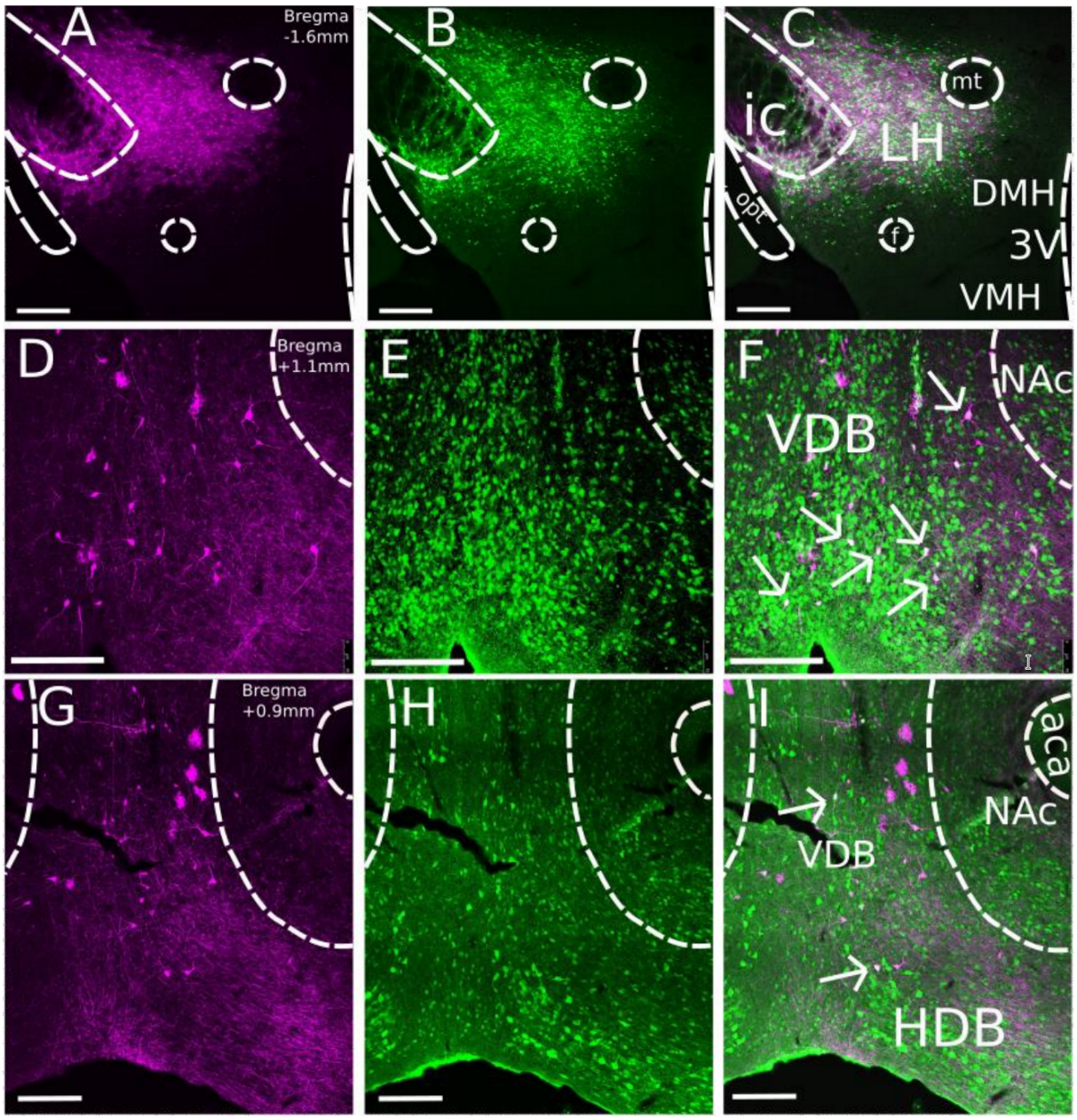


Figure 7. Tracing and identifying DBB neurons that receive direct inputs from LH.

(A-I) Coronal slices of Cre-dependent mCherry+ mouse brain after unilateral injection of anterograde tracer AAV1-Cre-GFP into the LH. Antibody staining against choline acetyltransferase (ChAT) is shown. Magenta = mCherry, Green = ChAT. Scale bar- 250μM. Aca = anterior commissure anterior commissure anterior limb; DMH = dorsomedial hypothalamus; HDB = horizontal limb of the diagonal band of Broca f = fornix; ic = internal capsule; LH = lateral hypothalamus; mt = mammillothalamic tract; Nac = nucleus accumbens; opt = optic tract; VMH = ventromedial hypothalamus; VDB = ventral limb of the diagonal band of Broca; 3V = third ventricle

Figure 7. Tracing and identifying DBB neurons that receive direct inputs from LH.



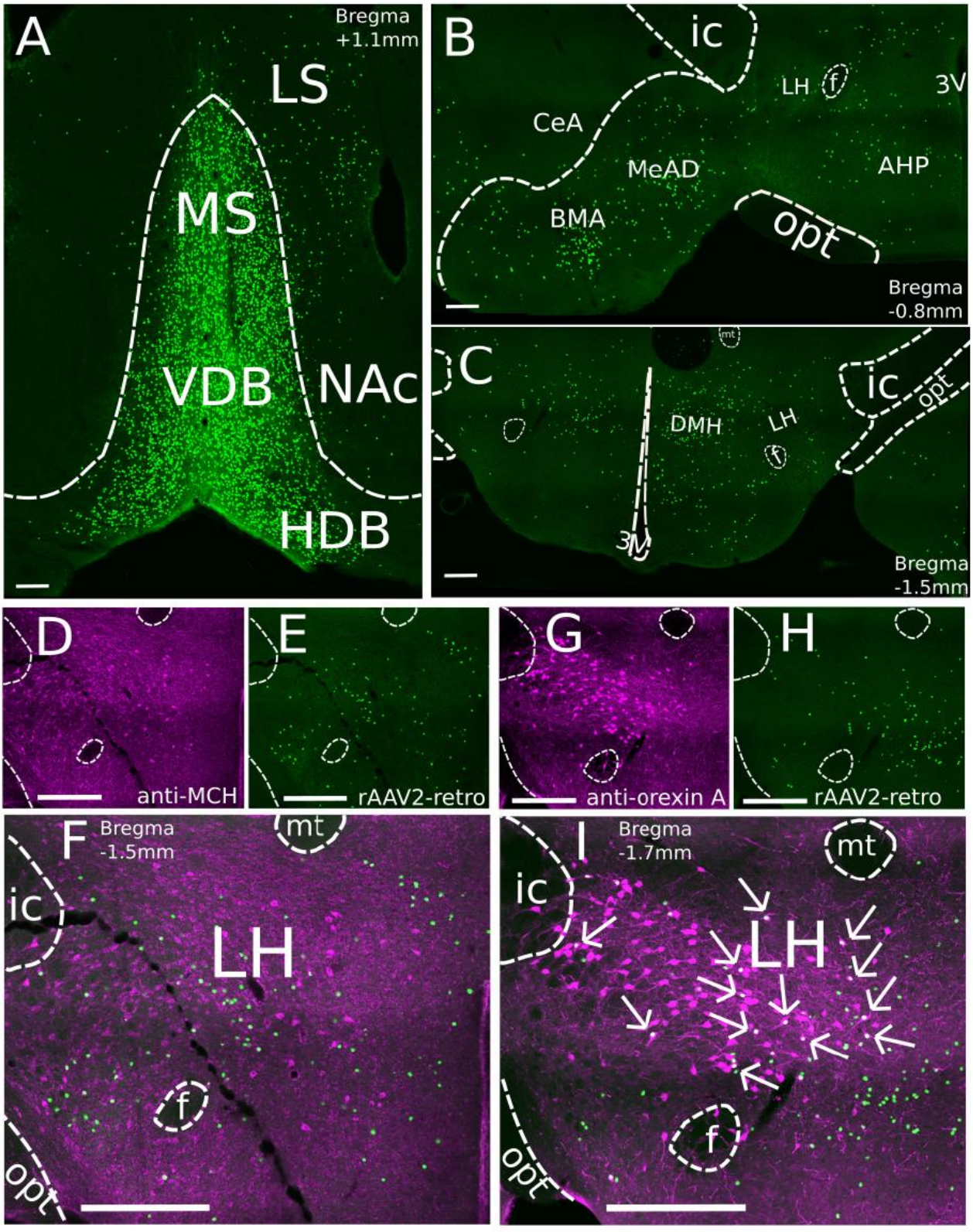
The primary objective of this study was not quantitative analysis of the proportions of types of each postsynaptic neuron. Thus, I did not pursue further exploration of the nature of the DBB neurons, having established the monosynaptic connection. This will be an avenue of study for future researchers.

The second strategy used a retrogradely transported virus, rAAV2-retro, carrying a plasmid encoding Cre and H2B::Venus [337]. Injection into the DBB revealed selective expression within the DBB and upstream expression within the LH (**Figure 8A-C**); antibody staining revealed that these neurons were not substantially colocalized with a melanin-concentrating hormone (**Figure 8D-F**). As expected, I did identify a subset of presynaptic neurons in the LH that colocalized with orexin A (the image with greatest colocalization is presented in **Figure 8G-I**). Previous research has demonstrated a role for orexin in the basal forebrain in the sleep/wake cycle [363]. However, given that the only observed photostimulation-evoked current was inhibitory in the LH^{Pdx1} mice, it is likely that these neuropeptidergic neurons are not mediating the observed effect. Orexin is usually co-localized with glutamate release machinery, and MCH is typically not colocalized with the full complement of either GABA or glutamate release machinery [364,365].

Figure 8. Tracing LH neurons that send direct projections to DBB neurons.

(A-C) Coronal slices of mouse brain after midline injection of retrograde tracer rAAV2-retro carrying a plasmid encoding Cre and H2B::Venus (green) into the DBB, demonstrating selective expression in the MS/DBB and its presynaptic targets. (D-F) Antibody staining against orexin (purple) demonstrates minimal colocalization. (G-I) Antibody staining against melanin-concentrating hormone (MCH; purple) demonstrates no colocalization. Scale bar- 250μM. aca = anterior commissure anterior limb; AHP = anterior hypothalamic area, posterior part; BMA = basomedial amygdala; CeA = central nucleus of the amygdala; DMH = dorsomedial hypothalamus; HDB = horizontal limb of the diagonal band of Broca f = fornix; ic = internal capsule; LH = lateral hypothalamus; LS = lateral septum; MS = medial septum; mt = mammillothalamic tract; meAD = medial amygdaloid nucleus; Nac = nucleus accumbens; opt = optic tract; VMH = ventromedial hypothalamus; VDB = ventral limb of the diagonal band of Broca; 3V = third ventricle

Figure 8. Tracing LH neurons that send direct projections to DBB neurons.

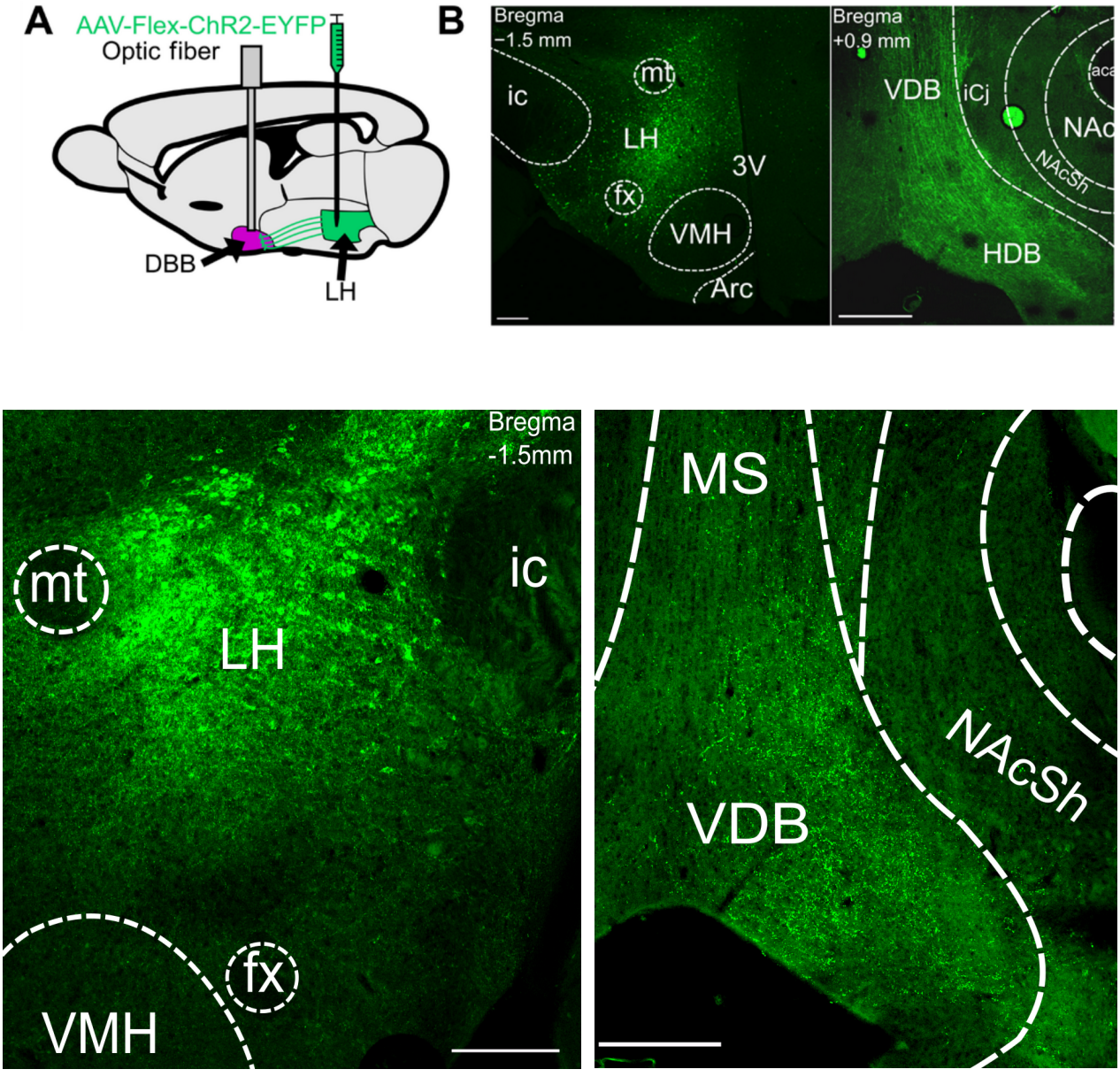


My data on feeding and predominant oIPSCs recorded from photostimulated LHPdx1-Cre neuron projections suggest a potential dominance of GABAergic projections in the LH→DBB pathway. Thus, I used vesicular GABA transporter (Vgat)-cre mice [326] to selectively target LH^{GABA} neurons (LH^{Vgat} or LH^{GABA}) for optogenetic manipulations. Targeting LH^{Vgat} neurons with AAV-FLEX-ChR2 ($LH^{Vgat\text{-}ChR2}$) revealed dense fibers in the DBB, as can be seen in the top row of **Figure 9**. **Figure 9A** shows the generalized virus injection strategy. Labeling LH^{Vgat} neurons with Synaptophysin::EGFP revealed specific GABAergic synaptic terminals in the DBB, as can be seen in the bottom row of **Figure 9**.

Figure 9. $LH^{VGAT\text{-}ChR2}$ and $LH^{Vgat\text{-}Synaptophysin::GFP}$ neurons project to and synapse in the DBB

(Top row) (A) Diagram demonstrating LH injection with AAV-FLEX-ChR2-EYFP and implantation of optic fiber over the DBB. (B) Representative coronal slices of LHVgat-ChR2-EYFP (left) and EYFP+ fibers in the DBB (right). (Bottom Row) Injection of LH Vgat neurons with FLEX-Synaptophysin::EGFP reveals strong synaptic projection to the MS and VDB/HDB, with only sparse connection LS or NAc/NAcSh. Scale bar- 250μM. aca = anterior commissure anterior limb; Arc = arcuate nucleus of the hypothalamus; fx = fornix; ic = internal capsule; iCj = islet of Cajal; LH = lateral hypothalamus; LS = lateral septum; MS = medial septum; mt = mammillothalamic tract; NAc = nucleus accumbens; NAcSh = nucleus accumbens shell; opt = optic tract; VMH = ventromedial hypothalamus; VDB = ventral limb of the diagonal band of Broca; 3V = third ventricle

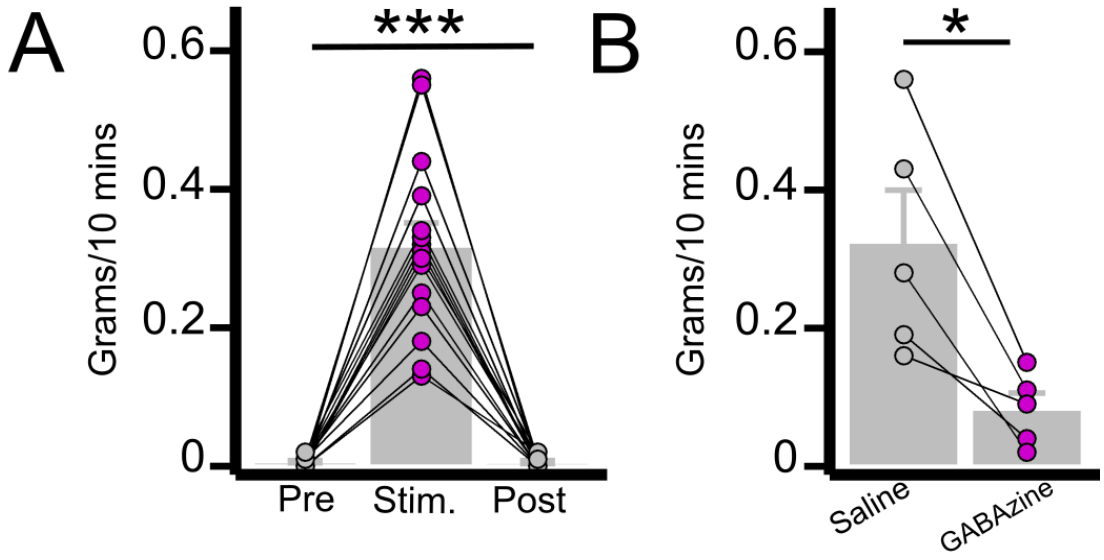
Figure 9. LH$^{VGAT\text{-}ChR2}$ and LH$^{Vgat\text{-}Synaptophysin::GFP}$ neurons project to and synapse in the DBB



Activation of $LH^{Vgat\text{-}ChR2}$→DBB projections induced rapid, light-locked feeding behavior, recapitulating our findings with $LH^{Pdx1\text{-}ChR2}$ mice **(Figure 10A)**. $LH^{Vgat\text{-}ChR2}$ light-induced feeding was prevented by per-microinjection of GABAzine, a GABA-A receptor antagonist, to the DBB **(Figure 10B)**. From these data, we conclude that the LH^{GABA}-DBB neurocircuit promotes feeding. Further, the local injection of GABAzine demonstrates that the feeding effect that we observed is not due to back-propagation of action potentials to LH^{Vgat} somata or axon collaterals to other regions.

Figure 10. Activation of LH$^{VGAT\text{-}ChR2}$-DBB projections induces feeding blockable by local GABAzine

(A) Food intake of fed mice during 10-min periods before (pre), during (stim),and after (post) photostimulation of LH^{GABA}→DBB projections (n = 15, P < 0.001). (B) Effect of DBB pretreatment with GABAzine (ɣ-aminobutyric acid type A (GABA-A) receptor antagonist) or saline on photostimulation-induced feeding (n=5, P=0.015).

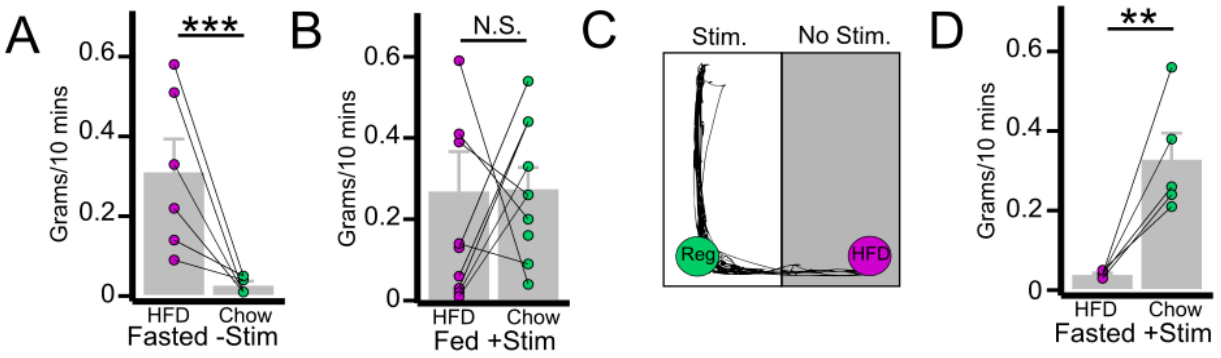


2.3.2. LH^{GABA}-DBB activation alters preference for palatable foods

Since feeding behavior is influenced by food palatability, we next explored whether the observed feeding was dependent on the evaluated palatability of the food to be consumed. This is particularly interesting given that activation of somatostatin-positive neurons (likely also GABAergic) in the basal forebrain selectively induced consumption of a palatable food for rodents, high-fat diet (HFD) [366]. In my experiments, as expected, control mice in the fasted (12 hours) state selectively consumed HFD instead of standard chow (**Figure 11A**). However, LH^{GABA}→DBB activation in the fed state elicited indiscriminate consumption of both chow and HFD (**Figure 11B**). This indicates an alteration in the evaluation of the palatability of the food; or, put another way, either appetite is increased such that the value of the foods cannot be distinguished as they both become highly palatable, or the standard chow's palatability was increased. To test the extent of altered food palatability caused by circuit activation, we next created a competition paradigm, where fasted mice must choose between chow paired with photostimulation and unpaired HFD; they uniformly preferred stimulation-paired chow (**Figure 11C,D**)

Figure 11. Activation of LH^{GABA}-DBB projections drives indiscriminate feeding

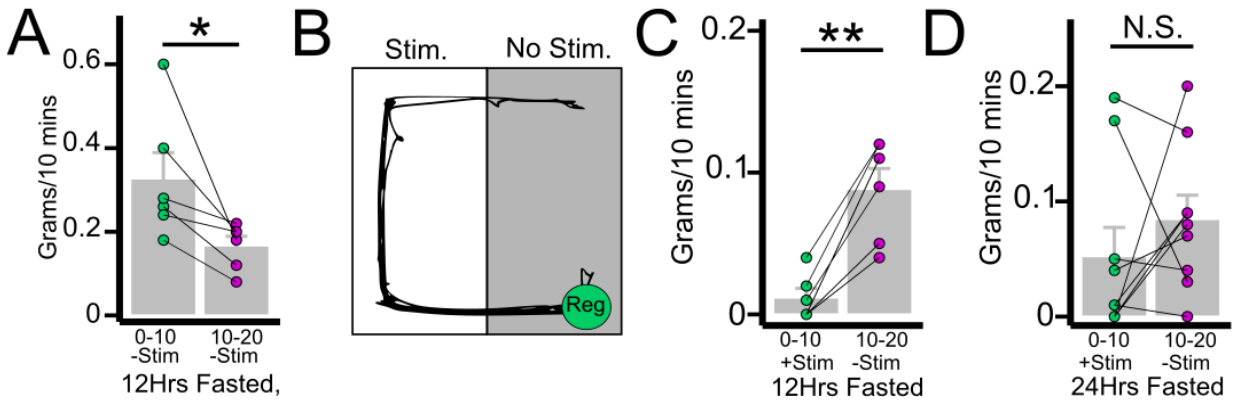
(A-B) Mouse preference for high-fat diet (HFD) or chow in the fasted state (E; n = 6, P = 0.019) and in the fed state with photostimulation (F; n = 9, P = 0.96). (C) Representative trace of a single mouse's movement during competitive choice test. Regular chow (reg) is paired with photostimulation. (D) photostimulation-paired chow consumption compared to nonpaired HFD consumption (n=5, P= 0.011).



Last, to test whether circuit activation is merely magnifying hunger or having other effects with positive valence, we extended the competition paradigm to force mice to choose either photostimulation or chow consumption over 10 min. The pairing was then removed, and food consumption over the next 10 min was measured to determine whether mice retained hunger after ceasing stimulation. As expected, in the control condition, mice approached food right away and ate food during both 10-min epochs (**Figure 12A**). However, when forced to choose between photostimulation and food, mice refrained from eating until stimulation was removed (**Figure 12B,C**). Prolonged fasting (24 hours) reduced this preference (**Figure 12D**), suggesting that activation is not sufficient to overcome intense physiologic hunger induced by 24-hour fasting.

Figure 12. Activation of LH^{GABA}-DBB projections is preferable to feeding, except in extreme hunger.

(A) Food consumption in 10-min increments upon food exposure after 12-hour fasting (n = 6, P = 0.028). (J) Representative trace of mouse movement when choosing between photostimulation or chow consumption after 12 hours (hrs) of fasting. (K and L) Food consumption when choosing between chow and photostimulation after 12 hours during the photostimulation period (0 to 10 min) and after photostimulation is ended (10 to 20 min) (K; n = 6, P = 0.001) and 24-hour fasting (L; n = 9, P = 0.34).



2.3.3. LH^{GABA}-DBB activation reduces anxiety

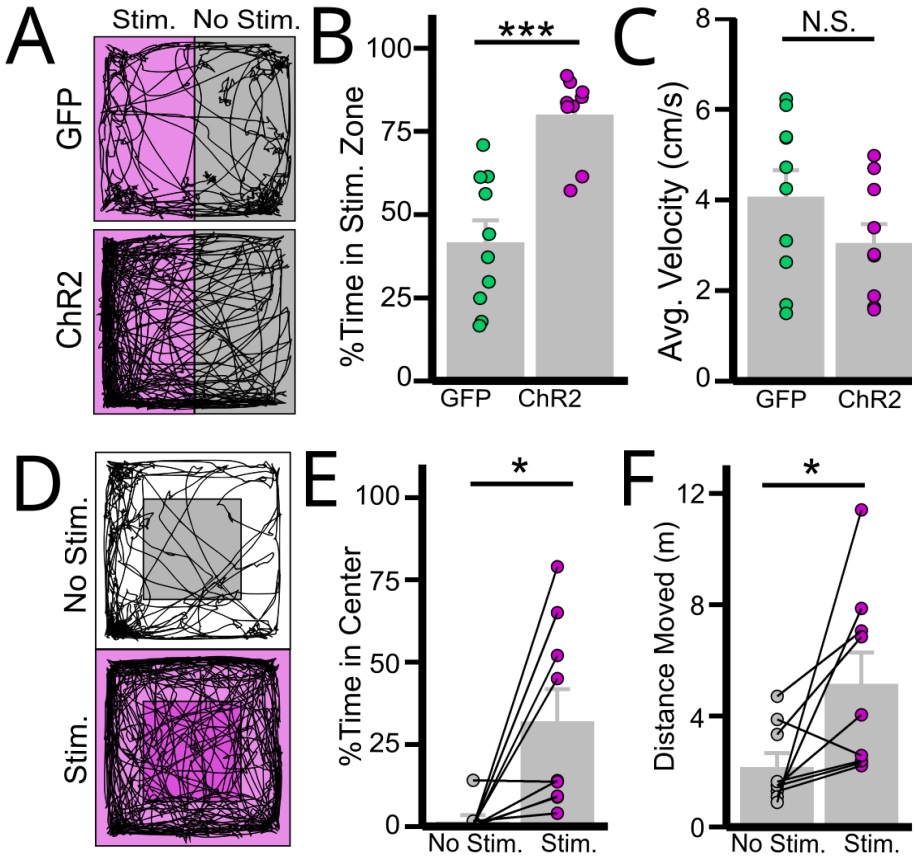
Given the results of the competition experiments, I next examined how circuit activation affects behavior in the absence of food altogether. First, I established that activation has a positive valence using the real-time place preference (RTPP) test. In the RTPP test, mice are allowed to move freely between sides of the chamber, with one side paired with photostimulation. $LH^{Vgat\text{-}ChR2}$ mice preferred the area coupled to photostimulation, whereas $LH^{Vgat\text{-}GFP}$ had no preference (**Figure 13A,B**). Notably, there was no difference in locomotion within the paired area (**Figure 13C**). I next tested mouse behavior on the open-field test (OFT), where the entire chamber is paired with photostimulation (**Figure 13D**); in this case, mice spent more time in the center and moved more in the OFT (**Figure 13E,F**).

It is interesting that in this particular test, there appeared to be bimodality to the response - this was not attributable to sex or litter, as far as I could discern. On the recorded video of mouse behavior, my subjective observation is that in the latter half of the test it becomes clear that the mice generally sit and 'stay put' in a randomly chosen location - sometimes that is within the designated center, and sometimes that is not. This may contribute to some extent of the disparity seen. Interestingly, food availability seems to reduce the observed increase in locomotion (likely because they are eating). This may also explain why the example movement trace in the absence (**Figure 13A**) of food covers greater area than in the presence (**Figure 11C) of food.**

It is important to note that the OFT, as used in this section, does not differentiate between a total increase in locomotion then leading to an increase in center time, versus a preferential increase in resting time in the center – a problem that has long been appreciated, but is often under-discussed, as a limitation to interpretations of the OFT [367]. The ratio of locomotion to percent time spent in the center is difficult to interpret, because as described above, the mouse' movement across time is not uniform and there may be artifacts of either latency to initiate moving, or the converse early fatigue leading to lack of future exploration. For example, a study using detailed manual ethological analysis of mice under the influence of diazepam and chlordiazepoxide versus a pulsed low frequency magnetic field found distinguishable effects between agents, despite all increasing center time 377. Future tests may be done where movement across time is separated into small chunks (e.g. 1 minute intervals), and the amount of locomotion per minute and percent center time can be shown as a trend. However, in each of the cases above, an increase amount of time spent in the center is still a proxy for relative behavioral disinhibiton (i.e. reduced anxiety) [368]. For the purposes of this experiment, the conclusion that increased percent time spent in the center reflects relative disinhibition/reduced anxiety is sufficient. Later experiments tackle anxiety/disinhibiton through other means.

Figure 13. Mice prefer activation of the LH$^{GABA-DBB}$ neurons and have reduced anxiety

(A to C) Effect of photostimulation of LHGABA→DBB projections on RTPP test (n = 14 per group). (A) Representative trace of mouse movement (black) in gray zone and photostimulation-paired magenta zone (stim zone). (B and C) Comparison between LHVgat-ChR2 mice and LHVgat-GFP mice on percentage of testing time spent in stim zone (B; P < 0.001) and average velocity in stim zone (C; P = 0.15). GFP, green fluorescent protein. (D to F) Ten-minute OFT in LHVgat-ChR2 mice (n = 9): Photostimulation effect on mouse movement (black trace) on OFT (D; center is shaded gray) and effect of photostimulation on percentage of time in center (E; P = 0.015) and distance traversed (F; P = 0.038).



To test the extent of positive valence encoded by photostimulation, I tested whether mice were willing to overcome the inherent anxiety of being in the center to receive photostimulation. They demonstrated preference for the center when it was paired with stimulation (**Figure 14**). However, this preference was quickly lost once photostimulation was removed, even after 7 days of training on the conditioned place preference (CPP) test. (**Figure 14**). These results suggest that the positive valence effect of circuit activation may be primarily related to its alleviation of anxiety, rather than independently rewarding.

Given the two major behavioral phenotypes observed—reduction in anxiety and increase in hunger—I next sought to examine the causal relationship between these two behaviors. First, we examined whether stimulation results in a greater amount of food consumed per minute or a faster food approach (i.e., reduced latency to onset of feeding). Notably, shorter latency to food consumption is a classic marker of reduced anxiety and is a standard metric used to judge the efficacy of anti-anxiety medication [369]. I found that photostimulation of fed mice reduced latency to onset of feeding as compared to mice that fasted for 24 hours (**Figure 15A**). However, it does not seem to indicate a substantial increase in hunger, as the total amount of food consumed was comparable and there was no difference in the feeding rate from onset of consumption to end of test (**Figure 15B,C**).

Figure 14. Activation of LH$^{Vgat\text{-}ChR2}$-DBB projections only induces transient center preference

(A-B) CPP test for photostimulation-paired center in LHVgat-ChR2 mice over 20 min per test (n = 9): mouse movement in chamber with (magenta) or without (gray) photostimulation-paired center (G), and effects of training on percentage of time spent in center initially without stimulation compared to the first training day (P = 0.007) and last training day to no-photostimulation extinction phase (24 hours after day 7; P < 0.001)

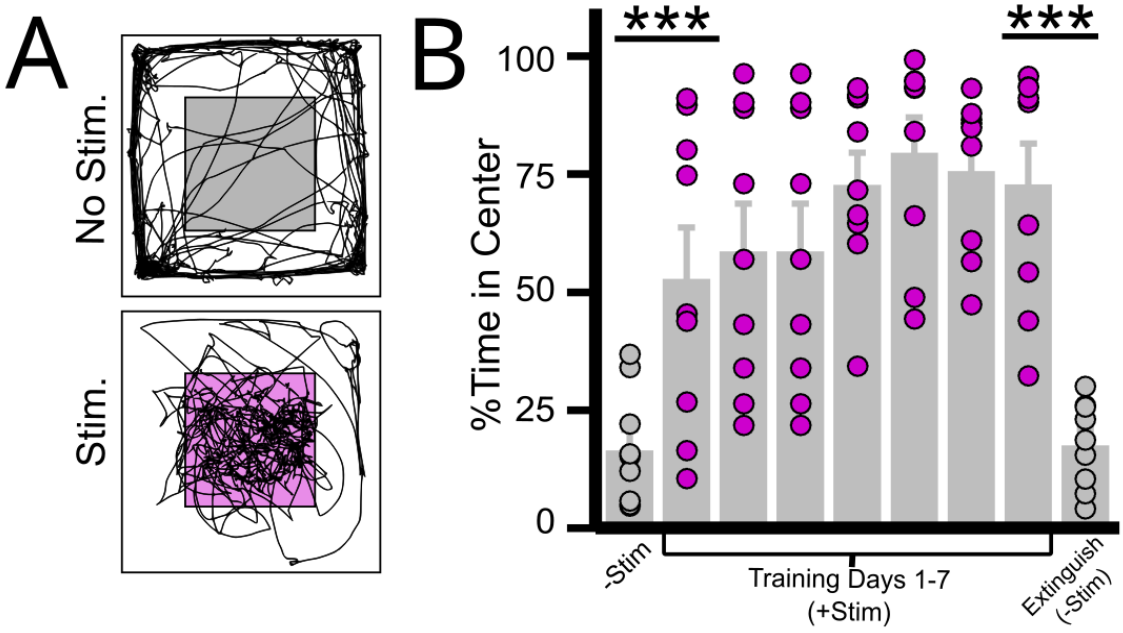
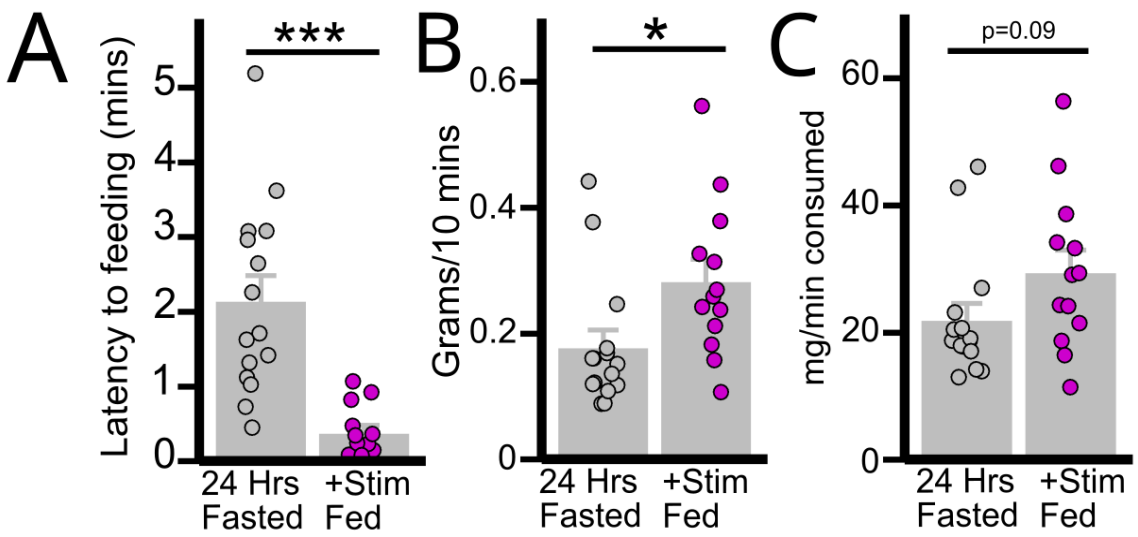


Figure 15. Activation of the circuit reduces anxiety so as to allow food consumption to occur

(A-C) Effect of photostimulation of LHGABA→DBB projections on feeding behaviors compared to 24-hour fasted mice (n = 15 per group): Effect of photostimulation on latency to feeding (A; P < 0.001) and food consumed (B; P = 0.022). (C) Food consumed per minute of feeding (P = 0.09).



To further examine the interaction between anxiety and hunger, I explored how placement of HFD on the open or closed arm of an elevated plus maze (EPM) with regular chow on the converse arm affected consumption after fasting **(Figure 16)**. It is well established that being on the open arm is aversive to rodents and that they strongly prefer the closed arm. My earlier data (**Figure 11**) indicate that mice naturally prefer HFD over chow when given equal access. Coincident with this, when HFD is located on the closed arm, fasted mice began eating HFD immediately and never touched the chow. However, in the flipped condition, there was increased latency to HFD approach, reduced HFD consumption, decreased latency to chow approach, and increased chow consumption.

Given that placing palatable food on the open arm reduced its value, I next isolated the effect of food placement on feeding behavior (**Figure 17A**). Fasted mice showed reduced latency and increased consumption when food was placed on the closed arm as opposed to the open arm (**Figure 17B,C)**. Last, activation of the LH^{GABA}→DBB neurocircuit markedly reduced the latency to feeding on the open arm while producing a comparable amount of food consumption (**Figure 17D,E**). From these results, I concluded that (i) anxiety imposed by environment substantially affects both feeding behavior and food choice and (ii) LH^{GABA}→DBB activation reduces this environmental anxiety to allow feeding to occur.

Figure 16. Competition between anxiogenic conditions and feeding behavior.

(A-F) Mice (n=20) were fasted overnight prior to all experiments and habituated to the elevated plus maze (EPM) prior to food placement; lines connect same mouse across conditions. (A-B) Schematic representing two experimental conditions: condition 1, regular chow (reg) is placed on the open arm of the EPM and high fat diet (HFD) on the closed arm; condition 2, regular chow is placed on closed arm and HFD on the open arm (C) Latency to regular chow consumption between the two conditions($p<0.001$) (D) Amount of regular chow consumed for 10 minutes after first bite ($p<0.001$) (E) Latency to HFD consumption between the two conditions ($p<0.001$) (F) Amount of HFD consumed for 10 minutes after first bite ($p<0.001$).

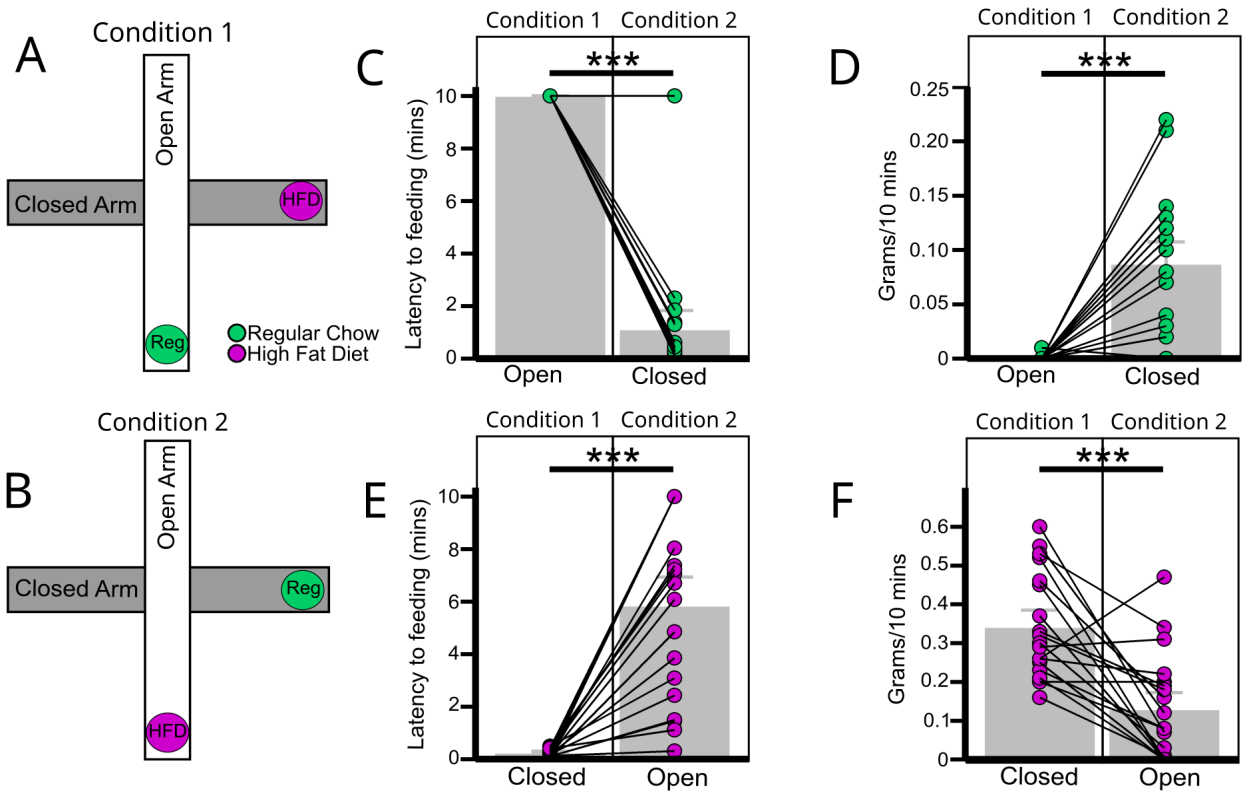
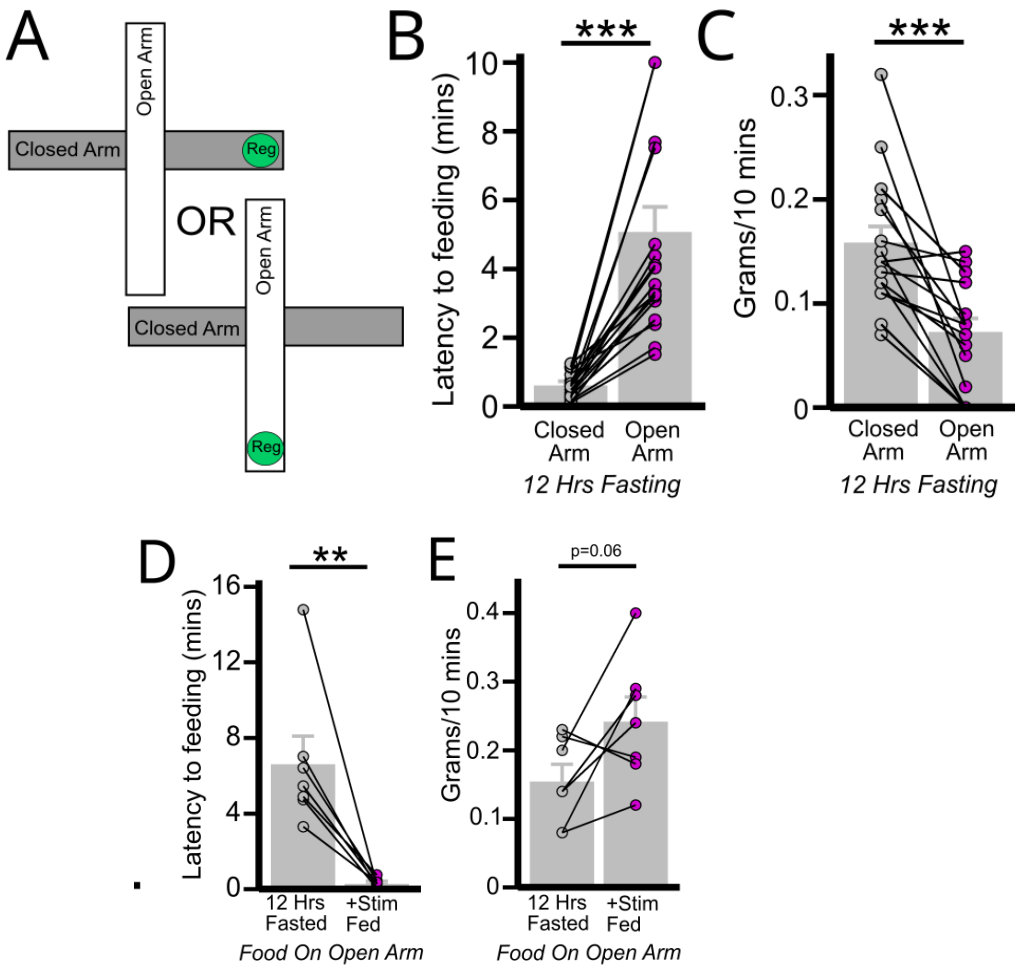


Figure 17. Activating the LH^{GABA}-DBB circuit reduces environmentally-driven anxiety to allow feeding

(A-E) Influence of food location on open or closed arm of EPM on fasted-refeeding response in control mice (n = 20). (A) Schematic showing two testing conditions: food placed on the closed (gray) arm or food placed on the open (white) arm. (B) Latency to the first bite of food in closed or open condition (P < 0.001). (C) Consumption 10 min following first bite (P < 0.001). (D-E) Influence of activation of L^{HGABA}→DBB on feeding when food was on open arm of EPM (n = 7). (D) Latency to the first bite of food on open arm when fasted or fed and with (+stim) laser stimulation (P = 0.004). (E) Consumption 10 min following the first bite (P = 0.06).

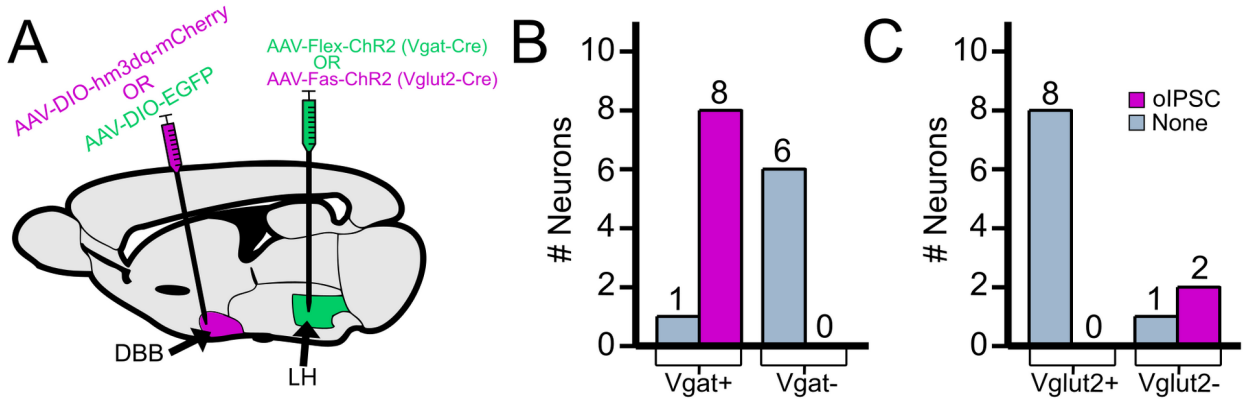


2.3.4. LH^{GABA} neurons specifically target DBB^{GABA} neurons

Having established the behavioral consequences of circuit activation, I next explored what types of neurons within the DBB are postsynaptic to LH^{GABA}→DBB projections. The DBB contains both glutamatergic and GABAergic neurons; the latter includes acetylcholine and somatostatin co-releasing neurons [370–372]. First, I used a double-injection method of ChR2 into the LH and a Cre-dependent fluorescent label in the DBB of Vgat-cre or Vglut2-cre mice and then Dr. Yungang Lu performed brain slice electrophysiology (**Figure 18A**). In Vgat-cre, I targeted the LH with AAV-FLEX-ChR2-EYFP and the DBB with AAV-DIO-hM3Dq-mCherry (marking LH^{Vgat} fibers with EYFP and DBB^{Vgat} neurons with mCherry). In Vglut2-cre, I targeted the LH with AAV-Fas-ChR2-mCherry and the DBB with AAV-FLEX-EGFP. The Fas-ChR2 vector is “Cre-Off,” such that only neurons that do not express Cre will express ChR2 . This means that $LH^{Non\text{-}Vglut2}$ fibers will be marked with mCherry and that DBB^{Vglut2} somata will be marked with enhanced green fluorescent protein (EGFP). Patching the fluorescent cell bodies in the DBB allowed molecular identification of the downstream target. From all neurons recorded, only DBB^{GABA} neurons were shown to receive inhibitory input (**Figure 18B,C)**.

Figure 18. Electrophysiological recordings in the DBB reveals an LHGABA-DBBGABA circuit

(A to C) DBB^{GABA} neurons receive direct synaptic input from LH^{GABA} neurons. (A) Schematic showing Vgat-cre mice receiving LH injection of AAV-FLEX-ChR2 ($LH^{Vgat\text{-}ChR2}$) and DBB injection of AAV-DIO-hM3Dq-mCherry ($DBB^{Vgat\text{-}hM3Dq}$), and Vglut2-cre mice receiving $LH^{Vglut2\text{-}Fas\text{-}ChR2}$:$DBB^{Vglut2\text{-}hM3Dq}$. (B-C) Brain slice electrophysiology of oIPSC in $DBB^{mCherry+}$ and $DBB^{mCherry-}$ neurons of $LH^{Vgat\text{-}ChR2}$:$DBB^{Vgat\text{-}hM3Dq\text{-}mCherry}$ mice (B; n = 3) and $LH^{Vglut2\text{-}Fas\text{-}ChR2}$:$DBB^{Vglut2\text{-}hM3Dq\text{-}mCherry}$ mice (C; n = 2).



I next sought to validate these data in vivo using a similar targeting strategy (**Figure 19A**). Vgat-cre mice received an injection of AAV-FLEX-ChR2 into the LH, and AAV-FLEX-DIO-hM3Dq-mCherry. *NOTE: hM3Dq is a a synthetic excitatory receptor that can be activated with clozapine-N-oxide (CNO); see **Figure 20** top for fluorescent pattern and CNO-induced c-fos expression.* Pretreatment of $LH^{Vgat\text{-}ChR2}$: $DBB^{Vgat\text{-}hM3Dq}$ mice with CNO substantially reduced the amount of photostimulation-induced feeding (**Figure 19B**). I also used the converse strategy, where Vglut2-cre mice received an injection of AAV-Fas-ChR2 in the LH and AAV-FLEX-hM3Dq-mCherry in the DBB (**Figure 20** bottom). Pretreatment of $LH^{Vglut2:Fas\text{-}ChR2}$:$DBB^{Vglut2\text{-}hM3Dq}$ mice with CNO had no effect on stimulation-induced feeding (**Figure 19C**).

Figure 19. Preactivation of DBB^{Vgat}, but not DBB^{Vglut2}, neurons prevents stimulation-induced feeding

(A) Schematic demonstrating targeting strategy. (B and C) Effect of CNO-mediated activation of DBB^{hM3Dq} on photostimulation-induced feeding in LH$^{Vgat\text{-}ChR2}$:DBB$^{Vgat\text{-}hm3Dq}$ mice [one-way analysis of variance (ANOVA) and Tukey post hoc test] (B; n = 11; saline:photostimulation, P < 0.001; saline:CNO-photostimulation, P = 0.07:photostimulation:CNO-photostimulation, P < 0.001), and LH$^{Vglut2\text{-}Fas\text{-}ChR2}$:DBB$^{Vglut2\text{-}hm3Dq}$ mice) (C; n = 8; saline:photostimulation, P = 0.002; saline:CNO-photostimulation, P = 0.006; photostimulation:CNO-photostimulation, P = 0.88).

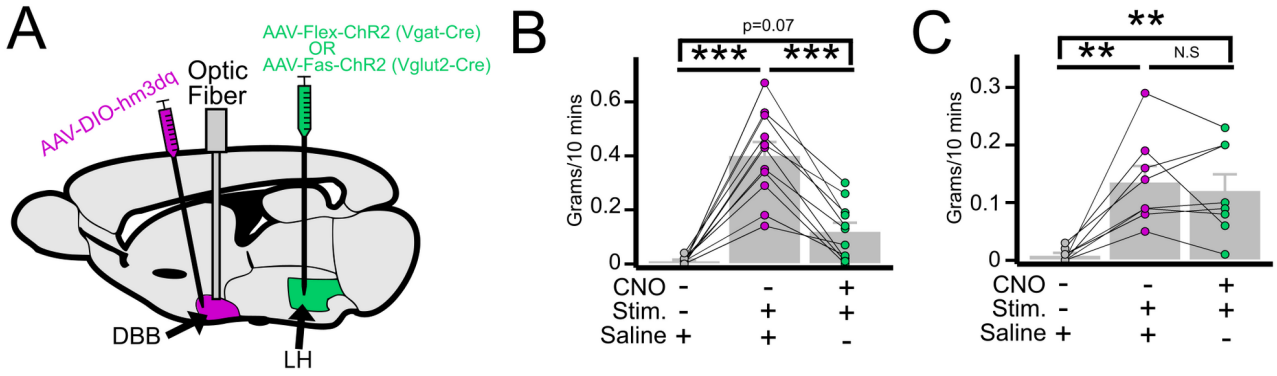
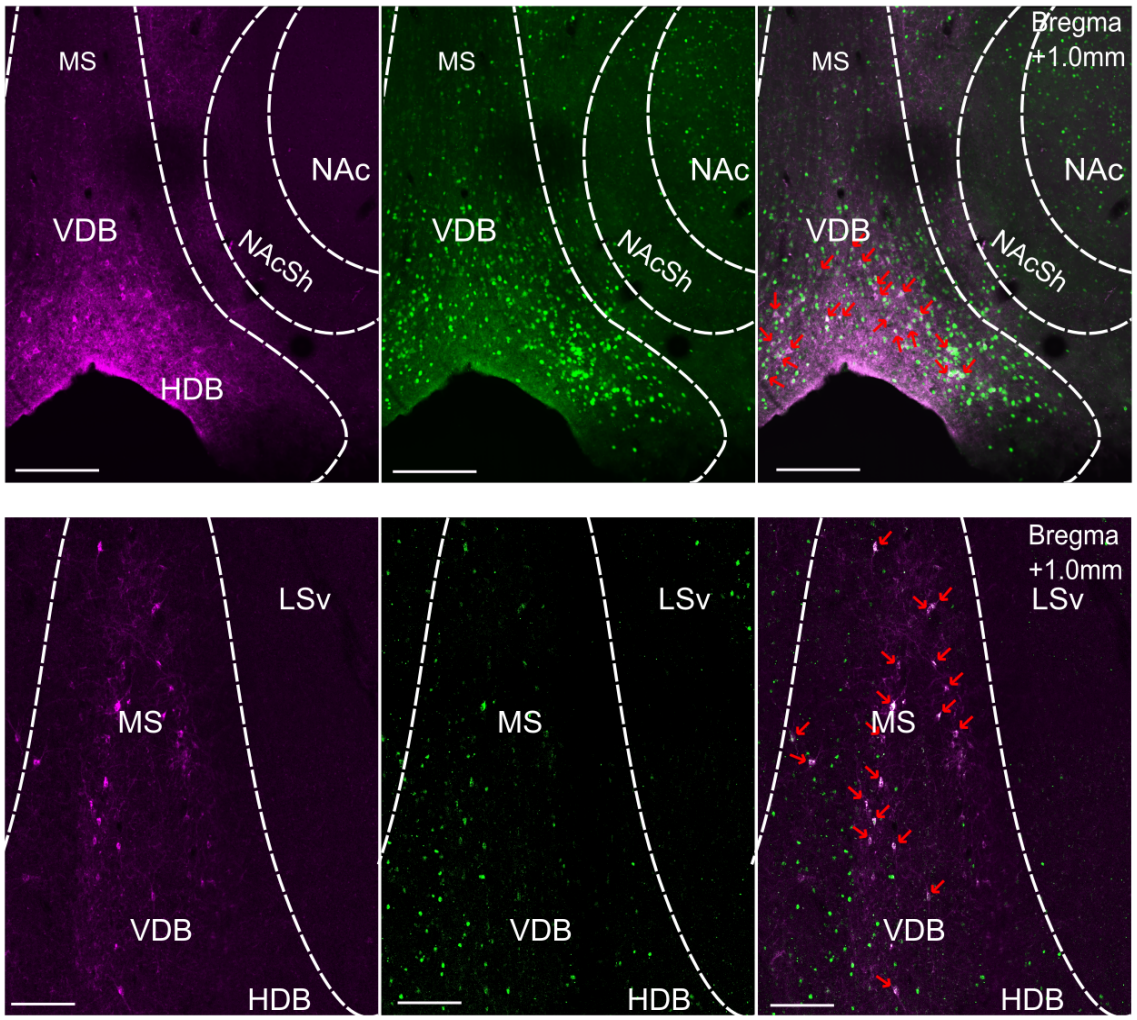


Figure 20. Administration of CNO induces c-Fos in DBB^{Vgat} and DBB^{Vglut2} neurons

(Top Row; scale bar = 250μm) Brain slices showing expression DIO-hm3Dq in DBB Vgat neurons (magenta); c-fos immunohistochemical staining (green) in these neurons 3 hours after injection of clozapine-N-oxide (CNO). (Bottom Row; scale bar = 100μm) Brain slices showing expression of DIO-hm3Dq DBB Vglut2 neurons (magenta) and c-fos expression (green) 3 hours after injection of CNO. Red Arrows indicate co-localization. HDB = horizontal limb of the diagonal band of Broca; LSv = lateral septum ventral part; MS = medial septum; NAc = nucleus accumbens; NAcSh = nucleus accumbens shell; VDB = ventral limb of the DBB

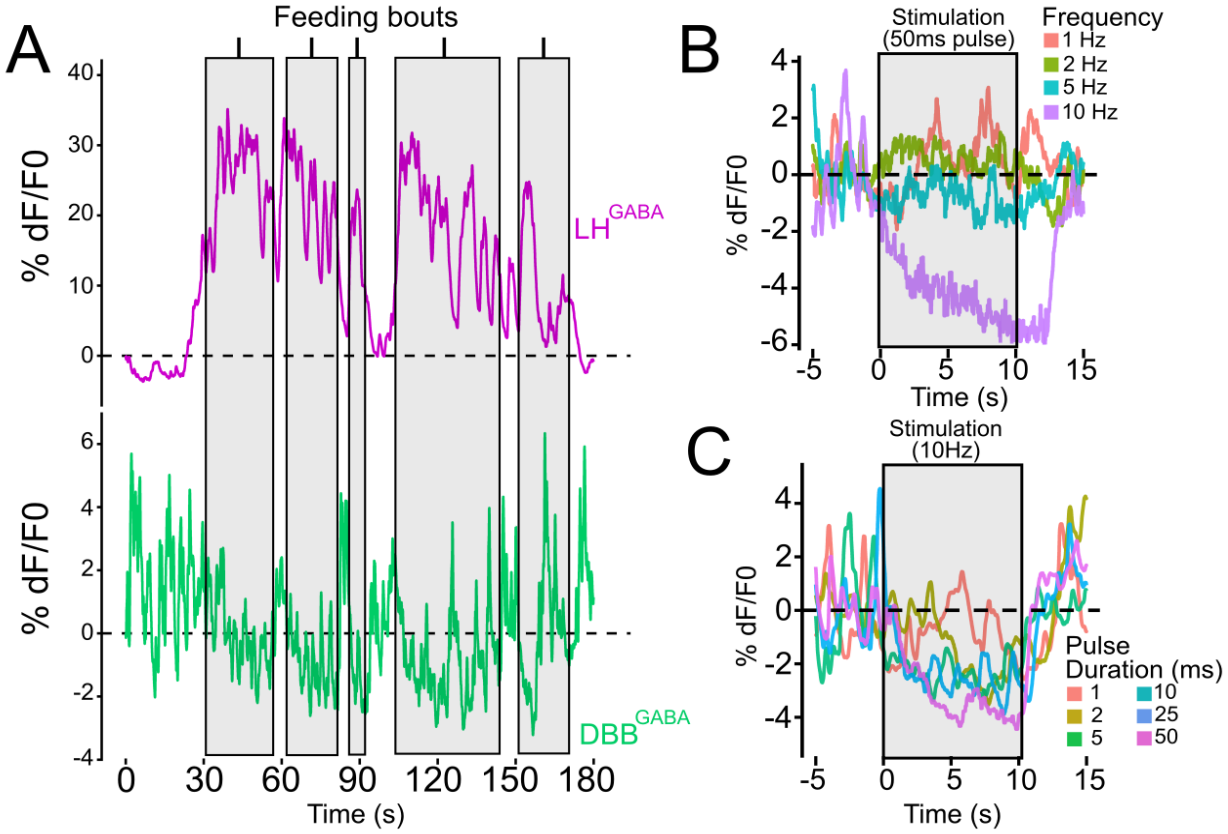


To further confirm the LH^{GABA}→DBB^{GABA} connection, I used real-time fiber photometry of calcium-dependent fluorescence from LH^{GABA} and DBB^{GABA} neurons expressing AAV-DIO-GCaMP6m [335]. This allows the identification of changes in neuronal population activity at the sub-second level (this particular protein easily allows determination of differences across 100ms). First, I sought to determine how these two populations of neurons respond to feeding. After fasting, simultaneous recording of $LH^{Vgat\text{-}GCaMP6m}$ and $DBB^{Vgat\text{-}GCaMP6m}$ neurons showed that, as LH^{GABA} neurons increased their activity (**Figure 21A top)**, DBB^{Vgat} neurons showed a concomitant decrease (**Figure 21A bottom**) specifically during the feeding bout.

Last, I sought to demonstrate in vivo that activation of $LH^{Vgat\text{-}ChR2}$ neurons produces a time-locked reduction in activity of DBB^{Vgat} neurons. Simultaneous photostimulation of $LH^{Vgat\text{-}ChR2}$ cell bodies and fiber photometry recording from $DBB^{Vgat\text{-}GCaMP6m}$ cell bodies demonstrated both a pulse frequency–dependent and a pulse duration–dependent reduction in DBB fluorescence (**Figure 21B,C**). Together, these results convinced us that LH^{GABA} neurons specifically inhibit DBB^{GABA} neurons in real time, with the strength of LH^{GABA} activation producing greater amounts of DBB^{GABA} inhibition.

Figure 21. Fiber photometry of LH^{Vgat} and DBB^{Vgat} neurons reveals inverse activity relationship

(A) Simultaneous recording of LH$^{Vgat\text{-}GCaMP6m}$ and DBB$^{Vgat\text{-}GCaMP6m}$ population activity during feeding bouts (shaded areas) recorded after 12hrs of fasting and free access to food. (B,C) Effect of photostimulation of LH$^{Vgat\text{-}ChR2}$ cell bodies on DBB$^{Vgat\text{-}GCaMP6}$ neuronal activity at various frequencies (B) and pulse durations (C).



2.3.5. DBB^{GABA} neurons are highly sensitive to anxiogenic environmental cues

Having demonstrated the specific connection between LH^{GABA} and DBB^{GABA} neurons, I then sought to look at the conditions in which DBB^{GABA} neurons are activated. I monitored activities of DBB$^{Vgat\text{-}GCaMp6m}$ neurons (**Figure 22A,B**) in several conditions. I observed that voluntary interaction with a novel object correlated with increased DBB$^{Vgat\text{-}GCaMP6m}$ activity during the period of active investigation of the novel object; this was repeatable across interactions and between mice (**Figure 22C** picture inset demonstrating what was coded as stimulus onset). In addition, both looming threat (**Figure 22D**) and loud sound (**Figure 22E**) stimuli increased DBB$^{Vgat\text{-}GCaMP6m}$ activity. Each of these is a sign of danger for a laboratory mouse [348–353] and indicates that these neurons are involved with heightened attention to anxiogenic stimuli.

Conversely, DBB$^{Vgat\text{-}GCaMP6m}$ neurons showed decreased activity during feeding bouts, consistent across feeding bouts and between mice (**Figure 23A,B**). Last, treatment with diazepam, an anti-anxiety benzodiazepine known to reduce latency to food consumption [369], reduced DBB$^{Vgat\text{-}GCaMP6m}$ activity (**Figure 23C,D**). Administering diazepam to fed mice caused feeding (**Figure 23E**). Further, administration to fasted mice caused indiscriminate feeding when given access to both chow and HFD simultaneously (**Figure 23F**). From these data, I concluded that DBB^{GABA} neurons respond to environmental cues of threat and reduce their activity during feeding. I also concluded that diazepam reduced DBB^{GABA} neuronal activity and recapitulated the indiscriminate feeding phenotype produced by LH^{GABA}→DBB^{GABA} activation.

Figure 22. Multi-modal anxiogenic stimuli cause an increase in DBB^{Vgat} activity

(A to E) Response of DBBVgat-GCaMP6m neuronal population to threatening environmental stimuli. (A) Schematic of DBBVgat-GCaMP6m injection and optic fiber placement strategy. (B) Brain slice with DBBVgat-GCaMp6m neurons (VDB); arrows indicate cell bodies. MS, medial septum. (C to E) DBBVgat-GCaMP6m neuronal activity change in response to stimulus (onset at 0 s) with gray traces indicating single interactions, magenta traces indicating averaged response, and heat map indicating normalized average responses per mouse in response to novel object interaction (C; inset shows stimulus onset), looming threat (D), & loud sound (E). MS = medial septum; NAc = nucleus accumbens; VDB = ventral limb of the DBB

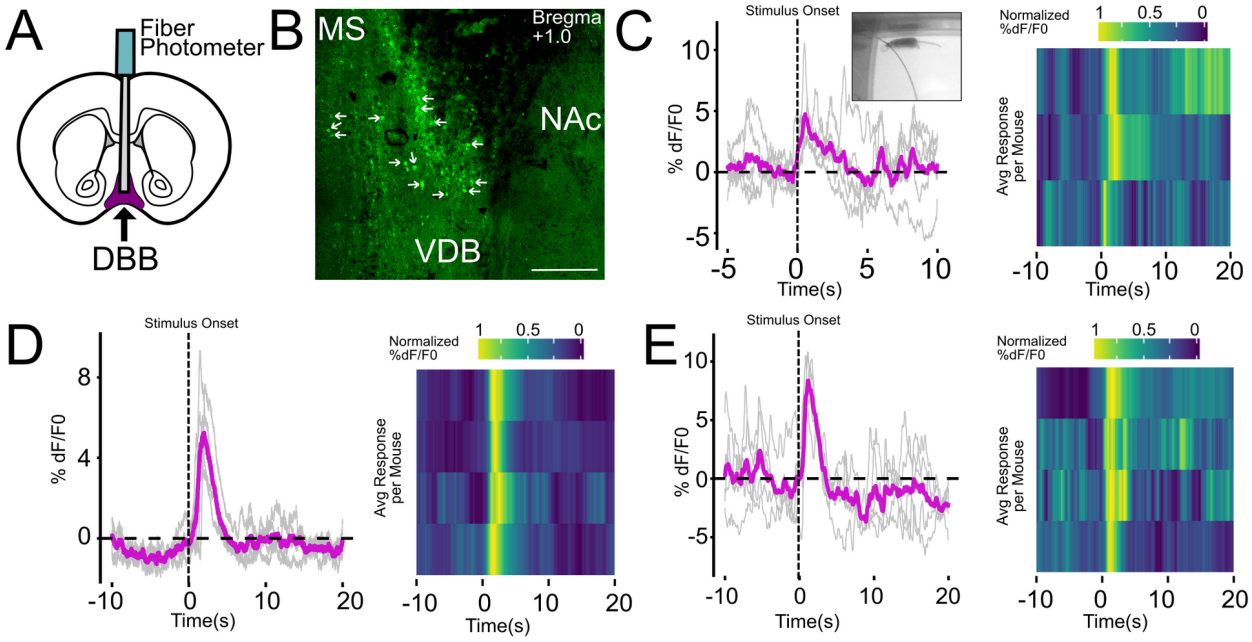
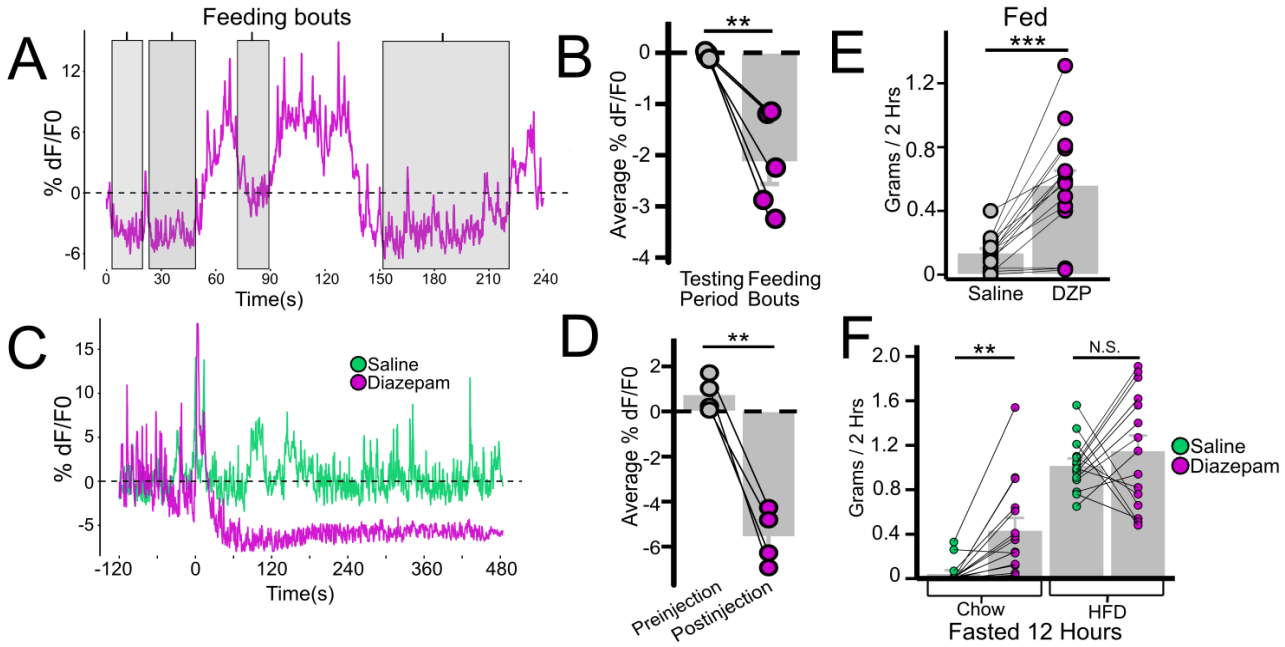


Figure 23. Feeding bouts cause a reduction in DBB^{Vgat} activity and diazepam recapitulates the effects of activating the LH^{GABA}-DBB^{GABA} circuit.

(A,B) Response of DBB$^{Vgat\text{-}GCaMP6m}$ neurons to feeding in the fasted condition with representative trace during feeding bouts (A; shaded gray) and averaged activity during feeding bout compared to average activity over relevant testing period (B; n = 5, P = 0.002). (C to D) Effect of diazepam (DZP) on DBB$^{Vgat\text{-}GCaMp6}$ neuronal activity and feeding behavior. (C) Effect of intraperitoneal DZP on DBB$^{Vgat\text{-}GCaMp6m}$ neuronal activity in comparison with intraperitoneal saline; (D; n = 4, P = 0.002) Averaged activity min before injection compared to average activity 3 to min after injection. (E) Effect of intraperitoneal injection of saline versus DZP on feeding (n = 15) when fed (P < 0.001). (F) Effect of DZP on fasting-induced food intake of chow and HFD when presented with free access to both simultaneously for 2 hours (chow, P = 0.001; HFD, P = 0.421).



2.4. Conclusions and significance

In this study, I found a previously unidentified LH^{GABA}→DBB^{GABA} pathway that promotes feeding by suppressing environmentally driven anxiety. This projection likely acts in concert with other known projections of LH^{GABA} neurons to coordinate successful feeding [286,294–296,298,302]. In vivo fiber photometry data show that DBB^{GABA} neurons respond to anxiogenic environmental stimuli and reduce activity during feeding. This is consistent with the known function of DBB neurons in promoting environment-responsive arousal [312,315,366,373,374]. My data thus suggest that LH^{GABA} neurons not only induce appetitive and consummatory behaviors but also alter sensitivity to the environment to promote feeding behavior.

Since the induced feeding state observed here is indiscriminate, this suggests that some of the altered sensitivity may result via altered evaluation of food value itself. This aligns with the converse phenotype of picky eaters who have anxious personalities and with a study showing that activation of forebrain somatostatin neurons increases anxiety with selective consumption of a calorie-dense diet [372,375]. I further explored this point and demonstrated that environmentally driven anxiety is a key mediator in the selection and proportion of low-value, but safe food consumed compared to high-calorie, but unsafe food. Moreover, the recapitulation of my circuit data with the effects of diazepam further points to the fundamental link between anxiety, food evaluation, and food consumption.

Notably, despite verifying LH targeting for included mice, because of inherent variation associated with stereotaxic injections and the continuous distribution of

GABAergic and PDX1 neurons between the LH and nearby regions, e.g., the dorsomedial hypothalamus, I could not completely rule out a slight contribution of nearby non-LH^{GABA} neurons in some mice tested in our study. Thus, it is possible that the DMH also plays a role in the findings discussed in these results.

From a pharmacologic perspective, my data are notable because one of the most troubling aspects of nearly all psychotropic agents for anxiety and depression is their strong tendency to induce hunger, weight gain, and metabolic disease [270]. These include drugs used for children with sensory processing disorders, such as risperidone for autism [376]. Further, given recent compelling data demonstrating that LH^{GABA} neurons can be activated by the mere presence of and consumption of rewarding foods and encode reward prediction regardless of hunger [296], it is conceivable that the LH^{GABA}→DBB^{GABA} neurocircuit may be involved in "stress eating," i.e., consumption of foods in the non-hunger state in order to reduce feelings of anxiety. A clinical example of this may be the compulsion to binge seen in binge-eating disorder after acute stress [131,269]. Mechanistic demonstration of the direct link between the hedonic LH and the anxiogenic DBB provides tantalizing evidence that this heretofore difficult-to-define behavior in humans may have firm grounding in neurobiology. Knowledge of the neurocircuitry mediating the tight connection between hunger and anxiety provides a mechanistic platform for specific development of behavioral interventions and therapeutics against eating and anxiety-related disorders.

Chapter 3. Real-time monitoring of the paraventricular hypothalamic nucleus and ventral lateral septum reveals tight inverse association between anxiety, stress-responses, and eating.

This chapter is based upon: Xu YZ*, Lu Y*, Cassidy RM*, Mangieri LR, Zhu C, Huang X, Jiang Z, Justice NJ, Xu Y, Arenkiel BR, Tong Q. Identification of a neurocircuit underlying regulation of feeding by stress-related emotional responses. Nature Communications 2019 10:3446 *Co-first authors

3.1. Rationale

In addition to the LH-DBB circuit discussed in Chapter 2, there are obviously many other neurocircuits that respond to hunger and anxiety that have neurodynamics reflecting the competition between these two affective states. More broadly in cognition and memory, these can be described as brain states. The septohippocampal connections have long been known to, once reaching a threshold of activity, induce fight-or-flight behavior or what may be called panic [377–379]. The destruction of the septal nuclei prevents learning or avoiding dangerous stimuli [380,381]. Further, it appears that the interaction between the BF and the septal nuclei may drive the presence of the rhythmic firing activity observed on electroencephalography (EEG) over the cortex and hippocampus associated with distinct arousal and attentional brain states: low synchronized firing rates produce theta and delta rhythms associated with memory recall, feeding, and passive observation, whereas high frequency firing rates produce gamma and alpha rhythms associated with attention [311,366,374,380,382,383]. Thus, much work conducted on the septal nuclei has focused on its role in entraining these observed EEG rhythms and establishing the role of these rhythms in producing the above observed brain states. The influence of the hypothalamus on this region in mediating the switching from one brain state to another has not been well-established.

However, as with the LH-DBB circuit, our lab possessed data indicating that hypothalamic nuclei also send projections to this region. Given our concurrent work showing the role of activation of PVH glutamatergic (PVH^{glut}) neurons in preventing

feeding and inducing self-grooming and defensive behaviors by their projection to glutamatergic neurons in the [VTA] [281,282], and the parallel LH inhibitory circuit from the LH-DBB, we decided to use a similar set of techniques as used in Chapter 3 to establish the role of the putative PVH-LSv circuit in mediating hunger, anxiety, and fight-or-flight responses.

Because of the complexity of these tools (particularly electrophysiology, behavioral assays, and fiber photometry data acquisition and analysis) and the relative levels of expertise possessed by each member, the three co-first authors of this paper contributed their expertise to generate and analyze data about this circuit's function at the electrophysiological (Yungang Lu), behavioral (Yuanzhong Xu), and real-time neural activity monitoring (myself) levels. In **section 3.2**, I have only introduced the methods relevant to the data discussed in detail in **section 3.3.** To provide context for this data, I have provided a brief summary of the findings produced by Drs. Xu and Lu at the beginning of **section 3.3** before discussing in detail the results of the fiber photometry experimentation. NOTE: *Dr. Xu performed the surgeries, brain slicing, and the behavioral experiments. We jointly developed the behavioral testing paradigms. I set up the equipment, wrote in-house code, analyzed the data sets, and produced all of the graphs.*

3.2. Material and Methods

NOTE: Only those methods that differ from **section 2.2** and which were involved in generating the data discussed in this section have been presented here.

Animal Models

The following transgenic Cre mouse line was used: *Sim1-Cre* (Single-minded 1 [384] , known to be expressed primarily within the PVH in a fashion similar to Pdx1-Cre).

The following knock-in mouse lines were used: vglut2^{flox} (to create the glutamate release knockout model, Sim1$^{Vglut2\ F/F,}$) [292,384] and Vgat-Cre.

In vivo fiber photometry experiments: Sim1-Cre mice with specific delivery of AAV-FLEX-GCaMP6m to the PVH and optic fiber implantation targeting PVH neurons and Vgat-Cre mice with specific delivery of AAV-FLEX-GCaMP6m to the LSv and optic fiber implantation targeting LSv were used for the in vivo fiber photometry Ca2+ imaging studies. In retrograde studies, rAAV2-retro-EF1a-Cre-Venus viruses were injected to the LSv of wild-type mice and the AAV-FLEX-GCaMP6m viruses were injected to the PVH. The recording was performed 6 weeks after the surgery. The GCaMP6m virus was provided by the Baylor NeuroConnectivity Core. The experiments were conducted at least 4 weeks after the surgery. We used the fiber photometry system from Doric Lenses to monitor Ca2+ signal from a group of PVH Sim1 neurons and LSv GABAergic neurons. Mice were acclimated to the behavioral chamber for at least 15 min prior to the beginning of each testing session. After baseline recording for 10 seconds, water spray was started by

spraying one time with water toward the head of mice with a sprayer and the recording continued for ~ 2 mins. For feeding studies, fasted or well-fed mice were acclimated in the cage for at least 5 mins before one high-fat diet pellet was introduced to the cage. Mouse behaviors including feeding were videotaped simultaneously with photometry recording. Data were acquired and analyzed in the same manner as in **section 2.2.6**. For water spray, loud sound, and light, the baseline was calculated from 10 seconds prior to stimulus onset, to 20 seconds after stimulus onset. Note that water spray consisted of two quick puffs into the face, light was an immediate switch from dark to light, and loud sound is as is described in section 2.2.6.

3.3. Results

3.3.1. Summary of findings collected by Dr. Xu and Dr. Lu on the description and function of the PVH-LSv circuit.

Through the work of Dr. Xu and Dr. Lu, our laboratory demonstrated the following key pieces of information about the PVH-LSv connection using Sim1-cre mice:

1. The PVH-LSv neurocircuit is glutamatergic and prevents feeding behavior by inducing aversion, anxiety, and the flight response. This connection is monosynaptic onto GABAergic neurons within the LSv. Microinjection of a glutamate receptor antagonist (DNQX+AP5) into the LSv increased the amount of time spent on the exposed arm of the EPM as well as induced feeding behavior.

2. Photostimulation of the PVH-LSv circuit induces self-grooming and escape behavior, best described as sprinting to the edge of the cage and jumping, or hiding in a hut. Further, it appears that the intensity of the signal is the primary determinant as to whether a mouse self-grooms or escapes, as the same mouse with low laser pulse duration grooms and at high pulse-duration jumps (though for some mice this was not scalable). Mice will also work to avoid laser stimulation on the RTPP and OFT. Conditioned place avoidance was not tested. Finally, circuit activation interrupts aggressive attacks during the resident-intruder assay in males, where the mouse with an optic fiber implant is exposed to an intruder in the home cage - something that normally induces attack.

3. Photostimulation of the PVH-LSv circuit prevents fasted mouse feeding; however, the inhibitory effect is reduced and eventually abolished as the mouse becomes progressively hungrier (6hrs fasting vs 12hr vs 20hr). Increased pulse durations still caused a reduced amount of eating for a given degree if hunger. At 6hrs fasting, mice consumed no food over 15 minutes; in 20hr fasting, there was an average 0.1g reduction in food consumption in the long pulse duration condition compared to short pulse duration (average ~0.2g total) over 15 minutes.

3.3.2. PVH and LSv neurons are sensitive to environmental stressors

In light of the above information, we next sought to explore the normal ethology of both PVH and LSv neuronal activity as related to feeding behavior and environmental anxiogenic cues, as established in my work on the LH-BF neurocircuit (**section 2.3.5**). To examine the function of the PVH-LSv projection in sensing environmental cues, we targeted PVH and LSv neurons for GCaMP6m expression and monitored their activity using fiber photometry. Three experimental setups were used:

3.3.2A PVH cell bodies were recorded after PVH^{Sim1} injection of GCaMP6m ($PVH^{Sim\text{-}GCaMP6}$)

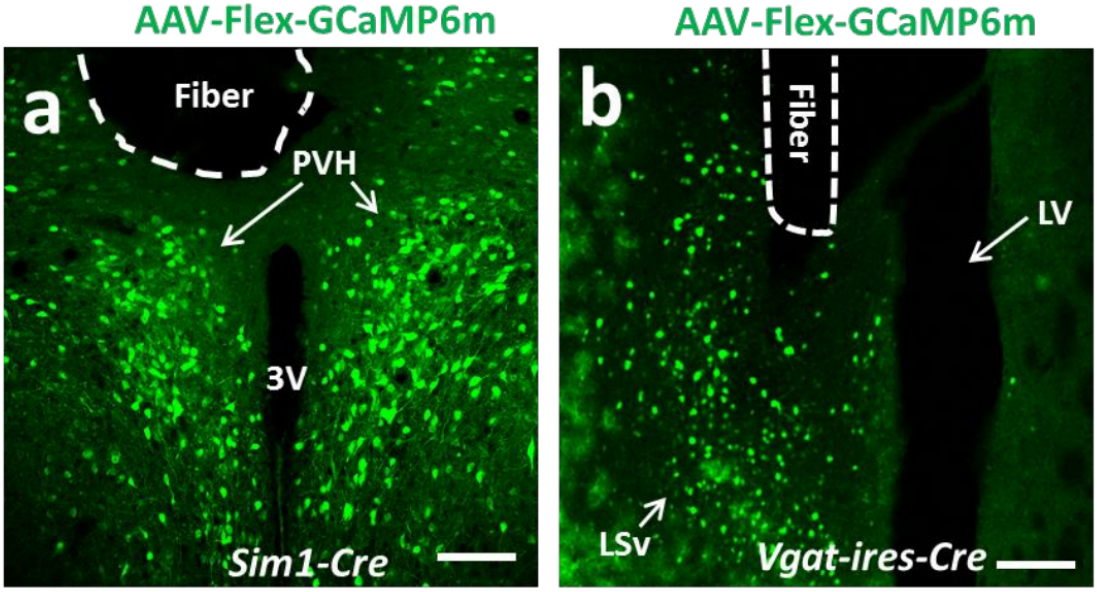
3.3.2B LSv cell bodies were recorded after LSv^{Vgat} injection of GCaMP6m ($Lsv^{Vgat\text{-}GCaMp6}$).

3.3.2C PVH cell bodies were recorded after LSv injection of retrograde AAV capsid bearing Cre (c.f. section 2.3.1), with simultaneous PVHLSv-targeting injection of GCaMP6m (PVHLSV-retro-GCaMp6). This allowed recording from neurons that send projections to

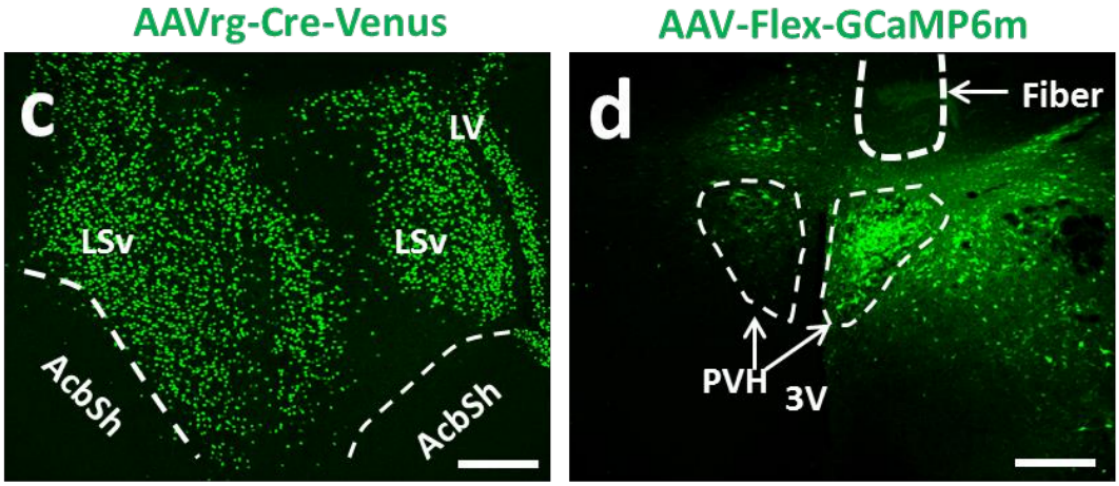
the LSv. These injection strategies and the placement of fibers can be seen in **Figure 24A-D**.

Figure 24. Targeting of GCaMP6m into the PVH and LSv for recording of real-time neural activity.

NOTE: Images taken from Dr. Yuanzhong Xu with permission. (A-D) Expression of Campagna and fiber photometer tract for recording of activity (A) Expression of GCaMP6 in PVHSim1 neurons (B) Expression of GCaMP6 in LSV^{Vgat} neurons (C) Expression of retroAAV-Cre in the LSv of a Cre-negative mouse (D) Expression of Cre-dependent GCaMP6 in the PVH of the



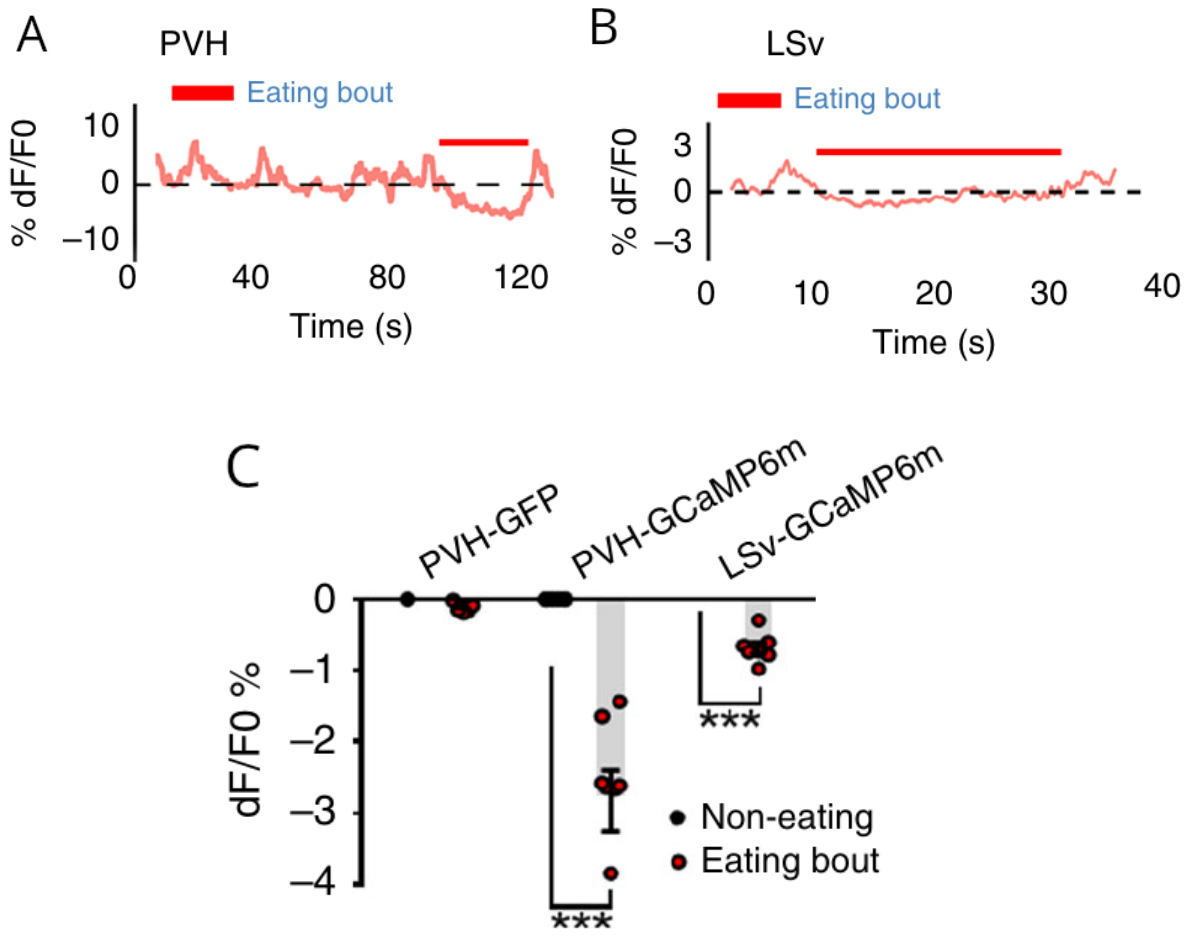
mouse infected with LSV retro-Cre. Scale bar = 100μM. AcbSh = accumbal shell; LSv = ventral lateral septum; 3V and LV = third and lateral ventricle. PVH = paraventricular hypothalamic nucleus.



Next, as in section 2.3.5 we monitored the activity of each of these regions during multiple ethologically relevant conditions. First, we examined the relative activity of the PVH and the LSv during fasted-refeeding. As seen in **Figure 25**, the PVH and LSv neurons both, over repeated trials, show an immediate and sustained drop in population-level activity during the feeding period. The reduction in activity stops as soon as the feeding bout has ceased. This is similar to the feeding-induced suppression of the DBB seen in **Figure 23**.

Figure 25. PVH^{Sim1} and LSv^{Vgat} neurons reduce in activity during feeding bouts

(A-C) Activity of PVH and LSv neurons during feeding bouts. (A) Representative trace of PVHSim1 neuronal population-level activity during feeding bouts. (B) Representative trace of

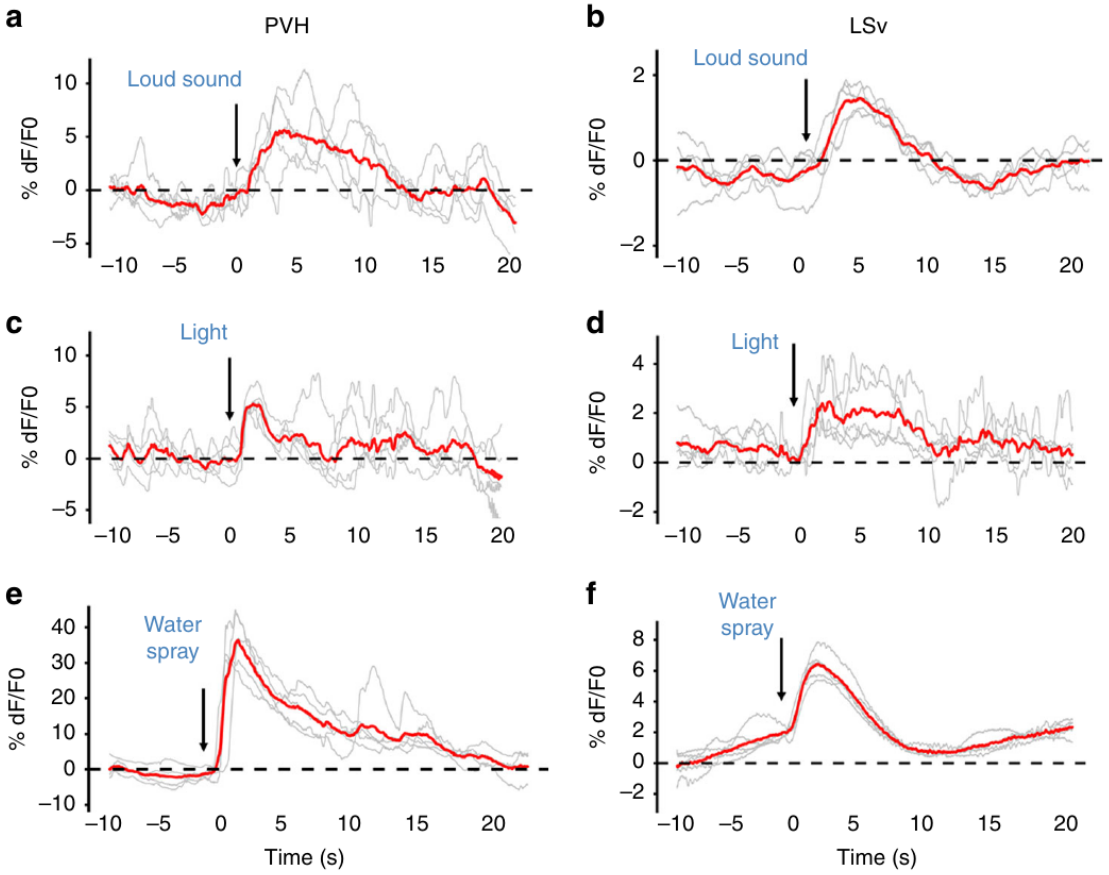


LSv^{Vgat} activity during a feeding bout. (C) Averaged neuronal activity overall for each neuronal region compared to average during feeding bout (n=8 per group).

Next, the PVH and LSv show tightly correlated activity levels during a variety of anxiogenic stimuli from the environment (‘environmental cues’). As observed in **Figure 26**, both the PVH and LSv respond to a sudden loud sound, a sudden shining of light in a dark room, and water spray (this induced grooming). These observations indicate it is the sudden anxiogenic stimulus and not grooming or a specific sensory modality driving the activity. This feeds into a more general concept that the observed behaviors of feeding and self-grooming and escape are not directly encoded by the LSv and DBB action, but instead are readouts of the change in brain state. Indeed, the context- and intensity-dependent intensity of expressed aversion, from self-grooming to escape activity, elicited by the PVH-LS circuit indicates that the primary function of the LSv is as a gate, which, when sufficiently activated, releases an action plan. That action plan is driven in this case by the activity of the PVH.

Figure 26. PVH^{Sim1} and LSv^{Vgat} neurons increase in activity during various anxiogenic environmental cues

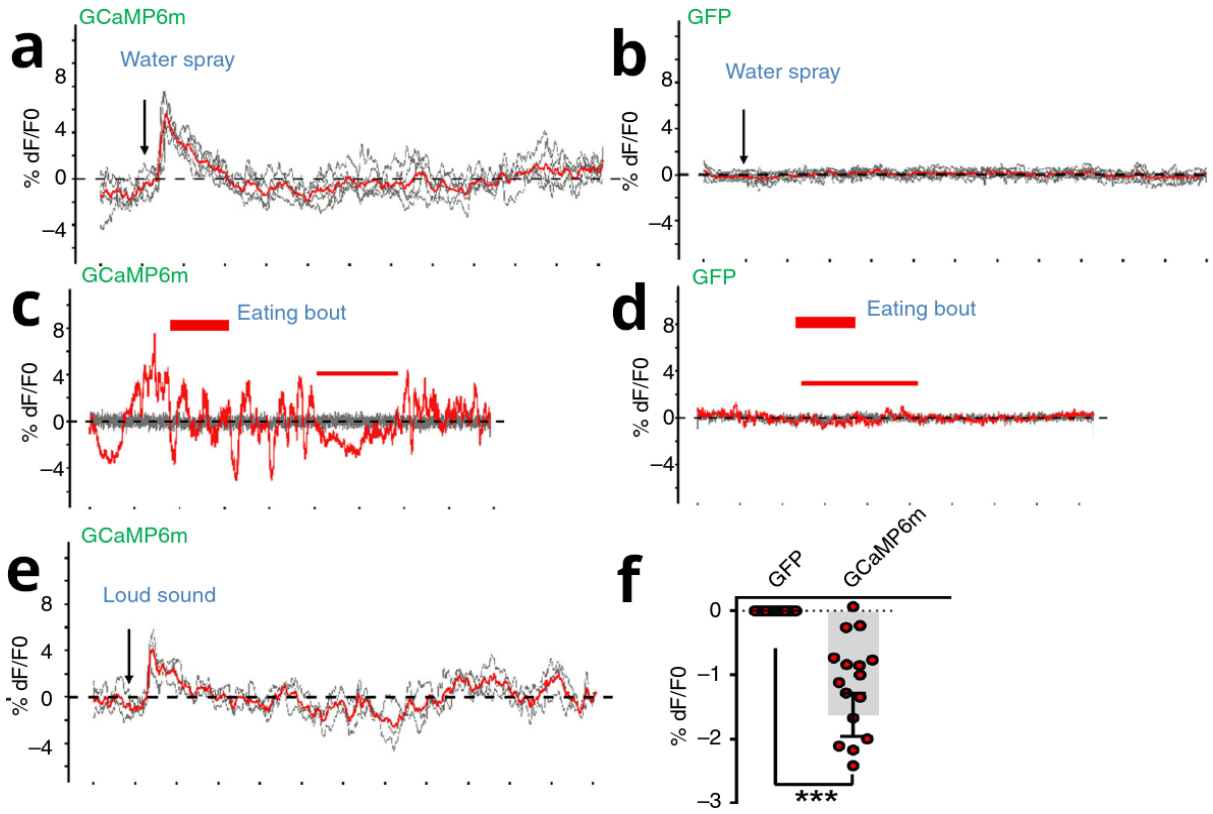
(a-f) In vivo Ca2+ fiber photometry measurements of PVH and LSv neuron activity during stress and feeding. Sim1-Cre (a, c, e, and g) and Vgat-Cre male mice (b, d, f, and h) with AAV-FLEX-GCaMP6m delivery to the PVH (a, c, e, and g) or LSv (b, d, f, and h) were implanted with optic fibers targeting the PVH or LSv for fiber photometry monitoring the in vivo activity of PVH and LSv neurons in freely moving mice. A loud sound (a and b), a brief light exposure in dark (c and d), water spray toward head (e and f) were associated with activation of PVH (a, c, e, and g) and LSv (b, d, f, and h).



Finally, we refined the GCaMP6-mediated monitoring of LSv-projecting PVH neurons by using retrograde tracers, as described in section 3.3.2. The retrograde AAV bearing a plasmid encoding cre, injected into the LSv with simultaneous injection of FLEX-GCaMP6 into the PVH and placement of a fiber over this area, allows highly specific monitoring of the $PVH^{LSv\text{-}projecting}$ neurons. This injection strategy can be seen in **Figure 24C,D**. In this setup, the $PVH^{LSv\text{-}GCaMp6}$ neurons showed a similar set of responses as seen in **Figure 26**, though the amplitude of response was less. Because of the numerous differences between experimental conditions, it would be inappropriate to draw conclusions about this difference in amplitude. However, what is clear is that the $PVH^{LSv\text{-}projecting}$ neuronal activity is tightly correlated with anxiogenic environmental cues as seen in **Figure 27**. This indicates the importance of this circuit for environmental monitoring. Notably, in the same conditions, mice with PVH infection of FLEX-GFP instead of FLEX-GCaMP6 showed no response to either water-spray or feeding bout

Figure 27. LSv-projecting PVH^{GCaMP6} neurons are activated by anxiogenic cues and inhibited by feeding

(a-f) In vivo Ca2+ fiber photometry measurements of LSv-projecting PVHGCaMP6 and PVHGFP neurons during stress and feeding. (a-b) Comparison of amplitude of response to water spray stimulus as described in section 2.2.6.3. in the (a) PVHGCaMP6 and (b) PVHGFP animals. Grey traces indicate individual recordings and red represents the averaged response (also for e). (c-d) Comparison of amplitude of response to initiation of feeding bout in mice fasted for 12hrs, comparing the (c) PVH^{GCaMP6} condition and (d) PVH^{GFP} condition. Grey is the Ca2+ independent signal, and red is the dependent (e) Demonstration of PVHGCaMP6 neurons responding to loud sound stimulus. (f) Comparison of the average %dF/F0 observed during a feeding bout minus overall %dF/F0 in the PVHGCaMP6 and PVHGFP conditions (n=20 per group, p<0.001)


a
GCaMP6m
Water spray
% dF/F0
8
4
0
−4
b
GFP
Water spray
% dF/F0
c
GCaMP6m
Eating bout
% dF/F0
d
GFP
Eating bout
% dF/F0
e
GCaMP6m
Loud sound
% dF/F0
f
GFP
GCaMP6m
% dF/F0
0
−1
−2
−3
***

3.4. Conclusions and Significance

In this study, we used the techniques refined in Chapter 2 to approach a previously untested circuit projecting from the PVH to the LSv. What we found was that activation of this circuit induced self-grooming and, at high intensities, fleeing behavior. Further, activation prevented feeding in the fasted condition and the activation of the circuit was aversive. Future work will need to demonstrate whether activation induces conditioned place aversion. Notably, activation of a PVH^{Sim1}-VTA neurocircuit studied in the Tong lab does produce conditioned place aversion [385]. My work demonstrated that the PVH and LSv both respond, in real-time, to a wide variety of anxiogenic environmental cues and are suppressed by feeding behavior. Overall, this work represents a compelling parallel and inversely functional circuit to the one I presented in Chapter 2 and provides new evidence emphasizing the centrality of the hypothalamus in driving basal forebrain and septal nuclei activity. This ties in quite nicely with data from our research showing that LH^{GABA} neurons inhibit PVH^{Glut} neurons to cause feeding [286,294], and converse evidence that the LSv^{GABA} neurons inhibit LH^{GABA} neurons during feeding [386].

Notably, previous work from our own lab and others has not been able to elicit such pronounced fleeing behavior by stimulation of PVH^{Glut} projections to other regions, such as the VTA [385], nor PVH^{PDYN} projections to the parabrachial nucleus or pre-locus coeruleus [387]. Finally, the fact that the intensity of circuit stimulation determined the progression from no response to self-grooming to jumping all show that the LSv is, at baseline, 'preventing' these behaviors from emerging, until it is activated.

This adds a unique piece of data to the concept suggested by Dr. Jeffrey Gray and Neil McNaughton in their magnum opus, *The neuropsychology of anxiety: an enquiry into the functions of the septo-hippocampal system*. In it, they claim that the septal nuclei act as the filter of incoming alertness-inducing stimuli. During a period of behavioral inhibition, these stimuli activate the PVH and the cortical sensory regions, but do not 'break through' to the septal nuclei and their hippocampal targets until sufficient ascending signal strength is attained [377]. This then causes the activation of hippocampally-encoded memory-related responses to these alertness-inducing stimuli [310]. Optogenetic activation of the presynaptic PVH neurons is sufficient to break through the filter, hence induce self-grooming and fleeing behavior.

Overall, both from the perspective of improving our understanding of the basic neurobiology of anxiety and fight-or-flight responses and from the perspective of understanding human disease anew, this work is an important advance in the field. As detailed above, the circuit has never before been functionally described and the detailed analysis will allow further circuit mapping in the future.

Chapter 4. Hypothalamic neurocircuits reciprocally controlling eating by modulation of environmentally-driven anxiety

This chapter represents original review and concluding remarks produced for this dissertation

4.1. Eating, hunger, anxiety, and satiety: what do these words even mean? The need for complexity.

Much of what is described in Chapters 2 and 3 was not possible to this degree of resolution until the 21st century. The confluence of rapid advances in genomics, gene editing, and protein engineering is responsible for the development of the tools described above (Cre-loxp, viral vectors, ChR2, GCaMP6, etc). The primary benefit is the cellular and molecular specificity *in vivo* that has heretofore been impossible; such specificity in fiber photometry and calcium imaging are akin to the advances in patch-clamp electrophysiology in the 1980s. However, this is in no way a negation of the complex and rigorous neuroanatomical and behavioral work done prior to these techniques. Limitation is substrate of creativity and often spurs lateral thinking and paradigm-shifting conclusions. In my limited experience, perhaps because of the highly technical nature of these advances requiring mastery of many diverse domains of science, there has been some neglect in recent high-impact publications in neuroscience of the more complex ideas developed in the 20th century, especially about behavior. Further, although these techniques afford incredibly useful insights into cellular and molecular characteristics of neurocircuits, there is a lack of subtlety in the interpretation of the output behavior.

This is particularly apparent in my own field of the neuroscience of eating behavior. The words *hunger* and *satiety* are easy to understand, but are extraordinarily broad. Hunger is generally used to refer to the fact that, when given access to food, the

animal eats the food. Satiety means that, when given access to the food, the animal does not eat the food. As pure behavioral descriptions, that is sufficient, but from the perspective of future translation of this information, they lack nuance. Further, emphasis on the elicited behavior alone misses many more subtle findings that challenge the participation of a particular nucleus in hunger or satiety.

4.2. Insights from the decerebrate rat on the challenges of interpreting starvation, obesity, and simplified measures of hunger and satiety

Some examples of why these distinctions are important arise from experiments with decerebrate animals, where the telencephalon and diencephalon have been disconnected from the brainstem. This results in functional decerebration, where all observed behavior is generated by the brainstem. Much of this work was performed by Harvey Grill, Ralph Norgren, and others in the late 1970s and early 1980s. The technique to produce a decerebrate rat is fairly simple though mechanically challenging to do safely: after anesthesia, a spatula is inserted just rostral to the superior colliculi and pushed until contact with the cranial floor is made, and sweeped left. A week later, the same is done to the right; this staging helps prevent destruction of the superior sagittal sinus and cranial nerves [388]. Completeness of transection was confirmed post mortem This results in complete disconnection of the cortex, thalamus, and hypothalamus from the body. Although such drastic surgery may seem more at home in *The Island of Dr. Moreau* by H.G. Wells than a laboratory, it offered the following valuable observations.

4.2.1. Aphagic and adipsic, but mobile with coordinated reactions to stimuli

As one may expect, decerebrate rats do not eat or drink and if left alone would probably starve to death [389,390]. They were sustained by tube feedings. They also do not have effective thermoregulation. The *prime facie* conclusion is that such extensive brain damage prevents coordination of any sort of behavior. However, surprisingly, these animals maintain a normal standing posture, groom spontaneously and correctly, and have an intact acoustic startle reflex with pre-pulse inhibition [391]. While they don't move spontaneously, they right themselves if turned over, squeak and run away if their paw is pin-pricked, vigorously bite the pincher if their paws are pinched, and will jump out of a cage and flee (with coordinated running) if their tail is pinched. [392,393] These responses were all very exaggerated in intensity compared to controls.

4.2.2. Taste discrimination, hunger, and satiety without taste association

Intact rats have facial responses indicating "liking" and "disliking" food when sucrose solution or sodium chloride *versus* quinine is placed on the tongue; further, they will ingest and consume food or reject it by spitting it out [389]. This suite of behaviors is collectively referred to as *taste reactivity*. Intraperitoneal injection of lithium chloride (LiCl) causes a nausea response; pretreatment with LiCl before administering sucrose causes the rat to reject sucrose taste on the next test, presumably because of the nausea association. This suite of behaviors is referred to as *bait shyness*. Decerebrate rats have the same taste reactivity as intact rats, but are not capable of developing bait shyness (i.e. LiCl pairing did not affect subsequent feeding) [388]. This is in keeping with the absence of

other sorts of classical conditioning such as to conditioned eye blink in the decerebrates. In the same study, the experimenters pretreated intact and decerebrate rats with a tube feeding. One hour later, administered sucrose solution every 5 seconds and monitored taste reactivity, with 30 second breaks. This was compared to the 24hr and 48hr fasted response. All fed rats initially accepted the solution, but eventually became satiated and rejected further solution. In the fasted condition, they accepted far more volume of solution before becoming satiated and rejecting. Increased sucrose concentration increased the amount of solution accepted in both conditions.

Other work by Grill demonstrated that decerebrate rats also show normal sympathetic control of gluconeogenesis. They injected 2-deoxy-D-glucose (2DG), a competitive hexo/glucokinase inhibitor that mimics acute hypoglycemia, intraperitoneally and thus elicited hyperglycemia in both decerebrates and controls (whereas saline did not); however, the amplitude of response appeared to be slightly lower (average jump of ~100mg/dL compared to ~150mg/dL) [394]. There is some possibility, of course, that the disconnected hypothalamus may still operate autonomously in response in changes to the blood detected via the median eminence, and cause sympathetic response purely via glucocorticoids.

4.2.3. An intact thalamus and hypothalamus restores voluntary locomotion but disrupts normal grooming and makes all tastes aversive

Comparison to animals with a preserved thalamus is even more striking. The surgery is more technical, requiring staged vacuum aspiration of the cerebral cortex,

striatum, basal forebrain (stopping at the preoptic area), septum, and hippocampus. Notably, the hypothalamus remains intact as well, but the shorthand nomenclature used was a *thalamic preparation* and *thalamic rats* [390].

Thalamic rats are still aphagic and adipsic and lack thermoregulation. They had most of the capabilities of the decerebrate rat. In addition, they spontaneously locomoted; however when moving they held their head low, back arched, and 'tip-toed' around. They had periods of quiescence and hyperactivity but overall were far more active than control rats. Surprisingly, their grooming behavior was grossly impaired compared to the decerebrate rat; their limbs would not make contact with the fur and over time they became disheveled. Perhaps even more surprisingly, the thalamic rats responded to all tastes as if they were quinine. The duration of the rejection response increased as the concentration of all solutions increased. They rejected all fluid, including water, thus making it impossible to test satiety.

4.2.4. Decerebrate and thalamic rat demonstrate the need to emphasize acute, subtle behaviors, rather than only chronic state changes

The above information is surprising. Decerebrate rats do not engage in any goal-oriented behaviors, but successfully engage in "housekeeping" ones (e.g. grooming, attacking a threat, taste discrimination, differentiating hunger and satiety by rejecting excess sucrose in the fed condition). Their responses are reflexive and intense. Thalamic rats show some goal-oriented behavior (e.g. spontaneous exploration), but have completely dysregulated housekeeping behavior and fail to successfully take in food. In

both cases, careful investigation showed results opposite of what would be expected. Although both rats would normally starve to death, the decerebrate rats (no hypothalamus) show hunger, whereas the thalamic rats show an excess of satiety). The conclusion varies wildly dependent on the level of readout.

Just as starvation here was not fully informative of whether the animal had a deficit in energy intake due to excess satiety or lack of hunger, obesity is also not necessarily informative of whether the animal has an excess of hunger or lack of satiety. Work in the Tong lab has previously demonstrated that knockout of beta-receptor on hematopoietic cells (primarily because of the loss of beta-receptor on the red blood cells) is sufficient to enhance high-fat diet-induced weight gain over several weeks, despite the fact that these mice consumed less food than controls. Sympathetic activation of the beta receptors induced increased glucose consumption by the red blood cells, increasing the animals' total daily energy expenditure [395].

4.2.5. Hypothalamic nuclei are primarily involved in dis/inhibiting other brain regions to release specific behaviors encoded by more primitive brain regions

As put by Garrett Stuber and Mark Rossi in the title of their 2018 review paper, all of the above evidence really means is that there are *Overlapping Brain Circuits for Homeostatic and Hedonic Feeding* [276]. Put another way, the fact that a given node elicits feeding is probably more representative of the fact that it is generally disinhibiting or activating the eating network subsequent to inhibition of some *other* network, and ultimately behavior. This may explain why the decerebrate rats were capable of

expressing hunger and satiety, whereas the thalamic rats were not; the former only retained the brainstem circuits that directly control the spinal cord to evoke many of these responses. The thalamic rats, by comparison, had several intact anxiety networks (such as the PVH connections to the VTA and PAG), but did not have intact appetitive networks (such as the VTA to nucleus accumbens - the latter was removed with the striatum). This produced a profound imbalance, where all taste-related sensory information produced the aversive response since it did not have a reciprocal, balanced appetitive network to inhibit it.

This model, which views hypothalamic nuclei as being involved in generating or inhibiting other areas to allow the release of particular behaviors, is akin to Jaak Panksepp's concept of "affective states" and affective state competition that he has reviewed extensively in many works 405–407. The utility of this model is it helps provide much more nuance and meaning to the vast array of behaviors elicited by optogenetics and observed by fiber photometry, and it calls for greater emphasis on the subtle differences in behavior observed during a given experiment, rather than a focus on chronic effects. Any given brain region has a unique set of inputs and outputs - however, there are common networks between them that ultimately allow release of a brainstem behavior. The medial forebrain bundle is a good example of this, as it clearly connects the disparate subcortical brain regions involved in what Panksepp would call the affective state of "seeking" i.e. willingness to explore and consume previously unknown substances and learn their properties. The VTA seems to be primarily responsible for the locomotive

and learning aspects of the "seeking" affective state; the lateral hypothalamus seems to be responsible for the consummatory aspects of the "seeking" affective state [304,306,396].

4.3. A proposed wiring diagram: LH GABA neurons inhibit multiple nodes in the PVH anxiety network to promote consumption

Obviously, the "seeking" affective state described above is a high level abstraction. Likely, such affective states and their underlying networks are determined by much more than just physical location (e.g. molecular characteristics and firing frequencies). Further, because of the current lack of nuance involved in distinguishing behaviors elicited by specific projections, so far only a small number of distinct affective states have been elucidated. For example, many researchers consolidate brain states to what are easily called appetition and aversion, formulated by William Craig over 100 years ago [397]. When these behaviors are not-goal directed, they have default outputs that can, for simplicity's sake, be called consumption and anxiety. Yet, this makes understanding the wiring diagrams of several nuclei more intelligible - rather than any projection mediating a necessary part of the seeking response itself, for example, it may instead be dampening or enhancing the activity of another structure.

It is in this framework that I have integrated the data presented here with other neurocircuit research into **Figure 28** [286,311,373,386,398–401]. What this demonstrates is that the LH^{GABA} and PVH^{Glut} nodes are are pretty clearly parallel to each other and drive opposite behaviors (consumption and anxiety). Whereas, the basal forebrain seems to be responsible for driving environment specific behaviors. This is in keeping with my findings

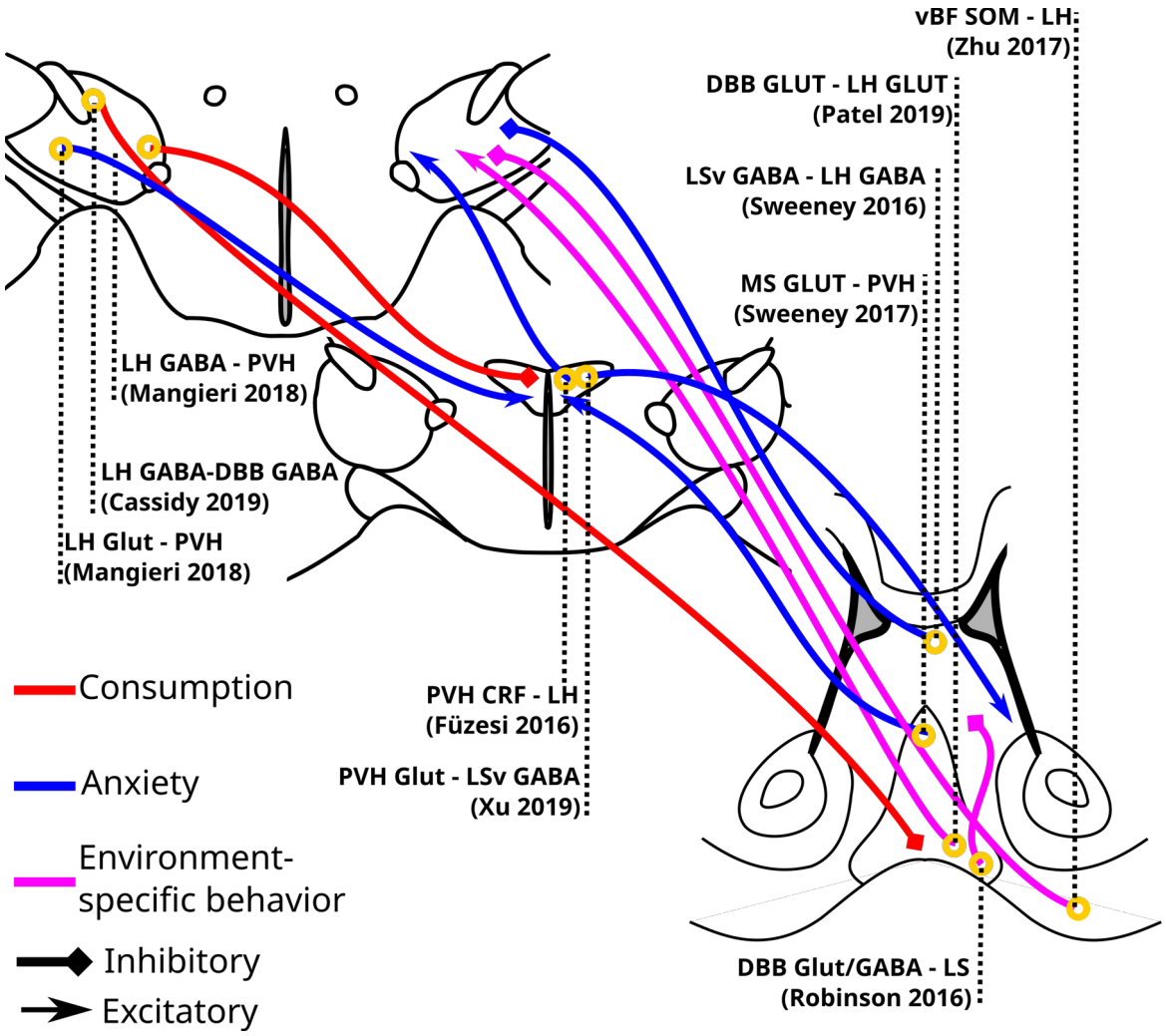
in Chapter 2 - the LH actively suppresses environment-driven responses in order to allow feeding to occur. Conversely, as in Chapter 3 activation of the PVH-LSv circuit causes non-specific fleeing behavior, regardless of the environment (e.g. intruder assay).

There is, of course, an incredible amount of complexity that is not conveyed in this wiring diagram - it is not meant to be exhaustive, but instead demonstrate the "competing states" theory. The LH GABA neurons are the only projections within this network that drive non-specific feeding, and the PVH projections conversely only drive non-specific anxiety. Although the medial and lateral septum projections to the LH were demonstrated to prevent feeding behavior [386,398] , unfortunately those publications did not evaluate any other behavior for the specific projection, even though it appears the diagonal band and septal nuclei are particularly context dependent in their control of behavior.

Figure 28 also emphasizes the basal forebrain's role as a "selector" of environment specific responses once either the consumption or anxiety response has been generated, via mechanisms *beyond* its classical interaction with the hippocampus [313]. For example, activation of the ventral-basal forebrain somatostatin projections to the LH causes specific consumption of fat, but not sucrose [372]. Activation of the DBB glutamatergic projections to the LH causes selective avoidance of food stimuli, but *not* general anxiety [373]. Perhaps most interestingly, activation of the local MS-DBB glutamatergic projections to the GABAergic septal neurons does not produce any behaviors (at least that have been published), but enhanced the signal strength of locomotion-induced theta rhythms in the

hippocampus [311]. In other tests, the septum-driven hippocampal theta rhythm is associated with multiple types of anxiety-related behaviors; further, there is a hippocampus glutamate -> LH glutamate circuit that also causes non-specific anxiety [402,403]. These observations add another network loop to the interrelationship between eating, anxiety, and responsiveness to the environment. Further, it should also be clear that there is great potential for the LH, which is activated by the mere act of eating food [296,309,400,404], to inhibit anxiety driven by environmental cues (encoded by the basal forebrain-septal nuclei - hippocampus loop) .

Figure 28. A network diagram of competing consumption and anxiety circuits within the LH, PVH, and basal forebrain



4.4. Summary and limitations

This dissertation began with describing the sobering reality that there has been unchecked explosion in metabesity in the United States. It ends with a wiring diagram relating neurocircuits inducing non-specific consumption and non-specific anxiety to those that drive context-specific behaviors. What is the connection? It appears to me the common view in the medical field that the reason people develop metabesity arises from one of three factors:

1. Completely external loci of control unrelated to behavior: genetics, reduced basal energy expenditure, specific disruption of peripheral homeostatic mechanisms
2. Impaired ratio of sensitivity to peripheral hunger signals (e.g. ghrelin) to satiety signals (leptin) drives irresistible eating
3. Dopaminergic-opioid (i.e. rewarding) drives eating of food in a non-hunger circumstance.

Certainly, there is robust and valuable evidence that warrants this focus. In mice, altering any of these knobs produces profound changes in hunger, satiety, obesity, and starvation. In humans, manipulating these variables also has acute effects. Yet, they rarely have lasting success. I believe this is not because these mechanisms are irrelevant in humans (clearly they are, as a few individuals do have great response), but because any success in one area is overridden by failure to treat another underlying driver of eating.

Put another way, manipulation of these mechanisms is ***necessary***, but not ***sufficient*** to reliably produce sustained weight loss.

In this work, I have presented two circuits showing reciprocity between systems that suppress eating (PVH glutamate) and activate with eating (LH GABA). They inhibit each other. These are part of broader networks that produce distinct affective states (here referred to as non-specific anxiety and non-specific consumption). Combined with the fact that the LH^{GABA} consummatory network is activated by food consumption, rather than hunger *per se*, it appears that this circuit may be intentionally exploitable. This exploitation manifests as 'stress eating', i.e. eating when one is not hungry nor particularly desirous of specific foods in order to reduce the sensation of stress/anxiety originating from these other networks. This is similar to self-medication with dopaminergic drugs observed clinically in depressed individuals.

4.4.1. Limitations

It is crucial to discuss the limitations of the techniques used in this study. First, many of the techniques utilized, though powerful, have substantial drawbacks. The genetically modified mice are an inbred strain, and although widely used within the fields of optogenetics and calcium imaging, may have substantial differences in their baseline behavior and response to manipulations compared even to other mice. It is increasingly understood that these inter-strain differences are particularly pronounced in behavior [338,350,405,406]. Thus, this caveat must be kept in mind while interpreting the translatability of any of these findings.

Second, all of the animal models used relied upon neurosurgically delivered viral vectors to deliver the gene encoding Cre-dependent ChR2, GCaMP6, etc. to the region of interest. All of the Cre lines used, except Sim1-Cre in Chapter 3, had Cre expression in off-target areas immediately adjacent to the region of interest (Vgat-Cre in the LH and DMH and zona incerta, Pdx1-Cre in the LH and DMH, Vglut2-Cre in the PVH and VMH, etc.). Although injection sites were verified, and in my subjective observation the few 'misses' that were excluded did not appear to show any photostimulation-related behavior, it is still very important to consider that these adjacent regions also may contribute to these findings. Unfortunately, as discussed in **Section 2.1.**, the hypothalamus has extraordinarily high cell-type heterogeneity. It appears that the basic developmental and neuroanatomical principles responsible for coordinating these regions' circuit participation requires much more research before such targeting techniques can be used. Although monosynaptic retrograde infection of presynaptic partners, as seen in the retro-AAV2 tracing experiment (**Figure 8**), can help increase the presynaptic specification to some degree, the technology still needs some improvement in order to allow full molecular specification both pre- and postsynaptically.

It is likely that the next few years will show extremely clever combination of Cre-*loxp* and Flp-*frt* (a system similar to Cre-loxp but with no crossover) systems to overcome these issues [407]. For example, in a Vgat-Flp animal, the postsynaptic region can be infected with retroAAV2-Cre, and the presynaptic region with DIO(Cre)-DIO(Flp)-ChR2. This plasmid requires both Cre excision AND Flp excision before it will be expressed, thus allowing only

presynaptic GABAergic neurons to be activated. Another example of use of this dual recombinase system is cell-fate mapping, such as examining a cross between ChAT-Cre and Vglut2-Flp in a mouse with Flp-dependent and Cre-dependent reporters [408].

Third, the natural limitation of any behavioral neuroscience endeavor is the difficulty in assessing the internal experience of the mouse. Mice cannot verbally communicate their experience, which limits correlation with human experience. Thus, there inherently is a substantial risk for either over-interpretation of the behaviors seen in a form of anthropomorphization, or an insufficient degree of attention paid to animal behavior, losing important subtle differences that would be easy to see in a human. In psychology, this problem is often discussed as the 'face validity' of the questions asked in a test to the actual experience they are attempting to capture. An excellent review of the challenges of face validity in rodent models of human psychiatric diseases can be found here [409].

My work described above certainly suffers from these limitations and the question of face validity. It is not possible yet to be certain how translatable these findings are to humans. Future work with humans in ethologically relevant conditions is necessary before conclusions on the interrelationship between anxiety and hunger as humans experience and express these affects. The next two sections will discus what data does exist with regards to the translation of rodent behavioral findings to human behavioral findings.

4.4.2. The evidence supporting translation of rodent behavior to human behavior

It is worth asking directly: is it valid to compare human and rodent behavior at all? Is it reasonable to infer internal experience from external behavior? On these points, I agree with Panksepp's discussion of this problem and his conclusion that when human and mice exhibit similar stereotyped mammalian behaviors (e.g. facial expressions in response to pain, eating, etc.), their internal experience, variously described as affect, emotion, or consciousness is also similar [410,411]. This conclusion is derived from several observations. The subcortical neuroanatomical organization of the mouse brain and human brain are similar, both in structure and in gene expression patterns during development – though obviously this is a generalization and a few subcortical areas are organized differently [412,413]. Further, electrode stimulation of mouse and human subcortical regions like the median forebrain bundle, as demonstrated by intracranial self-stimulation (ICSS) studies performed in the 1950s and 60s, produce similar reward-seeking behaviors [414–416]. Finally, the primary difference between human and mouse neuroanatomy arises from the expansion of the neocortex; damage to neocortical tissue in humans does not appear to alter the quality of affective experiences, but instead reduces the ability to regulate their expression. Evidence from patients with traumatic brain injury to the frontal lobe and frontotemporal dementia are demonstrative of this point [417–420]. Thus, it is reasonable to assume that manipulation of mouse subcortical tissue produces affective states similar to those experienced by humans. The translation

becomes more difficult once regions associated with cognition, like the prefrontal cortex and frontal cortex, are involved.

All of this being said, the analysis of mouse behavior and correlation of that to human experience still remains quite limited. One problem is the relative paucity of human-to-mouse translatable behavioral tests; because of the complex and rich information that is possible to derive from verbal report, it appears few studies observe human behavior absent communication.

4.4.3. Recent human experiments that replicate rodent behavior and a call for further validation and 'human-to-rodent' translation

There are several recent high quality and highly informative studies that have attempted to replicate rodent studies of affective states in humans directly [421]. One such study examined human exploratory behavior by tracking subjects with a GPS as they explored a central city area (a sort of natural open field test with clear boundaries and open areas) [422]. Four groups were tested in two comparisons: subjects diagnosed with agoraphobia compared to healthy controls; and subjects with no previous diagnosis who scored as high anxiety sensitivity versus low anxiety sensitivity on a validated scale for anxiety. The agoraphobic subjects and high anxiety sensitivity groups both showed a significantly reduced level of exploration of open areas compared to the controls. This correlates nicely with the open field test in rodent research, where a greater degree of avoidance of the center is described as increased levels of anxiety.

In another experiment, the influence of anxiety and pharmacological manipulation on open-arm exploration of an elevated plus maze (EPM) was tested [423]. Facilitated by the recent development of immersive virtual reality technology, humans evaluated to have high basal anxiety showed reduced exploration of the open arm of the EPM. The high anxiety humans, as compared to the low anxiety humans, also had higher peak concentrations of salivary alpha-amylase and salivary cortisol as well as increased heart rate, respiratory rate, and skin conductance in the immediate post-test period. Administration of lorazepam (a benzodiazepine anxiolytic) in a placebo controlled, randomized and double-blinded study significantly 1) reduced the latency to first open arm visit and 2) increased the amount of time spent on the open arm of the EPM as compared to placebo. By comparison, administration of yohimbe, a sympathomimetic anxiogenic drug had the exact opposite effects. The self-reported levels of anxiety were also reduced or enhanced by these drugs, respectively. The findings of this study correlate well with the assumptions made about the meanings of open-arm avoidance in the EPM in rodent behavioral neuroscience.

Behavioral observations of human infants also offer some insights into the basic nature of hunger and taste preference in humans, while avoiding the complexity associated with verbal report. When presented with sweet (sucrose) and bitter (quinine) solutions, they produce similar facial expression for liking and aversion as the decerebrated rats [424]. Further, several food neophobia (fear of new food) scales have been developed and tested in humans during food choice assays [425]. Although evidence is

limited, it appears that pathological manifestations of food neophobia – selective eating in children significant enough to lead to malnutrition – correlate with generalized anxiety [375,426]. This has some adjacency to the data discussed here, as latency to initiation of feeding behavior has been used as the metric of rodent neophobia and by proxy, a measure of general anxiety [369].

It appears promising that many of the findings in rodent behavioral neuroscience may translate to humans, and vice versa. Particularly with recent technological advancements in virtual reality and human behavior tracking, a widely expanded psychoactive pharmacological armamentarium, and non-invasive neurostimulation, it is becoming increasingly feasible to directly probe human neurocircuits and their behavioral outputs in tightly-controlled experiments. However, most of the behavioral tests performed in Chapters 2 and 3 have no validation in humans yet. Thus, the translation of their findings is limited. However, there is great opportunity to work on the interrelationship of human and rodent behavior and subsequently translate rodent findings with complex manipulations as described here, to human behavior and human disease.

4.5. Conclusion

The goal of this work has been to bring new appreciation to the role of the hypothalamus in mediating the interaction between affective states, with special reference to how hunger and its associated eating behavior may suppress negative affects like anxiety, and vice versa. Further, it has aimed to pose the hypothesis that

intentional manipulation of these eating neurocircuits to suppress anxiety is a potential driver of the epidemic of metabesity, similar in nature to how intentional manipulation of the endogenous reward neurocircuitry by use of substances of abuse was found to explain many aspects of addictions.

It is my hope that future work will elaborate on the LH-DBB and PVH-LS with greater nuance and more complex tests of behavior. There is now abundant technology, improving every year in specificity and sensitivity, to tackle challenging questions about the neurocircuits undergirding affective states and their interaction with each other. Similarly, it is now possible to perform similar experiments validating these behavioral findings in humans. My prediction is that eating will prove to be a potent suppressor of negative affective states like anxiety and stress both in rodents and in humans and that this effect relies upon hypothalamic neurocircuits, including the LH-DBB. Further, I predict that a behavioral and pharmacological treatment regimen directly targeting daily stress and anxiety, combined with existing dietary strategies and satiety pharmacology discussed in Chapter 1, will prove to be effective in treating metabesity.

Bibliography

1. Banting W. Letter on corpulence, addressed to the public. 1869. *Obes Res*. 1993;1(2):153-163. doi:10.1002/j.1550-8528.1993.tb00605.x.

2. Sacks FM, Bray GA, Carey VJ, Smith SR, Ryan DH, Anton SD, McManus K, Champagne CM, Bishop LM, Laranjo N, Leboff MS, Rood JC, Jonge L de, Greenway FL, Loria CM, Obarzanek E, Williamson DA. Comparison of Weight-Loss Diets with Different Compositions of Fat, Protein, and Carbohydrates. *N Engl J Med*. 2009;360(9):859-873.

3. Resources for Weight and Obesity - National Library of Agriculture. United States Department of Agriculture.

4. Nestle M, Jacobson MF. Halting the obesity epidemic: A public policy approach. *Public Health Rep*. 2000;115(1):12-24.

5. Bartrina JA. Public health and the prevention of obesity: Failure or success? *Nutr Hosp*. 2013;28(5):128-137.

6. Garrison LP, Carlson RJ, Carlson JJ, Kuszler PC, Meckley LM, Veenstra DL. A review of public policy issues in promoting the development and commercialization of pharmacogenomic applications: Challenges and implications. *Drug Metab Rev*. 2008;40(2):377-401. doi:10.1080/03602530801952500.

7. Anand P, Gray A. Obesity as market failure: Could a “deliberative economy” overcome the problems of paternalism? *Kyklos*. 2009;62(2):182-190. doi:10.1111/j.1467-6435.2009.00430.x.

8. Salas XR. The ineffectiveness and unintended consequences of the public health war on obesity. *Can J Public Heal*. 2015;106(2):e79-e81. doi:10.17269/CJPH.106.4757.

9. Bombak A. Obesity, health at every size, and public health policy. *Am J Public Health*. 2014;104(2):60-67. doi:10.2105/AJPH.2013.301486.

10. Gregg EW, Cadwell BL, Cheng YJ, Cowie CC, Williams DE, Geiss L, Engelgau MM, Vinicor F. Trends in the prevalence and ratio of diagnosed to undiagnosed diabetes according to obesity levels in the U.S. *Diabetes Care*. 2004;27(12):2806-2812. doi:10.2337/diacare.27.12.2806.

11. Flegal KM. Epidemiologic aspects of overweight and obesity in the United States. *Physiol Behav*. 2005;86(5):599-602. doi:10.1016/j.physbeh.2005.08.050.

12. Sempos C, Fulwood R, Haines C, Carroll M, Anda R, Williamson DF, Remington P, Cleeman J. The Prevalence of High Blood Cholesterol Levels Among Adults in the United States. *J Am Med Assoc*. 1989;262(1):45-52. doi:10.1001/jama.1989.03430010057031.

13. Burt VL, Cutler JA, Higgins M, Horan MJ, Labarthe D, Whelton P, Brown C, Roccella EJ. Trends in the prevalence, awareness, treatment, and control of hypertension in the adult us population: Data from the health examination surveys, 1960 to 1991. *Hypertension*. 1995;26(1):60-69. doi:10.1161/01.HYP.26.1.60.

14. Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of Obesity Among Adults and Youth: United States, 2015–2016. *NCHS Data Brief*. 2017;(288):2015-2016. https://www.cdc.gov/nchs/products/databriefs/db288.htm.

15. Gregg EW, Cheng YJ, Cadwell BL, Imperatore G, Williams DE, Flegal KM, Narayan KMV, Williamson DF. Secular trends in cardiovascular desease risk factors according to body mass index in US adults. *J Am Med Assoc*. 2005;293(15):1868-1874. doi:10.1001/jama.293.15.1868.

16. Eliasson B, Attvall S, Taskinen M, Smith U. The insulin resistance syndrome in smokers is related to smoking habits. *Arterioscler Thromb Vasc Biol*. 1994;14:1946-1950.

17. ATTVALL S, FOWELIN J, LAGER I, VON SCHENCK H, SMITH U. Smoking induces insulin resistance—a potential link with the insulin resistance syndrome. *J Intern Med*. 1993;233(4):327-332. doi:10.1111/j.1365-2796.1993.tb00680.x.

18. Cluette-Brown J, Mulligan J, Doyle K, Hagan S, Osmolski T, Hojnacki J. Oral nicotine induces an atherogenic lipoprotein profile. *Proc Soc Exp Biol Med*. 1986;182(3):409-413. doi:10.3181/00379727-182-3-RC1.

19. Benowitz NL. The role of nicotine in smoking-related cardiovascular disease. *Prev Med (Baltim)*. 1997;26(4):412-417. doi:10.1006/pmed.1997.0175.

20. Fuente-Martín E, Mellado-Gil JM, Cobo-Vuilleumier N, Martín-Montalvo A, Romero-Zerbo SY, Contreras ID, Hmadcha A, Soria B, Bermudo FM, Reyes JC, Bermúdez-Silva FJ, Lorenzo PI, Gauthier BR. Dissecting the brain/islet axis in metabesity. *Genes (Basel)*. 2019;10(5). doi:10.3390/genes10050350.

21. Irl B. H, Evert A, Fleming A, Gaudiani LM, Guggenmos KJ, Kaufer DI, McGill JB, Verderese CA, Martinez J. Culinary Medicine: Advancing a Framework for Healthier Eating to Improve Chronic Disease Management and Prevention. *Clin Ther*. 2019;41(10):2184-2198. doi:10.1016/j.clinthera.2019.08.009.

22. Li M, Kane J, Konu O. Nicotine, Body Weight and Potential Implications in the Treatment of Obesity. *Curr Top Med Chem*. 2005;3(8):899-919. doi:10.2174/1568026033452203.

23. Gurwitz D. The therapeutic potential of nicotine and nicotinic agonists for weight control. *Expert Opin Investig Drugs*. 1999;8(6):747-760. doi:10.1517/13543784.8.6.747.

24. Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of obesity among adults and youth: United States, 2011-2014. *Signif Heal Stat Sel Reports from Fed Agencies*. 2016; (219):91-101.

25. Pulgaron ER, Delamater AM. Obesity and type 2 diabetes in children: Epidemiology and treatment. *Curr Diab Rep*. 2014;14(8):1-21. doi:10.1007/s11892-014-0508-y.

26. de Vreede JJM, Gorgels APM, Verstraaten GMP, Vermeer F, Dassen WRM, Wellens HJJ. Did prognosis after acute myocardial infarction change during the past 30 years? A meta-analysis. *J Am Coll Cardiol*. 1991;18(3):698-706. doi:10.1016/0735-1097(91)90792-8.

27. Shih HP, Zhang X, Aronov AM. Drug discovery effectiveness from the standpoint of therapeutic mechanisms and indications. *Nat Rev Drug Discov*. 2018;17(1):19-33. doi:10.1038/nrd.2017.194.

28. Kesselheim AS. An empirical review of major legislation affecting drug development: past experiences, effects, and unintended consequences. *Milbank Q*. 2011;89(3):450-502. doi:10.1111/j.1468-0009.2010.00608.x.

29. Berndt ER, Aitken ML. Brand loyalty, generic entry and price competition in pharmaceuticals in the quarter century after the 1984 Waxman-Hatch legislation. *Int J Econ Bus*. 2011;18(2):177-201. doi:10.1080/13571516.2011.584423.

30. Lichtenberg FR. Benefits and Costs of Newer Drugs: An Update. *Manag Decis Econ*. 2007;28:485-490. doi:10.1002/mde.

31. Atella V, Piano Mortari A, Kopinska J, Belotti F, Lapi F, Cricelli C, Fontana L. Trends in age-related disease burden and healthcare utilization. *Aging Cell*. 2019;18(1):1-8. doi:10.1111/acel.12861.

32. Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, Hayflick L, Butler RN, Allison DB, Ludwig DS. A potential decline in life expectancy in the United States in the 21st century. *N Engl J Med*. 2005;352(11):1138-1145. doi:10.1056/NEJMsr043743.

33. Woolf SH, Schoomaker H. Life Expectancy and Mortality Rates in the United States, 1959-2017. *Jama*. 2019;322(20):1996. doi:10.1001/jama.2019.16932.

34. Murphy SL, Xu J, Kochanek KD, Arias E. Mortality in the United States, 2017. *NCHS Data Brief*. 2018.

35. Bacon L. End the war on obesity: Make peace with your patients. *MedGenMed Medscape Gen Med*. 2006;8(4):4-6.

36. McCracken E, Monaghan M, Sreenivasan S. Pathophysiology of the metabolic syndrome. *Clin Dermatol*. 2018;36(1):14-20. doi:10.1016/j.clindermatol.2017.09.004.

37. Sundström J, Risérus U, Byberg L, Zethelius B, Lithell H, Lind L. Clinical value of the metabolic syndrome for long term prediction of total and cardiovascular mortality: Prospective, population based cohort study. *Br Med J*. 2006;332(7546):878-881. doi:10.1136/bmj.38766.624097.1F.

38. Bremer AA, Mietus-Snyder M, Lustig RH. Toward a unifying hypothesis of metabolic syndrome. *Pediatrics*. 2012;129(3):557-570. doi:10.1542/peds.2011-2912.

39. Duclos M. Osteoarthritis, obesity and type 2 diabetes: The weight of waist circumference. *Ann Phys Rehabil Med*. 2016;59(3):157-160. doi:10.1016/j.rehab.2016.04.002.

40. Stefan N, Häring HU, Hu FB, Schulze MB. Metabolically healthy obesity: Epidemiology, mechanisms, and clinical implications. *Lancet Diabetes Endocrinol*. 2013;1(2):152-162. doi:10.1016/S2213-8587(13)70062-7.

41. Camhi SM, Must A, Gona PN, Hankinson A, Odegaard A, Reis J, Gunderson EP, Jacobs DR, Carnethon MR. Duration and stability of metabolically healthy obesity over 30 years. *Int J Obes*. 2019;43(9):1803-1810. doi:10.1038/s41366-018-0197-8.

42. Mongraw-Chaffin M, Foster MC, Anderson CAM, Burke GL, Haq N, Kalyani RR, Ouyang P, Sibley CT, Tracy R, Woodward M, Vaidya D. Metabolically Healthy Obesity, Transition to Metabolic Syndrome, and Cardiovascular Risk. *J Am Coll Cardiol*. 2018;71(17):1857-1865. doi:10.1016/j.jacc.2018.02.055.

43. Espinosa De Ycaza AE, Donegan D, Jensen MD. Long-term metabolic risk for the metabolically healthy overweight/obese phenotype. *Int J Obes*. 2018;42(3):302-309. doi:10.1038/ijo.2017.233.

44. Bradshaw PT, Monda KL, Stevens J. Metabolic syndrome in healthy obese, overweight, and normal weight individuals: The atherosclerosis risk in communities study. *Obesity*. 2013;21(1):203-209. doi:10.1038/oby.2012.173.

45. Bell JA, Hamer M, Sabia S, Singh-Manoux A, Batty GD, Kivimaki M. The natural course of healthy obesity over 20 years. *J Am Coll Cardiol*. 2015;65(1):101-102. doi:10.1016/j.jacc.2014.09.077.

46. Rey-López JP, De Rezende LF, De Sá TH, Stamatakis E. Is the Metabolically Healthy Obesity Phenotype an Irrelevant Artifact for Public Health? *Am J Epidemiol*. 2015;182(9):737-741. doi:10.1093/aje/kwv177.

47. Bays HE, Toth PP, Kris-Etherton PM, Abate N, Aronne LJ, Brown WV, Gonzalez-Campoy JM, Jones SR, Kumar R, La Forge R, Samuel VT. Obesity, adiposity, and dyslipidemia: A consensus statement from the National Lipid Association. *J Clin Lipidol*. 2013;7(4):304-383. doi:10.1016/j.jacl.2013.04.001.

48. Bays H, Ballantyne C. Adiposopathy: why do adiposity and obesity cause metabolic disease? *Future Lipidol*. 2006;1(4):389-420. doi:10.2217/17460875.1.4.389.

49. ANDERSSON BB, Wallin G, HEDNER T, Ahlberg A -C A-C, Andersson OK. Acute Effects of Short-term Fasting on Blood Pressure, Circulating Noradrenaline and Efferent Sympathetic Nerve Activity. *Acta Med Scand*. 1988;223(6):485-490. doi:10.1111/j.0954-6820.1988.tb17685.x.

50. Chan JL, Heist K, DePaoli AM, Veldhuis JD, Mantzoros CS. The role of falling leptin levels in the neuroendocrine and metabolic adaptation to short-term starvation in healthy men. *J Clin Invest*. 2003;111(9):1409-1421. doi:10.1172/JCI200317490.

51. Watts NB, Digirolamo M. Carbohydrate tolerance improves with fasting in obese subjects with noninsulin-dependent (Type II) diabetes. *Am J Med Sci*. 1990;299(4):250-256. doi:10.1097/00000441-199004000-00006.

52. Hall KD, Bemis T, Brychta R, Chen KY, Courville A, Crayner EJ, Goodwin S, Guo J, Howard L, Knuth ND, Miller B V., Prado CM, Siervo M, Skarulis MC, Walter M, Walter PJ, Yannai L. Calorie for calorie, dietary fat restriction results in more body fat loss than carbohydrate restriction in people with obesity. *Cell Metab*. 2015;22(3):427-436. doi:10.1016/j.cmet.2015.07.021.

53. Li C, Ostermann T, Hardt M, Lüdtke R, Broecker-Preuss M, Dobos G, Michalsen A. Metabolic and psychological response to 7-day fasting in obese patients with and without metabolic syndrome. *Forsch Komplementarmed*. 2013;20(6):413-420. doi:10.1159/000353672.

54. Stange R, Pflugbeil C, Michalsen A, Uehleke B. Therapeutic fasting in patients with metabolic syndrome and impaired insulin resistance. *Forsch Komplementarmed*. 2013;20(6):421-426. doi:10.1159/000357875.

55. Lingvay I, Guth E, Islam A, Livingston E. Rapid improvement in diabetes after gastric bypass surgery: Is it the diet or surgery? *Diabetes Care*. 2013;36(9):2741-2747. doi:10.2337/dc12-2316.

56. Goldhamer A, Lisle D, Parpia B, Anderson S V., Campbell TC. Medically supervised water-only fasting in the treatment of hypertension. *J Manipulative Physiol Ther*. 2001;24(5):335-339. doi:10.1067/mmt.2001.115263.

57. Goldhamer AC, Lisle DJ, Sultana P, Anderson S V., Parpia B, Hughes B, Campbell TC. Medically supervised water-only fasting in the treatment of borderline hypertension. *J Altern Complement Med*. 2002;8(5):643-650. doi:10.1089/107555302320825165.

58. Nicoll R, Henein MY. Caloric restriction and its effect on blood pressure, heart rate variability and arterial stiffness and dilatation: A review of the evidence. *Int J Mol Sci*. 2018;19(3). doi:10.3390/ijms19030751.

59. Michalsen A, Li C. Fasting therapy for treating and preventing disease - Current state of evidence. *Forsch Komplementarmed*. 2013;20(6):444-453. doi:10.1159/000357765.

60. Longo VD, Mattson MP. Fasting: Molecular mechanisms and clinical applications. *Cell Metab*. 2014;19(2):181-192. doi:10.1016/j.cmet.2013.12.008.

61. Steven S, Hollingsworth KG, Al-Mrabeh A, Avery L, Aribisala B, Caslake M, Taylor R. Very low-calorie diet and 6 months of weight stability in type 2 diabetes: Pathophysiological changes in responders and nonresponders. *Diabetes Care*. 2016;39(5):808-815. doi:10.2337/dc15-1942.

62. Liu B, Page AJ, Hatzinikolas G, Chen M, Wittert GA, Heilbronn LK. Intermittent fasting improves glucose tolerance and promotes adipose tissue remodeling in male mice fed a high-fat diet. *Endocrinology*. 2019;160(1):169-180. doi:10.1210/en.2018-00701.

63. Cahill GF. Fuel Metabolism in Starvation. *Annu Rev Nutr*. 2006;26(1):1-22. doi:10.1146/annurev.nutr.26.061505.111258.

64. Redman LM, Smith SR, Burton JH, Martin CK, Il'yasova D, Ravussin E. Metabolic Slowing and Reduced Oxidative Damage with Sustained Caloric Restriction Support the Rate of Living and Oxidative Damage Theories of Aging. *Cell Metab*. 2018;27(4):805-815.e4. doi:10.1016/j.cmet.2018.02.019.

65. Duška F, Tůma P, Mokrejš P, Kuběna A, Anděl M. Analysis of factors influencing nitrogen balance during acute starvation in obese subject with and without type 2 diabetes. *Clin Nutr*. 2007;26(5):552-558. doi:10.1016/j.clnu.2007.01.011.

66. Duška F, Anděl M, Kuběna A, Macdonald IA. Effects of acute starvation on insulin resistance in obese patients with and without type 2 diabetes mellitus. *Clin Nutr*. 2005;24(6):1056-1064. doi:10.1016/j.clnu.2005.08.008.

67. Schwingshackl L, Chaimani A, Schwedhelm C, Toledo E, Pünsch M, Hoffmann G, Boeing H. Comparative effects of different dietary approaches on blood pressure in hypertensive and pre-hypertensive patients: A systematic review and network meta-analysis. *Crit Rev Food Sci Nutr*. 2019;59(16):2674-2687. doi:10.1080/10408398.2018.1463967.

68. Zafar MI, Mills KE, Zheng J, Regmi A, Hu SQ, Gou L, Chen LL. Low-glycemic index diets as an intervention for diabetes: A systematic review and meta-analysis. *Am J Clin Nutr*. 2019;110(4):891-902. doi:10.1093/ajcn/nqz149.

69. Felber JP, Meyer HU, Curchod B, Maeder E, Pahud P, Jéquier E. Effect of a 3-day fast on glucose storage and oxidation in obese hyperinsulinemic diabetics. *Metabolism*. 1981;30(2):184-189. doi:10.1016/0026-0495(81)90170-0.

70. Arnett DK, Blumenthal RS, Albert MA, Buroker AB, Goldberger ZD, Hahn EJ, Himmelfarb CD, Khera A, Lloyd-Jones D, McEvoy JW, Michos ED, Miedema MD, Muñoz D, Smith SC, Virani SS, Williams KA, Yeboah J, Ziaeian B. 2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol*. 2019;74(10):e177-e232. doi:10.1016/j.jacc.2019.03.010.

71. Garber AJ, Abrahamson MJ, Barzilay JI, Blonde L, Bloomgarden ZT, Bush MA, Dagogo-Jack S, DeFronzo RA, Einhorn D, Fonseca VA, Garber JR, Garvey WT, Grunberger G, Handelsman Y, Hirsch IB, Jellinger PS, McGill JB, Mechanick JI, Rosenblit PD, Umpierrez GE. Consensus statement by the American association of clinical endocrinologists and American college of endocrinology on the comprehensive type 2 diabetes management algorithm – 2019 executive summary. *Endocr Pract*. 2019;25(1):69-100. doi:10.4158/CS-2018-0535.

72. Tricò D, Natali A, Arslanian S, Mari A, Ferrannini E. Identification, pathophysiology, and clinical implications of primary insulin hypersecretion in nondiabetic adults and adolescents. *JCI insight*. 2018;3(24). doi:10.1172/jci.insight.124912.

73. Page MM, Johnson JD. Mild Suppression of Hyperinsulinemia to Treat Obesity and Insulin Resistance. *Trends Endocrinol Metab*. 2018;29(6):389-399. doi:10.1016/j.tem.2018.03.018.

74. Williams KJ, Wu X. Imbalanced insulin action in chronic over nutrition: Clinical harm, molecular mechanisms, and a way forward. *Atherosclerosis*. 2016;247(May):225-282. doi:10.1016/j.atherosclerosis.2016.02.004.

75. Kopp W. Diet-Induced Hyperinsulinemia as a Key Factor in the Etiology of Both Benign Prostatic Hyperplasia and Essential Hypertension? *Nutr Metab Insights*. 2018;11:117863881877307. doi:10.1177/1178638818773072.

76. Srikanthan K, Feyh A, Visweshwar H, Shapiro JI, Sodhi K. Systematic review of metabolic syndrome biomarkers: A panel for early detection, management, and risk

stratification in the West Virginian population. *Int J Med Sci*. 2016;13(1):25-38. doi:10.7150/ijms.13800.

77. Kord-Varkaneh H, Fatahi S, Alizadeh S, Ghaedi E, Shab-Bidar S. Association of Serum Leptin with All-Cause and Disease Specific Mortality: A Meta-Analysis of Prospective Observational Studies. *Horm Metab Res*. 2018;50(7):509-520. doi:10.1055/a-0620-8671.

78. Frühbeck G, Catalán V, Rodríguez A, Gómez-Ambrosi J. Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. *Adipocyte*. 2018;7(1):57-62. doi:10.1080/21623945.2017.1402151.

79. Swainson MG, Batterham AM, Hind K. Age- and sex-specific reference intervals for visceral fat mass in adults. *Int J Obes*. 2020;44(2):289-296. doi:10.1038/s41366-019-0393-1.

80. White U, Ravussin E. Dynamics of adipose tissue turnover in human metabolic health and disease. *Diabetologia*. 2019;62(1):17-23. doi:10.1007/s00125-018-4732-x.

81. Ghaben AL, Scherer PE. Adipogenesis and metabolic health. *Nat Rev Mol Cell Biol*. 2019;20(4):242-258. doi:10.1038/s41580-018-0093-z.

82. Heinonen S, Jokinen R, Rissanen A, Pietiläinen KH. White adipose tissue mitochondrial metabolism in health and in obesity. *Obes Rev*. 2019;(August):1-23. doi:10.1111/obr.12958.

83. Musleh Ali ARH, Al-Kassas W, Husnain Haider K. Fatty Acid Escape Hypothesis: The Pathway to Type-2 Diabetes. *Diabetes Res – Open J*. 2019;5(1):8-17. doi:10.17140/droj-5-140.

84. Vishvanath L, Gupta RK. Contribution of adipogenesis to healthy adipose tissue expansion in obesity. *J Clin Invest*. 2019;129(10):4022-4031. doi:10.1172/JCI129191.

85. Hall JE, Do Carmo JM, Da Silva AA, Wang Z, Hall ME. Obesity-Induced Hypertension: Interaction of Neurohumoral and Renal Mechanisms. *Circ Res*. 2015;116(6):991-1006. doi:10.1161/CIRCRESAHA.116.305697.

86. Sigler MH. The mechanism of the natriuresis of fasting. *J Clin Invest*. 1975;55(2):377-387. doi:10.1172/JCI107941.

87. Di Pino A, DeFronzo RA. Insulin Resistance and Atherosclerosis: Implications for Insulin Sensitizing Agents. *Endocr Rev*. 2019;(May 2018):1447-1467. doi:10.1210/er.2018-00141.

88. Li C, Sadraie B, Steckhan N, Kessler C, Stange R, Jeitler M, Michalsen A. Effects of A One-week Fasting Therapy in Patients with Type-2 Diabetes Mellitus and Metabolic Syndrome - A Randomized Controlled Explorative Study. *Exp Clin Endocrinol Diabetes*. 2017;125(9):618-624. doi:10.1055/s-0043-101700.

89. Sanyal D, Raychaudhuri M. Hypothyroidism and obesity: An intriguing link. *Indian J Endocrinol Metab*. 2016;20(4):554-557. doi:10.4103/2230-8210.183454.

90. Franco MC, Antico Arciuch VG, Peralta JG, Galli S, Levisman D, López LM, Romorini L, Poderoso JJ, Carreras MC. Hypothyroid phenotype is contributed by mitochondrial complex I inactivation due to translocated neuronal nitric-oxide synthase. *J Biol Chem*. 2006;281(8):4779-4786. doi:10.1074/jbc.M512080200.

91. Vaidya B, Pearce SHS. Management of hypothyroidism in adults. *Bmj*. 2008;337(7664):284-289. doi:10.1136/bmj.a801.

92. Naviaux RK. Metabolic features and regulation of the healing cycle—A new model for chronic disease pathogenesis and treatment. *Mitochondrion*. 2019;46(August):278-297. doi:10.1016/j.mito.2018.08.001.

93. Révész D, Milaneschi Y, Verhoeven JE, Penninx BWJH. Telomere length as a marker of cellular aging is associated with prevalence and progression of metabolic syndrome. *J Clin Endocrinol Metab*. 2014;99(12):4607-4615. doi:10.1210/jc.2014-1851.

94. De Cabo R, Carmona-Gutierrez D, Bernier M, Hall MN, Madeo F. The search for antiaging interventions: From elixirs to fasting regimens. *Cell*. 2014;157(7):1515-1526. doi:10.1016/j.cell.2014.05.031.

95. Srikanthan P, Hevener AL, Karlamangla AS. Sarcopenia exacerbates obesity-associated insulin resistance and dysglycemia: Findings from the national health and nutrition examination survey III. *PLoS One*. 2010;5(5). doi:10.1371/journal.pone.0010805.

96. Evans W. Symposium: Sarcopenia: Diagnosis and Mechanisms Functional and Metabolic Consequences of Sarcopenia 1. *J Nutr*. 1997;127(5):998S–1003S. doi:doi.org/10.1093/jn/127.5.998S.

97. Kalinkovich A, Livshits G. Sarcopenic obesity or obese sarcopenia: A cross talk between age-associated adipose tissue and skeletal muscle inflammation as a main mechanism of the pathogenesis. *Ageing Res Rev*. 2017;35:200-221. doi:10.1016/j.arr.2016.09.008.

98. Kirwan JP, Solomon TPJ, Wojta DM, Staten MA, Holloszy JO. Effects of 7 days of exercise training on insulin sensitivity and responsiveness in type 2 diabetes mellitus. *Am J Physiol - Endocrinol Metab*. 2009;297(1). doi:10.1152/ajpendo.00210.2009.

99. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JRB, Newgard CB, Lopaschuk GD, Muoio DM. Mitochondrial Overload and Incomplete Fatty Acid Oxidation Contribute to Skeletal Muscle Insulin Resistance. *Cell Metab*. 2008;7(1):45-56. doi:10.1016/j.cmet.2007.10.013.

100. Ley SH, Hamdy O, Mohan V, Hu FB. Prevention and management of type 2 diabetes: Dietary components and nutritional strategies. *Lancet*. 2014;383(9933):1999-2007. doi:10.1016/S0140-6736(14)60613-9.

101. Gershuni VM, Yan SL, Medici V. Nutritional Ketosis for Weight Management and Reversal of Metabolic Syndrome. *Curr Nutr Rep*. 2018;7(3):97-106. doi:10.1007/s13668-018-0235-0.

102. Miller VJ, Villamena FA, Volek JS. Nutritional Ketosis and Mitohormesis: Potential Implications for Mitochondrial Function and Human Health. *J Nutr Metab*. 2018;2018:1-27. doi:10.1155/2018/5157645.

103. Urbain P, Strom L, Morawski L, Wehrle A, Deibert P, Bertz H. Impact of a 6-week non-energy-restricted ketogenic diet on physical fitness, body composition and biochemical parameters in healthy adults. *Nutr Metab*. 2017;14(1):1-11. doi:10.1186/s12986-017-0175-5.

104. Nymo S, Coutinho SR, Jørgensen J, Rehfeld JF, Truby H, Kulseng B, Martins C. Timeline of changes in appetite during weight loss with a ketogenic diet. *Int J Obes*. 2017;41(8):1224-1231. doi:10.1038/ijo.2017.96.

105. Longo VD, Panda S. Fasting, Circadian Rhythms, and Time-Restricted Feeding in Healthy Lifespan. *Cell Metab*. 2016;23(6):1048-1059. doi:10.1016/j.cmet.2016.06.001.

106. Gill S, Panda S. A Smartphone App Reveals Erratic Diurnal Eating Patterns in Humans that Can Be Modulated for Health Benefits. *Cell Metab*. 2015;22(5):789-798. doi:10.1016/j.cmet.2015.09.005.

107. Fujita N, Takei Y. Alcohol consumption and metabolic syndrome. *Hepatol Res*. 2011;41(4):287-295. doi:10.1111/j.1872-034X.2011.00787.x.

108. Koloverou E, Panagiotakos DB, Pitsavos C, Chrysohoou C, Georgousopoulou EN, Metaxa V, Stefanadis C, Skoumas Y, Katinioti N, Papadimitriou L, Masoura C, Vellas S, Lentzas SY, Kambaxis M, Palliou K, Metaxa V, Ntzouvani A, Mpougatsas D, Skourlis N, Papanikolaou C, Kouli GM, Christou A, Zana A, Ntertimani M, Kalogeropoulou A, Pitaraki E, Laskaris A, Hatzigeorgiou M, Grekas A, Vassiliadou C, Dedoussis G, Toutouza-Giotsa M, Tselika C, Poulopoulou S, Toutouza M. Effects of alcohol consumption and the metabolic syndrome on 10-year incidence of diabetes: The ATTICA study. *Diabetes Metab*. 2015;41(2):152-159. doi:10.1016/j.diabet.2014.06.003.

109. Fan AZ, Russell M, Dorn J, Freudenheim JL, Nochajski T, Hovey K, Trevisan M. Lifetime alcohol drinking pattern is related to the prevalence of metabolic syndrome. The Western New York Health Study (WNYHS). *Eur J Epidemiol*. 2006;21(2):129-138. doi:10.1007/s10654-005-5457-y.

110. Clerc O, Nanchen D, Cornuz J, Marques-Vidal P, Gmel G, Daeppen JB, Paccaud F, Mooser V, Waeber G, Vollenweider P, Rodondi N. Alcohol drinking, the metabolic syndrome and diabetes in a population with high mean alcohol consumption. *Diabet Med*. 2010;27(11):1241-1249. doi:10.1111/j.1464-5491.2010.03094.x.

111. Rodríguez-Monforte M, Sánchez E, Barrio F, Costa B, Flores-Mateo G. Metabolic syndrome and dietary patterns: a systematic review and meta-analysis of observational studies. *Eur J Nutr*. 2017;56(3):925-947. doi:10.1007/s00394-016-1305-y.

112. Davidson TL, Jones S, Roy M, Stevenson RJ. The cognitive control of eating and body weight: It's more than what you "think." *Front Psychol*. 2019;10(FEB). doi:10.3389/fpsyg.2019.00062.

113. DiFeliceantonio AG, Small DM. Dopamine and diet-induced obesity. *Nat Neurosci*. 2019;22(1):6-7. doi:10.1038/s41593-018-0304-0.

114. Grabovac I, Smith L, Stefanac S, Haider S, Cao C, Waldhoer T, Jackson SE, Yang L. Health Care Providers' Advice on Lifestyle Modification in the US Population: Results from the NHANES 2011-2016. *Am J Med*. 2019;132(4):489-497.e1. doi:10.1016/j.amjmed.2018.11.021.

115. Müller MJ, Enderle J, Pourhassan M, Braun W, Eggeling B, Lagerpusch M, Glüer CC, Kehayias JJ, Kiosz D, Bosy-Westphal A. Metabolic adaptation to caloric restriction and subsequent refeeding: The Minnesota Starvation Experiment revisited. *Am J Clin Nutr*. 2015;102(4):807-819. doi:10.3945/ajcn.115.109173.

116. Fothergill E, Guo J, Howard L, Kerns JC, Knuth ND, Brychta R, Chen KY, Skarulis MC, Walter M, Walter PJ, Hall KD. Persistent metabolic adaptation 6 years after "The Biggest Loser" competition. *Obesity*. 2016;24(8):1612-1619. doi:10.1002/oby.21538.

117. Franklin JC, Schiele BC, Brozek J, Keys A. Observations on human behavior in experimental semistarvation and rehabilitation. *J Clin Psychol*. 1948;4(1):28-45. doi:10.1002/1097-4679(194801)4:1<28::AID-JCLP2270040103>3.0.CO;2-F.

118. Keys A, Menott A, Karvonen MJ, Aravanjs C, Blackburn H, Buzina R, Djordjevic BS, Dontas AS, Fidanza F, Keys MH, Kromhout D, Nedeljkovic S, Punsar S, Seccareccia F, Toshima H. The diet and 15-year death rate in the seven countries study. *Am J Epidemiol*. 1985;124(6):903-915. doi:10.1093/aje/kwx101.

119. Yerushalmy J, Hilleboe HE. Fat in the diet and mortality from heart disease; a methodologic note. *N Y State J Med*. 1957;57(14):2343-2354.

120. Wang DD, Hu FB. Dietary Fat and Risk of Cardiovascular Disease: Recent Controversies and Advances. *Annu Rev Nutr*. 2017;37(1):423-446. doi:10.1146/annurev-nutr-071816-064614.

121. Westerterp-Plantenga MS, Luscombe-Marsh N, Lejeune MPGM, Diepvens K, Nieuwenhuizen A, Engelen MPKJ, Deutz NEP, Azzout-Marniche D, Tome D, Westerterp KR. Dietary protein, metabolism, and body-weight regulation: Dose-response effects. *Int J Obes*. 2006;30(SUPPL. 3):16-23. doi:10.1038/sj.ijo.0803487.

122. Piatti PM, Monti LD, Magni F, Fermo I, Baruffaldi L, Nasser R, Santambrogio G, Librenti MC, Galli-Kienle M, Pontiroli AE, Pozza G. Hypocaloric high-protein diet improves glucose oxidation and spares lean body mass: Comparison to hypocaloric high-carbohydrate diet. *Metabolism*. 1994;43(12):1481-1487. doi:10.1016/0026-0495(94)90005-1.

123. Drenick EJ, Swendseid ME, Blahd WH, Tuttle SG. Prolonged Starvation as Treatment for Severe Obesity. *J Am Med Assoc*. 1964;187(2):100-105. doi:10.1001/jama.1964.03060150024006.

124. Munro JF, Maccuish AC, Goodall JAD, Fraser J, Duncan LJP. Further experience with prolonged therapeutic starvation in gross refractory obesity. *Br Med J*. 1970;4(5737):712-714. doi:10.1136/bmj.4.5737.712.

125. Cahill GF. Starvation in Man. *N Engl J Med*. 1970;282(12):668-675.

126. Thomson TJ, Runcie J, Miller V. Treatment of obesity by total fasting for up to 249 days. *Lancet*. 1966;288(7471):992-996. doi:10.1016/s0140-6736(66)92925-4.

127. Stewart WK, Fleming LW. Features of a successful therapeutic fast of 382 days' duration. *Postgrad Med J*. 1973;49(569):203-209. doi:10.1136/pgmj.49.569.203.

128. Gebhard RL, Prigge WF, Ansel HJ, Schlasner L, Ketover SR, Sande D, Holtmeier K, Peterson FJ. The role of gallbladder emptying in gallstone formation during diet-induced rapid weight loss. *Hepatology*. 1996;24(3):544-548. doi:10.1053/jhep.1996.v24.pm0008781321.

129. Finnell JS, Saul BC, Goldhamer AC, Myers TR. Is fasting safe? A chart review of adverse events during medically supervised, water-only fasting. *BMC Complement Altern Med*. 2018;18(1):1-9. doi:10.1186/s12906-018-2136-6.

130. Telch CF, Agras WS. The effects of a very low calorie diet on binge eating. *Behav Ther*. 1993;24(2):177-193. doi:10.1016/S0005-7894(05)80262-X.

131. Polivy J, Herman CP. Dieting and Binging. A Causal Analysis. *Am Psychol*. 1985;40(2):193-201. doi:10.1037/0003-066X.40.2.193.

132. Brandhorst S, Longo VD. Protein Quantity and Source, Fasting-Mimicking Diets, and Longevity. *Adv Nutr*. 2019;10(4):S340-S350. doi:10.1093/advances/nmz079.

133. Anton SD, Moehl K, Donahoo WT, Marosi K, Lee SA, Mainous AG, Leeuwenburgh C, Mattson MP. Flipping the Metabolic Switch: Understanding and Applying the Health Benefits of Fasting. *Obesity*. 2018;26(2):254-268. doi:10.1002/oby.22065.

134. Browning JD, Baxter J, Satapati S, Burgess SC. The effect of short-term fasting on liver and skeletal muscle lipid, glucose, and energy metabolism in healthy women and men. *J Lipid Res*. 2012;53(3):577-586. doi:10.1194/jlr.P020867.

135. Heilbronn LK, Smith SR, Martin CK, Anton SD, Ravussin E. Alternate-day fasting in nonobese subjects: Effects on body weight, body composition, and energy metabolism. *Am J Clin Nutr*. 2005;81(1):69-73. doi:10.1093/ajcn/81.1.69.

136. White J V., Guenter P, Jensen G, Malone A, Schofield M. Consensus statement: Academy of nutrition and dietetics and American society for parenteral and enteral nutrition: Characteristics recommended for the identification and documentation of adult malnutrition (undernutrition). *J Parenter Enter Nutr*. 2012;36(3):275-283. doi:10.1177/0148607112440285.

137. Shetty P. Malnutrition and undernutrition. *Med (United Kingdom)*. 2006;34(12):524-529. doi:10.1016/j.mpmed.2018.12.012.

138. NB R, TT A, GF. C. Gluconeogenesis and its disorders in man. In: Hanson R, Mehlman M, eds. *Gluconeogenesis: Its Regulation in Mammalian Species*. New York: Wiley; 1976:515–30.

139. Sapir DG, Owen OE, Cheng JT, Ginsberg R, Boden G, Walker WG. The effect of carbohydrates on ammonium and ketoacid excretion during starvation. *J Clin Invest*. 1972;51(8):2093-2102. doi:10.1172/JCI107016.

140. Hannaford MC, Leiter LA, Josse RG. Protein wasting due to acidosis of prolonged fasting. *Am J Physiol - Endocrinol Metab*. 1982;6(3). doi:10.1152/ajpendo.1982.243.3.e251.

141. Soeters MR, Soeters PB, Schooneman MG, Houten SM, Romijn JA. Adaptive reciprocity of lipid and glucose metabolism in human short-term starvation. *Am J Physiol - Endocrinol Metab*. 2012;303(12). doi:10.1152/ajpendo.00397.2012.

142. Knapik JJ, Meredith CN, Jones BH, Suek L, Young VR, Evans WJ. Influence of fasting on carbohydrate and fat metabolism during rest and exercise in men. *J Appl Physiol*. 1988;64(5):1923-1929. doi:10.1152/jappl.1988.64.5.1923.

143. Cahill GF, Herrera MG, Morgan AP, Soeldner JS, Steinke J, Levy PL, Reichard GA, Kipnis DM. Hormone-fuel interrelationships during fasting. *J Clin Invest*. 1966;45(11):1751-1769. doi:10.1172/JCI105481.

144. Klein S, Sakurai Y, Romijn JA, Carroll RM. Progressive alterations in lipid and glucose metabolism during short-term fasting in young adult men. *Am J Physiol - Endocrinol Metab*. 1993;265(5 28-5). doi:10.1152/ajpendo.1993.265.5.e801.

145. Johnson RH, Walton JL. Fitness, Fatness, and Post-Exercise Ketosis. *Lancet*. 1971;297(7699):566-568. doi:10.1016/S0140-6736(71)91164-0.

146. Cox PJ, Clarke K. Acute nutritional ketosis: Implications for exercise performance and metabolism. *Extrem Physiol Med*. 2014;3(1):1-9. doi:10.1186/2046-7648-3-17.

147. Carnevale Schianca GP, Rossi A, Sainaghi PP, Maduli E, Bartoli E. The Significance of Impaired Fasting Glucose Versus Impaired Glucose Tolerance. *Diabetes Care*. 2003;26(5):1333-1337. doi:10.2337/diacare.26.5.1333.

148. Malin SK, Kirwan JP. Fasting hyperglycaemia blunts the reversal of impaired glucose tolerance after exercise training in obese older adults. *Diabetes, Obes Metab*. 2012. doi:10.1111/j.1463-1326.2012.01608.x.

149. Hernandez AR, Truckenbrod LM, Federico QP, Campos KT. Metabolic switching is impaired by aging and facilitated by ketosis independent of glycogen. *bioRxiv*. 2019:1-41. doi:10.1101/2019.12.12.874297.

150. Evert AB, Dennison M, Gardner CD, Timothy Garvey W, Karen Lau KH, MacLeod J, Mitri J, Pereira RF, Rawlings K, Robinson S, Saslow L, Uelmen S, Urbanski PB, Yancy WS. Nutrition therapy for adults with diabetes or prediabetes: A consensus report. *Diabetes Care*. 2019;42(5):731-754. doi:10.2337/dci19-0014.

151. Dall TM, Storm M V., Semilla AP, Wintfeld N, O'Grady M, Venkat Narayan KM. Value of lifestyle intervention to prevent diabetes and sequelae. *Am J Prev Med*. 2015;48(3):271-280. doi:10.1016/j.amepre.2014.10.003.

152. Leverence RR, Williams RL, Sussman A, Crabtree BF. Obesity Counseling and Guidelines in Primary Care. A Qualitative Study. *Am J Prev Med*. 2007;32(4). doi:10.1016/j.amepre.2006.12.008.

153. Foster GD, Wadden TA, Makris AP, Davidson D, Sanderson RS, Allison DB, Kessler A. Primary care physicians' attitudes about obesity and its treatment. *Obes Res*. 2003;11(10):1168-1177. doi:10.1038/oby.2003.161.

154. Bornhoeft K. Perceptions, Attitudes, and Behaviors of Primary Care Providers Toward Obesity Management: A Qualitative Study. *J Community Health Nurs*. 2018;35(3):85-101. doi:10.1080/07370016.2018.1475792.

155. Kahan S, Manson JE. Obesity Treatment, Beyond the Guidelines. Practical Suggestions for Clincial Practice. *J Am Med Assoc*. 2019:E1-E2. doi:10.1001/jama.2019.2352.

156. Ma J, Yank V, Xiao L, Lavori PW, Wilson SR, Rosas LG, Stafford RS. Translating the diabetes prevention program lifestyle intervention for weight loss into primary care: A randomized trial. *JAMA Intern Med*. 2013;173(2):113-121. doi:10.1001/2013.jamainternmed.987.

157. Barnes RD, Ivezaj V. A systematic review of motivational interviewing for weight loss among adults in primary care. *Obes Rev*. 2015;16(4):304-318. doi:10.1111/obr.12264.

158. Camps SGJA, Verhoef SPM, Westerterp KR. Weight loss, weight maintenance, and adaptive thermogenesis. *Am J Clin Nutr*. 2013;97(5):990-994. doi:10.3945/ajcn.112.050310.

159. Rosenbaum M, Hirsch J, Gallagher DA, Leibel RL. Long-term persistence of adaptive thermogenesis in subjects who have maintained a reduced body weight. *Am J Clin Nutr*. 2008;88(4):906-912. doi:10.1093/ajcn/88.4.906.

160. Martin CK, Heilbronn LK, De Jonge L, DeLany JP, Volaufova J, Anton SD, Redman LM, Smith SR, Ravussin E. Effect of calorie restriction on resting metabolic rate and spontaneous physical activity. *Obesity*. 2007;15(12):2964-2973. doi:10.1038/oby.2007.354.

161. Weinsier RL, Nagy TR, Hunter GR, Darnell BE, Hensrud DD, Weiss HL. Do adaptive changes in metabolic rate favor weight regain in weight-reduced individuals? An examination of the set-point theory. *Am J Clin Nutr*. 2000;72(5):1088-1094. doi:10.1093/ajcn/72.5.1088.

162. Stice E, Durant S, Burger KS, Schoeller DA. Weight suppression and risk of future increases in body mass: Effects of suppressed resting metabolic rate and energy expenditure. *Am J Clin Nutr*. 2011;94(1):7-11. doi:10.3945/ajcn.110.010025.

163. Thomas DM, Martin CK, Redman LM, Heymsfield SB, Lettieri S, Levine JA, Bouchard C, Schoeller DA. Effect of dietary adherence on the body weight plateau: A mathematical model incorporating intermittent compliance with energy intake prescription. *Am J Clin Nutr*. 2014;100(3):787-795. doi:10.3945/ajcn.113.079822.

164. Sainsbury A, Wood RE, Seimon R V., Hills AP, King NA, Gibson AA, Byrne NM. Rationale for novel intermittent dieting strategies to attenuate adaptive responses to energy restriction. *Obes Rev*. 2018;19(December):47-60. doi:10.1111/obr.12787.

165. Feung J. *The Obesity Code*. 1st ed. Greystone Books; 2016.

166. Luscombe ND, Clifton PM, Noakes M, Farnsworth E, Wittert G. Effect of a high-protein, energy-restricted diet on weight loss and energy expenditure after weight stabilization in hyperinsulinemic subjects. *Int J Obes*. 2003;27(5):582-590. doi:10.1038/sj.ijo.0802270.

167. Müller MJ, Enderle J, Bosy-Westphal A. Changes in energy expenditure with weight gain and weight loss in humans. *Curr Obes Rep*. 2016;5(4):413-423. doi:10.1007/s13679-016-0237-4.

168. Camps SGJA, Verhoef SPM, Westerterp KR. Leptin and energy restriction induced adaptation in energy expenditure. *Metabolism*. 2015;64(10):1284-1290. doi:10.1016/j.metabol.2015.06.016.

169. Hunter GR, Fisher G, Neumeier WH, Carter SJ, Plaisance EP. Exercise training and energy expenditure following weight loss. *Med Sci Sports Exerc*. 2015;47(9):1950-1957. doi:10.1249/MSS.0000000000000622.

170. Wing RR, Bolin P, Brancati FL, Bray GA, Clark JM, Coday M, Crow RS, Curtis JM, Egan CM, Espeland MA, Evans M, Foreyt JP, Ghazarian S, Gregg EW, Harrison B, Hazuda HP, Hill JO, Horton ES, Van Hubbard S, Jakicic JM, Jeffery RW, Johnson KC, Kahn SE, Kitabchi AE, Knowler WC, Lewis CE, Maschak-Carey BJ, Montez MG, Murillo A, Nathan DM, Patricio J, Peters A, Pi-Sunyer X, Pownall H, Reboussin D, Regensteiner JG, Rickman AD, Ryan DH, Safford M, Wadden TA, Wagenknecht LE, West DS, Williamson DF, Yanovski SZ. Cardiovascular effects of intensive lifestyle intervention in type 2 diabetes. *N Engl J Med*. 2013;369(2):145-154. doi:10.1056/NEJMoa1212914.

171. Wadden TA, Butryn ML, Hong PS, Tsai AG. Behavioral treatment of obesity in patients encountered in primary care settings: A systematic review. *JAMA - J Am Med Assoc*. 2014;312(17):1779-1791. doi:10.1001/jama.2014.14173.

172. Leung AWY, Chan RSM, Sea MMM, Woo J. An overview of factors associated with adherence to lifestyle modification programs for weight management in adults. *Int J Environ Res Public Health*. 2017;14(8). doi:10.3390/ijerph14080922.

173. Christensen DB, Williams B, Goldberg HI, Martin DP, Engelberg R, Logerfo JP. Assessing Compliance to Antihypertensive Medications Using Computer-Based Pharmacy Records. *Med Care*. 1997;35(11):1164-1170. doi:10.1097/00005650-199711000-00008.

174. Brown MT, Bussell JK. Medication adherence: WHO cares? *Mayo Clin Proc*. 2011;86(4):304-314. doi:10.4065/mcp.2010.0575.

175. Breckenridge A, Aronson JK, Blaschke TF, Hartman D, Peck CC, Vrijens B. Poor medication adherence in clinical trials: Consequences and solutions. *Nat Rev Drug Discov*. 2017;16(3):149-150. doi:10.1038/nrd.2017.1.

176. Karumbi J, Garner P. Directly observed therapy for treating tuberculosis (Review). *Cochrane Database Syst Rev*. 2015;(5). doi:10.1002/14651858.CD003343.pub4.www.cochranelibrary.com.

177. Deitel M. History of Bariatric Surgery. In: M. Korenkov, ed. *Bariatric Surgery*. Berlin: Springer-Verlag; 2012:1-217. doi:10.1007/978-3-642-16245-9_1.

178. O'Brien PE, Hindle A, Brennan L, Skinner S, Burton P, Smith A, Crosthwaite G, Brown W. Long-Term Outcomes After Bariatric Surgery: a Systematic Review and Meta-analysis of Weight Loss at 10 or More Years for All Bariatric Procedures and a Single-Centre Review of 20-Year Outcomes After Adjustable Gastric Banding. *Obes Surg*. 2019;29(1):3-14. doi:10.1007/s11695-018-3525-0.

179. Jakobsen GS, Småstuen MC, Sandbu R, Nordstrand N, Hofsø D, Lindberg M, Hertel JK, Hjelmesæth J. Association of bariatric surgery vs medical obesity treatment with long-term medical complications and obesity-related comorbidities. *JAMA - J Am Med Assoc*. 2018;319(3):291-301. doi:10.1001/jama.2017.21055.

180. Pareek M, Bhatt DL, Schiavon CA, Schauer PR. Metabolic Surgery for Hypertension in Patients with Obesity. *Circ Res*. 2019;124(7):1009-1024. doi:10.1161/CIRCRESAHA.118.313320.

181. Berger ER, Huffman KM, Fraker T, Petrick AT, Brethauer SA, Hall BL, Ko CY, Morton JM. Prevalence and Risk Factors for Bariatric Surgery Readmissions. *Ann Surg*. 2018;267(1):122-131. doi:10.1097/SLA.0000000000002079.

182. Kim GW, Lin JE, Blomain ES, Waldman SA. Antiobesity pharmacotherapy: New drugs and emerging targets. *Clin Pharmacol Ther*. 2014;95(1):53-66. doi:10.1038/clpt.2013.204.

183. George M, Rajaram M, Shanmugam E. New and emerging drug molecules against obesity. *J Cardiovasc Pharmacol Ther*. 2014;19(1):65-76. doi:10.1177/1074248413501017.

184. McLaren DG, Han S, Murphy BA, Wilsie L, Stout SJ, Zhou HHH, Roddy TP, Gorski JN, Metzger DE, Shin MK, Reilly DF, Zhou HHH, Tadin-Strapps M, Bartz SR, Cumiskey AM, Graham TH, Shen DM, Akinsanya KO, Previs SF, Imbriglio JE, Pinto S. DGAT2 Inhibition Alters Aspects of Triglyceride Metabolism in Rodents but Not in Non-human Primates. *Cell Metab*. 2018;27(6):1236-1248.e6. doi:10.1016/j.cmet.2018.04.004.

185. Li X, Bello NT. Anorectic state of obesity medications in the United States. Are leaner times ahead? *Expert Opin Pharmacother*. 2020;21(2):167-172. doi:10.1080/14656566.2019.1692815.

186. Bray GA. Use and abuse of appetite-suppressant drugs in the treatment of obesity. *Ann Intern Med*. 1993;119(7 II):707-713. doi:10.7326/0003-4819-119-7_part_2-199310011-00016.

187. Kornstein SG, Simon JA, Apfel SC, Yuan J, Barbour KA, Kissling R. Effect of Flibanserin Treatment on Body Weight in Premenopausal and Postmenopausal Women with Hypoactive Sexual Desire Disorder: A Post Hoc Analysis. *J Women's Heal*. 2017;26(11):1161-1168. doi:10.1089/jwh.2016.6230.

188. Silverstone T. Appetite suppressants: a review. *Drugs*. 1992;43(6):820-836.

189. Zimbron J, Khandaker GM, Toschi C, Jones PB, Fernandez-Egea E. A systematic review and meta-analysis of randomised controlled trials of treatments for clozapine-induced obesity and metabolic syndrome. *Eur Neuropsychopharmacol*. 2016;26(9):1353-1365. doi:10.1016/j.euroneuro.2016.07.010.

190. LeRiche WH, van Belle GE. A long-term study on the use of appetite suppressants. *J Can Med Assoc*. 1961;85(12):673-676.

191. Abenhaim L, Moride Y, Brenot F, Rich S, Benichou J, Kurz X, Higenbottam T, Oakley C, Wouters E, Aubier M, Simonneau G, Bégaud B. Appetite-suppressant drugs and

the risk of primary pulmonary hypertension. *N Engl J Med*. 1996;335(9):609-616. doi:10.1056/NEJM199608293350901.

192. Paumgartten FJR, Pereira SSTC, de Oliveira ACAX. Safety and efficacy of fenproporex for obesity treatment: a systematic review. *Rev Saude Publica*. 2016;50:25. doi:10.1590/S1518-8787.2016050006208.

193. Srivastava G, Apovian CM. Current pharmacotherapy for obesity. *Nat Rev Endocrinol*. 2018;14(1):12-24. doi:10.1038/nrendo.2017.122.

194. Jessen A, Buemann B, Toubro S, Skovgaard IM, Astrup A. The appetite-suppressant effect of nicotine is enhanced by caffeine. *Diabetes, Obes Metab*. 2005;7:327-333.

195. Tonstad S. Rimonabant: A cannabinoid receptor blocker for the treatment of metabolic and cardiovascular risk factors. *Nutr Metab Cardiovasc Dis*. 2006;16(2):156-162. doi:10.1016/j.numecd.2005.10.011.

196. Sam AH, Salem V, Ghatei MA. Rimonabant: From RIO to Ban. *J Obes*. 2011;2011. doi:10.1155/2011/432607.

197. Ravussin E, Smith SR, Mitchell JA, Shringarpure R, Shan K, Maier H, Koda JE, Weyer C. Enhanced Weight Loss With Pramlintide/Metreleptin: An Integrated Neurohormonal Approach to Obesity Pharmacotherapy. *Obesity*. 2009;17(9):1736-1743.

198. Lawson EA, Marengi DA, Desanti RL, Holmes TM, Schoenfeld DA, Tolley CJ. Oxytocin reduces caloric intake in men. *Obesity*. 2015;23(5):950-956. doi:10.1002/oby.21069.

199. Lawson EA. The effects of oxytocin on eating behaviour and metabolism in humans. *Nat Rev Endocrinol*. 2017;13(12):700-709. doi:10.1038/nrendo.2017.115.

200. Kuppens RJ, Donze SH, Hokken-Koelega ACS. Promising effects of oxytocin on social and food-related behaviour in young children with Prader–Willi syndrome: a randomized, double-blind, controlled crossover trial. *Clin Endocrinol (Oxf)*. 2016;85(6):979-987. doi:10.1111/cen.13169.

201. Pilitsi E, Farr OM, Polyzos SA, Perakakis N, Nolen-Doerr E, Papathanasiou AE, Mantzoros CS. Pharmacotherapy of obesity: Available medications and drugs under investigation. *Metabolism*. 2019;92:170-192. doi:10.1016/j.metabol.2018.10.010.

202. Charles ED, Neuschwander-Tetri BA, Pablo Frias J, Kundu S, Luo Y, Tirucherai GS, Christian R. Pegbelfermin (BMS-986036), PEGylated FGF21, in Patients with Obesity

and Type 2 Diabetes: Results from a Randomized Phase 2 Study. *Obesity*. 2019;27(1):41-49. doi:10.1002/oby.22344.

203. Sanyal A, Charles ED, Neuschwander-Tetri BA, Loomba R, Harrison SA, Abdelmalek MF, Lawitz EJ, Halegoua-DeMarzio D, Kundu S, Noviello S, Luo Y, Christian R. Pegbelfermin (BMS-986036), a PEGylated fibroblast growth factor 21 analogue, in patients with non-alcoholic steatohepatitis: a randomised, double-blind, placebo-controlled, phase 2a trial. *Lancet*. 2018;392(10165):2705-2717. doi:10.1016/S0140-6736(18)31785-9.

204. Daneschvar HL, Aronson MD, Smetana GW. FDA-Approved Anti-Obesity Drugs in the United States. *Am J Med*. 2016;129(8):879.e1-879.e6. doi:10.1016/j.amjmed.2016.02.009.

205. Hughes TE, Kim DD, Marjason J, Proietto J, Whitehead JP, Vath JE. Ascending dose-controlled trial of beloranib, a novel obesity treatment for safety, tolerability, and weight loss in obese women. *Obesity*. 2013;21(9):1782-1788. doi:10.1002/oby.20356.

206. van Boekel G, Loves S, van Sorge A, Ruinemans-Koerts J, Rijnders T, de Boer H. Weight loss in obese men by caloric restriction and high-dose diazoxide-mediated insulin suppression. *Diabetes, Obes Metab*. 2008;10(12):1195-1203. doi:10.1111/j.1463-1326.2008.00878.x.

207. Jensterle M, Salamun V, Kocjan T, Vrtacnik Bokal E, Janez A. Short term monotherapy with GLP-1 receptor agonist liraglutide or PDE 4 inhibitor roflumilast is superior to metformin in weight loss in obese PCOS women: A pilot randomized study. *J Ovarian Res*. 2015;8(1):1-8. doi:10.1186/s13048-015-0161-3.

208. Lustig RH, Rose SR, Burghen GA, Velasquez-Mieyer P, Broome DC, Smith K, Li H, Hudson MM, Heideman RL, Kun LE. Hypothalamic obesity caused by cranial insult in children: Altered glucose and insulin dynamics and reversal by a somatostatin agonist. *J Pediatr*. 1999;135(2 I):162-168. doi:10.1016/S0022-3476(99)70017-X.

209. Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, Rubin JR, Mowers J, White NM, Hochberg I, Downes M, Yu RT, Liddle C, Evans RM, Oh D, Li P, Olefsky JM, Saltiel AR. An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice. *Nat Med*. 2013;19(3):313-321. doi:10.1038/nm.3082.

210. Herbert C, Lassalle G, Alcouffe C, Bono F. Approaches targeting the FGF-FGFR system: a review of the recent patent literature and associated advanced therapeutic agents. *Pharm Pat Anal*. 2014;3(6):585-612. doi:10.4155/ppa.14.45.

211. Liu J, Wang Y, Lin L. Small molecules for fat combustion: targeting obesity. *Acta Pharm Sin B*. 2019;9(2):220-236. doi:10.1016/j.apsb.2018.09.007.

212. Sels JJE, Huijberts MSP, Hr B. Miglitol , a new alpha-glucosidase inhibitor. *Expert Opin Pharmacother*. 1999;1(1):149-156.

213. Senese R, Cioffi F, Petito G, Goglia F, Lanni A. Thyroid hormone metabolites and analogues. *Endocrine*. 2019;66(1):105-114. doi:10.1007/s12020-019-02025-5.

214. Gomez G, Stanford FC. US health policy and prescription drug coverage of FDA-approved medications for the treatment of obesity. *Int J Obes*. 2018;42(3):495-500. doi:10.1038/ijo.2017.287.

215. Zhuo C, Xu Y, Liu S, Li J, Zheng Q, Gao X, Li S, Jing R, Song X, Yue W, Zhou C, Upthegrove R. Topiramate and metformin are effective add-on treatments in controlling antipsychotic-induced weight gain: A systematic review and network meta-analysis. *Front Pharmacol*. 2018;9(NOV):1-10. doi:10.3389/fphar.2018.01393.

216. Vincent JB. The potential value and toxicity of chromium picolinate as a nutritional supplement, weight loss agent and muscle development agent. *Sport Med*. 2003;33(3):213-230. doi:10.2165/00007256-200333030-00004.

217. Arch JRS. Challenges in β3-adrenoceptor agonist drug development. *Ther Adv Endocrinol Metab*. 2011;2(2):59-64. doi:10.1177/2042018811398517.

218. Thomas JM, Dourish CT, Tomlinson JW, Hassan-Smith Z, Higgs S. Effects of the 5-HT2C receptor agonist meta- chlorophenylpiperazine on appetite, food intake and emotional processing in healthy volunteers. *Psychopharmacology (Berl)*. 2014;231(12):2449-2459. doi:10.1007/s00213-013-3409-x.

219. Dayabandara M, Hanwella R, Ratnatunga S, Seneviratne S, Suraweera C, de Silva VA. Antipsychotic-associated weight gain: Management strategies and impact on treatment adherence. *Neuropsychiatr Dis Treat*. 2017;13:2231-2241. doi:10.2147/NDT.S113099.

220. Bray GA, Heisel WE, Afshin A, Jensen MD, Dietz WH, Long M, Kushner RF, Daniels SR, Wadden TA, Tsai AG, Hu FB, Jakicic JM, Ryan DH, Wolfe BM, Inge TH. The science of

obesity management: An endocrine society scientific statement. *Endocr Rev*. 2018;39(2):79-132. doi:10.1210/er.2017-00253.

221. Yanovski SZ, Yanovski JA. Long-term drug treatment for obesity: A systematic and clinical review. *JAMA - J Am Med Assoc*. 2014;311(1):74-86. doi:10.1001/jama.2013.281361.

222. Khera R, Murad MH, Chandar AK, Dulai PS, Wang Z, Prokop LJ, Loomba R, Camilleri M, Singh S. Association of pharmacological treatments for obesity withweight loss and adverse events a systematic review and meta-analysis. *JAMA - J Am Med Assoc*. 2016;315(22):2424-2434. doi:10.1001/jama.2016.7602.

223. Ziauddeen H, Chamberlain SR, Nathan PJ, Koch A, Maltby K, Bush M, Tao WX, Napolitano A, Skeggs AL, Brooke AC, Cheke L, Clayton NS, Sadaf Farooqi I, O'rahilly S, Waterworth D, Song K, Hosking L, Richards DB, Fletcher PC, Bullmore ET. Effects of the mu-opioid receptor antagonist GSK1521498 on hedonic and consummatory eating behaviour: A proof of mechanism study in binge-eating obese subjects. *Mol Psychiatry*. 2013;18(12):1287-1293. doi:10.1038/mp.2012.154.

224. de Silva VA, Suraweera C, Ratnatunga SS, Dayabandara M, Wanniarachchi N, Hanwella R. Metformin in prevention and treatment of antipsychotic induced weight gain: A systematic review and meta-analysis. *BMC Psychiatry*. 2016;16(1):1-10. doi:10.1186/s12888-016-1049-5.

225. Jandacek RJ. Review of the effects of dilution of dietary energy with olestra on energy intake. *Physiol Behav*. 2012;105(5):1124-1131. doi:10.1016/j.physbeh.2011.12.018.

226. Seghatol FF, Rigolin VH. Appetite suppressants and valvular heart disease. *Curr Opin Cardiol*. 2002;17(5):486-492. doi:10.1097/00001573-200209000-00007.

227. Dowden H, Munro J. Trends in clinical success rates and therapeutic focus. *Nat Rev Drug Discov*. 2019;18(7):495-496. doi:10.1038/d41573-019-00074-z.

228. Harrison RK. Phase II and phase III failures: 2013-2015. *Nat Rev Drug Discov*. 2016;15(12):817-818. doi:10.1038/nrd.2016.184.

229. Adlersberg D, Mayer ME. Results of prolonged medical treatment of obesity with diet alone, diet and thyroid preparations, and diet and amphetamine. *Endocrinology*. 1949;9(November):275-284. doi:10.1210/jcem-9-3-275.

230. Cutting WC, Mehrtens HG, Tainter ML. Actions and uses of dinitrophenol: promising metabolci applications. *J Am Med Assoc*. 1933;101(3):193-195.

231. A. B. STOCKTON, W. C. CUTTING, M. L. TAINTER, Tainter ML, Stockton AB, Cutting WC. Dinitrophenol in the Treatment of Obesity. *J Am Med Assoc*. 1935;105(5):332-337.

232. Harris SC, Ivy AC, Searle LM. The mechanism of amphetamine-induced loss of weight. *J Am Med Assoc*. 1947;134(17):1468-1475.

233. Geisler J. 2,4 Dinitrophenol as Medicine. *Cells*. 2019;8(3):280. doi:10.3390/cells8030280.

234. LeDuc CA, Leibel RL. Auto-Regulation of Leptin Neurobiology. *Cell Metab*. 2019;30(4):614-616. doi:10.1016/j.cmet.2019.09.006.

235. Corbin KD, Driscoll KA, Pratley RE, Smith SR, Maahs DM, Mayer-Davis EJ. Obesity in type 1 diabetes: Pathophysiology, clinical impact, and mechanisms. *Endocr Rev*. 2018;39(5):629-663. doi:10.1210/er.2017-00191.

236. Chou K, Perry CM. Metreleptin: First global approval. *Drugs*. 2013;73(9):989-997. doi:10.1007/s40265-013-0074-7.

237. Quarta C, Sánchez-Garrido MA, Tschöp MH, Clemmensen C. Renaissance of leptin for obesity therapy. *Diabetologia*. 2016;59(5):920-927. doi:10.1007/s00125-016-3906-7.

238. Chan K, Truong D, Shangari N, O'Brien PJ. Drug-induced mitochondrial toxicity. *Expert Opin Drug Metab Toxicol*. 2005;1(4):655-669. doi:10.1517/17425255.1.4.655.

239. Jangaard NO, Pereira JN, Pinson R. Metabolic effects of the biguanides and possible mechanism of action. *Diabetes*. 1968;17(2):96-104. doi:10.2337/diab.17.2.96.

240. Vigneri R, Maddux B, Goldfine ID. The effect of phenformin and other adenosine triphosphate (ATP)-lowering agents on insulin binding to IM-9 human cultured lymphocytes. *J Cell Biochem*. 1984;24(2):177-186. doi:10.1002/jcb.240240208.

241. Gardner MLG, Bevan JS. Multiple actions of phenylethylbiguanide on respiration by rat liver mitochondria. *Biochem Pharmacol*. 1977;26(8):717-721. doi:10.1016/0006-2952(77)90214-3.

242. Grundlingh J, Dargan PI, El-Zanfaly M, Wood DM. 2,4-Dinitrophenol (DNP): A Weight Loss Agent with Significant Acute Toxicity and Risk of Death. *J Med Toxicol*. 2011;7(3):205-212. doi:10.1007/s13181-011-0162-6.

243. He L, Wondisford FE. Metformin action: Concentrations matter. *Cell Metab*. 2015;21(2):159-162. doi:10.1016/j.cmet.2015.01.003.

244. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia*. 2017;60(9):1577-1585. doi:10.1007/s00125-017-4342-z.

245. Qaseem A, Barry MJ, Humphrey LL, Forciea MA, Fitterman N, Boyd C, Horwitch C, Iorio A, Kansagara D, Manaker S, McLean RM, Vijan S, Wilt TJ. Oral pharmacologic treatment of type 2 diabetes mellitus: A clinical practice guideline update from the American college of physicians. *Ann Intern Med*. 2017;166(4):279-290. doi:10.7326/M16-1860.

246. Schultes B, Oltmanns KM, Kern W, Fehm HL, Born J, Peters A. Modulation of hunger by plasma glucose and metformin. *J Clin Endocrinol Metab*. 2003;88(3):1133-1141. doi:10.1210/jc.2002-021450.

247. Fruehwald-Schultes B, Oltmanns KM, Toschek B, Sopke S, Kern W, Born J, Fehm HL, Peters A. Short-term treatment with metformin decreases serum leptin concentration without affecting body weight and body fat content in normal-weight healthy men. *Metabolism*. 2002;51(4):531-536. doi:10.1053/meta.2002.31332.

248. Doogue MP, Begg EJ, Moore MP, Lunt H, Pemberton CJ, Zhang M. Metformin increases plasma ghrelin in Type 2 diabetes. *Br J Clin Pharmacol*. 2009;68(6):875-882. doi:10.1111/j.1365-2125.2009.03372.x.

249. Gudat U, Convent G, Heinemann L. Metformin and exercise: No additive effect on blood lactate levels in healthy volunteers. *Diabet Med*. 1997;14(2):138-142. doi:10.1002/(SICI)1096-9136(199702)14:2<138::AID-DIA311>3.0.CO;2-S.

250. Kristensen JM, Lillelund C, Kjøbsted R, Birk JB, Andersen NR, Nybo L, Mellberg K, Balendran A, Richter EA, Wojtaszewski JFP. Metformin does not compromise energy status in human skeletal muscle at rest or during acute exercise : A randomised , crossover trial. *Physiol Rep*. 2019;7(e14307). doi:10.14814/phy2.14307.

251. Braun B, Eze P, Stephens BR, Hagobian TA, Sharoff CG, Chipkin SR, Goldstein B. Impact of metformin on peak aerobic capacity. *Appl Physiol Nutr Metab*. 2008;33(1):61-67. doi:10.1139/H07-144.

252. Konopka AR, Laurin JL, Schoenberg HM, Reid JJ, Castor WM, Wolff CA, Musci R V., Safairad OD, Linden MA, Biela LM, Bailey SM, Hamilton KL, Miller BF. Metformin inhibits mitochondrial adaptations to aerobic exercise training in older adults. *Aging Cell*. 2019;18(1). doi:10.1111/acel.12880.

253. Seifarth C, Schehler B, Schneider HJ. Effectiveness of metformin on weight loss in non-diabetic individuals with obesity. *Exp Clin Endocrinol Diabetes*. 2013;121(1):27-31. doi:10.1055/s-0032-1327734.

254. Bray GA, Edelstein SL, Crandall JP, Aroda VR, Franks PW, Fujimoto W, Horton E, Jeffries S, Montez M, Mudaliar S, Pi-Sunyer FX, White NH, Knowler WC. Long-term safety, tolerability, and weight loss associated with metformin in the diabetes prevention program outcomes study. *Diabetes Care*. 2012;35(4):731-737. doi:10.2337/dc11-1299.

255. Xu T, Brandmaier S, Messias AC, Herder C, Draisma HHM, Demirkan A, Yu Z, Ried JS, Haller T, Heier M, Campillos M, Fobo G, Stark R, Holzapfel C, Adam J, Chi S, Rotter M, Panni T, Quante AS, He Y, Prehn C, Roemisch-Margl W, Kastenmuller G, Willemsen G, Pool R, Kasa K, Van Dijk KW, Hankemeier T, Meisinger C, Thorand B, Ruepp A, De Angelis MH, Li Y, Wichmann HE, Stratmann B, Strauch K, Metspalu A, Gieger C, Suhre K, Adamski J, Illig T, Rathmann W, Roden M, Peters A, Van Duijn CM, Boomsma DI, Meitinger T, Wang-Sattler R. Effects of metformin on metabolite profiles and LDL cholesterol in patients with type 2 diabetes. *Diabetes Care*. 2015;38(10):1858-1867. doi:10.2337/dc15-0658.

256. Wulffelé MG, Kooy A, De Zeeuw D, Stehouwer CDA, Gansevoort RT. The effect of metformin on blood pressure, plasma cholesterol and triglycerides in type 2 diabetes mellitus: A systematic review. *J Intern Med*. 2004;256(1):1-14. doi:10.1111/j.1365-2796.2004.01328.x.

257. Yang X, So WY, Ma RCW, Kong APS, Man HL, Yu L, Chow CC, Ozaki R, Ko GTC, Chan JCN. Low HDL cholesterol, metformin use, and cancer risk in type 2 diabetes: The Hong Kong diabetes registry. *Diabetes Care*. 2011;34(2):375-380. doi:10.2337/dc10-1509.

258. Zhou L, Liu H, Wen X, Peng Y, Tian Y, Zhao L. Effects of metformin on blood pressure in nondiabetic patients: A meta-analysis of randomized controlled trials. *J Hypertens*. 2017;35(1):18-26. doi:10.1097/HJH.0000000000001119.

259. Thomopoulos C, Katsimagklis G, Makris T. Metformin and blood pressure lowering: a questioned association. *J Hypertens*. 2017;35(1):27-28. doi:10.1097/HJH.0000000000001146.

260. Freed SC. Psychic factors in the development and treatment of obesity. *J Am Med Assoc*. 1947;133(6):369-373.

261. Cobb LK, Appel LJ, Franco M, Jones-Smith JC, Nur A, Anderson CAM. The relationship of the local food environment with obesity: A systematic review of methods, study quality, and results. *Obesity*. 2015;23(7):1331-1344. doi:10.1002/oby.21118.

262. Ford PB, Dzewaltowski DA. Disparities in obesity prevalence due to variation in the retail food environment: Three testable hypotheses. *Nutr Rev*. 2008;66(4):216-228. doi:10.1111/j.1753-4887.2008.00026.x.

263. Campbell KJ, Crawford DA, Salmon J, Carver A, Garnett SP, Baur LA. Associations between the home food environment and obesity-promoting eating behaviors in adolescence. *Obesity*. 2007;15(3):719-730. doi:10.1038/oby.2007.553.

264. Limbers CA, Young D, Beaujean AA. The Emotional Eating Scale adapted for children and adolescents: Factorial invariance across adolescent males and females. *Eat Behav*. 2016;22:164-169. doi:10.1016/j.eatbeh.2016.06.012.

265. Braden A, Musher-eizenman D, Watford T, Emley E, Musher-eizenman D. Eating when depressed, anxious, bored, or happy: Are emotional eating types associated with unique psychological and physical health correlates? *Appetite*. 2018;125:410-417. doi:10.1016/j.appet.2018.02.022.

266. Kauffman BY, Shepherd JM, Bakhshaie J, Zvolensky MJ. Anxiety sensitivity in relation to eating expectancies among college students. *J Am Coll Heal*. 2019;0(0):1-5. doi:10.1080/07448481.2019.1656216.

267. Freyhan FA. Obesity-Reply. *J Am Med Assoc*. 1947;133(14):1032.

268. Sumithran P, Prendergast LA, Delbridge E, Purcell K, Shulkes A, Kriketos A, Proietto J. Long-term persistence of hormonal adaptations to weight loss. *N Engl J Med*. 2011;365(17):365. doi:10.1056/NEJMoa1105816.

269. Naish KR, Laliberte M, MacKillop J, Balodis IM. Systematic review of the effects of acute stress in binge eating disorder. *Eur J Neurosci*. 2019;50(3):2415-2429. doi:10.1111/ejn.14110.

270. Domecq JP, Prutsky G, Leppin A, Sonbol MB, Altayar O, Undavalli C, Wang Z, Elraiyah T, Brito JP, Mauck KF, Lababidi MH, Prokop LJ, Asi N, Wei J, Fidahussein S, Montori VM, Murad MH. Drugs commonly associated with weight change: A systematic review and meta-analysis. *J Clin Endocrinol Metab*. 2015;100(2):363-370. doi:10.1210/jc.2014-3421.

271. Weiss RD, Griffin ML, Mirin SM. Drug abuse as self-medication for depression: An empirical study. *Am J Drug Alcohol Abuse*. 1992;18(2):121-129. doi:10.3109/00952999208992825.

272. Nestler EJ, Lüscher C. The Molecular Basis of Drug Addiction: Linking Epigenetic to Synaptic and Circuit Mechanisms. *Neuron*. 2019;102(1):48-59. doi:10.1016/j.neuron.2019.01.016.

273. Wansink B. From mindless eating to mindlessly eating better. *Physiol Behav*. 2010;100(5):454-463. doi:10.1016/j.physbeh.2010.05.003.

274. Nestler EJ. Is there a common molecular pathway for addiction? *Nat Neurosci*. 2005;8(11):1445-1449. doi:10.1038/nn1578.

275. Burnett CJ, Li C, Webber E, Tsaousidou E, Xue SY, Brüning JC, Krashes MJ. Hunger-Driven Motivational State Competition. *Neuron*. 2016;92(1):187-201. doi:10.1016/j.neuron.2016.08.032.

276. Rossi MA, Stuber GD. Overlapping Brain Circuits for Homeostatic and Hedonic Feeding. *Cell Metab*. 2018;27(1):42-56. doi:10.1016/j.cmet.2017.09.021.

277. Cassidy RM, Tong Q. Hunger and satiety gauge reward sensitivity. *Front Endocrinol (Lausanne)*. 2017;8(MAY):1-14. doi:10.3389/fendo.2017.00104.

278. Li XY, Han Y, Zhang W, Wang SR, Wei YC, Li SS, Lin JK, Yan JJ, Chen AX, Zhang X, Zhao ZD, Shen WL, Xu XH. AGRP neurons project to the medial preoptic area and modulate maternal nest-building. *J Neurosci*. 2019;39(3):456-471. doi:10.1523/JNEUROSCI.0958-18.2018.

279. Ralevski A, Horvath TL. Developmental programming of hypothalamic neuroendocrine systems. *Front Neuroendocrinol*. 2015;39:52-58. doi:10.1016/j.yfrne.2015.09.002.

280. van der Klaauw AA, Croizier S, Mendes de Oliveira E, Stadler LKJ, Park S, Kong Y, Banton MC, Tandon P, Hendricks AE, Keogh JM, Riley SE, Papadia S, Henning E, Bounds R, Bochukova EG, Mistry V, O'Rahilly S, Simerly RB, Minchin JEN, Barroso I, Jones EY, Bouret SG, Farooqi IS. Human Semaphorin 3 Variants Link Melanocortin Circuit Development and Energy Balance. *Cell*. 2019;176(4):729-742.e18. doi:10.1016/j.cell.2018.12.009.

281. Simerly RB. *Organization of the Hypothalamus*. Fourth Edi. Elsevier Inc.; 2015. doi:10.1016/B978-0-12-374245-2.00013-9.

282. Polex-Wolf J, Yeo GSH, O'Rahilly S. Impaired prohormone processing: A grand unified theory for features of Prader-Willi syndrome? *J Clin Invest*. 2017;127(1):98-99. doi:10.1172/JCI91307.

283. Pessoa L. A Network Model of the Emotional Brain. *Trends Cogn Sci*. 2017;21(5):357-371. doi:10.1016/j.tics.2017.03.002.

284. Petrovich GD. Lateral hypothalamus as a motivation-cognition interface in the control of feeding behavior. *Front Syst Neurosci*. 2018;12(April):1-7. doi:10.3389/fnsys.2018.00014.

285. Berthoud HR, Münzberg H. The lateral hypothalamus as integrator of metabolic and environmental needs: From electrical self-stimulation to opto-genetics. *Physiol Behav*. 2011;104(1):29-39. doi:10.1016/j.physbeh.2011.04.051.

286. Mangieri LR, Lu Y, Xu Y, Cassidy RM, Xu Y, Arenkiel BR, Tong Q. A neural basis for antagonistic control of feeding and compulsive behaviors. *Nat Commun*. 2018;9(1):1-15. doi:10.1038/s41467-017-02534-9.

287. Xu P, He Y, Cao X, Valencia-Torres L, Yan X, Saito K, Wang C, Yang Y, Hinton A, Zhu L, Shu G, Myers MG, Wu Q, Tong Q, Heisler LK, Xu Y. Activation of Serotonin 2C Receptors in Dopamine Neurons Inhibits Binge-like Eating in Mice. *Biol Psychiatry*. 2017;81(9):737-747. doi:10.1016/j.biopsych.2016.06.005.

288. Kim ER, Wu Z, Sun H, Xu Y, Mangieri LR, Xu Y, Tong Q. Hypothalamic Non-AgRP, Non-POMC GABAergic neurons are required for postweaning feeding and NPY

hyperphagia. *J Neurosci*. 2015;35(29):10440-10450. doi:10.1523/JNEUROSCI.1110-15.2015.

289. Xu YY, Lu Y, Xu P, Mangieri LR, Isingrini E, Xu YY, Giros B, Tong Q. VMAT2-mediated neurotransmission from midbrain leptin receptor neurons in feeding regulation. *eNeuro*. 2017;4(3):1-15. doi:10.1523/ENEURO.0083-17.2017.

290. Fan S, Dakshinamoorthy J, Kim ER, Xu Y, Huang C, Tong Q. An Indirect Action Contributes to C-Fos Induction in Paraventricular Hypothalamic Nucleus by Neuropeptide y. *Sci Rep*. 2016;6(September 2015):1-10. doi:10.1038/srep19980.

291. Tong Q, Ye CP, Jones JE, Elmquist JK, Lowell BB. Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. *Nat Neurosci*. 2008;11(9):998-1000. doi:10.1038/nn.2167.

292. Song J, Xu Y, Hu X, Choi B, Tong Q. Brain expression of Cre recombinase driven by pancreas-specific promoters. *Genesis*. 2010;48(11):628-634. doi:10.1002/dvg.20672.

293. Xu Y, O'Brien WG, Lee CC, Myers MG, Tong Q. Role of GABA release from leptin receptor-expressing neurons in body weight regulation. *Endocrinology*. 2012;153(5):2223-2233. doi:10.1210/en.2011-2071.

294. Wu Z, Kim ER, Sun H, Xu Y, Mangieri LR, Li DP, Pan HL, Xu Y, Arenkie BR, Tong Q. Gabaergic projections from lateral hypothalamus to paraventricular hypothalamic nucleus promote feeding. *J Neurosci*. 2015;35(8):3312-3318. doi:10.1523/JNEUROSCI.3720-14.2015.

295. Nieh EH, Matthews GA, Allsop SA, Presbrey KN, Leppla CA, Wichmann R, Neve R, Wildes CP, Tye KM. Decoding neural circuits that control compulsive sucrose seeking. *Cell*. 2015;160(3):528-541. doi:10.1016/j.cell.2015.01.003.

296. Sharpe MJ, Marchant NJ, Whitaker LR, Richie CT, Zhang YJ, Campbell EJ, Koivula PP, Necarsulmer JC, Mejias-Aponte C, Morales M, Pickel J, Smith JC, Niv Y, Shaham Y, Harvey BK, Schoenbaum G. Lateral Hypothalamic GABAergic Neurons Encode Reward Predictions that Are Relayed to the Ventral Tegmental Area to Regulate Learning. *Curr Biol*. 2017;27(14):2089-2100.e5. doi:10.1016/j.cub.2017.06.024.

297. Nieh EH, Vander Weele CM, Matthews GA, Presbrey KN, Wichmann R, Leppla CA, Izadmehr EM, Tye KM. Inhibitory Input from the Lateral Hypothalamus to the

Ventral Tegmental Area Disinhibits Dopamine Neurons and Promotes Behavioral Activation. *Neuron*. 2016;90(6):1286-1298. doi:10.1016/j.neuron.2016.04.035.

298. Li Y, Zeng J, Zhang J, Yue C, Zhong W, Liu Z, Feng Q, Luo M. Hypothalamic Circuits for Predation and Evasion. *Neuron*. 2018;97(4):911-924.e5. doi:10.1016/j.neuron.2018.01.005.

299. Venner A, Anaclet C, Broadhurst RY, Saper CB, Fuller PM. A Novel Population of Wake-Promoting GABAergic Neurons in the Ventral Lateral Hypothalamus. *Curr Biol*. 2016;26(16):2137-2143. doi:10.1016/j.cub.2016.05.078.

300. Venner A, Luca R De, Sohn LT, Bandaru SS, Verstegen AMJ, Arrigoni E, Fuller PM, Venner A, Luca R De, Sohn LT, Bandaru SS, Verstegen AMJ, Arrigoni E. An Inhibitory Lateral Hypothalamic-Preoptic Circuit Mediates Rapid Arousals from Sleep Article An Inhibitory Lateral Hypothalamic-Preoptic Circuit Mediates Rapid Arousals from Sleep. *Curr Biol*. 2019:1-14. doi:10.1016/j.cub.2019.10.026.

301. Weissbourd B, Ren J, DeLoach KE, Guenthner CJ, Miyamichi K, Luo L. Presynaptic Partners of Dorsal Raphe Serotonergic and GABAergic Neurons. *Neuron*. 2014;83(3):645-662. doi:10.1016/j.neuron.2014.06.024.

302. Jennings JH, Rizzi G, Stamatakis AM, Ung RL, Stuber GD. The Inhibitory Circuit Architecture of the Lateral Hypothalamus Orchestrates Feeding. *Science (80-)*. 2013;341(6153):1517-1522. doi:10.1126/science.1241812.

303. Hao S, Yang H, Wang X, He Y, Xu HH, Wu X, Pan L, Liu Y, Lou H, Xu HH, Ma H, Xi W, Zhou Y, Duan S, Wang H. The Lateral Hypothalamic and BNST GABAergic Projections to the Anterior Ventrolateral Periaqueductal Gray Regulate Feeding. *Cell Rep*. 2019;28(3):616-624.e5. doi:10.1016/j.celrep.2019.06.051.

304. Stuber GD, Wise RA. Lateral hypothalamic circuits for feeding and reward. *Nat Neurosci*. 2016;19(2):198-205. doi:10.1038/nn.4220.

305. Jennings JH, Ung RL, Resendez SL, Stamatakis AM, Taylor JG, Huang J, Veleta K, Kantak PA, Aita M, Shilling-Scrivo K, Ramakrishnan C, Deisseroth K, Otte S, Stuber GD. Visualizing hypothalamic network dynamics for appetitive and consummatory behaviors. *Cell*. 2015;160(3):516-527. doi:10.1016/j.cell.2014.12.026.

306. Bonnavion P, Mickelsen LE, Fujita A, de Lecea L, Jackson AC, Lecea L de, Jackson AC, 1Laboratory, de Lecea L, Jackson AC. Hubs and spokes of the lateral hypothalamus:

cell types, circuits and behaviour. *J Physiol*. 2016;594(22):6443-6462. doi:10.1113/JP271946.

307. Mickelsen LE, Kolling FW, Chimileski BR, Fujita A, Norris C, Chen K, Nelson CE, Jackson AC. Neurochemical heterogeneity among lateral hypothalamic hypocretin/orexin and melanin-concentrating hormone neurons identified through single-cell gene expression analysis. *eNeuro*. 2017;4(5). doi:10.1523/ENEURO.0013-17.2017.

308. Konadhode RR, Pelluru D, Shiromani PJ. Neurons containing orexin or melanin concentrating hormone reciprocally regulate wake and sleep. *Front Syst Neurosci*. 2015;8(JAN):1-9. doi:10.3389/fnsys.2014.00244.

309. Carus-Cadavieco M, Gorbati M, Ye L, Bender F, Van Der Veldt S, Kosse C, Börgers C, Lee SY, Ramakrishnan C, Hu Y, Denisova N, Ramm F, Volitaki E, Burdakov D, Deisseroth K, Ponomarenko A, Korotkova T. Gamma oscillations organize top-down signalling to hypothalamus and enable food seeking. *Nature*. 2017;542(7640):232-236. doi:10.1038/nature21066.

310. Durkin TP, Cazala P, Garcia R. Transsynaptic Mechanisms Controlling Cholinergic Neuronal Activation in the Septohippocampal and nBM-Cortical Pathways: Differential Roles in Memory and Attentional Processes? In: *The Behavioral Neuroscience of the Septal Region*. ; 2000:146-174. doi:10.1007/978-1-4612-1302-4_7.

311. Robinson J, Manseau F, Ducharme G, Amilhon B, Vigneault E, El Mestikawy S, Williams S. Optogenetic activation of septal glutamatergic neurons drive hippocampal theta rhythms. *J Neurosci*. 2016;36(10):3016-3023. doi:10.1523/JNEUROSCI.2141-15.2016.

312. Goard M, Dan Y. Basal Forebrain Activation Enhances Cortical Coding of Natural Scenes. *Nat Neurosci*. 2009;12(11):1444-1449. doi:10.1038/nn.2402.

313. Gielow MR, Zaborszky L. The Input-Output Relationship of the Cholinergic Basal Forebrain. *Cell Rep*. 2017;18(7):1817-1830. doi:10.1016/j.celrep.2017.01.060.

314. Carey RJ. A further localization of inhibitory deficits resulting from septal ablation. *Physiol Behav*. 1968;3(5):645-649. doi:10.1016/0031-9384(68)90128-5.

315. Johansson ÅK, Bergvall ÅH, Hansen S. Behavioral disinhibition following basal forebrain excitotoxin lesions: Alcohol consumption, defensive aggression,

impulsivity and serotonin levels. *Behav Brain Res*. 1999;102(1-2):17-29. doi:10.1016/S0166-4328(98)00159-4.

316. Sharma R, Sahota P, Thakkar MM. Nicotine administration in the cholinergic basal forebrain increases alcohol consumption in C57BL/6J mice Rishi. *Alcohol Clin Exp Res*. 2014;38(5):1315-1320. doi:10.1111/acer.12353.

317. Nolley DA. Hyperdipsia after septal and diagonal band of Broca lesions. *Psychon Sci*. 1972;26(3):131-132. doi:10.3758/BF03335455.

318. Ching Liang Shen. Efferent projections from the lateral hypothalamus in the guinea pig: An autoradiographic study. *Brain Res Bull*. 1983;11(3):335-347. doi:10.1016/0361-9230(83)90170-3.

319. Bernardis LL, Bellinger LL. The lateral hypothalamic area revisited: Neuroanatomy, body weight regulation, neuroendocrinology and metabolism. *Neurosci Biobehav Rev*. 1993;17(2):141-193. doi:10.1016/S0149-7634(05)80149-6.

320. Zaborszky L, Cullinan WE. Hypothalamic axons terminate on forebrain cholinergic neurons: an ultrastructural double-labeling study using PHA-L tracing and ChAT immunocytochemistry. *Brain Res*. 1989;479(1):177-184. doi:10.1016/0006-8993(89)91350-4.

321. Cullinan WE, Záborszky L. Organization of ascending hypothalamic projections to the rostral forebrain with special reference to the innervation of cholinergic projection neurons. *J Comp Neurol*. 1991;306(4):631-667. doi:10.1002/cne.903060408.

322. Lee SH, Dan Y. Neuromodulation of Brain States. *Neuron*. 2012;76(1):209-222. doi:10.1016/j.neuron.2012.09.012.

323. Arrigoni E, Mochizuki T, Scammell TE. Activation of the basal forebrain by the orexin/hypocretin neurones. *Acta Physiol*. 2010;198(3):223-235. doi:10.1111/j.1748-1716.2009.02036.x.

324. Morin AJ, Beaudet A. Origin of the neurotensinergic innervation of the rat basal forebrain studied by retrograde transport of cholera toxin. *J Comp Neurol*. 1998;391(1):30-41. doi:10.1002/(SICI)1096-9861(19980202)391:1<30::AID-CNE3>3.0.CO;2-S.

325. Herman AM, Ortiz-Guzman J, Kochukov M, Herman I, Quast KB, Patel JM, Tepe B, Carlson JC, Ung K, Selever J, Tong Q, Arenkiel BR. A cholinergic basal forebrain feeding circuit modulates appetite suppression. *Nature*. 2016;538(7624):253-256. doi:10.1038/nature19789.

326. Vong L, Ye C, Yang Z, Choi B, Chua S, Lowell BB. Leptin Action on GABAergic Neurons Prevents Obesity and Reduces Inhibitory Tone to POMC Neurons. *Neuron*. 2011;71(1):142-154. doi:10.1016/j.neuron.2011.05.028.

327. Lindeberg J, Ebendal T. Use of an internal ribosome entry site for bicistronic expression of Cre recombinase or rtTA transactivator. *Nucleic Acids Res*. 1999;27(6):1552-1554. doi:10.1093/nar/27.6.1552.

328. Liguz-Lecznar M, Skangiel-Kramska J. Vesicular glutamate transporters (VGLUTs): The three musketeers of glutamatergic system. *Acta Neurobiol Exp (Wars)*. 2007;67(3):207-218.

329. Nagy A. Cre recombinase: The universal reagent for genome tailoring. *Genesis*. 2000;26(2):99-109. doi:10.1002/(SICI)1526-968X(200002)26:2<99::AID-GENE1>3.0.CO;2-B.

330. Schnütgen F, Doerflinger N, Calléja C, Wendling O, Chambon P, Ghyselinck NB. A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. *Nat Biotechnol*. 2003;21(5):562-565. doi:10.1038/nbt811.

331. Saunders A, Johnson CA, Sabatini BL. Novel recombinant adeno-associated viruses for Cre activated and inactivated transgene expression in neurons. *Front Neural Circuits*. 2012;6(JULY 2012):1-10. doi:10.3389/fncir.2012.00047.

332. Atasoy D, Aponte Y, Su HH, Sternson SM. A FLEX switch targets channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *J Neurosci*. 2008;28(28):7025-7030. doi:10.1523/JNEUROSCI.1954-08.2008.

333. Zhu H, Roth BL. DREADD: A chemogenetic GPCR signaling platform. *Int J Neuropsychopharmacol*. 2015;18(1):1-6. doi:10.1093/ijnp/pyu007.

334. Nakai J, Ohkura M, Keiji I. A high signal-to-noise Ca2+ probe composed of a single green fluorescent protein. *Nat Biotechnol*. 2001;19:137-141. http://biotech.nature.com.

335. Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS. Ultra-sensitive fluorescent proteins for imaging neuronal activity. *Nature*. 2013;499(7458):295-300. doi:10.1038/nature12354.

336. Antonova I, Arancio O, Trillat A, Wang H, Zablow L, Udo H, Kandel ER, Hawkins RD. Rapid increase in clusters of presynaptic proteins at onset of long-lasting potentiation. *Science (80-)*. 2001;294(5546):1547-1550. doi:10.1126/science.1066273.

337. Tervo DGR, Hwang BY, Viswanathan S, Gaj T, Lavzin M, Ritola KD, Lindo S, Michael S, Kuleshova E, Ojala D, Huang CC, Gerfen CR, Schiller J, Dudman JT, Hantman AW, Looger LL, Schaffer D V., Karpova AY. A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons. *Neuron*. 2016;92(2):372-382. doi:10.1016/j.neuron.2016.09.021.

338. Kahle M, Horsch M, Fridrich B, Seelig A, Schultheiß J, Leonhardt J, Irmler M, Beckers J, Rathkolb B, Wolf E, Franke N, Gailus-Durner V, Fuchs H, de Angelis MH, Neschen S. Phenotypic comparison of common mouse strains developing high-fat diet-induced hepatosteatosis. *Mol Metab*. 2013;2(4):435-446. doi:10.1016/j.molmet.2013.07.009.

339. Stemmer K, Kotzbeck P, Bauer M, Neff C, Muller TD, Pfluger PT, Seeley RJ, Divanovic S. Thermoneutral housing is a critical factor for immune function and diet-induced obesity in C57BL/6 nude mice. *Int J Obes*. 2015;39(5):791-797. doi:10.1038/ijo.2014.187.Thermoneutral.

340. Heinrichs SC. Mouse feeding behavior: Ethology, regulatory mechanisms and utility for mutant phenotyping. *Behav Brain Res*. 2001;125(1-2):81-88. doi:10.1016/S0166-4328(01)00287-X.

341. Zeeni N, Nadkarni N, Bell JD, Even PC, Fromentin G, Tome D, Darcel N. Peripherally injected cholecystokinin-induced neuronal activation is modified by dietary composition in mice. *Neuroimage*. 2010;50(4):1560-1565. doi:10.1016/j.neuroimage.2010.01.065.

342. Goulding EH, Schenk AK, Juneja P, MacKay AW, Wade JM, Tecott LH. A robust automated system elucidates mouse home cage behavioral structure. *Proc Natl Acad Sci U S A*. 2008;105(52):20575-20582. doi:10.1073/pnas.0809053106.

343. Sheward WJ, Maywood ES, French KL, Horn JM, Hastings MH, Seck JR, Holmes MC, Harmar AJ. Entrainment to feeding but not to light: Circadian phenotype of VPAC 2

receptor-null mice. *J Neurosci*. 2007;27(16):4351-4358. doi:10.1523/JNEUROSCI.4843-06.2007.

344. Pitts S, Perone E, Silver R. Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice. *Am J Physiol - Regul Integr Comp Physiol*. 2003;285(154-1):57-67. doi:10.1152/ajpregu.00023.2003.

345. Graziano MSA. Ethological Action Maps: A Paradigm Shift for the Motor Cortex. *Trends Cogn Sci*. 2016;20(2):121-132. doi:10.1016/j.tics.2015.10.008.

346. Whishaw IQ, Faraji J, Mirza Agha B, Kuntz JR, Metz GAS, Mohajerani MH. A mouse's spontaneous eating repertoire aids performance on laboratory skilled reaching tasks: A motoric example of instinctual drift with an ethological description of the withdraw movements in freely-moving and head-fixed mice. *Behav Brain Res*. 2018;337(October):80-90. doi:10.1016/j.bbr.2017.09.044.

347. Anderson DJ, Perona P. Toward a science of computational ethology. *Neuron*. 2014;84(1):18-31. doi:10.1016/j.neuron.2014.09.005.

348. Grupe D, Nitschke J. Uncertainty and Anticipation in Anxiety. *Nat Rev Neurosci*. 2013;14(7):488-501. doi:10.1038/jid.2014.371.

349. Chesler EJ, Wilson SG, Lariviere WR, Rodriguez-Zas SL, Mogil JS. Influences of laboratory environment on behavior [1]. *Nat Neurosci*. 2002;5(11):1101-1102. doi:10.1038/nn1102-1101.

350. Crabbe JC, Wahlsten D, Dudek BC. Genetics of mouse behavior: Interactions with laboratory environment. *Science (80-)*. 1999;284(5420):1670-1672. doi:10.1126/science.284.5420.1670.

351. Belzung C, El Hage W, Moindrot N, Griebel G. Behavioral and neurochemical changes following predatory stress in mice. *Neuropharmacology*. 2001;41(3):400-408. doi:10.1016/S0028-3908(01)00072-7.

352. Wahlsten D, Metten P, Phillips TJ, Boehm SL, Burkhart-Kasch S, Dorow J, Doerksen S, Downing C, Fogarty J, Rodd-Henricks K, Hen R, McKinnon CS, Merrill CM, Nolte C, Schalomon M, Schlumbohm JP, Sibert JR, Wenger CD, Dudek BC, Crabbe JC. Different data from different labs: Lessons from studies of gene-environment interaction. *J Neurobiol*. 2003;54(1):283-311. doi:10.1002/neu.10173.

353. Lewejohann L, Reinhard C, Schrewe A, Brandewiede J, Haemisch A, Görtz N, Schachner M, Sachser N. Environmental bias? Effects of housing conditions, laboratory environment and experimenter on behavioral tests. *Genes, Brain Behav*. 2006;5(1):64-72. doi:10.1111/j.1601-183X.2005.00140.x.

354. Kim J, Lee S, Fang Y, Shin A, Park S, Hashikawa K, Bhat S, Kim D, Sohn J, Lin D, Suh GSB. Rapid, biphasic CRF neuronal responses encode positive and negative valence. *Nat Neurosci*. 2019;22:576-585. doi:https://doi.org/10.1038/s41593-019-0342-2.

355. Ferrero DM, Lemon JK, Fluegge D, Pashkovski SL, Korzan WJ, Datta SR, Spehr M, Fendt M, Liberles SD. Detection and avoidance of a carnivore odor by prey. *Proc Natl Acad Sci U S A*. 2011;108(27):11235-11240. doi:10.1073/pnas.1103317108.

356. Dent CL, Isles AR, Humby T. Measuring risk-taking in mice: Balancing the risk between seeking reward and danger. *Eur J Neurosci*. 2014;39(4):520-530. doi:10.1111/ejn.12430.

357. Pérez-Gómez A, Bleymehl K, Stein B, Pyrski M, Birnbaumer L, Munger SD, Leinders-Zufall T, Zufall F, Chamero P. Innate predator odor aversion driven by parallel olfactory subsystems that converge in the ventromedial hypothalamus. *Curr Biol*. 2015;25(10):1340-1346. doi:10.1016/j.cub.2015.03.026.

358. Dewan A, Cichy A, Zhang J, Miguel K, Feinstein P, Rinberg D, Bozza T. Single olfactory receptors set odor detection thresholds. *Nat Commun*. 2018;9(1):1-12. doi:10.1038/s41467-018-05129-0.

359. Silva L, Antunes A. Vomeronasal Receptors in Vertebrates and the Evolution of Pheromone Detection. *Annu Rev Anim Biosci*. 2017;5(1):353-370. doi:10.1146/annurev-animal-022516-022801.

360. Takahashi LK. Olfactory systems and neural circuits that modulate predator odor fear. *Front Behav Neurosci*. 2014;8(MAR):1-13. doi:10.3389/fnbeh.2014.00072.

361. Paxinos G, Franklin K. *The Mouse Brain in Stereotaxic Coordinates, Compact | 978-0-12-374244-5 | Elsevier*.; 2008.

362. Zingg B, Chou X lin, Zhang Z gang, Mesik L, Liang F, Tao HW, Zhang LI. AAV-Mediated Anterograde Transsynaptic Tagging: Mapping Corticocollicular Input-Defined Neural Pathways for Defense Behaviors. *Neuron*. 2017;93(1):33-47. doi:10.1016/j.neuron.2016.11.045.

363. Villano I, Messina A, Valenzano A, Moscatelli F, Esposito T, Monda V, Esposito M, Precenzano F, Carotenuto M, Viggiano A, Chieffi S, Cibelli G, Monda M, Messina G. Basal forebrain cholinergic system and orexin neurons: Effects on attention. *Front Behav Neurosci*. 2017;11(January):1-11. doi:10.3389/fnbeh.2017.00010.

364. Blanco-Centurion C, Bendell E, Zou B, Sun Y, Shiromani PJ, Liu M. VGAT and VGLUT2 expression in MCH and orexin neurons in double transgenic reporter mice. *IBRO Reports*. 2018;4(May):44-49. doi:10.1016/j.ibror.2018.05.001.

365. Schneeberger M, Tan K, Nectow AR, Parolari L, Caglar C, Azevedo E, Li Z, Domingos A, Friedman JM. Functional analysis reveals differential effects of glutamate and MCH neuropeptide in MCH neurons. *Mol Metab*. 2018;13(May):83-89. doi:10.1016/j.molmet.2018.05.001.

366. Espinosa N, Alonso A, Morales C, Espinosa P, Chávez AE, Fuentealba P. Basal Forebrain Gating by Somatostatin Neurons Drives Prefrontal Cortical Activity. *Cereb Cortex*. 2019;29(1):42-53. doi:10.1093/cercor/bhx302.

367. Cummins RA, Walsh RN. The Open-Field Test: A Critical Review. *Psychol Bull*. 1976;83(3):482-504.

368. Gould TD, Dao DT, Kovacsics CE. The Open Field Test. In: *Mood and Anxiety Related Phenotypes in Mice*. Vol 42. Neuromethods; 2009:1-20. doi:10.1007/978-1-60761-303-9.

369. Bodnoff SR, Suranyi-Cadotte B, Quirion R, Meaney MJ. A comparison of the effects of diazepam versus several typical and atypical anti-depressant drugs in an animal model of anxiety. *Psychopharmacology (Berl)*. 1989;97(2):277-279. doi:10.1007/BF00442264.

370. Gritti I, Henny P, Galloni F, Mainville L, Mariotti M, Jones BE. Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. *Neuroscience*. 2006;143(4):1051-1064. doi:10.1016/j.neuroscience.2006.09.024.

371. Saunders A, Granger AJ, Sabatini BL. Corelease of acetylcholine and GABA from cholinergic forebrain neurons. *Elife*. 2015;2015(4):1-13. doi:10.7554/eLife.06412.

372. Zhu C, Yao Y, Xiong Y, Cheng M, Chen J, Zhao R, Liao F, Shi R, Song S. Somatostatin Neurons in the Basal Forebrain Promote High-Calorie Food Intake. *Cell Rep*. 2017;20(1):112-123. doi:10.1016/j.celrep.2017.06.007.

373. Patel JM, Swanson J, Ung K, Herman A, Hanson E, Ortiz-Guzman J, Selever J, Tong Q, Arenkiel BR. Sensory perception drives food avoidance through excitatory basal forebrain circuits. *Elife*. 2019;8:1-28. doi:10.7554/eLife.44548.

374. Anaclet C, Pedersen NP, Ferrari LL, Venner A, Bass CE, Arrigoni E, Fuller PM. Basal forebrain control of wakefulness and cortical rhythms. *Nat Commun*. 2015;6:1-14. doi:10.1038/ncomms9744.

375. Farrow C V., Coulthard H. Relationships between sensory sensitivity, anxiety and selective eating in children. *Appetite*. 2012;58(3):842-846. doi:10.1016/j.appet.2012.01.017.

376. McDougle CJ, Scahill L, Aman MG, McCracken JT, Tierney E, Davies M, Arnold LE, Posey DJ, Martin A, Ghuman JK, Shah B, Chuang SZ, Swiezy NB, Gonzalez NM, Hollway J, Koenig K, McGough JJ, Ritz L, Vitiello B. Risperidone for the core symptom domains of autism: Results from the study by the Autism Network of the Research Units on Pediatric Psychopharmacology. *Am J Psychiatry*. 2005;162(6):1142-1148. doi:10.1176/appi.ajp.162.6.1142.

377. Gray JA, McNaughton N. *The Neuropsychology of Anxiety: An Enquiry into the Functions of the Septo-Hippocampal System*. Vol 33. 2nd ed. Oford University Press; 2003.

378. Veening JG, Böcker KBE, Verdouw PM, Olivier B, De Jongh R, Groenink L. Activation of the septohippocampal system differentiates anxiety from fear in startle paradigms. *Neuroscience*. 2009;163(4):1046-1060. doi:10.1016/j.neuroscience.2009.06.064.

379. Battaglia FP, Benchenane K, Sirota A, Pennartz CMA, Wiener SI. The hippocampus: Hub of brain network communication for memory. *Trends Cogn Sci*. 2011;15(7):310-318. doi:10.1016/j.tics.2011.05.008.

380. Colom L V. Septal networks: Relevance to theta rhythm, epilepsy and Alzheimer's disease. *J Neurochem*. 2006;96(3):609-623. doi:10.1111/j.1471-4159.2005.03630.x.

381. Solari N, Hangya B. Cholinergic modulation of spatial learning, memory and navigation. *Eur J Neurosci*. 2018;48(5):2199-2230. doi:10.1111/ejn.14089.

382. Ma J, Shen B, Stewart LS, Herrick IA, Leung LS. The septohippocampal system participates in general anesthesia. *J Neurosci*. 2002;22(2):2-7. doi:10.1523/jneurosci.22-02-j0004.2002.

383. Colgin LL. Rhythms of the hippocampal network. *Nat Rev Neurosci*. 2016;17(4):239-249. doi:10.1038/nrn.2016.21.

384. Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang CY, Mountjoy K, Kishi T, Elmquist JK, Lowell BB. Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell*. 2005;123(3):493-505. doi:10.1016/j.cell.2005.08.035.

385. Mangieri LR, Jiang Z, Lu Y, Xu YYY, Cassidy RM, Justice N, Xu YYY, Arenkiel BR, Tong Q. Defensive behaviors driven by a hypothalamic-ventral midbrain circuit. *eNeuro*. 2019;6(4):1-19. doi:10.1523/ENEURO.0156-19.2019.

386. Sweeney P, Yang Y. An inhibitory septum to lateral hypothalamus circuit that suppresses feeding. *J Neurosci*. 2016;36(44):11185-11195. doi:10.1523/JNEUROSCI.2042-16.2016.

387. Li MM, Madara JC, Steger JS, Krashes MJ, Balthasar N, Campbell JN, Resch JM, Conley NJ, Garfield AS, Lowell BB. The Paraventricular Hypothalamus Regulates Satiety and Prevents Obesity via Two Genetically Distinct Circuits. *Neuron*. 2019;102(3):653-667.e6. doi:10.1016/j.neuron.2019.02.028.

388. Grill HJ, Norgren R. Chronically decerebrate rats demonstrate satiation but not bait shyness. *Science (80-)*. 1978;201(4352):267-269. doi:10.1126/science.663655.

389. Grill HJ, Norgren R. The taste reactivity test. I. Mimetic responses to gustatory stimuli in neurologically normal rats. *Brain Res*. 1978;143(2):263-279. doi:10.1016/0006-8993(78)90568-1.

390. Grill HJ, Norgren R. The taste reactivity test. II. Mimetic responses to gustatory stimuli in chronic thalamic and chronic decerebrate rats. *Brain Res*. 1978;143(2):281-297. doi:10.1016/0006-8993(78)90569-3.

391. Davis M, Gendelman PM. Plasticity of the acoustic startle response in the acutely decerebrate rat. *J Comp Physiol Psychol*. 1977;91(3):549-563. doi:10.1037/h0077345.

392. Fox JE. Habituation and prestimulus inhibition of the auditory startle reflex in decerebrate rats. *Physiol Behav*. 1979;23(2):291-297. doi:10.1016/0031-9384(79)90370-6.

393. Grill HJ, Norgren R. Neurological tests and behavioral deficits in chronic thalamic and chronic decerebrate rats. *Brain Res*. 1978;143(2):299-312. doi:10.1016/0006-8993(78)90570-X.

394. Dirocco RJ, Grill HJ. The forebrain is not essential for sympathoadrenal hyperglycemic response to glucoprivation. *Science (80-)*. 1979;204(4397):1112-1114. doi:10.1126/science.451558.

395. Kim ER, Fan S, Akhmedov D, Sun K, Lim H, O'Brien W, Xu Y, Mangieri LR, Zhu Y, Lee CC, Chung Y, Xia Y, Xu Y, Li F, Sun K, Berdeaux R, Tong Q. Red blood cell β-adrenergic receptors contribute to diet-induced energy expenditure by increasing O2 supply. *JCI insight*. 2017;2(14):1-13. doi:10.1172/jci.insight.93367.

396. Bernardis LL, Bellinger LL. The lateral hypothalamic area revisited: Ingestive behavior. *Neurosci Biobehav Rev*. 1996;20(2):189-287. doi:10.1016/0149-7634(95)00015-1.

397. Craig W. Appetites and aversions as constituents of instincts. *Proc Natl Acad Sci U S A*. 1917;3(12):685-688. doi:10.1073/pnas.3.12.685.

398. Sweeney P, Li C, Yang Y. Appetite suppressive role of medial septal glutamatergic neurons. *Proc Natl Acad Sci U S A*. 2017;114(52):13816-13821. doi:10.1073/pnas.1707228114.

399. Xu YY, Lu Y, Cassidy RM, Mangieri LR, Zhu C, Huang X, Jiang Z, Justice NJ, Xu YY, Arenkiel BR, Tong Q. Identification of a neurocircuit underlying regulation of feeding by stress-related emotional responses. *Nat Commun*. 2019;10(1). doi:10.1038/s41467-019-11399-z.

400. Cassidy RM, Lu Y, Jere M, Tian J Bin, Xu YYYY, Mangieri LR, Felix-Okoroji B, Selever J, Xu YYYY, Arenkiel BR, Tong Q. A lateral hypothalamus to basal forebrain neurocircuit promotes feeding by suppressing responses to anxiogenic environmental cues. *Sci Adv*. 2019;5(3). doi:10.1126/sciadv.aav1640.

401. Füzesi T, Daviu N, Wamsteeker Cusulin JI, Bonin RP, Bains JS. Hypothalamic CRH neurons orchestrate complex behaviours after stress. *Nat Commun*. 2016;7(May):1-14. doi:10.1038/ncomms11937.

402. Korotkova T, Ponomarenko A, Monaghan CK, Poulter SL, Cacucci F, Wills T, Hasselmo ME, Lever C. Reconciling the different faces of hippocampal theta: The role of theta oscillations in cognitive, emotional and innate behaviors. *Neurosci Biobehav Rev*. 2018;85(September 2017):65-80. doi:10.1016/j.neubiorev.2017.09.004.

403. Jimenez JC, Su K, Goldberg AR, Luna VM, Biane JS, Ordek G, Zhou P, Ong SK, Wright MA, Zweifel L, Paninski L, Hen R, Kheirbek MA. Anxiety Cells in a Hippocampal-Hypothalamic Circuit. *Neuron*. 2018;97(3):670-683.e6. doi:10.1016/j.neuron.2018.01.016.

404. Barbano MF, Wang HL, Morales M, Wise RA. Feeding and reward are differentially induced by activating GABAergic lateral hypothalamic projections to VTA. *J Neurosci*. 2016;36(10):2975-2985. doi:10.1523/JNEUROSCI.3799-15.2016.

405. Post AM, Weyers P, Holzer P, Painsipp E, Pauli P, Wultsch T, Reif A, Lesch KP. Gene-environment interaction influences anxiety-like behavior in ethologically based mouse models. *Behav Brain Res*. 2011;218(1):99-105. doi:10.1016/j.bbr.2010.11.031.

406. Rodgers RJ, Cole JC. Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. *Physiol Behav*. 1993;54(4):729-736. doi:10.1016/0031-9384(93)90084-S.

407. Fenno LE, Mattis J, Ramakrishnan C, Hyun M, Lee SY, He M, Tucciarone J, Selimbeyoglu A, Berndt A, Grosenick L, Zalocusky KA, Bernstein H, Swanson H, Perry C, Diester I, Boyce FM, Bass CE, Neve R, Huang ZJ, Deisseroth K. Targeting cells with single vectors using multiple-feature Boolean logic. *Nat Methods*. 2014;11(7):763-772. doi:10.1038/nmeth.2996.

408. Nasirova N, Quina LA, Agosto-Marlin IM, Ramirez JM, Lambe EK, Turner EE. Dual recombinase fate mapping reveals a transient cholinergic phenotype in multiple populations of developing glutamatergic neurons. *J Comp Neurol*. 2020;528(2):283-307. doi:10.1002/cne.24753.

409. Goswami S, Rodríguez-Sierra O, Cascardi M, Paré D. Animal models of post-traumatic stress disorder: Face validity. *Front Neurosci*. 2013;7(7 MAY):1-14. doi:10.3389/fnins.2013.00089.

410. Panksepp J, Biven L. *The Archaeology of the Mind: Neuroevolutionary Origins of Human Emotions*.; 2012.

411. Panksepp J. The basic emotional circuits of mammalian brains: Do animals have affective lives? *Neurosci Biobehav Rev*. 2011;35(9):1791-1804. doi:10.1016/j.neubiorev.2011.08.003.

412. Birgner C, Nordenankar K, Lundblad M, Mendez JA, Smith C, Le Grevès M, Galter D, Olson L, Fredriksson A, Trudeau LE, Kullander K, Wallén-Mackenzie Å. VGLUT2 in dopamine neurons is required for psychostimulant-induced behavioral activation. *Proc Natl Acad Sci U S A*. 2010;107(1):389-394. doi:10.1073/pnas.0910986107.

413. Strand AD, Aragaki AK, Baquet ZC, Hodges A, Cunningham P, Holmans P, Jones KR, Jones L, Kooperberg C, Olson JM. Conservation of regional gene expression in mouse and human brain. *PLoS Genet*. 2007;3(4):0572-0583. doi:10.1371/journal.pgen.0030059.

414. Wise RA. Addictive Drugs and Brain Stimulation Reward. *Annu Rev Neurosci*. 1996;19(1):319-340. doi:10.1146/annurev.neuro.19.1.319.

415. Bishop MP, Elder ST, Heath RG. Intracranial Self-Stimulation in Man. *Science (80-)*. 1963;140(3565):394-396.

416. Olds ME, Fobes JL. The Central Basis of Motivation: Intracranial Self-Stimulation Studies. *Annu Rev Psychol*. 1981;32(1):523-574. doi:10.1146/annurev.ps.32.020181.002515.

417. Santillo AF, Lundblad K, Nilsson M, Waldö ML, Van Westen D, Lätt J, Nordström EB, Vestberg S, Lindberg O, Nilsson C. Grey and white matter clinico-anatomical correlates of disinhibition in neurodegenerative disease. *PLoS One*. 2016;11(10):1-19. doi:10.1371/journal.pone.0164122.

418. Sheelakumari R, Bineesh C, Varghese T, Kesavadas C, Verghese J, Mathuranath PS. Neuroanatomical correlates of apathy and disinhibition in behavioural variant frontotemporal dementia. *Brain Imaging Behav*. 2019. doi:10.1007/s11682-019-00150-3.

419. Osborne-Crowley K, McDonald S. A review of social disinhibition after traumatic brain injury. *J Neuropsychol*. 2018;12(2):176-199. doi:10.1111/jnp.12113.

420. Cohen-Zimerman S, Chau A, Krueger F, Gordon B, Grafman J. Machiavellian tendencies increase following damage to the left dorsolateral prefrontal cortex. *Neuropsychologia*. 2017;107:68-75. doi:10.1016/j.neuropsychologia.2017.11.007.

421. Kirlic N, Young J, Aupperle RL. Animal to human translational paradigms relevant for approach avoidance conflict decision making. *Behav Res Ther*. 2017;96:14-29. doi:10.1016/j.brat.2017.04.010.

422. Walz N, Mühlberger A, Pauli P. A Human Open Field Test Reveals Thigmotaxis Related to Agoraphobic Fear. *Biol Psychiatry*. 2016;80(5):390-397. doi:10.1016/j.biopsych.2015.12.016.

423. Biedermann S V., Biedermann DG, Wenzlaff F, Kurjak T, Nouri S, Auer MK, Wiedemann K, Briken P, Haaker J, Lonsdorf TB, Fuss J. An elevated plus-maze in mixed reality for studying human anxiety-related behavior. *BMC Biol*. 2017;15(1):1-13. doi:10.1186/s12915-017-0463-6.

424. Hetherington MM. Understanding infant eating behaviour – Lessons learned from observation. *Physiol Behav*. 2017;176:117-124. doi:10.1016/j.physbeh.2017.01.022.

425. Alley TR. *Conceptualization and Measurement of Human Food Neophobia*. Elsevier Ltd; 2018. doi:10.1016/b978-0-08-101931-3.00009-4.

426. Zucker N, Copeland W, Franz L, Carpenter K, Keeling L, Angold A, Egger H. Psychological and psychosocial impairment in preschoolers with selective eating. *Pediatrics*. 2015;136(3):e582-e590. doi:10.1542/peds.2014-2386.

Vita

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