LATERAL HYPOTHALAMIC NEUROTENSIN NEURONS ORCHESTRATE INGESTIVE BEHAVIORS

By

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ABSTRACT

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Food and water are necessary for survival but can only be obtained via ingestive behaviors (feeding, drinking and moving). Survival thus depends on the ability of the brain to coordinate the need for water and energy with appropriate behaviors to modify their intake as necessary for homeostasis. However, the balance of these behaviors also inherently determines body weight, and imbalances contribute to the development of weight disorders such as obesity and anorexia nervosa. The lateral hypothalamic area (LHA) of the brain is anatomically positioned to coordinate the sensation of osmotic and energy status with goal-directed ingestive behaviors necessary to maintain homeostasis and body weight, and, hence, may hold insight into potential treatment for energy balance disorders. This work was done to evaluate how neurotensin expressing LHA neurons (LHA Nts neurons) coordinate ingestive behaviors. The central hypothesis of this thesis was that *the LHA neurotensin (Nts) neurons direct drinking behavior in response to physiologic need (thirst) via neural outputs to the lateral preoptic area (LPO), but do not modify feeding via LPO.* The rationale for this hypothesis was that the LHA neurons promotes drinking, but suppresses feeding. In order to understand the nature of the drinking regulation by LHA Nts neurons, we experimentally activated LHA Nts neurons and studied drinking behavior under well-hydrated and dehydrated conditions. We observed that activation of LHA Nts neurons promotes voracious drinking of water and water-based solutions and with the given choice, animals prefer water and palatable

solutions, regardless of the caloric content, rather than bitter and dehydrating solutions. We also found that activation increases the motivation to drink in well hydrated and thirsty animals beyond their perceived thirst. In addition, we showed that LHA Nts neural activation is not necessary for homeostatic and need based consumption. Furthermore, we mapped the connectivity of the LHA Nts neurons within the brain and provided a list of osmo- and energy-sensory and regulatory areas which are directly connected to LHA Nts neurons. Finally, we identified LHA Nts→LPO circuit that drives the drinking effect of LHA Nts neurons, but is not the route they suppress feeding. Overall, this work suggests that control of drinking and feeding by LHA Nts circuits is divergent and modulation of specific LHA Nts circuits may be useful therapeutic targets to treat dysregulated water intake.

To my biological and science family

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KEY TO ABBREVIATIONS

3V	:	third ventricle
4V	:	fourth ventricle
aca	:	anteriorcommissure / anterior part
AgRP	:	agouti-related peptide
ANG I	:	angiotensin I
ANG II	:	angiotensin II
ANOVA	:	analyses of variance
ANP	:	atrial natriuretic peptide
Aq	:	aquaduct
ARC	:	arcuate nucleus
AVP	:	arginine-vasopressin
BMI	:	body mass index
BNST	:	bed nucleus of stria terminalis
CART	:	cocaine- and amphetamine-regulated transcript
CEA	:	central amygdala
CNO	:	clozapine N-oxide
CNS	:	central nervous system
CPP	:	conditioned place preference
CRH	:	corticotropin-releasing hormone
DA	:	dopamine
D3V	:	dorsal third ventricle

DE	:	dehydrated
DKA	:	diabetic ketoacidosis
DMH	:	dorsomedial hypothalamic nucleus
DREADDs	:	designer receptors exclusively activated by designer drugs
EU	:	euhydrated
f	:	fornix
FR	:	fixed ratio
GABA	:	gamma-aminobutyric acid
GAD	:	glutamate decarboxylase
Gal	:	galanin
GFP	:	green fluorescent protein
GHSR	:	growth hormone secretagogue receptor
GLP-1	:	glucagon-like peptide-1
GLP1R	:	glucagon-like peptide 1 receptor
HA	:	hemagglutinin
HCRT	:	hypocretin
HHS	:	hyperglycemic hyperosmolar state
hr	:	hour
IC	:	inferior colliculus
ic	:	Internal capsule
i.p	:	intraperitonal
LC	:	locus ceruleus
LHA	:	lateral hypothalamic area

lHb	:	lateral habenula
LPS	:	lipopolysaccharide
LT	:	lamina terminalis
LV	:	lateral ventricle
LepR	:	leptin receptor
LepRb	:	leptin receptor, long form
LPO	:	lateral preoptic area
MC4R	:	melanocortin-4 receptor
MCH	:	melanin concentrating hormone
MDMA	:	3,4-Methylenedioxymethamphetamine
mlf	:	medial longitudinal fasciculus
MM	:	medial mammallary nucleus
MnPO	:	median preoptic area
MPO	:	medial preoptic area
mt	:	mammillothalamic tract
NAc	:	nucleus accumbens
NAc C	:	nucleus accumbens / core part
NAc Sh	:	nucleus accumbens / shell part
NARP/Nptx2	:	neuronal pentraxin
NOS	:	nitric oxide synthase
NPY	:	neuropeptide Y
Nts	:	neurotensin
NTS	:	nucleus of solitary tract

NtsR1	:	neurotensin receptor 1
NtsR2	:	neurotensin receptor 2
o/n	:	overnight
OVLT	:	organum vaculasum latera terminalis
OX	:	orexin
PAG	:	periaqueductal gray
PB	:	parabrachial nucleus
PBS	:	phosphate buffered saline
PFA	:	perifornical hypothalamic area
PMV	:	ventral premammillary nucleus
POA	:	preoptic area
POMC	:	proopiomelanocortin
PR	:	progressive ratio
PVH	:	paraventricular nucleus of hypothalamus
RAAS	:	renin-angiotensin-aldosterone system
RM-ANOVA	:	repeated measures-analyses of variance
Scp	:	superior cerebellar peduncle
SFO	:	subfornical organ
SNc	:	substantia nigra / compact part
SNr	:	substantia nigra / reticular part
SO	:	supraoptic nucleus
S	:	supplementary
T2D	:	type 2 diabetes

TH	:	tyrosine hydroxylase
TRH	:	thyrotropin releasing hormone
UCN3	:	urocortin 3
vGAT	:	vesicular GABA transporter
VEH	:	vehicle
VMH	:	ventromedial hypothalamus
vHP	:	ventral hippocampus
VTA	:	ventral tegmental area
YFP	:	yellow fluorescent protein
ZI	:	zona incerta

CHAPTER 1. LATERAL HYPOTHALAMIC CONTROL OF ENERGY BALANCE

Authors: Gizem Kurt, Hillary L. Woodworth, and Gina M. Leinninger

This chapter is a modified version of a published book in Colloquium Series on Integrated Systems Physiology: From Molecule to Function (Morgan & Claypool)[1].

1.1 IMPLICATIONS OF THE LATERAL HYPOTHALAMIC AREA IN ENERGY BALANCE

1.1.1 Homeostasis and Ingestive Behavior

Perhaps the most fundamental theme of physiology is homeostasis: the maintenance of a relatively stable internal environment necessary to support life. Two essential components for homeostasis are adequate stores of energy (derived from caloric intake) and fluid (water), both of which are essential for cell, system and bodily health. However, the very physiological processes used to sustain life (e.g. respiration, thermogenesis, movement, digestion) constantly tap bodily reserves of energy and water so that they must be continually replenished. Because food and water cannot be synthesized within the body they must be replaced via ingestion. Preservation of energy and fluid homeostasis, thus, requires that animals constantly assess their internal environment, detect need for energy and/or water and then execute the appropriate feeding and/or drinking behaviors to obtain these resources from the environment. The feelings of hunger and thirst serve to communicate the body's need for food and water to the brain so that it can coordinate the appropriate ingestive behavior (feeding or drinking) to restore homeostasis. An important byproduct of this process is regulation of body

weight, which is a visible proxy for homeostasis and whether adequate resources are available to support bodily health. For example, fasting induced hunger or dehydration-induced thirst increase the motivation to find and ingest food and water, respectively[2,3]. Failure to obtain these resources results in acute weight loss that initially strengthens the drives to obtain them, and to avoid prolonged depletion of energy and fluid reserves that would compromise survival. Resource excess is coordinated with behavioral responses to limit intake: stomach fullness or increased body fat cue the cessation of feeding[4,5] while plasma hypotonicity biases for salt vs. water intake to restore fluid homeostasis[6]. Thus, individuals vigilantly monitor fluid and energy status and coordinate appropriate ingestive behaviors that impact body weight and survival. While work over the past decades indicates that the brain is crucial for orchestrating drive states, behavior and body weight, the precise neural circuits underlying these processes remain incompletely understood. Herein I will address the role of a particular part of the brain, the lateral hypothalamic area (LHA), in coordinating energy balance, homeostasis and, hence, in the physiology underlying health and survival.

1.1.2 What is Energy Balance and How Does it Relate to Health?

Energy homeostasis is often referred to and illustrated as “energy balance,” to convey the inter- dependent relationship between energy intake and expenditure that determines body weight and health (Figure 1-1A). Energy intake consists of calories consumed through food and caloric- liquids, such as milk, juices, or sugar-laden sports drinks and soda. Energy expenditure refers to the calories that are consumed by the body to support basal metabolism and behavior, in the form of voluntary physical activity. However, this “calories in – calories out” definition is an over simplified version of what is

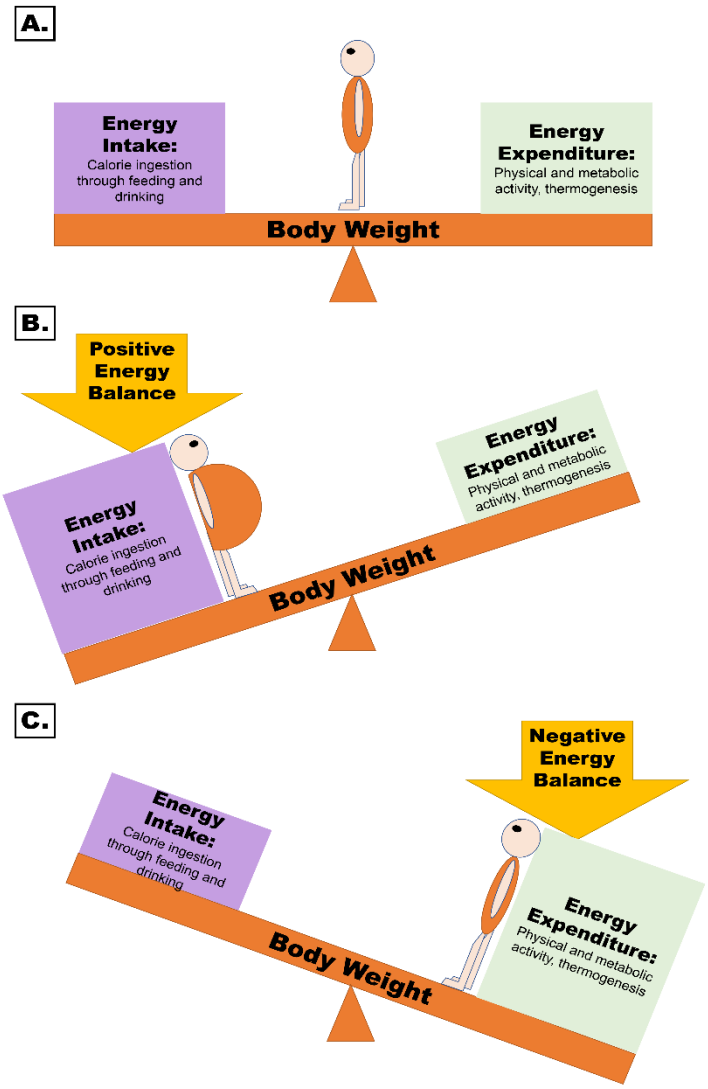

Figure 1-1. Energy balance. (A) The interdependent relationship between energy intake and expenditure that determines body weight. **(B)** Positive energy balance with increased energy intake and decreased energy expenditure leading to weight gain. **(C)** Negative energy balance with decreased energy intake and increased energy expenditure leading to weight loss.

going on in our bodies and undermines the complex interactions between peripheral cues, brain, genes, environment and unique metabolism of the individual. The interaction of all these factors contributes to a delicate energy balance with a homeostatic set-point. Under normal physiologic conditions, deviations from energy homeostasis are countered by adjustment of bodily mechanisms and/or behaviors to facilitate return to the energy balance set-point. Yet, this process can be disrupted, due to alterations in hormonal signaling, metabolic rate or ingestive behaviors, and can lead to positive or negative energy balance[7–14]. Positive energy balance is marked by higher calorie intake than calorie expenditure, leading to increase in body weight (Figure 1-1B) whereas negative energy balance is marked by lower calorie intake than calorie expenditure, leading to decrease in body weight (Figure 1-1C).

Excess positive energy balance leading to obesity is a major health concern, as the worldwide prevalence of obesity and overweight has increased dramatically over the past decades[15,16]. While there are rare monogenetic causes of obesity[17–29], most obesity cases are due to increased caloric intake[30] and decreased average physical activity levels[31,32]. Notably, the abundance and ease of access to palatable, calorie-rich, and inexpensive foods is considered to be the major driver of obesity in developed countries[33]. Indeed, the United States of America (U.S.) is experiencing an obesity epidemic[16,34], with self-reported adult obesity rates exceeding 35% of the population in many states (Figure 1-2). While geographic and socioeconomic factors may also play roles in the development of overweight and obesity[35], the incidence rates are high across demographics and regions of the U.S. Additionally, the growing occurrence of U.S.

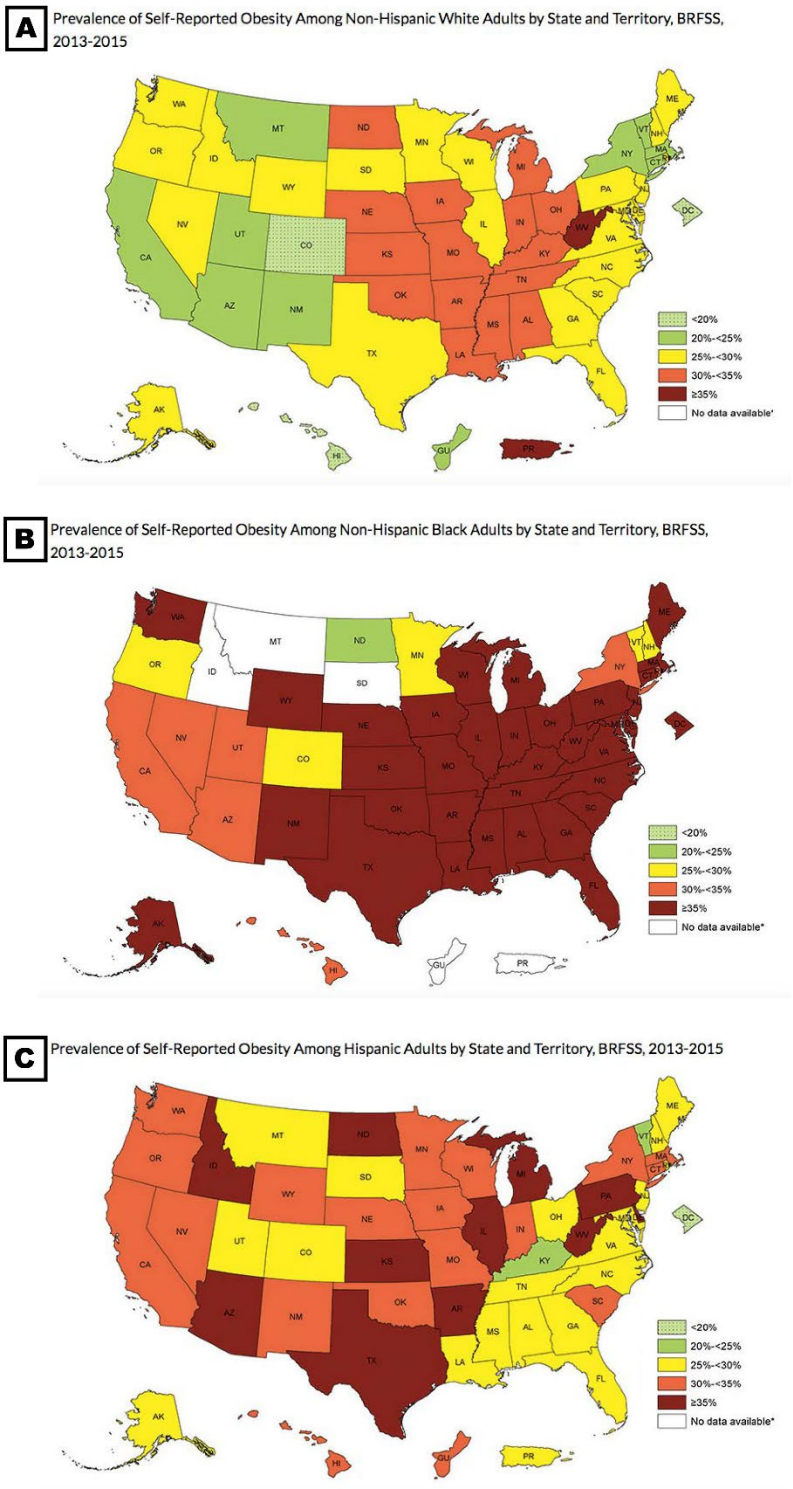

Figure 1-2. Self-reported obesity. *CDC adult obesity prevalence maps*[251] *obtained*

Figure 1-2 (*cont'd*) *from CDC website on August 10th, 2017 and available online at* https://www.cdc.gov/obesity/data/prevalence-maps.html. **(A)** Prevalence of self reported obesity among non-Hispanic white adults by state and territory 2013-2015. **(B)** Prevalence of self-reported obesity among non-Hispanic black adults by state and territory 2013-2015. **(C)** Prevalence of self-reported obesity among Hispanic white adults by state and territory 2013-2015.

childhood and adolescent obesity (Figure 1-3) puts these individuals at risk for early development of chronic obesity-linked conditions (Figure 1-4)**,** such as type-2 diabetes, cardiovascular disease, stroke and cancer that require lifelong management and which can reduce lifespan[36,37].

Despite the alarming increase in the rates and health consequences of obesity, the recommended and most commonly prescribed treatment has remained the same over decades: diet and exercise[38]. This treatment can be effective, and many dieters can lose weight acutely, but sustained weight loss requires dramatic, lifelong behavior changes that are hard to maintain. Moreover, weight loss via diet and exercise is not a sustainable approach unless the lifestyle alterations are consistent and permanent. Inconsistent compliance, or so called "yo-yo dieting" (meaning being on and off diet) often occurs, but can be detrimental, as it can slow down weight loss and cause adaptive biological responses that lead to weight regain[39,40]. It has also been shown that yo-yo dieting elevates heart disease, stroke and diabetes risk[39]. Due to the difficulty maintaining diet and exercise treatment, obesity is defined as a chronic and relapsing condition[40]. A second approach to treat obesity is the use of pharmacotherapies[40]. There are several

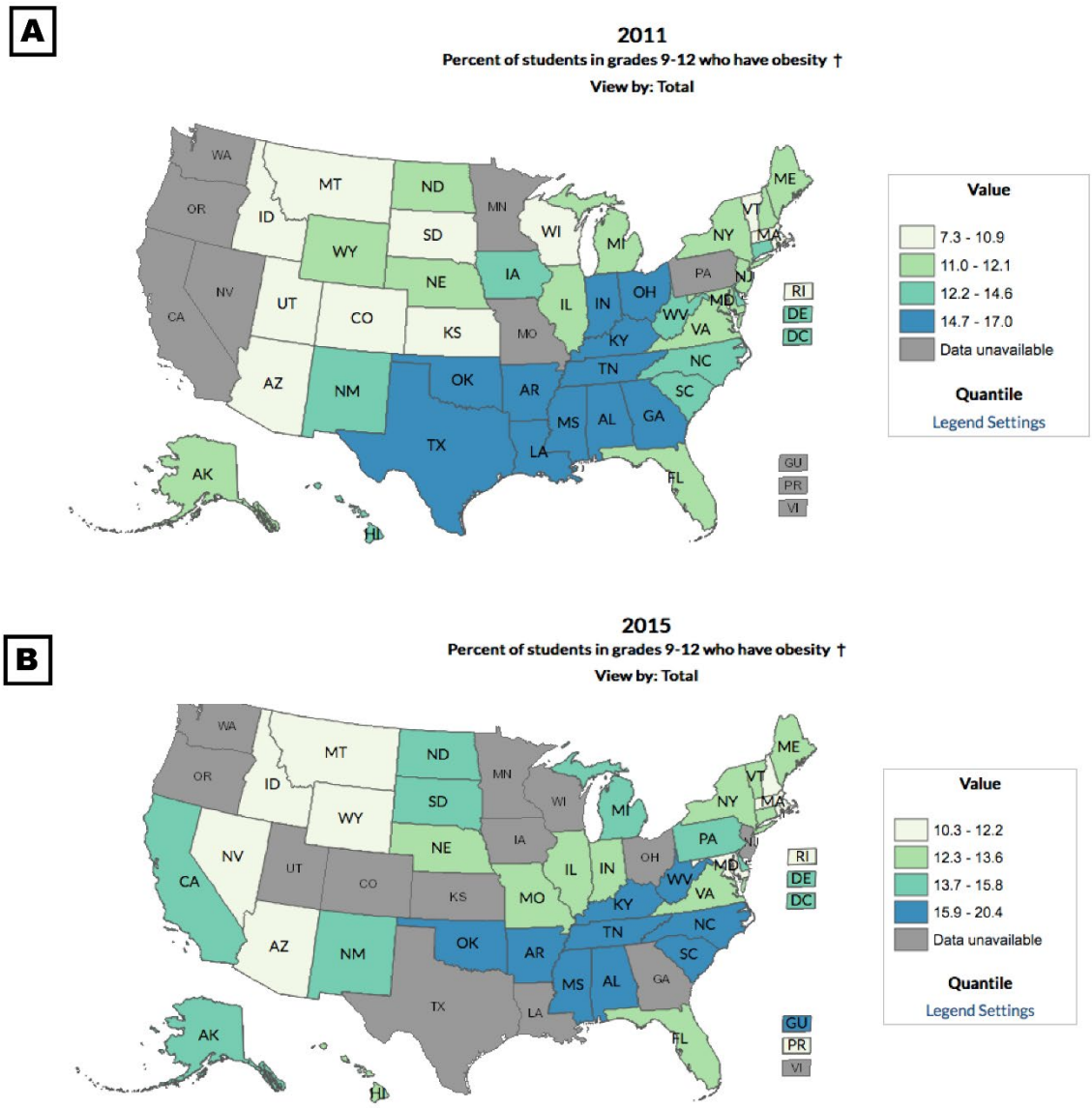

Figure 1-3. Obesity in students in grades 9-12. *CDC Nutrition, Physical Activity, and Obesity: Data,Trends and Maps*[252] *obtained from CDC website on August 10th, 2017 and available online at* https://www.cdc.gov/nccdphp/dnpao/data-trends-maps/index.html **(A)** Percent of students in grades 9-12 who have obesity by US states, 2011. **(B)** Percent of students in grades 9-12 who have obesity by US states, 2015.

FDA approved pharmacotherapy agents on the market, targeting fat absorption, glucagon-like peptide-1 (GLP-1) and gamma-aminobutyric acid (GABA) receptors, serotonergic, dopaminergic and adrenergic systems[40]. Although up to 6–8% total body weight loss might be achieved by use of these drugs, most of them are not approved for long term use and have severe side effects, such as increasing the risk of heart disease, headaches, insomnia, seizures, nausea, dizziness, constipation, diarrhea, fatigue, hypoglycemia[40]. The only other treatment alternatives are gastric bypass or device implementation surgeries, which are highly invasive, costly and only recommended for patients with body mass index (BMI) over 35[40–42]. Even with these invasive approaches, the failure rates range from 5-30% with relapse commonly observed after around 1.5years post-surgery[40]. In addition to the risk of failure and relapse as well as the very invasive procedure with potential surgery complications, there is also a less talked dark psychosocial side of bariatric surgery. In the long term, increased relapsed or de novo depression as well as suicide risk have been reported in post-surgery patients[43]. There is also an elevated risk of developing de novo alcohol abuse disorder in a minor but significant portion of post-surgery patients[44]. Hence, it is crucial to understand peripheral and brain mechanisms controlling the body weight to develop better strategies to effectively treat obesity and its health consequences.

1.1.3 What is Osmotic Balance and How Does it Relate to Health?

Water intake is crucial for maintaining cellular osmolality and survival; thus, thirst is an essential drive to maintain health. Inappropriate body water can occur due to insufficient intake, (causing dehydration) or intake beyond homeostatic need., but either imbalance can be lethal. Dehydration has been associated with decline in cognitive

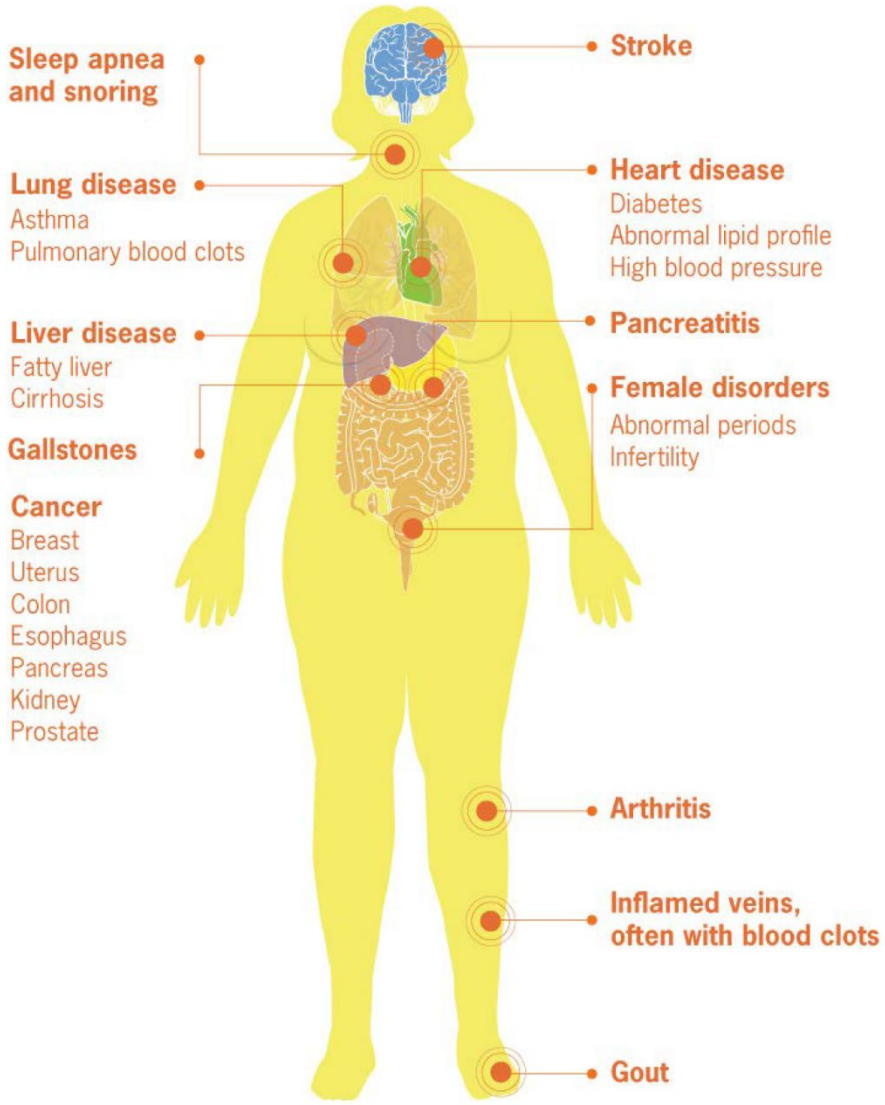

Figure 1-4. Obesity complications. *CDC Medical Complications of Obesity*[253] *obtained from CDC website on August 10th, 2017 and available online at* https://www.cdc.gov/vitalsigns/adultobesity/infographic.html

function, increased risk of cardiovascular disease, chronic kidney diseases, hypertension, stroke, reduced physical performance and headaches[45–47]. Prolonged dehydration is lethal. Osmolality imbalance can also occur due to excessive water intake after extreme

exercise, use of MDMA (also known as Ecstasy or Molly) or in psychogenic polydipsia. Excessive water intake can lead to water intoxication and cause hyponatremia, neurologic symptoms, seizures, coma and death[48–52].

Decline of thirst sensation with aging is of particular concern, as it renders the large population of elderly individuals prone to dehydration-induced sequalae[53,54]. It has been repeatedly shown that following dehydration due to exercise, extreme temperatures, polyuria, side effects of the drugs or overnight dehydration due to no access to water, the elderly have lower thirst sensation and drinking compared to young individuals[45,46,55]. Similarly, elderly persons demonstrate reduced thirst sensation and water consumption under osmotic thirst conditions created by hyperosmotic stimuli, i.e. hypertonic saline infusion, although there are also conflicting literature on osmotic thirst in the elderly[46,53,55]. Besides the reduction in thirst perception, decreased baroreceptor sensitivity with aging has also been observed, contributing to the dysregulation of water balance in elderly persons[55]. Elderly patients with type 2 diabetes (T2D) are particularly vulnerable to osmotic disruption. In T2D patients, the combination of polyuria and reduced thirst sensation can elevate hyperglycemic state and lead to diabetic ketoacidosis (DKA) or hyperglycemic hyperosmolar state (HHS), diabetic complications that have high morbidity and mortality[56–58]. Besides the health consequences, the economic burden of dehydration associated health problems is also heavy. In 1999, it was estimated that the cost of dehydration-associated hospitalizations in patients over 65 years old was more than 1 billion dollars[59]. Given that the size of the elderly population continues to increase, it is likely that these serious health and economic tolls will increase commensurately.

Despite the exigency of water for survival and related health consequences, we still know very little about the physiological mechanisms by which the brain coordinates the need for water with the behavior to get it. Therefore, it is essential that we learn about the brain circuits controlling thirst and hydration, to inform development of much-needed pharmacological strategies to combat dysregulated water balance in vulnerable populations such as the elderly, children, athletes and patients with polydipsia, T2D and obesity.

1.1.4 The Brain Coordinates Energy and Osmotic Balance

The brain monitors energy cues coming from the periphery and the environment and adjusts behaviors as needed to restore or maintain homeostasis. The arcuate nucleus (ARC)[60], paraventricular nucleus (PVH)[61–63], ventromedial hypothalamus (VMH) [64–66], lateral hypothalamic area (LHA) of the hypothalamus and the hindbrain (mainly the neurons of nucleus of solitary tract (NTS))[67] have all been shown to contribute to body weight regulation.

The ARC is an area where peripheral cues are rapidly sensed and can be integrated. The ARC serves as a “first responder” due to its position adjacent to the median eminence, a relatively leaky area of the blood brain barrier; here, contents from the circulation can more readily and rapidly enter the ARC compared to other brain regions. ARC neurons are grouped into 2 broad categories: orexigenic neurons promoting food intake (agouti-related peptide (AgRP) and neuropeptide Y (NPY) neurons) and anorexigenic neurons suppressing food intake (proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons)[60]. Hunger signals, such as the hormone ghrelin, activate AgRP neurons, promoting food seeking and intake,

whereas energy surfeit signals (i.e. glucose, leptin, insulin) inhibit AgRP neurons[68,69]. NPY is co-expressed in ARC AgRP neurons and also promotes consumption[70,71]. On the other hand, energy surfeit cues activate ARC POMC neurons leading to suppression of intake and promotion of energy expenditure[60,72]. Similarly, CART in the ARC promotes energy expenditure and elevates physical activity and ARC CART expression mainly colocalizes with POMC expression[73,74]. Besides the ARC neurons, specialized glial cells in the ARC, tanyctes, have been shown to be indispensable for leptin entry to the brain[75,76]. Another area that is heavily indicated in feeding control is the NTS, which is mainly indicated in receiving, integrating and relaying the vagal inputs coming from the gastrointestinal tract[67]. Besides the satiation signals carried through vagal nerve, NTS neurons can also sense peripheral energy state cues, including. leptin, glucose, ghrelin and GLP-1[67,77]. The LHA is one of the main contributors to feeding and energy homeostasis and it will be separately discussed later in this chapter.

Just like the energy status of the body, hydration and osmotic status are constantly monitored and fine-tuned by communication between the periphery and the brain. Humans, as all terrestrial species, have to drink water to maintain appropriate osmolality, and to compensate for water lost through essential bodily processes such as respiration, urination, sweating, salivation and by other means. Failure to regulate hydration compromises the cellular and tissue function, especially in the central nervous system (CNS). The perception of thirst is therefore critical to ensure appropriate water levels necessary for optimal health and survival.

At the peripheral level, the baroreceptors and the kidneys are the main players that detect volume and osmolality fluctuations; if deviations occur, they trigger a cascade of

events to inform the brain to promote necessary actions to restore fluid homeostasis. The kidneys are the main sensors and triggers of a hormonal pathway called the renin-angiotensin-aldosterone system (RAAS)[55,78]. In the RAAS system, the cascade of events is as follows: renin is secreted by the kidney in response to hypovolemia, leading to conversion of angiotensinogen to angiotensin I (ANG I) and then angiotensin II (ANG II), which in turn stimulates aldosterone release. Besides stimulating aldosterone release, which enhances Na^+ reabsorption in kidneys, ANG II also promotes vasoconstriction to combat reduced blood flow to the tissues and triggers arginine-vasopressin (AVP) release from the posterior pituitary, which promotes water retention[78]. On the other hand, with hypervolemia and the detection of stretching via heart baroreceptors, atrial natriuretic peptide (ANP) secretion is promoted, resulting in reduction in Na^+ reabsorption in kidneys, hence, increasing the water excretion[78].

In the brain, osmo-sensitive regions in the hypothalamus monitor plasma osmolality and control thirst (intake) and arginine-vasopressin (AVP) production and release (excretion)[78–80]. Several brain regions have been shown to contribute to the regulation of the hydration and osmolality, including the circumventricular organs (subfornical organ (SFO), organum vasculasum latera terminalis (OVLT) and the median preoptic area (MnPO), AVP-producing areas, the paraventricular nucleus of hypothalamus (PVH) and supraoptic nucleus (SO), the nucleus of solitary tract (NTS) and the parabrachial nucleus (PB)[79,80]. The main focus in water balance research to date has been on the circumventricular regions (SFO, OVLT and MnPO) that can directly sense osmolality. Several of these SFO, OVLT and MnPO neural populations have been marked as thirst promoting neurons, including glutamatergic neruons in the SFO and nitric oxide

synthase (NOS) expressing neurons in the MnPO [81–84]. Inhibitory neurons in these regions are also implicated in thirst, including GABAergic neurons in the SFO and glucagon-like peptide 1 receptor (GLP1R) expressing GABAergic neurons in the MnPO [83,84]. The brain can also detect anticipated fluctuations in osmolality and trigger the appropriate behavior to compensate any change in the immediate future[85,86] . Moreover, the brain assigns the dehydrated state a negative valence and promotes the actions to avoid it[62]. In addition to these circumventricular regions, the LHA has also been implicated in drinking behavior, and the LHA-drinking literature will be discussed later in this this chapter.

1.1.5 Discovery of a Role for the Lateral Hypothalamic Area (LHA) in Energy Balance

The first study to suggest a function for the LHA resulted from an experimental accident, and serves as a reminder to let the data (not a preconceived hypothesis) lead understanding of the underlying physiology. Scientists had been studying animals with brain lesions in specific hypothalamic sub-regions, reasoning that the observed deficits in "lesioned" animals indicate what behavior and physiology is normally controlled via the site. Lesions within the ventromedial hypothalamus (VMH) caused striking overeating and subsequent development of obesity, and as a result of these experiments the VMH was declared a "satiety center" whose intact function is necessary for normal body weight (Figure 1-5). Anand and Brobeck sought to further define the VMH mechanisms that coordinate energy balance, so they generated a cohort of "VMH-lesioned" rats. They

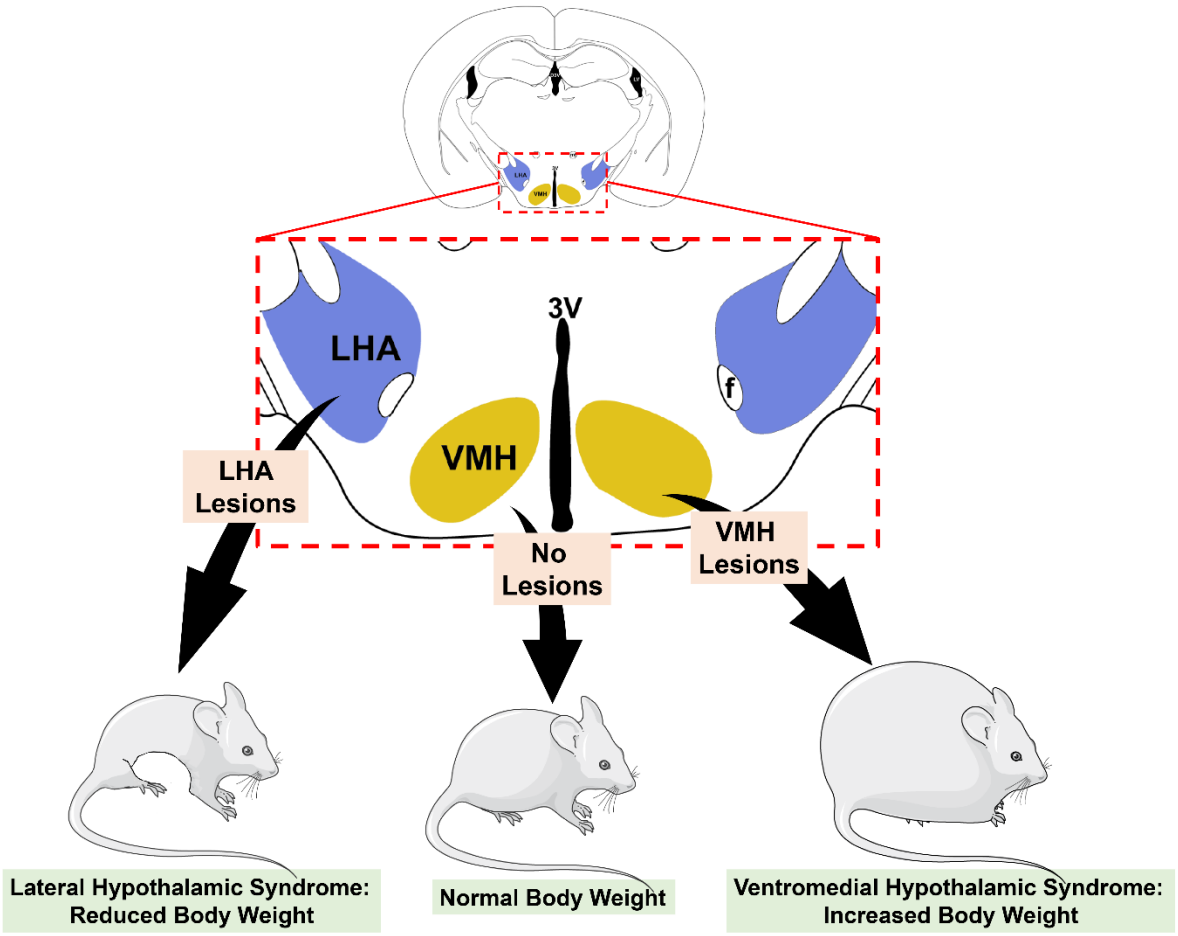

Figure 1-5. Impact of the VMH and LHA lesions on body weight in rodents. *Coronal brain section as modified from the Paxinos and Franklin (2001) mouse brain atlas*[137]*. Rodent illustrations*[254] *were modified from Smart Servier Medical Art on October 24th, 2017, available online at* http://smart.servier.com/category/general-items/animals/

expected to observe the hyperphagia and obesity characterized in prior VMH lesion studies, but their experimental rats unexpectedly exhibited such severe, self-imposed aphagia that they died of starvation unless they were force-fed by the experimenters[87]. Subsequent examination of the brains from these rats revealed the source of the

discrepancy: the lesions were not targeted to the VMH as intended, but instead were within the LHA. This serendipitous experiment promoted a view of LHA as the "feeding center"[87], thought to counteract the effects of the VMH "satiety center." LHA lesions were subsequently made in cats[88,89] and monkeys[89] and produced similar feeding suppression as those made in rats. By contrast, electrical stimulation of the LHA increases feeding[88], exploratory behaviors and intestinal motility in cats [90]. In rats, electrical stimulation of the LHA not only increased feeding but also the motivation to obtain food, determined by the rats willingness to press a lever and cross an electrical shock grid to obtain food [91]. Similarly, activating the LHA of goats triggered feeding and locomotion[92], which might reflect the fact that movement is necessary for these animals to procure food.

Taken together, the early lesion and activation studies were interpreted to support a "dual center hypothesis" in which the LHA and VMH exert antagonistic control of feeding. These descriptions are now recognized as oversimplifications, since the LHA and VMH are now accepted to contribute to many aspects of physiology beyond just feeding. Nonetheless, these loss and gain-of function manipulations provided the first clue that specific hypothalamic sub-regions control strikingly different behaviors, and that there must be different, brain-region specific mechanisms to regulate feeding and energy balance.

1.1.6 "Lateral Hypothalamic Syndrome" Suggests an Essential Role for the LHA in Coordinating Behavior

The LHA was initially deemed a "feeding center" because animals with LHA lesions will not voluntarily consume food[93,94]. Less emphasized, but equally important, is that LHA-lesioned animals also lose the motivation to drink water, and their resulting

dehydration causes death well before starvation[93,94]. However, rats with LHA lesions can be kept alive if they are administered food and water via stomach tubes[93–95]; this indicates that loss of action via the LHA impairs coordination of the need for resources and the motivation to ingest them, but it does not compromise the body's ability to utilize ingested resources. Intriguingly, the force-fed and force-hydrated LHA-lesioned rats termed as having "lateral hypothalamic syndrome" eventually recovered sufficient ingestive behavior to maintain survival via four distinct stages[94] reviewed below and in (Table 1-1).

- Stage-1: LHA-lesioned rats exhibited total aphagia and adipsia, and their survival depended on experimenter-administered food and water via stomach tubes.
- Stage 2: Rats ate small amounts of moistened, palatable food, so were considered to exhibit anorexia as opposed to aphagia. Rats remained adipsic and required experimenter-administered fluids.
- Stage 3: Rats voluntarily consumed some dry food as long as they were kept hydrated and would eat enough moistened palatable foods to support regulation of body weight without experimenter-administered nutrition. However, rats still did not voluntarily drink water.
- Stage 4: Rats were considered "recovered" because they accepted dry food and drank water, thus they no longer required force feeding/hydration to live. The recovered animals maintained sufficient body weight for survival, but their weight was lower compared to those with intact LHA action[94,96,97].

Stage	Defining Characteristic	Wet Palatable Food Consumption	Regulation of Body Weight and Food Intake on Wet Palatable Food	Dry Food Consumption with Hydration	Water Consumption. Survival with Water and Dry Food Intake.
1	Adipsia, Aphagia	✗	✗	✗	✗
2	Adipsia, Anorexia	✓	✗	✗	✗
3	Adipsia, Dehydration Aphagia	✓	✓	✓	✗
4	Recovery	✓	✓	✓	✓

Table 1-1. Lateral hypothalamic syndrome. Hallmarks of the lateral hypothalamic syndrome stages[94]. *Modified from Teitelbaum, P. & Epstein, A. N. (1962) The lateral hypothalamic syndrome: recovery of feeding and drinking after lateral hypothalamic lesions. Psychological Review. 69(2), 74-90.*

These data suggested that the LHA is important for energy *and* fluid balance, but that other brain sites can, in time, sufficiently regulate ingestive behavior to permit survival. However, upon careful study, even the rats that had "recovered" from lateral hypothalamic syndrome remained unable to appropriately respond to altered energy or fluid status with appropriate intake behavior. For example, normal rats respond to peripheral cues of insufficient energy status (e.g. low blood sugar or fasting) by eating more food, but the recovered lateral hypothalamic rats did not adjust feeding in response to these stimuli[98]. Likewise, recovered lateral hypothalamic rats did not counter dehydration with increased drinking behavior, and in fact only exhibited prandial drinking (e.g. water intake to facilitate chewing and swallowing of food)[94,99,100]. Similarly, they did not respond to dipsogenic

stimuli (i.e. thirst-inducing treatments such as hypertonic saline or polyethylene glycol) with appropriate drinking behavior, and hence also exhibited abnormal urinary water output[101],[102,103]. Fascinatingly, if these rats had the choice of receiving water through stomach tubes or the mouth they preferred it via the stomach[104], suggesting a pervasive, diminished motivation to voluntarily drink. The phenotype of lateral hypothalamic syndrome was similar in young and adult rats[105,106], but recovery was impaired in juvenile rats compared to adults, emphasizing the importance of an intact LHA for development and survival[105,107].

At face value these data suggest that the LHA is important for the *physiologic motivation* to consume. However, perhaps a more parsimonious interpretation is that the LHA is necessary to coordinate changes in metabolic and fluid status with appropriate ingestive behavior to resolve them. This also makes the LHA unique from other mediobasal hypothalamic sites with documented roles in energy balance, but not for fluid intake. Thus, the LHA is distinctive because it adaptively modifies both ingestive behaviors necessary for homeostasis and survival.

1.1.7 Physiologic and Pharmacologic Regulation of the LHA

Experimental lesions and electrical stimulation implicated the LHA in motivated ingestive behavior, but the activity of LHA neurons is also regulated by endogenous and exogenous stimuli relevant to maintaining energy and fluid balance. For example, early studies hinted that the LHA might modify peripheral glycemic control in response to alterations in plasma insulin and glucagon levels [108,109]. Indeed, central treatment with glucose[110,111], free fatty acids[112], and insulin[111] does modify the activity of some LHA neurons. These peripheral cues convey ample energy status to the brain, and

presumably the LHA detects these signals and modifies output ingestive behavior accordingly. Moreover, the LHA was also implicated in coordinating the motivation to obtain food, including willingness to work for it. Surveillance of LHA neurons in monkeys shows that their activity changes during a lever pressing-task to obtain food[113]. These data suggest that some LHA neurons are regulated as part of the process of harmonizing resource need and behavior. Indeed, electrical stimulation of the LHA increases rats' motivation to learn, which mimics the impact of food deprivation upon neural activity [114]. Consistent with a role for the LHA in water intake, osmolality changes are also detected within the LHA[110,115], and may serve to couple thirst with drinking behavior needed to resolve it. Intriguingly, pharmacological data suggest that feeding and drinking might be regulated via ligands acting upon distinct subsets of LHA neurons. For example, administration of adrenergic reagents into the LHA of sated rats induces feeding [116,117], whereas the injection of a cholinergic reagent into the LHA resulted in drinking behavior[116,117]. Cholinergic reagents also trigger drinking when injected into the preoptic area[118] and lateral septal nucleus[119], but fail to do so if administered to recovered LHA-lesioned rats. These data suggest that LHA-mediated control of drinking in the LHA occurs via a distinct mechanism compared to that via other brain areas.

1.1.8 "Neuronal Diversity in the LHA and Implications for Energy Balance

Initial studies of the LHA manipulated the entire region, but such bulk regulation is unlikely to occur physiologically due to the cellular heterogeneity of this region. Indeed, it is now recognized that the LHA contains many molecularly distinct populations of neurons, which are differentially implicated in control of feeding, drinking, locomotor activity, goal-directed behaviors, sleep / arousal or responses to stress or

inflammation[120–131]. As a result of these findings, the early designation of the LHA as a "feeding center" has fallen out of favor because it vastly under-represents the myriad ways in which the LHA can modify behavior to contribute to energy and fluid homeostasis. However, the molecular phenotyping of subsets of LHA neurons has enabled development of genetic methods to specifically identify LHA populations and study their contributions to physiology. Subsets of LHA neurons also receive information concerning energy status that may be important for appropriately coordinating feeding and other motivated behaviors. Some LHA neurons express receptors for the orexigenic hormone ghrelin[132,133], while separate LHA neurons express receptors for the feeding suppressing hormone leptin[134,135], indicating that the LHA directly intercepts circulating cues with opposing results upon energy balance. The LHA also receives dense input from the ARC[136], and thereby receives indirect information regarding peripheral energy status. Taken together, this work suggests that the LHA is uniquely positioned to integrate specific peripheral energy cues with appropriate motivated behaviors to adapt resource intake. Given that the LHA responds to anorectic, orexigenic and dipsogenic cues, there are likely distinct neural mechanisms by which the LHA can coordinate motivated behaviors and homeostasis. Indeed, several populations of neurons have been described in the LHA that vary in neurotransmitter and neuropeptide content, projection targets and function. Similar to the underlying premise of lesion studies, understanding how disruption of specific LHA populations or pathways compromises homeostasis will inherently reveal how they coordinate normal physiology. These findings may suggest the development of novel strategies to promote weight loss and maintenance of healthy body weight necessary to overcome the obesity epidemic.

1.2 ANATOMY AND COMPOSITION OF THE LHA

1.2.1 Location of the LHA and Implications for its Function

The architecture of the hypothalamus has been well described in rodents, per the rat and mouse brain atlases generated by Paxinos and Watson[137,138]. The rodent hypothalamus, much like in primates or humans, primarily consists of nuclei (sub-regions containing-densely populated neurons) distributed along the rostral-caudal and dorsal-

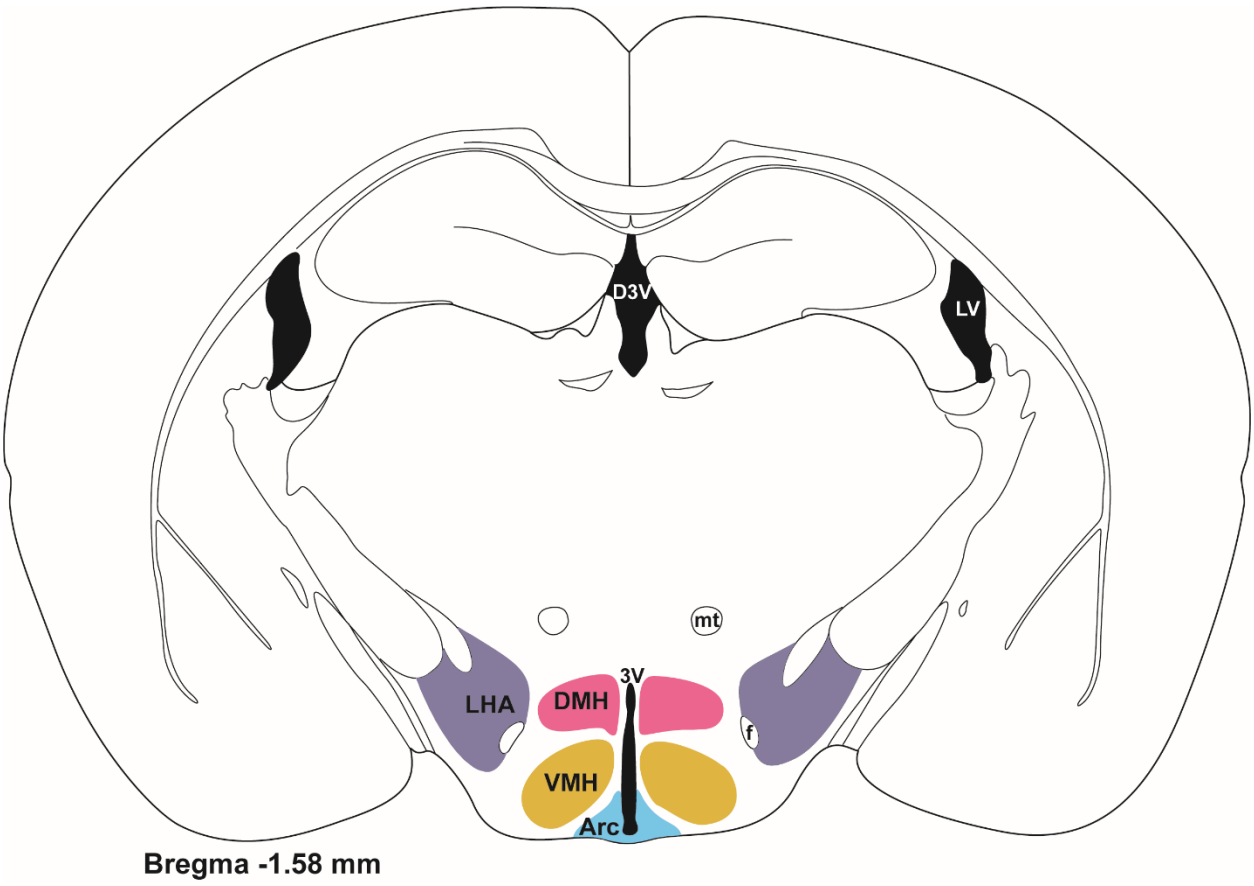

Figure 1-6. The localization of the LHA and mediobasal nuclei. Mouse coronal brain section at Bregma -1.58mm[137]. Arcuate nucleus (Arc), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), lateral hypothalamic area (LHA), 3rd ventricle (3V), fornix (f), mammillothalamic tract (mt), lateral ventricle (LV), dorsal 3rd ventricle (D3V).

ventral aspects bordering the third ventricle (3V). These “mediobasal hypothalamic nuclei” are grouped into the more ventral hypothalamic nuclei [the arcuate (ARC), ventral premammillary (PMV) and ventromedial (VMH) nuclei] and the more dorsal nuclei [the dorsomedial nucleus (DMH) and more rostrally the paraventricular nucleus (PVH)]. In contrast to the mediobasal structures, the LHA is considered an “area” rather than a nucleus because it spans across nearly the entire rostral-caudal continuum of the hypothalamus, with no easily definable cyto-architectural borders (Figure 1-6). In lieu of clear boundaries, the LHA is differentiated from the mediobasal nuclei by being generally dorsal to the PMV and VMH, but lateral to the DMH and PVH. The LHA is bordered dorsally by the zona incerta (ZI) and medially by the mammillothalamic tract (mt), which serves as an approximate landmark to visually differentiate the LHA from the DMH and PVH. The fornix (f) is roughly considered be within the medial-ventral extent of the LHA, and the portion of the LHA that lies just above and around the fornix is often referred to as “the perifornical hypothalamic area” (PFA). There is no observable boundary or tract between the LHA and the most rostral extent of the hypothalamus, the preoptic area (POA), making it particularly difficult to distinguish the precise margins of the LHA and the adjacent lateral preoptic area (LPO). Perhaps the most defining architectural feature of the LHA is that, in contrast to the mediobasal hypothalamic nuclei, it is not positioned adjacent to the 3V. This has important ramifications for the LHA in detecting peripheral stimuli. Since the 3V adjoins the median eminence, the permissive portion of the blood-brain barrier, it contains peripheral hormonal and nutrient cues that are thus easily and quickly accessed via the cells of the adjacent mediobasal nuclei. Due to its distant position from the 3V, the LHA lacks such “first-line”, direct access to these peripheral

cues. The LHA does have the capacity to directly respond to some circulating energy balance cues, such as leptin, ghrelin, insulin and glucose, but this occurs at a slower time course compared to that of mediobasal nuclei, presumably via transfer from capillaries reaching into the LHA[139]. However, the LHA also receives significant neural inputs from other brain areas, including from areas that may be more quickly or directly regulated by systemic signals. This had led to the idea that the LHA is an important coordinating center, receiving afferent input concerning energy status and sending projections to brain sites capable of modifying behavior. Furthermore, it is now clear that there are many distinct types of neurons contained within the LHA, which appear to have different connectivity and contributions to physiology.

1.2.2 Molecularly-Defined Populations of Neurons within the LHA

Given the expansive reach of the LHA, it is perhaps not surprising that this area is cellularly heterogenous, containing numerous different populations of neurons as well as resident glia. Subsets of LHA neurons have been described via their molecular signature, and while all of these populations have yet to be fully understood, it is clear that these neurons also differ in connectivity and function. A wealth of evidence supports the idea that subsets of LHA neurons coordinate unique aspects of energy and fluid balance, hence requiring systematic testing of each population and its projections to determine their function. Hence, any understanding of how the LHA contributes to physiology, and in particular energy balance, must separately evaluate the role of the molecularly-specified LHA subpopulations (Figure 1-7). To date, LHA neurons have been defined via their neuropeptide expression, classical transmitter signaling and expression of specific receptors[123,140,141].

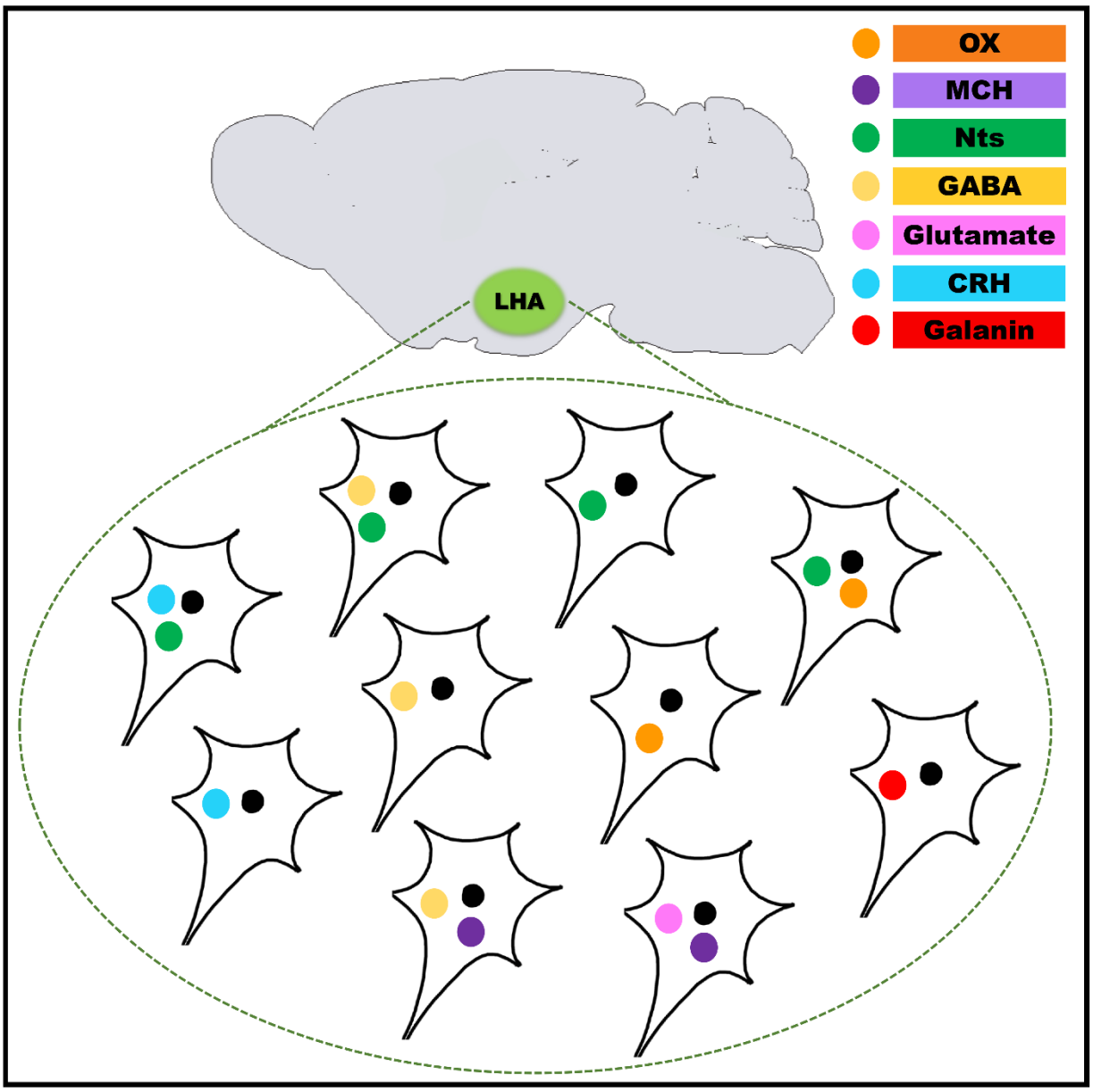

Figure 1-7. LHA heterogeneity. Schematic description of the major neurochemically-defined populations in the LHA. Co-localized dots indicate reported co-localizations of the neurotransmitters and neuropeptides. Orexin (OX), melanin-concentrating hormone (MCH), neurotensin (Nts), gamma-amino butyric acid (GABA), Glutamate, corticotropin-releasing hormone (CRH), Galanin.

The first documented molecular marker of a population of LHA neurons was melanin concentrating hormone (MCH)[142], whose expression is primarily confined to cell bodies within the LHA but also a few within the bordering ZI[143]. Although this population is most generally referred to as “MCH neurons”, MCH-containing neurons can be further subdivided into subsets that differ in their co-expression of nesfatin, CART or proteins to support synthesis and release of the classical neurotransmitters GABA or glutamate[140,144,145]. MCH itself plays important roles in control of arousal and energy balance [146–152].

Another major population of LHA neurons discovered were those that express the orexigenic neuropeptides orexin (OX)/hypocretin (HCRT) -A and/or –B, and these neurons are separate from the MCH neurons and are only expressed within cell bodies of the LHA[120,153]. OX also plays a critical role in wakefulness and arousal, such that loss of OX neurons or OX signaling causes narcolepsy[154,155]. The presiding view of OX neurons is that they promote arousal necessary to support goal-directed behaviors, including feeding and moving, and do so via their broad projections throughout the brain. Consistent with this, experimental activation of OX neurons increases arousal along with feeding, locomotor activity, and energy expenditure[156]. OX neurons are most activated during awake/alert periods, but are also activated by cues of energy depletion including fasting, ghrelin, and low glucose, which may serve to promote arousal necessary to obtain food and restore homeostasis when peripheral energy stores are low. In addition to containing OX they also contain and release glutamate, dynorphin and neuronal pentraxin (NARP/Nptx2), that could differentially modify downstream neurons[157–160].

A large population of LHA neurons expresses the neuropeptide, neurotensin (Nts),

and these are distinct from the MCH or OX-containing populations[133,140] (Figure 1-7). Unlike MCH and OX neurons that are specific to the LHA, Nts-expressing neurons are found in many sites throughout the brain; thus, Nts is a marker of a subset of LHA neurons, but is not a specific LHA marker[161–165]. LHA Nts neurons also appear to be neurochemically heterogeneous, with subsets that co-express the long form of the leptin receptor (LepRb) and/or GABA[128,133,166]. Some Nts neurons also co-express galanin, and these may project locally within the LHA to inhibit neighboring OX neurons[167,168]. Nts neurons may also be projection specified, since non-galanin containing Nts neurons project to the VTA, where pharmacologic Nts administration produces anorexia and locomotor activity[128,166,169]. Since Nts is an established modulator of DA signaling[170–172], the LHA may provide an endogenous source of Nts input to the VTA that impacts motivated behaviors. Nts signals via binding to the G protein coupled receptors Nts receptor-1 (NtsR-1) or -2 (NtsR-2). Pharmacologic Nts suppresses feeding and promotes weight loss[173–176]via an NtsR-1 dependent mechanism, however the functional role of LHA Nts neurons, and Nts signaling from them, has not been completely defined. Study of Nts neurons, including those in the LHA, has been somewhat hampered by the fact that immunoreagents do not reliably detect Nts-expressing soma without the use of colchicine treatment to inhibit axonal transport and concentrate the Nts within the cell bodies. This technical limitation may account for why characterization of the extensive population of LHA Nts neurons lagged behind that of MCH and OX neurons, which are readily detectable via standard immunolabeling methods. The development of mice that express Cre recombinase within Nts neurons (*Nts*^{Cre} mice) enables the facile detection and manipulation of Nts neurons using Cre-lox technology, and have begun to reveal how

Nts neurons contribute to physiology[166].

A sizeable population of LHA neurons express markers for the synthesis and vesicular packaging and release of GABA, hence referred to as LHA GABA neurons. Study of LHA GABA neurons or their function was limited by the fact that neither GABAergic markers or GABA itself can be detected via immunolabeling, but the establishment of Cre-recombinase lines for the vesicular GABA transporter-2 (vGAT2) and glutamate decarboxylase-2 (GAD2) have allowed multiple groups to visualize and interrogate the function of LHA GABA neurons[177,178]. There is considerable heterogeneity within the LHA GABA population[123], raising the possibility that specified subsets of LHA GABA neurons coordinate specific aspects of physiology. However, LHA GABA neurons do not appear to overlap with MCH or OX[177,178] (Figure 1-7), though they may overlap with some galanin (Gal) and/or Nts cells[123,128,179].

There is also a cluster of glutamate expressing neurons in the LHA[123]. OX neurons co-express glutamate, as may some MCH neurons, but there are also LHA glutamate neurons that lack any currently recognized neuropeptide or receptor markers[180,181]. Hence, like the LHA GABAergic neurons, the LHA glutamatergic subset is neurochemically diverse.

Besides these major LHA populations, there are other subpopulations of neurons such as the ones expressing the orexigenic peptide Gal [140,167,179,182], CART, corticotropin-releasing hormone (CRH)[183,184], thyrotropin releasing hormone (TRH), urocortin 3 (UCN3), tyrosine hydroxylase (TH), enkephalin and parvalbumin[185]. The expression of receptors has also been used to define the subgroups and function of LHA neurons along with the neuropeptide content. For example, the long form of the leptin receptor (LepRb),

which is essential for body weight control[24–26], is expressed in a group of LHA neurons (LHA LepRb neurons). At least some portion of adipose-derived hormone leptin action is mediated via LHA LepRb neurons, including subsets that co-express Nts and/or Gal[132,167]. LepRb neurons that co-express Nts vs. Gal appear to have differences in projection targets and leptin-mediated function, suggesting that they may be functionally distinct populations. Additionally, some LHA neurons express another receptor implicated in the control of energy balance, the melanocortin-4 receptor (MC4R). These LHA MC4R neurons are also neurochemically diverse: some of them contain Nts, others do not. While MC4R within the PVH is vital for regulation of feeding, including in part via leptin regulation, melanocortin action via the LHA MC4R cells does not alter feeding but may instead contribute to regulation of blood glucose levels, another important aspect of homeostasis[186,187].

1.2.3 Peripheral Regulators of LHA Neurons

1.2.3.1 Leptin

Leptin is made by white adipocytes, secreted into the blood, and this circulating pool of leptin can access the brain to act at neurons expressing LepRb. Leptin thus delivers the message of peripheral energy status to the brain, such that the more fat/leptin produced the more negative feedback should be induced (e.g. suppression of feeding, induction of energy expenditure) [188,189]. Intact leptin signaling via LepRb is essential for regulation of body weight, evidenced by the profound hyperphagia and obesity caused in mouse and man due to lacking the gene products for leptin (*ob/ob* [27,28] mice and humans [19,27,29]) or LepRb (*db/db* mice [20,21]). Leptin effects have been well characterized within the ARC, where leptin activates the anorectic POMC neurons that express LepRb, and

inhibits the activity of LepRb-expressing AgRP/NPY neurons to mitigate their orexigenic actions[190]. However, leptin actions within the ARC or other mediobasal hypothalamic regions do not explain the entirety of leptin regulation. The discovery of LepRb-expressing cells within the LHA, first via *in-situ* hybridization [191] and later using leptin receptor reporter mice [132,192], suggested that leptin might exert specific actions via the LHA as opposed to mediobasal sites. Indeed, peripheral leptin [188] or leptin infusion into the LHA suppresses feeding and promotes weight loss that depends on action via LHA LepRb neurons [132]. Intriguingly, LepRb expression in the LHA overlaps with GABA [132], Nts [166] and Gal [167,168] expressing neurons, but not with OX or MCH neurons [132]. However, leptin acts via LHA LepRb neurons that synapse onto OX neurons, thereby indirectly inhibiting OX neurons [132] and regulation of OX expression [166,193–195]. Leptin function may differ via LHA LepRb neurons expressing Nts vs. Gal [166,168] and GABAergic neurons [196]. Leptin modifies preference for sucrose and fat consumption via LHA Gal neurons [167]. Leptin acts via LHA Nts neurons to inhibit OX neurons and also acts via projections to the VTA to modify DA signaling and motivated locomotor activity [128,133,168]. Leptin is also involved in the metabolic stress response mediated by OX neurons[131], and indirectly suppresses the activity of LHA MCH neurons that may help to counteract orexigenic tone[197,198]. Together these data suggest that leptin acts via LHA LepRb neurons that in turn modify activity of key LHA populations and the DA system to modify behaviors relevant to energy balance. Loss of leptin action via the LHA promotes weight gain and dysfunction in target systems, demonstrating the unique yet vital role of leptin action via the LHA and its necessity for normal energy balance [133,166,167,179].

1.2.3.2 Ghrelin

The “hunger” hormone ghrelin is made by the stomach in response to energy

deficit and released to the circulation. Ghrelin levels are thus elevated during hunger states before a meal, but diminish upon feeding and restoration of circulating energy levels [199]. The ghrelin receptor (growth hormone secretagogue receptor, GHSR) is expressed in many brain regions, including the LHA [200], and circulating ghrelin can both access and modify the activity of the LHA [201]. Ghrelin acts in the brain to promote feeding, and in rodents it also triggers feeding associated foraging and hoarding behaviors[199], which are mediated, in part, via midbrain DA signaling [202–204]. Ghrelin may act specifically via LHA OX neurons that project to the VTA, thereby indirectly modifying DA signaling. Indeed, ghrelin increases the firing frequency and overall number of activated LHA Ox neurons [194,205]**.** Furthermore, ghrelin-induced regulation of feeding and the motivation to obtain palatable food (especially fat rich food) is partially mediated by LHA OX neurons [206,207]. While there may be minimal GHSR expression on OX neurons[140], ghrelin may still exert effects via GHSR-expressing ventral hippocampal (vHP) and ARC neurons that project to LHA OX neurons, and which have been demonstrated to modify feeding behavior [208,209]. Nonetheless, ghrelin action is primarily mediated via OX neurons, as it does not alter the activity of other major LHA populations, including MCH or Nts neurons [133,205,210].

1.2.3.3 Glucose

Similar to leptin and ghrelin, circulatory glucose levels inform the brain about the energy status of the body. Although increased circulating glucose and leptin both indicate energy surplus, glucose levels fluctuate rapidly and serve as an acute signal of immediate energy status, while leptin communicates long-term energy status and is modulated in a more gradual manner. Given that these peripheral signals convey distinct information, it

stands to reason that they may be detected by, and directly modulate, different sets of LHA neurons. Within the cellularly heterogeneous LHA, some neurons are glucose sensitive whereas others are unresponsive to changes in glucose [110]. Distinct LHA neural populations expressing OX [194,211,212], MCH [211,212], and markers of GABA synthesis [177] have been shown to be glucose sensitive. Elevated glucose strongly inhibits a subpopulation of OX neurons via a tandem-pore- K+ channel mechanism[212]. The glucose sensitive OX neurons in the LHA regulate depolarization of other LHA neural populations under hypoglycemic conditions [213], suggesting they may be the primary glucose sensors in the LHA while the others are indirectly regulated. Leptin blunts the activation of OX neurons in response to low glucose via GABA and Nts-mediated signaling, presumably from upstream LHA Nts-LepRb neurons [196]. Furthermore, it is highly probable that the glucose-sensitive OX neurons link the LHA with DA-mediated motivated feeding behavior, as decreased glucose elevates LHA OX neuron-mediated excitatory input onto the VTA DA neurons [196]. Additionally, some GABAergic LHA neurons are directly inhibited by elevated glucose levels [177], though their contributions to energy balance have yet to be determined. By contrast, glucose depolarizes MCH neurons [211] via a K_{ATP} channel mechanism, which may contribute to modulating glucose homeostasis [214].

1.2.3.4 Dehydration

The brain receives information about the hydration status of the body through two direct mechanisms: 1) via circulating hormones, such as vasopressin, angiotensin II and aldosterone and 2) via specialized circumventricular regions within the lamina terminalis (LT) that directly sense serum osmolality[6]. The LHA is a dehydration-sensitive brain region, as thirst increases neuronal activation within a subset of LHA neurons [215].

However, the LHA is not one of the circumventricular organs or part of the LT, and it is positioned far from the blood-brain barrier or ventricles via which hydration-status hormones might be rapidly conveyed. Thus, rather than directly detecting hydration status, it is more likely that the LHA receives indirect feedback on fluid status from LT neurons or hormone-responsive neurons throughout the brain. It remains to be determined what precise circuits convey the dehydration "message" to the LHA, but it increases the expression of CRH [125,127] and Nts within the LHA of rodents[125] that correlates with the intensity of dehydration-induced anorexia (a phenomenon in which water-deprived rodents cease eating, and only after sating their thirst will they ingest food)[125]. In rats, most of the dehydration-responsive LHA CRH neurons co-express Nts, while comparatively few contain OX and/or MCH [126], suggesting that LHA Nts neurons may be a specialized population for detecting aberrant fluid homeostasis. However, once animals are rehydrated, OX neurons are selectively activated, suggesting their involvement in relieving the anorexic effect of dehydration[126]. Given that LHA Nts neurons project to and inhibit OX neurons, there is rationale to support Nts neurons as the indirect "dehydration sensors" of the LHA that inhibit OX neurons and perhaps OX-mediated food seeking until water is restored, though this remains to be systematically tested.

1.3 ROLES OF LHA NTS NEURONS IN REGULATING FEEDING AND ENERGY EXPENDITURE

1.3.1 Overview of the LHA in Control of Feeding and Energy Expenditure

Of the many behaviors and physiological functions regulated by the LHA, feeding is perhaps the most well-studied. Indeed, even though feeding and drinking were equally blunted by LHA lesions, the field has primarily focused on the role of the LHA in feeding

Specific Roles of LHA Neurons in Feeding							
Physiologic Effects	**MCH**	**Orexin**	**Neurotensin**	**Galanin**	**CRH**	**GABA**	**Glutamate**
Homeostatic Food Intake	↑	↑	↓	↓ (LepRb+)	↓ ?	↑	↓
Palatable Food Intake	↑	↑	↓			↑	↓
Body Weight	↑	↑	↓				
Motivation to Obtain Food	↑	↑	↓	↑		↑	
Food Seeking	↑	↑				↑	
Food Choice	+			+			
Learned Feeding	+	+					
Sex Differences	+						

Table 1-2. Specific roles of LHA neurons in feeding.

Specific Roles of LHA Neurons in Arousal, Physical Activity and Energy Expenditure						
Physiologic Effects	**MCH**	**Orexin**	**Neurotensin**	**Galanin**	**GABA**	**Glutamate**
Arousal	↓ ?	↑			↑	
Sleep	↑	↓				
Physical Activity	↓	↑	↑	↑	↑	Modulatory
Energy Expenditure	↓	↑	↑	↑		
BAT Activity	↓	↑				
BAT Differentiation		↑				
Thermogenesis	↓	↑				
Stress Response	↑	↑				

Table 1-3. Specific roles of neurons in arousal, physical activity and energy expenditure.

rather than water intake, which promoted its initial moniker as a “feeding center.” Since then the field has come to recognize that the LHA coordinates a vast array of physiologic

processes, and cannot be considered a center for any one behavior. However, feeding remains the single most studied behavior controlled via the LHA, in part due to its potential relevance for understanding the pathogenesis and possible treatment of obesity and eating disorders. Here I provide a summary of how LHA neurons modify feeding, focusing on populations defined by their neurotransmitter or neuropeptide content in Table 1-2**.** Subsequently I will focus on the current understanding of how LHA Nts neurons control feeding behavior, and what remained unknown that led to the research of this thesis.

Energy is expended via the combination of basal metabolic rate (e.g. energy consuming processes necessary to support life) and volitional physical activity. Since individuals must be awake to engage in movement, arousal / sleep status has direct bearing on the amount of volitional physical activity and energy expenditure. I provided a summary of how LHA neurons modify these aspects of energy expenditure in Table 1-3, and will therefore focus on reviewing the current understanding of how LHA Nts neurons modify energy expenditure.

1.3.2 Neurotensin (Nts) Neurons in Control of Feeding and Energy Expenditure

Pharmacologic Nts has been implicated in suppressing feeding and promoting weight loss, but also in regulating locomotor activity, social behavior, sleep, body temperature, blood pressure, nociception and response to addictive drugs [134,216–220]. There are many populations of Nts neurons through the brain, yet it remained unclear which Nts neurons mediated the disparate physiological actions of Nts, including the anorectic effect. The development of *Nts*^{Cre} mice has begun to enable systematic testing of anatomically-distinct Nts populations, including those in the LHA[166], to define their

physiological importance. Activation of all LHA Nts neurons suppresses food intake via a NtsR-1 dependent mechanism, including limiting fasting-induced refeeding and motivated sucrose responding [221]. Simultaneously, activation of LHA Nts neurons increases locomotor activity, hence the increased energy expenditure and modest anorexia is sufficient to cause weight loss [128,221] . At least some LHA Nts neurons project to and release Nts to the VTA, which promotes activation of VTA DA neurons and DA release into the NAc via an NtsR-1-dependent mechanism[128]. Given that NTSR-1 is directly expressed on VTA DA neurons, and pharmacologic Nts treatment within the VTA activates DA neurons and DA release to the NAc that suppresses feeding, it is likely that LHA Nts → VTA neurons may directly mediate DA-dependent anorexia and locomotor activity[222–226].

LHA Nts neurons can be differentiated via their projection targets and, to some extent, their molecular phenotypes. Some LHA Nts neurons project locally and inhibit neighboring OX neurons [168] while others project to the VTA where pharmacologic Nts administration produces anorexia and locomotor activity[221]. A subset of LHA Nts neurons expresses LepRb, is activated by leptin, and co-expresses markers of GABA synthesis [133,166,169]. Developmental loss of LepRb specifically from LHA Nts neurons results in reduced striatal DA action, increased adiposity, and disrupted feeding response to leptin[133,166]. Thus, LHA Nts neurons may be useful to promote weight loss behaviors including via suppression of food intake, while disruption of Nts-mediated DA signaling might contribute to the development of obesity. Consistent with this view, genetically obese mice and rats exhibit reduced Nts mRNA and protein in the hypothalamus, but there is insufficient data to evaluate whether Nts is selectively decreased in the LHA, and

whether this is a cause or effect of long-term hyperphagia [227–229]. Some LHA Nts → VTA projections have been reported to induce reward seeking via a glutamatergic mechanism, suggesting that some LHA Nts neurons express glutamate [230]. However, close examination of the distribution of these LHA Nts neurons suggests that they may be a more rostral population compared to the LHA Nts neurons implicated in suppressing feeding, which are located more caudally within the perifornical LHA [133,166,221]. Additionally, in rats Nts is highly expressed within LHA CRH neurons [126], which are known to regulate stress responses [231,232] and feeding [233]. Moreover, the LHA Nts neurons are responsive to inflammation-induced stress induced by lipopolysaccharide (LPS) injections as shown by cFos mapping [122]. However, there is much yet unknown about how LHA Nts circuits mechanistically act to link stress and feeding. Understanding how Nts engages the DA system to modify motivated behaviors, including the specific role of LHA Nts neurons, will be important for discerning the therapeutic potential for Nts in regulating energy balance.

Peripheral injection of Nts analogs or NtsR-1 agonists are sufficient to suppress food intake and either induce weight loss or prevent weight gain in lean rodents [173,234]. Interestingly, these compounds also restrain feeding and reduce body weight in genetically obese rodents, indicating that Nts may have translational potential as an anti-obesity agent[173,234]. In both of these studies however, the obese animal models were genetically deficient in leptin signaling; given the overlap between leptin and Nts action, inducing Nts signaling might have simply rescued their disrupted leptin-induced Nts signaling to potentiate weight loss. The anorectic potential of Nts in diet-induced obesity, which is the common cause of human obesity, has yet to be examined. Peripheral Nts

or intra-VTA Nts does reduce operant responding for sucrose [235,236], suggesting that Nts may indeed be capable of suppressing intake of palatable, obesogenic foods. Furthermore, mice genetically lacking NtsR-1 display increased sucrose preference and susceptibility to weight gain on palatable diet but not chow, implicating the requirement of NtsR-1 for restraint of hedonic intake[169,237].

Chemogenetic activation of LHA Nts neurons increases locomotor activity, at least in part by increasing mesolimbic DA signaling[128,221]. LHA Nts neurons project to the VTA, and so can directly engage a subpopulation of VTA DA neurons that co-express NtsR-1. Activation of the LHA Nts → VTA NtsR-1 circuit induces DA into the NAc that directly modifies locomotor activity and potentiates energy expenditure and weight loss [222] [128]. However, ablation of the VTA NtsR-1-DA neurons results in profoundly elevated physical activity and energy expenditure that disrupts energy balance, and leads to low adiposity and body weight [222]. The locomotor-inducing effects of Nts via the VTA are unique, as Nts administered into the ventral pallidum has no effect on locomotor activity, while infusion into the striatum inhibits the locomotor response to psychostimulants [238–240]. Since LHA Nts neurons directly provide Nts to the VTA, they are endogenous modulators of DA-mediated locomotor behavior. Thus, the LHA Nts→VTA circuit regulates locomotor activity and increases energy expenditure, which along with Nts-anorectic effects, supports weight loss. LHA Nts neurons may also modify locomotor activity via their local inhibitory regulation of OX neurons, and intact LHA Nts → OX regulation is necessary for adaptive locomotor responses to leptin and ghrelin that are mediated via LHA Nts and OX neurons, respectively[133,166,168].

1.4 ROLES OF LHA NTS NEURONS IN DRINKING BEHAVIOR

1.4.1 Overview of the LHA in Control of Drinking

LHA lesion and stimulation studies identified an essential role for the LHA in modifying water intake, yet surprisingly, little investigation has followed into how the LHA modifies water seeking and intake behavior. Defining the fundamental neural mechanisms underlying drinking behavior, including the contributions of the LHA in this process, is paramount for developing therapies to treat water intake disorders that threaten survival. Below I summarized the modest literature concerning how specific neuropeptide and neurotransmitter-defined LHA populations contribute to LHA-dependent drinking behavior in Table 1-4 and will focus on reviewing the current understanding of how LHA Nts neurons control drinking behavior.

Specific Roles of LHA Neurons in Drinking						
Physiologic Effects	**MCH**	**Orexin**	**Neurotensin**	**CRH**	**GABA**	**Glutamate**
Water Intake	↑ ?	↑	↑		↑	
Rewarding Liquid Intake	↑	↑			↑	↓ ?
Dehydration		↑ mRNA ↓ Activation	↑ mRNA ↑ Activation	↑ mRNA ↑ Activation		
Motivation to Obtain Water		↑ ?	↑ ?			
Motivation to Obtain Rewarding Liquids		↑				

Table 1-4. Specific roles of LHA neurons in drinking.

1.4.2 Neurotensin (Nts) Neurons in Control of Drinking

Experimental activation of LHA Nts neurons increases water intake but suppresses feeding [221]. Thus, unlike the other LHA populations that non-specifically promote food and water consumption, LHA Nts neurons preferentially induce water intake over feeding,

suggesting that at least some of these neurons may coordinate drinking behavior necessary for fluid homeostasis. Consistent with this possibility, dehydration increases expression of Nts mRNA in the LHA [127], suggesting that osmolality status regulates LHA Nts neuronal function. Since experimental activation of LHA Nts neurons promotes Nts release, and exogenous Nts promotes drinking, then dehydration-induced upregulation of Nts could serve as a physiologic signal to drive water seeking and intake once water becomes available [128,241]. Intriguingly, LHA Nts-induced polydipsia is not blunted by NtsR-1 antagonists or in mice lacking NtsR-1[221]. Alternately, Nts-mediated drinking could be mediated via NtsR-2 or via an Nts-independent mechanism, but these possibilities have yet to be tested. Furthermore, LHA Nts-mediated drinking likely occurs via a leptin-independent population of LHA Nts neurons, as mice lacking LepRb in LHA Nts LepRb neurons do not exhibit any disruptions in drinking or bodily fluid content [133]. This raises the possibility that LHA Nts LepRb neurons might mediate the anorectic actions of LHA Nts neurons, while a separate Nts-containing population modifies drinking via a leptin-independent mechanism. In any case, these data offer a first potential hint for a specific mechanism by which the LHA could modulate motivated water intake. Going forward it will be important to determine if LHA Nts neurons specifically coordinate physiologic water need and drinking behavior necessary for fluid homeostasis.

1.5 GOALS OF THE DISSERTATION

Drinking and feeding are compulsory behaviors for survival. The brain surveys water and energy status and coordinates ingestive behavior to resolve imbalance. Any uncoupling of need and intake, however, leads to disease, such as anorexia, bulimia and obesity, increasing mortality[242–244]. Inappropriate water intake is also deleterious;

dehydration or excessive drinking beyond regulatory need is associated with cardiovascular dysfunction, coma and death[48,49,245,246]. Despite the prevalence and serious consequences of uncoordinated intake there are no effective pharmacological treatments to normalize deranged ingestion. Thus, there is crucial need to define the neural circuits that orchestrate intake to identify relevant treatments for ingestive disorders.

Since the LHA of the brain is essential for motivated feeding[88,93,141] and drinking[65,93,247] it may hold the key to understanding ingestion. The LHA has been difficult to study, however, because it contains many *molecularly distinct populations of neurons*. Yet, the LHA neurons characterized to date all promote feeding, and do not explain how the LHA suppresses feeding and regulates drinking behavior. In contrast, our lab has characterized a separate population of LHA neurons that express the neuropeptide Nts[128,166,168], and activation of these LHA Nts neurons promotes voracious water intake and restrains feeding. Intriguingly, my data suggest that LHA Nts neurons' control of ingestive behaviors may be projection specified, since they project densely to two sites in the brain: the ventral tegmental area (VTA) implicated in controlling motivated feeding[141,248] and the lateral preoptic area (LPO) that modifies drinking[249,250]. Collectively, these data informed the central hypothesis of my PhD thesis: **LHA Nts neurons direct drinking behavior in response to physiologic need (thirst) via neural outputs to the LPO, but do not modify feeding via LPO**. Hence, the goals of this dissertation are:

1. Determine how activation of all LHA Nts neurons impacts homeostatic and motivated drinking behavior (Chapter 2).

Hypothesis: Activation of LHA Nts neurons increases homeostatic and thirst-induced drinking as well as the motivation to drink.

Method: I combined *Nts*^{Cre} mice and cre-mediated activatory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to selectively activate all LHA Nts neurons, so as to decipher their roles in homeostatic and motivational regulation of drinking.

2. Identify LHA Nts neural circuits that could coordinate ingestive need and intake (Chapter 3).

Hypothesis: LHA Nts neurons are anatomically well positioned to receive nutrient and osmolality cues and to modify feeding and drinking behavior.

Method: I applied cre-dependent viral anterograde and retrograde tracing techniques by use of *Nts*^{Cre} reporter mice lines to map monosynaptic inputs to LHA Nts neurons and the brain areas LHA Nts neurons are projecting to. I also applied projection-site specific viral retrograde tracing approaches to determine if LHA Nts neurons send overlapping or separate projections to the VTA and the LPO, that are implicated in modulating feeding and drinking, respectively. These data reveal a novel and prominent LHA Nts →LPO circuit whose function has yet to be described.

3. Determine how inhibition of LHA Nts neurons modifies physiologic intake (Chapter 3).

Hypothesis: LHA Nts neuronal activation is required to direct the appropriate ingestive response to thirst or hunger.

Method: I used *Nts*^{Cre} mice and cre-dependent inhibitory DREADDs to suppress the activity of LHA Nts neurons in dehydrated or fasted mice, then measured intake when

water and food are restored. These data reveal that activation of LHA Nts neurons is not required for physiologic, need-based drinking and feeding behavior, suggesting that these neurons may primarily augment motivational (not homeostatic) ingestive behaviors.

4. Determine how activation of the LHA Nts projections to the LPO regulates ingestive behavior compared to activation of all LHA Nts neurons (Chapter 3).

Hypothesis: While activation of all LHA Nts neurons promotes drinking and suppresses feeding, activation of the LHA Nts → LPO circuit selectively promotes drinking, but does not suppress feeding.

Method: I expressed cre-mediated activating DREADDs in *Nts*^{Cre} mice and then administered the DREADD ligand clozapine-N-oxide (CNO) selectively in the LPO to specifically activate the LHA Nts→LPO projections. Using this method, I characterized the specific ingestive behaviors regulated by the LHA Nts→LPO circuit and compared to activating all LHA Nts neurons via peripheral injection of CNO.

By completing these goals, I revealed that subsets of LHA Nts neurons are anatomically positioned to coordinate energy and fluid balance cues with behavioral output via projections to the VTA and LPO. LHA Nts neurons promote voracious drinking via outputs to the LPO and by modifying the motivation to drink, but do not mediate the previously observed suppressive effects on feeding. These data suggest that distinct, projection specified subsets of LHA Nts neurons could modify motivated drinking and feeding behavior, and establish a novel, physiologic mechanism that may differentially modify drinking and feeding. These data could open the door to develop treatments to specifically treat drinking or feeding disorders, or for agents that could suppress food

intake while simultaneously increasing water intake. The translational implications of these findings for treating disrupted ingestive behaviors will be discussed in Chapter 4.

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CHAPTER 2. ACTIVATION OF LATERAL HYPOTHALAMIC AREA NEUROTENSIN - EXPRESSING NEURONS PROMOTES DRINKING

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ABSTRACT

Animals must ingest water via drinking to maintain fluid homeostasis, yet the neurons that specifically promote drinking behavior are incompletely characterized. The lateral hypothalamic area (LHA) as a whole is essential for drinking behavior but most LHA neurons indiscriminately promote drinking and feeding. By contrast, activating neurotensin (Nts)-expressing LHA neurons (termed LHA Nts neurons) causes mice to immediately drink water with a delayed suppression of feeding. We therefore hypothesized that LHA Nts neurons are sufficient to induce drinking behavior and that these neurons specifically bias for fluid intake over food intake. To test this hypothesis we used designer receptors exclusively activated by designer drugs (DREADDs) to selectively activate LHA Nts neurons and studied the impact on fluid intake, fluid preference and feeding. Activation of LHA Nts neurons stimulated drinking in water-replete and dehydrated mice, indicating that these neurons are sufficient to promote water intake regardless of homeostatic need. Interestingly, mice with activated LHA Nts

neurons drank any fluid that was provided regardless of its palatability, but if given a choice they preferred water or palatable solutions over aversive (quinine) or dehydrating (hypertonic saline) solutions. Notably, acute activation of LHA Nts neurons robustly promoted fluid but not food intake. Overall, our study confirms that activation of LHA Nts neurons is sufficient to induce drinking behavior and biases for fluid intake. Hence, LHA Nts neurons may be important targets for orchestrating the appropriate ingestive behavior necessary to maintain fluid homeostasis.

2.1. INTRODUCTION

Water intake is necessary to maintain cellular osmolality and homeostasis, therefore drinking is an essential behavior for survival. Dysregulation of drinking behavior, either too much or too little water, endangers health. Excessive water intake after extreme exercise, use of the drug Ecstasy or psychogenic polydipsia can lead to hyponatremia and severe neurological consequences that can cause death[2–5]. Inadequate water intake promotes life-threatening cardiovascular impairment[6,7] and may be due to voluntary or involuntary restriction of drinking. For example, individuals with anorexia purposely limit fluid intake to control body weight[8], while elderly persons have impaired ability to sense thirst that leads to unintentionally reduced drinking[9,10].

Despite the exigency of water there is still much to understand about how drinking behavior is regulated. The brain contributes to fluid homeostasis by monitoring serum osmolality and, in turn, coordinates appropriate peripheral actions and drinking behavior needed to restore any imbalance[11]. Neurons in the lateral terminalis (LT) are the first-line osmolality sensors and detect whether there is a “need” of water[12–16] but they do not themselves modulate water handling. Instead, LT neurons send afferents to numerous

brain regions that, in sum, control water intake and fluid handling. For example, LT projections to the supraoptic nucleus and paraventricular hypothalamus (PVH) coordinate release of the hormone arginine vasopressin to regulate peripheral fluid handling[17–19] and these circuits are vital for whole-body fluid balance. Yet, the brain regions that orchestrate the behavioral drinking response remain incompletely defined.

The lateral hypothalamic area (LHA) is a neuronally heterogeneous brain region that is positioned to orchestrate drinking because it receives inputs from LT neurons[20–23] and is known for controlling ingestive behavior. Indeed, electrical stimulation of the whole LHA induces drinking[24] whereas lesion of the LHA causes adipsia, aphagia and death from self-inflicted dehydration and starvation[25,26]. Hence, the LHA contributes to both ingestive behaviors, but most research has focused on how LHA neurons regulate feeding[27]. By comparison, the role of the LHA in mediating water intake behavior has been less studied. The best characterized LHA populations are those expressing the neuropeptides melanin-concentrating hormone (MCH)[28,29] or orexin (OX)[30,31] and the large population of LHA neurons expressing the classical neurotransmitter GABA[32,33]. These LHA populations are mostly distinct from each other [34] as no overlap has been detected between OX and MCH neuronal populations[35] as well as between GABA and MCH or OX expressing ones[27,32,33], although some MCH and OX neurons express the GABA synthesis pathway enzymes, but they lack the expression of vesicular secretion pathway proteins [36]. These MCH, OX and GABA populations are activated by specific peripheral cues and pathways, but they all generally promote ingestive behavior and do not appear to specifically coordinate feeding vs. drinking. For example, MCH neurons in the LHA promote intake of both food and non-aversive liquids[37–39]. Since MCH neurons

lack inputs from osmosensory centers[40], and preferentially increase intake of palatable solutions but not water[39], it is likely that MCH neurons primarily direct reward-based drinking rather than drinking to resolve osmolality imbalance. Activation of the adjacent OX neurons broadly induces intake of food, water and any rewarding liquids[41–43], hence these neurons do not bias for food or liquid intake. Experimentally activating the entire population of LHA GABA neurons also generally increases ingestive behaviors, promoting intake of solid food, caloric-liquids and gnawing at non-caloric objects (e.g. wood, the home cage)[33,44,45]. It is now recognized, however, that LHA GABA neurons are heterogeneous[33] and it is unlikely that they are all physiologically activated by the same cues or at the same time. Thus, while the LHA neurons studied to date generally promote food and liquid intake, it remained possible that specific subsets of LHA neurons might contribute uniquely to feeding and drinking.

Here we investigated the role of a specific subset of LHA GABA neurons that co-express the neuropeptide Neurotensin (Nts)[35,46–48] (referred to as LHA Nts neurons) in ingestive behavior. Nts is considered an anorectic neuropeptide and has been primarily studied in the context of feeding[49–52]. Since dehydration increases Nts expression in the LHA[53] and pharmacologic Nts promotes drinking[54], we reasoned that LHA Nts neurons might contribute to drinking behavior. Indeed, we previously showed that activating LHA Nts neurons causes an immediate, voracious increase in water intake with a delayed suppression of feeding[55]. This divergent effect on ingestive behavior is strikingly different from the general increase in drinking and feeding induced by activating MCH, OX or all LHA GABA neurons. It remained unclear, however, if LHA Nts neurons generally promote fluid intake or if they selectively promote physiologically meaningful or palatable liquids

over unpalatable or dehydrating ones. We, therefore, investigated the nature of the fluid intake triggered by activation of LHA Nts neurons and whether activation of LHA Nts neurons is sufficient to bias intake of any fluid, regardless of its palatability, over food intake.

2.2. MATERIALS AND METHODS

2.2.1. *Animals*

Mice were bred and housed on a 12-hour light and12-hour dark cycle. Mice had *ad libitum* access to rodent chow (Teklad 7913) and water unless noted otherwise. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and National Institute of Health guidelines. Male adult mice (14-90 wk old) were studied, and despite this substantial age range we found no alteration of LHA Nts-induced drinking behavior with aging (data not shown). Most tests were performed in a single cohort of mice between 50-90 weeks of age. Sucrose preference was performed in a separate cohort of mice between 14-35 weeks of age.

2.2.2. *Surgery*

Nts^{Cre} mice[46] (n=8, 2-3 months old) were stereotaxically injected with 300 nL of AAV-hSyn-DIO-hM3D(Gq)-mCherry, serotype 2 (University of North Carolina Vector Core) into each side of the LHA according to coordinates from the Paxinos and Franklin mouse brain atlas[56] (anteroposterior: -1.34, mediolateral: ±1.05, dorsoventral: -5.20). These mice were tested via a crossover design, such that each mouse received vehicle or clozapine N-oxide (CNO) injections. Due to equipment malfunction during the final assay (sucrose preference) the data for this cohort of mice could not be recovered. Since

the mice had been euthanized the assay could not be repeated, so a new cohort of *Nts*^{Cre} mice with bilateral LHA injections of AAV-hSyn-DIO-hM3D(Gq)-mCherry was generated for the sucrose preference experiment (n=7).

2.2.3. Acute Drinking, Osmolality and Body Temperature Measurements

All measurements were taken during the light cycle. The provided food and liquids (either water, 0.014% Quinine solution or 2% NaCl solution) and body weights were measured and urine was collected before and 4 hr after intraperitoneal injections of vehicle (VEH) [1X 0.2M pH 7.4 sterile PBS] or CNO [0.3 mg/kg]. All measurements were conducted in static home cages and provided food pellets and liquid bottles were manually weighted by use of an electronic scale before the start of the procedure and at the end of test to determine the consumed amounts. The temperature of the animals was measured before and after injection of VEH or CNO using a digital rectal thermometer. Urine osmolalities were measured using a Wescor 5520 Vapro Vapor Pressure Osmometer. For most measures n=8, but there is reduced sample size for this urine osmolality analysis due to difficulty in obtaining fresh urine samples (Figure 2-1E, VEH=6, CNO=7). The concentration of NaCl (2%) was chosen based on prior literature that this solution is acutely palatable to mice (such as during the 4-hour window assessed here) but prolonged access induces osmotic dehydration[57,58]. Several concentrations of quinine solution were screened to identify the minimum quinine concentration that caused all mice to prefer water, and hence was aversive (Supp. Figure 2-2). Based on this assessment 0.014% quinine was selected for testing because it produced quinine aversion (< 50% preference for quinine) in all mice.

2.2.4. Euhydration vs. Dehydration Experiment

Liquid, food and body weight measurements were taken manually as indicated in section 2.3, just prior to the dark cycle, then mice were either euhydrated (EU) or dehydrated (DE) overnight (~18 hr). Mice received VEH or CNO injections in the morning and then were either given water (EU) or remained dehydrated (DE) for 5 hours, after which liquid, food and body weight measurements were taken. Thus, this experiment had three test groups (n=8 for each): EU-EU control group (overnight euhydrated, 5-hour euhydrated), DE-EU (overnight dehydrated, 5-hr eu/rehydrated) dehydration motivated group, DE-DE (overnight dehydrated, 5-hr dehydrated) prolonged dehydration control group.

2.2.5. 2-Bottle Choice Tests

2 bottles, each containing water, were provided for 48 hours to acclimatize mice to the two-bottle arrangement. The position of the bottles in the cage was swapped every 24-hours to control for any place preference effects; observance of place preference during the water pre-testing would be cause for exclusion, but this was not noted in any of the mice. Next, one of the water bottles was replaced with a bottle containing the test solution so that mice had a choice of either water or 0.014% Quinine hydrochloride dihydrate (Sigma) solution, or water or 2% NaCl (Sigma), or water or 1% sucrose (Sigma). During 2-bottle preference testing the mice received twice daily injections of either VEH or CNO in the morning (9:30-10:00 am) and just before the dark cycle (5:30-6:00 pm). Since this was a crossover design, all mice served as their own controls, receiving both treatments. Mice were given at least 24-hrs before repeating the experiment with the alternate treatment (e.g. 2 bottles of water for 48-hrs, then water vs. other solution for 48-

hrs while being treated). Consumed liquids and food as well as body weight changes were determined by manually weighing the provided liquid bottles, food pellets and animals by use of a digital scale as in section 2.2.3.

2.2.6. Verification of hM3D(Gq)-mCherry expression in LHA

After conclusion of experiments, mice received a lethal i.p. dose of pentobarbital (Fatal Plus, Vortech) followed by transcardial perfusion with 0.2M PBS (pH 7.4) and then 10% formalin (Fisher Scientific, Pittsburgh, PA). Brains were extracted, post-fixed in 10% formalin, then dehydrated in 30% sucrose solution prior to coronal sectioning (30 µm) using a sliding microtome (Leica, Buffalo Grove, IL). Sections from each brain were collected into four separate series. One series of sections from each mouse was assessed via immunofluorescence microscopy as previously described[55] to verify mice in which hM3D(Gq)-mCherry expression was targeted to, and confined within the LHA. The mCherry was detected using a rabbit dsRed primary antibody (1:1000, cat #: AB_10013483, Clontech), followed by anti-rabbit secondary antibody conjugated to Alexa-568 (1:200, cat #: AB_2534017, LifeTech). Images were collected with an Olympus BX53 fluorescence microscope outfitted with transmitted light as well as FITC and Texas Red filters, by use of Cell Sens software and a Qi-Click 12 Bit cooled camera. Images were analyzed using Photoshop software (Adobe). Mice with bilateral mCherry expression in the LHA, without spread to neighboring brain regions, were included in the final dataset. Additionally, one mouse with unilateral mCherry expression confined to the LHA was included in the final dataset, because it demonstrated a comparable induction of drinking following CNO activation (Figure S2-1, mouse DQ60 identified by magenta arrow). This is consistent with our previous observations of comparable CNO-induced

drinking in mice with bilateral or a very well-targeted unilateral expression of hM3D(Gq)-mCherry expression in the LHA (data not shown). Importantly, CNO does not induce drinking in control mice that do not express hM3D(Gq)-mCherry in the LHA[55], thus the effects observed in this study are not due to off-target actions of CNO.

2.2.7. ***Statistics***

2-tailed student's t tests and 2-way ANOVA with 95% confidence intervals and alpha=0.05 were applied for the analyses by use of GraphPad Prism 7.0. All t-tests are paired unless indicated otherwise. For all data analyzed by ANOVA, either Sidak`s or Dunnett's multiple comparisons post-hoc test was applied depending on whether every group was compared to each other or to a control group, respectively. For body temperature analyses, repeated measures 2-way ANOVA (RM-ANOVA) was applied. For the effects of hydration status/motivation tests, 2-way ANOVA was used to compare each hydration condition to the control (EU-EU) condition and within each condition. Treatment effects between two groups were analyzed by t-tests, including acute water, 2% NaCl and quinine solution intake data as well as 2-bottle preference tests.

2.3. RESULTS

2.3.1. ***Activation of LHA Nts neurons induces water consumption but not feeding***

Nts^{Cre} mice were injected in the LHA with AAV-hSyn-DIO-hM3D(Gq)-mCherry so that hM3D(Gq)-mCherry expressing LHA Nts neurons could be selectively activated via treatment with CNO (Figure 2-1A). First, the acute effects of VEH (control) or CNO-induced activation of LHA Nts neurons were assessed 4 hours after treatment. VEH treatment did not cause any alteration in body weight, but mice with CNO-mediated

activation of LHA Nts neurons had significantly higher body weights (Figure 2-1B, $p < 0.001$). At first glance these data seemed contradictory with prior work showing that chemogenetic activation of LHA Nts neurons decreased body weight after 24 hr [55]. We hypothesized that the short-term (4 hr) LHA Nts-induced weight gain could be due to the weight of immediately ingested water and/or food, which may have been missed in the prior study that only assessed long-term effects on body weight (24 hrs or longer). Indeed, CNO-induced activation of LHA Nts neurons promoted voracious drinking over 4 hours (Figure 2-1C), but did not induce food intake during this time (Figure 2-1D). CNO treatment also led to a significant reduction in urine osmolality, indicating that LHA Nts-activated mice actually consumed water (Figure 2-1E). Thus, the acutely increased weight of LHA Nts-activated mice may be explained by the weight of the water they ingested. Over a longer time course, however, LHA Nts-induced drinking, locomotor activity and energy expenditure concomitant with a delayed restraint of feeding can produce weight loss [55]. We reasoned that LHA Nts-induced energy expenditure and locomotor behavior might increase body temperature, which could drive mice to ingest water in an effort to cool the body[59]. Body temperature was increased in both VEH and CNO-treated mice 15 minutes after injection as compared to pre-injection temperature (Figure 2-1F). VEH-treated control mice decreased body temperature 2 hrs after treatment, as might be expected of mice that are no longer stressed and have resumed the low arousal and activity state typical of mice during the light cycle [60,61]. By contrast, body temperature of CNO-treated mice remained elevated two hours later, consistent with the prior finding that activating LHA Nts neurons increases physical activity and

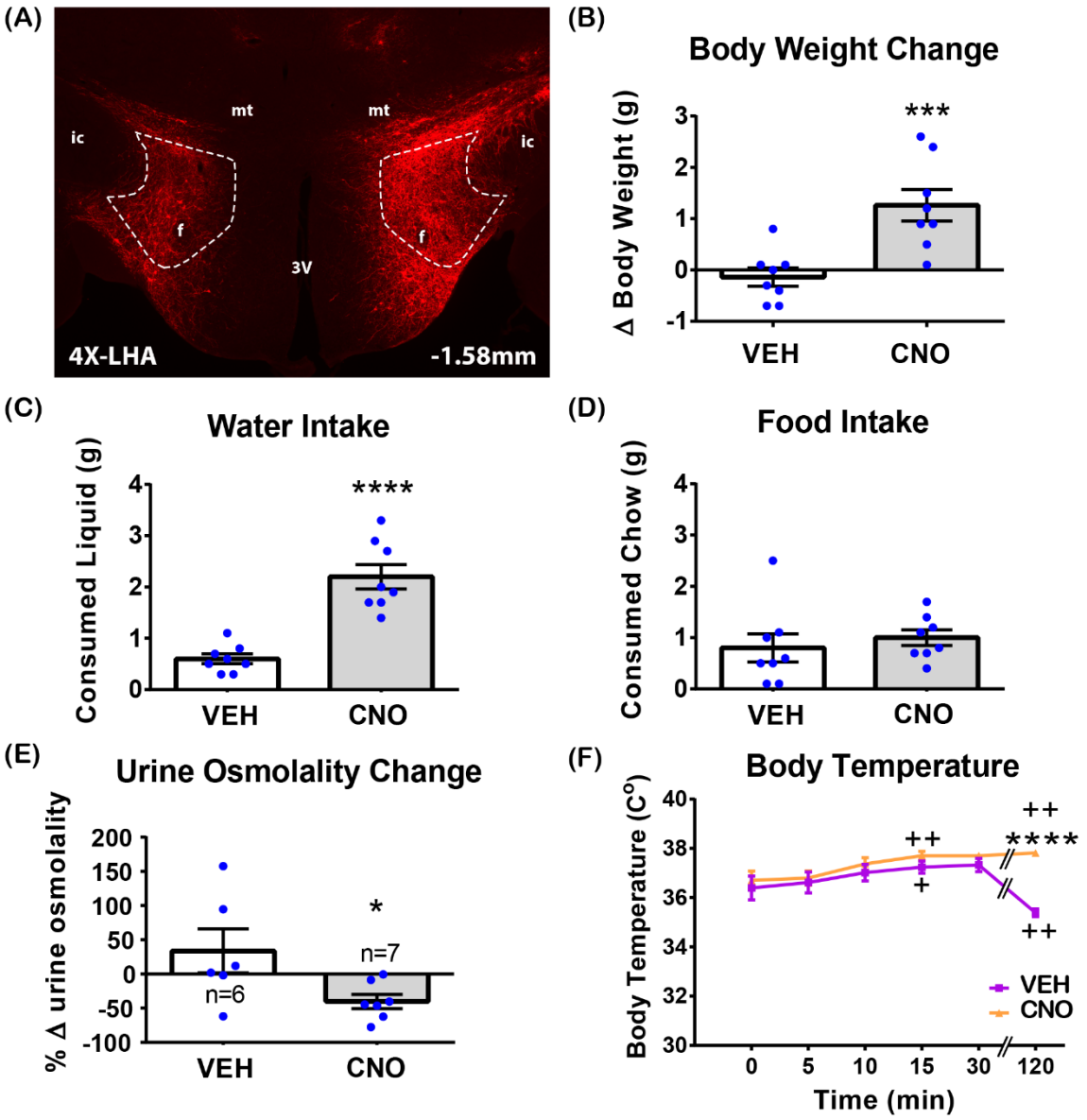

Figure 2-1. Acute activation of the LHA Nts neurons promotes water intake, but not food intake. (A) Representative image of m-Cherry immunofluorescence in the LHA of *Nts*^{Cre} mice, confirming bilateral expression of hM3Dq-mCherry within LHA Nts neurons. Area within the dashed lines indicates the LHA. 3V - 3rd ventricle, mt-mammillothalamic tract, f- fornix, ic - internal capsule. 4X magnification. **(B-F)** Unless otherwise indicated on the graph, n=8. Significant differences were determined by unpaired t-tests. **(B)** Body

Figure 2-1 (*cont'd*) weight change in 4 hrs after i.p. VEH and CNO injections. p=0.0003. **(C)** Consumed water in 4 hrs after i.p. VEH or CNO injections. p<0.0001. **(D)** Consumed food in 4 hrs after i.p. VEH or CNO injections. p=0.5908. **(E)** Urine osmolality change in 4hours after i.p. VEH or CNO injections. p=0.0395. **(F)** Rectal temperature after i.p. injection of VEH or CNO. "+" indicates a time at which temperature was significantly different from t=0 and "*" indicates significant difference between VEH and CNO at the same time point. 2-way RM-ANOVA for change within a treatment group, t=0 vs t=i: F (5, 70) = 6.967, p<0.0001; (time) F (5, 70) = 6.572, p<0.0001; (treatment) F (1, 14) = 5.917, p=0.0290. Sidak`s multiple comparison: (VEH) t=0 vs t=5min p=0.9680, t=0 vs t=10min p=0.4706, t=0 vs t=15min p=0.0354, t=0 vs t=30min p=0.1552, t=0 vs t=120min p=0.0092; (CNO) t=0 vs t=5min p= 0.9680, t=0 vs t=10min p= 0.1162, t=0 vs t=15min p=0.0041, t=0 vs t=30min p= 0.0692, t=0 vs t=120min p=0.0036. 2-way RM-ANOVA for CNO versus VEH: F (5, 35) = 16.38, p<0.0001; (time) F (5, 35) = 4.173, p=0.0044; (treatment) F (1, 7) = 38.71, p=0.0004. Sidak`s multiple comparison: VEH vs CNO at t=5min p= 0.6008, VEH vs CNO at t=10min p= 0.0763, VEH vs CNO at t=15min p= 0.0763, VEH vs CNO at t=30min p= 0.2674, VEH vs CNO at t=120min p<0.0001. *p<0.05; ***p<0.001; ****p<0.0001.

energy expenditure. Overall, these data reveal that acute activation of LHA Nts neurons specifically increases body temperature and promotes water, but not food, consumption.

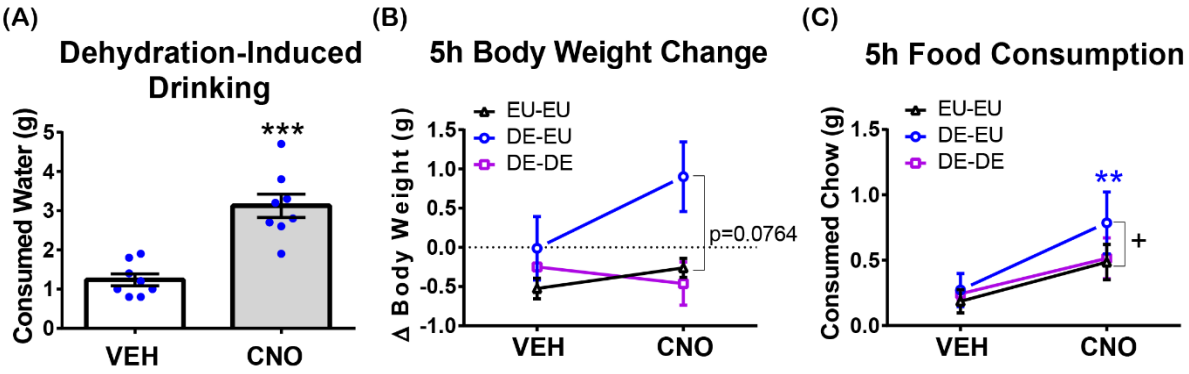

Figure 2-2. Acute activation of the LHA Nts neurons increases drinking beyond physiologic need. n=8 for all panels. EU-EU: mice with access to water for the entire study. DE-EU: mice that were dehydrated overnight then restored water just after treatment with VEH or CNO. DE-DE: mice that were dehydrated overnight and after treatment with VEH or CNO. **(A)** Dehydration-induced drinking 5 hours after i.p. injection of VEH or CNO in overnight dehydrated (DE) mice upon restoration of water (DE-EU mice). ***p=0.0005. **(B)** Body weight change 5 hours after i.p. injection of VEH or CNO. 2-way RM-ANOVA (2x3) for interaction of hydration status and injection condition, F (2, 14) = 1.171, p=0.3387. Hydration status: F (2, 14) = 15.58, p=0.0003. Dunnett's multiple comparisons test for the difference between EU-EU (CNO) and DE-EU (CNO), p=0.0764. Injection condition: F (1, 7) = 2.112, p=0.1894 **(C)** Total food intake 5 hours after i.p. injection of VEH or CNO in overnight dehydrated. Dunnett's multiple comparisons test for the difference between EU-EU (CNO) and DE-EU (CNO), p=0.0359. Sidak`s multiple comparisons test for the difference between DE-EU (VEH) and DE-EU (CNO), p=0.0018. 2-way RM-ANOVA (2x3) for interaction of hydration status and injection condition, F (2, 12) = 1.414, p=0.2809. Injection condition: F (1, 6) = 4.13, p=0.0884. Hydration status: F (2, 12) = 1.989, p=0.1795. “+” indicates significant difference between groups within a

Figure 2-2 (*cont'd*) treatment and "*" indicates significant difference within the group between different treatments.

2.3.2. Activation of LHA Nts neurons augments dehydration-induced drinking

We next investigated if activation of LHA Nts neurons can promote drinking behavior in the face of physiologic water need, e.g. dehydration. Water bottles were removed from the cages of mice expressing hM3D(Gq)-mCherry in LHA Nts neurons just prior to the dark cycle (when mice drink most of their daily water), thereby causing dehydration. The following morning the dehydrated mice were treated with VEH or CNO then water bottles were returned to assess drinking. As expected, dehydrated VEH-treated control mice drank water when it was restored, but CNO-mediated activation of LHA Nts neurons promoted significantly more water intake (Figure 2-2A). We next evaluated whether physiologic hydration status impacted LHA Nts neuronal control of feeding and body weight. CNO-mediated activation of LHA Nts neurons produced comparable food intake and body weight in consistently euhydrated (EU-EU) or dehydrated (DE-DE) mice (Figures 2-2B, C). By contrast, activation of LHA Nts neurons in dehydrated mice that are restored water (DE-EU mice) caused them to drink (Figure 2-2A) and to eat more food compared to CNO-treated euhydrated (EU-EU) controls (Figure 2-2C). In general, dehydration suppresses feeding in rodents (dehydration anorexia), but once water is restored they will drink and then eat[62]. Accordingly, the CNO-induced drinking in formerly dehydrated mice (DE-EU) resolves their dehydration-induced anorexia to reinstate feeding. Although re-hydrated LHA Nts activated mice consumed

more water and food they only exhibited a trend for higher body weight compared to the euhydrated (EU-EU) and dehydrated (DE-DE) controls. These data suggest that activation of LHA Nts neurons can induce water intake even beyond physiologic need.

2.3.3. ***Activation of LHA Nts neurons promotes liquid intake regardless of palatability***

LHA Nts neurons robustly induce water intake but we sought to determine if they also induce intake of other consumable liquids, such as palatable or aversive solutions. To test this, mice expressing hM3D(Gq)-mCherry in LHA Nts neurons were treated with VEH or CNO, then their water bottle was replaced with one containing either hypertonic saline (2% NaCl, Figures 2-3A-C) or 0.014% quinine solution (Figure 2-3D-F). Liquid intake, food intake and body weight were measured 4 hours later. Acute activation of LHA Nts neurons significantly increased intake of 2% NaCl that increased body weight, but did not induce food intake (Figures 2-3A-C). Since mice find 2% NaCl palatable in the short term[63], these data show that activation of LHA Nts neurons can promote intake of palatable liquids, not just water. Similarly, CNO-mediated activation of LHA Nts neurons also promoted consumption of non-palatable quinine solution compared to VEH-treated controls (Figure 2-3D) and acutely increased body weight (Figure 2-3E). Intriguingly, only the CNO-stimulated quinine intake was accompanied by an increase in feeding (Figure 2-3F), while consumption of an innocuous liquid (water) or a palatable liquid (2% NaCl) did not. It is possible that mice eat chow to counteract the bitter taste of the quinine. Overall, these data suggest that activation of LHA Nts neurons promotes intake of any available water-based solution regardless of its taste.

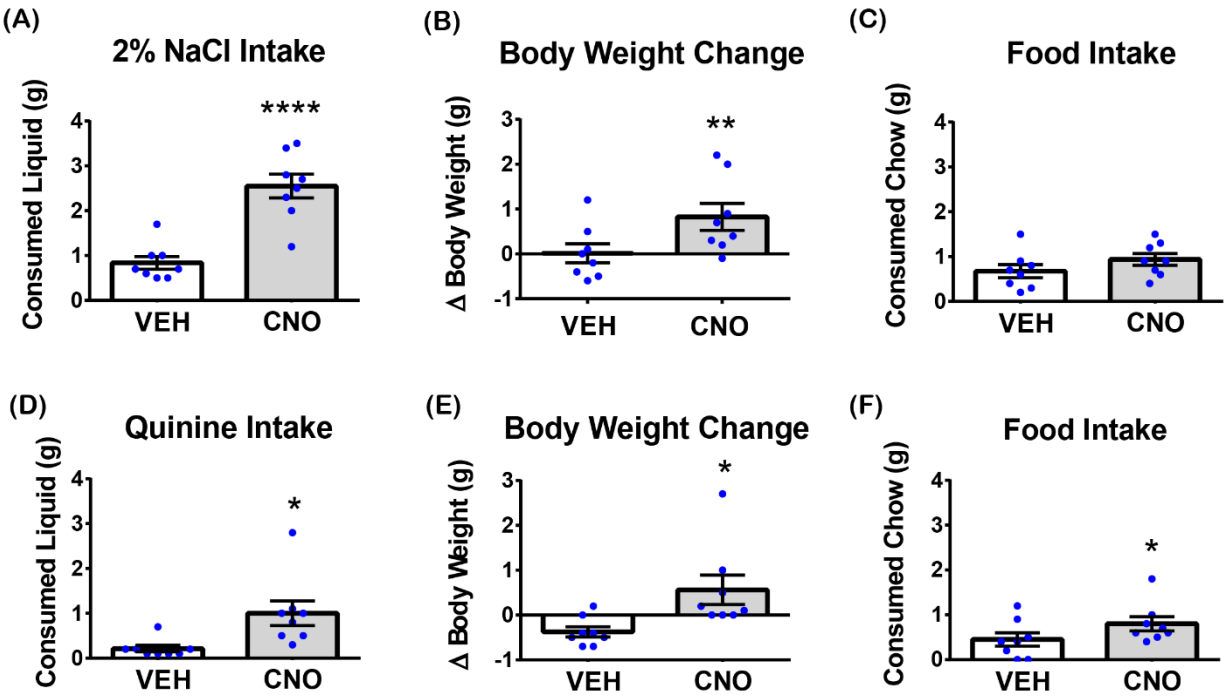

Figure 2-3. Acute activation of LHA Nts neurons promotes intake of available liquids. Graphed data shows the mean ± SEM, n=8. Significant differences were determined by unpaired t-tests. **(A)** 2% NaCl solution intake 4 hours after i.p. VEH or CNO injections, $p<0.0001$. **(B)** Body weight change 4 hours after i.p. VEH or CNO injections during acute 2% NaCl consumption, $p=0.0051$. **(C)** Food consumption 4 hours after i.p. VEH or CNO injections during acute 2% NaCl consumption, $p=0.1031$. **(D)** 0.014% quinine solution intake 4 hours after i.p. VEH or CNO injections, $p=0.0272$. **(E)** Body weight change 4 hours after i.p. VEH or CNO injections during acute 0.014% quinine consumption, $p=0.0358$ **(F)** Food consumption 4 hours after i.p. VEH or CNO injections during acute 0.014% quinine consumption, $p=0.0495$. *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

2.3.4. Mice with activated LHA Nts neurons prefer water or palatable solutions

LHA Nts neurons are sufficient to drive intake of innocuous (water), palatable and aversive liquids, but this could occur via distinct mechanisms. LHA Nts neurons might promote liquid intake by increasing the physiological drive to obtain any liquid, possibly in an effort to cool the body. Alternately, activation of LHA Nts neurons might enhance preference for a liquid, which could also spur intake. To explore the latter possibility, mice expressing hM3D(Gq)-mCherry in LHA Nts neurons were treated with VEH or CNO during 2-bottle preference tests over 48 hrs. First mice were given 48-hrs of *ad libitum* access to water and 2% NaCl. Although 2% NaCl is acutely palatable, prolonged consumption causes osmotic dehydration, an unpleasant state that rodents are eager to resolve by drinking water[62]. Hence, in this extended access experiment, 2% NaCl is a physiologically dehydrating liquid causing the unpleasant state. VEH and CNO-treated mice exhibited a similar preference to prolonged hypertonic 2% NaCl (< 50% preference) indicating that LHA Nts-activated mice do not prefer 2% NaCl to water (Figure 2-4A). Yet, activation of LHA Nts neurons resulted in a significant increase in water intake compared to 2% NaCl over 48-hrs, showing that induction still spurred drinking behavior (Figure 2-4B). No significant changes in body weight (Figure 2-4C) or feeding were observed 48 hrs later (Figure 2-4D). Thus, these data show that LHA Nts neurons (unlike other orexigenic LHA populations) selectively promote intake of water that does not promote weight gain.

We then tested if activation of LHA Nts neurons modified preference for a liquid that is normally not preferred due to its bitter taste, 0.014% quinine. Preference for

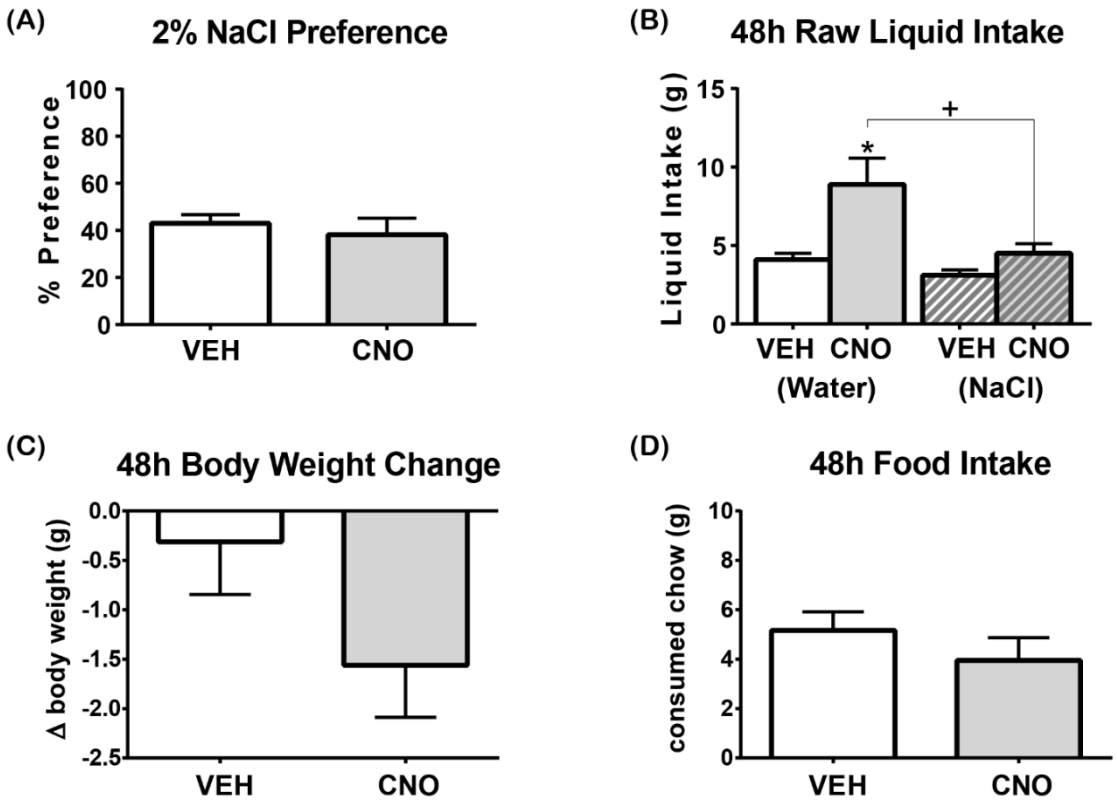

Figure 2-4. Activation of LHA Nts neurons do not change NaCl solution preference. Graphed data shows the mean ± SEM, n=8. 48-hr 2-bottle preference test in which one bottle contains water and the other contains 2% NaCl. All statistical tests are two-tailed paired t-test with alpha: 0.05. **(A)** 2% NaCl solution preference [t(7)=0.7271, p=0.4908]. **(B)** Water intake [t(7)=3.334, p= 0.0125] and 2% NaCl solution intake [t(7)=2.287, p=0.0560]. **(C)** Body weight change over 48-hours [t(7)=1.657, p=0.1414] **(D)** Food intake [t(7)=1.189, p=0.2732]. "+" indicates significant difference between the consumption of different liquids with the same treatment and "*" indicates significant difference in consumption of the same liquid between different treatments. *p<0.05, +p<0.05

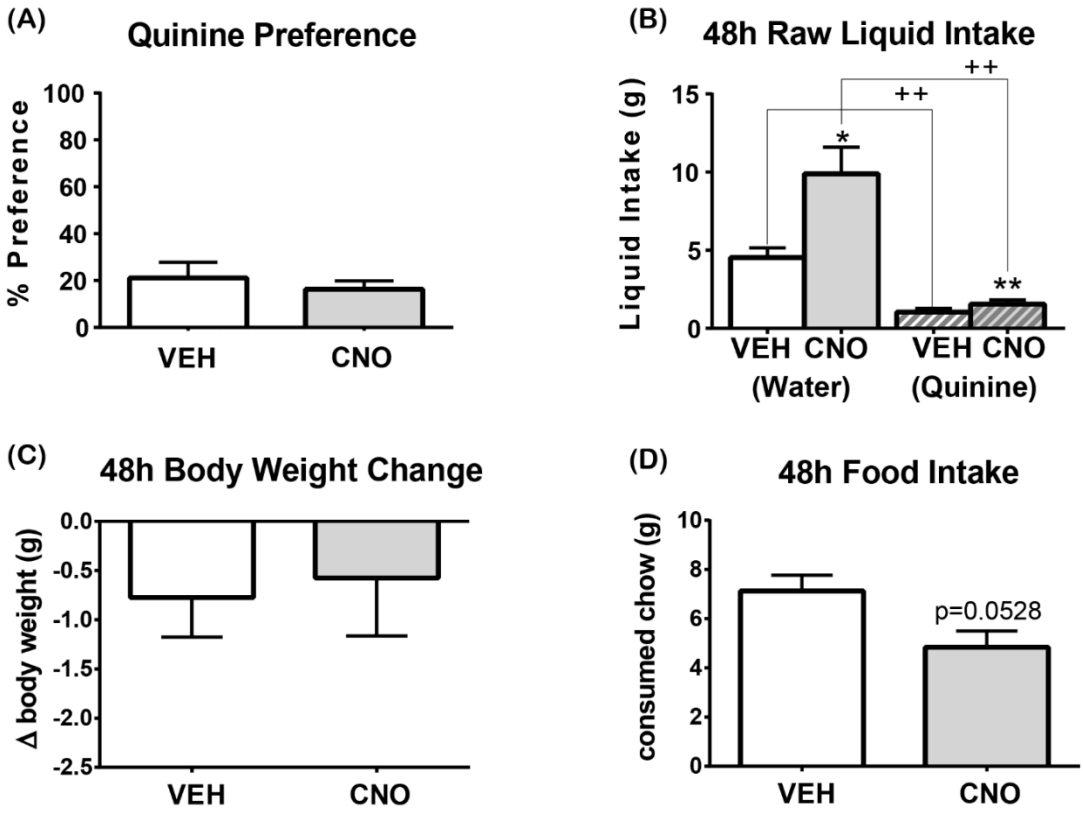

Figure 2-5. Activation of LHA Nts neurons do not change quinine solution preference. Graphed data shows the mean ± SEM, n=8. 48-hr 2-bottle preference test in which one bottle contains water and the other contains 0.014% quinine. All statistical tests are two-tailed paired t-test with alpha: 0.05. **(A)** 0.014% quinine preference, [t(7)=0.8685, p=0.4139]. **(B)** Water intake [t(7)=2.833, p=0.0253] and 0.014% quinine solution intake, [t(7)=5.274, p=0.0012]. VEH-induced water vs quinine consumption: t(7)=4.41, p=0.0031. CNO-induced water vs quinine consumption: t(7)=4.549, p=0.0026. **(C)** Body weight change in 48-hr [t(7)=0.2587, p=0.8033]. **(D)** Food intake, [t(7)=2.328, p=0.0528]. "+" indicates significant difference between the consumption of different liquids with the same treatment and "*" indicates significant difference in consumption of the same liquid between different treatments. *p<0.05, **p<0.01, ++ p<0.01.

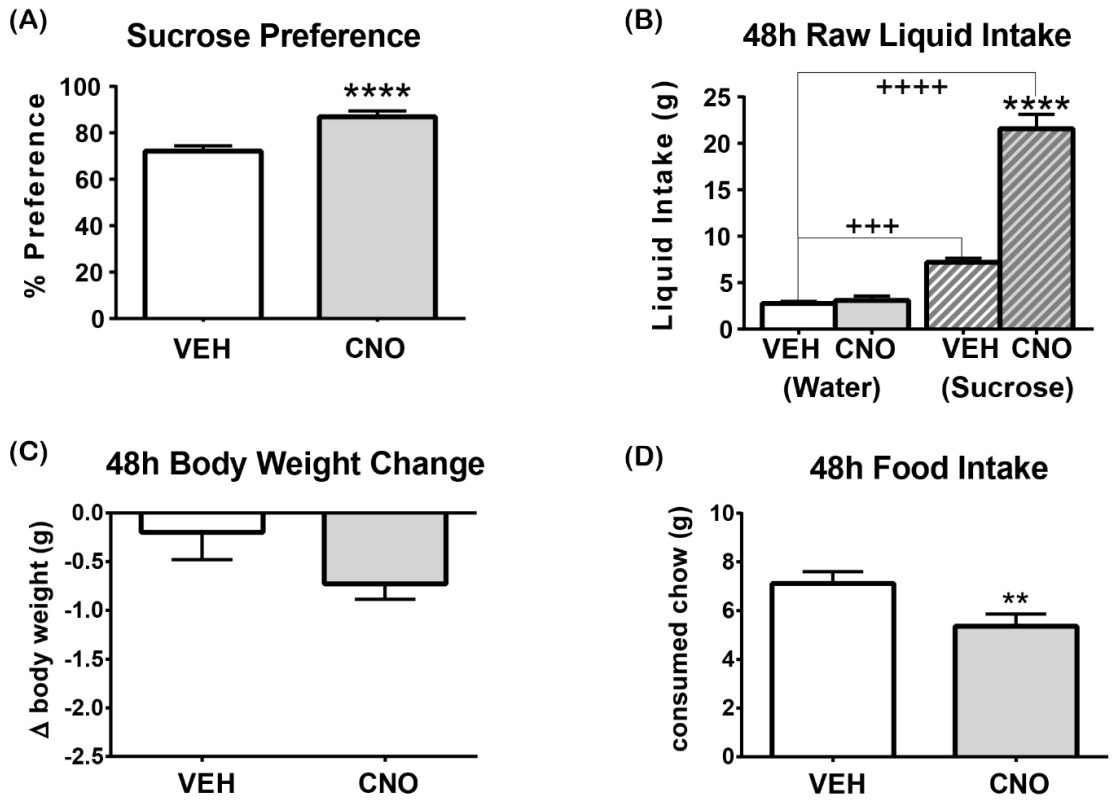

Figure 2-6. Sucrose preference is increased when LHA Nts neurons are activated. Graphed data shows the mean ± SEM, n=7. 48-hr 2-bottle preference test in which one bottle contains water and the other contains 1% sucrose. All statistical tests are two-tailed paired t-test with alpha: 0.05. **(A)** Sucrose preference, ****p<0.0001. [t(6)=16.48, p<0.0001] **(B)** 1% sucrose intake [t(6)=11.74 , p<0.0001] and water intake [t(6)=1.202, p=0.2746]. VEH-induced water vs 1% sucrose solution consumption: t(6)=8.955, p=0.0001. CNO-induced water vs 1% sucrose solution consumption: t(6)=9.896, p<0.0001. **(C)** Body weight change in 48-hr, [t(6)=1.584, p=0.1642]. **(D)** Food intake [t(6)=4.822, p=0.0029]. "+" indicates significant difference between the consumption of different liquids with the same treatment and "*" indicates significant difference in consumption of the same liquid between different treatments. **p<0.01, ****p<0.0001, +++p<0.001, ++++p<0.0001.

quinine was similarly very low (<30%) in VEH and CNO-treated mice, indicating that this solution is highly non-preferable compared to water (Figure 2-5A). CNO-mediated activation of LHA Nts neurons robustly stimulated water intake, showing that this system remains sufficient to induce drinking, and even modestly increased quinine intake (Figure 2-5B). Despite the increased liquid intake, mice with activated LHA Nts neurons did not exhibit weight gain over 48 hrs (Figure 2-5C) and, in fact, showed a nearly significant reduction in feeding (Figure 2-5D, $p = 0.0528$). Thus, although activation of LHA Nts neurons will promote intake of quinine if it is the only liquid available, water is still preferred over this unpalatable substance. Together, the quinine and 2% NaCl data confirm that activation of LHA Nts neurons does not indiscriminately promote liquid intake, but does increase drinking drive that demands to be satisfied, as possible.

Lastly, we investigated whether the increased drinking drive that occurs due to activating LHA Nts neurons could modify preference for palatable solutions, in this case 1 % sucrose solution. VEH and CNO-treated mice prefer sucrose to water (>50% preference) but CNO-mediated activation of LHA Nts neurons significantly increased sucrose preference compared to the VEH-treated controls (Figure 2-6A). While LHA Nts activation promoted increased intake of sucrose and water, the mice consumed significantly more sucrose solution than water (Figure 2-6B). Importantly, the mice did not gain weight over this time despite increased caloric intake from the sucrose (Figure 2-6C). LHA Nts activated mice also consumed less food (Figure 2-6D), perhaps because they were ingesting extra calories via the sucrose solution and no longer needed to derive as many calories from food. In sum, these data indicate that activation of LHA Nts

neurons is sufficient to induce drinking behavior, but that water or palatable solutions are preferred over physiologically dehydrating or unpalatable liquids.

2.4. DISCUSSION

The LHA has long been recognized as essential for feeding and drinking behaviors, yet there remains much to understand about how the molecularly diverse LHA populations mediate these behaviors. Most of the characterized LHA populations promote intake of both food and water[33,37–39,41–45,53], but other recently described populations restrain feeding without modulating water intake[64–66]. Thus, ingestive behaviors can be separately regulated by some LHA populations, but a population that biased for drinking behavior was yet to be described. Here we found that LHA Nts neurons are such a population because they strongly induce drinking but not feeding. Moreover, activation of LHA Nts neurons is sufficient to induce drinking behavior regardless of fluid balance status or the palatability of a single provided liquid. However, LHA Nts-induced drinking is informed by the physiologic valence and palatability of whatever liquids are available to drink, such that palatable or non-dehydrating liquids are preferred. Hence, LHA Nts neurons are a novel LHA population to bias for drinking over feeding, and may be useful targets to treat aberrant fluid balance states that threaten health, such as dehydration or excessive water ingestion.

Given that LHA Nts neurons can promote drinking behavior it will be important to understand which precise signals mediate this physiology. Although we refer to them as “LHA Nts neurons”, the neurons express Nts and some also express the neuropeptides galanin[65,67], CRH[53] and the neurotransmitter GABA[47]. It remains to be determined how each of these releasable transmitters contributes to LHA Nts-induced physiology and if

there is a certain signal that mediates drinking behavior. CRH is a logical candidate via which LHA Nts neurons might regulate drinking behavior, based on its regulation in dehydration anorexia[68]. The role of LHA Nts-induced Nts itself will be particularly important to investigate, given that Nts has been documented both as a dipsogenic[54] and anorectic[49–52] neuropeptide. LHA Nts-induced promotion of drinking may be partially mediated through the release of Nts and actions via the neurotensin receptor 1 (NtsR1) [55]. Alternatively, Nts released from LHA Nts neurons may act via neurotensin receptor 2 (NtsR2). In the future, reagents to modulate the expression and function of NtsR1 or NtsR2 in a site-specific manner will be useful to determine if Nts modulates drinking behavior via either of these receptor isoforms. Furthermore, in the current study we investigated the immediate drinking effect of activation of LHA Nts neurons that is divergent from the feeding suppression observed as an after effect hours later in chronic activation [55]. Going forward, it is also important to identify differences in underlying intracellular cascades and downstream target neurons. It might be surmised that if the disparate regulation of feeding and drinking is due to the distinct intracellular and downstream paths within the LHA Nts neurons, the drinking promoting effects might be triggered via immediate release of neurotransmitters/neuropeptides to the direct action site whereas feeding suppressing effects may be due to longer processes such as gene transcriptions or modifications, or maybe due to indirect action on other neurons that regulate feeding. A possibility for the indirect regulation of feeding could be via modulation of activity of LHA OX neurons by LHA Nts neurons [46,48,69].

Our data show that LHA Nts neurons are sufficient for inducing drinking but further studies are required to determine if they are also necessary for drinking behavior and

water homeostasis. The brain must coordinate osmosensory status with motivated behavior (e.g. the act of seeking and consuming water) to appropriately regulate bodily water homeostasis. Given that lesioning the LHA causes adipsia[25,26] that persists even in the face of severe dehydration, this area likely contributes to coordinating physiologic water need and intake behavior. Indeed, the LHA receives input from osmosensory regions and projects to numerous brain areas that could modulate goal directed behaviors, including the ventral tegmental area (VTA)[35,55,70], the locus coeruleus[64], the preoptic area[71,72] and the paraventricular hypothalamic nucleus (PVH)[73]. Thus, it is not unreasonable that Nts neurons within the LHA might contribute to coordinating physiologic drinking, and their role in this process will be important to determine. Since osmoregulatory-linked drinking behavior is crucial for survival, however, it is highly likely that there are redundant mechanisms to modulate drinking behavior, including within the LHA. For example, LHA Orexin neurons are modulated by changes in fluid balance, and experimental activation of Orexin neurons induces water as well as food intake[41,53].

It is also possible that LHA Nts neurons mediate purely motivational aspects of drinking behavior, hence they may be sufficient to induce drinking but not required for maintenance of water homeostasis. Indeed, LHA Nts neurons project to midbrain reward centers [35,55,70] that modulate goal-directed behaviors, which could be a mechanism to modulate drinking behavior. Increased sucrose preference (Figure 2-6A) might be hinting to this possible motivational aspect as sucrose solution is rewarding for rodents and activation of LHA Nts neurons promoted further increase in sucrose solution intake and preference. It is especially tempting to speculate about motivational aspects due to a recent work indicating recruitment of dopamine (DA) systems leading increase in

dopamine levels in nucleus accumbens shell in a need- and taste-based manner upon dehydration and sodium depletion[74]. However, we did not test the motivation to obtain liquids in the current paper and in the future, it might be interesting to apply a motivational test (such as progressive ratio in operant paradigm) both for water and palatable solutions. On the other hand, it is important to note that the activation of LHA Nts neurons suppresses the motivation to obtain sucrose pellets in fasted mice [55]. Therefore, it is likely that the nature of regulation of calorically relevant food and liquids by LHA Nts neurons might be different and there might be an underlying hedonic and motivational mechanism driving the liquid intake. Besides, it might be considered that rather than the caloric value (as caloric intake is suppressed with LHA Nts neural activation seen in food intake[55]), it may be the palatable taste of the sucrose solution that is causing a further increased sucrose preference. Under this rationale, it would be intriguing to see how the preference of a pleasurable but calorically irrelevant solution (such as saccharin solution) would be affected after LHA Nts activation. One might speculate if there is a hedonic aspect, the preference of an artificially sweetened solution would be as high as sucrose solution; however, if it is the contribution of the calories in the solution and post-ingestive effects driving the increase in sucrose preference, then preference for the artificially sweetened solution would not expected to change by LHA Nts neural activation. In the future, to decipher the contribution of hedonics and motivation to drinking driven by LHA Nts neurons and to address the mentioned speculations, further studies including pleasurable but calorically irrelevant solutions (e.g. artificial sweeteners) and liquid foods (e.g. Ensure by Abbott Laboratories), tests measuring motivation and liking (such as progressive ratio in operant testing and conditioned place preference/aversion) could be done.

On the other hand, the possibility of the nature of this regulation may not be related to motivation, but might be purely modulating the physiologic drinking circuits should not be ruled out. Thirst regulatory roles of LT areas[5,59] and crosstalk between subfornical organ (SFO), median preoptic area (MnPO) and organum vasculosum of the latera terminalis (OVLT) have been well studied, especially in the recent years with the development of molecular tools and Cre/LoxP technology[12,75]. Recently, it was demonstrated that SFO[76,77] as well as MnPO and OVLT[77] circuits encode an aversive status associated with thirst. In addition, recent work showed an anticipatory brake on drinking as soon as fluid ingestion starts via inhibition of nitric oxide synthase-expressing drinking-promoting SFO neurons[14] by inhibitory glucagon-like peptide receptor 1 expressing MnPO neurons[75]. The connection of these LT areas with the LHA has been documented before[20–23]. Moreover, a functional connection between the SFO and the LHA has been minimally studied by observing a link between adrenergic receptor activity in the LHA and angiotensin II-induced drinking through SFO[78–80], yet an extensive study to investigate this potential circuit has not been done. Similarly, the LHA is one of the areas where MnPO and OVLT neurons send dense projections[16]. Yet, to our knowledge, nature of those potential circuits connecting the SFO, MnPO and OVLT drinking circuits to the LHA has not been investigated extensively. Therefore, it is intriguing to investigate potential LHA-LT circuits to regulate drinking behavior in detail. Given that the activation of LHA Nts neurons increase water consumption in dehydrated mice beyond physiologic need (Figure 2-2A), one may speculate that LHA Nts circuits may modify the regular control and breaks of the LT systems directing drinking behavior and delaying the thirst quenching or maybe possibly putting a brake on the anticipatory drinking circuits via

acting on GABAergic MnPO neurons inhibiting drinking-promoting SFO neurons[14,75]. In the future to investigate these possibilities, circuit specific activation and inhibition studies might be applied.

Our current study focused on understanding how LHA Nts neurons modify drinking behavior, but going forward will be important to determine if these neurons also engage endocrine mechanisms to modulate fluid balance. The two most well studied hormones in water balance are angiotensin II and vasopressin. Systemic and central angiotensin injection promotes drinking[81–83], and it is possible that this system might intersect with LHA Nts neurons to induce water intake. Given that circulating angiotensin signaling via osmosensory regions [78–80] precedes changes in adrenergic signaling in the LHA, the physiologic effects of angiotensin II-may occur prior to any induction of LHA Nts neurons. On the other hand, vasopressin neurons are important contributors to peripheral water balance, and dehydration-induced increases in vasopressin decrease excretion of water through the kidneys to conserve water[5]. By contrast, LHA Nts-induced drinking leads to water-excretion, as shown by decreased urine osmolality (Figure 2-1E). Therefore, it is possible that activation of LHA Nts neurons might suppress vasopressin neurons so as to dissipate the increased water load via excretion. Going forward, it will be of interest to determine if LHA Nts neurons project to, and perhaps inhibit, vasopressin neurons found within the supraoptic nucleus (SO) and paraventricular hypothalamic nucleus (PVH). Though beyond the scope of this study, circuit tracing and temporal endocrine assessments will be useful to determine if LHA Nts neurons engage and modulate these endocrine drinking systems.

We cannot yet determine whether LHA Nts-induced drinking is a response to elevated body temperature in an effort to cool the body. Our current data indicate that activation of LHA Nts neurons may sustain increased body temperature in mice. This possibility is consistent with prior work showing that activation of LHA Nts neurons increases locomotor activity and energy expenditure[55], both of which might maintain elevated body temperature as we saw in this study. Notably, however, our data suggest that LHA Nts neurons do not contribute to the Nts-mediated hypothermia that is observed with systemic or central Nts treatment[84–86]. This is important because it suggests that LHA Nts neurons do not mediate all of the disparate physiology that has been ascribed to Nts[87], and that they may exert discrete facets of Nts-mediated signaling. Given that hypothermia and hypotension are induced by systemic and central Nts, the absence of LHA Nts-induced hypothermia raises hope that LHA Nts neurons may avoid such adverse physiologic effects. In sum, the unique role of LHA Nts neurons in specifically promoting drinking behavior suggests that these neurons serve a unique role amongst the LHA populations and within Nts signaling. Future studies to elaborate the signals and circuits whereby LHA Nts neurons modulate behavior may be useful to inform development of treatments for maladaptive drinking behaviors.

ACKNOWLEDGEMENTS

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APPENDIX

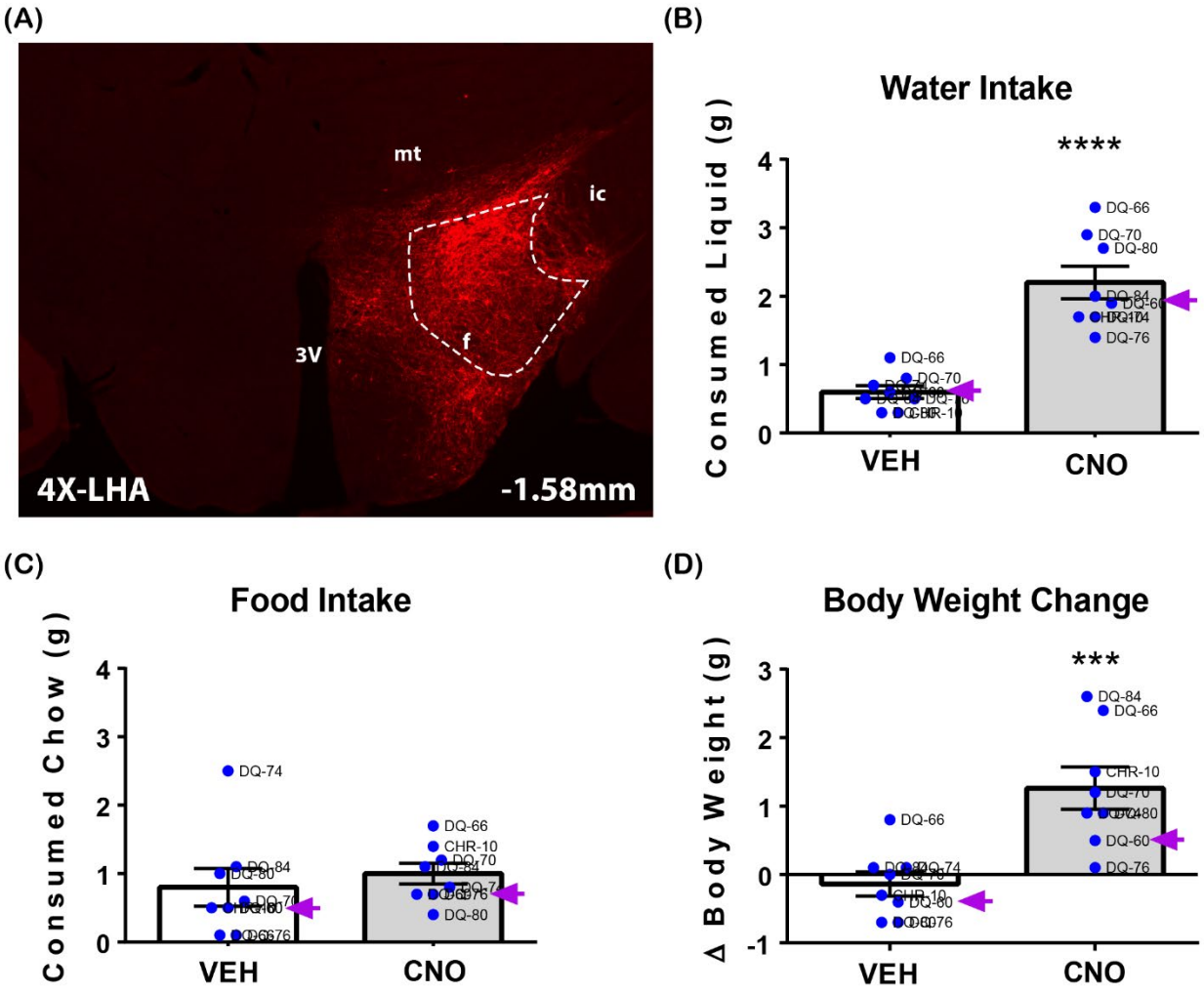
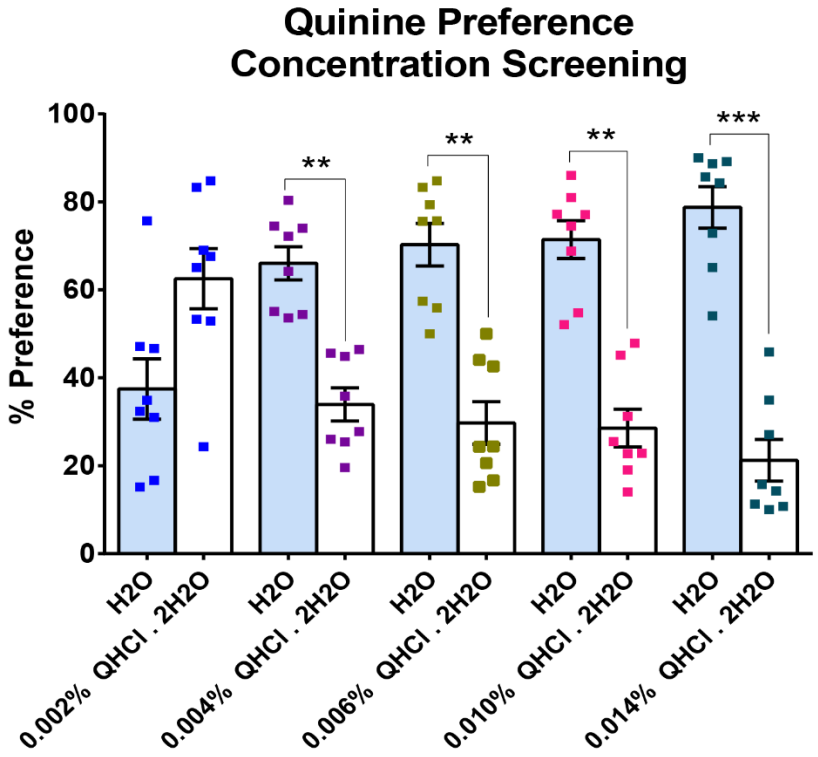


Figure S2-1. Comparison of bilateral or unilateral expression of hM3D(Gq)-mCherry. (A) Immunofluorescent detection of hM3Dq-mCherry in the LHA of the *Nts*^{Cre} mouse that had robust, unilateral hM3Dq-mCherry expression in throughout the LHA with minimal spread beyond it (mouse ID: DQ-60). Area within the dashed lines indicates the LHA. 3V - 3rd ventricle, mt-mammillothalamic tract, f- fornix, ic - internal capsule. **(B-D)** Data from all mice included in the study, which all had bilateral LHA Nts neuron-induced hM3D(Gq)-mCherry except for the unilateral expressing mouse (DQ-60, magenta arrow). Each data point identifies a mouse. **(B)** Total water intake, **(C)** total food intake and **(D)**

Figure S2-1 (*cont'd*) body weight change 4 hours after i.p. injection of VEH or CNO. Note that DQ-60 had comparable drinking, feeding and body weight change to mice with bilateral hM3D(Gq)-mCherry expression and so was included in the study. These data suggest that unilateral activation of LHA Nts neurons may be sufficient to modulate drinking behavior.

(A)



(B)

	0.002%		0.004%		0.006%		0.010%		0.014%	
Mouse ID	%water	%QHCl.2H2O	%water	%QHCl.2H2O	%water	%QHCl.2H2O	%water	%QHCl.2H2O	%water	%QHCl.2H2O
CHR-10	31.0	69.0	64.2	35.8	84.8	15.2	86.0	14.0	90.0	10.0
DQ-60	34.9	65.1	74.0	26.0	75.6	24.4	74.5	25.5	88.7	11.3
DQ-66	46.7	53.3	72.2	27.8	75.7	24.3	77.2	22.8	84.3	15.7
DQ-70	47.1	52.9	80.4	19.6	83.3	16.7	81.0	19.0	89.2	10.8
DQ-74	32.4	67.6	74.5	25.5	79.4	20.6	77.1	22.9	85.7	14.3
DQ-76	15.2	84.8	53.6	46.4	50.0	50.0	52.1	47.9	65.1	34.9
DQ-80	16.7	83.3	54.4	45.6	55.9	44.1	54.8	45.2	54.1	45.9
DQ-84	75.7	24.3	55.1	44.9	57.4	42.6	68.8	31.3	72.9	27.1

Figure S2-2. Screening of quinine HCl dihydrate solution preference. (A) 2-bottle preference test over 48 hr comparing water and solutions of quinine hydrochloride dihydrate (QHCl.2H2O). Graphed data shows the average percent quinine preference ± SEM, n=8. Blue bars indicate water, white bars indicate the quinine solution and each point represents a mouse. *Nts*^{Cre} mice prefer water over quinine starting 0.004% QHCl.2H2O in water. Paired t-tests, 0.002%: t(7)=1.826, p=0.1106; 0.004%: t(7)=4.265,

Figure S2-2 (*cont'd*) p=0.0037; 0.006%: t(7)=4.198, p=0.0040; 0.010%: t(7)=4.988, p=0.0016; 0.014%: t(7)=6.101, p=0.0005. **(B)** Table of Individual the percent preferences for each mouse during screening. Bolded blue numbers indicate mice with close to 50% or above preference for quinine solution.

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CHAPTER 3. A CIRCUIT TO BEHAVIORAL ANALYSIS OF HOW LATERAL HYPOTHALAMIC AREA NEUROTENSIN NEURONS MEDIATE MOTIVATED DRINKING BEHAVIOR

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This chapter is a modified version of a primary research paper manuscript prepared for submission.

ABSTRACT

Feeding and drinking are essential for survival, yet it remains unclear how the brain specifically coordinates the motivation to drink vs eat. The lateral hypothalamic area (LHA) modulates the motivation to ingest, but most of the neurochemically-defined populations of LHA neurons simultaneously promote drinking and feeding. By contrast, we recently showed that activation of LHA neurotensin (Nts)-expressing neurons (LHA Nts neurons) specifically biases for water intake while restraining feeding. Here we explored the neural mechanisms by which LHA Nts neurons divergently regulate ingestive behavior. We show that LHA Nts neurons are anatomically positioned to coordinate both drinking and feeding since they receive inputs from energy and fluid-sensing brain regions. Intriguingly, LHA Nts neurons send distinct projections to the ventral tegmental area (VTA) and the lateral proptic area (LPO), regions that are implicated in modulating

feeding and drinking behavior, respectively. Activation of all LHA Nts neurons is sufficient to increase the motivation to drink, but not eat, although activation is not required to maintain homeostatic ingestion. Finally, we show that the LHA Nts → LPO circuit is sufficient to mediate drinking behavior, but this circuit does not suppress feeding as observed after activating all LHA Nts neurons. Overall, our study is the first to identify LHA Nts neuronal circuitry that contribute to these LHA neurons unique ability to selectively augment the motivation to drink vs. eat. Specific manipulation of LHA Nts→LPO circuitry may be useful to improve fluid intake without disrupting feeding or general homeostasis.

3.1. INTRODUCTION

Food and water are necessary for survival, so animals must engage ingestive behaviors (feeding and drinking) to obtain them. The brain therefore constantly surveys osmolality and energy status and adjusts drinking or feeding as needed to maintain homeostasis. However, discoordination of these processes endangers fluid and energy balance, and can contribute to life threatening ingestive disorders including obesity, anorexia, polydipsia and chronic dehydration[1–3]. Strangely, despite the fact that eating and drinking are the most common and frequent of all animal behaviors, the underlying neural mechanisms coordinating them remain incompletely understood. Defining the neural circuits that orchestrate drinking or feeding behavior is therefore essential to understand how animals thrive and survive, and necessary to tailor proper treatments to drinking or feeding imbalances that threaten health.

The lateral hypothalamic area (LHA) is anatomically positioned to coordinate osmotic and energy sensing with goal-directed ingestive behavior[4–8] and indeed was initially deemed a “feeding center” because rodents with LHA lesions lose all motivation to eat food[9,10]. This designation, however, obscured the equally important role of the LHA in drinking behavior. Indeed, LHA-lesioned rodents also lose all motivation to drink water, and in fact die from dehydration before starvation[11,12]. Thus, the LHA modifies both ingestive behaviors necessary for survival. Yet research has largely characterized how the LHA impacts feeding, whereas LHA-mediated drinking has remained enigmatic. The discovery of molecularly- and projection-specified populations of neurons within the LHA suggested that some of them might specifically coordinate drinking vs feeding. Most of the characterized LHA populations promote intake of both food and water[13–23], but others restrain feeding without modulating water intake[24–26] suggesting that ingestive behaviors can indeed be separately regulated by some LHA populations. However, the LHA populations studied to date do not explain how the LHA specifically induces drinking behavior vs. food intake.

Recently, LHA neurons expressing the neuropeptide neurotensin (Nts) have emerged as a possible population to bias for drinking vs feeding. These “LHA Nts neurons” co-express the inhibitory classical neurotransmitter GABA, but constitute a subpopulation of all LHA GABAergic neurons[27–32] that have been shown to promote feeding behaviors[14,17,22,33]. Interestingly, LHA Nts neurons are heterogeneous, and at least some subpopulations have been described that differ in their expression of the long form of the leptin receptor (LepRb)[28], their responses to energy (leptin) or dehydration cues[32,34], and projections to the ventral tegmental area (VTA)[32]. Indeed, several lines of

evidence suggest that subsets of LHA Nts neurons might differentially control ingestive behaviors. Anorectic leptin action is partially mediated by the subset of LHA Nts neurons that co-express LepRb, and loss of LepRb from these neurons impairs leptin-mediated suppression of feeding, decreases locomotor activity and energy expenditure and promotes adiposity[27,30]. Moreover, experimental activation of LHA Nts neurons suppresses feeding[35], which depends on signaling via neurotensin receptor-1 (NtsR1)[31]. These observations identified a novel role for LHA Nts neurons in mediating anorexia, in contrast to the activation of adjacent orexin/hypocretin or melanin concentrating hormone (MCH)-expressing LHA neurons[36,37], or the bulk activation of all GABAergic neurons[14,17,22,33] that prompt food intake. Yet notably, experimental activation of all LHA Nts neurons also promotes voracious drinking. Thus, LHA Nts neurons are unique from other LHA neurons because they differentially regulate ingestive behaviors, promoting drinking but not feeding[35,38]. Given the heterogeneity of LHA Nts neurons, we hypothesized that there may be distinct, projection-specified subsets of LHA Nts neurons dedicated to each ingestive behavior, such that activation of a specific subpopulation can promote drinking vs. feeding. Here we investigated the neural circuitry by which LHA Nts neurons influence drinking behavior and provide evidence of a dedicated circuit by which LHA Nts neurons can mediate fluid intake. These findings are the first to reveal how the LHA can selectively modulate the motivation drink, and advances understanding of how this brain region coordinates specific ingestive behaviors required for survival.

3.2. MATERIALS AND METHODS

3.2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University in accordance with Association for

Assessment and Accreditation of Laboratory Animal Care and National Institute of Health guidelines. Mice were bred and housed on a 12-hr light/12-hr dark cycle schedule with *ad libitum* access to chow (Teklad 7913) and water, except during fasting and dehydration experiments. Male and female adult mice (12–140 wk old) were used in the studies.

3.2.2. Surgery

Male and female *Nts*^{Cre} [30], *Nts*^{Cre}*;HA*, *Nts*^{Cre}*;L10-GFP* and wild type C57/Bl6 mice were stereotaxically injected with cre-dependent vectors at a rate of 100 nL/min. For anterograde tracing, *Nts*^{Cre} mice (n=4) received a unilateral LHA injection of 250nL of Adenoviral-Synaptophysin-mCherry (graciously provided by Dr. Martin G. Myers Jr, University of Michigan), then were euthanized 2 wks later. To label neurons that monosynaptically project to LHA Nts neurons, *Nts*^{Cre}*;L10-GFP* and wild type mice (controls) were injected into the LHA with cre-dependent helper TVA virus coding for hSyn-TVA-rabiesB19G (200-300 nL). After 3-4 wks mice received 200-400nL of pseudotype rabies virus (EnvA) - ΔG-mCherry into the LHA (to label all inputs to LHA Nts neurons) or into the VTA or LPO (to only label the inputs of LHA Nts neurons that project to each site). Monosynaptic tracers were graciously provided by Dr. Martin G. Myers Jr, University of Michigan. Mice receiving both vectors in the LHA were sacrificed 10 days after the last surgery (n=3 *Nts*^{Cre}*;L10*, n=2 *Nts*^{Cre}*;L10-GFP* with no helper virus, n=1 wild type with both viruses). Mice injected in the LHA-LPO (n=3 *Nts*^{Cre}*;L10,* n=1 *Nts*^{Cre}*;L10-GFP* with no helper virus) and LHA-VTA (n=3 *Nts*^{Cre}*;L10-GFP* , n=1 *Nts*^{Cre}*;L10-GFP* with no helper virus) were sacrificed 4wks after the last surgery.

For simultaneous labeling of LHA Nts inputs to the VTA and LPO, *Nts*^{Cre}*;HA* mice (n=3), were unilaterally injected with AAV5-EF1a-DIO-eYFP-WPRE-hGH and AAV5-

EF1a-DIO-mCherry (graciously provided by Dr. Michelle Mazei-Robison, Michigan State University) into the LPO (250nL) and VTA (160nL). Injections were counter-balanced between brain regions to control for any differences in retrograde efficiency of the AAV-eYFP and AAV-mCherry. Mice were sacrificed 12wks after surgery to allow for complete labeling. LHA (anteroposterior: −1.34, mediolateral:±1.05, dorsoventral: −5.20), VTA (anteroposterior: −3.20, mediolateral:±0.48, dorsoventral: −4.65) and LPO (anteroposterior: +0.42, mediolateral:±0.75, dorsoventral: −5.60) coordinates were taken from the Paxinos and Franklin mouse brain atlas[39].

For chemogenetic experiments, *Nts*^{Cre} and wild type mice received bilateral LHA injections (500 nL per side) of AAV2-hSyn-DIO-hM3D(Gq)-mCherry and AAV2-EF1a-DIO-hM4D(Gi)-mCherry (University of North Carolina Vector Core and Addgene). For LPO cannulation surgeries, the LPO was cannulated on one side at 0^{o} angle (anteroposterior: +0.42, mediolateral:±0.75) and the other side at 20^{o} angle (anteroposterior: +0.42, mediolateral:±2.79) with 4.60mm stainless steel cannula and with 1.00mm projection dummy.

3.2.3. Home Cage and Metabolic Cage Drinking and Feeding Experiments in DREADD-Expressing Mice

For the chemogenetic inhibition of LHA Nts neurons, body weight and composition (percentage of lean fat and fluid mass) were analyzed using a Bruker Minispec LF50 prior to and after housing in TSE metabolic cages (PhenoMaster, TSE Systems). Mice were acclimatized for 1 day to the TSE cages, followed by twice daily (9:30 am/5:30 pm) sham injections. Mice then received 2 days of twice-daily i.p. VEH treatment (0.2M pH 7.4 sterile PBS), followed by 2 days of i.p. clozapine N-oxide (CNO) (0.3 mg/kg) injections. Food

and water consumption, total activity, respiratory exchange ratio (RER) and energy expenditure were tracked. Total of n=16 *Nts*^{Cre} mice expressing inhibitory DREADD (n=9 females, n=5 males) and n=10 WT control mice (n=4 females, n=6 males) were analyzed in Bruker, TSE and the home cage experiments. Since no significant difference between male and female consumption was observed, female and male consumption data were combined and analyzed by normalizing against the initial body weights and shown as percent fraction of body weight.

For the basal consumption, fasting and dehydration experiments, mice injected with activatory and inhibitory DREADDs in the LHA were housed in their home cages, where they received i.p. injection or cannula infusion (LPO cannulated activatory DREADD expressing mice) of VEH or CNO. Injection of bromophenol blue into the LPO of WT mice confirmed that a 100 nL volume was contained to the LPO (data not shown), hence for LPO-specific injections we administered 0.3 mg/kg CNO in100nL per side. The NtsR1 antagonist, SR48692 (cat # SML0278, Sigma), was prepared in 1% Tween as described previously[35], and i.p. injected at 0.3 mg/kg, 30 minutes before VEH/CNO infusions. Body weight, food and water bottle weights were manually recorded before and after any treatments using an electronic balance. Total of n=14 *Nts*^{Cre} expressing activatory DREADD (n=9 females, n=5 males) and n=5 WT control mice (n=4 females, n=1 male) were used in systemic activation studies in home cages. Also, total of n=10 LPO cannulated *Nts*^{Cre} mice expressing activatory DREADD (n=8 females, n=2 males) and n=4 LPO cannulated WT control mice (n=4 females) were used in home cage ingestion studies. Because the unilateral LHA Nts→LPO circuit activation was enough to produce voracious drinking (data not shown), we analyzed bilaterally and unilaterally (due

to occlusion of a single cannula) VEH/CNO infused mice together in the final analyses. LPO cannulated mice received once a day (9:30am) cannula infusions while other groups received twice a day (9:30 am/5:30 pm) i.p. injections.

For dehydration-induced drinking experiments, water bottles were removed from the home cages just before the start of the dark cycle (5:30-6:30pm), food and body weights were recorded to keep track of the ad libitum eating and how much weight mice lost. In the following morning, mice received i.p. VEH or CNO injections and were given access to water. Water bottle, food and body weights were recorded at 3/4hr, 8hr and 24hr time points.

For 2% NaCl solution induced dehydration, mice were given *ad libitum* 2% NaCl solution for 4 days without access to water. On the 5^{th} day they received i.p. VEH/CNO just before the start of dark cycle, and 2% NaCl solutions were removed and water bottles were given back. Body weights, food and water consumption were recorded 1hr, 2hr and 16hr post injection at dark cycle.

For fasting-induced re-feeding experiments, food was removed just before the start of the dark cycle (5:30-6:30pm), and water bottle and body weights were recorded to keep track of the ad libitum drinking and how much weight mice lost. In the following morning, mice received i.p. VEH or CNO injections and were given access to food. Water bottle, food and body weights were recorded at 3/4hr, 8hr and 24hr time points.

At least 2 days of break was given in between the experiments as the recovery period of mice to re-gain their lost weight and re-hydrate as well as the wash out period for the drugs in between the experiments before switching the injection groups. Any mice that lost more than 20% or their body weight or showed signs of fatigue were taken out

of the experiments. During fasting-induced re-feeding, n=2 *Nts*^{Cre} mice (activatory DREADD: 1 defective bottle and 1 fatigue and dehydration) and n=2 *Nts*^{Cre} mice (inhibitory DREADD: 2 fatigue and dehydration) were removed from the study, leaving n=14 mice in each group. In the LPO cannulated cohort, n=1 *Nts*^{Cre} mouse (activatory DREADD: 1 cannula displacement) was excluded from the study.

3.2.4. 2-Bottle Choice Tests

48-hr 2-bottle preference tests were conducted as previously described[38]. Briefly, prior to the test, each single-housed mouse was acclimatized to the two-bottle setup by giving 2 bottles filled with water for at least 24hrs. To eliminate any interference of a potential place preference in the test results, every 24hrs the position of the bottles was swapped. For the actual tests, one bottle was filled with water while the other one was filled with the tested solution. Mice were given a choice of either 0.5% sucrose (Sigma) solution or 0.01% saccharin (Sigma) solution for 48h, and body, food and liquid bottles weights were collected every 12hr by use of an electronic balance. Mice were injected twice/day either with VEH or CNO in the morning (9:30-10:30am) and just before the dark cycle (5:30-6:30pm) with a crossover design, in which all mice served as their own controls. A minimum 24hr break was given in between each 48hr treatment period before switching injection groups.

3.2.5. Operant Conditioning

Male and female *Nts*^{Cre} mice expressing activatory DREADDs were single-housed and trained in operant chambers (Med Associates, St. Albans, VT) to work for water reward (50uL per reward) for 1hr daily or until mice received a maximum of 50 rewards. Animals were dehydrated overnight by removal of water from their home cages throughout the training period and trained in the operant chambers the next morning. 1h

after the end of the daily training session they were returned to their home cage, where they had access to ad libitum water to rehydrate. Body weights were tracked to confirm hydration status (e.g. regain of any lost weight) before the start of dehydration for the next day`s training session, right before the dark cycle. On the first day, mice were magazine trained with 50uL water reward delivery at every 15 seconds so that they would learn where the water was dispersed and acclimatized to the chambers. Then, they were trained for fixed ratio-1 (FR-1) schedule in which they learned to nose poke at the active poke hole for water reward and 1 poke was equal to 1 reward with a 5 seconds time-out. To eliminate the impact of side preference, the active and the inactive poke hole (right or left) was counterbalanced. FR-1 training continued until mice earned a minimum of 10 rewards with 75% accuracy for 3 consecutive days and the ones failed to meet the criteria for 4 weeks were taken out of the study. N=6 mice passed the accuracy and minimum reward criteria, and were switched to FR-3 schedule for 3 days, where 1 reward requires 3 active hole nose pokes. Finally, mice were trained in progressive ratio (PR) schedule, in which each successive reward required more nose pokes than the previous reward, as described before[27,35]. Briefly, the order for the response ratio was 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95..etc and the formula for calculation of the response ratio was $PR=[5e^{(R*0.2)}]-5$, where R=(number food rewards earned+1). PR schedule under dehydrated status continued until the mice had a 3-day stable breakpoint (the maximum nose pokes paid for the final reward). Once the breakpoints were stabilized, overnight dehydrated mice received VEH or CNO injections half an hour before each session and tested in PR ratio. Then, a 1 wk break was given for mice to fully rehydrate and the PR test was repeated under euhydration until the mice had a 3-day stable breakpoint. Next,

euhydrated mice received VEH or CNO injections 30 min before each session and were tested again. Each mouse received 3 days of VEH and 3 days of CNO injections and the PR test was repeated in each of these days. 1 mouse brain could not be analyzed to verify LHA-confined DREADD-mCherry expression due to tissue loss, but 5 of them were verified viral expression. 1 mouse`s drinking information after the operant session was missing for the well-hydrated condition due to malfunctioned water bottle; therefore, the post-operant drinking analyses for that group was done with n=5 mice

3.2.6. Conditioned Place Preference for Water

For conditioned place preference (CPP) protocol, single-housed male and female activatory DREADD expressing Nts^{Cre} mice (n= 8) were tested in CPP boxes (San Diego Instruments) with two main compartments with a narrow central separation area. Each compartment had different visual and tactile cues. On the pre-test day (day 1), euhydrated mice were allowed to explore the box freely for 15 min. After day 1, each compartment was either assigned to water presence or absence by application of counterbalanced and unbiased strategy in which preferred side and non-preferred side was assigned to water in about half of the mice[40]. During 4 days (day 2-5) of conditioning, animals were overnight dehydrated by removal of water bottles from the cages. In the morning of the day 2 and day 4, mice were placed into the water-paired side without any access to the other compartment for 30 min and, similarly, in the morning of day 3 and day 5, they were placed into the water-unpaired side for 30 min with no access to the other compartment. After each session, mice were rehydrated with ad libitum access to water in their home cages until the start of overnight dehydration and body weights were recorded to keep track of hydration after each session. On the post-test day (day 6) euhydrated mice

received either i.p. VEH or CNO injections 30 minutes before the test and then were placed in CPP boxes and allowed to explore the box freely for 15 min. Time-spent and locomotor activity in both chambers were recorded by the manufacturer's software, and (paired – unpaired) time spent with VEH and CNO injections was compared in pre- and post-tests.

3.2.7. Immunostaining for Viral Tracing Experiments and Verification of Viral Expression in LHA for DREADD studies

Mice used in the tracing experiments received a lethal i.p. dose of pentobarbital (Fatal Plus, Vortech) followed by transcardial perfusion with 0.2M PBS (pH 7.4) and then 10% formalin (Fisher Scientific, Pittsburgh, PA) at the end of the indicated timelines in section 3.2.2. After the extraction of brains, they were post-fixed in 10% formalin, followed by dehydration in 30% sucrose solution before coronal sectioning (30 μm) using a sliding microtome (Leica, Buffalo Grove, IL). Brain sections were collected as four separate sets. For each mouse, a single set of brain each mouse was immunostained and evaluated by use of fluorescence (AdSyn-mCherry and modified Rabies virus tracings) or laser scanning confocal microscopy (retrograde AAV tracings from LPO and VTA) as previously described[35].

Mice used in inhibitory functional studies underwent the same perfusion protocol. Mice used in activatory functional studies received either VEH or CNO injections 90 minutes prior to perfusion or decapitation to assess activation levels in the LHA Nts neurons by use of cFos immunostaining to verify activation of DREADD expressing LHA Nts neurons after CNO injections.

hM3D(Gq)-mCherry and hM4D(Gi)-mCherry expressions were assessed by immunofluorescence staining in order to verify the targeting. A rabbit dsRed primary antibody (1:1000, cat #: AB_10013483, Clontech) and an anti-rabbit secondary antibody conjugated to Alexa-568 (1:200, cat #: AB_2534017, Life Technologies) were used to detect the viral expressions. Mice with bilateral and robust unilateral mCherry expression in confined into the LHA were included in the final analyses. The reason why mice with robust unilateral expression was included was due to the previous verification that those mice exhibit comparable consumption behaviors with activation[38]. cFos was detected with a primary goat cFos polyclonal (1:500, cat #: sc-52-G, Santa Cruz) and an anti-goat secondary antibody conjugated with Alexa-488.

Similar to DREADD expression analyses, AdSyn-mCherry, monosynaptic retrograde modified rabies and retrograde AAV LPO/VTA tracing sections were immunostained with the same dsRed primary and secondary antibodies. Monosynaptic retrograde modified Rabies tracing sections were also immunostained for GFP detection to visualize Nts neurons by use of a chicken polyclonal GFP primary antibody (1:2000, cat #: ab13970, Abcam) and an anti-chicken secondary antibody conjugated with Alexa-488 (1:200, cat#: 703-545-155, Jackson ImmunoResearch). Retrograde AAV LPO/VTA tracing sections were also stained for HA detection to visualize the Nts expressing neurons (mouse anti-HA monoclonal primary antibody (1:1000, cat #: MM-101P, Covance) and anti-mouse secondary antibody conjugated with Alexa-647 (1:200, cat# A31571, Invitrogen-Thermo Fisher).

AdSyn-mCherry, modified Rabies virus and DREADD expression images were collected with an Olympus BX53 fluorescence microscope outfitted with transmitted light

as well as FITC and Texas Red filter sets, by use of Cell Sens software and a Qi-Click 12 Bit cooled camera. Retrograde AAV tracing images were collected by Nikon C2 Laser Scanning Confocal Microscope by use of FITC, Texas Red filters and Cy5 filter sets with Nikon Plan Fluor 10X/ 0.30 and Plan Apo λ 20X/0.75 and NIS elements AR 5.00.00 software. All images were analyzed by Photoshop software (Adobe).

3.2.8. RNAScope

C57Bl/6J wild type mice (n=2 per LPO and VTA) were perfused and brains were extracted, post-fixed and sectioned as described in section 3.2.7. Three free floating sections of LPO and VTA per mouse were selected for application of RNAScope single-plex assay (catalog #322360, Advanced Cell Diagnostics). The manufacturer's protocol was followed. Sections were washed in 1X PBS and incubated in Pretreatment 1 (H_2O_2) at RT until bubbling stops (45-60 min) followed by 0.5X PBS wash and mounting on positively charged slides. Following washing in dH_2O and drying at 60°C oven, they were incubated in 1X Pretreatment 2 (Target Retrieval Agent) for 5-10 min at 99-104°C and then, washed with dH_2O, dried at RT, dipped in 100% EtOH and air dried. Then, they were incubated in Pretreatment III solution (Protease Plus) for 15 min at 40 °C, followed by dH_2O wash. Sections were then incubated in NtsR1 (cat #: 422411, Advanced Cell Diagnostics) and NtsR2 (cat #: 452311, Advanced Cell Diagnostics) probes for 2 h in a humidified oven at 40 °C. After amplification steps (Amp1-6), hybridization was visualized by application of Fast-Red-A and Red-B (60:1) for 10-15minutes. Finally, after washing Fast-Red solution, slides were dehydrated by dipping into xylene and cover-slipped with antifade mounting agent.

3.2.9. Statistics

Paired and unpaired 2-tailed student's t test, 2-way ANOVA and repeated measures ANOVA (RM-ANOVA) with 95% confidence intervals and alpha=0.05 were applied for the analyses by use of GraphPad Prism 7.0. For ANOVA analyses, either Tukey`s or Sidak's multiple comparisons post-hoc test was applied. The effect sizes were calculated by Cohen`s d.

For activatory DREADD analyses, paired t-test was used to analyze basal consumption, fasting-induced refeeding and 2-bottle preference tests. Operant PR test and CPP data were analyzed by paired and unpaired t-test, respectively. GraphPad in-built outlier test detected no outliers in operant data. CPP analysis was done by use of the spent time difference between water paired site and water unpaired side (paired – unpaired). For site-specific activation experiments, VEH and CNO groups are compared by paired t test and for comparisons of the effect of NtsR1 antagonist, 2-way ANOVA with Tukey`s post-hoc tests were applied.

For the inhibitory DREADD analyses, body composition and 2% NaCl dehydration results were analyzed by paired t-test, while the TSE and other home cage need-based consumption results were analyzed by repeated measures 2-way ANOVA with Tukey`s (comparison of all groups) or Sidak`s (comparison within genotype) post hoc tests were applied.

3.3. RESULTS

3.3.1. LHA Nts neurons are anatomically positioned to coordinate ingestive behaviors

We first examined if LHA Nts neurons project to brain regions known to modulate drinking and/or feeding. *Nts*^{Cre} mice were injected in the LHA with an adenovirus

expressing cre-dependent synaptophysin-mCherry[41], thereby inducing mCherry in the soma, axon and synaptic terminals of LHA Nts neurons. An unbiased survey of the entire brain revealed LHA Nts mCherry-labeled terminals in multiple brain regions (Figure 3-1A-C & Table S3-1). Of these, the lateral preoptic area (LPO), a brain region implicated in drinking behavior [42–44], received the highest density of LHA Nts projections (Figure 3-1C.i). Many LHA Nts neurons projections were also identified in brain regions that modify the motivation to eat and move, such as the ventral tegmental area (VTA), substantia nigra compacta (SNc) (Figure 3-1C.ii), periaqueductal gray (PAG) (Figure 3-1C.iii), and parabrachial nucleus (Figure 3-1C.iv). Thus, LHA Nts neurons engage brain regions known to modulate feeding and drinking.

Based on prior work showing that LHA Nts neurons are heterogeneous[27,28], we hypothesized that different subsets of LHA Nts neurons might project to brain regions implicated in drinking vs. feeding behavior. To examine this possibility, we investigated LHA Nts neurons that project to the LPO (in light of its important role in drinking behavior) vs. the VTA (with a well-established role in modulating motivated feeding). First, we verified that LPO and VTA neurons are capable of being modulated by Nts released from LHA Nts projections, which requires signaling via the G-protein coupled neurotensin receptor-1 or -2 (NtsR1 or NtsR2). Indeed, the LPO and VTA contain many neurons expressing NtsR1, as well as modest expression of NtsR2 (Figure 3-1D). These data indicate that LHA Nts projections could functionally modulate target LPO and VTA neurons. Next, we investigated whether discrete or overlapping LHA Nts neurons project to the LPO and VTA. *Nts^{Cre};HA* reporter mice received LPO and VTA injections of

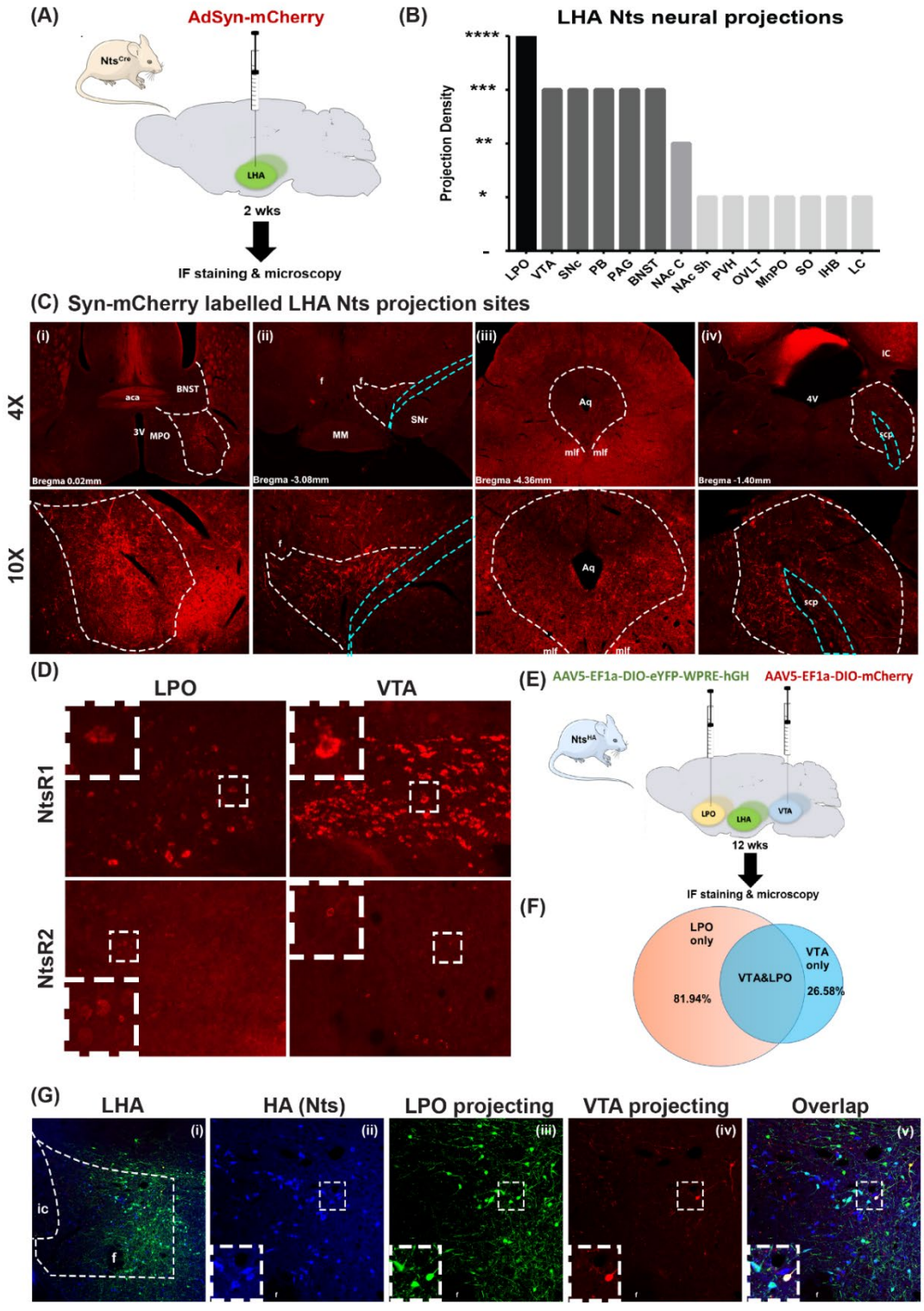

Figure 3-1. LHA Nts neurons send projection to feeding and drinking regulatory

Figure 3-1 ***(cont`d)*** **brain areas. (A)** Schematic representation of AdSyn-mCherry anterograde tracing experiment. **(B)** LHA Nts projection density graph. *Nts*^{Cre} mice (n=4) were injected with cre-dependent adenoviral synaptophysin-mCherry virus and density of the projection sites throughout the brain was assessed semi-quantitatively by assessing the total number of synaptic buttons and "eye-balling" in a given area and rating from the densest to least dense within a single brain. Then it was compared to other brains and assessed for the other 3 brains. **** shows the densest projections, * indicates the least dense and – indicates no projections. LPO: lateral preoptic area, VTA: ventral tegmental area, SNc: substantia nigra/ compact part, PB: parabrachial nucleus, PAG: periaqueductal gray, BNST: bed nucleus of stria terminalis, NAc C: nucleus accumbens/core part, NAc Sh: nucleus accumbens/shell part, PVH: periventricular nucleus of hypothalamus, OVLT: organum vasulasum latera terminalis, MnPO: median preoptic area, SO: Supraoptic nucleus, lHB: lateral habenula, LC: Locus ceruleus. **(C)** LHA Nts neurons send densest projections to LPO (**C.i**- white dashed), followed by VTA (**C.ii**-white dashed), SNc (C.ii- light blue dashed), PAG (**C.iii**-white dashed) and PB (**C.iv**-white dashed). aca: anteriorcommissure/anterior part, 3V: third ventricle, 4V: fourth ventricle, f: fornix, Aq: aquaduct, scp: superior cerebellar peduncle, mlf: medial longitudinal fasciculus, MPO: medial preoptic area, MM: medial mammallary nucleus, SNr: substantia nigra/reticular part, IC: inferior colliculus **(D)** LPO and VTA neurons have potential to respond Nts inputs. NtsR1 and NtsR2 mRNA (red) was detected in wild type mice LPO and VTA brain sections (n=2 mice) by RNAScope assay. 20X images. NtsR1: neurotensin receptor 1, NtsR2: neurotensin receptor 2. **(E)** Schematic representation of dual retrograde tracing experiment. **(F)** Venn diagram showing percentage of overlapping

Figure 3-1 ***(cont'd)*** and distinct LHA Nts projections to VTA and LPO. **(G)** Partially overlapping LHA Nts neural populations send projections to LPO and VTA. eYFP and mCherry tagged retrograde viruses were injected into LPO and VTA, respectively, in Nts;HA mice (n=3). HA signal (blue) indicates Nts expressing neurons, YFP signal (green) indicates LPO projecting neurons, and mCherry signal (red) indicates VTA projecting neurons. Overlap of YFP, mCherry and HA signals (white) indicates the same neuron sending projections to both areas whereas overlap of only YFP and HA (light blue) or only mCherry and HA (magenta) signals indicates distinct neurotensin expressing neurons projecting to either LPO or VTA, respectively, but not to both areas. **(G.i)** 10X magnification. Area circled in white dashed line indicates LHA. **(G.ii-v)** 20X magnification. White rectangle indicates the magnified section on lower left corners in original photos. **(G.ii)** Neurotensin expressing neurons. **(G.iii)** LPO projecting neurons **(G.iv)** VTA projecting neurons. **(G.v)** Overlap of all three signals. f: fornix, ic: internal capsule.

retrograde AAVs that express either cre-dependent eYFP or mCherry. This method permitted simultaneous visualization of LHA Nts soma (via immunofluorescent labeling of HA), along with any eYFP and/or mCherry indicative of soma that project to the LPO or VTA (Figure 3-1E & G). We found that most LHA Nts neurons projecting to the VTA also project to the LPO (73.42$\pm$9.17%) (Figure 3-1F). However, the majority of LHA Nts neurons project to the LPO but do not simultaneously project to the VTA (81.94$\pm$15.52%) (Figure 3-1F). We also observed a small proportion of LHA Nts neurons that project to the VTA but not the LPO. In sum, these data reveal that some LHA Nts neurons differentially target the LPO vs. the VTA, raising the possibility that they may be part of

distinct circuits coordinating the drinking and feeding behaviors associated with these regions.

If subsets of LHA Nts neurons are dedicated to orchestrating drinking vs. feeding they may be modulated by changes in osmolality and energy status. Indeed, the LHA receives such information *indirectly* via inputs from osmolality-sensing neurons in the lateral terminalis (LT) [45] and a variety of energy sensing areas[5,7,8,46], but it was unknown if LHA Nts neurons specifically receive these inputs. We therefore used a genetically-modified rabies tracing system[47] to identify the upstream regulators of LHA Nts neurons. First, we labeled the presynaptic inputs to all LHA Nts neurons by injecting the cre-dependent helper virus and the pseudotyped rabies virus directly into the LHA of *Nts^{Cre};GFP* mice (Figure 3-2A). The specificity of the dual-viral approach was confirmed by the absence of mCherry expression in *Nts^{Cre};GFP* mice that only received the rabies-mCherry virus (Figure S3-1A & C-E). Similarly, wild type (WT) mice that received both the cre-dependent helper and pseudotyped rabies viruses had no mCherry-labeled neurons at the tracing sites, confirming the cre-specificity of the system (Figure S3-1A & B) However, in *Nts^{Cre};GFP* mice that received both viruses in the LHA, many LHA Nts neurons labeled with GFP also expressed mCherry indicative of successful rabies labeling (Figure 3-2C). In these mice we observed mCherry-expressing neurons in multiple brain regions beyond the LHA (Figure 3-2D-F). Notably, LHA Nts neurons receive monosynaptic inputs from LT neurons, including those in the subfornical organ (SFO) (Figure 3-2D.i), the median preoptic area (MnPO) and the organum vasculasum latera

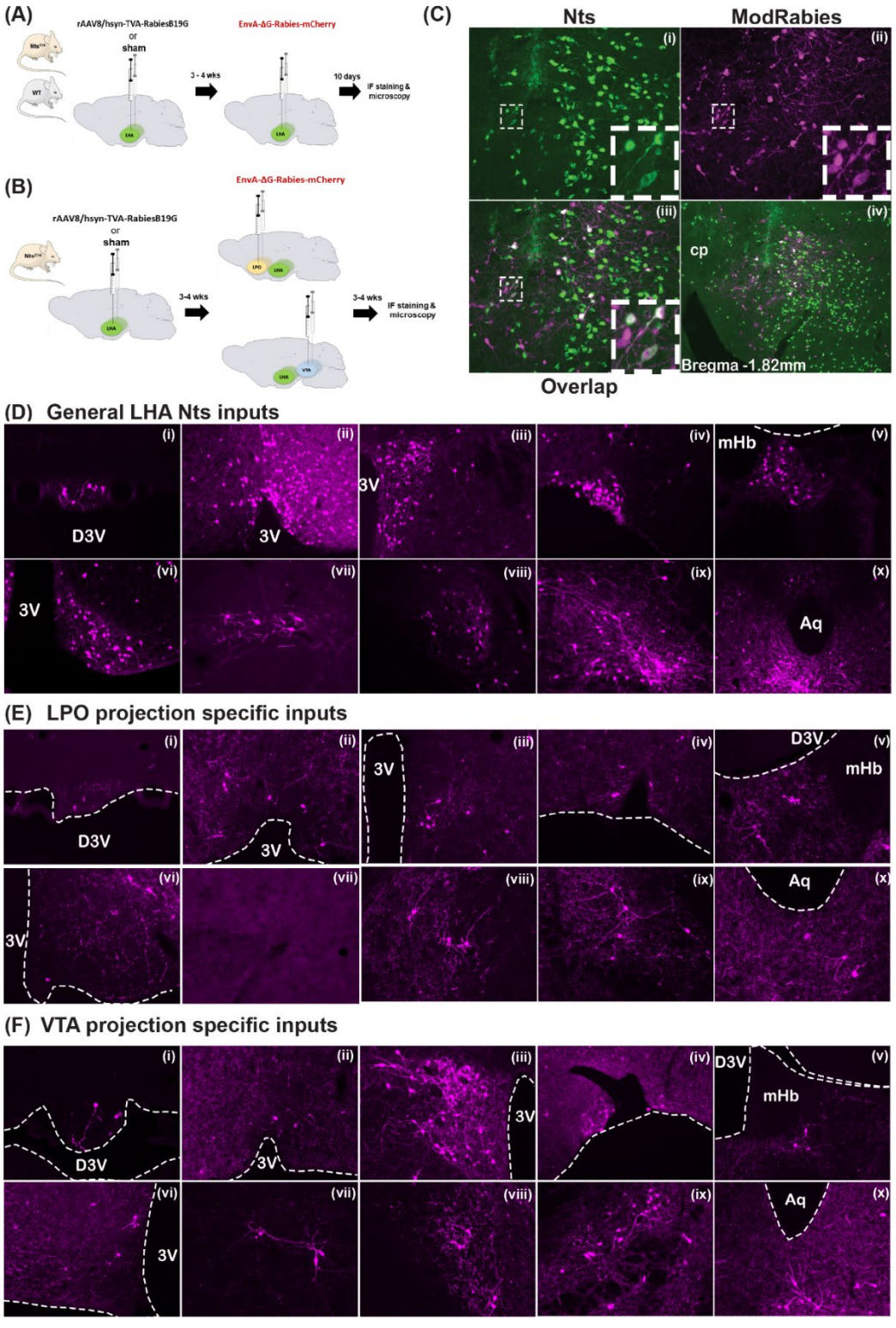

Figure 3-2. LHA Nts neurons receive direct inputs from energy and hydration

Figure 3-2 ***(cont`d)*** **sensing/regulatory areas.** Direct inputs to LHA Nts neurons were detected by cre-mediated monosynaptic rabies tracing from the LHA in *Nts^{Cre};GFP* mice. Mice first received cre-dependent helper virus (TVA) into the LHA followed by modified rabies virus (ModRabies-mCherry) into the LHA for general monosynatic tracing from LHA Nts neurons **(A)** and helper virus injected into the LHA followed by injection of modified rabies virus into either the LPO or the VTA for projection site specific tracing from LHA Nts neurons **(B)**. **(C)** Confirmation of the expression of modified rabies virus (ModRabies) in LHA Nts neurons. 20X **(C.i-iii)** and 10X **(C.iv)** LHA immunofluorescence staining. **(C.i)** Green: GFP (Nts), **(C.ii)** Magenta: mCherry (modRabies), White: colocalization. **(C.iii)** Overlap (white) indicates primarily transduced Nts neurons, magenta indicates neurons synapsing onto Rabies-expressing Nts neurons. **(D)** Areas sending direct projections to LHA Nts neurons. **(D.i)** SFO, Bregma: -0.46mm, **(D.ii)** MnPO & OVLT, Bregma: 0.50mm, **(D.iii)** PVH, Bregma: -0.70mm, **(D.iv)** SO, Bregma: -0.70mm, **(D.v)** lHb, Bregma: -1.58mm, **(D.vi)** ARC, Bregma: -1.58 mm, **(D.vii)** NTS, Bregma: -7.48mm **(D.viii)** CEA, Bregma: -1.58mm, **(D.ix)** PB, Bregma: -5.02mm, **(D.x)** PAG & DR, Bregma: -4.48mm. There are partially overlapping LHA Nts circuits that may control feeding and drinking. Projection site specific full circuits were detected by injecting cre-dependent helper virus into the LHA of *Nts^{Cre}* mice, followed by injection of modified Rabies virus into either LPO (+/+ n=3, -/+ n=1) or VTA (+/+ n=3, -/+ n=1). 20X LHA immunofluorescence showing expression in LHA Nts neurons (magenta). **(E)** LPO projection specific LHA Nts neural inputs. **(E.i)** SFO, Bregma: -0.34mm, **(E.ii)** MnPO & OVLT, Bregma: 0.50mm, **(E.iii)** PVH, Bregma: -0.82mm, **(E.iv)** SO, Bregma: -0.58mm, **(E.v)** lHb, Bregma: -1.94mm, **(E.vi) Figure 3-2** ***(cont'd)*** ARC, Bregma: -1.82 mm, **(E.vii)**

NTS, Bregma: -6.48mm, **(E.viii)** CEA, Bregma: -1.46mm, **(E.ix)** PB, Bregma: -4.84mm, **(E.x)** PAG & DR, Bregma: -4.04mm. **(F)** VTA projection specific LHA Nts neural inputs. **(F.i)** SFO, Bregma: -0.46mm, **(F.ii)** MnPO & OVLT, Bregma: 0.50mm, **(F.iii)** PVH, Bregma: -0.46mm, **(F.iv)** SO, Bregma: -0.82mm, **(F.v)** lHb, Bregma: -1.22mm, **(F.vi)** ARC, Bregma: -1.58 mm, **(F.vii)** NTS, Bregma: -6.64mm, **(F.viii)** CEA, Bregma: -1.82mm, **(F.ix)** PB, Bregma: -5.20mm, **(F.x)** PAG & DR, Bregma: -4.48mm.

terminalis (OVLT) (Figure 3-2D.ii) that are known to sense osmolality changes and lead to changes in drinking behavior[48–52]. Neurons in the paraventricular nucleus of hypothalamus (PVH) and the supraoptic nucleus (SO) also project to LHA Nts neurons (Figure 3-2D.iii & iv); these regions are implicated in both drinking and feeding control[53–56] and are the main sources of the osmolality regulatory hormone vasopressin[57]. Inputs to LHA Nts neurons also originated from the lateral habenula (lHb), linked to motivation, aversion[58] and alcohol intake[59–61] (Figure 3-2D.v). Additionally, LHA Nts neurons receive direct inputs from multiple brain regions linked with regulation of feeding, including the arcuate nucleus (ARC) (Figure 3-2D.vi), the nucleus of solitary tract (NTS) (Figure 3-2D.vii), the central amygdallar nucleus (CEA) (Figure 3-2D.viii), the parabrachial nucleus (PB) (Figure 3-2D.ix) and the periaqueductal gray (PAG) [62–65] (Figure 3-2D.x). Hence, LHA Nts neurons receive inputs from osmosensory and energy sensing-regions of the brain, and so could conceivably be regulated by changes in fluid and energy balance.

Next, we used projection-specified rabies tracing to determine whether LHA Nts neurons that project to the LPO and VTA receive differential input from osmolality and energy-sensing regions. The cre-dependent helper virus was delivered to the LHA of

Nts^{Cre} mice, followed by LPO or VTA injection of pseudotyped rabies virus (Figure 3-2B). This approach labeled the LHA Nts soma that project to the LPO or VTA, as well as the presynaptic neurons that project onto them. We found that the LPO and VTA-projecting LHA Nts neurons receive a similar magnitude of inputs from areas such as the SFO, MnPO/OVLT, lHb, CEA, PB and PAG/DR (Figure 3-2E & F). In contrast, we observed that VTA-projecting LHA Nts neurons received more input from cells of the ARC, SO, PVH and NTS than LPO-projecting cells (Figure 3-2E & F). Taken together, these data hint that projection-specified LHA Nts neurons receive largely similar input, with only a few regions providing more biased input to the VTA-projecting LHA Nts neurons compared to the LPO-projecting population.

3.3.2. Effects of Activating LHA Nts Neurons on Drinking Behavior

We recently reported that a single chemogenetic activation of LHA Nts neurons during the light cycle (when rodents are normally sleeping) promotes immediate voracious drinking[35,38] followed by later suppression of feeding during the dark cycle[35]. Curiously, this enhancement of drinking did not extend to the dark cycle, when mice typically ingest most of their food and water. To understand if LHA Nts neuronal activation can also induce drinking behavior during the dark cycle we injected *Nts*^{Cre} mice bilaterally in the LHA with pAAV2-hSyn-DIO-hM3D(Gq)-mCherry to enable cre-mediated expression of activatory DREADDs in LHA Nts neurons. Mice were then monitored in home cages while treated during the light and dark cycles with the DREADD-ligand CNO to activate LHA Nts neurons (Figure 3-3A). Similar to previous reports, DREADD-mediated activation of LHA Nts neurons during the light cycle promoted voracious water drinking (

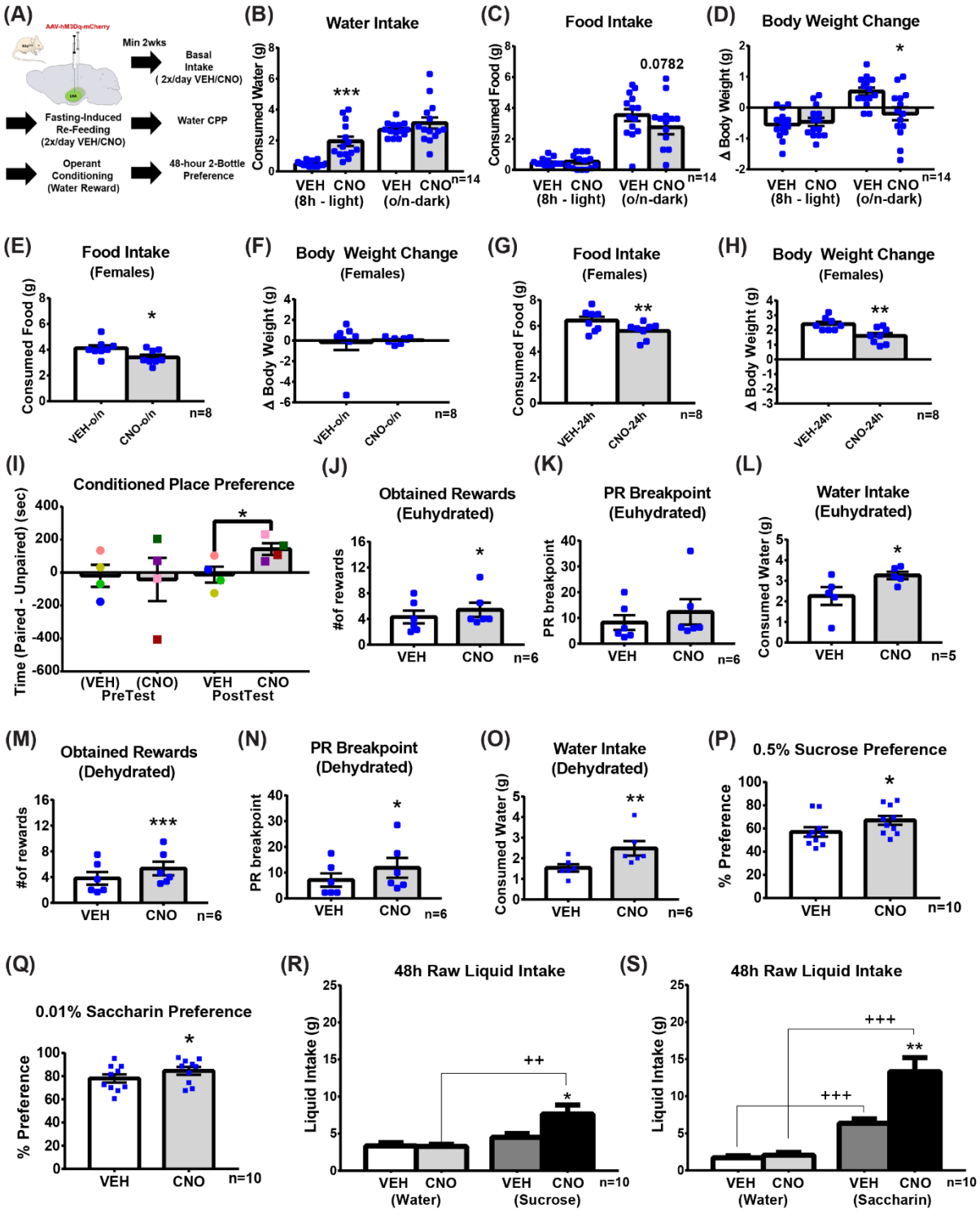

Figure 3-3. The effects of LHA Nts activation in light and dark cycle consumption, motivation to drink and solution preference. Cre-dependent DREADDq (AAV-hM3Dq-

Figure 3-3 ***(cont'd)*** mCherry) was injected bilaterally into the LHA of male and female *Nts*^{Cre} mice **(A)**. Total **(B)** water and **(C)** food intake and **(D)** body weight change 8hrs after (light cycle) and overnight (dark) following twice daily i.p. injection of VEH or CNO in well hydrated fed mice. Total **(E & G)** food intake and **(F & H)** body weight change after overnight (dark cycle) and 24hrs (light+dark cycle) following twice daily i.p. injection of VEH or CNO in overnight fasted female mice. **(I)** CPP 30mins after i.p. injection of VEH or CNO with water presence/absence conditioning in well-hydrated mice. **(J-O)** Operant training for water reward. Number of obtained rewards during PR session in **(J)** well-hydrated and **(M)** overnight dehydrated mice. Progressive ratio (PR) breakpoint for water reward 30mins after i.p. injection of VEH or CNO in **(K)** well-hydrated and **(N)** overnight dehydrated mice. **(L & O)** Home cage consumed water 5hrs following PR test session. **(P-S)** 48-hr 2-bottle preference tests in which one bottle contains water and the other contains either 0.5% sucrose or 0.01% saccharin solution. **(P)** 0.5% sucrose solution preference. **(Q)** 0.01% saccharin solution preference. **(R)** Water and 0.5% sucrose solution intake. **(S)** Water and 0.01% saccharin solution intake. * p<0.05, **/++ p<0.01, +++ p<0.001.

t(13)=4.958, p=0.0003) (Figure 3-3B). However, DREADD-mediated activation of LHA Nts neurons during the light cycle did not alter food intake (t(13)=0.5631, p=0.5830) (Figure 3-3C), confirming that LHA Nts neuronal activation only promotes drinking during this period. By contrast, DREADD-mediated activation of LHA Nts neurons just prior to the dark cycle did not augment drinking behavior overnight (t(13)=1.52 ,p=0.1524) (Figure 3-3B) whereas there was a trend for reduced feeding (t(13)=1.912, p=0.0782)

(Figure 3-3C), similar to the dark-cycle feeding restraint reported in prior studies[35]. The increased error of hand-weighing food from home cages could have prevented reaching the level of significance, whereas the previous study used automated metabolic cages that more precisely measure remaining chow[35]. Regardless, even interpreted conservatively, our present data confirm that activation of LHA Nts neurons does not promote feeding. Moreover the twice-daily activation of LHA Nts neurons led to a significant drop in body weight during the dark cycle ($t(13)=2.8$, $p=0.0150$) (Figure 3-3D), consistent with weight loss observed from a single light-cycle activation. WT mice injected in the LHA with pAAV2-hSyn-DIO-hM3D(Gq)-mCherry (controls) neither expressed mCherry or exhibited any changes in drinking or feeding with CNO treatment, confirming that CNO itself is not having off-target effects (Figure S3-2 A-C). Taken together, our data reveal that LHA Nts neurons preferentially induce water intake but not food intake. Moreover, LHA Nts neuronal activation motivates drinking during the light cycle (when motivation to drink is low) but not during the dark cycle when there is already a high circadian-mediated homeostatic drive to ingest water. These findings support the possibility that LHA Nts neurons may primarily influence the motivation to drink rather than homeostatic need to drink.

We therefore examined if activation of LHA Nts neurons impacts the motivation to drink in *Nts*^{Cre} mice expressing activatory DREADDs in LHA Nts neurons. As a first step, we used CPP to determine if activating LHA Nts neurons increases preference for water during the light cycle, when mice do not typically drink. Mice were alternately conditioned in one chamber containing a filled water bottle or the other chamber containing an empty water bottle. On the test day, the water bottles were removed from both chambers, then

normally hydrated (euhydrated) mice were given a light-cycle injection of VEH or CNO and given free access to both chambers. CNO-mediated activation of LHA Nts neurons increased preference for the chamber that was previously paired with water ($t(6)=2.578$, $p= 0.0419$) (Figure 3-3I), suggesting increased motivation/preference to drink water that spurs water-seeking. We next asked if activating LHA Nts neurons alters the willingness to work for water using a progressive ratio (PR) operant responding paradigm. In euhydrated mice, CNO-mediated activation of LHA Nts neurons modestly increased the number of obtained water “rewards” ($t(5)=2.758$, $p=0.0399$, Cohen`s $d=0.43$) (Figure 3-3J), although the increase in PR breakpoint (that measures the limit of how much animals will work for a reward) did not reach statistical significance ($t(5)=1.714$, $p=0.1472$) (Figure 3-3K). However, we noted that after operant training, when mice were returned to home cages with *ad libitum* water, the CNO-treated mice consumed significantly more water ($t(4)=3.211$, $p=0.0326$) (Figure 3-3L). We reasoned that because mice anticipated the forthcoming free access to water it may have tempered the extent to which they would work for PR water rewards during the testing session. We therefore dehydrated mice overnight prior to PR testing, thereby increasing osmolality and the motivation to obtain water. In this context CNO-mediated activation of LHA Nts neurons in dehydrated mice significantly increased the number of water rewards obtained ($t(5)=9.328$, $p= 0.0002$, Cohen`s $d=0.61$ ) (Figure 3-3M), the PR breakpoint ( $t(5)=3.525$, $p= 0.0168$, Cohen`s $d=0.59$) (Figure 3-3N), and after the test mice still consumed more *ad libitum* water in their home cages ($t(5)=4.227$, $p=0.0083$) (Figure 3-3O). Overall, these data confirm that activating LHA Nts neurons increases the motivation to drink, which accounts for their voracious water consumption during times of normally low water intake.

Given that LHA Nts neurons bias motivation for fluid intake, but not food intake, we next examined whether the caloric content of a liquid might impact LHA Nts-mediated drinking. *Nts*^{Cre} mice expressing activatory DREADDs in LHA Nts neurons were tested in 2-bottle preference tests for intake of water vs. a solution of sucrose (palatable, caloric) or saccharin (palatable, non-caloric). CNO-mediated activation of LHA Nts neurons increased preference of both sucrose ($t(9)=2.758$, $p=0.0222$) and saccharin solution ($t(9)=2.567$, $p=0.0303$) over water (Figures 3-3P and Q). CNO-treated mice also drank more of the palatable sucrose solutions compared to water (CNO-sucrose solution vs water: $t(9)=3.445$, $p= 0.0073$; CNO-saccharin solution vs water: $t(9)=5.428$, $p= 0.0004$), and had augmented intake of palatable solution compared to VEH-treatment (sucrose solution-VEH vs CNO: $t(9)=2.901$, $p= 0.0176$; saccharin solution-VEH vs CNO: $t(9)=4.062$, $p= 0.0028$) (Figures 3-3R and S). Thus, LHA Nts neuronal activation promotes intake of palatable solutions independent of their caloric content. Taken together with our previous work[35,38], our findings indicate that LHA Nts neurons are sufficient to motivate drinking behavior over feeding, and bias intake for the most palatable liquid available.

3.3.3. Inhibition of LHA Nts Neurons Alone does not Disrupt Drinking or Feeding

We next examined whether activation of LHA Nts neurons is required for drinking, feeding and the maintenance of homeostasis. To address this question, we expressed inhibitory DREADDs in the LHA of *Nts*^{Cre} mice to enable CNO-mediated suppression of LHA Nts neuronal activity over 24 hrs (Figure 3-4A). Mice were monitored in metabolic cages during treatment to assess any ingestive and/or metabolic changes. Although

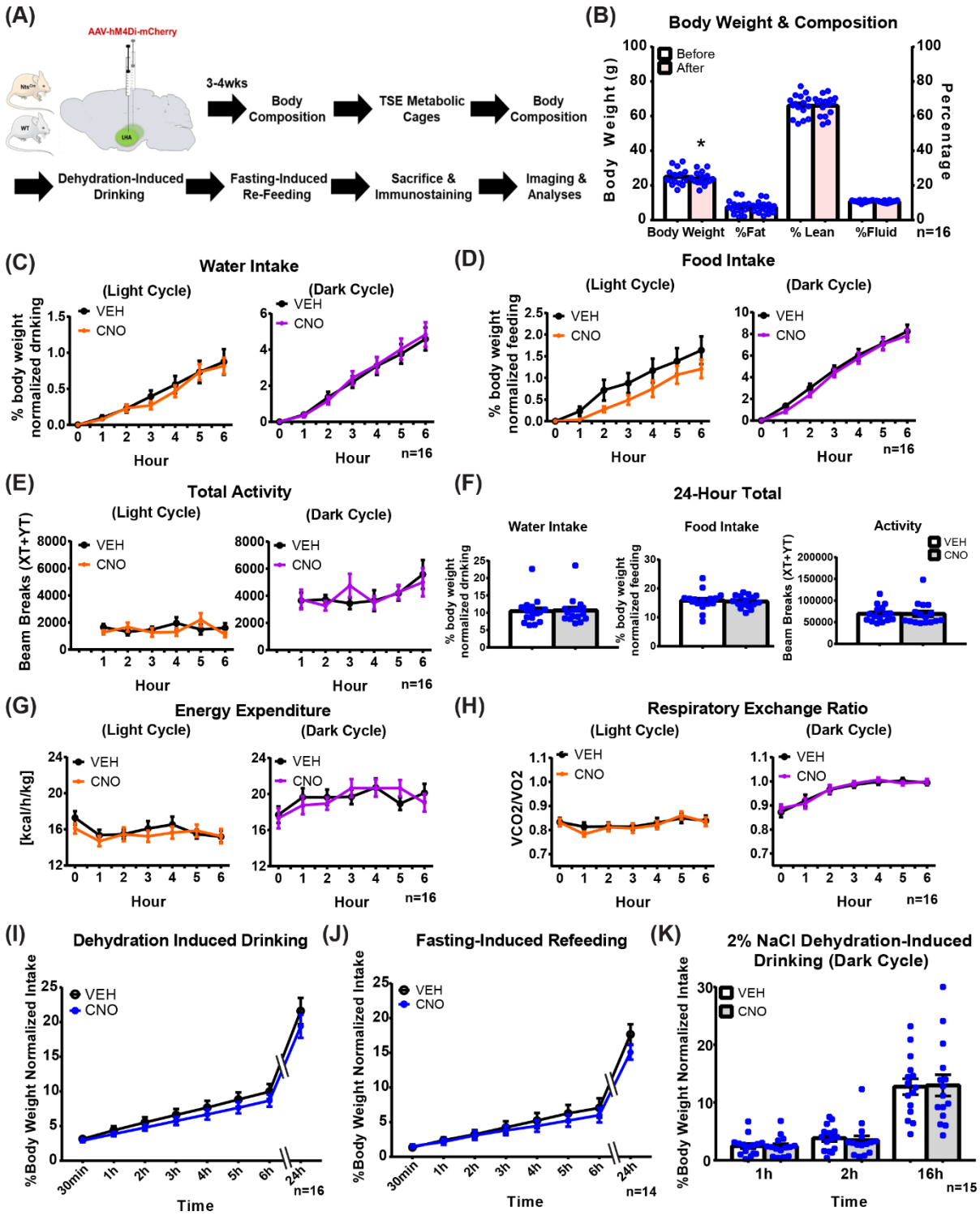

Figure 3-4. LHA Nts inhibition does not change the basal consumption and activity or need-based ingestion. Cre-dependent DREADDi (AAV-hM4Di-mCherry) was

Figure 3-4 ***(cont'd)*** injected bilaterally into the LHA of male and female *Nts*^{Cre} (n= 16) and WT mice (n=10). Analyses of *Nts*^{Cre} mice were shown in this figure. Analyses of WT mice can be found in Figure S3-3. t=0 marks the injection times on each graph and the indicated times are "time past post injection". **(A)** Experimental scheme. **(B)** Body composition was analyzed before and after i.p. VEH/CNO injections in TSE cages. Light and dark cycle post injection (t=0) cumulative **(C)** water and **(D)** food intake, hourly **(E)** total locomotor activity and **(G)** energy expenditure as well as **(H)** respiratory exchange ratio at each hour indicated were recorded in TSE metabolic cages with twice daily i.p. injection of either VEH or CNO **(F)** 24-hr total water and food intake as well as activity levels in TSE cages. **(I-K)** Need-based consumption tests in home cages. **(I)** Overnight dehydration-induced drinking. **(J)** Overnight fasting-induced re-feeding. **(K)** 96-hr 2% NaCl consumption induced dehydration drinking. * $p<0.05$.

CNO-mediated inhibition of LHA Nts neurons slightly decreased body weight ($t(15)=2.914$, $p= 0.0107$, Cohen`s d= -0.21) it did not produce any significant changes in body composition (percentage lean mass $t(15)=0.1226$, $p= 0.9041$; percentage fat mass $t(15)=0.2602$, $p= 0.7983$; percentage fluid mass $t(15)=1.232$, $p= 0.2371$) following inhibition of LHA Nts neurons (Figure 3-4B). A similar reduction in body weight was observed in AAV-DREADD-injected WT mice ($t(9)=3.41$, $p= 0.0077$, Cohen`s d=-0.58) (Figure S3-3A). Given that we've previously demonstrated that CNO has no effect in the absence of DREADD expression[35] (Figure S3-2A-C), we posit that the slight reduction in body weight groups might be due to the stress of repeated CNO injections or transfer into metabolic cages (which can be innately stressful compared to home cages). Moreover,

CNO-mediated inhibition of LHA Nts neurons did not have any effect on water intake (light cycle: F (1, 30) = 0.1778, p=0.6762; dark cycle: F (1, 30) = 0.02974, p=0.8642; 24 hours total: Sidak`s adjusted p=>0.9999 ) (Figure 3-4C & F) or food intake (light cycle: F (1, 30) = 1.962, p=0.1715; dark cycle: F (1, 30) = 0.362, p=0.5519; 24 hrs total: Sidak`s adjusted p= 0.9997) (Figure 3-4D & F). Neither did CNO-mediated inhibition of LHA Nts neurons alter locomotor activity (light cycle: F (1, 30) = 0.383, p=0.5407; dark cycle: F (1, 30) = 2.624e-005, p= 0.9959; 24 hrs total: Sidak`s adjusted p= >0.9999) (Figure 3-4E & F), energy expenditure (light cycle: F (1, 30) = 0.3914, p=0.5363; dark cycle: F (1, 30) = 0.003728, p=0.9517) (Figure 3-4G) or respiratory exchange ratio (RER) (light cycle: F (1, 30) = 0.08788, p=0.7689; dark cycle: F (1, 30) = 0.02199, p=0.8831) (Figure 3-4H). Thus, inhibition of LHA Nts neurons does not impede the maintenance of ingestive behaviors and metabolism.

Next, we tested whether chemo-inhibition of LHA Nts neurons impeded drinking or feeding in response to homeostatic challenge (e.g. dehydration or fasting). If LHA Nts neuronal activation is necessary for drinking behavior, then CNO-mediated inhibition should suppress drinking in dehydrated (thirsty) mice. Likewise, if activation of LHA Nts neurons is necessary for suppression of feeding then inhibiting LHA Nts neurons should lift the restraint and possibly cause overeating. Instead we found that CNO-mediated inhibition of LHA Nts neurons did not modify dehydration or fasting-induced ingestion (dehydration-induced drinking F (1, 30) = 0.7885, p=0.3816; fasting-induced feeding (F (1, 30) = 0.1213, p=0.7301) (Figures 3-4I & J). Since complete water deprivation might be too strong of a homeostatic deviation to be overcome by LHA Nts neurons, we also exposed mice to a more gradual cause of dehydration: exposure to hyperosmotic 2%

NaCl solution. Similar to water deprivation, CNO-mediated inhibition of LHA Nts neurons did not impede drinking in response to this hyperosmotic dehydration (1hr: t(14)=0.1007, p= 0.9212; 2hr: t(14)=0.4998, p= 0.6250; 16hr: t(14)=0.1658, p= 0.8707) (Figure 3-4K). Hence, while activation of LHA Nts neurons is sufficient to promote motivated drinking, activation of these neurons is not a required safeguard of fluid homeostasis.

3.3.4. LHA Nts → LPO Circuit Activation is Sufficient to Promote Drinking

The LPO has been implicated in modulating drinking behavior [42–44], but not feeding behavior. Since LHA Nts neurons most densely project to the LPO, we hypothesized that the LHA Nts→LPO circuit might specifically promote drinking. To test this, we injected AAV2-hSyn-DIO-hM3D(Gq)-mCherry into the LHA of *Nts*^{Cre} and WT mice (negative controls), and placed indwelling cannulas over the LPO (Figure 3-5A & B). First, mice were treated with systemic CNO to verify that activation of all LHA Nts neurons promoted drinking (Figure 3-3B). Next, mice received injections of VEH or CNO into the LPO so as to selectively activate the LHA Nts neurons projecting to this site. Similar to CNO-mediated activation of all LHA Nts neurons, activation of the LHA Nts→LPO projecting neurons was sufficient to promote drinking behavior in well hydrated and fed mice in the light cycle (t(9)=4.793, p=0.0010) (Figure 3-5C) but not during the dark cycle (t(9)=0.3563, p=0.7298) (Figure 3-5E). Conversely, activating all LHA Nts neurons has no effect on food intake in the light cycle (Figure 3-3C), whereas CNO-mediated activation of the LHA Nts→LPO projecting neurons caused an acute increase in food intake during this timeframe (t(9)=3.498, p= 0.0067) (Figure 3-5D). However, activation of the Nts→LPO circuit did not alter food intake during the dark cycle (t(9)=1.231, p= 0.2495) (Figure 3-5F), when activation of LHA Nts neurons can restrain feeding [35 38] and (Figure

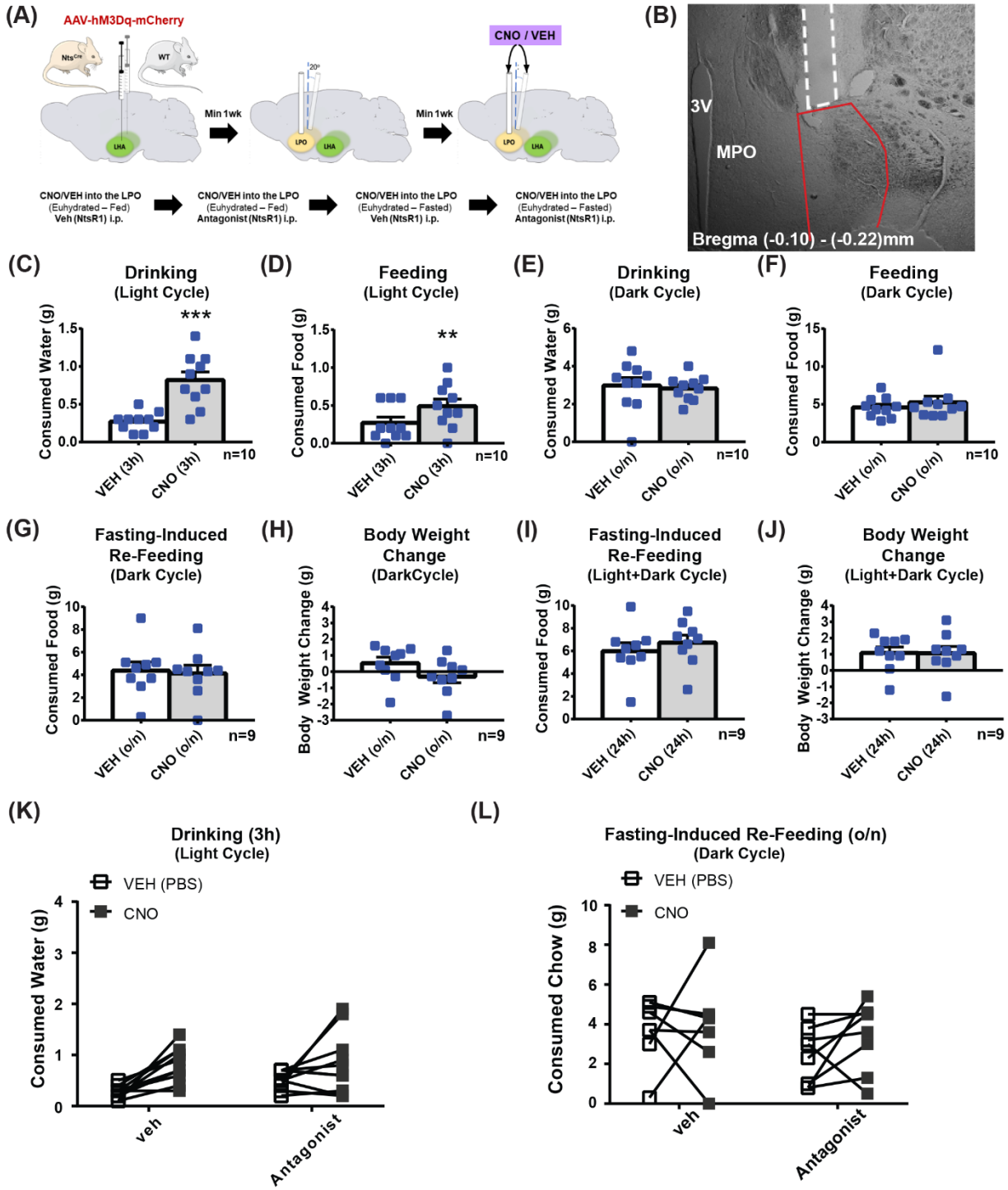

Figure 3-5. Projection site-specific activation of LHA Nts neurons. Cre-dependent DREADDq (AAV-hM3Dq-mCherry) was injected bilaterally into the LHA of male and female Nts^{Cre} mice(n=10) and indwelling cannulas were placed bilaterally over the LPO

Figure 3-5 ***(cont'd)*** for projection site specific activation of LHA Nts neurons **(A)**. VEH or CNO was infused to the projection sites via cannula. NtsR1 antagonist (SR48692) and its vehicle (Veh) were delivered via i.p. injection 30minutes before CNO or VEH infusion. **(B)** Verification of the correct placement of LPO cannula (4X magnification). Dashed line shows the place of the cannula and area circled in red shows the LPO. Injector extends 1mm below the cannula. Total **(C)** water and **(D)** food consumption 3hrs after VEH/CNO delivery in well hydrated-fed mice. Total overnight (o/n) (dark cycle) **(E)** water and **(F)** food consumption after VEH/CNO delivery in well hydrated-fed mice. Total **(G)** food consumption and **(H)** body weight change o/n (dark cycle) after VEH/CNO delivery in fasted mice. Total **(I)** food consumption and **(J)** body weight change 24hrs after VEH/CNO delivery in fasted mice. Total **(K)** water consumption 3h after VEH/CNO delivery in well hydrated-fed mice injected with Veh or NtsR1 antagonist. Total **(L)** water consumption 3hrs after VEH/CNO delivery in fasted mice injected with Veh or NtsR1 antagonist.

3-3E). These data suggest that the LHA Nts→LPO circuit invokes drinking but does not contribute to the suppression of feeding previously described by activating all LHA Nts neurons. If anything, the drinking invoked by the LHA Nts→LPO circuit may simultaneously spur food intake, consistent with the normal linkage of drinking and eating. In light of prior findings that activating all LHA Nts neurons restrains fasting-induced refeeding[35], we reasoned that any feeding effects mediated by the LHA Nts→LPO circuit might be more evident after fasting. Indeed, systemic CNO-mediated activation of all LHA Nts neurons suppressed fasting-induced feeding during the dark cycle ($t(7)=3.464$, $p=0.0105$) (Figure 3-3E) and over 24 hrs ($t(7)=3.591$, $p=0.0089$) (Figure 3-3G) leading

to reduction of body weight in 24hrs (t(7)=4.619, p=0.0024) (Figure 3-3H). Yet, activation of the LHA Nts→LPO circuit did not suppress fasting-induced refeeding in the dark cycle (t(8)=0.2405, p= 0.8160) (Figure 3-5G) nor over 24 total hrs (t(8)=0.8746, p=0.4073) (Figure 3-5I). Notably, no effect of CNO was observed in WT mice after LPO-specific delivery (Figure S3-4).

Intriguingly, while NtsR1 is essential for LHA Nts neuron-mediated feeding[35], it may not contribute to regulation of drinking[35]. We therefore reasoned that even though NtsR1 is expressed in the LPO (Figure 3-1D), NtsR1 signaling may not be necessary for the LHA Nts→LPO circuit to stimulate drinking. To test this, we injected the NtsR1 antagonist (SR48692) i.p. 30mins prior to LPO specific delivery of CNO/VEH. There was no significant effect of NtsR1 antagonist on water intake (F (1, 36) = 1.356, p= 0.2518) (Figure 3-5K), consistent with prior data that NtsR1 is not necessary for drinking behavior[35]. Additionally, systemic treatment with the NtsR1 antagonist prior to activation of the LHA Nts→LPO circuit did not restrain fasting-induced feeding (F (1, 28) = 2.332, p=0.1380) (Figure 3-5L). Overall, these data suggest that LHA Nts → LPO circuit activation is sufficient to promote drinking via an NtsR1-indepenent mechanism. In combination with previous work, our findings suggest that the NtsR1-dependent effects on feeding are mediated by LHA Nts neurons projecting outside of the LPO.

3.4. DISCUSSION

The LHA is an essential hub for ingestive behavior, and most LHA neural subpopulations generally promote feeding and drinking. It remained unclear, however, if these behaviors could be differentially regulated. Our initial characterization of LHA Nts neurons suggested that they may be unique from the majority of LHA populations in that they divergently regulate drinking and feeding. Here we examined the neuronal

mechanisms and behaviors underlying LHA Nts neuronal bias for water intake. We detailed the circuit architecture and a potential NtsR1-independent mechanism by which LHA Nts neurons promote the motivation to drink, but confirm that their activation is not obligatory for homeostasis. We also provide the first evidence of an LHA Nts→LPO circuit mediating drinking behavior, but this circuit does not appear to mediate the suppression of feeding observed when activating all LHA Nts neurons. These findings shed light on how the LHA can orchestrate specific ingestive behaviors, and notably, provide insight into the regulation of LHA-mediated drinking that had been largely ignored.

Finding monosynaptic afferents to LHA Nts neurons coming from the LT[4–8,66], ARC[67,68], NTS[69], PVH[54], PB[70,71] and PAG[72] (Figure 3-2D) is consistent with previous work showing inputs to LHA from these areas. Projections from LT areas, i.e. SFO, OVLT and MnPO, are particularly interesting, given the established role of these areas in osmolarity sensing and regulation of homeostatic and anticipatory drinking[48–52]. Chemogenetic stimulation of SFO neurons promotes drinking[50,73,74] in part via projections to MnPO neurons[75], and activating a specific MnPO subpopulation that projects to the LHA circuit has been shown to increase blood pressure and drinking behavior via negative reinforcement[49,76]. However, since activating LHA Nts neurons is neither aversive nor rewarding, they likely lie in a separate circuit targeted by yet-to-be defined LT neurons. Since LHA Nts neurons engage the VTA, it is possible that they modify mesolimbic signaling that governs goal-mediated behaviors. Intriguingly, replenishing water following dehydration increases dopamine in the nucleus accumbens[77], indicating a potential mesolimbic circuit for control of motivation to drink. Given that we observed some overlap

of LHA Nts neurons projecting to the LPO and the VTA (Figure 3-1F &G), these neurons might integrate LPO-mediated drinking behavior and mesolimbic action.

Given the well-established roles of ARC and NTS neurons in modulating feeding[62–65], projections from these regions to LHA Nts neurons are likely candidates for invoking anorectic actions of LHA Nts neurons. Going forward it will be important to identify the specific neural populations in these regions that project to LHA Nts neurons to understand how they contribute to feeding behavior. For example, the POMC/CART population in the ARC might stimulate LHA Nts neurons to decrease motivation to eat, while the ARC AgRP/NPY population might suppress LHA Nts neurons to lift the brake on orexigenic neurons to promote feeding and food seeking behaviors. Since NTS inputs to the LHA Nts neurons were only observed in the LHA Nts neurons projecting to the VTA, but not the LPO, we hypothesize that the NTS→LHA Nts→VTA circuit probably modifies feeding rather than drinking. The neurochemical identity of these NTS neurons remains to be determined, but glucagon like protein 1 (Glp1)-expressing neurons are likely candidates because they suppress motivational feeding in part via acting on LHA Nts neurons[78]. The role of PAG inputs to LHA Nts neurons is currently unclear, but modulation of GABAergic PAG neurons has recently been shown to alter food reward[79] and feeding[80]. However, LHA Nts neurons, which are a subset of all LHA GABA neurons, also project to the PAG (Figure 3-1C), suggesting reciprocal regulation of these regions. Activation of an LHA GABA→PAG circuit increases feeding[80], opposite of effects via LHA Nts neurons, making it unlikely that the LHA Nts → PAG neurons overlap with this characterized orexigenic pathway.

Our data show that the PVH, PB and lHb send projections to LHA Nts neurons, regions that are considered to be at the interface of feeding[55,81,82] and drinking[34] behavior, and could impact behaviors mediated via the LPO and/or VTA. Future studies will be required to test how each of these LHA Nts-projections impacts ingestive behavior, and should be informed by previous work. For example, some GABAergic LHA neurons project to the PVH and promote feeding[54]. Given the role of Nts in suppressing feeding, it is likely that LHA Nts→ PVH neurons are either a subset amongst other orexigenic LHA GABA neurons or that they lie in a separate pathway. Alternately, LHA Nts → PVH neurons might also contribute to drinking behavior, as PVH corticotropin-releasing hormone (CRH) expressing neurons are regulated by dehydration[34,83] and PVH magnocellular neurons express Arginine-vasopressin (AVP)[57] that stimulates drinking. Similarly, SO magnocellular neurons are involved in AVP production and secretion[57] so the SO→LHA Nts connection observed here might also serve to promote drinking. Given the connectivity of LHA Nts neurons with the PB, lHb and CEA, regions that can mediate taste aversion or anorexia, these circuits are also likely candidates to exert LHA Nts-mediated feeding restraint [82,84,85].

Here we report that activation of LHA Nts neurons is not only sufficient to promote drinking, but it does so by increasing motivation to drink and seek water (Figure 3-3J-O). Since dehydration and hyperosmolality are aversive states[49,76], one can see the advantage of having a circuit that increases the motivation to drink. Moreover, thinking about suppression of feeding and promotion of drinking together in the natural environment, this would certainly be an important evolutionary advantage under periods when water is not as accessible (e.g. draught) and animals are under chronic dehydration

where finding water should be prioritized over finding food. Because dehydration is life-threatening on a faster timescale than starvation, it is possible that LHA Nts circuits evolved to prioritize drinking behavior to ensure survival. Moreover, because LHA Nts neurons increase preference for palatable caloric and non-caloric liquids (Figure 3-3P-S), the stimulated fluid intake is more likely to be due to the liking and/or wanting of fluid rather than to address energy status. This is an important observation in terms of evaluating therapeutic potential of LHA Nts circuits: it suggests that invoking LHA Nts neurons will not necessarily promote drinking high-calorie beverages that could lead to weight gain, as non-caloric beverages might be equally palatable and sought-after. Because LHA Nts neurons can promote drinking, but are not necessarily required for fluid homeostasis, modulating LHA Nts neurons might have potential to correct aberrant drinking drive. For example, the coordination of osmolality/thirst and the motivation to consume water is disrupted during aging and in persons with diabetes, putting individuals at higher risk of serious complications, such as cognitive dysfunction, increased risk of cardiovascular disease, hyperosmolar hyperglycemia, chronic kidney diseases, hypertension and stroke[2,86,87]. Having a potential circuit to modify by pharmacologic intervention to promote drinking in these patients with a bonus of suppression of feeding to promote weight loss, especially in diabetic and obese patients, certainly would have therapeutic potential. To our knowledge, there has been no study investigating the correlation between alterations in Nts and aging-induced decline of thirst. It is possible that reduced Nts signaling might contribute to this state, although our findings suggest that loss of Nts signaling via NtsR1 does not impede drinking. These data suggest that LHA Nts neurons might influence drinking instead via NtsR2, or alternately, Nts-

independent mechanisms. Given that LHA Nts neurons also express GABA and other neuropeptides[28], there are multiple releasable signals that could influence behavior via the LPO and in other projection sites.

While LHA Nts neurons are sufficient to promote drinking, inhibition of LHA Nts neurons does not alter homeostatic and need based consumption; hence, LHA Nts circuits are not essential for ingestive behavior. Likewise, an independent study also found that chemo-inhibition of LHA Nts neurons had no deleterious impact on metabolism, although it could modify response to stress[88]. This may seem at odds with historical studies showing an indispensable role of the LHA in feeding and drinking regulation[11,89]. Yet, regulation of body hydration and osmolality is essential for survival, so it is plausible that circuit redundancy evolved to ensure maintenance of drinking for survival of the species. Indeed, we already know that there are LT circuits to control osmoregulatory drinking, as discussed previously. Also, other LHA neurons can modulate drinking behavior. For instance, activation of LHA Orexin/Hypocretin and MCH neurons has been most described in promoting feeding, but also increases fluid intake[15,3420,90,91]. Thus, it is likely that any suppression of LHA Nts circuit activity that might diminish motivated drinking can be compensated by these or other drinking circuits to ensure survival. Indeed, the finding that LHA Nts activation is not essential for homeostasis suggests that these neurons could be targets to improve disrupted motivation for water without endangering maintenance of homeostasis.

Our focus here was on how LHA Nts neurons could motivate drinking, but in the future, it will also be important to understand which LHA Nts circuits are responsible for suppression of feeding. A strong candidate for feeding modification is LHA Nts→VTA

circuit (Figure 3-1C). Decades of work have illuminated the important role of VTA in motivated behaviors and reward processing[92,93] via release of dopamine (DA) into the nucleus accumbens and prefrontal cortex[92]. Other LHA populations, including LHA GABA[85,94,95] and Orexin/Hypocretin[96,97] neurons[27,41]project to DAergic[98] and GABAergic[17,18] VTA neurons and influence motivated feeding. Likewise, LHA Nts neurons also project to the VTA, where a subset of VTA DA neurons expresses NtsR1, the NtsR isoform implicated in restraining feeding. Therefore, it is plausible that activation of the LHA Nts→VTA circuit might be responsible for suppression of feeding via Nts acting on DAergic VTA neurons. This hypothesis merits study in the future to determine if the LPO and VTA indeed mediate divergent ingestive behaviors.

We found that the dense projections of LHA Nts neurons in the LPO are sufficient to promote drinking, narrowing down at least on mechanism by which LHA Nts neurons mediate fluid intake. While prior work has confirmed that the LHA sends projections to the LPO[88,99], to our knowledge this is the first report describing a functional role for LHA input to the LPO. We cannot at this time rule out that other LHA Nts projection targets also mediate drinking behavior, but the LPO is at least an important contributor. Our finding that inhibition of NtsR1 does not impede LHA Nts→LPO mediated drinking is also instructive, and points toward NtsR1-independent mechanisms at play in the LPO. Besides expressing Nts, all LHA Nts neurons contain GABA[28,31], which could mediate inhibitory regulation of the LPO. Additionally, CRH[34], galanin[25,28,100] and CART[28] are also co-expressed in some LHA Nts neurons and could potentially modulate targets, although it is yet unknown if the LPO-projecting LHA Nts neurons contain these neuropeptides. Going forward, it will be helpful to characterize the neurochemical profile of the LHA

Nts→LPO circuit. Defining the LHA neurotransmitters and neuromodulators responsible for LHA Nts→LPO mediated drinking, and which neurons in the LPO they act upon, is essential to understand how this circuit mechanistically modifies drinking behavior and if it might be leveraged to correct aberrant drinking behaviors.

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APPENDIX

Area	Overall Rating	Area	Overall Rating	Area	Overall Rating
Dorsal Peduncular Cortex (DP)	*	Magnocellular Preoptic Nucleus (MCPO)	-	Lateral Habenular Nucleus, medial part (LHbM)	*
Infralimbic cortex (IL)	*	Periventricular Hypothalamic Nucleus (Pe)	*	Posterior Hypothalamic Area (PH)	*
Lateral Septal Nucleus (LS)	*	Caudate Putamen - Striatum (Cpu)	-	Periaqueductal Gray (PAG)	***
Nucleus of the Vertical Limb of the Diagonal Band (VDB) (Diagonal Band Nucleus (NDB))	**	A14 Dopamine cells area around Pe	*	Ventral Tegmental Area (VTA), Rostral part (VTAR), Parabrachial pigmented nucleus of VTA (PBP), Paranigral Nucleus of the VTA (PN)	***
Medial Septal Nucleus (MS)	*	Extended Amygdala (EA)	**	Substantia nigra compacta (SNc), Dorsal tier (SNcD)	***
Nucleus of the Horizontal Limb of the Diagonal Band (HDB) (Diagonal Band Nucleus (NDB))	***	Anterior Amygdaloid Area (AA)	*	Substantia nigra reticular part (SNr)	-
Organum Vasculosum of the Lamina Terminalis (OVLT)	*	Globus Pallidus (GP)	-	Ventral tuberomammillary nucleus (VTM)	*
Ventral Pallidum (VP)	*	Subfornical Organ (SFO)	?	pre-Edinger-Westphal nucleus (prEW)	-
Substantia innominata (SI), basal part	*	Stria terminalis (st)	*	Mesencephalic Reticular formation (mRt), Pararubral nucleus (PaR), Retrorubral field (RRF)	***
Accumbens nucleus, core (NAcC)	**	Antroventral Thalamic Nucleus (AV)	-	Dorsal Raphe nucleus (DR)	*
Accumbens nucleus, shell (NAcSh)	*	Paraventricular Thalamic Nucleus (PV), Anterior Part (PVA), Posterior part (PVP)	*	Lateral lemniscus (ll)	**
Median Preoptic Area (MnPO)	*	Paraventricular Hypothalamic Nucleus (Pa)	*	Just above Longitudunal fasciculus of the pons (fp) (maybe) Reticulotegmental nucleus of the Pons (RtTg)	*
Medial Preoptic Area (MPO)	-/*	Supraoptic Nucleus (SO)	*	Subpeduncular tegmental nucleus (SPTg)	*
Ventromedial Preoptic Nucleus (VMPO)	-	Central Amygdaloid Nucleus (CEA), Amygdoloid nucleus (A)	*	Parabrachial Nucleus (PB) (or Pedunculotegmental nucleus – PTg and mRT mix)	***
Antroventral Periventricular Nucleus (AVPe)	-	Paraxiphoid nucleus of Thalamus (PaXi) & Reunience Thalamic Nucleus (Re) (can not differentiate them well)	-/*	Subcoeruleous nucleus (SubC)	*
Lateral Preoptic Area (LPO)	****	Arcuate Hypothalamic Nucleus (ARC)	-	Locus coeruleus (LC)	*
Bed Nucleus of Stria Terminalis (BNST) – ventral part	***	Dorsomedial Hypothalamic Nucleus (DM)	*	Dorsal Tegmental Nucleus (DTg)	-
Ventrolateral Preoptic Nucleus (VLPO)	*	Ventromedial Hypothalamic Nucleus (VMH)	-	Anterodorsal thalamic nucleus (AD)	-
		Parapyramidal nucleus (PPy)	*	Corpus collosum (cc)	-

Table S3-1. Projection sites of LHA Nts neurons. **** indicates the highest and * indicates the lowest density. – indicates absence of axonal terminals.

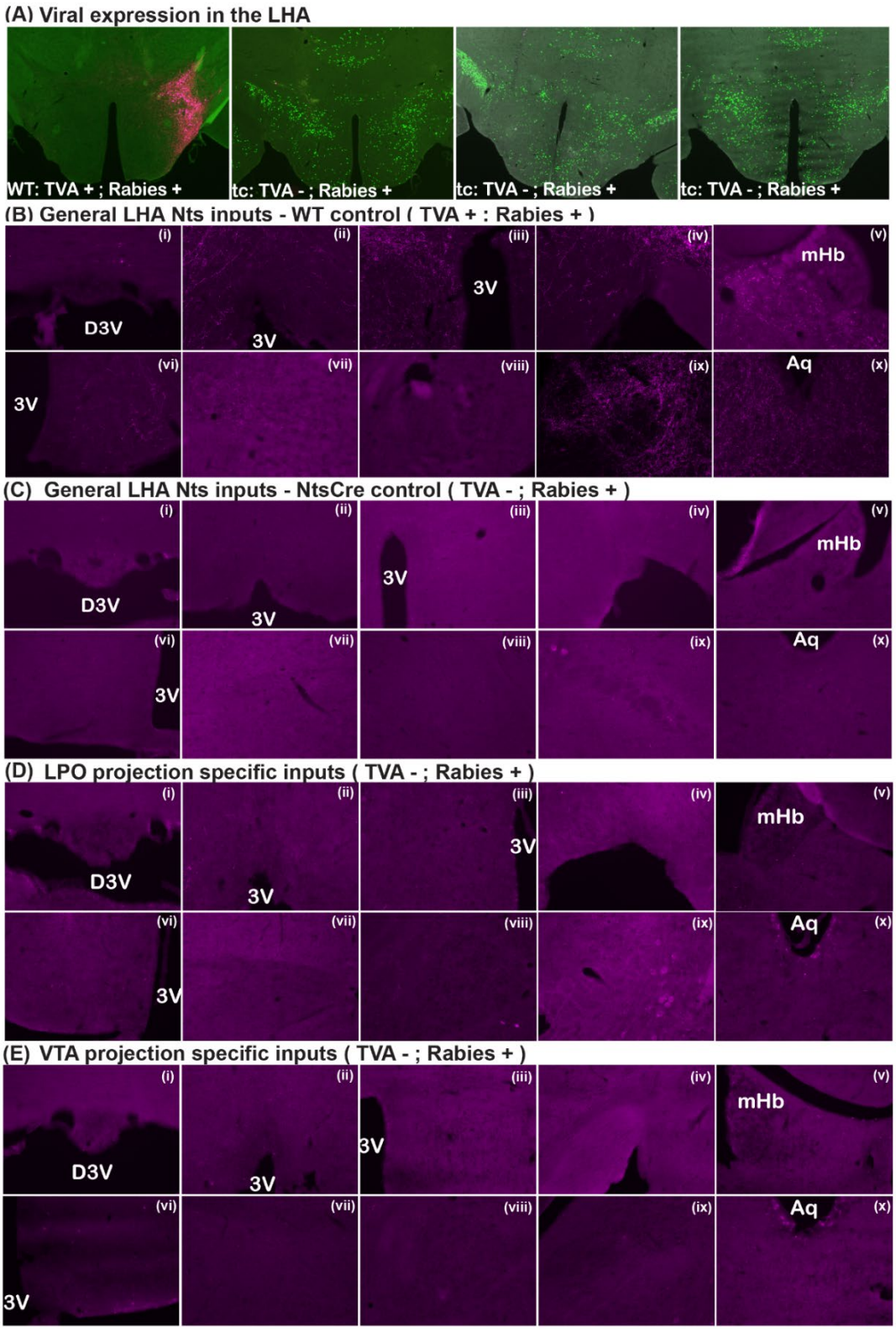

Figure S3-1. Monosynaptic retrograde rabies tracing controls. WT mice received

Figure S3-1 ***(cont`d)*** both cre-dependent helper (TVA+) and modified Rabies (Rabies +) viruses **(A & B)**. *Nts^{Cre};GFP* mice first received a sham surgery for (TVA -) into the LHA followed by modified Rabies virus (Rabies +) into the LHA for general monosynatic tracing from LHA Nts neurons **(A & C)** or following sham surgery, modified Rabies virus into either the LPO **(A & D)** or the VTA **(A & E)** for projection site specific tracing controls. **(A)** 4X representative images for viral expression in the LHA in all control groups. **(B)** 20X control images of LHA: LHA (TVA +;Rabies+) injected WT mice for the areas found to be sending direct projections to LHA Nts neurons (n=1). **(C)** 20X control images of LHA: LHA (TVA -;Rabies+) injected *Nts^{Cre}* mice for the areas found to be sending direct projections to LHA Nts neurons (n=2). **(D)** 20X control images of LHA: LPO (TVA -;Rabies+) injected *Nts^{Cre}* mice for LPO projection specific LHA Nts neural inputs controls (n=1). **(E)** 20X control images of LHA: VTA (TVA -;Rabies+) injected *Nts^{Cre}* mice for VTA projection specific LHA Nts neural inputs controls (n=1). From **B-E (i)** SFO, **(ii)** MnPO & OVLT, **(iii)** PVH, **(iv)** SO, **(vi)** ARC, **(vii)** NTS, **(viii)** CEA, **(ix)** PB, **(x)** PAG & DR.

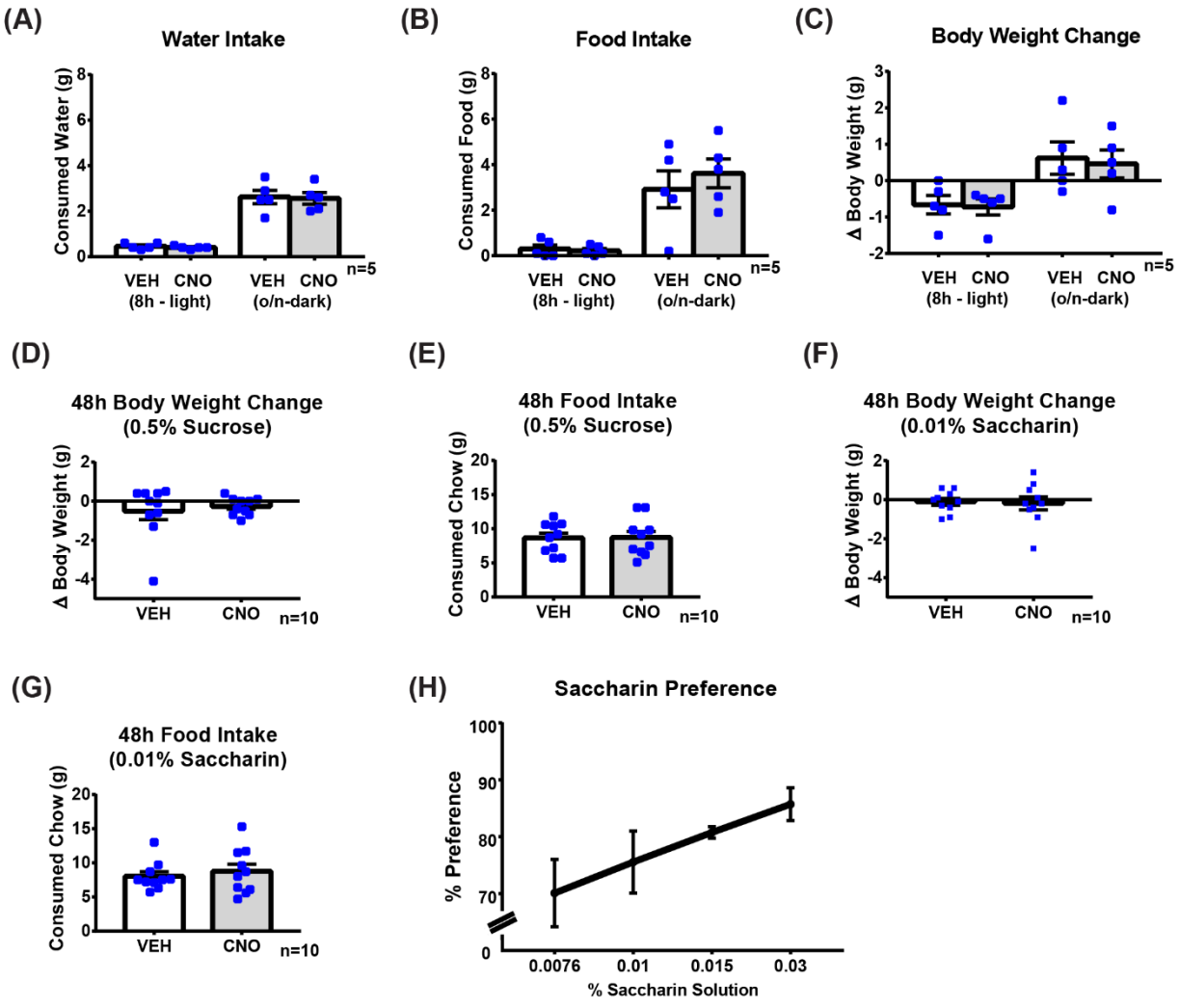

Figure S3-2. The effects of LHA Nts activation in consumption and 48-hr 2-bottle preference. (A-C) Cre-dependent DREADDq was injected bilaterally into the LHA of male and female WT mice. Total **(A)** water and **(B)** food intake and **(C)** body weight change 8hrs after in the light cycle and overnight (o/n) in the dark cycle following twice daily i.p. injection of VEH or CNO in well hydrated fed mice. **(D-H)** 48-hr 2-bottle preference tests in Nts^{Cre} mice expressing DREADDq in LHA Nts neurons. **(D)** Body weight change and **(E)** food intake in 48-hr 2-bottle 0.5% sucrose solution preference test. **(F)** Body weight change and **(G)** food intake in 48-hr 2-bottle 0.01% saccharin solution preference test.

Figure S3-2 ***(cont`d)*** **(H)** Saccharin solution percentage versus average preference curve generated for determination of optimal saccharin percent.

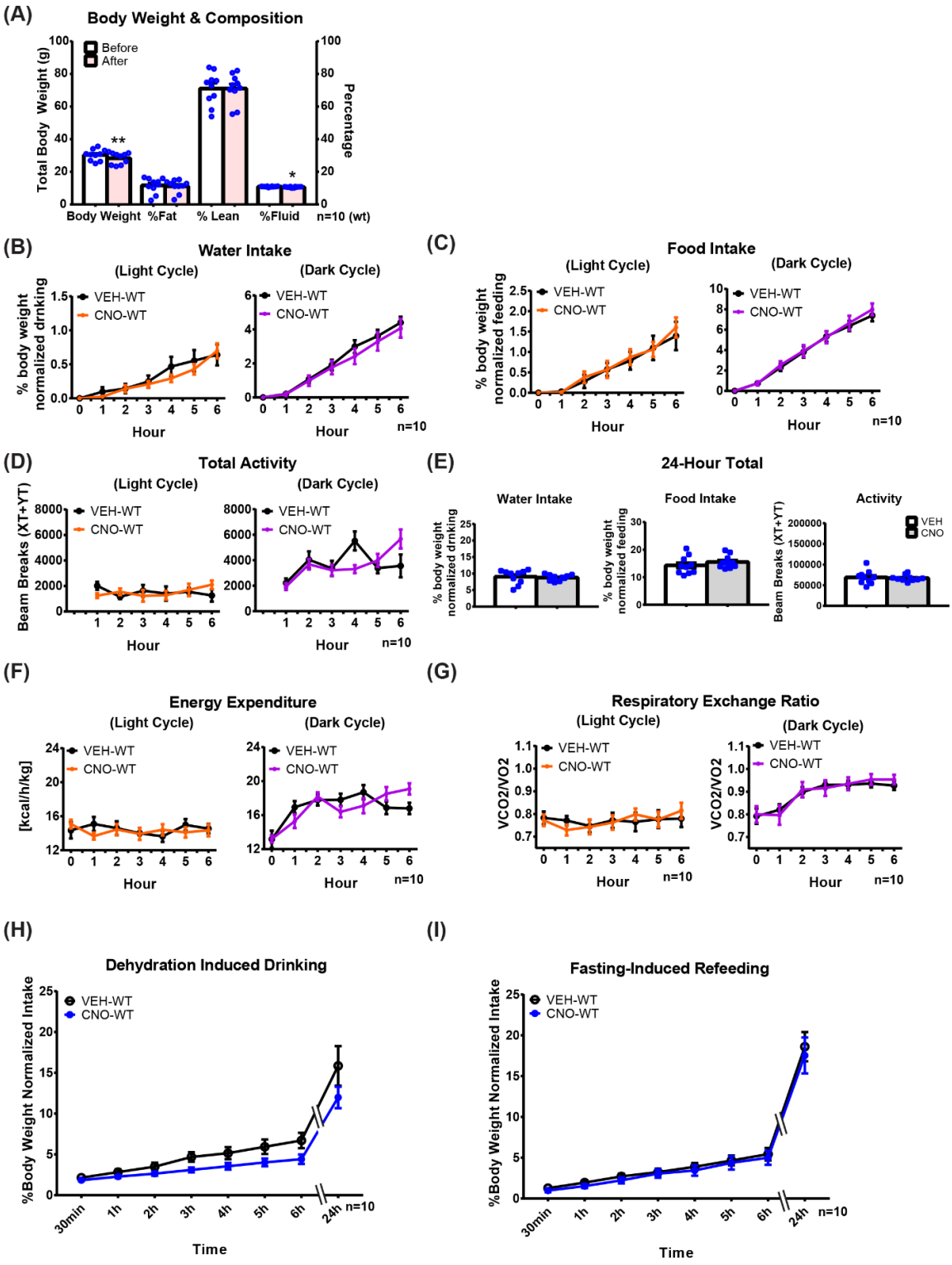

Figure S3-3. LHA Nts inhibition does not change the basal consumption and

Figure S3-3 *(cont`d)* activity or need-based ingestion. Cre-dependent DREADDi was injected bilaterally into the LHA of male and female Nts^{Cre} (n= 16) and WT mice (n=10). Analyses of WT mice were shown in this figure. t=0 marks the injection times on each graph and the indicated times are “time past post injection”. **(A)** Body composition was analyzed before and after i.p. VEH/CNO injections in TSE cages. Light and dark cycle post injection cumulative **(B)** water and **(C)** food intake, hourly **(D)** total locomotor activity and **(F)** energy expenditure as well as **(G)** respiratory exchange ratio at each hour indicated were recorded in TSE metabolic cages with twice daily i.p. injection of either VEH or CNO. **(E)** 24-hr total water and food intake as well as activity levels in TSE cages. **(H-I)** Need-based consumption tests in home cages. **(H)** Overnight dehydration-induced drinking. **(I)** Overnight fasting-induced re-feeding. * $p<0.05$, ** $p<0.01$.

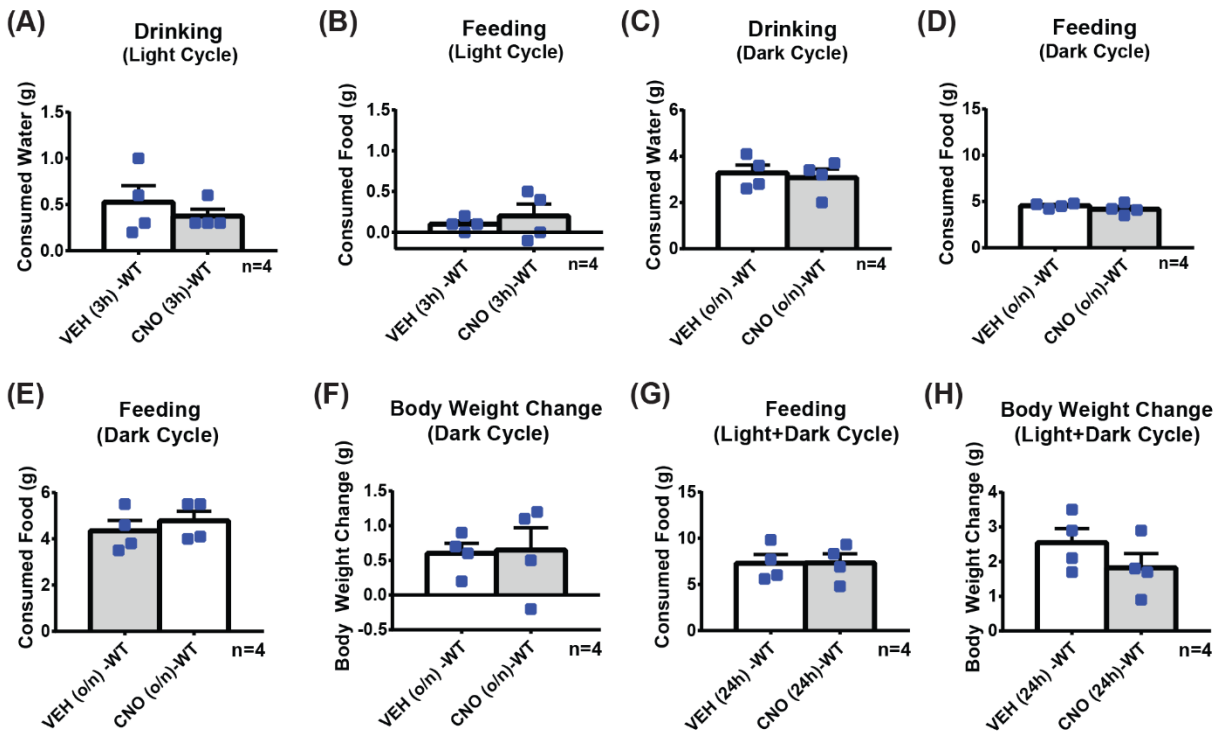

Figure S3-4. Projection site-specific activation of LHA Nts neurons. Cre-dependent DREADDq was injected bilaterally into the LHA of male and female WT mice (n=4) and indwelling cannulas were placed bilaterally over the LPO for projection site specific activation of LHA Nts neurons. VEH or CNO was infused to the projection sites via cannula. Total water and food consumption **(A-B)** 3hrs (light cycle) and **(C-D)** o/n (dark cycle) after VEH/CNO delivery in well hydrated-fed WT mice. Total food consumption and body weight change **(E-F)** o/n (dark cycle) and **(G-H)** 24hrs (light+dark cycle) after VEH/CNO delivery in fasted WT mice.

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CHAPTER 4. SUMMARY, DISCUSSION, AND TRANSLATIONAL IMPLICATIONS

4.1. SUMMARY OF RESULTS

The overall goal of this PhD thesis was to understand the role of LHA Nts neurons in drinking behavior. The main hypothesis of the work was that *the LHA Nts neurons direct drinking behavior in response to physiologic need (thirst) via neural outputs to the LPO, but do not modify feeding via LPO*.

In Chapter 2, we studied the acute experimental activation of LHA Nts neurons by use of transgenic cre-loxP mice and DREADD technologies, and its effects on drinking behavior. Our hypothesis for this Chapter was that *the activation of LHA Nts neurons increases homeostatic and thirst-induced drinking*. By utilizing Nts^{Cre} mice and activatory DREADDs, we activated all LHA Nts neurons by systemic injection of the DREADD ligand, CNO. We discovered that acute activation of LHA Nts neurons was sufficient to promote voracious drinking in well hydrated mice without modifying their feeding, and also augmented drinking in thirsty mice (Figure 4-1). We also found that activation of LHA Nts neurons increased body temperature, consistent with an independent report that optogenetic or chemogenetic activation of LHA Nts neurons caused hyperthermia[1]. Another important finding in this study was that acute activation of these neurons promoted the intake of any solution (bitter or acutely palatable) if it was the only fluid available; however, if mice had a choice of fluids, activation of LHA Nts neurons caused an increase in preference for a palatable solution (sucrose), but not for dehydrating or bitter solutions (hyperosmotic NaCl and quinine). Moreover, LHA Nts-activated mice increased their intake of water and palatable solutions, but not dehydrating and bitter

solutions. Taken together, these data confirmed that activation of LHA Nts neurons broadly promotes fluid intake. Moreover, these data pointed to LHA Nts neurons in possibly coordinating motivated drinking behavior, since they could further augment intake of palatable substances.

In Chapter 3, we built upon these findings to define the neurocircuitry of LHA Nts neurons, their requirement for ingestive behaviors and whether they change motivation to drink. Additionally, we compared the effects of activating all LHA Nts neurons with selective activation of the LHA Nts→LPO projections, thereby revealing the contributions of this specific circuit within the sum of all LHA Nts neuronal action. We explored 4 hypotheses in this Chapter (1 per study). For the studies of LHA Nts connectivity our hypothesis was that *LHA Nts neurons are anatomically well positioned to receive nutrient and osmolality cues and to modify feeding and drinking behavior*. We investigated this hypothesis using cre-mediated, viral retrograde and anterograde tracers to map the neuronal inputs to LHA Nts neurons and their projections throughout the brain. These experiments confirmed our hypothesis, showing that LHA Nts neurons receive afferents from osmo- and energy- sensory and regulatory regions and they were send efferents to brain regions known to modify drinking and feeding behavior. Intriguingly, while we found partial overlap between LHA Nts neurons projecting to the LPO and VTA (implicated in modulating drinking and feeding, respectively), but also that more LHA Nts neurons solely projected to the LPO than to the VTA. Given that the LPO is important for drinking behavior, these data prompted further exploration of the LHA Nts→LPO circuit to define its specific contributions to LHA Nts neuronal action (addressed later).

In the Chapter 3 study examining the requirement of LHA Nts neurons for ingestive behaviors our hypothesis was that *the LHA Nts neuronal activation is required to direct the appropriate ingestive response to thirst or hunger*. Here we used a cell specific chemogenetic approach (i.e. expressing inhibitory DREADDs in the LHA of Nts^{Cre} mice) to suppress activity of LHA Nts neurons during homeostasis and osmotic and energy balance challenges. In contrast to our hypothesis, these experiments revealed that LHA Nts neural activation was not required for neither homeostatic nor need-based (i.e. thirst and fasting) drinking and feeding. In hindsight, this null finding suggests that maintaining fluid and energy homeostasis is likely so important so as not to be dependent on any single neuronal population or circuit. Given the exigency of drinking and feeding for survival, it makes biological sense that LHA Nts neurons may be just one of several neural pathways that contribute to these behaviors. Indeed, circuit redundancy likely evolved to safeguard homeostasis. However, even though activation of LHA Nts neurons is not solely required for fluid and energy balance, these data do not discount that activation is sufficient and important to modify these behaviors.

For the Chapter 3 studies on the role of LHA Nts neurons in motivated drinking our hypothesis was that *the activation of LHA Nts neurons increases motivation to drink.* Here again we used Nts^{Cre} mice expressing activatory DREADDs in the LHA to permit activation of all LHA Nts neurons, and tested how this impacted conditioned place preference for a “water-providing” location (CPP) and motivation to work for water (progressive ratio schedule operant responding). We found that activation of LHA Nts neurons increased the preference for a water-paired environment, which we suggest is analogous to promoting water seeking. Activating LHA Nts neurons also increased the

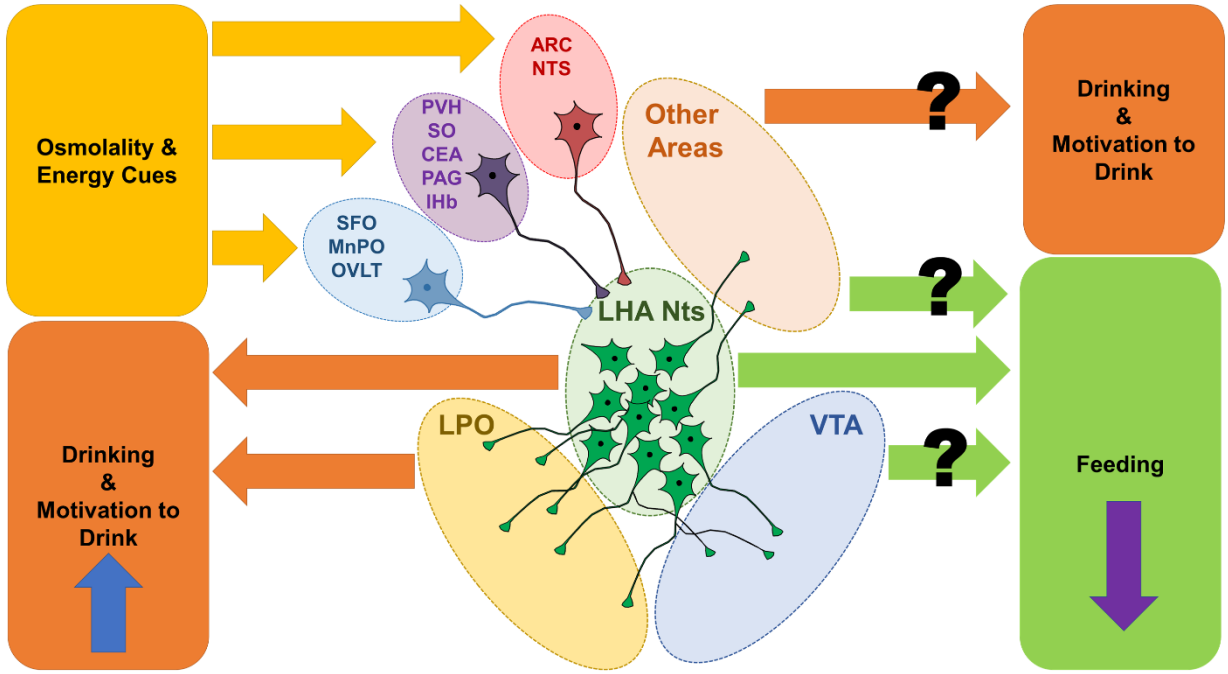

Figure 4-1. Summary of Major Research Results. LHA Nts neurons receive direct monosynaptic inputs from osmolality and energy status sensory areas and send projections to osmolality and energy balance regulatory areas. They send the densest projections to LPO and via activation of all LHA Nts neurons as well as LHA Nts → LPO projections, they promote drinking and motivation to drink. However, LHA Nts → LPO circuit does not mediate LHA Nts activation-induced suppression of feeding.

amount that mice worked to obtain water, indicating that they modulate the motivation to drink. We also used 2-bottle solution preference tests to confirm that LHA Nts-mediated drinking is not dependent on the caloric content of the liquid. Overall, these data confirm the hypothesis that LHA Nts neurons increase motivation to drink and suggest that LHA Nts neural control of drinking might not be directly about homeostatic drinking but might

be more about increasing the thirst perception and motivation to search and consume water when the water need is urgent.

Finally, in Chapter 3 we tested the hypothesis that *activation of all LHA Nts neurons promotes drinking and suppresses feeding, but activation of the LHA Nts→LPO circuit selectively promotes drinking, but does not suppress feeding.* We tested this by using the same chemogenetic approach to facilitate activation of LHA Nts neurons, but by selectively delivering CNO in the LPO, we confined activation to the LHA Nts→LPO projections. Consistent with our hypothesis, we found that activation of LHA Nts→LPO circuit was sufficient to promote drinking, but this circuit did not suppress feeding. Since pharmacological inhibition of NtsR1 did not impede LHA Nts→LPO mediated drinking, it suggests that NtsR1-independent mechanisms mediate drinking behavior via this pathway. In sum, the work in Chapter 3 revealed the role of LHA Nts neurons in motivational and selective drinking behavior and a novel brain circuit that through activation of LHA Nts projections in the LPO promotes drinking (Figure 4-1).

4.2. DISCUSSION

4.2.1. Limitations and Technical Considerations of this Work

We leveraged viral cre-lox mediated tools throughout this thesis to specifically modulate LHA Nts neurons and test our hypotheses. Therefore, our results are limited by the efficiency of the viral tools used in these experiments. In order to minimize the limitations, we applied several controls for each virally-mediated tool and technique.

The genetically-modified rabies tracing system has gained popularity for identifying the synaptic input neurons of specific neural populations, thus was ideal for our goal to map the inputs to LHA Nts neurons. For our modified rabies tracing studies (Chapter 3),

we used non-cre expressing WT mice as controls to verify the cre specificity of the system, namely that the dual viral, cre-dependent system could not label circuits in the absence of cre expression. We also verified that both viruses are required for tract tracing by injecting some *Nts*^{Cre} mice with only the modified rabies, which indeed could not trace neurons without previous injection of the AAV helper virus that encodes proteins requisite for rabies infections and transsynaptic spread. We also reasoned that successive, weeks-apart injections of both component vectors into the same brain region might substantially damage cells, and might permit them to be infected by rabies virus despite lacking the necessary rabies receptor protein. Indeed, WT mice receiving dual injections in the LHA did exhibit modest levels of tissue disruption and some mCherry expression indicative of rabies-mCherry infection. However, because these neurons lacked cre and cre-dependent expression of the protein necessary for transsynaptic spread, we observed no mCherry elsewhere in the brain (Figure S3-1). We addressed the potential dual-injection damage issue by separating the injections so that the AAV helper was injected in the LHA while the rabies virus was injected in an LHA Nts projection region of interest (in this case, the LPO or VTA). This method avoided potential damage of injecting into the same site, and as an added bonus, provided more circuit information (e.g. identifying the neuronal inputs to projection-specified subsets of LHA Nts neurons.). These controls vouch that the genetically modified rabies tracing tools were specific in our hands. However, we acknowledge that there may be lower viral transduction efficiencies in the projection site-specific modified rabies approach, which could have led to a failure to detect all neurons/brain areas sending afferents to LHA Nts neurons projecting specifically to LPO

or VTA. Therefore, there is a chance that the slight distinctions observed on the input circuit level might have been due to such an experimental artifact.

We used well-established viral tools for chemogenetic studies, i.e. DREADDs[2,3], to modify the activity of LHA Nts neurons and understand their function in ingestive behavior. Even though these tools were shown to be efficient to study behavior, there are still limitations in using them. Recently, it has been shown that the DREADD-ligand CNO, initially reported to be inert, may have off target effects due to the actions of its metabolite, clozapine[4,5]. Therefore, it is important to verify that the CNO dosage used for chemogenetic studies does not exert any effects when injected into non-DREADD expressing mice. For this reason, we included non cre-expressing WT controls in this study that received the same CNO treatments as DREADD-expressing *Nts*^{Cre} mice (Figures S3-4 - S3-6, S3-8 & S3-9). These controls suggest that CNO has no significant off-target effects on ingestive behaviors in mice lacking DREADD receptors, consistent with a previous report from our lab[6]. Thus, the CNO-treatment induced alterations in behaviors reported in this thesis were not due to off-target effects mediated by back metabolism to clozapine.

Another caveat of this work is that we have not fully excluded sex-differences in LHA Nts neuronal control of ingestive behaviors. Our initial work on these neurons, the studies presented in Chapter 2, only used male mice. However, we recognized that it was important to investigate potential sex-specific regulation in drinking and feeding via LHA Nts circuits. Indeed, our finding of dense LHA Nts neurons to the LPO suggested that sex differences could come into play, as the LPO is a sub-area of the preoptic area, some parts of which are sexually dimorphic[7–9]. Therefore, we incorporated female mice into our

subsequent studies detailed in Chapter 3. We observed a similar promotion of drinking (Figure 3-3B) and suppression of feeding (Figure 3-3E & G) in females following activation of LHA Nts neurons and no difference between male and female ingestion in LHA Nts inhibition studies. Since we did not detect any differences between male and female mice, data obtained from both sexes were pooled for analysis.

4.2.2. Future Directions

The culmination of this thesis work shows that LHA Nts neurons promote motivational drinking behavior, but not feeding, and that the drinking is preferentially directed toward consumption of water and palatable water-based solutions. We also provide evidence that LHA Nts neurons are not essential for homeostatic and need-based ingestion, but they are well connected within the CNS to be able to modify ingestive behaviors. Moreover, we have identified a novel LHA Nts→LPO circuit that drives the drinking effect of LHA Nts neurons, separate from the feeding regulation by these neurons.

Here we focused on the mechanism by which LHA Nts neurons promote drinking, and identified at least one pathway (the LHA Nts→LPO circuit) that contributes to this behavior via a NtsR1-independent mechanism. It will be important to understand whether this LHA Nts→LPO circuit interfaces with known osmo-regulatory (neuro)hormonal mechanisms. For example, does activation of LHA Nts neurons induce changes in AVP that contribute to drinking behavior? Alternately, do endogenous changes in AVP modulate LHA Nts neuronal activity to increase motivation to drink? Exploring these questions will be important, but technically challenging since there is no reliable assay for rodent AVP levels. It will also be important to examine whether LHA Nts neurons engage

AVP-expressing neurons, or if they lie in a parallel circuit with AVP signaling pathways. Given that osmo-sensory regions project to both LHA Nts neurons and PVH and SO neurons known to express AVP, it is possible that LHA Nts neurons and PVH/SO neurons might be simultaneously modulated to drive the motivational and neuroendocrine aspects of drinking behavior, respectively. Further investigation into these directions is warranted to understand if LHA Nts neurons are solely implicated in motivated drinking or are recruited in endogenous drinking regulation to encourage the motivation to drink along with neurohormonal mechanisms to modify peripheral water handling. Additionally, it will be important to determine if ANG II, a potent stimulator of drinking, can regulate the activity of LHA Nts neurons, as LHA injection of ANG II has been reported to promote drinking via a yet uncharacterized LHA population[10].

Going forward, it will be important to delineate the signaling by which LHA Nts projections mediate ingestive behavior. LHA Nts neurons are not a uniform population[11], and exhibit significant molecular heterogeneity. There may be specific subsets of LHA neurons, with distinguishable molecular signatures, that mark the LHA Nts neurons that are regulated by dehydration vs. energy status. Indeed, future research should examine whether candidate LHA Nts projections identified here (e.g. to the LPO and VTA) differ in their molecular signature. This knowledge could suggest molecular approaches to modulate the activity of projection-specified LHA Nts neurons to further elucidate their contributions to ingestive behavior.

4.3. TRANSLATIONAL IMPLICATIONS OF THIS RESEARCH

The essential role of the LHA in regulating ingestive behavior and energy balance has been known for more than a half century[12,13] yet how it mediates this vital physiology

still remains unclear. This is due to the LHA's marked cellular heterogeneity and targeting of diverse regions throughout the brain, as the roles of each must be probed to understand what actions they mediate. Defining and understanding the myriad LHA circuits and their contribution to normal physiology, as well as how disruptions contribute to disease pathogenesis, could identify treatments for ingestive disorders. Yet to date, these circuits have mainly been studied in rodent models, largely due to the array of cre-lox genetic and viral tools that can be used in rodents to identify and manipulate specific neurons and circuitry. Such mechanistic studies of the LHA are simply not possible in humans. Studies of the human hypothalamus have been limited to imaging studies, which lack the cellular resolution of rodent studies, but provide vital information about the role of the hypothalamus in ingestive disorders[14,15]. For example, an imaging study of euhydrated and dehydrated humans found that although several brain areas significantly decreased in size in dehydrated state, only the volume change of hypothalamic/thalamic tissue was correlated with serum osmolality levels[16]. Even though there are not many human studies about the role of the brain in water and energy balance, these studies still support decades of accumulated results from rodents, signifying the translational value of rodent studies to decipher the central circuits controlling ingestive behaviors.

Similarly, the role of neurotensin and its contributions have also been largely studied in rodent systems to date, with little known about neurotensin regulation in humans. Although there are a few studies about neurotensin-body weight relation in humans[17–22], to our knowledge, Nts or Nts receptors have never been studied in hydration disorders. However, Nts has been implicated in schizophrenia, in which psychogenic polydipsia is a common co-morbidity[23,24]. Nts levels are lower in the cerebrospinal fluid

(CSF) of schizophrenic patients but are restored following antipsychotic treatment[25,26]. In addition, *NTSR1* and *NTS* gene mutations have also been found in schizophrenic patients, although no causative association with the disease has been identified to date[25,26]. Thus, there is much yet to be learned about the role of Nts in human physiology.

The basic science work of this thesis, while confined to rodents, advances understanding of the physiological control of drinking and could have implications for treatment of disrupted ingestive behaviors in humans. Our finding that the activation of LHA Nts neurons and a novel LPO projecting circuit are sufficient to promote drinking refines the field's knowledge of how drinking is mediated, and what circuitry might be targeted to modify motivated drinking. Moreover, we showed that selective activation of all LHA Nts increases motivation to drink and intake of hydrating palatable liquids, but inhibition of these neurons does not disrupt baseline homeostatic and need-based consumption. From a translational perspective, these results may be relevant in treating chronic dehydration, which is commonly observed in aged individuals due to diminished perception of thirst and in diabetic patients with polyuria. The chronic dehydration in both of these conditions puts individuals at risk of life-threatening cognitive, cardiovascular, and hyperglycemic complications[27–29], increases the number of hospitalizations and compounds economic burden on the health system[30]. Urging such prone individuals to simply drink more water is not effective, since the lack of awareness of dehydration and the blunted drive to drink mean that individuals do feel the need to drink, and so do not. Thus, in these conditions of blunted motivation for water, it is vital to increase the motivation to drink so that individuals will restore homeostasis and prevent the development of the life-threatening sequalae of osmolality disruption. Thus, our work

demonstrating an LHA Nts neural circuit that can increase motivation to drink without disrupting the baseline physiology or promoting higher caloric intake, could be very promising for treating individuals prone to dehydration. Hence, this work points to the therapeutic potential of targeting LHA Nts circuits to overcome dysregulated drinking. In the future, defining the precise mechanisms by which LHA Nts circuits modify drinking behavior could point the way to the development of safe pharmacotherapy tools targeting these circuits that could be useful to improve human health.

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