THE ROLE OF LATERAL HYPOTHALAMIC NEUROTENSIN NEURONS IN ADAPTIVE ENERGY BALANCE

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ABSTRACT

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The lateral hypothalamic area (LHA), receives cues of energy and fluid status from the body and coordinates appropriate feeding, drinking and activity (e.g. adaptive responses) to ensure survival. The LHA contains many distinct populations of neurons, however, and it remains unclear how each of these contribute to energy balance. Here we sought to understand how LHA neurons expressing the neuropeptide neurotensin (LHA Nts neurons) coordinate distinct behaviors necessary for adaptive response and control of body weight. While activation of most LHA Neurons increases both feeding and drinking, activation of LHA Nts neurons specifically promotes drinking but reduces feeding. LHA Nts neurons may exert these divergent actions via distinct circuits, as they have been shown to modulate dopamine (DA) signaling and local orexin (OX) neurons. Consistent with this, we have distinguished two projection-specific and molecularly distinct subsets of LHA Nts neurons. One subset co-expresses Nts and the long form of the leptin receptor (LepRb), is activated by leptin and projects to the ventral tegmental area (VTA) and substantia nigra compacta (SNc); we refer to these as Nts^{LepRb} neurons. A separate subset of LHA Nts neurons lacks LepRb, is activated by dehydration and does not project to the VTA or SNc; we refer to these as Nts^{Dehy} neurons. Intriguingly, however, we found all LHA Nts neurons are similar in that they express the inhibitory neurotransmitter, GABA. We next investigated the role of the Nts^{LepRb} subpopulation for adaptive response by studying mice lacking leptin signaling via Nts^{LepRb} neurons. Loss of leptin regulation only via Nts^{LepRb} neurons induced obesity, blunted adaptive response to leptin and to ghrelin (a hormonal activator of OX neurons) and dysregulated DA signaling. Finally, we

defined the necessity of LHA Nts neurons for energy balance by genetically ablating or chemogenetically inhibiting them in adult mice. Prolonged loss of LHA Nts neurons decreased drinking, locomotor activity and deranged OX expression in target neurons that led to increased adiposity. By contrast, LHA Nts inhibition preserved OX expression but still blunted locomotor activity. Together these data suggest that LHA Nts neurons modulate physical activity that is not dependent on OX, but that the LHA Nts→OX circuit is necessary for regulation of drinking and adiposity. Collectively, our data show that LHA Nts neurons are necessary for regulation of adaptive energy balance, and that distinct subpopulations of LHA Nts neurons may control ingestive and locomotor behavior via OX-dependent and independent pathways. This work suggests that there may be unique LHA Nts circuits to regulate drinking, motivated feeding ingestive disorders such as obesity, anorexia nervosa, psychogenic polydipsia and dehydration.

To my family, lab mates and mentors

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KEY TO ABBREVIATIONS

3V	third ventricle
ANOVA	analysis of variance
ARC	arcuate nucleus
CNO	clozapine-N-oxide
CPu	caudate/putamen
DA	dopamine
DAT	dopamine transporter
Dlk1	delta-like 1
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
EGFP	enhanced green fluorescent protein
f	fornix
FG	fluorogold
FR	fixed ratio
GABA	gamma-aminobutyric acid
Gal	galanin
GFP	green fluorescent protein
GHSR	growth hormone secretagogue receptor
i.p.	intraperitoneal

ICV	intracerebroventricular
ISH	in situ hybridization
LepRb	leptin receptor
LHA	lateral hypothalamic area
LPO	lateral preoptic area
LRKO	LepRb knocked out
MCH	melanin-concentrating hormone
mt	mammillothalamic tract
NAc	nucleus accumbens core
NAsh	nucleus accumbens shell
Nts	neurotensin
NtsR1	neurotensin receptor 1
OX	orexin/hypocretin
PCR	polymerase chain reaction
POA	preoptic area
PR	progressive ratio
PVN	paraventricular hypothalamic nucleus
RER	resting energy expenditure
RT	reverse transcription
SEM	standard error of the mean

SN	substantia nigra
STN	subthalamic nucleus
TH	tyrosine hydroxylase
VMH	ventromedial hypothalamus
VTA	ventral tegmental area
ZI	zona incerta

CHAPTER 1 An Introduction: Body Weight Regulation – Neuronal Populations in the Lateral Hypothalamus

1.1 Introduction

An organism's survival depends on its ability to sense nutrient status and accordingly regulate intake and energy expenditure behaviors. Uncoupling of energy sensing and behavior, however, underlies energy balance disorders such as anorexia or obesity. The hypothalamus regulates energy balance, and the lateral hypothalamic area (LHA) is poised to coordinate peripheral cues of energy status and behaviors that impact weight. The LHA modulates food intake, thus was dubbed the 'feeding center' of the brain, but is also an essential regulator of drinking, locomotor behavior, arousal/sleep and autonomic output. There are several populations of LHA neurons that are defined by their neuropeptide content and contribute to energy balance. LHA neurons that express the neuropeptides melanin-concentrating hormone (MCH) or orexins/hypocretins (OX) are best defined and these neurons play important roles in regulating ingestion, arousal, locomotor behavior and autonomic function via distinct neuronal circuits. Recently, another major population of LHA neurons containing the neuropeptide Neurotensin (Nts) has been implicated in coordinating anorectic stimuli and behavior to regulate hydration and energy balance. Intriguingly, there are subsets of Nts neurons that are 'tuned' to specific cues of energy status; this heterogeneity potentially allows for more discrete control and rectification of energy imbalance. Understanding the neuronal circuits via which the LHA coordinates energy sensing and behavior and the role that Nts neurons and the underlying sub-populations has the potential to inform pharmacological strategies to modify behaviors and treat energy balance disorders.

1.1.1 The Periphery and the Brain Act in Concert to Regulate Energy Balance

Food and water are essential for survival, and organisms have developed physiological systems to ensure that the body maintains sufficient stores of these resources [1]. Such systems must synthesize two crucial processes: energy sensing (to determine the resource needs of the body) and appropriate output behaviors that are organized by the brain (to resolve bodily need). For example, resource deficits such as fasting, or dehydration increase the motivation to find and ingest food and water, respectively. Resource excess is also coordinated with an appropriate behavioral response: stomach fullness or increased energy reserves (e.g. body fat) cue the cessation of feeding while also promoting physical activity and fat burning to resolve energy excess [2;3]. At their essence, such physiologic 'drive' systems thus match bodily need and behavior to ensure survival. These systems must also be dynamic, since bodily resource needs fluctuate considerably each day (from periods of repletion to deficit and back again), and must continually survey the energy and hydration status of the body to detect and resolve any imbalance. Further, physiologic systems that modulate drive to drink, eat and move inherently regulate energy balance: the caloric intake and amount of energy expended that together determine the weight of the organism. Extreme deficits in energy intake impair survival, while excesses in energy can promote metabolic disease and morbidity. Thus, survival and energy balance are irrevocably linked, and rely on constant, dynamic communication between the periphery and the brain.

How then does the body convey messages that can be 'read' by the brain, and how does the brain interpret these into behaviors to correct energy imbalance? A major step forward in understanding this process was the discovery of circulating hormones that communicate energy status, and how loss of communication between the periphery and brain promotes disease. One such body-to-brain regulator is the hormone leptin, which is produced

in adipose tissue and acts to suppress feeding and promote energy expenditure via neurons in the brain that express the long form of the leptin receptor, LepRb [4-7]. Loss of either peripheral leptin production or central LepRb expression promotes overeating, decreases energy output and leads to severe obesity in rodents [4-6] and humans [8;9], revealing the crucial role of this periphery/brain regulatory system. The hormone ghrelin also mediates powerful control of energy balance via regulation in the brain. Ghrelin is produced by the stomach during periods of energy deficit and acts via brain neurons expressing the growth hormone secretagogue receptor (GHSR) to stimulate feeding [10;11]. Increased ghrelin action via GHSR promotes over-feeding and potentiates weight gain [12]. These examples demonstrate that peripheral cues access and regulate the brain to either promote or inhibit feeding behaviors, and thus regulate energy balance.

Normal energy balance relies on the appropriate synergism of peripheral cues and behavior, but uncoupling these deranges energy balance. Indeed, individuals with anorexia nervosa self-restrict their feeding despite intact cues signaling energy need [13]. Similarly, tastiness can trump satiation: few among us are invulnerable to the attractive sight and smell of a dessert, despite having just consumed an ample meal and being energy replete. As such, normal weight and obese individuals may over consume palatable, calorie-dense foods despite the presence of energy excess signals that should inhibit intake [14]. Thus, eating disorders or obesity occur when the need to eat no longer matches the desire to eat [15], incurring serious health tolls including increased morbidity. Yet despite the increasing severity of anorexia in youth [16] and the obesity pandemic [17;18], there remain limited pharmacologic treatments to modify energy imbalances [19;20]. Modifying diet and exercise remains the gold standard treatment for disordered energy balance, but these lifestyle changes are difficult to maintain long term, yield modest improvements in body weight and prove largely ineffective at improving functional

outcomes and life expectancy [19;21-23]. Surgical interventions such as gastric banding or gastric bypass are effective in promoting weight loss in obese individuals, but these procedures are highly invasive, and many individuals regain weight in subsequent years [24;25]. It is therefore imperative to identify strategies to restore normal energy balance function to treat the millions of individuals suffering from these diseases. Identifying the brain mechanisms that coordinate energy cues and appropriate behavioral response will suggest tractable pharmacological pathways to treat feeding and energy balance disorders.

While many areas of the brain contribute importantly to the regulation of feeding and metabolism, this review will focus on the role of the lateral hypothalamic area (LHA) in controlling energy balance for three reasons. 1) The LHA modifies intake of natural and pharmacologic rewards and physical activity, and such function via the LHA is required for survival. 2) The LHA is anatomically positioned to receive peripheral cues and regulate motivated behaviors. 3) Distinct neuronal populations within the LHA are 'tuned' to specific energy cues and induce cue-appropriate behavioral responses. Thus, understanding the precise neurochemistry, connectivity and function of the LHA neuronal subpopulations will suggest mechanisms by which to suppress or enhance feeding, drinking and energy expenditure as required to restore energy balance. Modifying action via the LHA therefore has potential to improve a spectrum of health problems.

1.1.2 The Lateral Hypothalamic Area (LHA) is a Crucial Regulator of Energy Balance

The hypothalamus as a whole has long been recognized to modulate body weight, water balance, body temperature and the sympathetic nervous system [26]. Hetherington and Ranson were the first to imply that each sub-region of the hypothalamus controls specific facets of

energy balance, demonstrating that selective lesion of the ventromedial nucleus of the hypothalamus (VMH) caused profound overeating and obesity. The VMH was hence deemed an essential 'satiety center' of the brain [27;28] and inspired many labs to study 'hypothalamic obesity' caused by VMH lesions It was in this context that Bal K. Anand, (while working at Yale with Brobeck) was using stereotaxic techniques to lesion the VMH of rats and, by his account, "…was much disconcerted to find that my rats immediately after such lesions completely stopped eating and would die of starvation". This phenotype was completely opposite of the hyperphagia and obesity expected due to lesion of the VMH [29]. As it turned out, Anand and Brobeck had made a (fortuitous) targeting error, missing the VMH, but instead ablating the LHA in their experimental rats. The resulting LHA-lesioned rats had the ability to move, eat and drink, but lost all inclination to do so: as a result, they all died of self-inflicted starvation and dehydration [30-32]. By contrast, electrical stimulation of the LHA promotes feeding and drinking behaviors, as well as increasing physical activity [33-35]. Collectively, these seminal loss and gain of function experiments suggested that the LHA is a 'feeding center' that acts in opposition to the VMH satiety center and led to the 'dual center hypothesis' of feeding regulation [36;37]. Today the LHA is still regularly described as the feeding center of the brain, which often overshadows its other crucial roles for regulating drinking, physical activity, alertness/arousal and coordination of sensory stimuli with appropriate output behaviors [38]

The fact that LHA-lesioned animals imminently died of starvation and dehydration complicated their use to determine how the LHA promoted feeding. Teitelbaum and Stellar found that rats with LHA lesions could only be kept alive via force-feeding them liquid nutrients three times per day [39]. This regimen was a serious toll for Teitelbaum (the last daily treatment was at 2:00 AM!) and he grew desperate for a way to induce the animals to feed themselves. He recalled another time he'd had to stay up till the wee hours dealing with rats, while

performing husbandry of a rat colony during his assistantship: “I used to stop, munch chocolate bars, and offer the rats some. I soon discovered that shortly before my break, many rats were lined up at the front of each cage, all waiting for their treat. Later, I remembered this when trying to tempt aphagics to eat. Nevertheless, it was a thrill to see a rat, being kept alive by tube-feeding, refusing food and water for two months postoperatively, suddenly gobble up bits of chocolate.” [40] Thus, Teitelbaum found that LHA-lesioned rats eschewed normal foods, but could be coaxed to eat sufficient calories in the form of palatable substances (i.e. evaporated milk, cookies, milk chocolate but not bittersweet chocolate) to permit their survival [41]. Eventually, the lesioned rats overcome their aphagia, resume normal feeding and regain weight. Importantly, this discovery suggested that loss of LHA function didn’t negate the ability to feed, but blunted the motivation to feed, even when food is desperately needed for survival. Further, it identified the LHA as an important center for feeding drive, though it is not the sole mediator of motivated ingestion; other neuronal systems exert some (presumably lesser) drive that can, in time, be sufficient to mediate survival [41]. Intriguingly, drinking drive remains particularly impaired in LHA-lesioned animals even after their ‘recovery’. These seemingly normal rats do not coordinate physiologic perturbations (e.g. high salt-intake/dehydration, hunger, altered food valuation) with appropriately paired drinking or feeding behavior [38;42;43]. Close observation revealed that drinking is strictly time-locked with feeding bouts in these rats, “…as if the animal were drinking not to quench its thirst but simply to wet its mouth…perhaps just as a means to wet food and make it swallow-able” [43]. Thus, while other neuronal systems can mediate aspects of motivational drive, the LHA is crucial for pairing physiologic cues and drive response.

Anatomists challenged the notion that the ‘cell-poor’ LHA could itself regulate motivation, arguing that it was actually due to lesion or stimulation of the diffuse fiber systems passing through the LHA. Coursing through the LHA are nigro-striatal dopamine fibers as well as axons

of passage within the medial forebrain bundle (mfb), each of which terminate in brain centers associated with reward and motivation [44]. Indeed, these tracts regulate motivation, and disruption of the mfb or dopamine-containing neurons blunts feeding, drinking and movement behavior, similar to LHA lesions [42;44-47]. Two crucial findings, however, solidified a specific role for LHA neurons in regulating motivation relevant to energy balance. First, stimulation of the LHA still induces motivated feeding even in rats with a severed mfb [47]. Secondly, neurotoxins that selectively ablate LHA cell bodies, but spare axons passing through the LHA, results in aphagia and adipsia similar to the original lesions that disrupted both cells and fibers [48;49]. Thus, these data confirm that neurons of the LHA directly modulate motivated behaviors. It was subsequently determined that LHA neurons are anatomically linked with neural systems that regulate reward and goal-directed behaviors, including direct projections onto midbrain dopamine neurons that release dopamine into the forebrain [50]. Collectively these classical lesion, stimulation and anatomical studies established the LHA as a powerful coordinator of the drive to eat, drink and move. Such methodologies could not, however, fully elucidate the mechanisms by which the LHA mediated these effects: how does the LHA receive status cues (e.g. resource/energy need) from the body and how does it translate these into appropriate output behaviors?

1.1.3 Connectivity and Neuronal Diversity in the LHA: Implications for Energy Balance

The strikingly different phenotypes produced by lesion of the LHA (aphagia, adipsia, weight loss) or the medial hypothalamic regions (hyperphagia, obesity) suggests that these regions differ in neurochemistry and/or their anatomical engagement of brain systems that regulate behavior. Medial hypothalamic nuclei, such as the VMH, arcuate nucleus (ARC), dorsomedial hypothalamus (DMH) and paraventricular hypothalamic nucleus, (PVN) are compact, cell-dense and have well-defined projection targets throughout the brain. The LHA, by

contrast, encompasses a large swath of tissue over the entire rostral-caudal extent of the hypothalamus. The sheer expanse of the LHA, coupled with the fact that it lacks obvious cellular architecture, complicated anatomical and functional studies. The LHA can, however, be roughly subdivided into regions based on proximity to fiber tracts. For example, the mamillothalamic tract lies at the medial limit of the LHA, where it borders with the DMH and later the PVN. The fornix is a large and easily identifiable tract that runs through the ventral aspect of the LHA, and the area just above and surrounding the fornix is often referred to as the perifornical area of the LHA. Pioneering work by the Saper group utilized these anatomical landmarks in combination with neuronal tract tracing methods to determine the precise connectivity of LHA subregions with the rest of the brain [51;52]. While these studies characterized some subregion-specific projection targets, as a whole, they demonstrate that the LHA projects broadly throughout the forebrain, midbrain and hindbrain regions, each of which is implicated in distinct facets of physiologic control. The lack of a unified output region, however, suggests that LHA-mediated regulation of behavior and energy balance is complicated and not homogenous in nature.

The next leap in understanding the LHA's role in energy balance was the discovery of its hetero-cellularity, and the resulting concept that specific populations of LHA neurons coordinate discrete energy cues and behavioral response. Indeed, it is clear that there are many distinct populations of neurons within the LHA that differ in molecular signature. The full extent of LHA neurons are yet to be characterized, but three substantial populations of neurons have been described and can be defined by their expression of a specific neuropeptide: neurons containing melanin concentrating hormone (MCH), the orexins/hypocretins (OX) and neurons containing neurotensin (Nts). Intriguingly, these subpopulations are molecularly and spatially distinct (Figure 1-1) suggesting that each population may also differ in connectivity and functional

output. The emergence of molecular techniques that enable site-specific manipulation of genetically-distinct neuronal populations has allowed the field to probe the roles of MCH, OX and Nts neurons, and suggests that each of these populations have roles in regulating energy balance. While there are also smaller populations of neurons within the LHA expressing other neuropeptides and neurotransmitters, we will focus on the emerging and distinct roles of MCH, OX and Nts neurons on coordinating peripheral energy cues and behaviors, and their respective contributions to energy balance.

1.2 Melanin Concentrating Hormone (MCH) Neurons

1.2.1 General Overview of Melanin Concentrating Hormone (MCH) Neurons

Melanin concentrating hormone (MCH) is a 19-amino acid cyclic neuropeptide that was first documented in the pituitary of teleost fish, enabling them to change skin color and blend into their environment [53;54]. Soon after MCH was identified in the brains of rats [55] and humans [56], where it is primarily found within neuronal cell bodies of the LHA as well as small number of neurons in the zona incerta [57]. Most often these are solely referred to as MCH neurons, but they also contain the classical (fast) neurotransmitters GABA or glutamate via which they can inhibit synaptic contacts [58]. Additional sub-populations of MCH neurons can be differentiated by their co-expression of nesfatin [59] or the neuropeptide cocaine-amphetamine-regulated transcript (CART) [58;60-63]. CART co-expression signifies a distinct MCH population that projects to forebrain sites involved in behavior modulation, while non-CART expressing MCH neurons preferentially project to caudal brainstem and spinal cord [61;62]

MCH acts via neurons expressing the Gi/o-protein coupled receptor MCH Receptor-1 (MCHR-1), thus MCH action is inhibitory [64-68]. Higher order mammals and humans (but not rodents) also express a Gq-coupled MCH Receptor-2 that activates target neurons and may exert opposite actions to MCHR-1 [69-71]. MCHR-1 is highly expressed within neurons of the cerebral cortex, olfactory tubercle, limbic structures (hippocampus, septum, nucleus of the diagonal band, bed nucleus of the stria terminalis, amygdala) forebrain (caudate-putamen, nucleus accumbens core and shell) and the arcuate nucleus [72]. MCH neurons also project to areas implicated in regulating feeding, such as the parabrachial nucleus [73] and PVN [74], but project sparsely to regions that regulate arousal, such as the dorsal raphe, ventrolateral periaqueductal gray, locus coeruleus and preoptic area [64;68;72;75;76]. Though the LHA as a whole densely projects into the dopamine (DA)-enriched ventral tegmental area (VTA) and regulates DA-mediated ingestive and locomotor behaviors [77], MCH neurons do not regulate the VTA [72]. Rather, MCH neurons engage the DA system via projections to the nucleus accumbens (NA), where MCHR-1 is expressed on dopamine receptor-1 (D1R) and dopamine receptor-2 (D2R)-expressing neurons [78]. MCH neurons also project to hindbrain regions including the nucleus of the solitary tract, dorsal motor nucleus of the vagus and ventral medulla sympathetic premotor areas [57;79].

1.2.2 MCH plays a role in feeding and drinking regulation.

Central injection of MCH into the brain increases feeding in rodents and promotes obesity [80-82]. MCH treatment, however, does not preferentially stimulate intake of palatable food or sucrose, suggesting a role for MCH in regulating general ingestive behavior, but not necessarily in hedonic aspects of feeding [83;84]. Consistent with this, MCH expression is increased in hungry animals, including fasted or hyperphagic leptin-deficient mice (ob/ob), compared to normal controls [81]. Similarly, mice that genetically overexpress MCH are

hyperphagic and gain weight [85]. In contrast, genetically engineered mice that lack MCH eat less, are lean and exhibit improved metabolic profiles throughout aging [86-88]. Mice lacking MCHR-1 are also lean with less body fat than controls, but this is primarily due to their increased locomotor activity and energy expenditure. While one might expect decreased feeding in MCHR-1 deficient mice (due to loss of orexigenic MCH action), they actually display mild overeating. In this case the modest hyperphagia may be required to support their increased energy expenditure, but at any rate, is not sufficient to produce obesity [89;90]. Blocking acute action of MCHR-1 via selective antagonists, however, does suppress feeding, meal size and weight gain in normal weight and obese rodents [76;91;92]. These findings have accordingly spurred interest in development of brain-permeable MCHR-1 antagonists to reduce food intake and promote weight loss.

MCH action in the NA shell is particularly important for regulating motivation and reinforcement for drugs of abuse and natural rewards, including food. Selective administration of MCH in the NA increases feeding, and conversely, delivery of an MCHR-1 antagonist in this region inhibits food intake [93]. Genetic deletion or pharmacologic antagonism of MCHR-1 also blunts cue-induced responding for food, suggesting a deficit in learning processes that drive motivated feeding [94]. MCH neurons sense nutrient status and accordingly promote the motivation to feed in order to maintain euglycemia [95]. Indeed, activation of MCH neurons promotes intake of sweetened liquids along with DA output into NA [96]. Thus, MCH neuronal signaling via MCHR-1 in the NA is sufficient to coordinate energy need and feeding, and may be a tractable pathway to modulate feeding in energy balance disorders.

Central administration of MCH increases water intake in the presence or absence of food [84;97]. Woods and colleagues demonstrated, however, that MCH does not specifically promote water intake, and also increases ingestion of ethanol, sucrose solution and food [98]. Therefore, MCH is likely a general inducer of intake behavior (eating and drinking). Feeding and drinking are time-coupled behaviors, and so-called 'prandial drinking' occurs just prior to, during and following bouts of feeding [99]. MCH action may influence the desire to drink in order to wet the mouth or via a DA-mediated reward mechanism, but does not seem to have a role in thirst per se [100].

Based upon the hypophagia of mice lacking MCH, it was hypothesized that MCH deletion could curb feeding to promote weight loss in obesity. The Maratos-Flier group tested this by genetically deleting MCH in mice that are deficient for leptin, and hence are hyperphagic and obese. The resulting double MCH/leptin knock-out mice were leaner than leptin-depleted controls, but did not exhibit any blunting of feeding. Instead, the reduced adiposity of MCH/leptin knock-out mice was due to their increased energy expenditure [101]. Indeed, MCH neurons act via polysynaptic connections to the hindbrain and spinal cord to regulate brown adipose tissue, the vital tissue for promoting thermogenesis and basal metabolic rate [102]. MCH neurons presumably inhibit thermogenic energy expenditure via this pathway [57;79]. By contrast, blockade of MCH signaling increases brown adipose tissue mass and thermogenesis and reduces body weight [103]. Genetic deletion of MCHR-1 in mice also promotes hyperactivity via changes in the NA [104], suggesting that the combined increase of thermogenesis and physical activity supports weight loss and leanness. Interestingly, ablation of MCH neurons in adult obese mice does not decrease their feeding or body weight [105], suggesting that developmental disruption of MCH neurons is essential for modifying energy balance.

1.3 Orexin/Hypocretin (OX) Neurons

1.3.1 General Overview of Orexin (OX) Neurons

In 1998 separate research groups reported the discovery of two neuropeptides produced from the same gene product: one group dubbed them the hypocretins [106] and the other referred to them as orexins [107]. We will utilize the orexin (OX) designation due to its simple abbreviation. OX action is transduced via two Gq protein coupled receptors, orexin receptor-1 (OXR-1) and Orexin receptor-2 (OXR-2) [107-109]. OXR-1 binds OX-A and -B with equal affinities, but OXR-2 preferentially binds OX-B. Strikingly, OX neurons project broadly throughout the brain [110] and virtually every brain region contains at least one of the two OX receptors, suggesting that central OX action controls a wide array of functions [111]. Although OX neurons are located in a similar distribution to MCH neurons, they appear to be regulated by completely different stimuli and inputs, so studies of the LHA often contrast the roles of MCH and OX neurons. Compared to MCH neurons OX neurons are spontaneously active. The OX and MCH neuronal systems may not be entirely divergent, however, since at least some OX neurons project to and regulate MCH neurons [112].

1.3.2 OX regulation of feeding and drinking.

Central OX administration acutely promotes feeding [107;113;114] though more modestly compared to other orexigenic neuropeptides [115]. OX neurons also regulate the mesolimbic reward system and intake of natural and drug rewards via direct projections onto VTA dopamine neurons [116;117]. OX neurons are activated during cue-induced feeding [118;119], and in turn they activate VTA DA neurons and promote DA release into the NA and prefrontal cortex [120-122]. OX regulation via this mesolimbic circuit promotes ingestion of highly salient substances (e.g. high fat diet, drugs of abuse) but not of comparatively bland chow or aversive substances [122-127]. OX

specifically promotes motivated response (work) for palatable foods that is attenuated by OXR antagonists [119;128;129]. OX enhances the activation of DA neurons via a similar mechanism to cocaine, suggesting that OX is required for structural changes in the DA system that increase motivated drive, reward craving and intake [130]. Both OX and glutamate release are required for cue-induced reinstatement (seeking) of rewards via the VTA. Given that OX and glutamate are released from same neuron, it is proposed that OX acutely modulates reward intake while glutamate mediates long-term modifications known to underlie addiction to drugs and natural rewards [125].

In normal weight animals the synaptic inputs onto OX neurons are predominantly excitatory, but fasting (e.g. low leptin tone) increases the excitatory inputs onto OX neurons. Leptin treatment attenuates the fasting-induced increase in excitatory tone, presumably restoring (diminishing) activation of OX neurons to normal levels [131]. While it is tempting to infer that leptin solely acts to oppose OX neurons, more recent data suggest that leptin and OX act cooperatively to coordinate energy sensing and behavior in the long term. For example, in the obese state the bias of excitatory inputs onto OX neurons shifts, such that synaptic tone is primarily inhibitory. Leptin treatment reverses the inhibitory synaptic bias to OX neurons, in essence, promoting restored activation of OX neurons [132]. At some level leptin and OX signaling may be synergistic, since disruption of one system also deranges the other. Indeed, loss of leptin or LepRb promotes obesity and decreases OX expression compared to normal weight animals [133;134]. Similarly, chronic OX overexpression or treatment with OXR-2 agonists improves leptin sensitivity, and suppresses palatable food intake and weight gain [135]. Parsing the acute and developmental interactions of leptin and OX signaling will be important to fully understand dynamic regulation of feeding via these systems.

Central OX treatment in rats increases drinking, while water deprivation increases OX expression [136]. Similarly, pharmacogenetic activation of OX neurons increases water intake [137] but genetic ablation of OX neurons decreases drinking (as well as feeding, locomotor, wakefulness) suggesting drive reduction [138-140]. Mice lacking OX also drink less sucrose, although their preference for it is unaffected [141]. Consistent with this, pharmacological antagonism of OXRs reduces all liquid intake (water, ethanol and sucrose), suggesting that the OX system promotes general drinking behavior, regardless of the liquid's caloric or rewarding value [142]. Activation of OX neurons is linked with bodily fluid status, such that OX neurons are inactive during dehydration, but are activated just after drinking/re-hydration occurs [100]. OX neurons may, therefore, enhance the motivation to drink when fluid is available to resolve the water imbalance.

Mechanistically, OX neurons may modulate drinking behavior via projections to, and excitatory regulation of the subfornical organ [136;143] as well as projections to the medulla [114]. OX neurons may also modify drinking behavior via projections into the mesolimbic and striatal systems, which are implicated in motivational drinking and drinking secondary to psychiatric dysfunction (psychogenic polydipsia).

Central OX increases locomotor activity, mainly in the form of grooming and food seeking behaviors [113;144;145]. OX neurons are maximally activated during exploration, grooming and feeding, [146], and pharmacogenetic-mediated activation of OX neurons increases locomotor activity [137], linking OX action with locomotor drive. Inflammatory challenge, however, reduces OX activation [147;148] and produces the lethargy characteristic of acute and chronic illness [149;150]. OX regulates somatic movement [151] but primarily controls motivated locomotor activity via

activation of VTA DA neurons and DA release into the NA [152]. Inhibitors of DA signaling thus blunt OX-mediated locomotor activity [153]. Rodents lacking OX are hypoactive, exhibit less motivated wheel running and the decreased volitional energy expenditure promotes weight gain [140;154].

1.4 Neurotensin (Nts) Neurons

1.4.1 General Overview of Neurotensin (Nts) Neurons

In 1973 Carraway and Leeman isolated a 13-amino acid peptide from bovine hypothalamus. Upon finding that intravenous Nts injection dilated blood vessels, lowered blood pressure and caused cyanosis in rats they coined this peptide neurotensin (Nts) to reflect its pressor function [155]. Most Nts is expressed within the intestine or adrenal gland, and accounts for the bulk of the Nts released into the plasma. While Nts is rapidly degraded in the circulation, a limited amount of circulating Nts may access the brain [156;157 158]. However, approximately 10% of bodily Nts expression is produced within the brain, is enriched in synaptosomes [159;160] and is released after neuronal depolarization via a calcium dependent mechanism, signifying that in the brain Nts is a peptide neurotransmitter [161]. Nts in the brain is also rapidly degraded by membrane-bound angiotensin converting enzyme, proline endopeptidase and prohormone convertases-1 and -2, suggesting that it mediates short-acting signal transduction that is quickly inactivated [162-165]. Using *in situ* hybridization or radioimmunolabeling Nts was identified throughout the nervous system, including within the spinal cord, hindbrain (nucleus of solitary tract, LC, parabrachial nucleus), midbrain (periaqueductal gray, VTA, SN) limbic system (amygdala, hippocampus), forebrain (caudate putamen, NA), thalamus, and within the hypothalamus, particularly the preoptic area, PVN and the LHA [166-170]. Immunohistochemical detection of Nts, however, requires treating rodents with colchicine, an anterograde transport

inhibitor that leads to accumulation of proteins within the cell body, but which is inherently toxic and prevents study of these neurons in normal physiologic context [166;167;171;172]. As such, the technical limitations of identifying Nts neurons have restricted study of their roles in physiology and behavior.

Nts binds to neurotensin receptor-1 (NtsR1) and NtsR2, both of which are Gq-coupled protein receptors [173;174]. A third receptor, neurotensin receptor-3 (NtsR3, also called sortillin) is a single transmembrane receptor with unclear function, but NtsR-3 does not specifically transduce Nts signals [175]. NtsR1 has high affinity for Nts and is predominantly expressed on neurons [176], including on dopamine-expressing neurons in the midbrain [177]. NtsR2 exhibits low affinity Nts binding, is antagonized by the antihistamine levocabastine and is expressed on a few neurons, but primarily within astrocytes [178-181]; [177]. Nts specifically promotes activation of NtsR-expressing neurons in the prefrontal cortex, VLPAG, SN and in VTA DA neurons [182-184]. Nts action via NtsRs induces a non-selective cation current to promote neuronal depolarization [185]. Additionally, Nts interacts with D2Rs on DA neurons to block their inhibitory effects, thus Nts acts via dual mechanisms to promote the activation of DA neurons [186-189]. Indeed, within the VTA Nts-containing axon terminals are primarily apposed with DA neurons [190] [191] and Nts acts via the VTA to promote DA release into the NA and modify reward behavior [192;193] [194] . Both NtsR1 and NtsR2 are implicated in regulating DA neurons [195-197], but treatment with NtsR1-sepecific antagonists blocks Nts-mediated DA release from midbrain neurons, suggesting that NtsR1 is the predominant modulator of VTA DA neurons [198;199] [177].

The development of mice that express cre recombinase in Nts neurons (*Nts*^{Cre} mice) permitted the facile identification of Nts neurons throughout the brain, including a large

population of Nts neurons within the LHA [200]. These *Nts*^{Cre} mice, when bred onto a cre-mediated green fluorescent reporter line, identify LHA Nts neurons that are distinct from adjacent neurons expressing MCH or OX (Figure 2 A), similar to previous reports that identified Nts, OX and MCH via in situ hybridization or colchicine-mediated immunostaining [100;200;201]. Nts neurons in the LHA are also more abundant, by far, than MCH or OX neurons (Figure 2 B). Given the important roles for MCH and OX neurons, the comparative multitude of Nts neurons suggests that they have a sizeable physiologic impact. LHA Nts neurons, however, are not a homogenous population; there are subpopulations of Nts-containing neurons within the LHA with distinct molecular signatures, though these have yet to be fully characterized. Some LHA Nts neurons co-express the long form of the leptin receptor (LepRb) and are activated by leptin [200] and some of these neurons also co-express the inhibitory neuropeptide galanin and/or melanocortin-4 receptor [201]. Other subpopulations of Nts neurons contain CRH [100] or MCHR-1 [72]. Additionally, LHA Nts neurons have been reported to co-express either the classical neurotransmitter GABA or glutamate [200;202]. As a whole, LHA Nts neurons project densely within the LHA to OX neurons and also to the VTA, via which they likely regulate DA neurons [191]. Indeed, activation of LHA Nts neurons causes release of Nts to the VTA that potentiates the activation of VTA DA neurons and DA release to the nucleus accumbens via an NtsR1-dependent mechanism [194]. Mice self-stimulate LHA Nts neurons, including those that project to the VTA, presumably because it is rewarding [202]. Nts action also regulates the activity of OX neurons via mechanisms that remain to be determined [200;203]. Thus, LHA Nts neurons exert control of OX neurons and VTA DA neurons that could (as established above) modulate feeding, drinking and locomotor activity. While the precise roles of LHA Nts neurons in these physiologic behaviors have yet to be fully elucidated, there is a large literature to suggest that central Nts can indeed modify behaviors relevant to energy balance.

1.4.2 Role of Neurotensin (Nts) Neurons in Regulating Feeding

Central Nts has been considered an anorectic neuropeptide, but appears to have site-specific effects on feeding. Pharmacologic Nts in the SN and VTA modestly decreases food intake in satiated and food-deprived rodents [204-208]. Administration of Nts into the LHA or ventral striatum, however, does not alter feeding, suggesting that Nts mediates other aspects of behavior via these regions [205;209]. NtsR1 is the essential receptor isoform for Nts-mediated suppression of feeding [210;211]. Brain permeable NtsR1-specific agonists accordingly decrease feeding and body weight in normal mice, as well as in leptin-deficient obese mice, suggesting that Nts action via NtsR-1 may be useful in treating obesity [211]. Loss of Nts expression might therefore be expected to promote feeding and exacerbate weight gain, and indeed hyperphagic, obese rodents have reduced Nts expression in the brain, including the hypothalamus that may have contributed to the disease state [212-217].

Nts neurons are regulated by the anorectic hormone leptin, suggesting coordinated roles of Nts and leptin to modify feeding and body weight. Chronic leptin treatment decreases food intake and body weight as expected, and also decreases Nts expression within the LHA [218;219]. By contrast, acute leptin treatment of hypothalamic-derived cell lines increases Nts expression [220]. Nts potentiates leptin-mediated inhibition of feeding via NtsR-1 [221;222] but mice deficient in NtsR1 have an impaired anorectic response to leptin, confirming that leptin and Nts/NtsR1 synergistically modify feeding [223]. Intriguingly, the leptin/NtsR1 system may have more impact in regulating non-homeostatic feeding: while mice lacking NtsR1 exhibit normal chow intake, they over consume palatable, high-fat diet or a sucrose solution that promotes obesity [191]. The LHA is the site of leptin and Nts synergy: a subset of Nts neurons co-express LepRb, are exclusively found within the LHA, and represent the only Nts neurons in the brain that are directly activated by leptin. Deletion of LepRb specifically in LHA Nts-LepRb neurons promotes

mild hyperphagia and obesity in mice [200]. Furthermore, intact NtsR1 expression is required for LHA Nts^{LepRb} neurons to restrain feeding, indicating the functional integration of leptin and Nts/NtsR-1 action [191]. In this regard, stimulating NtsR1 neurons (similar to leptin-mediated Nts release from LHA Nts-LepRb neurons) may be useful to suppress feeding and body weight. Indeed, brain permeable NtsR1-specific agonists decrease feeding and body weight in normal mice, as well as in leptin-deficient obese mice, suggesting that Nts action via NtsR1 may be useful in treating obesity [211]. LHA Nts neurons, including Nts^{LepRb} neurons, likely exert some regulation of feeding via their projections to the VTA. Nts activates VTA neurons, promotes reinforcement [224] and rats will self-administer Nts as if it is rewarding [225]. Similarly, activation of LHA Nts neurons promotes reward responding [202] but, intriguingly, suppresses food intake [177]. Given that Nts-mediated anorexia is enhanced by co-administration with DA agonists [209], activation of LHA Nts neurons may stimulate VTA DA neurons to suppress feeding. LHA Nts neurons may also project to the parabrachial nucleus [226], and it is possible that Nts contributes to anorectic drive via this brain region [227]. Additionally, some LHA Nts neurons co-express MC4R and LepRb (but not OX or MCH) and may be regulated via melanocortins to modulate feeding. Together these data suggest that LHA Nts neurons can suppress feeding, but it remains unclear if they are necessary for feeding regulation and the precise mechanisms by which they control it.

1.4.3 Role of Neurotensin (Nts) Neurons in Regulating Drinking

Water deprivation or osmotic stimulation specifically increases Nts expression in the LHA [228;229] and experimental activation of LHA Nts neurons promotes voracious drinking [177]. These data suggest that at least some LHA Nts neurons may detect water deficit and coordinate drinking behavior to restore fluid homeostasis. Rats have a modest population of LHA Nts neurons that co-expresses CRH and are responsive to water deficit [230], but mice have very few

Nts-CRH co-expressing neurons, suggesting they detect water imbalance via distinct mechanisms. While it remains unclear how water deprivation affects the activity of LHA Nts neurons, it is clear that the OX neurons that they project to are inhibited by water deprivation. After drinking, however, activation of OX neurons is restored [100]. It is tempting to speculate that Nts neurons detect water deprivation and suppress OX neurons to promote the drive for water, while drinking behavior releases Nts action and permits OX activation. Consistent with this idea, central Nts treatment increases water intake in rats [204;231-233]. Nts may also have a general role in inhibiting intake of 'rewarding' liquids such as ethanol [234;235], sucrose [191] or thirst-induced water intake, which may be itself be pleasurable after dehydration. In sum, these data hint at a role for LHA Nts neurons in drinking behavior, but the underlying mechanisms and necessity for LHA neurons in regulating fluid balance remain unclear.

1.4.4 Role of Neurotensin (Nts) Neurons in Regulating Physical Activity

Similar to the site-specific effect of Nts in mediating feeding, Nts also exerts brain site-specific control of locomotor activity. Nts in the NA (which is not directly regulated by LHA Nts neurons) suppresses locomotor activity [171;236-239]. By contrast, Nts treatment in the VTA promotes locomotor activity along with DA output into NA and olfactory tubercle [240-243]. Chronic Nts administration into the VTA causes long-lasting sensitization and progressively increased locomotor activity even after treatment is suspended, suggesting that sustained, endogenous release of Nts remodels VTA circuits to modulate locomotor output [244;245]. Given that LHA Nts neurons project to and can activate NtsR-expressing DA neurons [191;200], they may promote locomotor activity via the VTA. Indeed, silencing the subset of LHA Nts neurons that co-express LepRb, and presumably silencing Nts release to the VTA, reduces locomotor activity and disrupts DA signaling [200]. Functionally, Nts action in the VTA may also exert antidepressant effects, as it increases forced swim efforts even at sub-threshold doses that do not promote

general locomotor increase [246]. Stress increases Nts in the VTA, perhaps to potentiate adaptive locomotor behaviors needed for survival [247;248]. Central or systemic Nts, however, diminishes locomotor effects, suggesting that Nts actions in the NA outweigh those via the VTA [241;249;250]. The cellular localization of NtsRs likely accounts for the differential control of locomotor activity via NA and VTA neurons. VTA DA neurons express NtsRs at the dendrites and soma, so Nts action via stimulatory NtsRs promotes activation of DA neurons and release of DA into the NA that induces locomotor activity. By contrast, Nts injected directly into NA acts via postsynaptic NtsRs on the dendrites and soma of GABAergic spiny neurons [251]. Increasing Nts action via the NA may be useful to suppress the excessive locomotor effects in schizophrenia, similar to the effects of antipsychotics [252]. LHA Nts neurons, however, do not project into the ventral or dorsal striatum, and thus likely promote locomotor activity via projections to, and regulation of VTA DA neurons.

LHA Nts neurons additionally project within the LHA and modulate OX neurons, which also regulate ambulatory activity. Some Nts neurons in the LHA are activated by inflammatory signals, but adjacent OX neurons are inhibited in these conditions, suggesting differential control of these neuronal populations. LHA Nts neurons project onto and inhibit OX neurons [253], thus inflammation or illness-mediated activation of Nts neurons suppresses the activity of OX neurons and decreases locomotor activity during these states [150;200;254]. Similarly, loss of action via Nts^{LepRb} neurons decreases locomotor activity and energy expenditure in mice that promotes obesity [200], and some portion of these effects are likely mediated via regulation of OX neurons [191;200]

To date there are no direct reports concerning the role of LHA Nts neurons in arousal. Burdakov described a large population of non-MCH, non-OX GABAergic neurons in the LHA that are spontaneously active during waking and sleeping periods, and it is possible that these are Nts neurons. There were four subtypes of these uncharacterized GABAergic neurons, each of which exhibited distinct electrophysiologic properties (firing rate/response). Similarly, LHA Nts neurons are heterogeneous, and at least some of them are GABAergic [255], so it is possible that these electrophysiologically-distinct populations are in fact subpopulations of LHA Nts neurons [256-258]. Central administration of Nts promotes alertness and prolongs latency to sleep stages, suggesting that LHA Nts neurons could play a role in sustained arousal [259]. Overall, these data suggest rationale for LHA Nts neurons to influence locomotor activity and possibly arousal, but this has yet to be explicitly studied.

1.5 Summary and Rationale for Studying LHA Nts Neurons in Energy Balance

The LHA is an essential brain region for coordinating feeding, drinking and energy expenditure behaviors that inherently modify energy balance and weight, but the underlying mechanisms are incompletely understood. The LHA receives a diverse array of peripheral cues that communicate bodily energy and fluid balance (e.g. hormones, nutrient levels, changes in osmolality) and contains multiple populations of neurons that project to virtually every region of the brain. The discovery of neuropeptide-specific populations within the LHA has catapulted the field's understanding of how the LHA can manage the formidable task of coordinating diverse ingestive and locomotor responses via specialized, cue-sensitive neurons that mediate actions via distinct neuronal projections. While the characterization of MCH and OX neurons have advanced understanding of how the LHA coordinates some behaviors, these populations

mainly increase feeding to support energy-consuming physical activity and alertness. In contrast, LHA Nts neurons are implicated in restraining feeding, increasing drinking and promoting physical activity, but this has not been directly studied due to the previous inability to easily detect and manipulate Nts neurons. Taken together, these studies hint that LHA Nts neurons may exert opposing roles to their MCH and OX-expressing neighbors. If this is true, then LHA Nts neurons may be unique targets for modifying behavior and treating specific ingestive disorders such as disruptions of feeding (obesity, anorexia nervosa) or drinking (dehydration, psychogenic polydipsia) that endanger health.

1.6 Goals of the Dissertation

The body of work presented here will explore the Nts population in the LHA in an effort to elucidate its specific role in ingestive behavior, energy balance and control of body weight. My central hypothesis is that there are distinct subpopulations of LHA Nts neurons that coordinate ingestive behaviors, and that LHA Nts neurons are essential for control of body weight (Figure 1-1). I will therefore use novel mouse models expressing Cre or FlpO recombinase in Nts neurons that enable me to specifically manipulate these neurons and define their phenotypes and contributions to ingestive behaviors and energy balance. Using these mice, I will pursue the following goals:

1: Define Subsets of LHA Nts That Could Coordinate Ingestive Behavior (Chapter 2)

Hypothesis: Separate populations of LHA Nts neurons are distinguishable via molecular, circuit and neurochemical criteria, and detect either cues of energy or fluid status.

Method: We utilized *Nts*^{Cre} and newly generated Nts^{FlpO} mice to identify LHA Nts neurons, distinguish their molecular expression, neurochemistry and responses to cues of energy or fluid status.

2: Establish the Contribution of LHA Nts^{LepRb} Neurons to Adaptive Energy Balance (Chapter 3)

Hypothesis: LHA Nts neurons that co-express the long form of the leptin receptor (Nts^{LepRb} neurons) are essential for leptin and ghrelin-mediated adaptations in energy balance.

Method: We studied mice with developmental deletion of LepRb specifically from LHA Nts^{LepRb} neurons to characterize how loss of action via Nts^{LepRb} neurons impacts neurocircuitry, cellular responses to leptin and ghrelin and control of energy balance.

3: Determine the Role of LHA Nts Neurons in Adaptive Energy Balance (Chapter 4).

Hypothesis: LHA Nts neurons are essential for control of ingestive behavior and body weight.

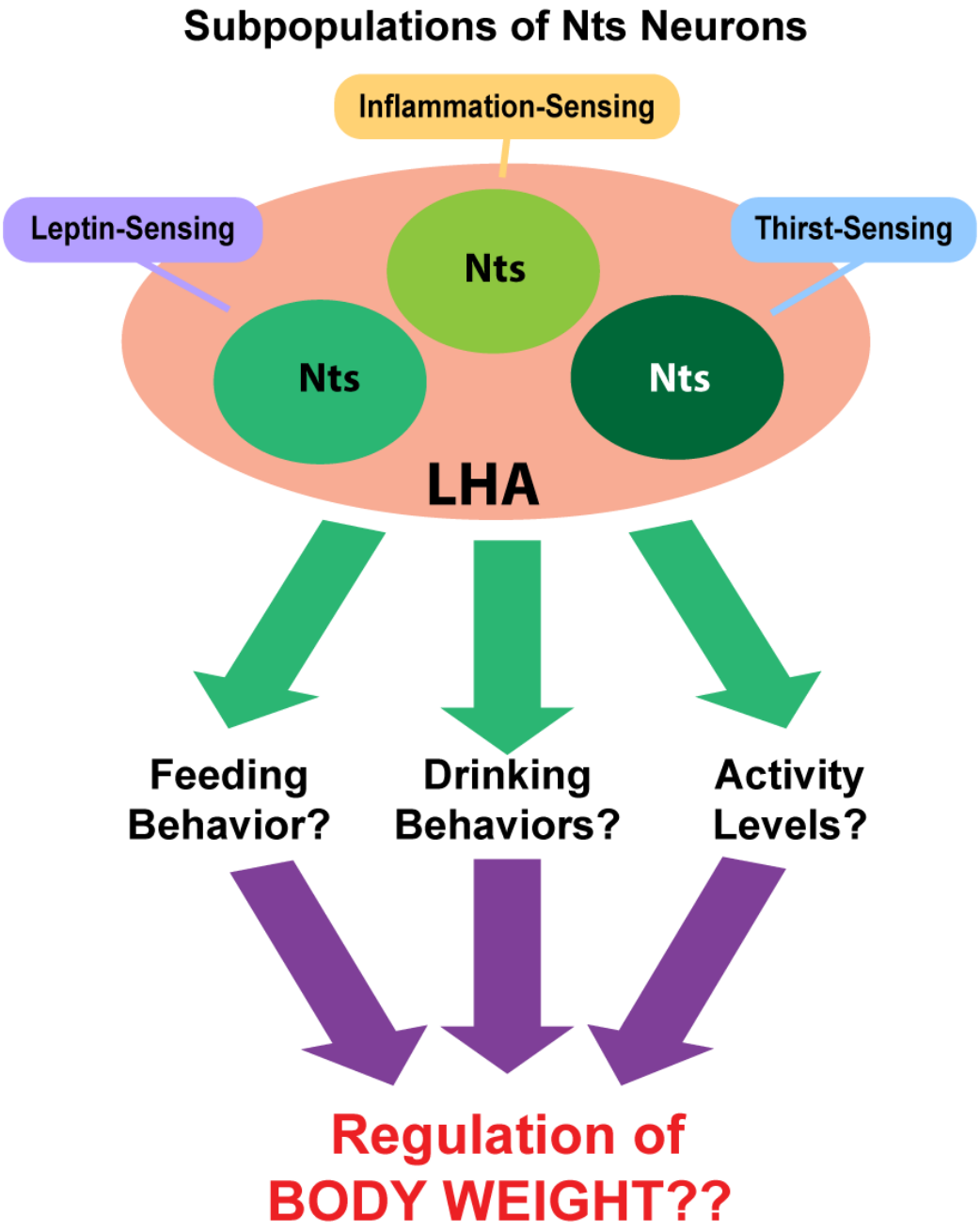
Method: We specifically lesioned LHA Nts neurons or suppressed their activity to reveal their requirement for control of feeding, drinking, movement and body weight.

Through these goals (Figure 1-1), we describe specific subsets of LHA Nts neurons positioned to coordinate distinct peripheral cues and ingestive behaviors. Furthermore, we demonstrate that LHA Nts neurons, in total, are required for appropriate control of energy balance and fluid homeostasis. Our work is novel in that it, for the first time, defines an obligate physiological role for LHA Nts neurons, and establishes neural mechanisms by they contribute

to adaptive behaviors necessary for health. These findings may lead to development of strategies to treat specific ingestive disorders, which will be discussed in Chapter 5.

1.7 Figures

Figure 1-1: Proposed Model of LHA Nts Neuronal Regulation of Energy Balance



Subpopulations of Nts neurons in the LHA may each contribution to regulation of energy balance independently by coordinating distinct peripheral signals and behaviors in response to different energy status conditions.

CHAPTER 2 Heterogeneity of Lateral Hypothalamic Neurotensin Neurons: Distinct Subsets Are Activated by Leptin or Dehydration

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This Chapter is an adaptation of a manuscript that has been prepared for submission

2.1 Abstract

The lateral hypothalamic area (LHA) is essential for ingestive behavior, but it remains unclear how LHA neurons coordinate feeding vs. drinking. Most LHA populations promote food and water consumption, but LHA neurotensin (Nts) neurons preferentially induce water intake while suppressing feeding. Here we identify two molecularly and projection-specified subpopulations of LHA Nts neurons that are positioned to coordinate either feeding or drinking. One subpopulation co-expresses the long form of the leptin receptor (LepRb) and is activated by the anorectic hormone leptin (Nts^{LepRb} neurons). A separate subpopulation lacks LepRb and is activated by dehydration (Nts^{Dehy} neurons). These molecularly distinct LHA Nts subpopulations also differ in connectivity: the Nts^{LepRb} neurons project to the ventral tegmental area (VTA) and substantia nigra compacta (SNc), but Nts^{Dehy} neurons do not. We then investigated whether LHA Nts neurons can be differentiated by expression of the inhibitory neurotransmitter GABA or the excitatory neurotransmitter glutamate (glut). Using a genetic dual recombinase approach to simultaneously label Nts neurons and GABA or glutamate-containing neurons, we confirm that all LHA Nts neurons are GABAergic. Collectively, our data identify two molecularly- and projection-specified subpopulations of LHA Nts neurons that intercept either leptin or dehydration cues, and hence these populations may separately regulate feeding or drinking behavior. In the future, selective regulation of these LHA Nts subpopulations might be useful to treat ingestive disorders such as polydipsia or obesity.

2.2 Introduction

The lateral hypothalamic area (LHA) of the brain receives cues of osmotic and energy status, and accordingly coordinates goal-directed ingestive behavior necessary for maintaining homeostasis [51;52;260-263]. Indeed, the LHA was initially deemed an essential "feeding center" because animals with LHA lesions lose all motivation to eat food [30;31]. Less emphasized, but equally important, is that animals with LHA lesions also lose the motivation to drink water, and their resulting dehydration causes death well before starvation[32]. Thus, the LHA as a whole modifies both types of ingestive behavior necessary for survival but the underlying mechanisms by which it does so remain incompletely understood.

The discovery of molecularly- and projection-specified populations of neurons within the LHA suggested that some of them might be specialized to coordinate drinking vs. feeding. However, most of the LHA populations studied to date indiscriminately promote intake of food and water. For example, LHA neurons expressing melanin concentrating hormone (MCH) promote intake of both substances, and do not specifically organize feeding vs. drinking [98]. A separate population of orexin/hypocretin-expressing LHA neurons regulate arousal-dependent behaviors, including feeding, drinking and locomotor activity, but do not specify any particular ingestive behavior per se [137]. Instead, animals with activated orexin/hypocretin neurons appear to consume more food and water simply because they are awake more, and have more opportunity to ingest. LHA neurons have also been distinguished by their expression of the classical neurotransmitters glutamate or GABA. Inhibiting LHA glutamate neurons in mice increases their intake of a palatable 'meal replacement" drink [264], but it is unclear if this is an effort to obtain fluid, calories or if both ingestive behaviors are modulated by these neurons. Similarly, activation of all LHA GABA neurons increases behaviors to obtain food and liquids, but also increases gnawing at non-caloric objects such as wood or the cage floor [265-267]; thus,

bulk activation of LHA GABA neurons cannot be considered to direct any specific ingestive behavior. While such en masse activation of the large LHA GABA population is unlikely to occur physiologically, there are functionally and molecularly distinct subpopulations of LHA GABA neurons that have yet to be fully characterized [194;255;265;268]. It is therefore possible that subsets of LHA GABA neurons might be activated by distinct physiologic cues, and hence differentially control food vs. water intake. However, the studies of LHA populations to date do not explain how the LHA specifically coordinates feeding or osmolality cues to direct the appropriate ingestive behavior.

We recently characterized a large, molecularly distinct population of LHA neurons that express the neuropeptide Neurotensin (Nts), but which are separate from the MCH or orexin/hypocretin neurons [177;269]. Unlike other LHA populations that promote both food and water consumption, experimental activation of LHA Nts neurons promotes voracious drinking but restrains feeding via incompletely understood mechanisms [177] Since LHA Nts neurons have been reported to contain glutamate [194] or GABA [200], we hypothesized that LHA Nts neurons might be molecularly and functionally heterogeneous, such that subsets of LHA Nts neurons exist to coordinate drinking vs. feeding. Indeed, some (but not all) LHA Nts neurons co-express GABA and the long form of the leptin receptor (LepRb) and are activated by the anorectic hormone leptin [200;269] we refer to these as Nts^{LepRb} neurons. This Nts^{LepRb} population comprises a small, but essential subset of LHA Nts neurons necessary to mediate the anorectic response to leptin and proper regulation of energy balance [269]. However, mice lacking LepRb in LHA Nts^{LepRb} neurons do not exhibit any disruptions in drinking or bodily fluid content, suggesting that LHA Nts-mediated drinking may be mediated via different LHA Nts neurons [269]. At least some LHA Nts neurons are responsive to physiologic changes in serum osmolality, as dehydration increases expression of Nts mRNA within the LHA [230]; we refer to these as Nts^{Dehy} neurons.

Exogenous Nts treatment also promotes drinking [270], though the endogenous sources of Nts mediating this effect remained unknown. Given that experimental activation of LHA Nts neurons promotes Nts release [177;194], and drinking [100;177;194;229], the dehydration-induced upregulation of LHA Nts could serve as a physiologic signal to drive water seeking and intake once water becomes available [228]. Taken together, these data suggest that some LHA Nts neurons can be activated by cues of energy status (leptin) or osmolality status (dehydration), and might comprise separate populations of LHA Nts neurons to coordinate feeding or drinking behavior. We therefore assessed whether LHA Nts^{LepRb} neurons and Nts^{Dehy} neurons are the same, or whether they are separate populations that are distinguishable via molecular, circuit and neurochemical criteria.

2.3 Materials and Methods

2.3.1 Animals

Adult male and female mice were used for studies. Some *Nts^{Cre};GFP* and *$LepRb^{Cre}$;GFP* mice were generated and treated with euhydration or dehydration at the University of Michigan, under the supervision of the Unit for Laboratory Animal Medicine (ULAM). These procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC). All other mice were generated from a breeding colony at Michigan State University, where mice were housed in a 12h light/12h dark cycle and had *ad libitum* access to water and chow diet (Teklad 7913). MSU mice were cared for by Campus Animal Resources (CAR) and all animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

2.3.2 Generation of *Nts^{FlpO} Knock*-In Mice

We modified the targeting vector used to generate *Nts^{Cre}* mice [200] to create an *Nts^{FlpO}* targeting vector. Briefly, the IRES-Cre was replaced with an IRES-FlpO sequence, such that it is inserted between the stop codon and the polyadenylation site of the sequence encoding the 3' end of the mouse *Nts* gene. An *frt*-flanked NEO cassette lied upstream of the IRES-FlpO for selection purposes. The linearized *Nts^{FlpO}* targeting vector was electroporated into mouse R1 embryonic stem (ES) cells (129sv background) and cells were selected with G418. DNA from ES cell clones was analyzed via qPCR for loss of homozygosity using Taqman primer and probes for the genomic *Nts* insertion sites (Nts-IRES: Forward: TGAAAAGGCAGCTGTATGAAAATAA, Nts-IRES: Reverse: TCAAGAATTAGCTTCTCAGTAGTAGTAGGAA, Nts-IRES: Probe: CCAGAAGGCCCTACATTCTCAAGAGG. *NGF* was used as a copy number control. Putative positive ES clones were expanded, confirmed for homologous recombination by Southern blot and injected into mouse C57BL/6 blastocysts to generate chimeras. Chimeric males were mated with C57BL/6 females (Jackson Laboratory), and germline transmission was determined initially via progeny coat color, then confirmed via conventional PCR for FlpO (as described below).

2.3.3 Breeding and Genotyping

The *Nts^{Cre};GFP* and *LepRb^{Cre};GFP* mice used for Figure 2-6 were generated and genotyped as described previously [255]. For all other experiments we utilized *Nts^{cre}* mice (Jackson stock #017525) that had been bred onto the C57/Bl6 background (Jackson #008327) mice for at least seven generations. To visualize Nts, vGat and vGlut2-expressing neurons, heterozygous *Nts^{cre}* mice, homozygous *Slc32a1$^{tm2(cre)Lowl}$* (Vong 2011) [Jackson stock #028862]

and *Slc17a6*$^{tm2(cre)Lowl}$ (Vong 2011) [Jackson stock # 028863] mice were crossed with homozygous *Rosa26*$^{EGFP\text{-}L10a}$ mice (Krashes 2014) and progeny heterozygous for both alleles were studied (*Nts*^{cre}*;GFP* mice, *vGat*^{Cre}*;GFP* and *vGlut2*^{Cre}*;GFP* mice respectively). To simultaneously detect Nts and vGat or vGlut2 we utilized a dual-recombinase strategy. Briefly, we interbred *Nts*^{FlpO} mice (to permit FlpO-mediated recombination) and *Slc32a1*$^{tm2(cre)Lowl}$ or *Slc17a6*$^{tm2(cre)Lowl}$ mice (that enable Cre-mediated recombination) to generate progeny that were heterozygous for FlpO and Cre. These mice were injected with FlpO- and Cre-dependent reporters to visualize Nts and vGAT/vGlut-expressing neurons as described below. Genotyping was performed using standard PCR using the following primer sequences: *Nts*^{cre}*:* common forward: 5' ATA GGC TGC TGA ACC AGG AA, Cre reverse: 5' CCA AAA GAC GGC AAT ATG GT and WT reverse: 5' CAA TCA CAA TCA CAG GTC AAG AA. *Rosa26*$^{EGFP\text{-}L10a}$: mutant forward: 5' TCT ACA AAT GTG GTA GAT CCA GGC, WT forward: 5' GAG GGG AGT GTT GCA ATA CC and common reverse: 5' CAG ATG ACT ACC TAT CCT CCC. Nts^{FlpO}: *Nts-FlpO-WT*: Forward: CCAGGAAGATATCCTTGATAACGTCAAT, Reverse: GCAAGAAACATCACATCCAATAAAGCA N, *Nts-FlpO-M:* Forward: TGACCTACCTGTGCTGGATGAT, Reverse: CCACGTTCTTGATGTCGCTGAA. vGat^{Cre}: VgatIRESCre Common Forward: CTTCGTCATCGGCGGCATCTG, VgatIRESCre -WT Reverse: CAGGGCGATGTGGAATAGAAA, VgatIRESCre -Mutant Reverse: CCAAAAGACGGCAATATGGT.

2.3.4 Stereotaxic Injections

Stereotaxic surgeries were performed as described previously [269] using coordinates from the mouse brain atlas of Paxinos and Franklin [271]. To facilitate detection of Nts containing cell bodies via Nts-IR, adult *Nts*^{cre}*;GFP*, *vGat*^{Cre}*;GFP* and *vGlut2*^{Cre}*;GFP* mice received injections of colchicine (10 µg in a volume of 500 nL) into the lateral ventricle (A/P -0.2, M/L -1.0, D/V -2.1),

and were euthanized via cardiac perfusion ~48 hours after treatment. For tract tracing studies, *Nts^{cre};GFP* mice were injected unilaterally with 75 nL of the retrograde tracer FluoroGold (FG) into the VTA (A/P: -3.2, M/L: +/-0.48, D/V: -4.65) or SNc (A/P: -3, M/L +/-1.3, D/V -4.7), and recovered for 7-10 days to allow for accumulation of FG in cell bodies of origin. Animals were only included in the study if the FG injection was targeted to, and confined within the VTA or SNc. To simultaneously identify Nts and vGAT or vGlut, adult *Nts^{FlpO};vGat^{Cre}* and *Nts^{FlpO};vGlut2^{Cre}* mice were injected in the LHA with 400 nL of AAV-FrtGFP adenovirus (provided by David Olson, University of Michigan) followed by 400 nL AAV-DREADDq (AAV-hM3Dq-mCherry; UNC Vector Core), which were infused at a rate of 100 nL/minute. LHA coordinates were A/P: -1.34, M/L +/-1.5, D/V -4.9, angle: 6°. Mice recovered for 2 – 3 weeks to permit sufficient time for recombinase-mediated reporter expression.

2.3.5 Leptin or Dehydration Treatment

Some FG-injected *Nts^{cre};GFP* mice were treated with PBS or recombinant mouse leptin (5 mg/kg, i.p.) purchased from the National Hormone and Peptide Program (Los Angeles Biomedical Research Institute, Los Angeles, CA), then were perfused 2 hours later. Males and females were studied but no notable differences between sexes were observed, so they were pooled for analysis: VTA-injected vehicle-treated n=6; female VTA-injected leptin-treated n = 10; SNc-injected vehicle-treated n = 5; SNc-injected leptin-treated n = 9.

Other FG-injected *Nts^{cre};GFP* mice underwent a dehydration-activation paradigm in which they were either had ad-lib access to water (euhydration) or the water bottle was removed for 12 hr during the dark cycle (when mice drink most of their daily water). Mice were perfused the following morning and brain sections were stained for cFos, GFP and FG. Due to the lack of

observable differences between sexes, males and females were pooled for analysis: VTA-injected, euhydrated n = 5; VTA-injected, dehydrated n = 9; SNc-injected, euhydrated n = 5, SNc-injected, dehydrated n = 9.

Adult male 8-12 wk old *Nts^{Cre};GFP* and *LepRb^{Cre};GFP* mice were also treated via euhydration or had water bottles removed for 24 hr (including during the dark cycle) to induce dehydration, then were perfused (Euhydrated *Nts^{Cre};GFP* n=7, *LepRb^{Cre};GFP* n = 5; Dehydrated *Nts^{Cre};GFP* n=4, *LepRb^{Cre};GFP* n = 3). Brains were analyzed for cFos and GFP, and 3 LHA sections from each mouse were analyzed using Photoshop to count the number of GFP-only labeled neurons and GFP neurons containing cFos. Graphed data represent the average percentage of GFP neurons containing cFos out of the total number of GFP-labeled neurons ± SEM. Significant differences between genotypes and treatments were determined via 2-way ANOVA with Bonferroni posttests.

2.3.6 Immunohistochemistry and Immunofluorescence

Mice were treated with a lethal dose of *i.p.* pentobarbital and perfused transcardially with either 10% formalin or 4% paraformaldehyde (Sigma-Aldrich 158127) containing 0.4% picric acid (Sigma-Aldrich 197378). Brains were removed, post-fixed in the same fixative overnight at 4˚C, then dehydrated with 30% sucrose in PBS for 2-3 days, and sectioned into 30 µm slices using a sliding microtome (Leica). Brain sections were then analyzed by immunohistochemistry and/or immunofluorescence as previously described [269]. For activation studies, brain sections first were exposed to either rabbit-anti pSTAT3 (1:500, Cell Signaling) or goat-anti cFos (1:500, Santa Cruz) followed by incubation with species specific Alexa-488 conjugated (Jackson ImmunoResearch, 1:200) or Alexa-568 conjugated antibodies (LifeTech, 1:200)) and visualization with DAB (Sigma). Immunofluorescent labeling was performed exposing sections

to primary antibodies, including chicken anti-GFP (1:2000, Abcam), rabbit anti-FG (1:500, Fluorochrome), rabbit-anti Nts (1:500, Phoenix) and/or anti-dsRed (1:1000, Clontech), followed by incubation with species-specific secondary antibodies conjugated to AlexaFluor 488 or 568 fluorophores (1:200, Life Technologies or Jackson ImmunoResearch). Immunolabeled brain sections were analyzed using an Olympus BX53 fluorescence microscope outfitted with transmitted light to analyze DAB-labeling, as well as FITC and Texas Red filters for IF. Microscope images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe).

2.4 Results

2.4.1 Validation of *Nts^{Cre};GFP* Mice to Identify LHA Nts Neurons

In situ hybridization (ISH) data from the Allen Brain atlas identifies a significant population of Nts-expressing cells within the LHA (Figure 2-1 A-B), which is consistent with previous ISH reports [230]. However, ISH-mediated detection is not ideal to permit further characterization of Nts cells, such as determination of co-expressed transcripts or responses to leptin or dehydration. Immunoreactivity (IR) is a more facile detection method that is compatible with co-expression and activation analyses, but Nts-IR only labels fibers, not cell bodies (Figure 2-1 B). In agreement with previous reports, we found that Nts-containing cell bodies could be detected via Nts-IR in animals that have been pre-treated with the axonal transport inhibitor, colchicine (Figure 2-1 D), but this treatment precludes assessment of normal physiologic responses [272]. However, the similar distribution of Nts labeling from ISH and colchicine-mediated Nts-IR (Figure 2-1 B vs 2-1 D), confirm that there is a large population of Nts-containing neurons within the LHA.

The recent development of *Nts*^{Cre} mice enables the facile detection and manipulation of mouse Nts neurons using Cre-Lox technology, hence we reasoned that this model would be useful to examine the molecular expression, projections, activation responses and neurochemistry of LHA Nts neurons without physiology-disrupting colchicine treatment. As a first step we verified the fidelity of *Nts*^{Cre} mice for identifying Nts neurons by crossing them onto a Cre-inducible GFP reporter line [273], to produce mice that express GFP selectively in Nts neurons (*Nts*^{Cre}*;GFP* mice). Adult *Nts*^{Cre}*;GFP* mice were treated with colchicine, and their brains were analyzed for GFP and Nts-IR. We examined two brain regions that have been shown via ISH to contain numerous Nts neurons in mice: the subthalamic nucleus (STN) and the LHA [274]. Similar to these ISH findings, *Nts*^{Cre}*;GFP* mice have dense populations of GFP-labeled neurons within the STN and LHA, and these co-label with Nts-IR (Figure 2-1 E-K). Intriguingly, Nts-IR is more highly-expressed within the LHA compared to the STN, hinting at a potentially important LHA function for this neuropeptide. In any case, these data verify that *Nts*^{Cre}*;GFP* mice reliably identify Nts neurons, and that we can use them to characterize features of LHA Nts neurons.

2.4.2 *Nts*^{Cre}*;GFP* Mice Confirm that Some LHA Nts Neurons Project to the VTA and SNc

LHA Nts neurons project to two adjacent midbrain regions, the ventral tegmental area (VTA) and the substantia nigra compacta (SNc) [191;200], which regulate motivated behaviors and motor function, respectively [275;276]. We therefore hypothesized that different subsets of LHA Nts neurons might project to the VTA or SNc to differentially control behavior. As a first step to addressing this hypothesis, we verified that we can identify LHA Nts neurons that project to the VTA or SNc. *Nts*^{Cre}*;GFP* mice were injected in either the VTA (Figure 2-2 A) or the SNc (Figure 2-2 C) with the retrograde tract tracer FluoroGold (FG), which is taken up by local terminals and transported retrogradely back to the cell bodies of origin. Examination of VTA-injected mice revealed many LHA cell bodies that have accumulated FG, some of which also contained GFP

and hence identify LHA Nts neurons that project to the VTA (Figure 2- 1 B, yellow arrows). There were also numerous LHA Nts neurons that did not accumulate FG from the VTA, indicating that these neurons do not project to the VTA (Figure 2-1 B, cyan arrows). Analysis of SNc-injected mice reveals some FG-labeled LHA Nts neurons that project to the SNc (Figure 2-1 D, yellow arrows) but also many LHA Nts neurons devoid of FG that do not synapse within the SNc (Figure 2-1 D, cyan arrows). Together, these data verify that that some LHA Nts neurons project to the VTA and SNc. In addition, the LHA Nts neurons lacking FG labeling hint that there are separate populations of LHA Nts neurons, which might project to either the VTA or SNc, or sites other than the midbrain.

2.4.3 Nts^{LepRb} neurons are a Subset of LHA Nts Neurons that Project to the Midbrain

At least one subset of LHA Nts neurons may be molecularly distinguished via their expression of LepRb (Nts^{LepRb} neurons), and we reasoned that they might project to either the VTA or SNc. To identify Nts^{LepRb} neurons, we treated *Nts^{Cre};GFP* mice with vehicle or leptin (5 mg/Kg *i.p.* 4hr), which induces phosphorylation of STAT3 (pSTAT3) specifically in LepRb-expressing neurons. While no pSTAT3 is observed in the LHA of vehicle treated mice (Figure 2-3 A-C), leptin treatment increases pSTAT3 (Figure 2-3 E, red arrows and blue-outlined red arrows). Some GFP-labeled LHA Nts neurons contain leptin-induced pSTAT3 and these are Nts^{LepRb} neurons (Figure 2-3 D-F, blue-outlined red arrows). However, many GFP-labeled LHA Nts neurons do not contain pSTAT3 (Figure 2-3 D-F, blue arrows). Together, these data confirm that there are at least two molecularly distinct populations of LHA Nts neurons: one LHA Nts population expresses LepRb and can be revealed by leptin-induced pSTAT3 treatment (the Nts^{LepRb} neurons), but another LHA Nts population lacks LepRb.

Next, we asked whether the molecularly-specified Nts^{LepRb} neurons are also projection-specified, targeting the VTA or SNc. As in Figure 2, we injected *Nts^{Cre};GFP* mice with FG to label LHA Nts neurons that project to the VTA or SNc, and also treated mice with leptin to permit pSTAT3-mediated identification of Nts^{LepRb} neurons. Via this paradigm we identified Nts^{LepRb} neurons that accumulated FG from the VTA, indicating that some Nts^{LepRb} neurons project to the VTA (Figure 2-3 G-K, white arrows). We also noted some Nts^{LepRb} neurons that project to the SNc (Figure 2-3 L-P, white arrows). In each case we also observed Nts^{LepRb} neurons that did not accumulate FG; these may be Nts^{LepRb} neurons that project to the midbrain region that wasn't injected with FG, or these neurons might project to a yet undetermined site outside of the midbrain (Figure 2-3 G-P, orange arrows). As expected, we also observed LHA Nts neurons lacking LepRb, some of which project to the VTA or SNc (Figure 2-3 G-P, yellow arrows), but other LHA Nts neurons did not accumulate FG and hence do not project to either midbrain region (Figure 2-3 G-P, cyan arrows). In sum, these data signify that Nts^{LepRb} neurons are a molecularly-specified subset of LHA Nts neurons that project to both the VTA and SNc.

2.4.4 Nts^{Dehy} Neurons are a Subset of LHA Nts Neurons that Do Not Project to the Midbrain

Since dehydration disrupts bodily osmolality and upregulates Nts mRNA in the LHA [230], we postulated that it might also modify the activity of some LHA Nts neurons. To test this hypothesis, we provided *Nts^{Cre};GFP* mice with *ad lib* water (Euhydration) or removed their water overnight, when mice consume most of their daily fluid (Dehydration). Brains were then examined for GFP (to identify Nts neurons) and cFos (a marker of recent neuronal depolarization). During euhydration we observed few LHA Nts neurons with cFos, suggesting that LHA Nts neurons are not activated during normal fluid balance (Figure 2-4 A-C). In contrast, overnight dehydration increased cFos within some (but not all) LHA Nts neurons

(Figure 2-4 D-F, blue outlined red arrows); these are Nts^{Dehy} neurons. These data demonstrate that a distinct physiologic cue, dehydration, activates a subset of LHA Nts neurons (Nts^{Dehy} neurons), but it remained unclear if Nts^{Dehy} neurons are distinct from Nts^{LepRb} neurons at a molecular or projection level.

To assess the midbrain projections of Nts^{Dehy} neurons, *Nts^{Cre};GFP* mice were injected with FG in the VTA or SNc (as in Figure 2-2), and then were dehydrated overnight to label Nts^{Dehy} neurons. Although we observed Nts^{Dehy} neurons in the LHA, none of these accumulated FG from the VTA or SNc (Figure 2-4 G-P). We did, however, observe FG accumulation within other LHA Nts neurons that were not activated by dehydration, confirming successful retrograde labeling. Together with our previous data (Figure 2-2), these data indicate that some LHA Nts neurons, including Nts^{LepRb} neurons, project to the midbrain, but Nts^{Dehy} neurons do not. Given that Nts^{Dehy} neurons and Nts^{LepRb} neurons have different projection targets, they must comprise distinct subsets of LHA Nts neurons.

2.4.5 Nts^{LepRb} and Nts^{Dehy} neurons Are Separate Subpopulations of LHA Nts Neurons

We reasoned that if Nts^{LepRb} neurons are distinct from Nts^{Dehy} neurons they would not be activated by dehydration. However, the requirement for leptin or dehydration to functionally identify Nts^{LepRb} and Nts^{Dehy} neurons meant that we could not simultaneously label these subsets in *Nts^{Cre};GFP* mice. Instead, to test our hypothesis, we analyzed cFos-IR to identify activated neurons in brains from euhydrated or dehydrated *Nts^{Cre};GFP* mice (where GFP identifies all LHA Nts neurons) and from *$LepRb^{Cre}$;GFP* mice (where GFP identifies LepRb neurons). Since the majority of LHA LepRb neurons co-express Nts, the *$LepRb^{Cre}$;GFP* mice identify the Nts^{LepRb} neurons as well as some non-Nts expressing neurons [200]. While dehydration significantly

increased the percentage of LHA Nts neurons containing cFos (Figure 2-5 A,C), it did not alter the proportion of LepRb neurons containing cFos compared to the euhydrated state (Figure 2-5 B,C). Since LHA LepRb neurons contain the subset of Nts^{LepRb} neurons, this means that the population of Nts^{LepRb} neurons is not activated by dehydration, but other non-LepRb containing Nts-containing neurons are activated by dehydration. These data signify that Nts^{LepRb} neurons are functionally distinct from Nts^{Dehy} neurons, as distinguished via their response to dehydration. Furthermore, these subpopulations of LHA Nts neurons are molecularly distinct, such that LepRb expression can be used to distinguish Nts^{LepRb} neurons from Nts^{Dehy} neurons. Taken together, data from Figures 2-3, 2-4 and 2-5 reveal that LHA Nts neurons are not a homogeneous population, but contain molecularly and projection-specified subsets of Nts^{LepRb} neurons and Nts^{Dehy} neurons, that are differentially activated in response to changes in energy or fluid balance, respectively.

2.4.6 Verification of Cre-Dependent Reagents to Determine Classical Neurotransmitter Content

Given the heterogeneity of LHA Nts neurons at the molecular and circuit level, we hypothesized that they might differ in other ways, such as in their classical neurotransmitter content. Indeed, neuropeptide-expressing neurons may contain either the classical/fast neurotransmitters GABA or glutamate, and release of the classical/fast neurotransmitter thereby determines whether synaptic targets are inhibited or activated [277]. However, there have been conflicting reports on the neurochemistry of LHA Nts neurons, with some studies indicating that LHA Nts neurons co-express the inhibitory neurotransmitter GABA [200] while others suggest they are glutamatergic [202] We therefore sought to define the classical neurotransmitter content of

LHA Nts neurons, and whether subpopulations might be neurochemically distinguishable due to containing GABA or glutamate.

As a first step we required a method to identify GABA and glutamate-containing cell bodies, since they cannot be detected using immunoreagents. *vGat*^{Cre} and *vGlut2*^{Cre} mice have been used to identify GABA and glutamate-expressing neurons, respectively, so we verified the fidelity of these lines for identifying classical neurotransmitter expression within the vicinity of the LHA. First, we crossed *vGat*^{Cre} and *vGlut2*^{Cre} mice onto a Cre-inducible GFP reporter line, such that any cells that express these transcripts during development will be labeled with GFP (*vGat*^{Cre}*;GFP* and *vGlut2*^{Cre}*;GFP* mice). One caveat of such developmental labeling, however, is that developing neurons may alter neurotransmitter expression over lifespan, which might confound interpretation of how they signal in the adult brain [278]. Thus, we also injected adult *vGat*^{Cre} and *vGlut2*^{Cre} mice with an AAV Cre-inducible red fluorescent protein (RFP), to visualize mature neurons containing GABA and glutamate via expression of RFP. We then compared the GFP- and AAV-RFP reporter expression with ISH data from the Allen Brain atlas to verify whether each method faithfully identified classical neurotransmitter expression in LHA-adjacent brain regions known to primarily contain glutamatergic neurons (the STN) or GABAergic neurons (the zona incerta, ZI). ISH for *vgat* confirms high expression within the zona incerta (ZI), but not the adjacent STN (Figure 2-6 A,D,E) and the *vGat*^{Cre}*;GFP* mice (Figure. 2-6 B,F,G) similarly exhibit numerous GFP-labeled vGAT neurons within the ZI but few in the STN (Figure 2-6 F,G). The *vGat*^{Cre} mice injected with AAV-RFP exhibited RFP-labeled vGAT cells in a similar distribution, with many RFP-labeled cell bodies in the ZI but none in the adjacent STN (Figure 2-6 C,H,I). In sum, these data confirm that Cre-mediated expression via reporter mice or AAVs can be used to visualize GABA neurons. In contrast, ISH for *vglut2* indicates minimal ZI expression, but robust labeling in the STN (Figure 2-6 J,M,N) and the distribution of GFP-

labeled vGlut2 neurons is similar from *vGlut2^{Cre}:eGFP* mice (Figure 6 K,O,P). Many GFP positive cells are present in the STN (Figure 2-5 O) in contrast to a marked absence of GFP-labeled cells in the STN (Figure 2-5 P). We observed some RFP expression within the STN of *vGlut^{Cre}* mice that were injected in the LHA with AAV-RFP (Figure 2-6 L,Q), but the AAV did not spread laterally enough to robust infect this region. Consistent with the lack of glutamate neurons in the ZI, we observed minimal RFP within the ZI even though the AAV-RFP appears to have transduced cells in adjacent regions (Figure 2-6 L,R). Taken together, these data demonstrate that the Cre-dependent GFP reporter and AAV-RFP reporters can be used along with *vGat^{Cre}* and *vGlut2^{Cre}* mice to accurately identify GABA and glutamate neurons, and hence we can use these models to examine the neurotransmitter content of the LHA.

2.4.7 *Nts^{FlpO}* Mice Identify LHA Nts Neurons Without the Use of Cre

To define the neurotransmitter content of LHA Nts neurons we must simultaneously label Nts and vGat or vGlut. However, the use of *vGat^{Cre}* and *vGlut2^{Cre}* mice necessary for detection of GABA and glutamate neurons precludes simultaneous Cre-dependent detection of Nts using *Nts^{Cre}* mice. To overcome this limitation, we generated a dual recombinase system, whereby Cre-mediated recombination identifies vGAT or vGlut-expressing cells, but FlpO-dependent recombination is used to visualize Nts expressing cells. To enable FlpO-dependent identification of Nts neurons, we modified the targeting vector that was used to generate *Nts^{Cre}* mice, and replaced the coding sequence for Cre-recombinase with FlpO recombinase. We then injected the resulting *Nts^{FlpO}* mice or control mice lacking Cre (WT) with an AAV that drives FlpO-inducible expression of GFP (AAV-FrtGFP) to verify the specificity of the FlpO-dependent system for visualizing LHA Nts neurons (Figure 2-7). No GFP-labeled cells were observed in the LHA, ZI or STN of WT mice injected with AAV-FrtGFP, confirming the FlpO-dependence for GFP expression (Figure 2-7 A-E). By contrast, injection of AAV-FrtGFP into the LHA of *Nts^{FlpO}*

mice resulted in GFP-labeled cell bodies within the LHA, ZI and STN (Figure 2-7 F-H). The distribution of GFP-labeled cells in *Nts*^{FlpO} mice is similar to that of *Nts*^{Cre}*;GFP* mice, though fewer cells are reported; these results are consistent with the limited recombination efficiency of Flp as compared to Cre [279]. However, the *Nts*^{FlpO} mouse model enables dual recombinase studies necessary to permit simultaneous detection of Nts and other signals that require Cre-mediated detection, such as vGAT and vGlut.

2.4.8 Determination of Classical Neurotransmitter Content Within LHA Nts Neurons Using the Dual Recombinase System

Next, we verified the fidelity of the dual recombinase system to simultaneously label Nts cells and vGat or vGlut-expressing cells. *Nts*^{FlpO} mice were crossed to *vGat*^{Cre} or *vGlut2*^{Cre} mice to produce *Nts*^{FlpO}*;vGat*^{Cre} and *Nts*^{FlpO}*;vGlut*^{Cre} mice respectively. As a first step we injected these mice in the ZI with AAV-Frt-GFP (to permit FlpO-mediated expression of GFP that identifies Nts neurons) and AAV-LoxP-RFP (to permit Cre-mediated expression of RFP for detection of vGat or vGlut neurons). Since the ZI primarily contains GABAergic but not glutamatergic neurons (Figure 2-6), the dual recombinase system should only result in GFP and RFP co-labeling of ZI cells in *Nts*^{FlpO}*;vGat*^{Cre} mice, but not in *Nts*^{FlpO}*;vGlut*^{Cre} mice. As anticipated, dual AAV injection into the ZI of *Nts*^{FlpO}*;vGlut*^{Cre} mice results in many GFP-labeled Nts neurons, none of which contain RFP-vGlut (Figure 2-8 A, B, cyan arrows). The GABAergic ZI is in fact devoid of RFP-vGlut, despite the robust induction of RFP in surrounding regions known to contain glutamate (Figure 2-8 A,B). By contrast, in dual AAV-injected *Nts*^{FlpO}*;vGat*^{Cre} mice, we observed that all of the GFP-labeled Nts cells co-label with RFP-vGat, indicating that ZI Nts neurons are GABAergic (Figure 2-8 C,D; white arrows indicate co-labeled cells, cyan arrows identify RFP-vGat cells that do not contain GFP-Nts). Taken together, these data confirm that the ZI Nts cells are GABAergic, but not glutamatergic, as would be expected of this

primarily GABAergic brain region. Furthermore, these data confirm the fidelity of the dual recombinase system to distinguish the classical neurotransmitter content of LHA Nts neurons.

Next, we used to dual recombinase system to determine whether subsets of LHA Nts neurons can be discriminated via their classical neurotransmitter expression. Dual AAV injection into the LHA of *Nts^{FlpO};vGlut^{Cre}* mice identifies GFP-Nts neurons (Figure 2-8 E,F, cyan arrows) and RFP-vGlut2 neurons (Figure 2-8 E,F, magenta arrows), but we did not observe any LHA cells that co-express both labels. By contrast, dual AAV injection into the LHA of *Nts^{FlpO};vGat^{Cre}* mice identified GFP-Nts neurons, most of which co-label with RFP-vGat (Figure 2-8 G,H, white arrows). We also observed many RFP-vGAT neurons that did not co-label with GFP-Nts (Figure 2-8, G, magenta arrows). Together these data suggest that LHA Nts neurons are predominantly GABAergic, and they comprise a subset within the larger population of LHA GABA neurons.

2.4.9 Determination of Classical Neurotransmitter Content of LHA Nts Neurons Using Colchicine-Mediated Nts-IR

The dual recombinase method suggests that LHA Nts neurons do not contain glutamate, based on the absence of neurons co-expressing both Nts-GFP and RFP-vGlut. However, this negative result could occur due to experimental artifact. For example, inefficient AAV-LoxP-RFP infection within *Nts^{FlpO};vGlut^{Cre}* mice might result in under-detection of LHA glutamate neurons. We therefore sought to validate the classical neurotransmitter content of LHA Nts neurons via a strategy that did not depend on AAV-mediated recombination. We attempted to generate dual-reporter mice, but commercially available FlpO and Cre reporter lines proved ineffective for simultaneous labeling of Nts and vGat or vGlut neurons. This also raised concern

that limited efficiency of FlpO-mediated recombination in *Nts*^{FlpO} mice might under-report LHA Nts neurons, and diminish the likelihood of detecting a small population of glutamatergic LHA Nts neurons.

As an alternate method, we treated *vGat*^{Cre}*;GFP* and *vGlut2*^{IRESCre}*;GFP* mice (validated in Figure 2-6) with colchicine, allowing for simultaneous visualization of GFP-labeled vGat and vGlut neurons and Nts-IR cell bodies (as in Figure 2-1). Similar to findings using the dual recombinase strategy, we observed numerous Nts-IR cell bodies within the LHA and ZI of colchicine-treated mice (Figure 2-9 A-F). While the ZI from *vGlut2*^{Cre}*;GFP* mice was devoid of GFP-vGlut2 neurons (Figure 2-9 E), *vGat*^{Cre}*;GFP* mice had many GFP-vGat neurons in the ZI, many of which also contained Nts-IR (Figure 2-9 F, white arrows). These data are consistent with the GABAergic phenotype of the ZI, and our findings using the dual recombinase system (Figure 2-8) that ZI Nts neurons are GABAergic but not glutamatergic. We observed many Nts-IR labeled cell bodies within the LHA (Figure 2-9 A-D), as well as GFP-labeled LHA glutamate neurons (Figure 2-9 C) and GFP-labeled GABA neurons (Figure 2-9 D). Despite the robust GFP-labeling induced in both lines, we did not observe any Nts-IR cell bodies within the LHA that co-localized with GFP-vGlut (Figure 2-9 C). By contrast, essentially all of the LHA Nts-IR cell bodies overlap with GFP-vGat (Figure 2-9 D, white arrows), but we also observed GFP-vGat neurons that did not contain Nts-IR (Figure 2-9 D, magenta arrows). In sum, colchicine-mediated Nts-IR recapitulated our findings using the dual-recombinase system: that LHA Nts neurons are GABAergic, but comprise a subset of the larger population of LHA GABA neurons.

2.5 Discussion

The LHA is essential for the motivation to eat and drink, but the neural mediators of these behaviors have yet to be fully understood. While most LHA populations promote food and

liquid intake, LHA Nts neurons divergently regulate ingestive behavior by suppressing feeding and promoting drinking [177;269] . Since LHA Nts neurons regulate ingestion in opposing directions, we hypothesized that there may be separate subpopulations of LHA Nts neurons to coordinate feeding vs. drinking behavior. Consistent with this, we have characterized two separate subpopulations of LHA Nts neurons that are differentially activated by leptin (Nts^{LepRb} neurons) or dehydration (Nts^{Dehy} neurons). While all LHA Nts neurons are GABAergic, the Nts^{LepRb} and Nts^{Dehy} subpopulations differ in molecular expression of LepRb and at the circuit level, and hence can be distinguished via these criteria. These data demonstrate, for the first time, the heterogeneity of LHA Nts neurons, and their specific responsiveness to either energy or fluid balance cues suggest that they may coordinate different ingestive behaviors (feeding vs. drinking). Going forward, it will be important to define the roles of Nts^{LepRb} neurons and Nts^{Dehy} neurons in coordinating ingestive behavior. The projection and molecular differences between neural subpopulations, as we have defined here, may enable development of molecular tools to selectively modulate either Nts^{LepRb} or Nts^{Dehy} neurons, and thereby to discern their receptive contributions to homeostasis.

LHA Nts neurons have been less studied compared to other LHA populations, largely because the methods to detect Nts-expressing cell bodies (ISH or colchicine-mediated Nts-IR) were difficult to employ. Furthermore, these methods impeded examination of any co-expressed markers, circuit differences or responses to physiologic status necessary to understand their function. We overcome these limitations by using Nts^{Cre} mice, which provide a non-invasive means of reliably identifying Nts neurons, and permit their study under normal, physiologic conditions. Using these mice, we found that ~12% of the LHA Nts neurons are Nts^{Dehy} neurons (Figure 2-5 C), and Nts^{LepRb} neurons make up a separate 15% of LHA Nts neurons [269]. While these are modestly sized populations, they can significantly influence

homeostasis; for example, mice lacking leptin-regulation via Nts^{LepRb} neurons have impaired ability to respond to energy balance cues and diminished dopamine signaling that cause them to become overweight [200;269]. Further characterization of the remaining 70% of LHA Nts neurons at the molecular and circuit level may provide insights about their function. For example, at least some LHA Nts neurons are activated by LPS-mediated inflammation and inhibit local orexin/hypocretin neurons, and these may contribute to illness-behavior [150]. Although these LPS-regulated LHA Nts neurons are distinct from LepRb-expressing neurons (data not shown), it remains unclear if they overlap with the Nts^{Dehy} population. Nts signaling is also implicated in regulation of analgesia, thermoregulation, stress and addiction, so it will be important to determine if/how the remaining LHA Nts neurons contribute to these diverse aspects of physiology. Characterizing the heterogeneity of LHA Nts neurons may also suggest intersectional or pharmacological strategies to target specific subpopulations of LHA Nts neurons, and hence selective physiological outputs.

LHA neurons project widely throughout the brain, and differentially modify behavior depending on what brain regions they target. Our finding that only Nts^{LepRb} neurons, and not Nts^{Dehy} neurons, project to the midbrain suggests that there are distinct LHA Nts neural mechanisms for leptin-mediated suppression of feeding vs. regulation of drinking and fluid balance. We previously showed that experimental activation of LHA Nts neurons causes release of Nts to the VTA, and dopamine release into the nucleus accumbens [194] that can modify motivated intake behavior [194;280;281]. Thus, at least some portion of anorectic leptin regulation via Nts^{LepRb} neurons occurs via their modulation of the mesolimbic dopamine signaling. This is consistent with the requirement of leptin action via Nts^{LepRb} neurons for adaptive energy balance in response to hormonal cues of energy status, and for regulating body weight and the integrity of the mesolimbic dopamine system, which are due in part to Nts

signaling via VTA neurons expressing neurotensin receptor-1 (NtsR1) [191;200]. In contrast, Nts^{Dehy} neurons must act via other yet-to-be determined projection targets, and do not directly modulate dopamine signaling to modify physiology. While the function of Nts^{Dehy} neurons and their projection sites remains to be established, the activation of Nts^{Dehy} neurons in response to dehydration suggests that they may coordinate fluid need with the motivation to drink, and perhaps to maintaining fluid homeostasis. The discovery of specific subsets of LHA Nts neurons data also provides context for understanding why experimental activation of all LHA Nts neurons results in diverging ingestive behaviors. Such activation simultaneously induces Nts^{LepRb} neurons that act via the VTA (and may be anorectic) as well as the Nts^{Dehy} neurons that regulate separate targets, and it is possible that these populations suppress feeding and promote drinking, respectively [177]. Since Nts^{LepRb} and Nts^{Dehy} neurons are induced by separate physiological cues (leptin or dehydration), it remains to be determined whether there are any physiological situations in which these subpopulations are concurrently activated. In any case, our data confirm that Nts^{LepRb} and Nts^{Dehy} neurons have distinct circuitry, thus projection-specific modulation may be a useful strategy to discern their respective contributions to ingestive behavior.

Our data reveal molecular and circuit heterogeneity of LHA Nts neurons, but surprisingly all LHA Nts neurons contain the same classical neurotransmitter, GABA. Thus, LHA Nts neurons presumably inhibit synaptic targets via release of GABA, as well as regulating postsynaptic and adjacent neurons via release of Nts. It remains to be determined if GABA and Nts are always co-released from LHA Nts neurons, and hence the importance of the dual neurotransmitter and neuropeptide signals for control of ingestive behavior. However, work from other LHA neurons suggests that different physiological stimuli bias the release of neurotransmitter vs. neuropeptide signals, and the receipt of these messages depends on the

repertoire of receptors expressed on target neurons, which can also vary [282-284]. In this sense, the dual Nts and GABA expression may permit signaling flexibility, such that LHA Nts neurons can adapt signaling in various contexts and via different circuits. Our finding that LHA Nts neurons are GABAergic is consistent with other reports of overlapping LHA Nts and GABAergic neurons [194;285] but contrasts with the reported population of glutamatergic LHA Nts neurons that directly project to the VTA [202]. This discrepancy may be due to characterization of different Nts neurons between this study and our own. We characterized LHA neurons in the vicinity of the perifornical region, roughly between Bregma -1.34 to 1.70 [271], but the reported glutamatergic LHA Nts neurons were identified around the "rostral lateral hypothalamus" corresponding to Bregma -0.4. In fact, Bregma -0.4 is well beyond the boundary of the perifornical LHA, and occurs at the rostral border of the LHA where it merges into the preoptic area. Hence, it is entirely possible that the perifornical LHA Nts neurons studied here are GABAergic, while the much more rostral population of LHA Nts neurons within the hypothalamus-preoptic continuum are glutamatergic. We did not assess LHA Nts neurotransmitter content in this rostral region, since it is well beyond the accepted LHA region defined by the presence of MCH and orexin/hypocretin neurons. However, in the future, we could use the dual recombinase system to define the neurochemistry of these, and other Nts neurons throughout the brain.

While all LHA Nts neurons contain GABA, they comprise only a minor subset of the vast population of LHA GABA neurons (Figure 2-8, 2-9). This may account for the strikingly different behaviors observed after experimental activation of LHA Nts neurons (suppression of feeding, increased drinking) vs. activation of all LHA GABA neurons (increased feeding, drinking and gnawing directed at non-biological objects) [177;194;266;267]. Since activation of LHA Nts and LHA GABA neurons promotes drinking, LHA Nts neurons contribute to at least some of the polydipsic effect. The orexigenic effect observed with activation of all LHA GABA neurons, however, likely

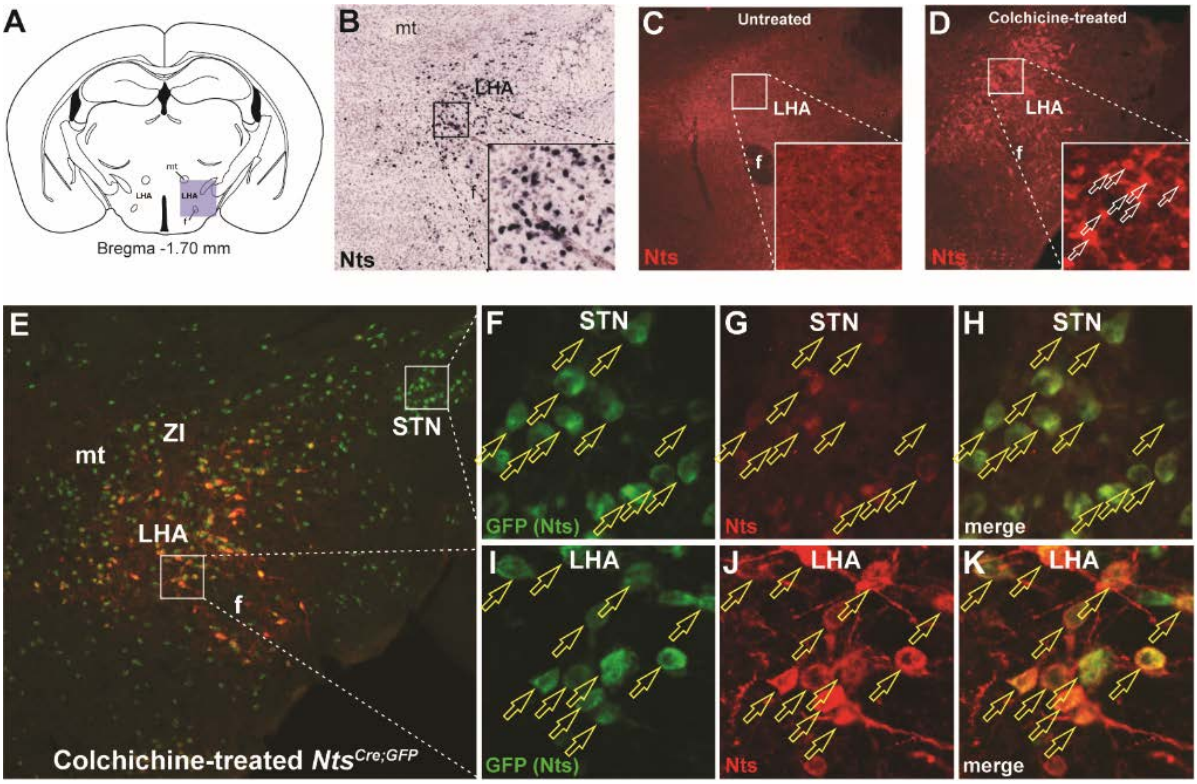
masks the anorectic effects mediated by the modest population of LHA Nts neurons (presumably Nts^{LepRb} neurons) encompassed within them. Our findings agree with reports that there are functionally-distinct subpopulations of LHA GABA neurons [265], and LHA Nts neurons identify a functionally unique subset within the larger population of all GABA neurons that suppresses feeding instead of promoting it. Additionally, the finding that LHA Nts neurons are a subset of LHA GABA neurons may explain the proposed differences in VTA regulation that have been ascribed to these populations. Some LHA GABA neurons project to the VTA, where they disinhibit VTA GABA neurons that in turn releases inhibition of DA neurons to facilitate DA release and feeding [266;286]. Some GABA-containing LHA Nts neurons, including Nts^{LepRb} neurons, also project to the VTA, and their precise synaptic targets are yet to be defined. However, NtsR1 is predominantly expressed by VTA dopamine neurons, suggesting that LHA Nts neurons might act via different targets then the general VTA-projecting LHA GABA neurons. Going forward, it will be important to distinguish how subsets of LHA Nts neurons and other LHA GABA neurons modify VTA signaling to understand how they differentially coordinate feeding behavior.

Taken together, our data reveal the heterogeneity of LHA Nts neurons, and suggest at least some molecular and projection differences between subpopulations that may be useful to modulate specific subsets. Since LHA Nts neurons are differentially regulated by energy status (leptin) vs fluid status (dehydration), and comprise separate subpopulations, our data intriguingly suggest that there are separate neural mechanisms to coordinate feeding and drinking necessary for homeostasis and survival. If true, then the molecular and projection features we report here may enable design of strategies to selectively modify the LHA Nts neurons that control feeding vs. those that modify drinking. Such strategies could prove useful

to treat life-threatening feeding disorders such as obesity or anorexia nervosa, or disrupted fluid balance, as commonly occurs due to aging-related loss of thirst or in psychogenic polydipsia.

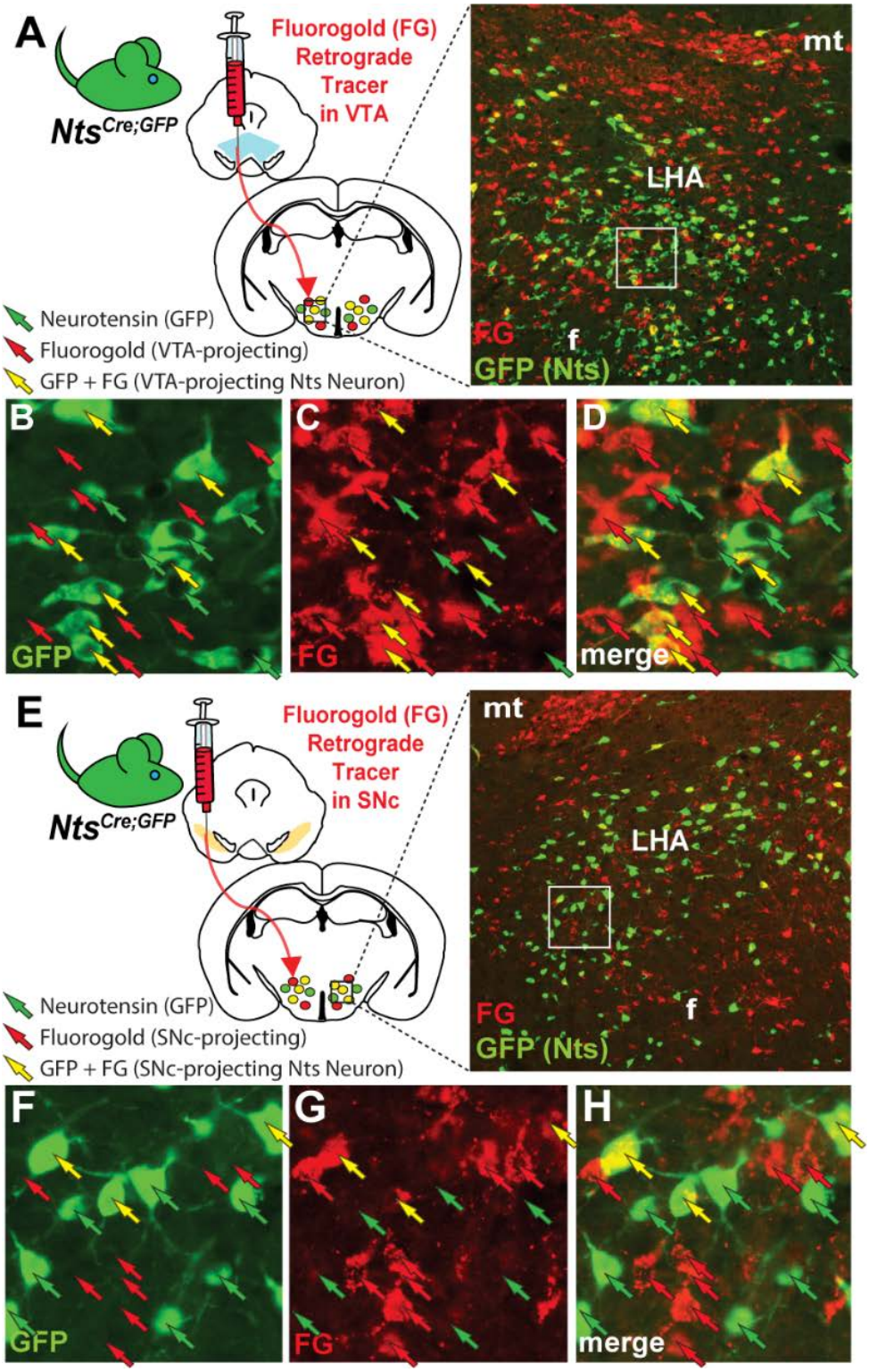
2.6 Figures

Figure 2-1: Validation of *Nts*^{Cre}*;GFP* Mice to Visualize LHA Nts Neurons



A) Representative portion of the LHA that contains B) *Nts*-expressing cell bodies detected via ISH (courtesy of the Allen Brain Atlas (Lein 2007), C) Nts-Immunoreactivity (IR) only identifies fibers within the LHA unless D) mice were pretreated with ICV colchicine, which inhibits axonal transport and permits detection of Nts-IR within cell bodies (white outline arrows). E) Nts-IR (red) in colchicine-treated *Nts*^{Cre}*;GFP* mice that express GFP in Nts neurons (green). F-H) Insets show the STN from E. F) Many GFP-labeled Nts cell bodies are found within the STN consistent with *Nts* ISH (Lein 2007),and the G) Nts-IR cell bodies in this region H) entirely overlap with the GFP (Nts) cells (yellow outline arrows). I-K) Insets show the LHA from E, where I) the GFP-labeled cell bodies and J) Nts-IR cell bodies K) overlap (yellow outline arrows). Together these data confirm that *Nts*^{Cre}*;GFP* mice correctly identify Nts-expressing cells, and can be used to visualize them.

Figure 2-2: LHA Nts Neuron Project to the VTA and SNc

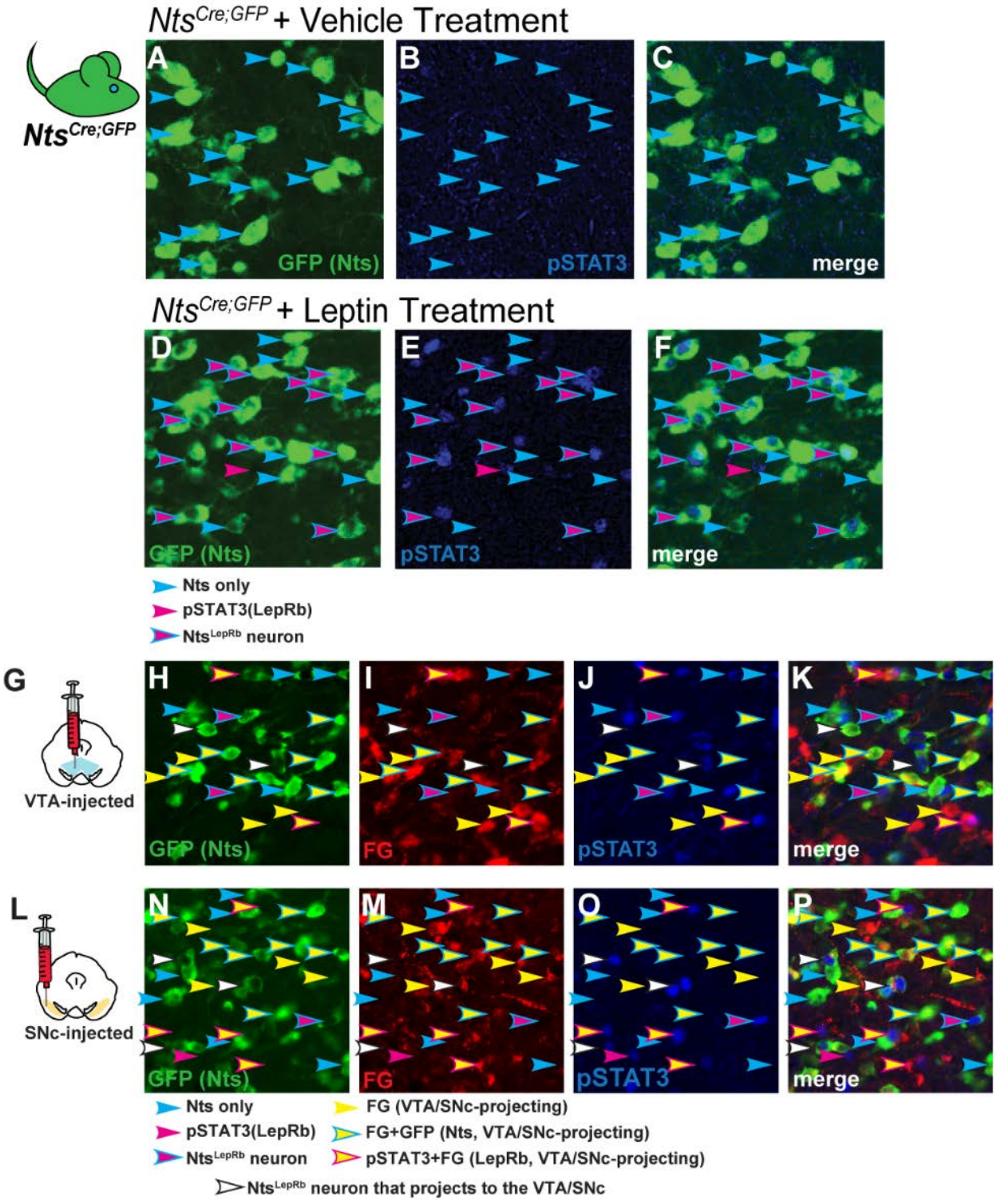


A) *Nts^{Cre};GFP* mice were injected in the VTA with the retrograde tract tracer FluoroGold (FG). B-D) Representative insets from the LHA showing B) GFP-labeled Nts cell bodies (green) and

Figure 2-2 (cont'd)

C) cell bodies that have accumulated FG (red) and from the VTA. D) Some GFP-labeled Nts cells contain FG, indicating LHA Nts neurons that project to the VTA (yellow arrows) while GFP-labeled Nts cells lacking FG do not project to the VTA (green arrows). Some non-Nts cells also project to the VTA (red arrows). E) *Nts^{Cre};GFP* mice received FG into the SNc. F-H) Insets from the LHA show F) GFP-labeled Nts cell bodies (green) and G) cell bodies that have accumulated FG (red) and from the SNc. The LHA contains cells co-labeled with Nts-GFP and FG indicating that they project to the SNc (yellow arrows), as well as GFP-labeled Nts neurons that lack FG and do not project to the SNc (green arrows). There are also non-Nts neurons that project to the SNc (red arrows). Together, these data demonstrate that many LHA Nts neurons project to the VTA and the SNc, but the projection density to the VTA is slightly more robust than to the SNc. VTA-injected n = 12, SNc-injected n = 11. Abbreviations: mt = mammillothalamic tract; f = fornix; LHA = lateral hypothalamic area.

Figure 2-3: Nts LepRb Neurons Project to the VTA and SNc

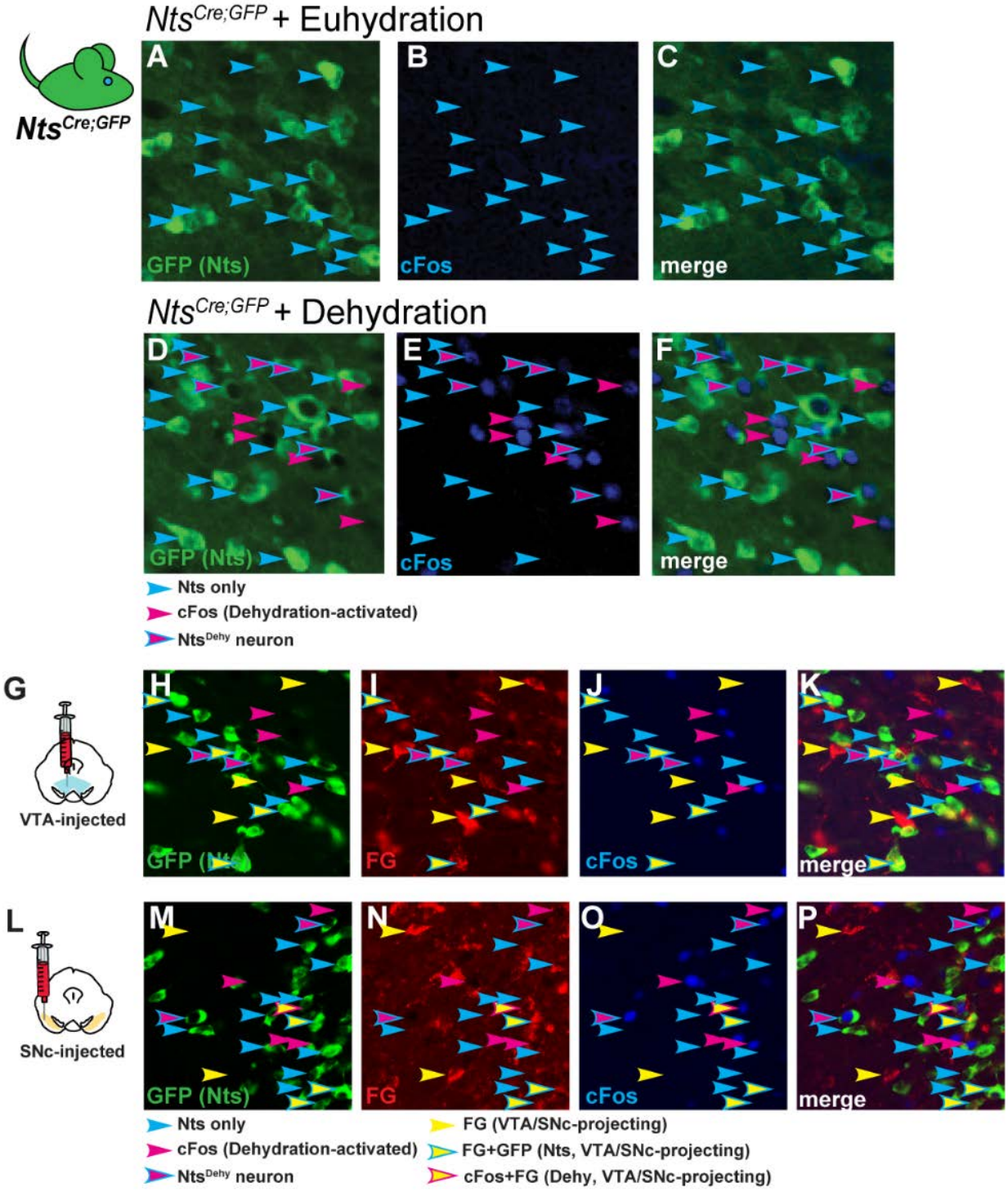


Nts^{Cre}*;GFP* mice were treated with A-C) vehicle or D-F) leptin (5mg/kg, IP, 2 hr) to permit detection of A) GFP-labeled Nts neurons (green) and B) phosphorylated STAT3 (pSTAT3), a marker for leptin-activated LepRb neurons (blue). Cyan arrows label Nts neurons without

Figure 2-3 (cont'd)

pSTAT3. Magenta arrows identify pSTAT3 that does not co-label with GFP (e.g. LepRb neurons that do not express Nts). Cyan-outlined magenta arrows identify GFP-labeled Nts neurons that co-localize with pSTAT3 and are Nts^{LepRb} neurons. G-P) *Nts^{Cre};GFP* mice received FG in the VTA or SNc (to identify midbrain projecting neurons) and were treated with leptin (5mg/kg, IP, 2 hr) to permit identification of LepRb neurons via induction of pSTAT3. Examination of the LHA from VTA-injected mice revealed H) GFP-labeled Nts neurons, I) FG-labeled neurons that project to the VTA and J) pSTAT3 neurons. K) Merged panels identify some neurons containing GFP, FG and pSTAT3 that are Nts^{LepRb} neurons that project to the VTA (white arrows). L-P) Examination of the LHA from SNc-injected mice reveal M) GFP-labeled Nts neurons, N) FG-labeled neurons that project to the SNc and O) pSTAT3 neurons. P) Merged panels identify some neurons containing GFP, FG and pSTAT3 that are Nts^{LepRb} neurons that project to the SNc (magenta-outlined yellow arrows). Key for other arrows: cyan arrows = Nts-GFP only neurons; magenta arrows = pSTAT3-only (LepRb) neurons; cyan-outlined magenta arrows = Nts^{LepRb} neurons that do not project to the VTA/SNc; yellow arrows = FG-only neurons that project to the VTA/SNc; cyan-outlined yellow arrows = Nts neurons that project to the VTA/SNc but do not contain LepRb; magenta-outlined yellow arrows = VTA/SNc projecting LepRb neurons that do no express Nts; white arrows = VTA/SNc-projecting Nts^{LepRb} neurons. These data demonstrate that at least some Nts^{LepRb} neurons project to the VTA and the SNc. VTA-injected vehicle-treated n=6; female VTA-injected leptin-treated n = 10; SNc-injected vehicle-treated n = 5; SNc-injected leptin-treated n = 9.

Figure 2-4: Dehydration-sensitive LHA Nts Neurons DO Not Project to the VTA or SNc

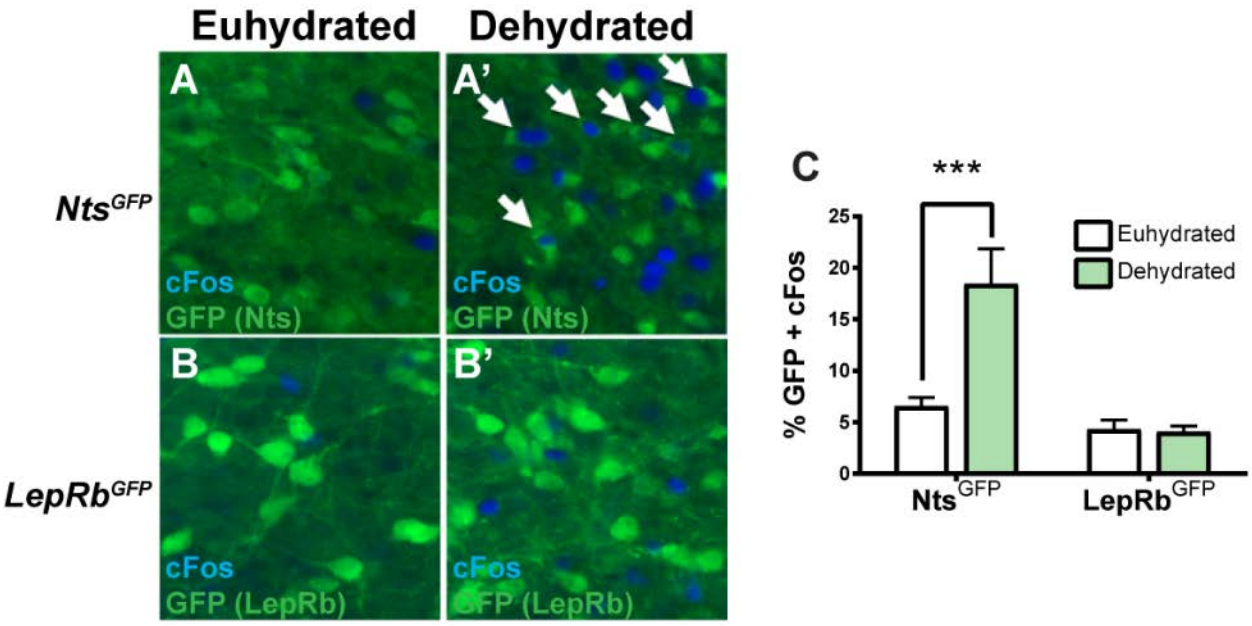


LHA NtsDehy Neurons Do Not Project to the VTA or SNc. A-F) NtsCre;GFP mice were given ad-lib water (Euhydrated) or were dehydrated overnight. Brains were assessed for GFP-labeled Nts neurons (green) and cFos, a marker of recent neuronal depolarization (blue). Cyan arrows label

Figure 2-4 (cont'd)

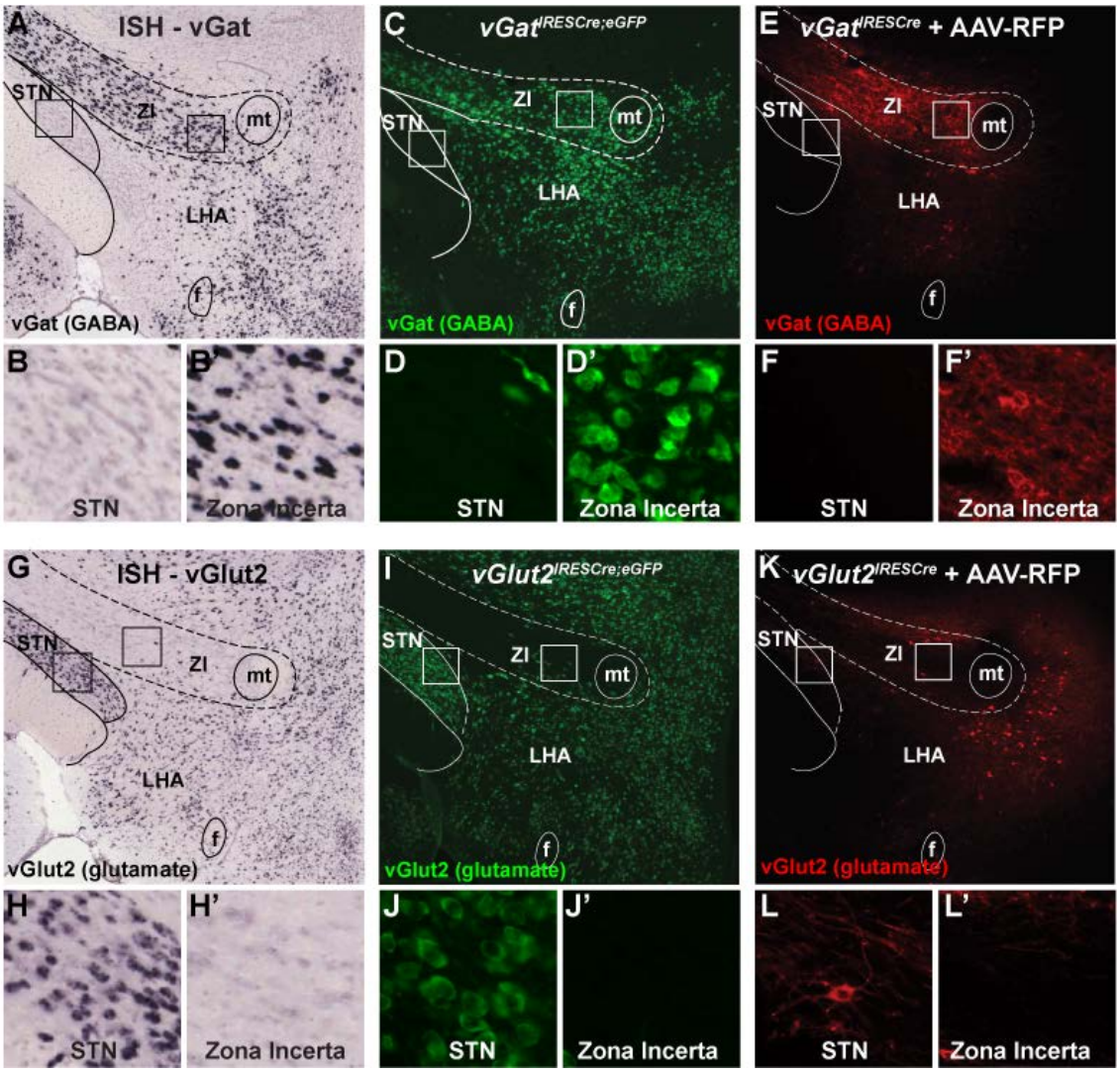
Nts-GFP neurons and magenta arrows identify dehydration activated neurons that do not express Nts-GFP. Cyan-outlined magenta arrows identify Nts-GFP neurons that co-express cFos (Nts^{Dehy} neurons). G-P) *Nts^{Cre};GFP* mice were injected with FG into the VTA or SNc (to identify midbrain projecting neurons) and dehydrated overnight (to identify dehydration-activated neurons via cFos-IR). Assessment of the LHA revealed some Nts^{Dehy} neurons (Cyan-outlined magenta arrows), but none of these accumulated FG. Similarly, L-P) Nts^{Dehy} neurons were found within the LHA of SNc-injected mice, but none of these contained FG. Key for arrows: cyan arrows = Nts-eGFP only neurons; magenta arrows = cFos-only (dehydration-activated) neurons; cyan-outlined magenta arrows = Nts^{Dehy} neurons that do not project to the VTA/SNc; yellow arrows = FG-only neurons that project to the VTA/SNc; cyan-outlined yellow arrows = Nts neurons that project to the VTA/SNc but are not activated by dehydration; magenta-outlined yellow arrows = dehydration-activated VTA/SNc projecting neurons that do no express Nts. No arrows are present to label VTA/SNc-projecting Nts^{Dehy} neurons because no such neurons were found. These data demonstrate that the subpopulation of LHA Nts^{Dehy} neurons does not project to the VTA or SNc. VTA-injected, euhydrated n = 5; VTA-injected, dehydrated n = 9; SNc-injected, euhydrated n = 5, SNc-injected, dehydrated n = 9.

Figure 2-5: LHA *Nts*^{LepRb} Neurons are Molecularly Distinct from *Nts*^{Dehy} Neurons



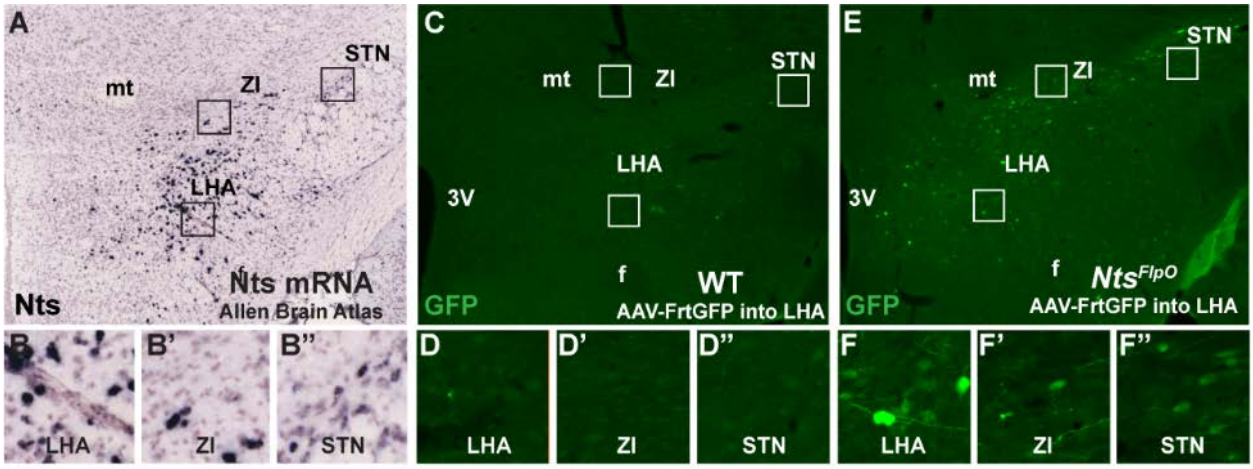
In order to further verify that the dehydration and leptin-sensitive subpopulations of LHA Nts neurons do not overlap we employed a mouse model that expresses GFP in all LepRb neurons (*LepRb;GFP* mice). *LepRb-GFP* mice and *Nts*^{Cre}*;GFP* mice were treated with dehydration (O.N) or given ad lib water and brains were immunostained for GFP and cFos (a marker of recent neuronal activation). A) Dehydration induces cFos expression within Nts neurons in the LHA. However, B) while cFos induction can been seen, there is no colocalization with LepRb neurons. C) Quantification of cFos induction in Nts vs LepRb neurons after dehydration. This data shows that dehydration does not activate LepRb neurons and therefor the LepRb expressing subpopulation of LHA Nts neurons is necessarily distinct from LHA Nts^{Dehy} neurons.

Figure 2-6: Visualization of LHA GABA and Glutamate Neurons



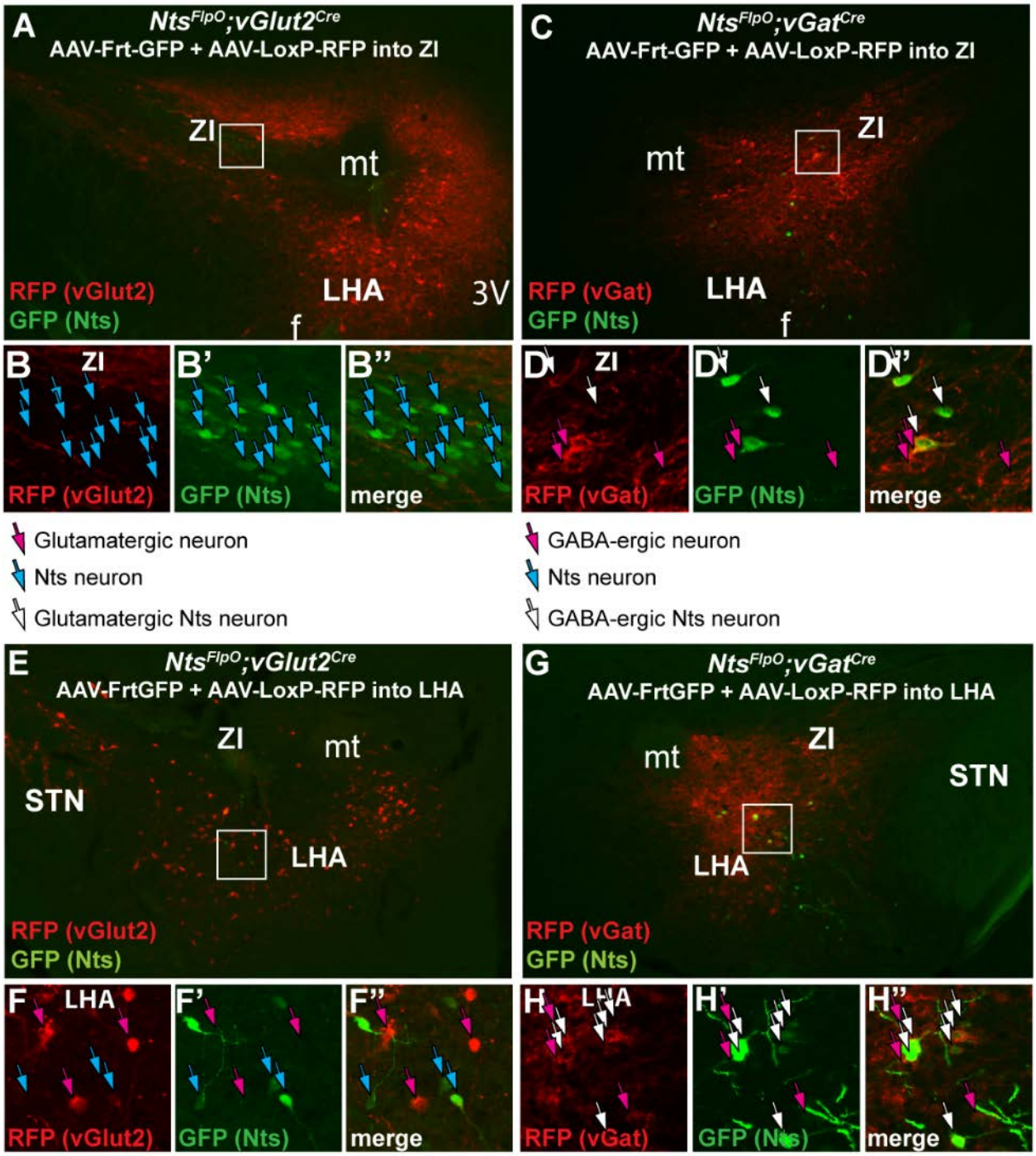
A) ISH for *vGat* expression from the Allen Brain Atlas (Lein 2007), B, B') Digital magnifications of the STN and ZI show that the STN lacks *vGa*t expression while the Zona Incerta (ZI) contains many *vGa*t-expressing cell bodies. C, D, D') *vGat^{Cre};GFP* mice show a similar distribution of vGat-GFP labeled cells as *vGat* ISH (A), including few vGat-GFP cells in the STN and many within the ZI. E, F, F') *vGat^{Cre}* mice injected with AAV Cre-inducible RFP have no RFP labeling in the STN but many ZI-labeled RFP cells, and mirror the distribution observed via *vGa*t ISH and from *vGat^{Cre};GFP* mice. G) ISH for *vGlut2* expression from the Allen Brain Atlas. Boxed regions are digitally magnified in H, H') and identify many *vGlut2* cell bodies in the STN but none within the ZI. I, J, J') *vGlut2^{Cre};GFP* mice identify a similar distribution of GFP-labeled *vGlut2* cells as the *vGlut2* ISH, including many GFP vGlut2-GFP cells in the STN but none in the ZI. K, L, L') Likewise, *vGlut2^{Cre}* mice injected in the LHA with AAV Cre-inducible RFP have some viral spread and vGlut2-RFP labeled cell bodies in the STN but none in the ZI. Collectively these data demonstrate that *vGat^{Cre}* and *vGlut2^{Cre}* mice can be used with Cre-inducible reporter mice or AAVs to reliably identify vGat and vGlut neurons. Abbreviations: mt = mammillothalamic tract; f = fornix; LHA = lateral hypothalamic area, STN = Sub-thalamic Nucleus, ZI = Zona Incerta.

Figure 2-7: FlpO-Mediated Detection of Nts Neurons



A-B'') *Nts* ISH identifies many Nts-containing cell bodies within the LHA, and some within the ZI and STN, courtesy of Allen Brain atlas (Lein 2007). C-D'') WT mouse injected with AAV-FrtGFP in the LHA shows no induced GFP expression within the LHA, ZI or STN. E-F'') *Nts*^{FlpO} mouse injected with AAV-FrtGFP in the LHA shows many GFP-labeled cell bodies around the injection site, similar to the distribution of *Nts* ISH (A). Many GFP-induced cell bodies are found within the LHA and some are observed within the ZI and STN. These data confirm the specificity of Nts^{FlpO} model and AAV-FrtGFP to identify Nts neurons in a Cre-independent manner. Abbreviations: mt = mammillothalamic tract; f = fornix; LHA = lateral hypothalamic area, STN = Sub-thalamic Nucleus, ZI = Zona Incerta.

Figure 2-8: Dual Recombinase Identification of Neurotransmitter Content of Nts Neurons

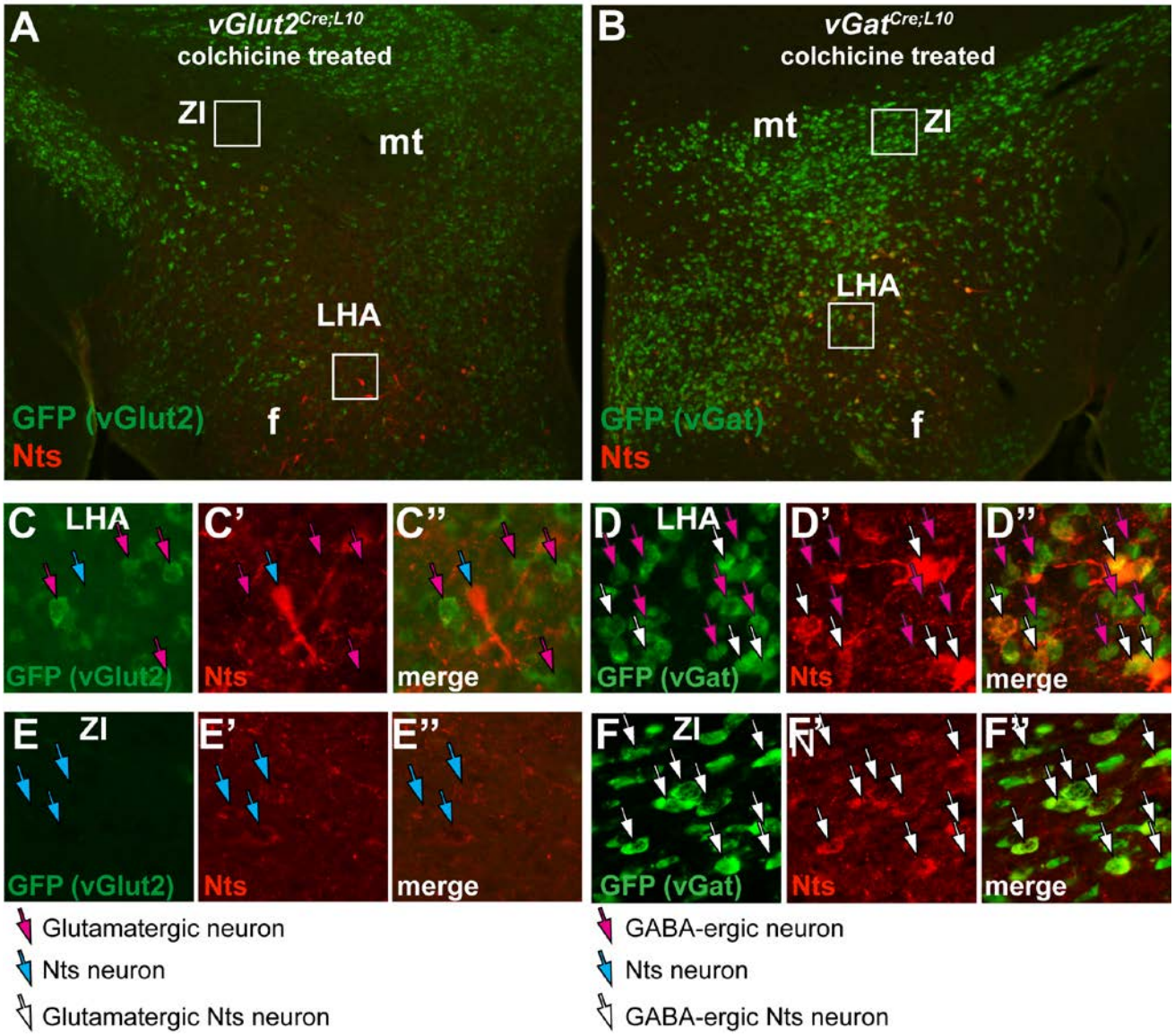


Content of Nts Neurons. A-B'') *Nts*^{FlpO}*;vGlut2*^{Cre} mice were injected in the ZI with AAV-FrtGFP (to identify Nts neurons, green) and AAV CreRFP (to identify vGlut2/glutamate neurons, red). Digital magnification of the boxed area in A revealed many GFP-Nts neurons (cyan arrows) but no vGlut2 neurons in the ZI, consistent with the GABAergic nature of this brain region. C-D'') Dual AAV injection into the ZI of *Nts*^{FlpO}*;vGat*^{Cre} mice identifies vGat/GABA neurons in the ZI (magenta arrows), and all observed Nts-vGat2 neurons contain vGat2 (white arrows). These

Figure 2-8 (cont'd)

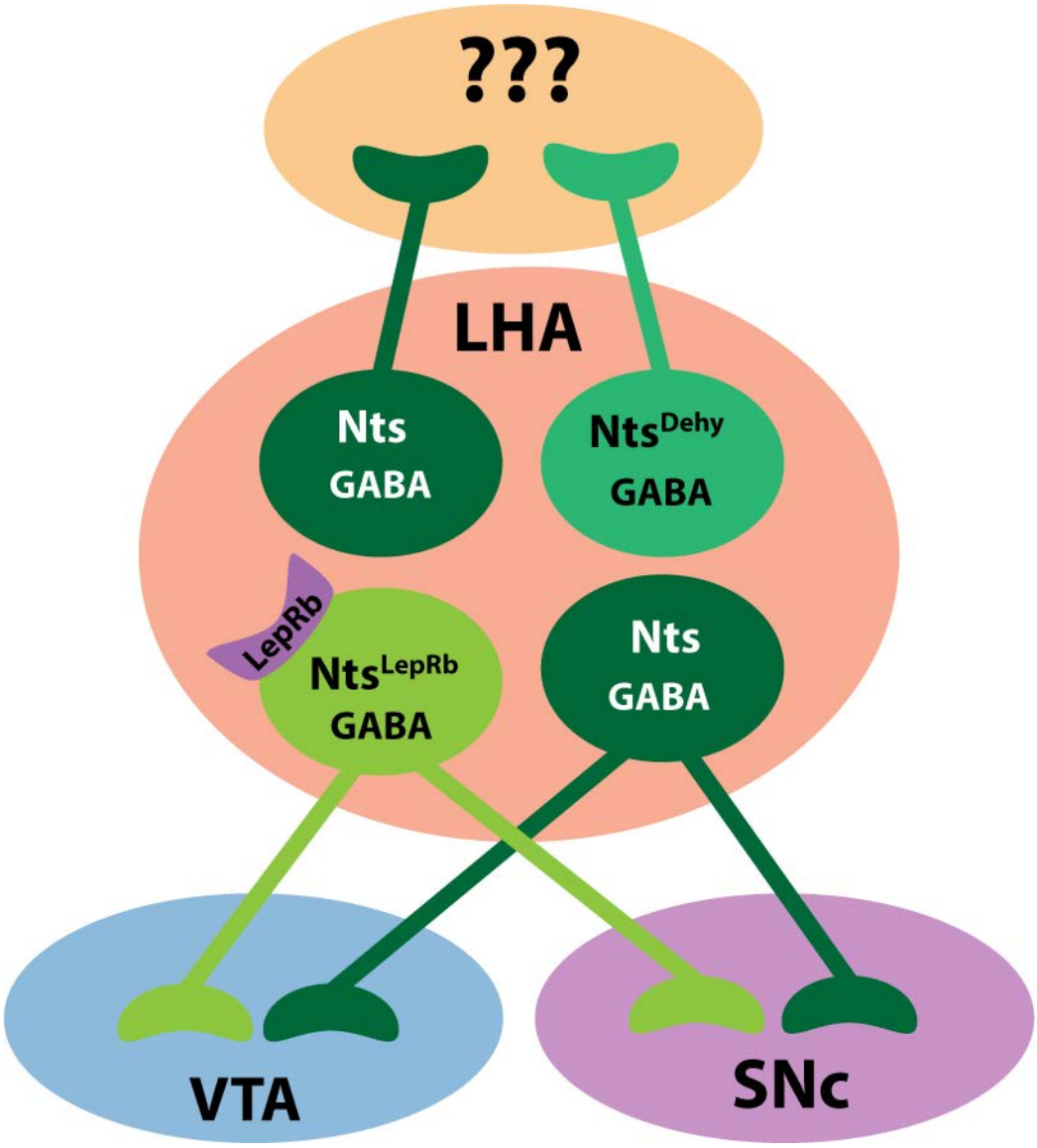
data confirm that the dual recombinase method correctly discerns GABA vs glutamate-containing areas of the brain while also permitting identification of Nts neurons. E-F'') Dual AAV injection into the LHA of *Nts^{FlpO};vGlut2^{Cre}* mice identifies RFP-vGlut2 neurons (magenta arrows) and GFP-Nts neurons (cyan arrows). No overlapping RFP-vGlu2 and GFP neurons were observed, indicating that LHA Nts neurons do not contain glutamate. G-H'') Dual AAV injection into the LHA of *Nts^{FlpO};vGat^{Cre}* mice revealed many RFP-vGat neurons (magenta arrows), and also GFP-Nts neurons that overlapped with RFP-vGat cells (white arrows). Together, these data confirm that LHA Nts neurons express GABA but not glutamate. *Nts^{FlpO};vGlut2^{cre}* n=5, *Nts^{FlpO};vGat^{Cre}* n=6). Abbreviations: mt = mammillothalamic tract; f = fornix; LHA = lateral hypothalamic area, STN = Sub-thalamic Nucleus, ZI = Zona Incerta.

Figure 2-9: Confirmation of Dual Recombinase Results With Colchicine Treatment



A, B) *vGlut2^{Cre};GFP* mice and *vGat^{Cre};GFP* mice were treated with colchicine to permit detection of Nts-IR (red) and GFP (green). C-C'') In *vGlut2^{Cre};GFP* mice, many GFP-vGlut2 cell bodies are found in the LHA (magenta arrows) along with Nts-IR cell bodies (cyan arrows), but no overlapping cells were found. E-E'') Analysis of the ZI from *vGlut2^{Cre};GFP* mice revealed a few Nts-IR neurons, but no vGlut2-GFP cells, consistent with the GABAergic neurochemistry of the ZI. D-D'') The LHA of *vGat^{Cre};GFP* mice contained many GFP-vGat cell bodies (magenta arrows) and Nts-IR cells that all co-labeled with GFP-vGat (white arrows). D-D') Similarly, co-labeling was observed in the GABAergic ZI, F-F') but not in *vGlut2^{Cre};GFP* mice. These data confirm that LHA Nts neurons contain vGat and are GABAergic, but do not contain vGlut/glutamate. *vGlut2^{Cre};GFP* mice n=4; *vGat^{Cre};GFP* mice n=5. Abbreviations: mt = mammillothalamic tract; f = fornix; LHA = lateral hypothalamic area, STN = Sub-thalamic Nucleus, ZI = Zona Incerta.

Figure 2-10: Model of LHA Nts Subpopulations and Their Neurotransmitter Content and Projection Targets



All LHA Nts neurons co-express the classical neurotransmitter, GABA and some project to the VTA and SNc. Of these, there are distinct subpopulations of LHA Nts neurons that respond to different anorectic cues; some are leptin-sensing (Nts^{LepRb} neurons) and some are dehydration-activated (Nts^{Dehy} neurons). Nts^{LepRb} neurons project the VTA and SNc where they can access the mesolimbic DA circuit, but Nts^{Dehy} neurons do not. Collectively, these data describe functionally, molecularly and projection-distinct subpopulations of LHA Nts neurons that may contribute to adaptive energy balance.

CHAPTER 3 Loss of Action via Neurotensin-Leptin Receptor Neurons Disrupts Leptin and Ghrelin-Mediated Control of Energy Balance

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3.1 Abstract

The hormones ghrelin and leptin act via the lateral hypothalamic area (LHA) to modify energy balance but the underlying neural mechanisms remain unclear. We investigated how leptin and ghrelin engage LHA neurons to modify energy balance behaviors and whether there is any cross-talk between leptin and ghrelin-responsive circuits. We demonstrate that ghrelin activates LHA neurons expressing Hypocretin/Orexin (OX) to increase food intake. Leptin mediates anorectic actions via separate neurons expressing the long form of the leptin receptor (LepRb), many of which co-express the neuropeptide neurotensin (Nts); we refer to these as Nts^{LepRb} neurons. Since Nts^{LepRb} neurons inhibit OX neurons, we hypothesized that disruption of the Nts^{LepRb} neuronal circuit would impair both Nts^{LepRb} and OX neurons from responding to their respective hormonal cues, thus compromising adaptive energy balance. Indeed, mice lacking leptin action via Nts^{LepRb} neurons, in a from-birth knock out model, exhibit blunted adaptive responses to leptin and ghrelin that discoordinate the mesolimbic dopamine system, and ingestive and locomotor behaviors, leading to weight gain. Collectively these data confirm that Nts^{LepRb} neurons are important neuronal hubs within the LHA for hormone-mediated control of ingestive and locomotor behaviors and the regulation of body weight.

3.2 Introduction

Adaptive energy balance is the process by which the brain detects changes in energy status and directs appropriate feeding and energy expenditure behaviors to resolve the imbalance. The lateral hypothalamic area (LHA) is crucial for this process, as demonstrated by rodents with LHA lesions that lose motivation to ingest and move even in the face of starvation [31;32]. Understanding of how the LHA mediates adaptive energy balance, however, has been complicated by the neuronal complexity of this brain region [287]. For example, LHA neurons containing the classical neurotransmitters GABA or glutamate modify ingestion, but yet-to-be defined subpopulations appear to control distinct aspects of feeding behavior [264-266]. LHA neurons can also be defined by their neuropeptide expression, such as populations that express the neuropeptides melanin concentrating hormone (MCH) or hypocretin/Orexin (OX) and play important roles in arousal and promoting feeding [81;106;288]. The LHA also contains neurons that express the neuropeptide neurotensin (Nts), which are implicated in suppressing feeding behavior in response to dehydration-anorexia [289], inflammation [150] or the anorectic hormone leptin [200]. Indeed, at least some LHA Nts neurons express the long form of the leptin receptor (LepRb) and are directly activated by leptin: we refer to these as Nts^{LepRb} neurons.

LHA neuronal populations may detect specific energy cues, and thus differentially control feeding and locomotor behavior to adapt energy balance. The LHA receives two important hormone regulators of adaptive energy balance: leptin and ghrelin. Leptin is made by adipose cells in proportion to energy stores, is released into the circulation and binds to LepRb-expressing neurons throughout the brain [2]. Leptin acts specifically via LepRb neurons in the LHA to modify feeding, physical activity and nutrient reward, and loss of leptin action via these neurons promotes weight gain [200;290]. Cells of the stomach and gastrointestinal tract secrete ghrelin, a hormone that acts via neurons expressing the growth hormone secretagogue receptor

(GHSR) to increase food intake [291]. LHA OX neurons express GHSR, are activated by ghrelin and mediate at least some part of the orexigenic response to this hormone [292-294]. Since leptin and ghrelin depolarize target neurons in the LHA [200;295] but promote opposing behaviors, it is tempting to speculate that they act via separate LHA populations. For example, there are leptin-regulated Nts^{LepRb} neurons and ghrelin-regulated OX neurons that each project to the dopamine-rich ventral tegmental area (VTA) [255;296] and promote dopamine release to the nucleus accumbens (NAc) that can modify feeding and locomotor activity. Furthermore, leptin and ghrelin action via the LHA engages the dopamine signaling system to suppress or promote feeding, respectively [191;200;297]. Thus, there may be separate leptin and ghrelin-mediated LHA circuits to coordinate peripheral need and dopamine-mediated behaviors required to restore energy balance. It remains possible, however, that there is also cross talk between leptin and ghrelin-mediated neural circuits to coordinate hormonal responses. Indeed, since Nts^{LepRb} neurons project to and inhibit OX neurons [200;298], leptin action via Nts^{LepRb} neurons might be able to suppress the ability of OX neurons to respond to ghrelin. Understanding precisely how leptin and ghrelin engage the LHA is crucial to understand the regulation of adaptive energy balance, and whether pharmacological modulation of these circuits might be a useful strategy to modify body weight.

Here we examined the neural mechanisms by which leptin and ghrelin coordinate adaptive energy balance via the LHA. We found that leptin and ghrelin act directly via separate LHA populations- Nts^{LepRb} and OX neurons, respectively. However, since Nts^{LepRb} neurons project to and inhibit OX neurons [200;298] we speculated that leptin action via Nts^{LepRb} neurons suppresses OX neurons and their ability to respond to ghrelin. In the face of increased adiposity this circuit arrangement would simultaneously enable leptin action while suppressing ghrelin-mediated signaling via the LHA, thus favoring weight loss behaviors. Loss of action via

Nts^{LepRb} neurons, however, could derange the response to both leptin and ghrelin. We therefore investigated whether loss of leptin signaling via Nts^{LepRb} neurons impairs the Nts^{LepRb} → OX neuronal circuit, and thus prevents adaptive energy responses to both leptin and ghrelin.

3.3 Materials and Methods

3.3.1 Animals

All procedures were approved by the Michigan State University and the University of Michigan Institutional Animal Care and Use Committees, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and National Institute of Health guidelines. Mice were bred in house, maintained on a 12h light/dark cycle with a*d libitum* access to food and water, unless otherwise noted in experimental methods. Male mice were used in all metabolic studies, and both males and females were used to examine the distribution of LHA neurons.

Nts^{cre} mice [200] [Jackson stock ♯ 017525], were crossed onto the C57/Bl6 line for seven generations to obtain fully backcrossed animals. To visualize Nts neurons, *Nts^{cre}* mice were crossed with $Rosa26^{EGFP\text{-}L10a}$ mice [273], and heterozygotes were studied (referred to as *Nts^{EGFP}* mice). Mice lacking LepRb expression in Nts neurons (LRKO mice) were generated similar to [200], but here only C57/Bl6 backcrossed animals were utilized. Briefly, mixed background $Lepr^{fl/fl}$ mice were crossed onto the C57/Bl6 background (Jackson #008327) for 7 generations. Next, backcrossed *Nts^{cre}* mice were bred with backcrossed $Lepr^{fl/fl}$ mice to generate $Nts^{cre/+}$;$Lepr^{fl/+}$ and $Nts^{+/+}$;$Lepr^{fl/+}$ mice, which were subsequently intercrossed to obtain $Nts^{cre/+}$;$Lepr^{fl/fl}$ study animals (LRKO) and $Nts^{+/+}$;$Lepr^{fl/fl}$ littermate controls (controls). Note that all resulting study mice are on

the C57/Bl6 background. Male LRKO and control study mice were single housed at 4 wk of age and studied between 8 and 37 wk of age.

DNA was extracted from tail biopsies of progeny and analyzed via standard PCR to identify study animals. *Nts*^{cre}*:* common forward: 5' ATA GGC TGC TGA ACC AGG AA, cre reverse: 5' CCA AAA GAC GGC AAT ATG GT and WT reverse: 5' CAA TCA CAA TCA CAG GTC AAG AA. *Rosa26*$^{EGFP\text{-}L10a}$: mutant forward: 5' TCT ACA AAT GTG GTA GAT CCA GGC, WT forward: 5' GAG GGG AGT GTT GCA ATA CC and common reverse: 5' CAG ATG ACT ACC TAT CCT CCC). *Lepr*$^{fl/fl}$ mice were genotyped to verify cre-mediated deletion and the absence of germline *Lepr* excision (referred to as LeprΔ): mLepR-105: 5' TGA ACA GGC TTG AGA ACA TGA ACA C, mLepR-65-A: 5' AGA ATG AAA AAG TTG TTT TGG GAC GAT, mLepR-106: 5' GGT GTC TGA TTT GAT AGA TGG TCT T). Mice that genotyped as LeprΔ were not used for studies or for breeding purposes and were euthanized. A terminal tail biopsy was collected at the conclusion of each study, and isolated DNA was used to verify genotype.

3.3.2 Reagents

Recombinant mouse leptin was purchased from the National Hormone and Peptide Program (Los Angeles Biomedical Research Institute, Los Angeles, CA), and mice were treated with 5 mg/kg leptin via intraperitoneal (IP) injection; this leptin dose optimally identifies leptin-responsive LepRb neurons throughout the hypothalamus [191]. Recombinant rat ghrelin was purchased from the PolyPeptide Group (Torrance, CA). Mice were treated with either intracerebroventricular (ICV, 3 µg) or IP (100 µg) ghrelin. In all cases, control injections consisted of equal volumes of vehicle solution. D-Amphetamine hydrochloride (Cayman

Chemical, Ann Arbor MI) was administered to assess activation of the dopamine system (4 mg/kg, IP).

3.3.3 Immunohistochemistry and immunofluorescence

Mice were anesthetized with a lethal dose of IP pentobarbital and transcardially perfused with PBS followed by 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Brains were removed, post-fixed overnight in 10% formalin and then dehydrated in 30% sucrose before coronal sectioning (30 µm) into four series using a freezing microtome (Leica, Buffalo Grove, IL). A single series of brain sections was analyzed for each immunostaining experiment. To identify Nts^{LepRb} neurons in the LHA, *Nts*^{EGFP} mice were treated with leptin (5 mg/kg, IP, 2h) and brains were analyzed for EGFP (Nts), OX and phosphorylated-STAT3 (pSTAT3; a specific marker of leptin-activated LepRb neurons [299]. To identify neurons activated by ghrelin, *Nts*^{EGFP} mice were treated with ghrelin (100 µg, IP, 4 hr) and brains were analyzed for EGFP (Nts), OX and cFos (a marker of neuronal depolarization). Leptin and ghrelin-induced neuronal activation in the NAc was assessed in LRKO and control mice treated for 4 hr with IP leptin, ghrelin or vehicle (as described above), and brains were analyzed via immunohistochemistry for cFos. Immunostaining was performed as previously described [255]. Briefly, brain sections were exposed to primary antibodies for either cFos (Santa Cruz, goat, 1:500) or pSTAT3 (Cell Signaling, rabbit, 1:500), followed by species-specific biotinylated antibodies (Jackson ImmunoResearch, 1:100), avidin-biotinylation reaction (Vectastain, Vector Laboratories) and diaminobenzidine (DAB) detection (Sigma). Other antibodies were subsequently added and visualized via immunofluorescence, using species-specific Alexa-488 conjugated (Jackson ImmunoResearch, 1:200) or Alexa-568 conjugated antibodies (LifeTech, 1:200). Antibodies used for immunofluorescence included GFP (Abcam, chicken, 1:2000), dsRed/Tomato (Clonetech, rabbit, 1:1000), Orexin-A (Santa Cruz, goat, 1:1000) and MCH (Santa Cruz, goat,

1:1000). Brains were analyzed using an Olympus BX53 fluorescence microscope outfitted with transmitted light to analyze DAB-labeled tissue, as well as FITC and Texas Red cubes. Microscope images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe, San Jose, CA). Cell counts were determined from microscope images, and an average of 6 LHA sections were counted from each brain. For counting purposes, the LHA was designated as the area below and lateral to the mammillothalamic tract (mt) and above the fornix. Each cell type was counted individually and only once (i.e. cells expressing Nts only were counted separately from those expressing Nts and pSTAT3). Counts were made in one series of brain sections, but were multiplied by 4 to obtain total number of neurons per mouse. Graphs represent the average number of neurons ± SEM.

3.3.4 Stereotaxic Injection for ICV Ghrelin Treatment and Tract Tracing

ICV cannulas were placed in LRKO and control mice to deliver ghrelin centrally. Mice were administered pre-surgical analgesic, then were anesthetized using vaporized isoflurane/O_2 and placed in a stereotaxic frame. After exposing the skull, an indwelling 26-gauge stainless steel cannula with a removable dummy injector (Plastics One, Roanoke, VA) was implanted into the ventricle. Coordinates to the lateral ventricle (from bregma) were A/P: -0.34, M/L: -1.0 and D/V: -2.4 in accordance with the atlas of Paxinos and Franklin [271]. Mice were allowed to recover for 1 week. For treatment, the dummy was replaced with an injector with a 2.45 mm projection used to deliver either 3 µL of sterile PBS or ghrelin (1 µg/µL), thus 3 µg total ghrelin was administered. Mice were excluded from analysis if their cannulas were misplaced or if they failed to gain weight and eat normally during the surgical recovery period prior to the study.

Cre-mediated tract tracing was performed in *Nts*^{Cre} and *Nts*$^{cre/+;Leprfl/fl}$ mice similar to above, but instead a guide cannula with a stylet was placed into the LHA (A/P: 1.34, M/L, 1.13 and D/V, 5.20 in accordance with the atlas of Paxinos and Franklin [271]. The stylet was removed and replaced by an injector, via which 250 nL of Adenoviral-Synaptophysin-mCherry (Ad-Syn-mCherry, graciously provided by Dr. Martin G. Myers Jr, University of Michigan) was injected into the LHA at a rate of 100 nL/min. After 5 min the injector and cannula were removed from the skull, and the incision was closed using VetBond surgical adhesive. Mice were then housed individually for 5–7 days to allow for viral-mediated expression of new proteins and their transport before euthanasia and tissue collection. Mice were only included for study if Syn-mCherry expressing cell bodies were confined to the LHA.

3.3.5 Metabolic Profiling:

LRKO and control mice were weaned at 4 weeks of age, maintained on standard rodent chow, and single housed at 7 wk of age. Body composition was measured between 8-10 wk of age using an NMR-based analyzer (Bruker Minispec L550, Billerica, MA). Mice were then placed in TSE PhenoMaster metabolic cages (TSE Systems, Chesterfield MO), which are home-cage sized cages outfitted with calorimetry sensors and beam-break sensors to detect –x, -y and –z plane movement. Ambient temperature was maintained at 20-23˚C throughout analysis and the airflow rate through the chambers was adjusted to maintain an oxygen differential around 0.3% at resting conditions. VO_2 and VCO_2 in each chamber were sampled for 3 min bouts rotating through each cage and –x, -y and –z beam breaks were recorded continuously. Mice were acclimated in chambers for 1 day then measurements were collected for three full 24 hr cycles. Data from the last full 12 hr light and 12 hr dark cycle were used for analysis. Mice were returned to their home cages for 1 wk, then were placed in TSE cages

containing a running wheel to assess their voluntary movement, including the amount of time spent on the wheel and running speed.

3.3.6 Sucrose preference testing

LRKO and control mice (10–16 wk) were single housed for at least one week prior to two-bottle sucrose preference testing. For baseline testing of water intake, mice were given two water bottles with sipper tubes, which were placed adjacent to the food hopper. The positions of the bottles were swapped each morning to control for any proximity preference for the bottle closest to the food hopper. Next, the content of one of the bottles was replaced with 1% sucrose, such that the mice had constant access to both water and sucrose-containing bottles. In one experiment, mice were treated every 12 hr for 2 days with vehicle, then with leptin (5 mg/kg, IP), and their daily liquid consumption was measured at 08:00 by weighing the bottles. In a separate experiment, mice were treated every 12 hr for 2 days with vehicle, followed by ghrelin (100 µg, IP), during which daily liquid consumption was measured at 17:00 by weighing the bottles. Data are reported as sucrose preference, which is the percentage of sucrose consumed out of total liquid consumed. Total water consumption during baseline testing days and during preference testing days was also calculated but no differences in water intake were observed between genotypes.

3.3.7 Operant Responding

Single-housed LRKO and control littermates ages 9 – 37 weeks were given 15 sucrose testing pellets (20 mg sucrose tablets, TestDiet, St. Louis, MO) in their home cage the night before beginning daily testing in operant chambers (Med Associates, St. Albans, VT) to prevent neophobia to sucrose rewards. Each session in the operant chambers lasted 1 hr or until mice

the received a maximum of 50 sucrose rewards. On the first training day a sucrose pellet was delivered into the magazine every 30 seconds for a maximum of 50 pellets, so that the mice learned where to obtain sucrose. Mice were then food restricted to 85% of their average food intake, during which they trained to nose-poke for sucrose pellets on a fixed ratio (FR)-1 schedule and then an FR-5 schedule. The food restriction was used to incentivize mice to learn the nose-poking task, and is commonly used for food-related operant testing. During FR-1 a nose poke in the active port results in delivery of one sucrose pellet to the magazine, followed by a 5-second timeout during which nose poking will not elicit a sucrose reward (to allow the mouse time to eat the pellet). No sucrose pellet is delivered in response to nose poking in the inactive port. The position of the active poke (right or left side of the chamber) was counterbalanced between mice. Mice were trained on FR-1 until they received a minimum of 20 rewards with ≥ 75% accuracy (active pokes out of total active and inactive pokes) for three consecutive days. Next the mice were trained for three days on an FR-5 schedule (5 active nose pokes required to receive a sucrose reward, with a 5 second time out). Mice were then restored *ad libitum* food in their home cages throughout progressive ratio (PR) testing. During PR testing the response ratio was calculated such that the number of responses required to earn a food reward followed the order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 and so on. The final ratio completed is the breakpoint. Mice were tested via PR until they maintained a stable breakpoint for a minimum of 3 days (defined as the point at which the number of earned rewards deviates by no more than 1 reward.) After achieving stable PR, each mouse was treated with IP vehicle, leptin (5 mg/kg) or ghrelin (100 µg) 1 hr prior to PR testing to assess how these hormones modify motivated responding. Mice received only one hormone per day, but each mouse received all of the treatments over the course of the experiment.

3.3.8 Gene Expression.

LRKO (n=10) and control mice (n=10) were euthanized and brains were microdissected to obtain the LHA, VTA and NAc. The microdissection of the LHA is performed such that it is enriched in MCH, OX and Nts neurons located above the fornix (the perifornical region) and just lateral and below the MT, roughly between Bregma -2.18 mm and -1.06 mm [271]. Two of the LHA samples from control mice were excluded from analysis because the dissections were too rostral, and were not centered on the perifornical region. The tissue samples were snap frozen on dry ice and stored at -80˚C for later processing. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and 200 ng samples were converted to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Sample cDNAs were analyzed in triplicate via quantitative RT-PCR for gene expression using TaqMan reagents and an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA). *GAPDH* expression was used as an internal control. Relative mRNA expression values are calculated by the $2^{-\Delta\Delta Ct}$ method, with normalization of each sample to the average ΔCt value from control mice. Two of the LHA samples from LRKO mice were compromised during tissue processing and were not included in the final results. Additionally, there was a pipetting error while preparing one of the control LHA samples for *Dlk1* RT-PCR, thus it was excluded from the final analysis.

3.3.9 Amphetamine-Induced Assessment of Striatal Activation

To assess activation of the dopamine system, animals were treated with D-amphetamine hydrochloride (4mg/kg, IP). Mice were perfused 2 hr after treatment, and brains were immunostained for cFos as described above.

3.3.10 Data analysis

Paired t-tests (to compare two groups) or one-way ANOVA with Bonferroni post-testing (for comparisons between multiple groups) were calculated using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Error bars depict ± standard error of the mean (SEM). Differences were considered significant for $p < 0.05$.

3.4 Results

3.4.1 Neurotensin Neurons are a Distinct Population of LHA Neurons and Some Respond to Leptin

As a first step, we examined the distribution of three neuropeptide-defined populations of LHA neurons that have been implicated in regulating energy balance: those containing MCH, OX or Nts. Since Nts-expressing neurons cannot be visualized using standard immunolabeling techniques, we examined brains from *Nts*^{EGFP} mice for expression of Nts-EGFP to identify Nts neurons, along with immunofluorescent labeling of MCH and OX neurons (Figure 3-1 A). While neurons expressing MCH, OX and Nts are co-distributed throughout the LHA, they do not overlap, indicating that they comprise three separate populations of neurons. Neuronal counts (Figure 3-1 B) reveal similarly sized MCH and OX neuronal populations within the LHA (MCH = 2596 ± 497 neurons, OX = 2779 ± 319 neurons, n = 4) but there are three times as many Nts-EGFP-labeled neurons (Nts = 8832 ± 494 neurons, n =4). These data confirm that LHA Nts neurons are a distinct, highly abundant neuronal population within the LHA with presumably unique contributions to physiology.

The LHA also contains neurons expressing LepRb, some of which co-express Nts [200]. Given the large number of LHA Nts neurons, we sought to clarify what proportion of them

express LepRb and thus can adaptively modify energy balance in response to leptin. We treated *Nts*^{EGFP} mice with leptin (5 mg/kg, IP, 2 h) and examined the distribution of Nts-EGFP neurons along with leptin-induced phosphorylated signal transducer and activator-3 (pSTAT3), which identifies neurons containing functional LepRb. We observed that some, but not all Nts-EGFP neurons contained pSTAT3 (Figure 3-1 C, filled arrows). We also observed pSTAT3-positive cells that did not co-label with Nts-EGFP (Figure 3-1 C, unfilled arrows), consistent with reports that some LHA LepRb neurons do not contain Nts [200;201]. Cell counts confirmed that approximately 15 % of all Nts-EGFP neurons co-expressed pSTAT3, and these are hence referred to as Nts^{LepRb} neurons. Thus, Nts^{LepRb} neurons represent a leptin-sensitive subset of all Nts neurons, and they are intermixed with but distinct from MCH and OX neurons (Figure 3-1 D).

3.4.2 LHA Nts and OX Neurons are Directly Regulated by Different Energy Balance Hormones

The orexigenic hormone ghrelin and the anorectic hormone leptin modify energy balance, in part via actions in the LHA. Leptin activates LepRb expressing neurons, including Nts^{LepRb} neurons (Figure 3-1). Neurons expressing the Gq-coupled GHSR can be activated by ghrelin, including OX neurons [293]. It remained unclear, however, if leptin and ghrelin act via completely separate or overlapping populations of LHA neurons (e.g. perhaps leptin and ghrelin regulate both Nts and OX neurons). To examine this, we first treated *Nts*^{EGFP} mice with either vehicle or leptin (5 mg/kg, IP, 4 h) and examined whether Nts and OX neurons exhibit leptin-induced pSTAT3. Leptin treatment significantly increased the percentage of Nts-EGFP neurons containing nuclear pSTAT3 (Figure 3-2 A, filled arrows and Figure 3-2 B: vehicle = 1.2 ± 0.6%, n=4; leptin =16.1 ± 1.5%, n=5, $p < 0.0001$), confirming that Nts^{LepRb} neurons respond to leptin. By contrast, very few OX neurons exhibited nuclear pSTAT3 with vehicle treatment, and this

percentage did not increase in response to leptin (Figure 3-2 A unfilled arrows and Figure 3-2 B; vehicle = 0.5 ± 0.3%, n=4, leptin =0.9 ± 0.3%, n=5). Next, we treated *Nts*^{EGFP} mice with either vehicle or ghrelin (100 µg, IP, 4 h) and examined whether Nts and OX neurons exhibit cFos, a marker of neuronal depolarization that has been used to identify ghrelin-activated neurons in rodents and primates [292;293;300]. Ghrelin treatment did not increase the percentage of Nts-EGFP neurons that co-express cFos compared to vehicle treatment, suggesting that ghrelin does not activate Nts neurons (Figure 3-2 C unfilled arrows and Figure 3-2 D: vehicle = 2.1 ± 0.5%, n=4, ghrelin = 2.9 ± 0.3%, n=4). Ghrelin treatment did, however, significantly increase the percentage of OX neurons that contain nuclear cFos (Figure 3-2 C filled arrows and Figure 3-2 D: vehicle = 9.2 ± 3.2%, n=4, ghrelin = 25.3 ± 4.3%, n=4), p = 0.005), consistent with previous reports that OX neurons are directly regulated by ghrelin. Together these data demonstrate that leptin specifically activates Nts^{LepRb} neurons while ghrelin specifically activates OX neurons, and thus that these hormones modify energy balance via separate LHA neuronal populations.

3.4.3 Generation of Mice to Study the Contribution of Nts^{LepRb} Neurons to Homeostasis and Motivated Behavior

Leptin action via LepRb is crucial for normal regulation of body weight [5;6;8], thus the subset of Nts neurons that respond to leptin, the Nts^{LepRb} neurons, likely contributes to energy balance. Since Nts^{LepRb} neurons are specific to, and confined within the LHA [200], they may control unique aspects of leptin action compared to non-Nts expressing LepRb neurons in other parts of the brain. Indeed, we previously examined the contribution of Nts^{LepRb} neurons in mixed-background mice with LepRb knocked out specifically from LHA Nts neurons (LRKO mice); these LRKO mice exhibit mild hyperphagia at young ages, decreased physical activity and blunted activation of the mesolimbic dopamine system that collectively cause increased body weight [200]. These data suggested that loss of leptin action specifically via Nts^{LepRb}

neurons might disrupt both homeostatic and dopamine-mediated motivated behaviors that impact energy balance, such as palatable feeding and non-obligate locomotor activity [297;301-303]. Such motivated behaviors must be assessed in pure-background mice to detect meaningful differences, and indeed we could not detect differences in palatable feeding or non-obligate activity in mixed-background LRKO mice (data not shown). Thus, to investigate the contribution of Nts^{LepRb} neurons to motivated behavior and energy balance, we backcrossed control and LRKO mice onto to the C67/Bl6 background, which is the most commonly used genetic background for assessment of motivated behaviors (Figure 3-3 A). As a first step we verified that backcrossed LRKO mice lack functional LepRb in Nts^{LepRb} neurons by visualizing vehicle and leptin-induced pSTAT3 immunoreactivity in backcrossed control and LRKO mice (5 mg/kg leptin, IP, 2 h). Vehicle treated control and LRKO mice had essentially no pSTAT3 within the LHA (Figure 3-3 B). Leptin treatment robustly induced pSTAT3 in the LHA of control mice, but much less so in the LHA of LRKO mice, confirming the loss of functional LepRb in Nts^{LepRb} neurons (Figure 3-3 B). LRKO mice do, however, exhibit some pSTAT3-positive neurons within the LHA, indicating that the non-Nts containing LHA LepRb neurons are intact. Loss of LepRb is specific to the LHA, the site of Nts^{LepRb} neurons, as demonstrated by the finding that leptin-induces similar amounts of pSTAT3 within other brain regions of control and LRKO mice, such as the arcuate nucleus (Figure 3-3 B, insets). These data confirm that backcrossed LRKO mice, like the previously generated mixed-background line [200], lack functional LepRb in Nts^{LepRb} neurons and thus are ideal models to examine the contributions of Nts^{LepRb} neurons in both homeostatic and motivated behaviors that impact energy balance. Backcrossed LRKO mice were thus used for all subsequent experiments.

3.4.4 Loss of Action via Nts^{LepRb} Neurons Disrupts Ghrelin Action in the LHA

Since Nts^{LepRb} neurons project to and inhibit OX neurons [200;298;304] we speculated that loss of leptin signaling via Nts^{LepRb} neurons might disrupt regulation of OX neurons, including their response to ghrelin, thus compromising adaptive energy balance. To test this, we treated control and LRKO mice with central vehicle and ghrelin (3 µg, ICV, 4 hr) and quantified the percentage of OX neurons that co-express nuclear cFos (OX:cFos) as a marker of activated OX neurons. As expected, ghrelin treatment significantly increased the percentage of OX:cFos neurons in control mice (Figure 3-3 C arrows and 3D: control mice, vehicle = 35.5 ± 2.0%, n =6; ghrelin = 43.0 ± 2.5%, n=5, p = 0.049). By contrast, ghrelin treatment did not increase the percentage of OX:cFos neurons in LRKO mice compared to vehicle treatment (Figure 3-3 C arrows and 3D: LRKO mice, vehicle = 44.5 ± 1.8%, n=3; ghrelin = 44.1 ± 2.4%, n=3). It is possible that the loss of inhibitory input from Nts^{LepRb} neurons leads to biased excitatory input onto OX neurons, such that they are at a maximal threshold of activation and cannot be further activated by other signals (such as ghrelin). Differences in ghrelin-mediated activation in control and LRKO mice are not due to altered numbers of OX neurons, since control and LRKO mice have equivalently-sized populations of OX neurons (Figure 3-3 E: control = 1002 ± 24 neurons, n = 11; LRKO = 985 ± 23 neurons, n = 6, p = 0.928). Thus, although leptin and ghrelin act via separate populations of LHA neurons, loss of leptin sensing via Nts^{LepRb} neurons that project to OX neurons impairs the ability of OX neurons to be activated by ghrelin. These data suggest that loss of leptin signaling via Nts^{LepRb} neurons may disrupt adaptive energy balance in response to both anorectic and orexigenic cues of energy balance.

3.4.5 Loss of Action via NtsLepRb Neurons Disrupts Energy Balance to Cause Weight Gain

Leptin and ghrelin are important for modifying homeostatic and motivated behaviors that impact energy balance [297;301-303]. Since our data suggest that LRKO mice have blunted leptin and ghrelin-mediated regulation of LHA neurons, we reasoned that their regulation of body weight is disrupted due to altered homeostatic and motivated behaviors. To test this, we compared control and LRKO mice in a battery of metabolic and behavioral tests. Indeed, adult backcrossed LRKO mice weigh more than control mice (Figure 3-4 A, control = 23.93 ± 0.42 g, LRKO = 25.39 ± 0.53 g, p=0.037), and have increased body fat (Figure 3-4 B, control = 3.89 ± 0.36 %, LRKO = 5.84 ± 0.77 %, p=0.024) similar to the overweight phenotype of mixed background LRKO mice [200]. Interestingly, adult LRKO mice do not become overweight due to increased chow intake (Figure 3-4 C, control = 3.79 ± 0.28 g, LRKO = 3.50 ± .22 g, p=0.422) or a deranged respiratory quotient (RQ), which would indicate altered metabolism of carbohydrate and fat compared to controls (Figure 3-4 D, control = 0.89 ± 0.01, LRKO = 0.88 ± 0.02 g, p=0.890.). Given the lack of increased caloric intake or altered usage, we examined whether the increased body weight of LRKO mice could be due to altered energy expenditure. Indeed, LRKO mice have significantly reduced spontaneous locomotor activity compared to control mice (Figure 3-4 E, control = 91,341 ± 6,505 activity counts, LRKO = 70,330 ± 4,669 activity counts, p=0.016) and exhibit a trend toward reduced spontaneous oxygen consumption/VO_2 (Figure 3-4 F, control = 3488 ± 118 mL/hr/kg, LRKO = 3283 ± 133 mL/hr/kg, p=0.257). These data suggest that deficits in energy expenditure promote weight gain in LRKO mice, and are consistent with data from mixed background mice [200]. Spontaneous locomotor activity contributes to the regulation of body weight [305] but we reasoned that deficits in motivated, non-volitional locomotor activity might also contribute to disrupted energy expenditure. Motivated locomotor activity can be assessed in rodents by giving them access to a running wheel, and wheel running promotes

activation of mesolimbic dopamine neurons and dopamine release into the striatum [306;307]. We therefore gave control and LRKO mice access to running wheels to determine if loss of leptin signaling through Nts neurons alters non-obligatory (volitional) locomotion. Indeed, LRKO mice spent significantly less time on running wheels than control mice (Figure 3-4 G, control = 204.5 ± 25.2 min, LRKO = 87.3 ± 32.5 min, p=0.009) and thus consumed less oxygen (Figure 3-4 H, control = 4766 ± 185 mL/hr/kg , LRKO = 3862 ± 148, p=0.001). Collectively, these data indicate that loss of leptin action via Nts^{LepRb} neurons disrupts regulation of body weight due to decreased energy expenditure, including impairing spontaneous and motivated locomotor activity that is mediated via the mesolimbic dopamine system.

3.4.6 Loss of Action via Nts^{LepRb} Neurons Disrupts Adaptive Feeding and Preference for Palatable Food

In addition to locomotor activity, the mesolimbic dopamine system also modifies the motivation to eat. Since loss of leptin action via Nts^{LepRb} neurons disrupts the mesolimbic dopamine system [200] and motivated locomotor activity (Figure 3-4), we reasoned that LRKO mice might also have altered motivation to eat. Motivated feeding is influenced by two factors: how much the food is preferred (or "liked") and how much the food is "wanted" [308]. We first investigated whether food preference is altered in LRKO mice via a two-bottle sucrose preference test. Control and LRKO mice did not exhibit any differences in total liquid intake (Figure 3-5 A: control = 9.13 ± 0.52 g vs. LRKO = 9.42 ± 0.47, p =0.696), or sucrose preference (Figure 3-5 B: control = 71.05 ± 1.91 % vs. LRKO = 72.11 ± 1.73 %, p =0.685), indicating that loss of action via Nts^{LepRb} neurons does not impede general sucrose preference or ingestion. We reasoned, however, that the disruption of leptin signaling via Nts^{LepRb} neurons might impair the ability to modify preference in response to changes in circulating leptin, such as might occur with increased adiposity. To examine the adaptive response to leptin, we treated control and

LRKO mice with vehicle or leptin (5 mg/kg, IP) during sucrose preference testing. As expected, leptin decreases feeding compared to vehicle treatment in control mice but LRKO mice do not significantly attenuate feeding in response to leptin (Figure 3-5 C: control = -3.13 ± 0.73 g vs. LRKO = -0.49 ± 0.79 g, p =0.023). Sucrose preference, however, did not differ significantly between leptin-treated control and LRKO mice (Figure 3-5 D: control = -1.86 ± 1.61 % vs. LRKO = 1.18 ± 1.27 %, p =0.168). In sum these data indicate that increased leptin suppresses feeding, in part, via LHA Nts^{LepRb} neurons, but that leptin does not modify sucrose preference or "liking" via Nts^{LepRb} neurons.

Increases in circulating ghrelin promote feeding, and this response is mediated, in part, via OX neurons [294;297]. Since ghrelin-mediated activation of OX neurons is impaired in LRKO mice we investigated whether this altered their feeding. Both control and LRKO mice increase chow intake in response to ghrelin treatment (Figure 3-5 E: control = 0.48 ± 0.15 g vs. LRKO = 0.69 ± 0.15 g, p =0.557, indicating that adaptive feeding responses to ghrelin remain intact. Ghrelin treatment also increases sucrose preference in control mice, as expected, but ghrelin-induced sucrose preference is significantly blunted in LRKO mice (Figure 3-5 F: control = 5.10 ± 1.11 % vs. LRKO = -0.45 ± 1.58 %, p =0.018). These data support a role for OX neurons in mediating sucrose preference, and suggests that disruption of the Nts^{LepRb} → OX circuit prevents ghrelin-mediated liking of palatable foods.

3.4.7 Loss of Action via Nts^{LepRb} Neurons Disrupts Adaptive "Wanting" of Palatable Food

Mesolimbic dopamine signaling can modify how much foods are "wanted" and thereby modify food intake. Since Nts^{LepRb} neurons and their OX projection targets both engage the

mesolimbic dopamine system [200;297], we investigated whether loss of action via these circuits impaired food wanting. We therefore assessed control and LRKO mice for their willingness to work for sucrose pellets in a progressive ratio (PR) operant task, in which the breakpoint indicates the animal's relative level of reward "wanting". At baseline, the breakpoint of control and LRKO animals is similar, indicating that they similarly want, and will work for palatable food (Figure 3-6 A: control = 44.55 ± 8.08 vs. LRKO = 33.00 ± 3.34, p =0.157). Leptin however, decreases operant responding for rewards [309], which might be mediated, in part, via Nts^{LepRb} neurons. To examine this hypothesis, we treated control and LRKO mice with vehicle and leptin during PR testing. As expected, control mice decrease their PR breakpoint in response to leptin, indicating suppressed sucrose wanting, but LRKO mice do not (Figure 3-6 B: control = -10.18 ± 4.15 vs. LRKO = 0.40 ± 1.67, p =0.015). Ghrelin treatment increases PR breakpoint in control mice, consistent with previous reports [294], but this increased wanting is blunted in ghrelin-treated LRKO mice (Figure 3-6 C: control = 26.67 ± 8.91 vs. LRKO = 7.60 ± 2.59, p =0.020). Together these data indicate that loss of action via the Nts^{LepRb} neuronal circuit impairs leptin and ghrelin from modifying the incentive salience of palatable foods, and thus prevents appropriate hormone-coordinated feeding.

3.4.8 Expression of LHA Signaling Peptides is Altered After Disruption of the Nts^{LepRb} Circuit

Our previous and current data confirm that loss of action via Nts^{LepRb} neurons prevents LHA neurons from being appropriately activated by leptin or ghrelin. Loss of neuronal activation could therefore impair adaptive response via preventing release of LHA signaling peptides that regulate postsynaptic target neurons, such as OX and mesolimbic dopamine neurons. To investigate this possibility, we isolated RNA from the LHA of control and LRKO mice, and assessed expression of neuropeptides and hormone receptors that are specifically expressed in

Nts^{LepRb} neurons and OX neurons. Indeed, the loss of Nts^{LepRb} neuronal action in LRKO mice coincides with diminished expression of *Nts* (Figure 3-7 A: control = 1.05 ± 0.13-fold, n=8 vs. LRKO = 0.62 ± 0.07, n=7, p =0.012), suggesting that Nts^{LepRb} neurons are no longer making sufficient Nts to mediate downstream signaling. Intriguingly, although at least some Nts^{LepRb} neurons also co-express the neuropeptide *galanin*, LRKO mice retain normal *galanin* expression. *LepRb* expression, however, is upregulated in LRKO mice, which likely reflects a compensatory effort to enhance leptin action via the population of non-Nts expressing LepRb neurons (Figure 3-3 B).

Since Nts^{LepRb} neurons project to and inhibit OX neurons, we hypothesized that expression of energy balance neuropeptides and receptors might be disrupted in OX neurons of LRKO mice. *OX* expression remains similar between control and LRKO mice, consistent with previous findings [200]. There is a slight increase, however, in *delta-like 1 (Dlk1),* which is expressed in OX neurons and can be released to modify neuronal signaling [310;311]. *GHSR* expression is also similar in control and LRKO mice, suggesting that the loss of ghrelin-response in LRKO mice is not due to lacking the ability to bind ghrelin directly, but likely due to disruption of other signaling mechanisms.

Nts^{LepRb} neurons also project to the VTA, where released Nts activates neurotensin receptor-1 (NtsR1) expressing dopamine neurons and induces dopamine release into the NAc [194;200]. Despite the loss of action via Nts^{LepRb} neurons in LRKO mice, however, they do not exhibit any differences in VTA expression of *tyrosine hydroxylase* (*TH*- the rate limiting marker of dopamine synthesis), the *dopamine active transporter (DAT)* or *NtsR1.* These data suggest that loss of action via Nts^{LepRb} neurons does not impair the functionality of VTA dopamine

neurons and their ability to respond to Nts, per se. Instead, it is possible that the loss of leptin and ghrelin mediated activation of LHA neurons results in diminished activation of VTA dopamine neurons, and therefore reduces dopamine release to the NAc that regulates motivated behaviors.

3.4.9 Loss of Action via the Nts^{LepRb} Circuit Disrupts the Mesolimbic Dopamine System

LRKO mice exhibit blunted motivated feeding and locomotor responses to leptin and ghrelin, thus we reasoned that these mice have diminished activation of the mesolimbic dopamine system. This could be solely due to diminished activation of the Nts^{LepRb} and OX neurons (Figures 3-2, 3-3) that project to the VTA to activate dopamine neurons and promote dopamine release into the NAc. Another possibility is that the developmental deletion of LepRb in LRKO mice causes them to develop fewer neuronal projections to the VTA with which to modify dopamine signaling. To investigate this possibility, we visualized the projections of LHA Nts neurons by injecting Nts^{Cre} and LRKO mice in the LHA with Ad-Syn-mCherry, which causes cre-dependent expression of the synaptophysin-mCherry fusion protein in Nts-expressing neurons. Importantly, Syn-mCherry is expressed within cell bodies and also localizes to axon terminals, thereby enabling detection of projections throughout the brain [191]. Injection of Ad-Syn-mCherry identifies similar numbers of cell bodies and projections within the LHA of control and LRKO mice (Figure 3-8 A, D) suggesting that there is no developmental defect in the number of Nts neurons or their local projections to OX neurons. By contrast, we observed that LRKO animals had fewer projections to the VTA than control mice (Figure 3-8 B,C,E,F). These data indicate that deletion of LepRb from Nts^{LepRb} neurons diminishes the development of projections to the VTA, and thus presumably reduces the activation of VTA dopamine neurons and dopamine release to the NAc. If this were true, we anticipated that leptin and ghrelin-mediated activation of the NAc would be decreased in LRKO mice compared to controls. To

investigate this, we examined cFos in the NAc shell (NAcSh) and Core (NAcC), where dopamine release modifies motivated feeding and locomotion. As a first step, we treated control and LRKO mice with amphetamine, which promotes DA release and induces cFos, to verify that LRKO mice can exhibit dopamine-mediated activation of the NAc. Indeed, amphetamine increases cFos in both control and LRKO mice, though it is reduced in the latter (Figure 3-8 G,K). These data are consistent with the reduced amphetamine-mediated locomotor activity of LRKO mice compared to controls, suggesting that they have reduced capacity to adapt to signals that should increase mesolimbic dopamine signaling [200]. Next, we queried the activation of the mesolimbic dopamine system in response to vehicle, leptin or ghrelin, to determine whether the blunted adaptive response to hormones corresponds with diminished NAc activation. Control and LRKO mice have similarly low levels of striatal cFos after vehicle treatment (Figure 3-8 H,L). Leptin treatment modestly increases cFos within the NAcC of control mice, but this response is absent in LRKO mice (Figure 3-8 I,M). Ghrelin treatment robustly increases cFos in the NAcC and NAcSh of control mice, but there is essentially no increase of cFos in ghrelin-treated LRKO mice (Figure 3-8 J,N). In sum, these data confirm that loss of action via the Nts^{LepRb} circuit disrupts leptin and ghrelin-mediated activation of mesolimbic dopamine signaling, and thus can blunt the adaptive motivated behaviors regulated by these hormones.

3.5 Discussion

Here we define neural mechanisms by which the LHA coordinates leptin and ghrelin-mediated adaptations in energy balance. Leptin specifically activates a subset of LHA Nts neurons that co-express LepRb, the Nts^{LepRb} neurons, to mediate weight loss behaviors. By contrast, ghrelin acts upon OX neurons to promote feeding. Since Nts^{LepRb} neurons project to

and inhibit OX neurons they can indirectly modify ghrelin-mediated regulation of OX neurons. Loss of action via Nts^{LepRb} neurons thus disrupts leptin and ghrelin-mediated adaptive responses, dis-coordinating appropriate ingestive and locomotor behavior that leads to weight gain. Collectively, these data reveal an important role for Nts^{LepRb} neurons in mediating adaptive energy balance and normal body weight.

Our findings establish Nts neurons as a major neuronal population within the LHA that are vital for mediating energy balance. Previous physiological study of LHA Nts neurons was impeded by the inability to immunohistochemically detect them, as this required function-impairing colchicine treatment. Similar limitations deterred understanding of LHA populations containing galanin (Gal), GABA or glutamate, but the recent development of cre-inducible mouse models enabled determination of their roles in energy balance [286;290;312]. We thus used a knock-in cre-inducible mouse model to identify Nts neurons within the LHA, and find that Nts neurons are co-distributed amongst, but distinct from, OX and MCH neurons. Our findings differ from a report that all OX neurons contain Nts [203], which was determined with a commercial antibody that is no longer available. However, our data agree with reports that Nts is required for leptin-mediated inhibition of OX neurons, suggesting that Nts is released from LepRb neurons and is not in fact co-expressed within OX neurons [295]. Furthermore, since Nts is an anorectic neuropeptide [222;313], it is unlikely that it would be co-expressed within orexigenic OX neurons. Indeed, Nts in the LHA has been linked to suppressing feeding in response to dehydration, inflammation and leptin [150;200;229]. Intriguingly, only 15% of LHA Nts neurons co-express LepRb and are activated by leptin (Nts^{LepRb} neurons). Thus, there are at least two subpopulations of LHA Nts neurons: the leptin-responsive Nts^{LepRb} neurons and other non-LepRb expressing Nts neurons with yet-to-be determined physiological roles. LHA Nts subpopulations may differ in neuropeptide or classical neurotransmitter content [194;201;202], though

at least the Nts^{LepRb} neurons are GABAergic [200]. Going forward it will be important to distinguish LHA Nts populations at the molecular and functional levels to determine their contributions to energy balance.

Our data demonstrate that leptin and ghrelin engage separate, but interconnected LHA circuits to exert adaptive energy balance (Figure 3-9). Leptin directly activates Nts^{LepRb} neurons, but not OX neurons since they lack LepRb [255]. Leptin regulates OX neurons indirectly, however, since Nts^{LepRb} neurons synapse upon and inhibit OX neurons [298]. Nts^{LepRb} neurons also project to the VTA, where Nts activates dopamine neurons to induce dopamine release into the NAc [194;200]. Therefore, leptin-regulated Nts^{LepRb} neurons modify energy balance through two separate neuronal circuits: 1) via inhibiting orexigenic OX neurons and 2) via Nts-mediated activation of mesolimbic dopamine neurons. While the specific contributions of these pathways have yet to be understood, leptin action via these Nts^{LepRb} projections is required to limit feeding, promote physical activity, and hence maintain normal body weight. By contrast, ghrelin increases the proportion of activated OX neurons, but does not activate Nts neurons or LepRb neurons [this manuscript] [293;295]. Collectively, these data suggest that anorectic leptin directly activates LepRb-expressing neurons, while ghrelin can activate GHSR-expressing OX neurons, in agreement with electrophysiological studies [288;295]. It remains unclear, however, whether all OX neurons express GHSR and can be directly activated by ghrelin. Indeed, we observed that ghrelin increases activation of some, but not all OX neurons, suggesting that there may be at least some non-GHSR expressing OX neurons with unique functions. OX neurons can be distinguished into glucose-excited and glucose-inhibited populations, and this latter population is more likely to be activated during energy depletion, when circulating ghrelin is high [314]. Our current work also does not exclude the possibility that ghrelin may also activate GHSR-expressing neurons outside of the LHA that project to and activate OX neurons. In any case,

ghrelin can either directly or indirectly promote activation of OX neurons, but does not activate the separate Nts^{LepRb} neurons. In the future it will be important to define the precise subpopulations of LepRb and OX neurons regulated by leptin and ghrelin, to fully understand how they dynamically modify adaptive energy balance.

Nts^{LepRb} neurons are required for normal energy balance, and loss of action via Nts^{LepRb} neurons in LRKO mice results in hypo-locomotion and increased body weight. Intriguingly, LRKO mice exhibit diminished physical activity but do not commensurately reduce food intake, thereby promoting weight gain. Reduced movement may result from disruption of the Nts^{LepRb} → OX and/or Nts^{LepRb} → VTA circuits, since both OX and dopamine signaling promote locomotor activity [305;315]. Indeed, leptin modifies dopamine -mediated running reward [301] and Nts activates VTA dopamine neurons to promote locomotor activity [244]. Since Nts^{LepRb} neurons directly engage the mesolimbic dopamine system, the Nts^{LepRb} → VTA circuit may signal via Nts to couple excess energy status with the motivation to engage in volitional exercise, thereby potentiating energy expenditure and weight loss. Restoring action via the Nts^{LepRb} → VTA circuit could promote the motivation to engage in physical activity and support weight loss.

To date there have been two populations of LepRb-expressing neurons reported in the LHA: those expressing Nts (Nts^{LepRb} neurons) or Gal (Gal^{LepRb} neurons.) While many LHA Gal neurons co-express LepRb [290], and at least some Gal neurons co-express Nts [201], it remained unclear whether Nts^{LepRb} and Gal^{LepRb} neurons were in fact overlapping populations. The phenotype of mice lacking LepRb in Gal^{LepRb} neurons (termed GRKO mice), however, contrasts from that of LRKO mice, suggesting that Nts^{LepRb} and Gal^{LepRb} neurons are distinct populations [200;290]. First, LRKO mice exhibit reduced spontaneous and volitional physical activity, but

locomotor activity is normal in GRKO mice. Second, loss of leptin action via Nts^{LepRb} neurons does not alter baseline sucrose preference, but GRKO mice have increased preference for palatable sucrose. Third, Gal expression is normal in the LHA of LRKO mice, but reduced in GRKO mice, suggesting that the LRKO and GRKO phenotypes result from different neurochemistry. Fourth, Nts^{LepRb} neurons directly project to the VTA, but Gal^{LepRb} neurons do not. Collectively, these findings suggest that Nts^{LepRb} and Gal^{LepRb} neurons are distinct neuronal populations, and mediate different aspects of adaptive energy balance. Nts^{LepRb} and Gal^{LepRb} and neurons are similar, however, in that they both project to and inhibit OX neurons [200;290]. It remains possible that they regulate distinct subpopulations of OX neurons, and thus differentially modify locomotor activity and nutrient drive.

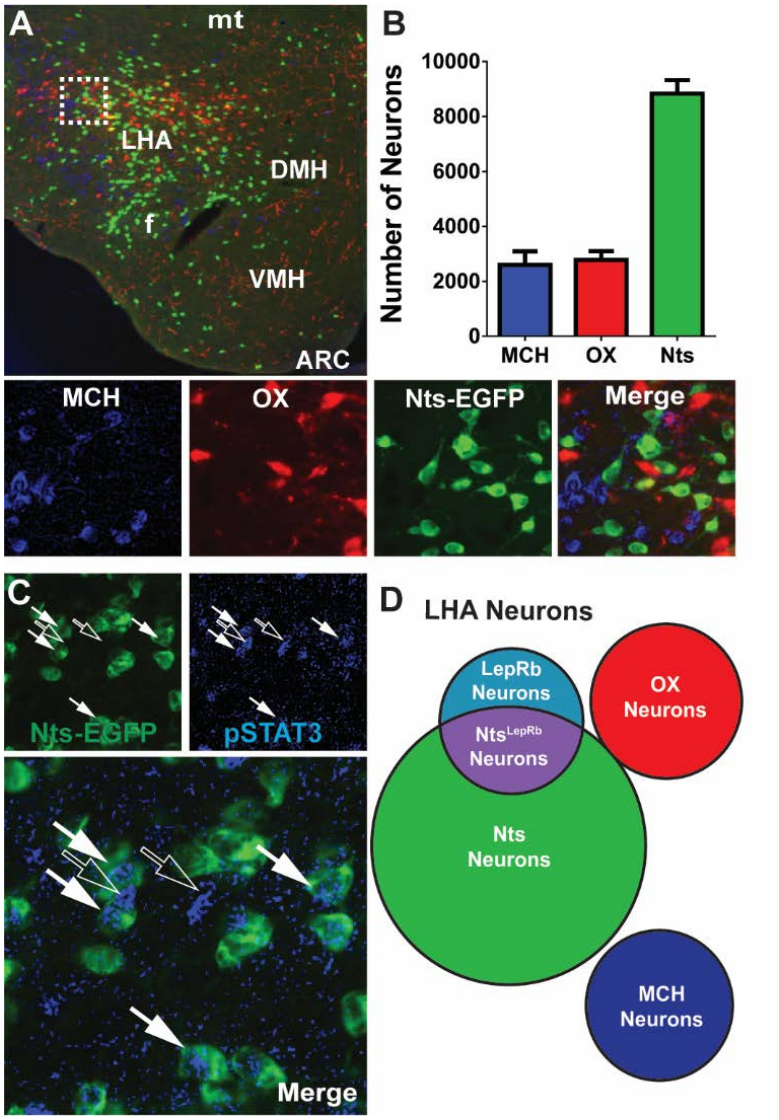
LRKO mice do not adapt their responding for sucrose rewards in response to leptin and ghrelin, likely due to reduced engagement of the mesolimbic dopamine system that governs the incentive salience of rewards. Nts^{LepRb} neurons project to the VTA directly and inhibit OX neurons that modify motivational drive via the VTA. Loss of action via Nts^{LepRb} neurons may therefore blunt appropriate responses to leptin, such as increases in leptin during obesity, and fail to suppress the motivation for palatable foods, thereby exacerbating intake and weight gain. Interestingly, ghrelin-mediated sucrose preference and operant responding for sucrose is blunted in LRKO mice, suggesting that loss of action via Nts^{LepRb} neurons disrupts both the ghrelin-mediated liking and wanting of sucrose. Our data suggest two mechanistic disruptions of the mesolimbic dopamine system in LRKO mice that may underlie altered food motivation. First, there is a reduction in Nts^{LepRb} projections to the VTA, resulting in diminished activation of the mesolimbic dopamine “wanting” system in response to leptin. Second, the loss of Nts^{LepRb} neuronal regulation of OX neurons causes them to be less responsive to ghrelin treatment, such that OX → VTA neurons may not be activated and induce mesolimbic dopamine release. We

acknowledge that the LRKO mice used in this study have developmental deletion of LepRb from Nts^{LepRb} neurons, and so do not necessarily reflect the requirement for LepRb in the adult LHA. Our data do, however, reveal a crucial role for LepRb in proper formation of LHA circuits and activation of the mesolimbic dopamine system, and suggest that intact signaling via these circuits is important for regulation of body weight. Indeed, while the central and systemic hormone treatments in this study engaged many intact LepRb and GHSR-expressing neurons throughout the brain, the loss of action from just the Nts^{LepRb} neurons confined to the LHA was sufficient to disrupt motivated responding and energy balance. These data confirm that Nts^{LepRb} neurons are significant contributors to hormone-mediated adaptive energy balance.

Collectively these data identify Nts^{LepRb} neurons as important controllers of OX neurons, the mesolimbic DA system and adaptive energy balance. Loss of action via Nts^{LepRb} neurons promotes overweight by preventing leptin-mediated weight loss behaviors. Simultaneously, loss of action via Nts^{LepRb} neurons also impairs the ability to appropriately respond to energy deprivation signals such as ghrelin, leading to a loss of recognition of when to eat, and when not to eat. Loss of action via Nts^{LepRb} neurons therefore deranges adaptive energy balance, and leads to inappropriate locomotor and ingestive behaviors that promote weight gain. In the future, determining the precise mechanisms by which Nts^{LepRb} neurons regulate OX and mesolimbic dopamine target neurons may reveal pharmacological approaches to restore appropriate regulation of adaptive energy balance.

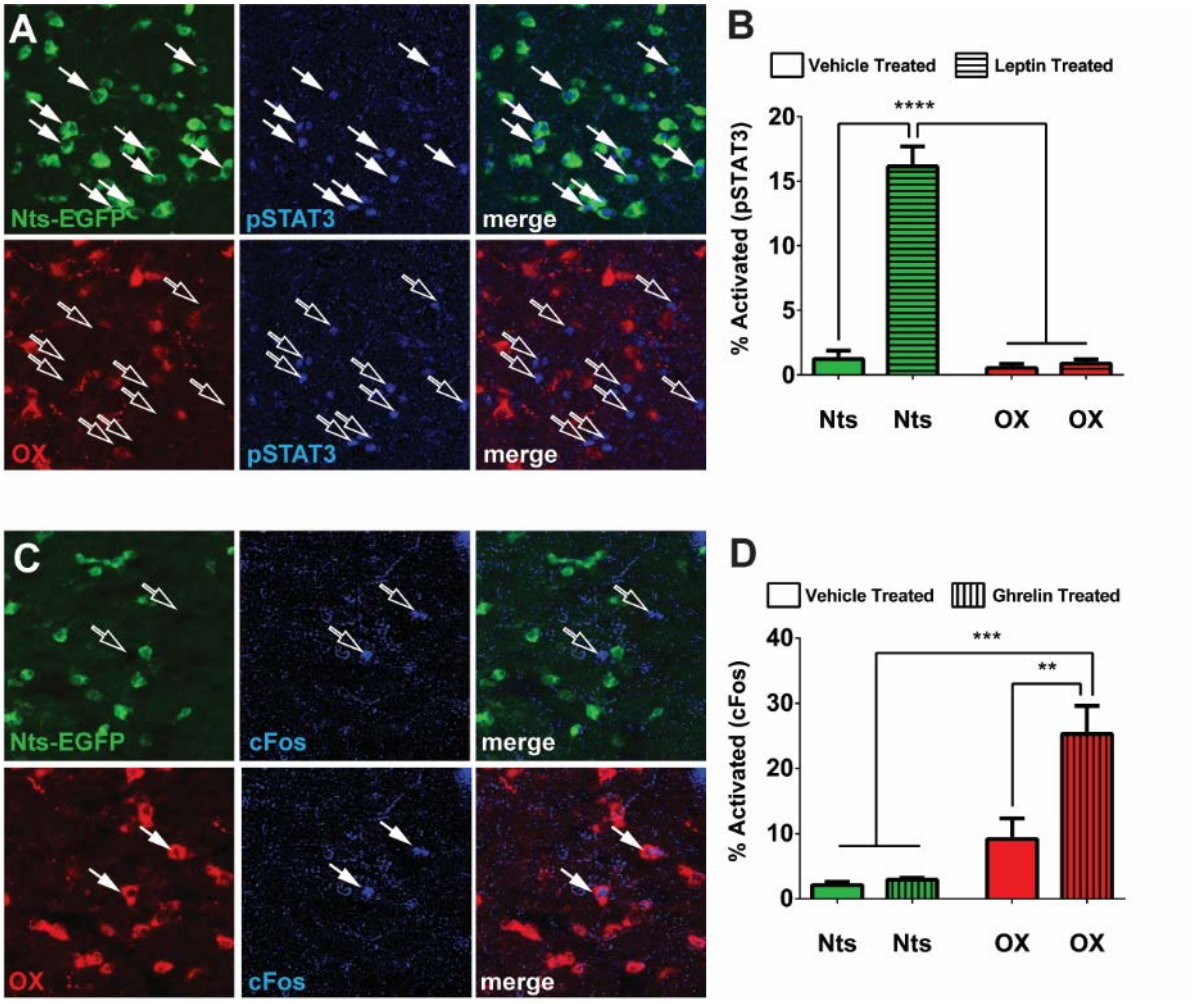
3.6 Figures

Figure 3-1: Distribution of Neuropeptide-Defined and LepRb Neuron in the LHA



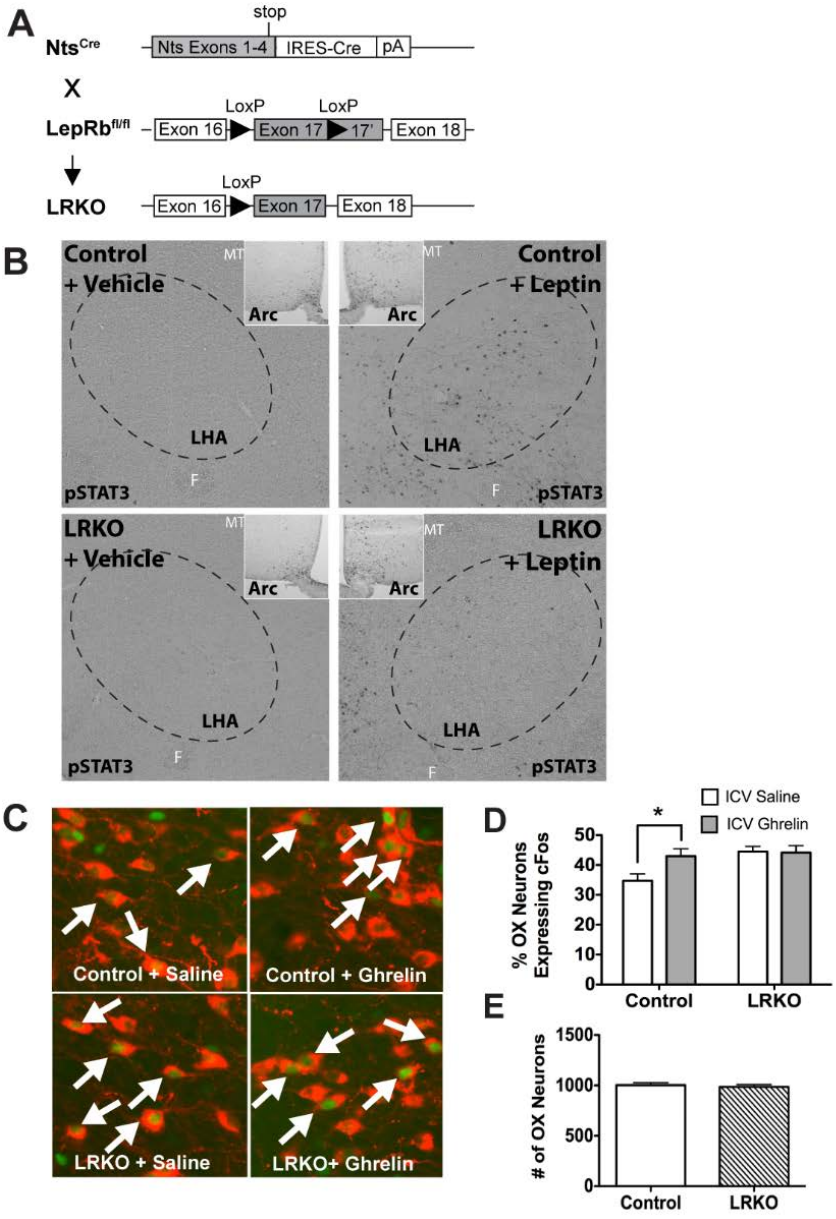
A) The distribution of LHA neurons was determined via immunofluorescent labeling of melanin concentrating hormone neurons (MCH, blue), orexin neurons (OX, red) and neurotensin neurons (Nts-EGFP, green) in the brains of *Nts*^{EGFP} mice (n = 4). The dashed-box area is enlarged in the panels below, showing the individual staining for each neuronal population and the merged image. Abbreviations: mt = mammillothalamic tract; f = fornix; DMH = dorsomedial hypothalamus; VMH = ventromedial hypothalamus; ARC = arcuate nucleus. B) Quantitation of the average number of MCH, OX and Nts neurons in the LHA ± SEM, n=4. C) *Nts*^{EGFP} mice were treated with leptin (5mg/kg, IP, 2 hr) and brains were analyzed via immunohistochemistry and immunofluorescence to identify Nts-EGFP neurons (green) and phosphorylated STAT3 (pSTAT3), a marker for LepRb activation (blue). Filled arrows identify Nts-EGFP neurons that co-localize with pSTAT3 and are Nts^{LepRb} neurons. Unfilled arrows identify LepRb neurons that do not express Nts. D) Schematic depicting the relative size and distribution of the MCH, OX, Nts, Nts^{LepRb} and LepRb neuronal populations in the LHA.

Figure 3-2: Nts and OX neurons Respond to Distinct Hormonal Cues



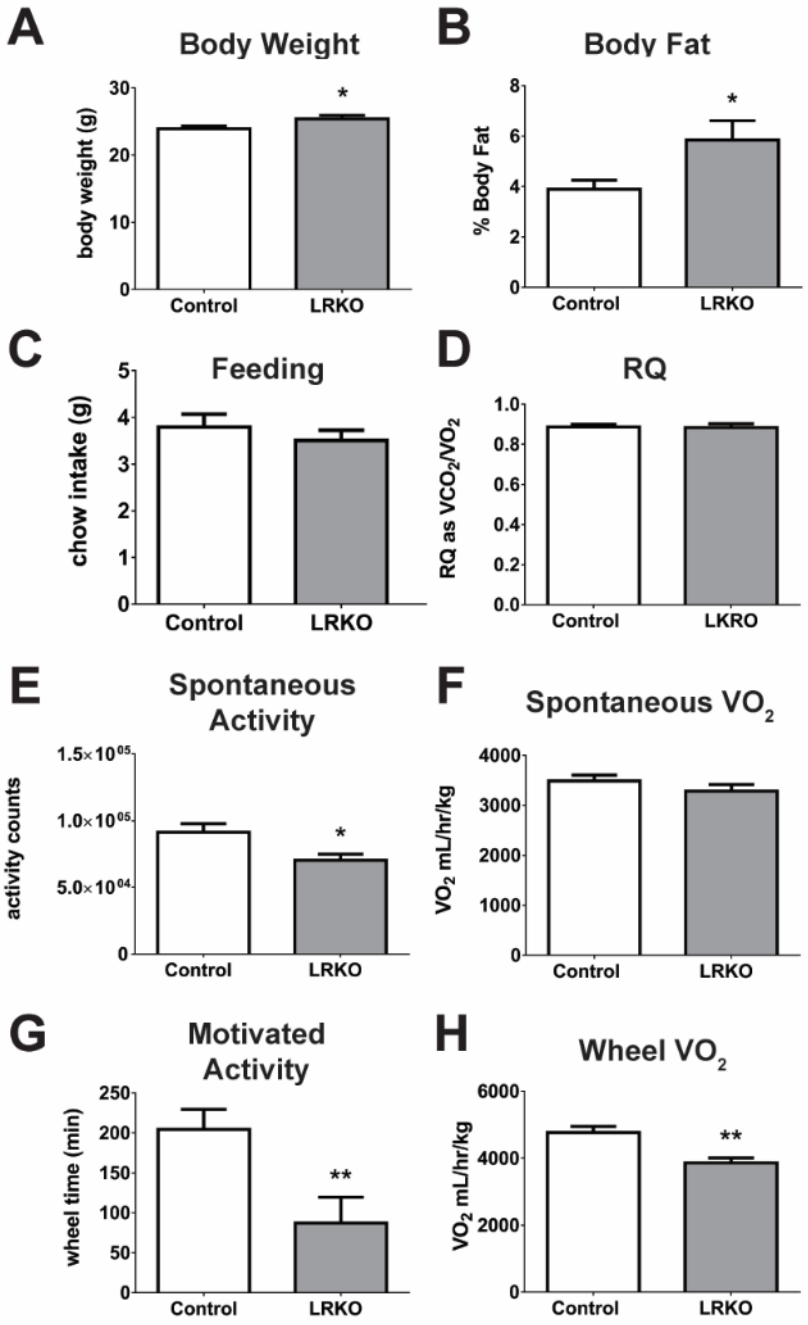
A) Male *Nts*^{EGFP} mice were treated with vehicle or leptin (5 mg/kg, IP, 2 hr) and brains were immunostained for Nts-EGFP (green), OX (red) and pSTAT3, a marker of leptin-activated LepRb neurons (blue). In the top panels, the filled arrows identify pSTAT3-labeled nuclei within Nts-EGFP neurons, which are leptin-activated Nts^{LepRb} neurons. In the bottom panels, the unfilled arrows identify the same pSTAT3-labeled nuclei, none of which are found within OX neurons. B) Quantification of the percentage of Nts and OX neurons that contain pSTAT3 (e.g. are activated by leptin) in response to vehicle or leptin treatment (vehicle n=4, leptin n=5). C) Male Nts^{EGFP} mice were treated with ghrelin (100 µg/treatment, IP, 4 hr) and brains were immunostained for Nts-EGFP (green), OX (red) and cFos, a marker of neuronal depolarization (blue). In the top panels, the unfilled arrows identify cFos-labeled nuclei that are not found within Nts neurons. In the bottom panels, the filled arrows identify the same cFos-labeled nuclei from the top panels, which are found within OX neurons. D) Quantification of the percentage of Nts and OX neurons that contain cFos (e.g. are activated by ghrelin) in response to vehicle or ghrelin treatment (vehicle n=4, leptin n=4). Graphed data represent average values ± SEM. Statistical differences were determined via one-way ANOVA, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Figure 3-3: Loss of Leptin Action via Nts^{LepRb} Neurons Blunts the Ghrelin-Mediated Activation of OX Neurons



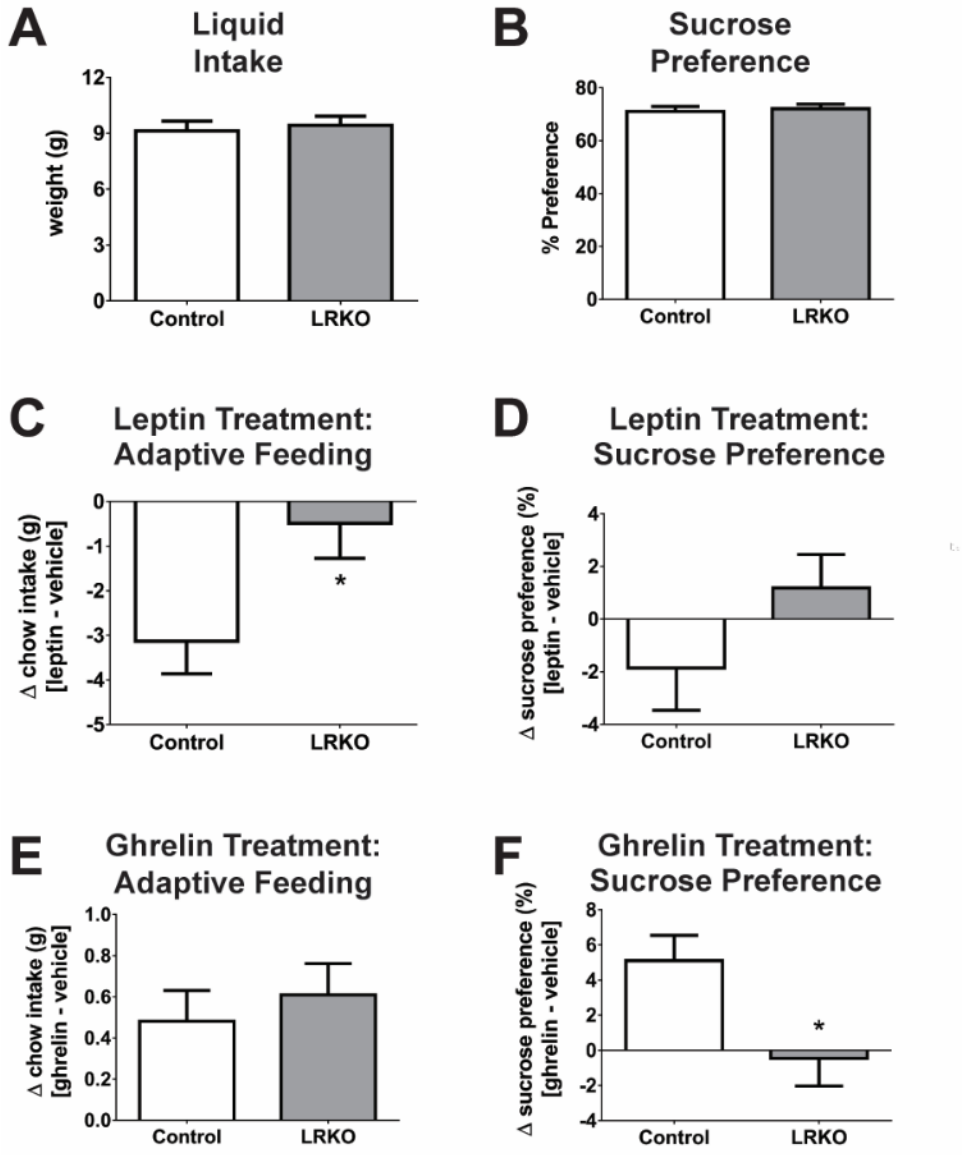
A) Generation of LRKO mice, which lack functional LepRb only in Nts neurons. B) Backcrossed Control (*Nts^{++};Lepr^{flfl}*) and LRKO (*Nts^{Cre+};Lepr^{flfl}*) mice were treated with vehicle or leptin (5 mg/kg, IP, 2 hr) and brains were immunostained for pSTAT3 to identify leptin-activated LepRb neurons. LRKO mice have fewer pSTAT3-positive neurons in the LHA compared to controls, confirming loss of functional LepRb from LHA Nts^{LepRb} neurons. C) Male control and LRKO mice were treated with saline or ghrelin (3 µg, ICV, 4 hr) and brains were analyzed via immunohistochemistry and immunofluorescence for OX (red) and cFos (green). Arrows identify OX neurons that contain cFos-labeled nuclei (e.g. OX:cFos cells), which are activated OX neurons. D) Quantitation of the percentage of OX neurons that contain cFos (OX:cFos) in treated control and LRKO mice. (Control + saline, n=6; control + ghrelin, n=6; LRKO + vehicle, n=3; LRKO + ghrelin, n=3). E) Quantitation of the total number of LHA OX neurons from three representative LHA sections of control mice (n = 12) and LRKO mice (n = 6). Graphed data represent average values ± SEM, * $p \leq 0.05$ by ANOVA.

Figure 3-4: Loss of Leptin Signaling via NtsLepRb Neurons Disrupts Energy Balance



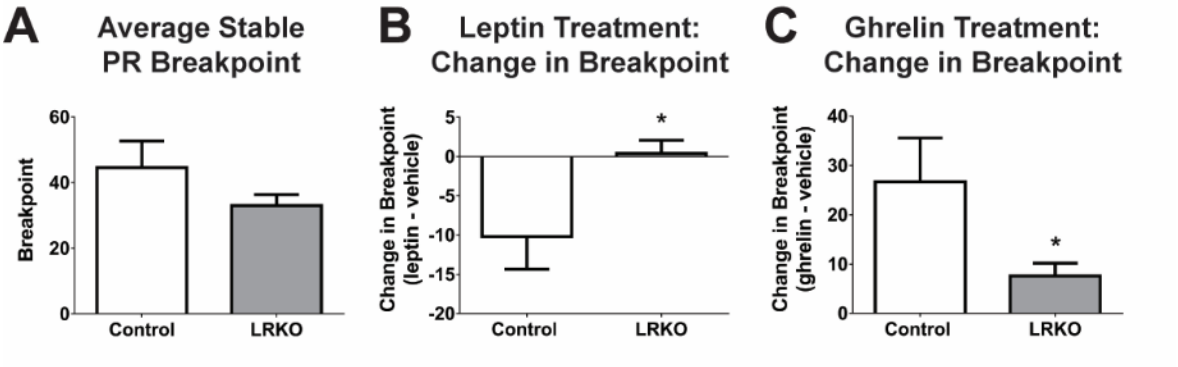
A-E) Energy balance was assessed in spontaneously moving adult (8-12 wk) male control (n = 13) and LRKO mice (n=14). A) Body weight and B) the percentage of body fat was increased in LRKO mice compared to controls, but C) chow intake and D) respiratory quotient are not significantly different. E) Spontaneous locomotor activity is decreased in LRKO animals relative to controls, and F) there is a trend for decreased spontaneous VO_2. When offered a running wheel, control mice exhibit increased G) wheel running time and H) VO_2 compared to LRKO mice (n = 10-12 per genotype). Graphed data represents average value ± SEM, *p<0.05, **p<0.01 by Student's t test.

Figure 3-5: Loss of Action via NtsLepRb Neurons Disrupts Adaptive Reward Preference



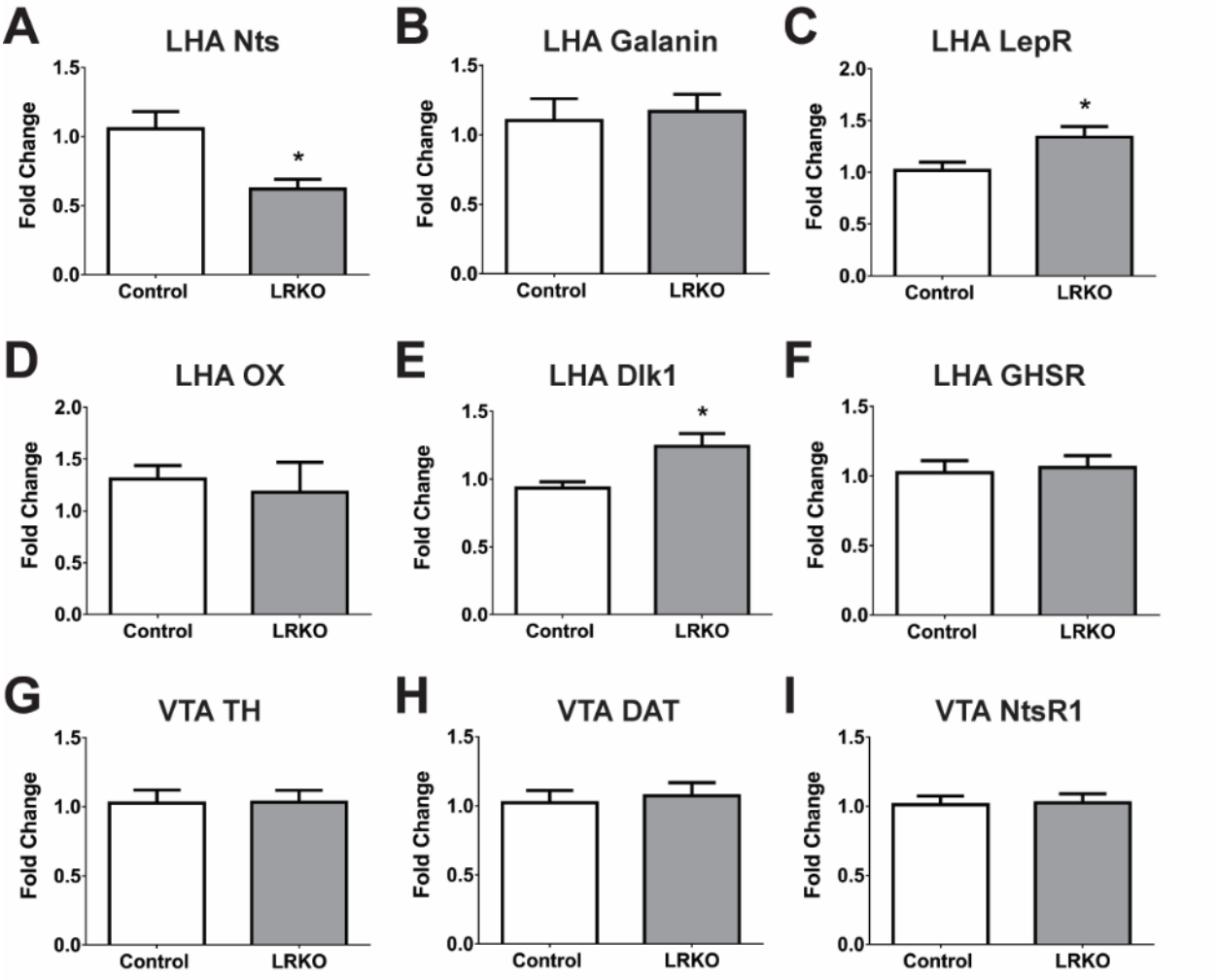
A) Total liquid intake and B) sucrose preference were similar in control and LRKO mice at baseline (control n=20, LRKO n=18). C) Control mice adaptively decrease chow intake in response to leptin but LRKO mice do not. D) There is no significant difference in leptin-mediated adaptive sucrose preference between control and LRKO mice (control n=14, LRKO n=11). E) Control and LRKO mice adaptively increase chow intake in response to ghrelin treatment (control n=11, LRKO n=11). F) Ghrelin treatment adaptively increases sucrose preference in control mice, but not in LRKO mice (control n=10, LRKO n=11). Graphed data represents average value ± SEM. *$p<0.05$, by Student's t test.

Figure 3-6: Loss of Action via NtsLepRb Neurons Disrupts Adaptive Reward Wanting



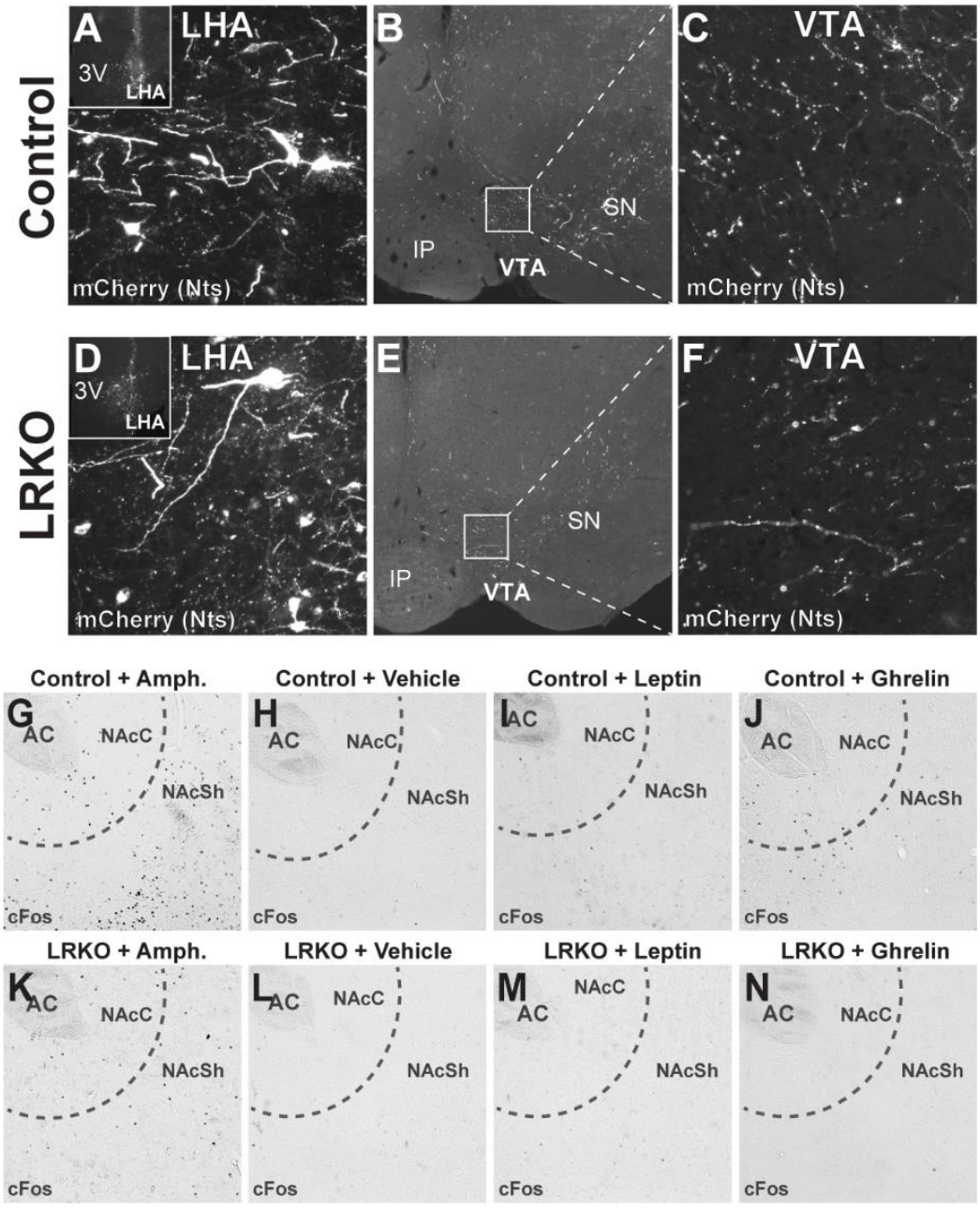
Adult male control and LRKO mice were tested via a progressive ratio (PR) paradigm for their willingness to work for sucrose rewards (9 – 37 wk old, control n=11, LRKO n=15). The PR breakpoint represents how much the sucrose reward is wanted. A) Control and LRKO mice have similar baseline PR breakpoints at baseline. B) Leptin treatment adaptively decreases the PR breakpoint in control mice, but not in LRKO mice that are unable to respond to leptin via Nts^{LepRb} neurons. C) Ghrelin treatment adaptively increases the PR breakpoint in control mice, but not in LRKO mice. Graphed data represents average value ± SEM, *$p<0.05$ by Student's t test.

Figure 3-7: Loss of Action via NtsLepRb Neurons Disrupts LHA Gene Expression



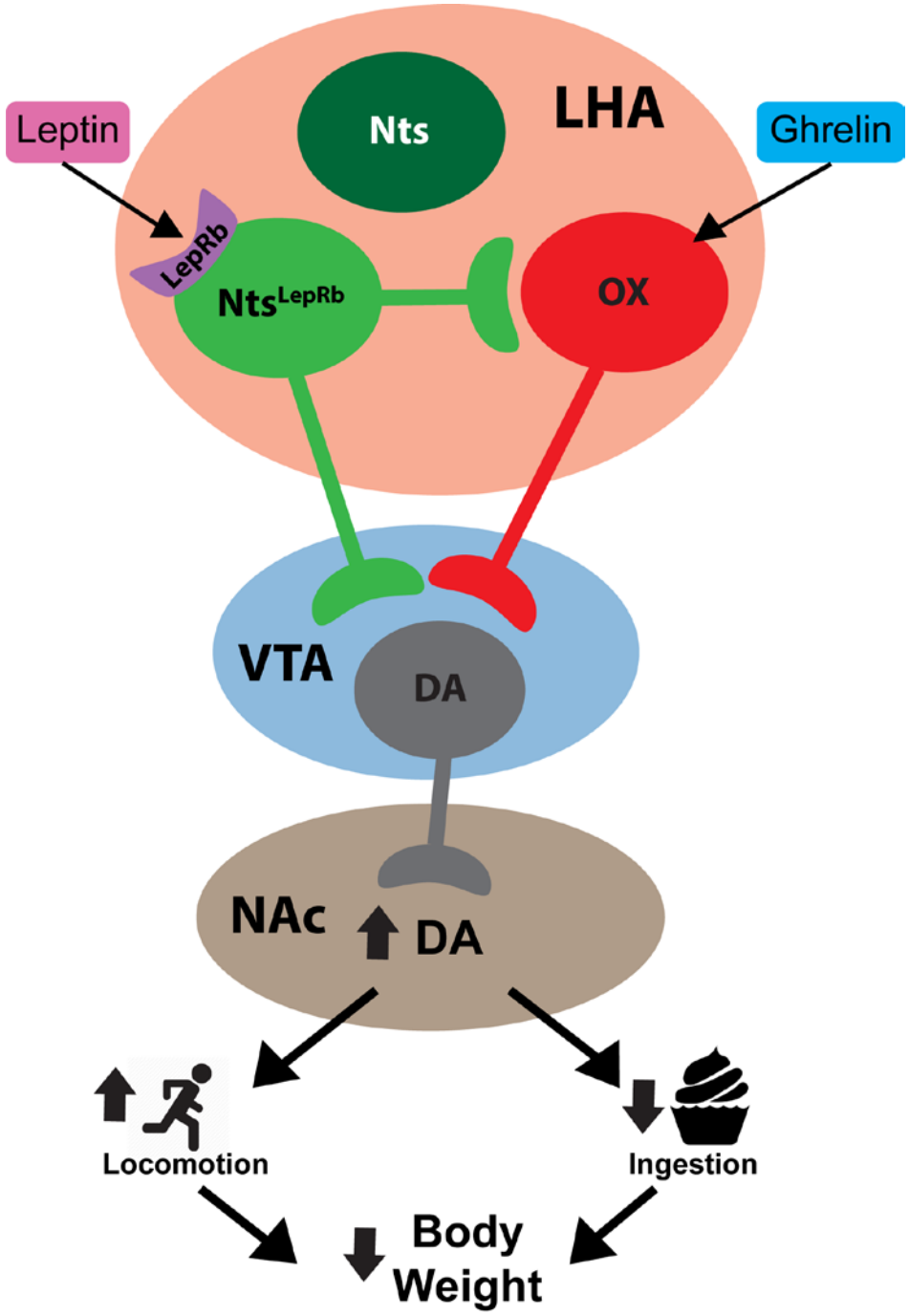
Gene expression was assessed in the brains of adult male (13 -19 wk) control and LRKO mice (n = 7-8 per genotype). A-C) Gene expression in the LHA for transcripts that are specific to LepRb neurons, including A) *Nts*, B) *galanin* and C) *LepR*. D-F) Gene expression in the LHA for transcripts that are specific to OX neurons, including D) *OX,* E) *Dlk1* and F) *GHSR*. G-I) VTA gene expression of transcripts found in mesolimbic dopamine neurons, including G) *TH*, H) *DAT* and I) *NtsR1*. Graphed data represents average value ± SEM. *$p<0.05$ by Student's t test.

Figure 3-8: Projections of LHA Nts Neurons and Activation of Mesolimbic DA Signaling



A-F) Immunofluorescent detection of synaptophysin-mCherry in adult (21-31 wk) male *Nts*^{Cre} mice (Control, n = 3) and LRKO mice (n = 4) mice following intra-LHA injection of the cre-inducible anterograde tract tracer, Ad-Syn-mCherry. A) Representative image of the LHA from a control mouse, showing Nts cell bodies and local projections within the LHA and B) projections to the VTA. C) Enlargement of the boxed area from panel B. D) Representative image of the Nts-containing cell bodies and location projections from a LRKO mouse, and E) projections to the VTA. F) Enlargement of the boxed area from panel E. Insets in A and D identify the injection site into the LHA. G-N) Immunohistochemical detection of cFos in the nucleus accumbens core (NAcC) and shell (NAcSh) of adult male control and LRKO mice following treatment with G, K) amphetamine (control n = 5, LRKO n = 5), H, L), vehicle (control n = 7, LRKO n = 6), I, M) leptin (control n = 5, LRKO n = 6) and J, N) ghrelin (control n = 4, LRKO n = 5). Mice were male, 17-23 wk of age.

Figure 3-9: Model Leptin-Mediated LHA Nts Neuronal Contribution to Energy Balance



LHA NtsLepRb neurons act as “command neurons” and regulate hormone-mediated adaptive feeding and activity both directly and by modulation of ghrelin-sensitive OX neurons.

CHAPTER 4 Lateral Hypothalamic Area Neurotensin Neurons are Required for Control of Energy Balance

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This chapter is an adaptation of a manuscript in preparation for submission Abstract

4.1 Abstract

The lateral hypothalamic area (LHA) is essential for motivated ingestive and locomotor behaviors that impact body weight. Yet, despite the clear necessity of the LHA as a whole, it remains unclear how the neurochemically-defined subpopulations of LHA neurons contribute to energy balance. In particular, the role of the large population of LHA neurotensin (Nts) neurons has remained ambiguous due to the lack of methods to easily visualize and modulate these neurons. Since some LHA Nts neurons are activated by leptin and other anorectic cues, and they modulate dopamine or local LHA Orexin neurons implicated in energy balance, we reasoned that LHA Nts neurons are necessary for control of motivated behaviors and body weight. To test this hypothesis, we used a genetic lesion technique to selectively ablate LHA Nts neurons in adult mice. Loss of LHA Nts neurons resulted in profoundly increased adiposity compared to mice with intact LHA Nts neurons, as well as diminished locomotor activity, energy expenditure and water intake. Loss of LHA Nts neurons also led to downregulation of Orexin, revealing important cross-talk between the LHA Nts and Orexin populations in maintenance of behavior and body weight. Chronic chemogenetic inhibition of LHA Nts neurons did not disrupt Orexin expression, but it suppressed locomotor activity and the adaptive response to leptin. Taken together, these data reveal independent and OX-dependent actions of LHA Nts neurons, and reveal their necessity in controlling energy balance.

4.2 Introduction

Obesity affects millions of individuals worldwide, predisposing them to chronic conditions and increased mortality [86]. Yet, there are few efficacious interventions for the disease and diet and exercise remain the most prescribed treatment. While such lifestyle changes can induce weight loss, they are difficult to maintain and are thwarted by bodily adaptations that oppose sustained weight loss [316-319]. Understanding how the brain regulates feeding and locomotor behaviors that impact body weight may suggest strategies to support weight loss and maintenance of healthy body weight.

Experimental brain lesions have been pivotal for determining how specific brain regions regulate physiology, and this method exposed the essential role of the lateral hypothalamic area (LHA) in control of body weight. Lesion of the entire LHA causes adipsia, aphagia and impaired motivation to move that leads to profound weight loss and death [30;31]. Global LHA lesion, however, disrupts all of the neurochemically and projection-specified populations of LHA neurons, obscuring determination of which specific neurons mediate facets of energy balance. For example, LHA neurons defined by their expression of the neuropeptide melanin concentrating hormone (MCH) are activated by glucose and facilitate feeding, but suppress locomotor activity and arousal [86;88;320]. Separate neurons expressing the neuropeptide Orexin/Hypocretin (OX) are activated by signals of energy deficit and promote arousal along with feeding, drinking, and locomotor activity [288;321]. LHA neurons are also defined by their expression of either GABA or glutamate; while GABA neurons promote voracious ingestive behavior in part via projections to the mesolimbic dopamine (DA) system, glutamate neurons suppress motivated intake via projections to the lateral habenula [264-266]. These LHA populations, however, do not explain the entirety of LHA actions, particularly how the LHA intercepts anorectic cues such as leptin or dehydration and coordinates appropriate ingestive behavior.

The LHA also contains a large population of neurotensin (Nts)-expressing neurons that are distinct from, and more numerous than OX and MCH neurons [269], yet, their requirement for energy balance was unknown. Unlike MCH and OX neurons, LHA Nts neurons specifically respond to feeding suppressing cues such as dehydration and the adipose derived hormone, leptin [200;229;269]. Indeed, a subset of LHA Nts neurons co-express the long form of the leptin receptor (LepRb), and developmental deletion of LepRb from these cells causes weight gain [200] . Activation of the entire population of LHA Nts neurons suppresses feeding and promotes voracious drinking and locomotor activity, indicating that LHA Nts neurons can differentially modify ingestive behavior compared to activation of neighboring MCH and OX neurons. Projection mapping from LHA Nts neurons suggests two circuit mechanisms by which they might modulate behavior and energy balance. At least some LHA Nts neurons project to the VTA where they modify DA release to the nucleus accumbens, where DA modulates motivated ingestive and locomotor behavior [194]. Alternately, LHA Nts neurons project to and inhibit OX neurons, which impedes OX-mediated regulation of feeding, including in response to the orexigenic hormone ghrelin [200]. Taken together, these data suggest that LHA Nts neurons may coordinate unique facets of energy balance, but it remained unclear whether they are necessary for maintaining normal body weight. We therefore used genetic lesion and chemogenetic inhibition to explore the specific requirement for LHA Nts neurons in control of behavior and body weight.

4.3 Materials and Methods

4.3.1 Reagents

CNO was obtained from the NIH as part of the Rapid Access to Investigative Drug Program funded by the NINDS. Aliquots of 40x CNO stock solution were made by diluting with PBS/10% beta-cyclodextrin (Sigma) and stored at -20˚C until use. CNO stock solution was diluted to 1X with

PBS (VEH) just prior to use. Recombinant leptin was purchased from the National Hormone and Peptide Program (Torrance, CA).

4.3.2 Animals

All procedures were approved by the Michigan State University and the University of Michigan Institutional Animal Care and Use Committees, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and National Institute of Health guidelines. Mice were bred in house, maintained on a 12h light/dark cycle with a*d libitum* access to food and water, unless otherwise noted in experimental methods. Male mice were used in all metabolic and chronic studies, and both males and females were used for acute leptin refeeding studies.

4.3.3 Mice

Nts^{cre} mice (Jackson stock #017525) were crossed with homozygous *Rosa26*$^{EGFP\text{-}L10a}$ mice (Krashes 2014) and progeny heterozygous for both alleles were used for study (*Nts*^{cre}*;GFP* mice). Heterozygous *Nts*^{cre} mice were also used for separate experiments. Genotyping was performed using standard PCR using the following primer sequences: *Nts*^{cre}*:* common forward: 5' ATA GGC TGC TGA ACC AGG AA, Cre reverse: 5' CCA AAA GAC GGC AAT ATG GT and WT reverse: 5' CAA TCA CAA TCA CAG GTC AAG AA. *Rosa26*$^{EGFP\text{-}L10}$: mutant forward: 5' TCT ACA AAT GTG GTA GAT CCA GGC, WT forward: 5' GAG GGG AGT GTT GCA ATA CC and common reverse: 5' CAG ATG ACT ACC TAT CCT CCC.

4.3.4 Generation and Phenotyping of LHA Nts-Ablated Mice

8-10 wk old *Nts*^{Cre}*;GFP* males received bilateral LHA stereotaxic injections of AAVs as described previously [269] using coordinates to target the LHA (A/P: 1.34, M/L, 1.13 and D/V, 5.20 in

accordance with the mouse brain atlas of Paxinos and Franklin [271]. One cohort of mice received 500 nL of AAV-DTA (lox-mCherry-loxDTA-WPRE-AAV, serotype 10), which induces Cre-dependent expression of cytotoxic Subunit A of Diphtheria Toxin in the presence that leads to cell death; we refer to these as LHA Nts-ablated mice. A separate group of mice received 500 nL of AAV-GFP (rAAV2/hSyn-DIO-eGFP, University of North Carolina Vector Core), which induces Cre-dependent GFP expression and leaves LHA Nts neurons intact; these are Controls. Mice were single-housed and assessed weekly for food intake and body weight. At 16 wk-post surgery, mice were analyzed for body composition using an NMR-based instrument (Minispec mq7.5, Bruker Optics). Mice were then acclimated for 24 hr within TSE cages (PhenoMaster, TSE Systems), followed by 4 days of continuously measurement of food and water intake, locomotor activity, and energy expenditure. Ambient temperature was maintained at 20-23˚C and the airflow rate through the chambers was adjusted to maintain an oxygen differential around 0.3% at resting conditions. Metabolic parameters including VO_2, respiratory exchange ratio, and energy expenditure were assessed via indirect calorimetry by comparing O_2 and CO_2 concentrations relative to a reference cage. After metabolic analysis the mice were assessed for 1% sucrose preference as described previously [269]. Preference was assessed by determining the % of total volume drunk that was sucrose solution. Mice were included in the final data-set if post-hoc analysis confirmed that injections were localized to, and contained within the LHA. Additionally, AAV-DTA injected mice were only included if they had visible loss of Nts-GFP neurons within the LHA but not in adjacent regions, as assessed via immunofluorescent detection of Nts-GFP. After post-hoc examination, 4/13 Nts-ablated and 3/8 control mice were excluded from analysis due to unilateral targeting and/or spread of the injection site outside of the LHA; thus, final bilateral groups consisted of LHA Nts-ablated n=9 and control n=5. For qualitative assessment of LHA cell types, a separate cohort of *Nts^{Cre};GFP* mice received unilateral LHA injections of AAV-DTA and were perfused either 2 or 10 wk later (6 and 6 mice, respectively). After post-hoc analysis and exclusion of mis-targeted animals, there were 4 well-targeted and ablated 2 wk mice and 4, 10 wk mice.

4.3.5 Gene Expression of LHA Nts-Ablated Mice

Nts^{Cre} mice received unilateral LHA injections of AAV-DTA. Mice were euthanized 2 or 10 wk post-surgery to recover the un-injected (control) and AAV-DTA injected (LHA Nts-ablated) sides of the LHA (2-week n=6, 10-week n=8). Tissue samples were immediately snap frozen on dry ice and stored at -80˚C for later processing. RNA was extracted using Trizol (Invitrogen) and 200 ng samples were converted to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Sample cDNAs were analyzed in triplicate via quantitative RT-PCR for gene expression using TaqMan reagents and an ABI 7500 (Applied Biosystems, Foster City, CA). Using *GAPDH* expression as an internal control, the relative mRNA expression values were calculated by the $2^{-\Delta\Delta Ct}$ method and the AAV-DTA injected values were normalized to the un-injected values. To verify AAV-DTA mediated ablation, the fold change in *Nts* was compared between the ablated and intact sides of the LHA. Mice were only included in final analysis if the ablated side fold change in *Nts* expression was greater than one standard deviation from the mean fold change of the control side. By this method, 4 mice were excluded from analysis (Final 2-week n=4, 10-week n=6).

4.3.6 Chemogenetic Inhibition of LHA Nts Neurons

Mice were injected bilaterally with 500 µL of AAV-hM4D-mCherry (UNC vector core) into the LHA and began analysis 3 wk later. Mice received twice-daily *i.p.* injections (8-9AM and 6-7PM) of VEH for two days to acclimatize them to treatment. Mice were then separated into two groups that received twice-daily treatments for 28 days: one group received VEH (controls) and the other received CNO (to inhibit LHA Nts neurons). Mice were analyzed within TSE cages during the first 5 days and then again for the final 6 days of treatment. In the interim, mice were housed in home cages and daily food intake and body weight were assessed via hand-measuring. Mice were only included in the final study if post hoc analysis confirmed that mCherry expression was localized to,

and confined within the LHA on both sides of the brain. Of 14 mice injected, 2 VEH-treated and 2 CNO-treated were exclude as misses (Chronic VEH-treated n = 7, Chronic CNO-treated n = 7).

4.3.7 Fasting-Induced Re-feeding

Chow was removed from home cages at 6PM and mice were given a clean cage bottom to prevent intake of food that may have fallen into the bedding. Mice had *ad lib* access to water during food-deprivation. The following morning between 8AM-9AM, fasted mice were treated with *i.p.* VEH or CNO to inhibit LHA Nts neurons. Then, 30 minutes later mice were treated with PBS or leptin (5 mg/kg, *i.p.)*. Chow was returned 30 min after PBS/leptin treatment, and food intake and body weight were measured 1, 3, 9 and 24 hr after return of food. The study was performed using a cross-over design, such that each mouse received every treatment (VEH/PBS, VEH/Leptin, CNO/PBS and CNO/Leptin) and could serve as its own control. Mice were given at least 2 full days of recovery from fasting between each trial to ensure complete weight regain.

4.3.8 Imaging and Quantification of Neuronal Populations

Immunofluorescence was performed as in [269]. Primary antibodies used included chicken anti-GFP (Abcam, 1:1000), goat-anti Orexin (Santa Cruz, 1:1000), goat-anti MCH (Phoenix Peptides, 1:1000), DLK1 (Santa Cruz, 1:500). Species-specific secondary antibodies were used for detection at 1:200 (Jackson ImmunoResearch or Thermo Fisher-Invitrogen) using either Alexa Fluor-488 or 586-conjugated fluorophores. Brain sections were analyzed using an Olympus BX53 fluorescence microscope outfitted with FITC and Texas Red filters. Microscope images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe). For quantification of LHA neurons, two representative sections were selected from each animal and counts were performed in an area defined as ventral and lateral

to the mt, and dorsal to the fornix. Since each brain is sectioned into four equally representative series of sections, the total number of each neurons is multiplied by four for final analysis.

4.3.9 Statistics

Students t-test (to compare two groups) or one-way ANOVA with Bonferroni post-testing (for comparisons between multiple groups) were used to determine significant differences between groups, and were calculated using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Error bars depict ± standard error of the mean (SEM). Differences were considered significant for $p < 0.05$. For all data, *p<0.05, **p<0.01 and ***p<0.001.

4.4 Results

4.4.1 Loss of LHA Nts Neurons Causes Obesity

To determine if LHA Nts neurons are required for the control of energy balance, we genetically lesioned them [177;322] (Figure 4-1-A). Adult *Nts*^{Cre} mice were injected unilaterally in the LHA with an AAV expressing Cre-dependent Diphtheria Toxin A subunit (AAV-DTA), such that the DTA expression selectively kills LHA Nts neurons. Adjacent cells remain intact because DTA lacks the B-subunit necessary to internalize other cells, so it is not transmitted outside of the Nts Cre-expressing neurons. We confirmed that AAV-DTA resulted in ablation of Nts neurons by 2 weeks post-surgery, while adjacent MCH neurons remained intact (Figure. 4-1-B). Next, we generated cohorts of mice bilaterally injected in the LHA with AAV-DTA (LHA Nts-Ablated mice) or with AAV-GFP, which drives Cre-dependent expression of GFP so that Nts neurons remain intact (Control). By 16 wk post-injection the LHA Nts-ablated mice were visibly larger than the controls (Figure 4-1-C,D). Intriguingly, the LHA Nts-ablated mice did not exhibit increased total body weight or Δ body weight over the course of study compared to controls, but the LHA Nts-ablated mice have a

significantly higher percentage of body fat (Figure 4-1-E-G). These data suggest that LHA Nts neurons are required to prevent excess adiposity and for maintaining appropriate body weight.

4.4.2 Loss of LHA Nts Neurons Blunts Water Intake and Locomotor Activity

Next, we sought to determine the cause of the obesity in LHA Nts-ablated mice, and whether it resulted from disrupted ingestive behavior and/or energy expenditure. LHA Nts-ablated mice ate similar amounts of chow as control mice, thus their obesity is not due to excess caloric intake (Figure 4-2 A,B). Interestingly, LHA Nts-ablated mice drank less water than controls, suggesting some disruption of ingestive behavior (Figure 4-2 B). Additionally, the LHA Nts-ablated mice displayed reduced ambulatory locomotion relative to control mice (Figure 4-2 C) as well as a decrease in VO_2 and a trend for lower VCO_2 (Figure 4-2 D,E,F). The respiratory exchange ratio (RER) was not different between groups suggesting that substrate usage is not changed with loss of LHA Nts neuronal function (data not shown). Taken together, these data suggest that loss of LHA Nts neurons diminishes locomotor activity and energy expenditure, which can promote the development of obesity.

4.4.3 Loss of LHA Nts Neurons Decreases Motivated Activity and Adaptive Energy Balance

One of the main projections of LHA Nts neurons is to the mesolimbic dopamine circuit that modifies the motivation to ingest and move [191]. We therefore reasoned that loss of LHA Nts neurons, in addition to disrupting homeostatic energy balance, might particularly impair motivated behaviors that impact body weight. To examine this, we gave control and LHA Nts-ablated mice access to running wheels within metabolic cages, allowing us to examine their non-obligatory, motivated locomotor activity (wheel running) and its effects on other metabolic parameters. As expected, control mice with intact LHA Nts neurons engaged the running wheels, but the LHA Nts-

Ablated exhibited vastly reduced running time, wheel rotations and shorter run bouts over 48 hr (Figure 4-3 A-C). The wheel-running control mice also consumed significantly more chow and water than LHA Nts-ablated mice (Figure 4-3 D,E). Since both feeding and drinking are elevated in the control mice during wheel-running as compared to baseline (Figure 4-2 A,B), which likely reflects the increased and fluid intake necessary to support increased energy demand due to the wheel running. Similarly, energy expenditure, VO_2 and VCO_2 was higher in control mice compared to LHA Nts-ablated mice, consistent with their increased energy output via wheel running (Figure 4-3 F-H). RER remained similar between groups, indicating that substrate usage is not altered even with the higher energy demands of the wheel-running control mice (Figure 4-3 I). These data reveal that loss of LHA Nts neurons impairs volitional motivated locomotor activity and associated energy expenditure, while mice with intact LHA Nts neurons choose to engage in exercise-like behavior. Since sustained exercise along with a healthy diet protects against weight gain [323], via LHA Nts neurons may be necessary for the motivation to exercise and ability to maintain normal body weight.

4.4.4 Loss of LHA Nts Neurons Disrupts Drinking but Not Sucrose Preference

LHA Nts neurons project to and release Nts into the ventral tegmental area (VTA), a brain region implicated in modifying the motivation to work for food rewards (e.g. dopamine-dependent "wanting") and their hedonic value (e.g. opioid-dependent "liking") [15;324]. While our data suggest that LHA Nts neurons are required for motivated running behavior, which is known to be DA-dependent [325], we also examined whether these neurons are required for hedonic intake. We therefore tested control and LHA Nts-ablated mice via a two-bottle sucrose preference assay, which is well established to identify hedonic intake [326]. Prior to receiving sucrose, LHA Nts-ablated mice consumed less water than control mice, consistent with our findings from metabolic cages (Figure 4 A). While LHA Nts-ablated and control mice both increased intake of sucrose over water, and exhibited similar sucrose preference, the LHA Nts-ablated mice consumed less total volume of each

liquid compared to controls (Figure 4-4 B-D). Together, these data indicate that LHA Nts neurons are important mediators of homeostatic drinking behavior, but that they are not required for hedonic consumption.

4.4.5 Lesion of LHA Nts Neurons Precedes Reduction in Orexin Expression

Some LHA Nts neurons synaptically modulate OX neurons [269;298;304], thus loss of LHA Nts neurons might impair OX function over time and indirectly contribute to the behavioral derangements and obesity of LHA Nts-ablated mice. We therefore investigated the impact of LHA Nts ablation at 2 wk post-injection, when AAV-DTA mediated ablation should be complete, yet control and LHA Nts-ablated mice exhibit comparable weight gain at this time point (Figure 4-5 A). We also examined mice 10 wk-post injection, when LHA Nts-ablated mice show a trend toward increased weight gain compared to controls (Figure 4-5 A). As a first step, we evaluated *Nts^{Cre};GFP* mice that were unilaterally injected with AAV-DTA for Nts-GFP neurons and MCH and OX peptide expression via immunofluorescence (IF); in these experiments, the contralateral un-injected LHA serves as an intact control. As observed previously (Figure 4-1 B), AAV-DTA injection depletes LHA Nts-GFP neurons at 2 wk relative to the control side but the MCH population remained intact (Figure 4-5 B,C). OX-IF appeared somewhat reduced, but bright, highly-expressing OX neurons were still apparent at 2 wk post-injection (Figure 4-5 B,C). Depletion of LHA Nts-GFP neurons was also evident at 10 wk post AAV-DTA injection compared to the control side, and MCH-IF was also somewhat reduced at this time. OX-IF, however, was more obviously diminished, with fewer brightly-labeled OX expressing cell bodies at 10 wk post-injection compared to the control LHA or 2 wk-ablated mice (Figure 4-5 D,E). Cell counts confirmed a significant reduction in Nts-GFP neurons in the ablated LHA compared to the control side at 2 and 10 wk (Figure 4-4 F). The number of IF-detected MCH and OX neurons was similar in the ablated and control LHA at 2 wk, but was significantly decreased at 10 wk (Figure 4-5 F). These data confirm that the genetic lesion method causes a rapid and sustained loss of LHA Nts neurons. Furthermore, our data verify that the LHA Nts lesion was specific, since these neurons were depleted by 2 wk post-injection, at which point adjacent MCH

and OX neurons remained intact. However, the reduction in OX-IF at 10 wk suggests that prolonged loss of LHA Nts neurons impaired OX expression, which may have contributed to the behavioral and energy balance impairments observed in LHA Nts ablated mice.

Since LHA Nts neurons act, in part, via projections onto OX neurons, sustained loss of LHA Nts input could disrupt the function and/or viability of OX-expressing neurons. To examine the repercussions of LHA Nts lesion on transcription, we assessed gene expression in *Nts*^{Cre} mice that received AAV-DTA in one side of the LHA, while the un-injected side served as an intact LHA control (Figure 4-5 A, B). As expected, *Nts* expression was significantly decreased in the Nts-ablated LHA at 2 and 10 wk after AAV-DTA injection. *MCH* expression remained unchanged between the control and Nts-ablated LHA at both time points, consistent with our previous observation of intact MCH-IF in LHA Nts lesioned mice (Figure 4-5-F). Conversely, we observed a trend for decreased *OX* expression in the Nts-ablated LHA at 2 wk post-injection, and a significant *OX* reduction by 10 wk (Figure 4-6 A,B). This downregulation of *OX* could represent a specific consequence of loss of LHA Nts signaling input, but could also result from general suppression of transcription in OX neurons and/or decreased viability. To assess this possibility, we measured *DLK-1* expression, a gene product that is co-expressed within OX neurons, but not by other LHA neurons [310]. *DLK-1* expression was similar in the intact and Nts-ablated LHA at 2 and 10 wk post-injection, indicating that loss of LHA Nts neurons does not generally impair gene expression within OX neurons. We also examined DLK1 protein expression via IF, which revealed similar numbers of DLK1 and OX+DLK1-expressing neurons in the Nts-ablated and control LHA, suggesting that at least these OX neurons remain intact (Figure 4-5 C). By contrast, we observed significantly fewer neurons with OX-IF in the Nts-ablated LHA compared to the control side. Taken together, these data indicate that lesion of LHA Nts neurons specifically downregulates OX transcription, which reduces OX peptide and hence impairs detection of the “OX neurons” via OX-IF. This is not necessarily indicative of OX neuronal cell death, however; the retention of DLK1 protein known to be co-

expressed within “OX neurons”, confirms that at least some of them remain intact. Thus, loss of LHA Nts neurons specifically leads to decreased OX expression without disrupting all function and viability of the “OX” population.

4.4.6 Chronic Inhibition of LHA Nts Neurons Decreases Locomotion

Our LHA Nts ablation data confirm that LHA Nts neurons are necessary for proper regulation of OX neurons, and that prolonged loss of LHA Nts neurons disrupts both Nts, OX and energy balance. While these findings reinforce the interdependence of Nts and OX neurons [269], they impaired determination of which facets of the LHA Nts-ablated phenotype are specifically due to loss of LHA Nts neurons (rather than loss of OX-mediated control.) To circumvent this issue, we used chemogenetics (DREADD technology) to selectively inhibit LHA Nts neurons for 4 wk, thereby avoiding long-term structural changes that might impair OX neurons and obfuscate LHA Nts effects. Adult *Nts*^{Cre} mice were bilaterally injected with Cre-inducible AAV-hM4Di-mCherry in the LHA to drive expression of inhibitory DREADD-mCherry selectively on LHA Nts-GFP neurons (Figure 4-7 A, B). We then treated mice with either vehicle or the DREADD agonist clozapine-N-oxide (CNO) to inhibit LHA Nts neurons, and examined how acute (24 hr) or chronic (4 wk) inhibition impacted energy balance (Figure 4-7 C,D). Neither acute nor chronic CNO-treatment altered body weight, adiposity, feeding or drinking relative to vehicle-treated controls (Figure 4-7 E-H). However, locomotor activity was significantly diminished by acute and chronic CNO treatment. While not significant, CNO-treated mice also showed a trend toward decreased ambulatory activity speed. Despite the reduction in locomotor activity, CNO-mediated inhibition of LHA Nts neurons did not alter respiration rates (VO_2, VCO_2) or substrate usage (RER) (Figure 4-7 K-M). These data indicate that inhibition of LHA Nts neurons specifically blunts locomotor activity.

4.4.7 Inhibition of LHA Nts Neurons Does Not Reduce OX

We next examined the distribution of Nts-DREADDi-mCherry neurons and OX-IF in chronically CNO-treated mice, to determine if prolonged inhibition of LHA Nts neurons impaired OX expression similar to the effect of LHA Nts ablation. Nts-DREADDi-mCherry was robustly expressed in the LHA from mice treated for 4-wk with Vehicle or CNO (Figure 4-8 A). We also observed a similar distribution of OX-IF labeled cells in both groups, although the OX-IF was somewhat less intense in the cell bodies of the CNO-treated mice compared to vehicle-treated animals (Figure 4-8 B). These data suggest that, unlike prolonged LHA Nts lesion, chronic inhibition of LHA Nts neurons does not significantly derange OX peptide expression. Thus, the inhibition model presumably reflects the behaviors governed by LHA Nts neurons alone, without effects mediated by disruption of OX neurons.

4.4.8 Inhibition of LHA Nts Neurons Disrupts Leptin-Sensitivity

The disruption of LHA Nts and downstream OX neurons after LHA Nts lesion prevented assessment of how LHA Nts neurons selectively contribute to adaptive energy balance in the lesion model. However, because OX neurons are preserved in mice with chronically inhibited LHA Nts neurons, they can be used to study the specific requirement for LHA Nts neuronal activity in mediating adaptive energy balance without the influence of OX neurons. Since ~15% of LHA Nts neurons express the long form of the leptin receptor (LepRb), we reasoned that suppression of LHA Nts neural activity would blunt the anorectic response to leptin [269]. To test this, we fasted mice expressing DREADDi-mCherry in LHA Nts neurons overnight, to increase their motivation to eat. Mice were then treated with either vehicle or CNO to inhibit LHA Nts neurons just prior to injection with PBS or leptin, and then chow was returned to their cages. As expected, leptin decreased re-feeding and prevented weight gain even in the hungry vehicle-treated mice at 3 and 9 hr post treatment (Figure 4-9 A-F). By comparison, CNO-mediated inhibition of LHA Nts neurons blunted

the anorectic effect of leptin at 3 and 9 hr post treatment. CNO-treated mice, however, gained less body weight, suggesting that leptin-mediated effects via some non-LHA Nts neurons can restrain weight regain even without suppressing feeding, presumably via increasing energy expenditure. Both vehicle and CNO-treated mice resumed normal feeding and body weight 24 hr after treatment, indicating that LHA Nts inhibition does not cause permanent impairments in homeostatic energy balance. These data confirm that inhibition of LHA Nts neurons impairs the adaptive anorectic response to leptin. LHA Nts neurons are thus required for the appropriate coordination of leptin and reduced feeding behavior, as might occur in response to elevated adiposity. Loss of function via LHA Nts neurons may therefore impair both locomotor activity and appropriate adaptive feeding in response to peripheral energy cues that could, over time, potentiate weight gain.

4.5 Discussion

We demonstrate that LHA Nts neurons are necessary for control of behavior and body weight, confirming an important role for this neuronal population in energy balance. In particular, loss of LHA Nts neurons decreases OX expression, drinking behavior, locomotor activity and energy expenditure, and leads to obesity. By comparison, inhibition of LHA Nts neurons blunts locomotor activity and the anorectic response to leptin without disrupting OX expression. Together, these data implicate a specific role for LHA Nts neurons in regulating locomotor activity and the adaptive response to leptin, as these effects were not contingent upon loss of action via downstream OX neurons. Since deficits in drinking and energy expenditure were only observed after sustained LHA Nts ablation and with OX reduction, these behaviors may depend on sustained communication between LHA Nts and OX neurons. In sum, our findings support different LHA Nts circuit mechanisms in controlling behaviors relevant to energy balance, and that chronic disruption of LHA Nts neurons may contribute to maladaptive behaviors that potentiate obesity.

Experimental lesions of the entire LHA revealed its important role in energy balance, but they masked the individual contributions of discrete, neurochemically-defined LHA populations. Due to the ability to easily detect MCH and OX via immunoreactivity, the roles of these neurons have been well studied within the LHA, and both are considered to be orexigenic, glucose responsive and to modulate arousal in opposing directions (MCH promotes sleep and limits locomotor activity, OX facilitates waking and locomotor activity)[327 320]. Genetic ablation has previously been used to discern the necessity of MCH or OX neurons for energy balance, and their distinct contributions to energy balance. Ablation of MCH neurons in adult mice caused significantly increased locomotor activity, improved glucose tolerance and leanness, but did not significantly alter food intake [328]. Conversely, ablation of adult OX neurons decreased arousal/wake time, locomotor activity and drinking, while it increased cataplexy and body weight, with no observed change in feeding [140] By comparison, we found that adult lesion of LHA Nts neurons decreased locomotor activity and drinking and increased adiposity, with no effect on homeostatic or hedonic feeding. The phenotypes resulting from LHA Nts or OX ablation are strikingly similar, and may be due to the fact that LHA Nts neurons synaptically regulate OX neurons and coordinate at least some physiology via OX neurons [135;269]. Hence, loss of LHA Nts neurons and the subsequent downregulation of OX presumably impairs OX-mediated functions, and accounts for the overlapping LHA Nts and OX-ablation phenotypes. Prolonged loss of action via the LHA Nts → OX circuit could also impair MCH neurons, which are synaptically regulated by OX neurons. These data suggest that LHA Nts neurons are crucial master controllers of OX neurons and perhaps the LHA as a whole.

LHA neurons have also recently been parsed by their classical neurotransmitter content, and at least some LHA Nts neurons co-express GABA [255]. Accordingly, we note some similarities between mice with genetic lesion of GABA neurons or disruption of LHA Nts neurons. For example, loss of LHA GABA neurons blunts fasting induced refeeding [265] and in our study fasted LHA Nts-inhibited mice had impaired anorectic responses to leptin (Figure 4-8). However, the phenotypes of

LHA GABA vs Nts-ablated neurons do not perfectly overlap. Interestingly, ablation of LHA GABA neurons did not affect locomotor behavior [265], while loss or inhibition of LHA Nts neurons resulted in substantial reductions in physical activity. While modulation of LHA GABA neurons must include the subset of LHA Nts-GABA neurons, the differences in observed ablation phenotypes suggest that the LHA GABA and LHA Nts neurons are not fully overlapping populations. Thus, there are likely to be some separate behaviors mediated via GABA and Nts neurons, which have yet to be disentangled.

Intriguingly, ablation of MCH, OX or LHA Nts neurons does not impair homeostatic feeding, despite the well-established roles of these peptides to modify food intake [140;328] (Figure 4-2). This may suggest that the LHA is more important for regulating adaptive feeding in response to changing peripheral signals of energy status, and for why we did not observe any reduction in homeostatic feeding of LHA Nts ablated mice. One important signal for adaptive energy balance is leptin, which communicates high peripheral energy stores to the brain and suppresses resultant feeding to prevent weight gain. At least some LHA Nts neurons co-express LepRb and mediate the adaptive leptin response. We therefore reasoned that loss of LHA Nts neurons would impair the adaptive feeding and body weight response to leptin. Indeed, inhibition of LHA Nts neurons blunted leptin-mediated suppression of feeding and resulted in increased body weight. Thus, loss of LHA Nts neurons disrupts leptin-mediated adaptive energy balance, particularly under conditions of increased appetitive drive (as in the fasted mice we tested). Individuals who lose weight via a long-term diet and exercise regime also have an increased appetitive drive, which may prompt feeding and weight regain [329]. We postulate that this could be due, in part, to diminished actions of LHA Nts neurons, and the inability to suppress feeding drive necessary for sustained weight loss. Furthermore, this would impair individuals from responding to elevated leptin, as might occur due to increased adiposity, and from being able to curb the motivation to eat.

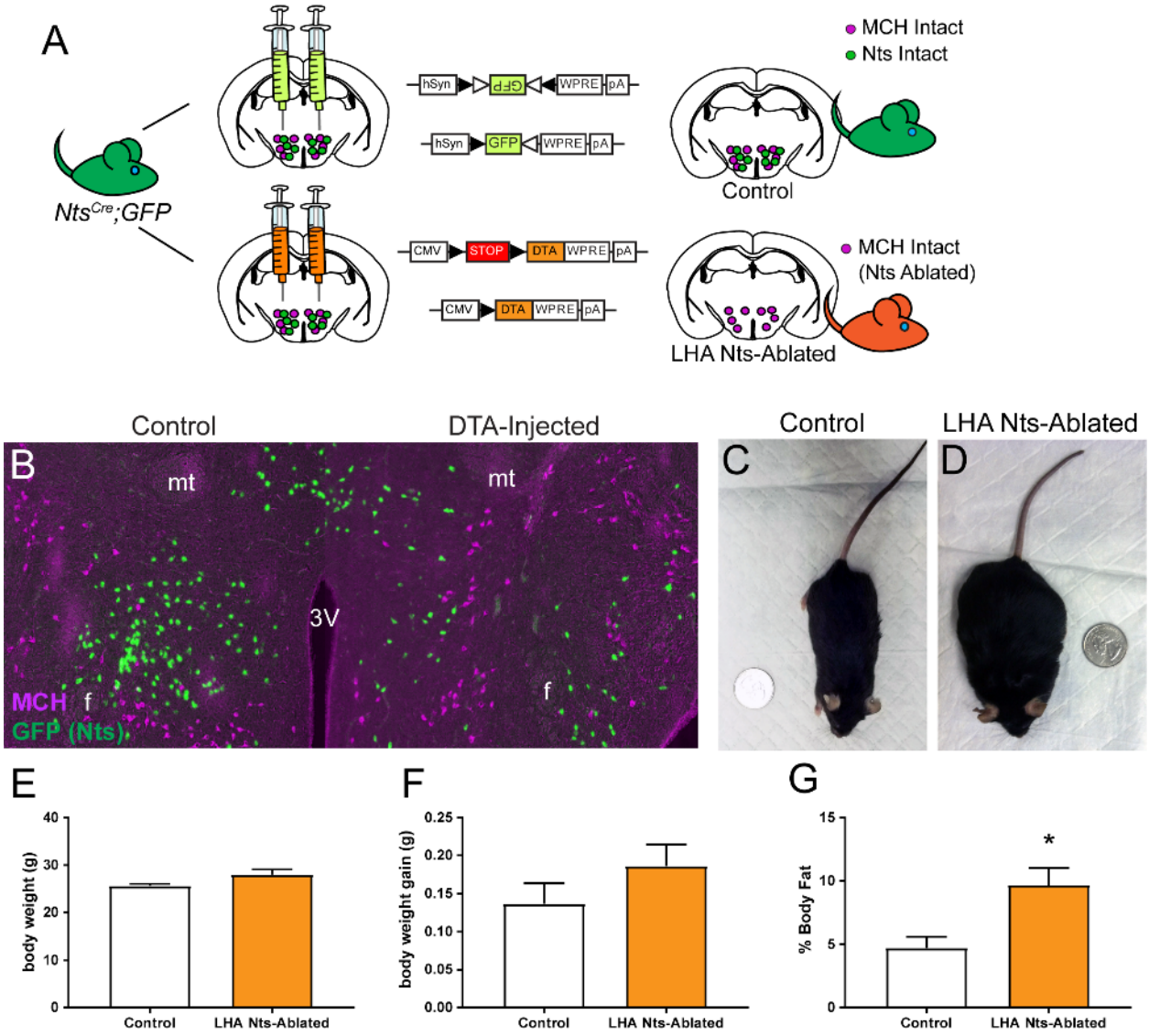
At least some LHA Nts neurons that project to the VTA, co-express LepRb, and presumably they coordinate anorectic leptin response with DA regulation[200;269]. Indeed, LHA Nts neurons regulate mesolimbic DA neurons in the VTA, and promote DA release to the nucleus accumbens that modulates motivated ingestion and physical activity [177;191;269]. Loss of LHA Nts neurons presumably impairs DA-dependent actions, which may be more apparent in conditions that elevate motivation. For example, LHA Nts-ablated mice exhibit reduced ambulatory locomotor activity, but have more profound deficits in motivated locomotor activity (e.g. wheel running). LHA Nts-ablated mice remained capable of moving, and did engage their running wheels, though to a lesser extent than control mice. Given that loss of mesolimbic DA blunts locomotor activity [330], it is possible that loss of the LHA Nts → VTA DA circuit contributes to the diminished physical activity, which over time may potentiate fat accumulation despite normal caloric intake (Figure 4-2 A). LHA Nts-Inhibited mice also demonstrated a decrease in activity, without any apparent downregulation of OX, providing rationale that this effect might be mediated via an OX-independent pathway. Similarly, the blunting of locomotor activity in both the LHA Nts-ablated and inhibited models suggests that at least some portion of LHA Nts-mediated control of physical activity occurs independently of OX, and may be mediated via projections to the VTA or other LHA Nts projection sites. While our data do not rule out a contribution of LHA Nts → OX neurons in regulating physical activity, this circuit is not the sole mediator of the behavior. By contrast, the blunted drinking and energy balance was only noted in the LHA Nts-ablated mice with downregulation of OX neurons, suggesting that these may be predominantly mediated via an LHA Nts → OX circuit. Going forward it will be crucial to dissect the circuit-specific roles of LHA Nts neurons, which may reveal specific targets to modify drinking, feeding and locomotor activity.

In contrast to global LHA manipulations, we selectively modulated LHA Nts neurons to reveal their contributions to energy balance, and their necessity to prevent obesity. A caveat of our studies is that LHA Nts neurons are not homogeneous, hence by modulating ***all*** LHA Nts neurons, we have

obscured the specific roles of any subpopulations of LHA Nts neurons. For example, LHA Nts neurons can be divided into at least two subpopulations: ~15% of LHA Nts neurons express LepRb and are regulated by leptin [200;269], while other LHA Nts neurons are regulated by dehydration [229]. As such, simultaneous loss of *all* LHA Nts neuronal subtypes may yield a mixed phenotype by disrupting function of LepRb-expressing neurons that modify feeding, and dehydration-modulated neurons that presumably coordinate drinking to restore fluid balance. Identification of genetic markers to discern molecularly-specified subpopulations of LHA Nts neurons will be necessary to selectively ablate them and reveal their specific functions. Alternately, ablating LHA Nts neurons that project to local OX neurons vs. those that project to the VTA may be useful to assess whether these circuits separately modify drinking vs feeding and locomotor behavior. While there is still much to be learned about how LHA Nts neurons modify physiology, our data reveal that they are necessary and important mediators for locomotor activity, adaptive feeding responses, drinking and control of normal adiposity. Thus, loss of action via LHA Nts neurons may contribute to behavioral deficits that lead to obesity. In the future, defining the signaling and circuit mechanisms by which LHA Nts neurons coordinate ingestive and locomotor behavior may suggest strategies to restore LHA Nts function and healthy body weight.

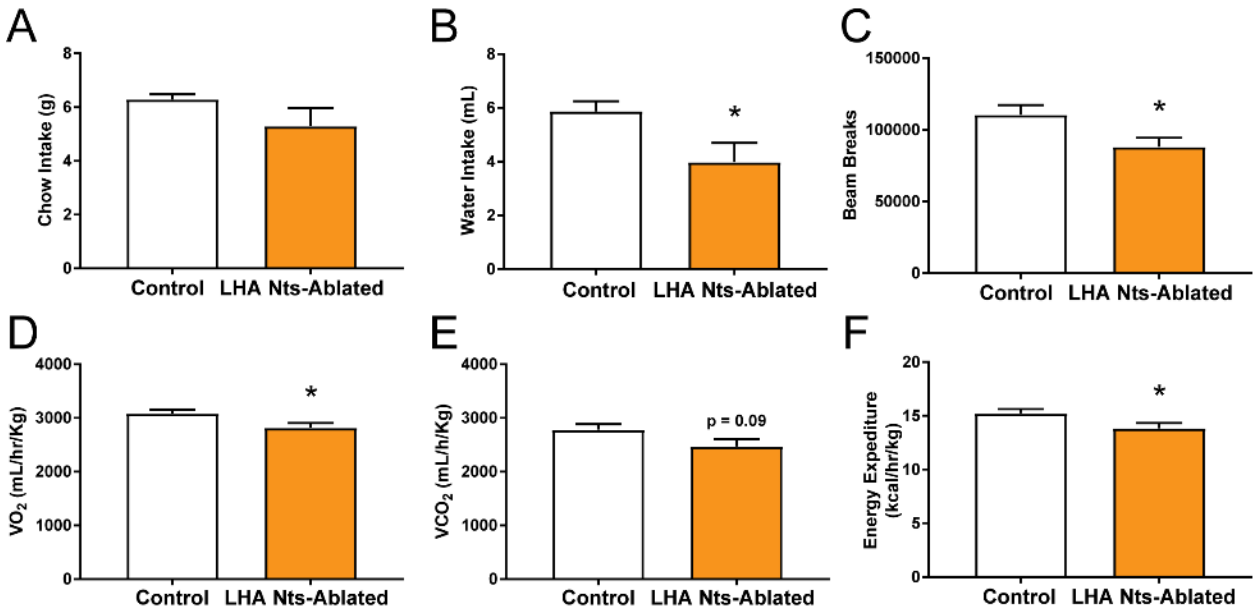
4.6 Figures

Figure 4-1: Ablation of LHA Nts Neurons Increases Adiposity



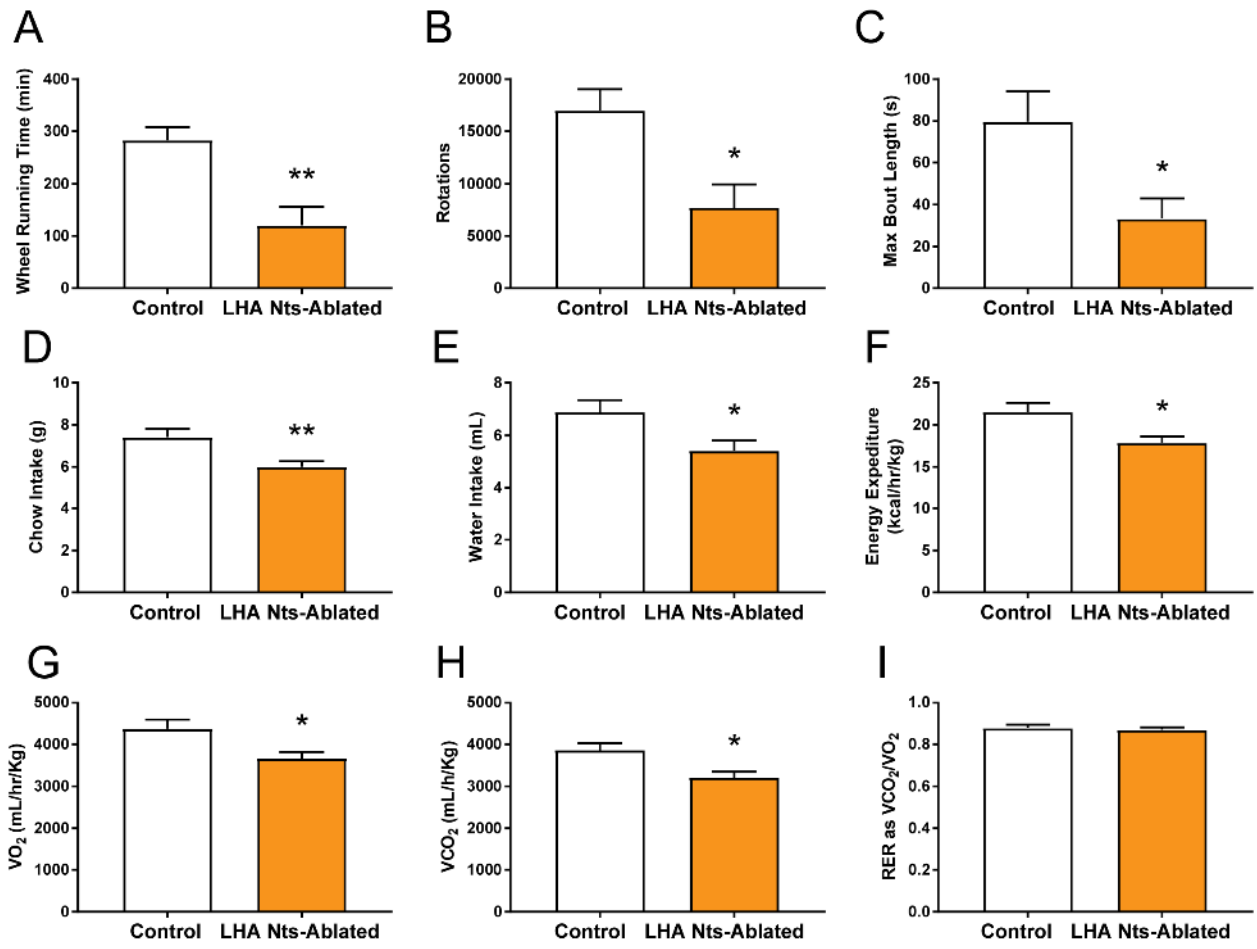
A) To examine the role of LHA Nts neurons in energy balance, we injected *Nts*^{Cre} mice bilaterally with a control virus or AAV-DTA to specifically ablate LHA Nts neurons (Nts-Ablated n=9, Control n=5). B) On the control side, Nts-GFP and MCH IF can be seen in the LHA. However, on the DTA-injected side, while MCH IF reveals an intact population of MCH neurons, there is a marked reduction in GFP IF demonstrating that this model specifically targets and ablated Nts neurons while avoiding other adjacent neuronal populations. C) A control mouse of average body weight is shown with a quarter for size reference. D) An LHA Nts-Ablated animals displays a much fattier body composition and oily looking for showing that specific loss of LHA Nts neurons clearly changes body composition. E, F) Interestingly, on average, body weight was not significantly different between ablated and control mice, nor was weight gained after surgery. However, G) body adiposity is significantly increased in ablated mice relative to controls.

Figure 4-2: Loss of LHA Nts Neurons Blunts Water Intake and Locomotion



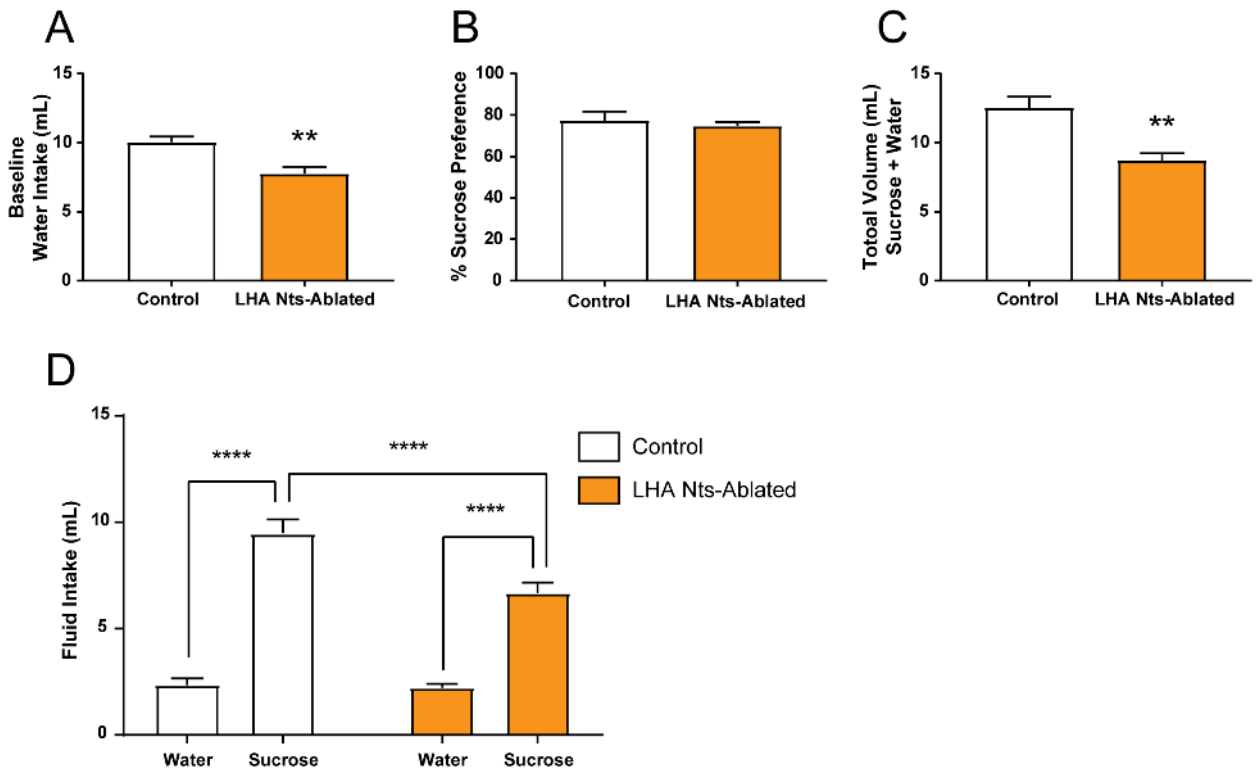
The Nts-Ablated animals generated as described in Figure 2-1 were studies for feeding, drinking and activity levels. A) The increased in body fat that we observed was not because of increased chow-feeding, in fact ablated mice ate slightly less than controls and B) drank significantly less water. To asses if locomotion led to the increase in body fat, mice were assessed in TSE metabolic cages which revealed that C) ablated mice exhibit a decrease in activity relative to controls and show a decrease in D) VO_2 and E) a trend toward and decrease in VCO_2 and a F) decrease in energy expenditure.

Figure 4-3: Motivational Locomotor Activity is Blunted by Loss of LHA Nts Neurons



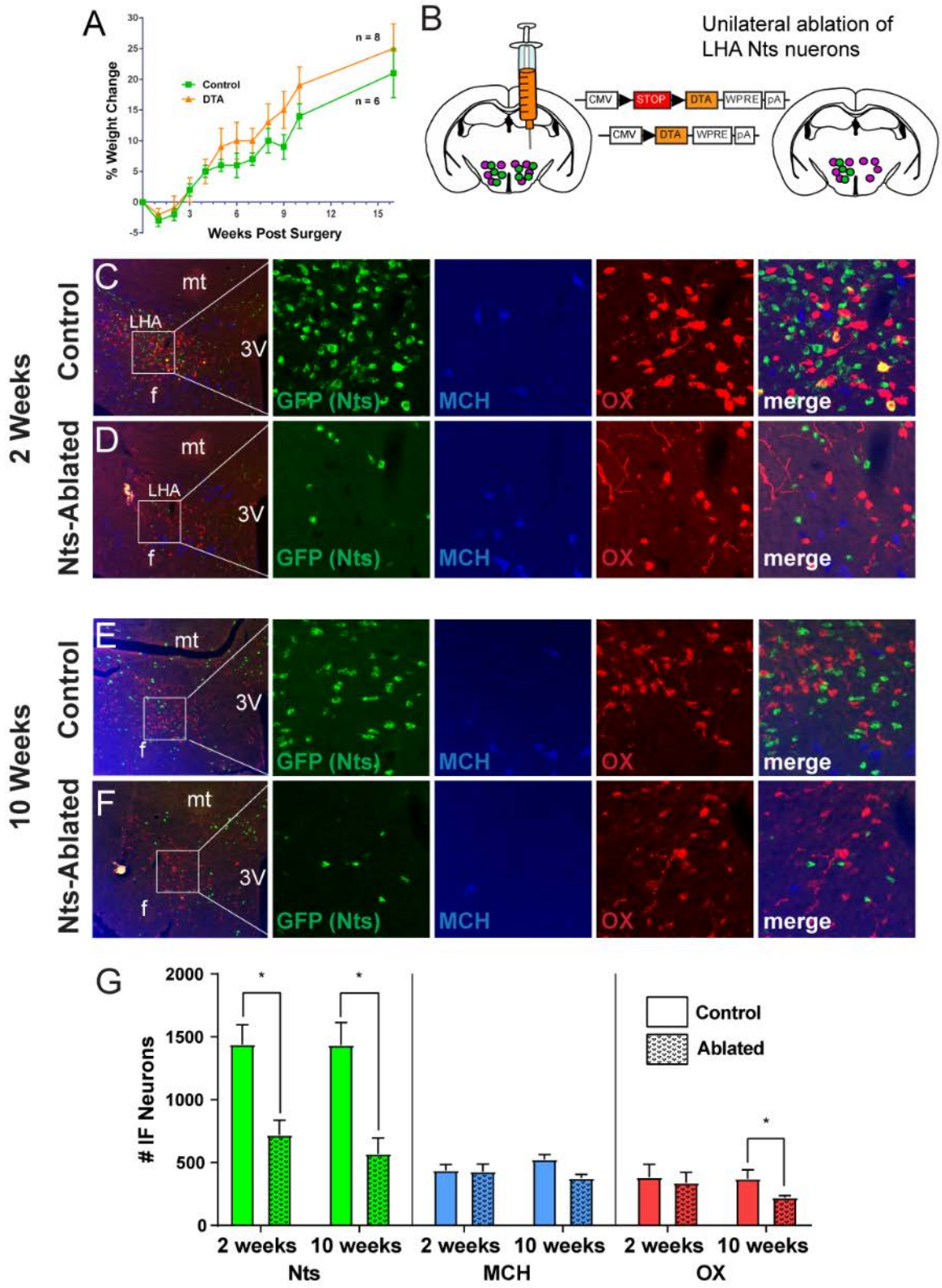
The Nts-Ablated animals generated as described in Figure 2-1, were studied for feeding, drinking and activity levels in metabolic cages as in Figure 2-2, but with the addition of a running wheel to measure non-obligatory (volitional) locomotion (LHA NTs-Ablated n=7; control n=5). A-C) When offered the opportunity to run in a running wheel, LHA Nts-Ablated mice spend significantly less time on wheels, run for fewer revolutions and in shorter bouts, when compared to control animals. D, E) Because they use less energy running than controls, LHA Nts-Ablated animals do not upregulate their intake of food or water relative to controls. F-I) As expected, ablated animals exhibit F) lower energy expenditure, VO_2 and VCO_2 relative to controls. I) No change in RER between groups suggests that these changes result from a motivation and not metabolic shift. Taken together these data show that loss of LHA Nts neurons blunts volitional locomotor activity and as such compensatory upregulation of feeding and metabolism are absent.

Figure 4-4: Loss of LHA Nts Neurons Decreases Drinking but Not Sucrose Preference



LHA Nts-Ablated and control animals (LHA Nts-Ablated n=6, Control n=5) were given a two-bottle choice sucrose preference test. A) Water consumption was assessed before animals were given a choice between sucrose and water and LHA Nts-Ablated animals drank less water than controls at baseline. B) However, overall preference for sucrose was not different between groups. C) During testing days (when mice had both water and sucrose available) the total volume drunk was decreased in ablated animals. D) Volumes of each liquid are shown here. Ablated mice increased their intake of sucrose relative to water, but not as much as control animals. Both groups drank the same volume of water on test days. These data show that loss of LHA Nts neurons does not decrease hedonic preference for sucrose, but it does lead to a decrease in overall drinking.

Figure 4-5: Time Course Showing Loss of LHA Neurons After Ablation of Nts Population

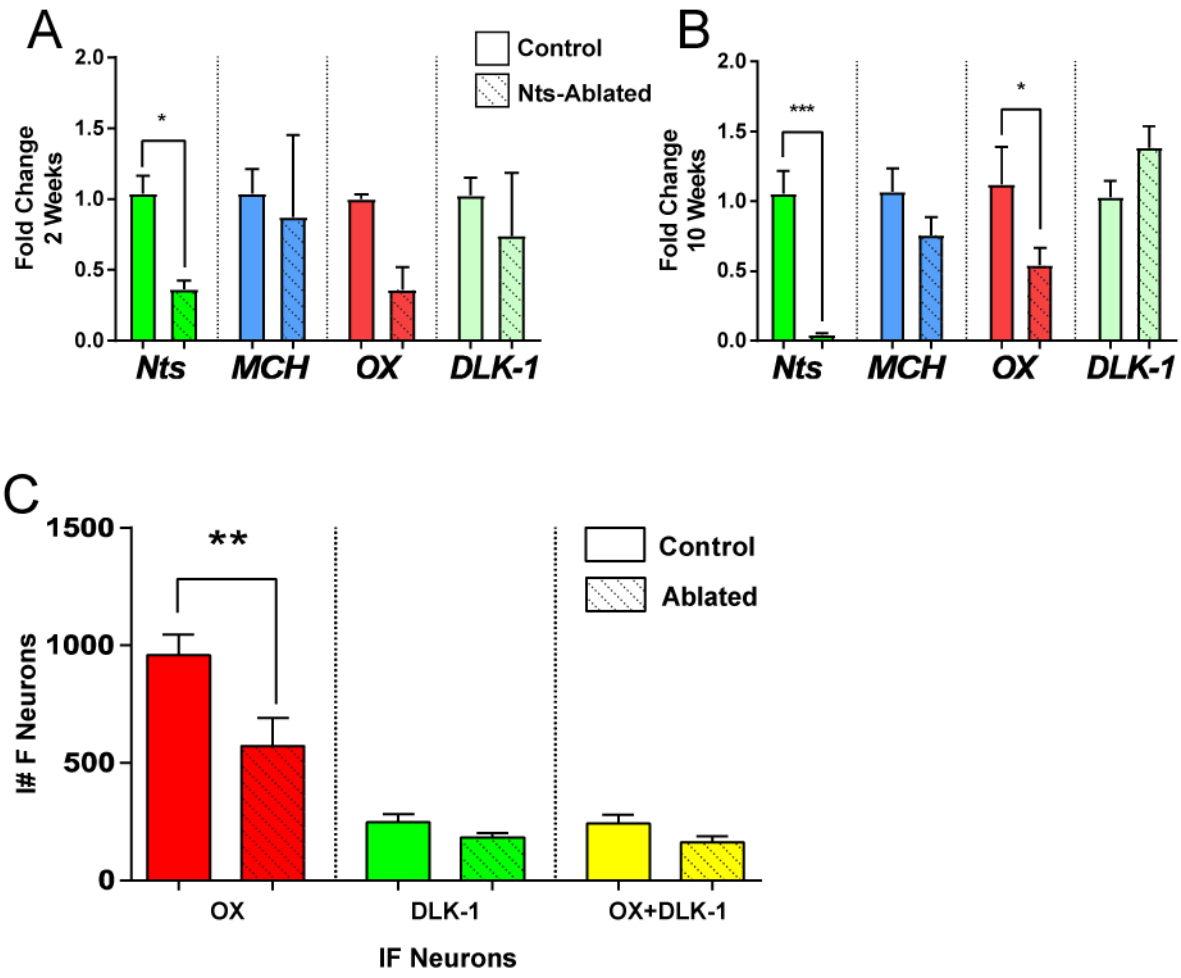


Mice from the longitudinal study described in Figure 1-3) were examined histologically to identify which were appropriately targeting to include for analysis. At that time, it was observed that not only

Figure 4-5 (cont'd)

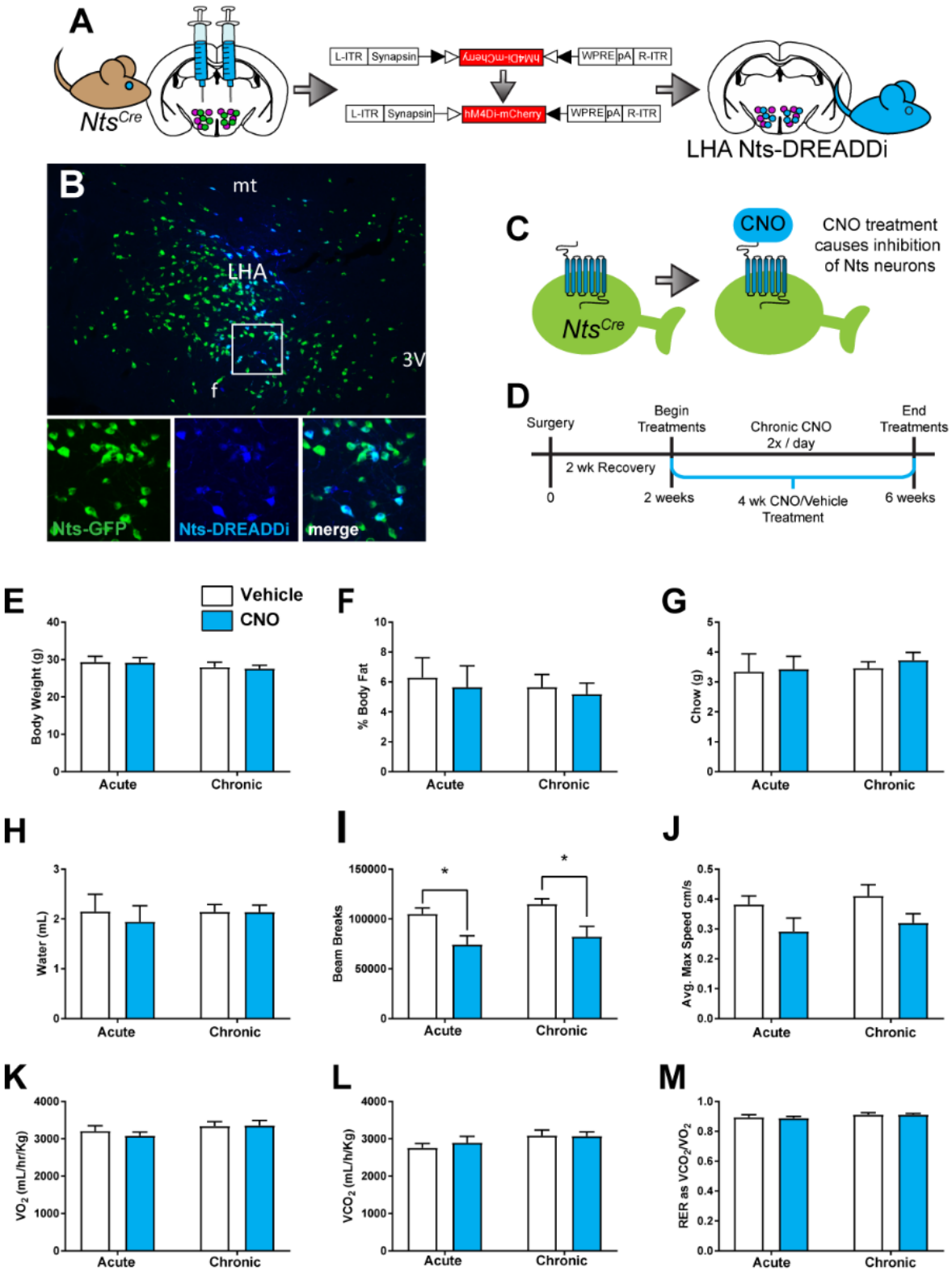
was there a decrease in Nts IF in well targeting animals, but also a decrease in OX IF. A) The longitudinal weights of study animals were plotted over time and while not significant, there is a divergence of body weights of ablated animals from controls at around 4 weeks post-surgery. To determine when the loss of each population occurred, mice were injected unilaterally with AAV-DTA and brains were examined at 2, 6 and 10 weeks post-injection. B, C) Brains show a dramatic decrease in Nts-GFP on the ablated vs the control hemisphere at 2 weeks, but MCH and OX IF remains mostly consistent on both sides. D, E) At 10 weeks post-surgery, Nts IF is decreased on the ablated side relative to controls and MCH remains intact. However, the OX IF has not decreased in number of neurons and some of the remaining neurons appear less bright than the control side. F) Quantification of LHA Nts, MCH and OX neurons at 2 and 10 weeks post-surgery. These data indicate that ablation of Nts neurons by AAV-DTA is complete or nearly complete by 2 weeks post-surgery but there is a slow loss of OX IF that occurs by 10 weeks.

Figure 4-6: Loss of LHA Nts Neurons Leads to a Decrease in OX Peptide Expression



To further investigate the impact of ablation of LHA Nts neurons on other LHA populations we generated unilateral LHA Nts-Ablated mice as described in Figure 4-5. A, B) Gene expression levels of *Nts, MCH OX* and *DLK-1* (a marker of OX neurons) were examined for fold change relative to controls. *Nts* expression is decreased relative to controls at both 2 and 10 weeks but *MCH* is not at either time point. At two weeks *OX* appears to be slightly, but not significantly decreased, but by ten weeks it is significantly decreased. Interestingly, DLK-1 expression is not decreased at two or 10 weeks post-surgery suggesting that loss of OX IF is not due entirely because of loss OX neurons, but because expression levels of the OX peptide have been downregulated. F) Counts of OX and DKL-1 neuronal immunofluorescence in unilaterally ablated animals show a significant decrease in OX IF, but not DKL-1 or cola belled neurons. Collectively these data suggest that loss of LHA Nts neurons disrupts LHA OX neurons leading to downregulation of the OX peptide.

Figure 4-7: Inhibition of LHA Nts Neurons Blunts Locomotion

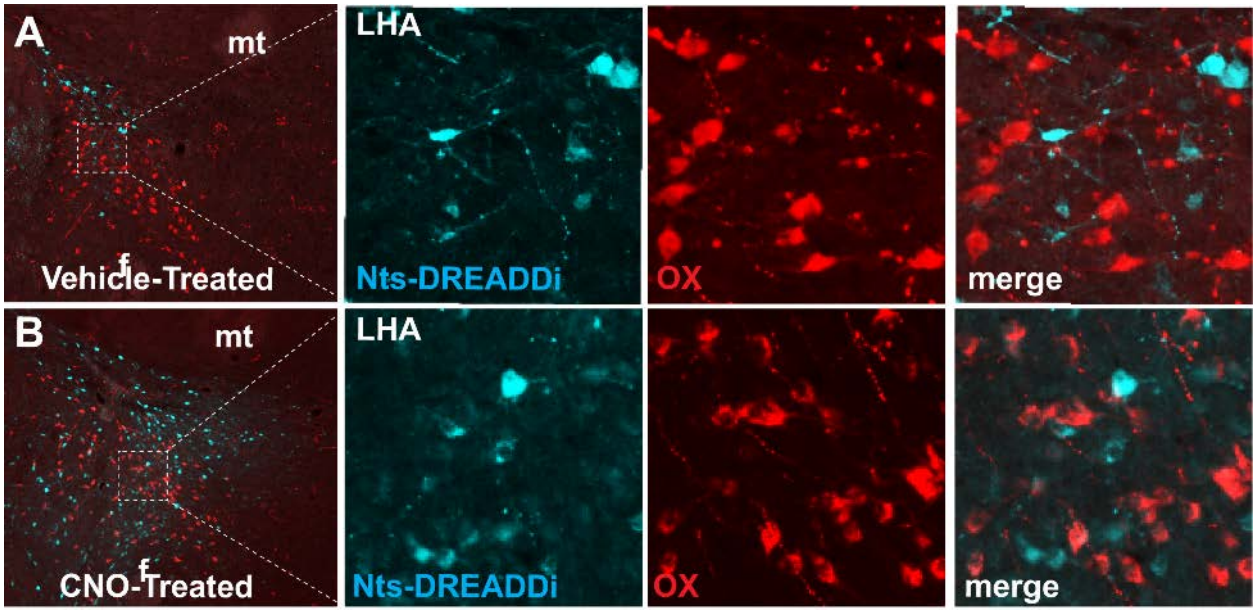


Bilateral injection of *Nts*^{Cre} mice with AAV-HM4D-mCherry into the LHA of *Nts*^{Cre} mice Cre-dependent expression of DREADD receptors to allow for chemical inhibition of LHA Nts neurons

Figure 4-7 (cont'd)

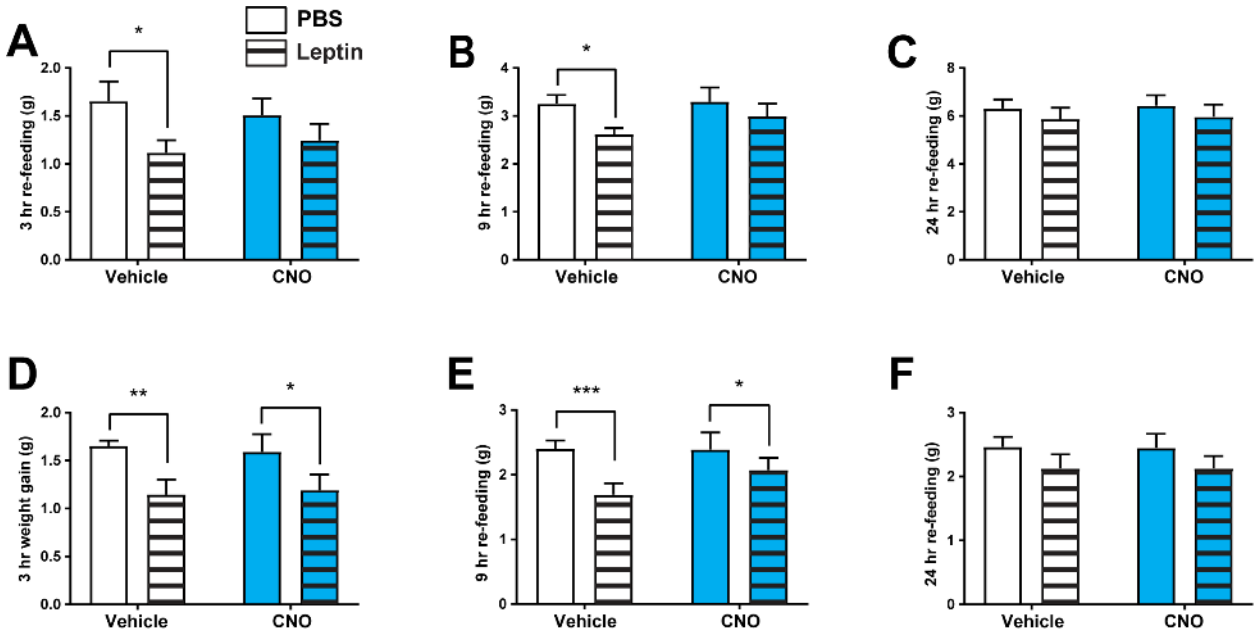
(CNO n=8; vehicle n=7). C) I.P. treatment with the agonist CNO in one group of injected mice inhibits Nts neurons, while a control group is treated with vehicle. D) Mice were treated 2x/day for 4 weeks with CNO/vehicle to chronically inhibit neurons and mice were examined for body composition and metabolic parameters at onset of treatment and after 4 weeks of treatment. E – H) Body weight, body fat, chow-feeding and water-drinking were not different between CNO and vehicle groups acutely or chronically. I) However, activity levels were decreased in CNO treated animals both acutely and chronically. J) A trend for a decrease in maximum activity speed can also be seen both acutely and chronically. K-M) Despite the decrease in locomotor activity, VO_2, VCO_2 and RER are not different among groups at either time point. This data demonstrates the inhibition of LHA Nts neurons decreases locomotion.

Figure 4-8: Inhibition of LHA Nts Neurons Does Not Reduce OX



Chemogenetic Inhibition of LHA Nts Neurons Disrupts LHA OX Neuronal Function. *Nts*^{Cre} mice received bilateral LHA injections of AAV-HM4D-mCherry to allow for chemical inhibition of LHA Nts neurons (CNO n=8; vehicle n=7). Mice were treated 2x/day for 4 weeks with CNO/vehicle to chronically inhibit neurons. B A) Viral induced expression of DREADD receptors is coupled with a fluorescent tag to show viral spread and targeting in Nts neurons (shown in cyan) and OX IF (shown in red). In the LHA of the vehicle treated animal, Nts-DREADDi fluorescence is visible and a robust population of LHA OX neurons. These exist near each other but do not overlap (merged panel). B) In a CNO-treated animal after chronic inhibition of Nts neurons we see a similar number of Nts-DREADDi IF neurons. The orexin population appears similar in number, but the cells appear dimmer – suggesting a possible downregulation of OX peptide due to chronic loss of input from LHA Nts neurons.

Figure 4-9: Chemogenetic Inhibition of LHA Nts Neurons Blunts Response to Leptin



Nts^{Cre} mice were bilaterally injected with AAV-HM4D-mCherry into the LHA and allowed to recover. Mice were food restricted O.N. and the following morning, they received I.P. CNO/Vehicle treatment followed by I.P. leptin/PBS (5mg/kg) and food was returned (crossover study design so each animal had all four treatments (n=10). A) Three hours after food was returned, leptin reduced food intake in vehicle treated mice. However, CNO treated mice did not respond as robustly to leptin. B) This same effect can be seen nine hours after food is returned, but by 24 hours C) there is not difference between treatment groups. D) Similarly, at 3 hours both vehicle and CNO treated mice show a leptin-induced blunting of re-feeding, but the effect in more profound in the vehicle treated mice when compared to controls and E) this pattern remains at 9 hours. F) 24 hours after treatment all groups have regained similar amounts of weights. These data show that inhibition of LHA Nts neurons blunts leptin-suppression off fasting-induced re-feeding.

CHAPTER 5 Summary, Discussion of Outcomes and Future Directions

5.1 Summary of Dissertation

The goal of this project was to understand the role of LHA Neurotensin (Nts) neurons in adaptive energy balance. Regulation of energy balance is crucial for survival, and coordination of energy status and appropriate response behaviors is necessary for maintaining this balance. Disorders like anorexia nervosa and obesity result when there is a loss of appropriate coordination between energy status and adaptive response behaviors. The Lateral Hypothalamic Area (LHA) receives energy and hydration status cues from the periphery and initiates adaptive response behaviors, thus may be important for properly coordinating energy balance. The LHA contains several population of neuropeptide- or neurotransmitter defined neurons that may each contribute to the regulation of energy balance. LHA populations that express orexin (OX) or melanin concentrating hormone (MCH) respond indiscriminately to thirst and hunger signals. By contrast, there were hints that the separate population of LHA Nts neurons might coordinate responses to cues of energy excess or thirst, and may exert important contributions to adaptive energy and fluid balance. Until recently, however, study of Nts neurons was hindered by a lack of reagents to easily detect Nts-expressing cells and manipulate them *in vivo*. To overcome this obstacle, this thesis describes the development and use of mouse models that allowed for facile visualization and modulation of LHA Nts neurons, and revealed their important, novel contributions to adaptive energy balance.

5.1.1 Chapter 2 - Heterogeneity of LHA Nts Neurons: Distinct Subsets are Activated by Leptin or Dehydration

Hypothesis: Separate population of LHA Nts neurons are distinguishable by molecular, circuit and neurochemical criteria and detect energy statue or fluid balance cues.

Defining the neurochemistry and projections of LHA neurons is essential for understanding the signaling mechanisms by which they contribute to energy balance. This work shows that there are at least two molecularly distinct populations of LHA Nts neurons: one co-expresses LepRb and responds to the anorectic hormone leptin (Nts^{LepRb} neurons) while a separate population lacks LepRb and is activated by dehydration (Nts^{Dehy} neurons) (Figure 2-2). The Nts^{LepRb} neurons project to the VTA and SN, but Nts^{Dehy} neurons do not, suggesting that these subpopulations of LHA Nts neurons exert effects via different pathways (Figure 2-3, 2-4). The subpopulations are, however, similar in their classical neurotransmitter content, as a newly developed dual-recombinase strategy revealed that LHA Nts neurons co-express GABA but not glutamate (Figure 2-8, 2-9). This suggests that LHA Nts neurons can release either Nts and/or GABA, which could have differential effects on downstream targets. Going forward, it will be important to discern if GABA and Nts are released at all projection sites of LHA Nts neurons, or whether there are conditions for differential release or action that may permit adaptive energy balance. Indeed, there is precedence for differential release, as OX neurons bias release of OX, glutamate or dynorphin depending on environmental status [284]. Furthermore, it is possible that release of Nts vs. GABA might be affected by obesity, resulting in impaired regulation of energy balance, and this will be important to test in the future. In sum, these data establish a framework for understanding the molecular and circuit mechanisms by which LHA Nts neurons may coordinate energy or fluid balance.

5.1.2 Chapter 3 - Loss of Action via Neurotensin-Leptin Receptor Neurons Disrupts Leptin and Ghrelin-Mediated Control of Energy Balance

Hypothesis: LHA Nts neurons that co-express the long form of the leptin receptor are essential for leptin and ghrelin-mediated adaptations in energy balance.

Here, the leptin-sensitive subpopulation of LHA Nts neurons (Nts^{LepRb} neurons) was examined to determine its specific role in energy balance. LHA Nts^{LepRb} neurons could mediate actions via their projections to local OX neurons or to the VTA, the seat of the mesolimbic DA system that modifies motivated behaviors. Study of mice genetically lacking LepRb in Nts neurons allowed determination of how loss of leptin signaling via otherwise intact LHA Nts neurons impacts energy balance. Leptin-disrupted mice exhibited increased body adiposity and weight, but not chow intake. They also had decreased locomotor behavior (both ambulatory and motivated) and a diminished ability to respond appropriately to the feeding-suppressing hormone, leptin. Importantly, the ability to respond appropriately to ghrelin (an orexigenic signal that activates OX neurons) was also disrupted. Since LHA Nts^{LepRb} neurons project to OX neurons [200] these data suggest that interrupting function of the LHA Nts^{LepRb} neurons also deranges downstream OX neurons. Taken together, these data reveal interdependence of OX and LHA Nts neurons in control of adaptive energy balance.

5.1.3 Chapter 4 – Lateral Hypothalamic Neurotensin Neurons are Essential for Control of Locomotor Activity and Body Weight

Hypothesis: LHA Nts neurons are essential for control of ingestive behavior and body weight

Here all LHA Nts neurons were ablated in adult animals to reveal their collective role in adaptive energy balance. Using a genetic lesion technique to specifically target and ablate LHA Nts neurons, it was found that loss of LHA Nts neurons increased adiposity and decreased locomotor

activity. Interestingly, acute ablation of LHA Nts neurons had no impact on adjacent MCH or OX neurons, but prolonged loss of LHA Nts neurons caused downregulation of OX at the transcript and peptide level. By contrast, another protein expressed within OX neurons, DLK-1, was unaffected by loss of LHA Nts neurons, and its retention suggests that the "OX" neurons remain intact despite their downregulation of OX. These data support a mechanism via which loss of LHA Nts neurons specifically deranges the expression of OX, but not all peptides, in downstream OX neurons. Additionally, DREADD technology was employed to inhibit LHA Nts neurons; since this method did not cause downregulation of OX, it specifically revealed the role of LHA Nts neurons, without consequences from loss of downstream OX action. Acute or chronic LHA Nts inhibition decreased locomotor activity, and blunted the anorectic response to leptin necessary for adaptive energy balance. These data reveal that LHA Nts neurons are necessary to coordinate appropriate locomotor activity and adaptive energy balance, and loss of these functions may predispose individuals to adiposity.

Taken together, the data generated from all of these studies establishes LHA Nts neurons as necessary for energy balance. Furthermore, this work identifies discrete LHA Nts subpopulations that may contribute in distinct ways to regulation of motivated locomotor behavior, motivated consumption and drinking behavior.

5.2 Discussion

5.2.1 Mouse models used in the study of LHA Nts neurons.

To facilitate the study of LHA Nts neurons, several mouse models were employed in this project, that either modulated all LHA Nts neurons or a specific subpopulation of them (Nts^{LepRb} neurons). A visual summary of the phenotypes of these models is included in Figure 5-2.

Assessment and comparison of these three models, when aligned with the current body of LHA research, builds a comprehensive description of LHA Nts neuronal function. As the LHA contains other neuronal populations that regulate energy balance, it is helpful to also consider the phenotypes obtained from the current work to that resulting from loss of all LHA neurons (LHA lesion), and specific ablation of MCH, OX, GABA or glutamate neurons. These are discussed below by phenotype.

Body composition: Genetic lesion of adult LHA Nts neurons (LHA Nts-ablated mice, Chapter 4) caused an obese phenotype. While the LHA Nts-ablated mice did not weigh significantly more than controls, their percent body fat was much higher, they looked very round and they had oily coats similar to diet-induced obese mice or those with congenital leptin deficiency that are profoundly obese. Mice with genetically disrupted leptin signaling specifically in Nts neurons (LRKO mice, Chapter 2) also had substantially increased adiposity and modest increases in body weight relative to controls. Likewise, increased body size/adiposity is observed with lesion of OX or glutamate neurons, many of which co-express OX [140;264]. Since loss of LHA Nts neurons was accompanied by a decrease in OX-IF (Figure 4-4), loss of input from LHA Nts neurons may cause dysregulation of downstream OX neurons that contributes to adiposity. This is consistent with data from Chapter 2 and prior work demonstrating that Nts neurons regulate OX neurons [200;298]. However, the significance of this finding was that loss of OX peptide expression must have contributed to the phenotype of LHA Nts-Ablated mice.

Locomotor Activity: Chemogenetic inhibition of LHA Nts neurons (LHA Nts-Inhibited mice) did not alter OX expression, suggesting that any phenotypes arising from this model were not influenced by OX dysfunction. The LHA Nts-Inhibited phenotype was more restricted than that of LHA Nts-Ablated mice, but both models exhibited decreased activity. These data suggest that LHA

Nts neurons modify locomotor activity that is not dependent on their regulation of OX neurons. By contrast, other phenotypes associated with LHA Nts ablation (e.g. blunted drinking and energy expenditure) may require action via an intact LHA Nts → OX circuit. LRKO mice also showed a decrease in activity at baseline. This could be mediated via LHA Nts neurons projections to the VTA, where they can modulate DA neurons expressing NtsR-1 [191;200], and indeed, LRKO mice have blunted DA signaling and DA-mediated locomotor activity [200]. To further assess motivated locomotion, Nts-ablated and LRKO mice were offered wheels to examine non-obligatory dopamine-mediated rewarding activity [306;325]. LRKO and Nts-ablated mice spent much less time on wheels than controls, revealing that LHA Nts neurons are necessary for motivated locomotion. Given that LHA Nts neurons project to and can activate NtsR1-expressing DA neurons[191;194;200], they may promote locomotor activity via the VTA. Going forward it will be important to selectively test whether locomotor behavior is mediated via LHA Nts projections to the VTA or other sites that have yet to be determined.

Feeding: No change in chow intake was found in any of the models relative to controls. However, as some LHA Nts neurons are activated by the feeding suppressing hormone, leptin [200;269], mice were tested for leptin response. In WT mice, leptin reduces fasting-induced re-feeding, which was blunted in Nts-Inhibited mice and LRKO mice (Figure 3-5 A, Figure 4-9). LRKO mice were also assessed for motivated intake of palatable rewards, and showed a failure to respond to leptin, including no leptin-mediated blunting of motivated responding for sucrose (Figure 3-6). Interestingly, ablation of LHA glutamate neurons increases motivated feeding similar to LRKO mice. Since many LHA glutamate neurons co-express OX, loss of action via the Nts → OX pathway may mirror the effect of glutamate neuron lesion due to suppression of OX/glutamate neurons [200]. Together, these data suggest a role for the Nts^{LepRb} subpopulation in regulation of motivated feeding, which may be mediated, in part, via regulation of (glutamatergic) OX neurons.

Drinking: LHA Nts-ablated mice show a drastic decrease in water intake (Figure 4-2), but this was not observed in LRKO mice that only have impaired Nts^{LepRb} neurons. An important consideration with LHA Nts-Ablated and LHA Nts-Inhibited models employed in this study, is that they target ***all*** LHA Nts neurons. As LHA Nts neurons are comprised of distinct Nts^{LepRb} and Nts^{Dehy} subpopulations (and likely more that have yet to be determined), this means that bulk LHA Nts ablation/inhibition will have produced mixed phenotypes due to the contributions of all of the LHA Nts subsets. Dehydration activated ~12% of all LHA Nts neurons, which are Nts^{Dehy} neurons (Figure 2-4), but did not alter regulation of LHA LepRb neurons that contain the Nts^{LepRb} subpopulation. Therefore, necessarily Nts^{LepRb} neurons are not dehydration sensitive and do not overlap with the Nts^{Dehy} subpopulation (Figure 2-5). Additionally, Nts^{LepRb} neurons project to the VTA and SNc where they are poised to modulate motivated behavior via DA neurons, but LHA Nts^{Dehy} neurons do not, suggesting that regulation of adaptive drinking behavior occurs by other, not yet characterized, circuits.

5.2.2 LHA Nts Neuronal Subtypes

Hormonal response was considered in chapters 3 and 4, as changes in peripheral energy status hormones are essential for the control of adaptive energy balance. Inhibition of LHA Nts neurons disrupts appropriate response to leptin (Figure 4-9) which agreed with data from LRKO mice with developmental LepRb deletion and hence loss of leptin regulation of the Nts^{LepRb} neurons (Figure 3-5, 3-6). While LHA Nts-inhibition did not cause a change in body composition, disruption of leptin signaling via Nts^{LepRb} neurons increased both weight and adiposity, which is again similar to OX or glutamate ablation [140;264]. By contrast, the disruption of leptin action via Nts^{LepRb} neurons (e.g. in LRKO mice) did not cause a change in drinking, as it did in the LHA Nts-ablated model. Together these models show a functionally different mechanism for regulation of activity vs. drinking by LHA Nts neurons, and agree with findings from Chapter 2 that leptin-sensitive Nts^{LepRb} neurons are

distinct from dehydration-sensitive Nts^{Dehy} neurons. As expected, LRKO mice lacking leptin action via Nts^{LepRb} neurons had an inability to respond appropriately to leptin-treatment (Figure 3-5, 3-6). Additionally, loss of LHA Nts-leptin signaling impaired appropriate response to the OX neuronal activator, ghrelin (Figure 3-5, 3-6). This is consistent with previous work demonstrating an LHA Nts → OX circuit by which LHA Nts neurons regulate the activity of OX neurons [200;298]. Similarly, work from Chapter 4 demonstrates that ablation of LHA Nts neurons leads to downregulation of OX peptide (Figure 4-4), providing further rationale that LHA Nts neurons are necessary for the function of OX neurons, and in particular, for OX expression and OX-mediated effects.

Taken together, data from the LRKO, LHA Nts-ablated and LHA Nts-inhibited models describe: 1) potential pathways via which LHA Nts neurons regulate negative energy balance and 2) subpopulations of LHA Nts neurons that are distinguishable by their activation cues, projections to the VTA and may modulates distinct aspects of adaptive energy balance. These results fit into a larger body of research on LHA neurons and particularly have implications regarding the regulation of the VTA DA reward circuit. The leptin-sensitive population of LHA Nts^{LepRb} neurons projects to the VTA, where it is poised to engage the mesolimbic DA circuit to modulate motivated consumption and locomotion. Furthermore, the definition of LHA Nts neurons as GABAergic (Chapter 2) suggests how LHA Nts neurons might modulate the activity of the VTA. Pharmacologic Nts is well established to activate VTA DA neurons [184;188], but this seems at odds with findings that LHA Nts neurons contain stimulatory Nts and inhibitory GABA. A possible circuit mechanism to explain these contradictory signals is that GABA and Nts act at different VTA target neurons. LHA Nts neurons may release GABA onto VTA GABA neurons, which inhibits them, and in turn causes disinhibition of downstream VTA DA neurons. These actions could reinforce the direct Nts-mediated activating effects on NtsR-1 expressing VTA DA neurons [194;331]. Our data cannot rule out that the leptin-sensitive LHA Nts^{LepRb} neurons may also act via local OX neurons, and prior evidence supports that at least some of them do [298]. Dehydration sensitive LHA Nts^{Dehy} neurons, however, do not project to

the VTA, and hence may contribute to drinking behavior regulation via a non-mesolimbic circuit. LHA Nts-mediated drinking could perhaps be due to projections to OX neurons or other yet to be characterized mechanisms.

5.2.3 Summary and Translational Potential

Collectively this work has described the functional contribution of LHA Nts neurons to energy balance and established that they are crucial mediators of adaptive response to peripheral energy and fluid status cues. Furthermore, this work confirms that there are discrete subpopulations of LHA Nts neurons, which presumably contribute distinctly to coordination of adaptive energy balance. About 15% of LHA Nts neurons are leptin-sensitive (Nts^{LepRb} neurons) and regulate locomotor activity and motivated food intake by accessing the mesolimbic DA circuit. Additionally, another population of LHA Nts neurons is sensitive to dehydration (Nts^{Dehy} neurons), but these do not project to the midbrain; thus, LHA Nts-mediated regulation of drinking behavior is modulated by a yet to be determined circuit.

These findings suggest that LHA Nts neurons, and perhaps specific subpopulations and circuits, may be potential therapeutic targets for separate ingestive disorders that threaten human health. One possible application may be to leverage the LHA Nts^{Dehy} neurons to regulate drinking behavior. Many individuals suffer from derangements in water balance that threaten survival, yet the incomplete understanding of control of water balance has prevented development of treatments to normalize drinking and fluid homeostasis. For example, aging is often accompanied by dysregulation of homeostatic control, including a decrease in thirst that can lead to dehydration [332]. Inability to recognize a need for fluids and a failure to drink, can lead to sodium and water imbalance and dangerous conditions that precipitate cardiovascular events and early death [333;334]. Since the subset of Nts^{Dehy} neurons respond to thirst, these neurons could potentially be manipulated

pharmacologically to treat age-related loss of thirst. Sadly, many age-related disorders are regarded as simply inevitable and geriatric patients can feel dismissed and isolated. If it were possible to enhance drinking behavior, it could extend, and improve quality of life elderly patients. Conversely, this circuit could be manipulated to treat overactive thirst, such is the case in psychogenic polydipsia, which can lead to catastrophic electrolyte imbalance and death [335].

Modulating LHA Nts neurons may be also be useful to treat energy balance disorders, such as obesity. Currently, the only widely available treatment has been diet and exercise or "lifestyle modification", the goal of which is to reduce caloric intake compared to caloric need. Caloric deficits obtained in this manner can produce weight loss. Unfortunately, compliance with this treatment is often a struggle and those who do initially have success often re-gain weight in the long term. One complication of maintained excess weight is that is brings about changes within the body and brain [317;319], that make maintenance of reduced weight particularly challenging. While there are current pharmacological interventions available that decrease weight, they are not used nearly as often as drugs for co-morbid disorders like type II diabetes [336] which implies a lack of physician confidence in existing pharmaceuticals. Additionally, efficacy of available drugs ranges from 2-8% loss of body weight over a year of therapy [337] which is not enough to reduce the body weight of someone with a BMI categorized as morbidly obese into a healthy BMI, so there is a clear need to better understand the neural circuitry that is implicated in obesity in order to improve available drug therapies.

5.2.4 Pharmacotherapeutic Targets

This work demonstrates that the leptin-sensitive subpopulation of LHA Nts neurons is necessary to suppress motivated feeding and increase physical activity, dual behaviors that can support weight loss. While these leptin-sensitive LHA Nts^{LepRb} neurons are induced with activation of all LHA Nts neurons, specifically activating just this subset might enhance anorectic and pro-

activity behaviors to enhance weight loss. Thus, further investigation of this circuit may identify strategies to help restrain feeding, encourage exercise motivation and ultimately facilitate weight loss. Data herein has shown that LHA Nts^{LepRb} neurons project to the VTA (Figure 2-3) where they can access and modulate DA neurons, a subset of which respond to Nts [177;194]. Mice lacking LepRb signaling in LHA Nts neurons have decreased activity and decreased mesolimbic activation after leptin treatment [269], promoting this LHA Nts → VTA DA circuit as a potential target for pharmacotherapeutic intervention. Activation of LHA Nts neurons suppresses feeding, but requires functional NtsR-1 [177]. However, LHA Nts-mediated induction of activity is NtsR-1-independent [177] suggesting that these two adaptive behaviors could potentially be targeted and manipulated by separate mechanisms. This could allow assistance with exercise compliance to be enhanced independently from NtsR1-agonist mediated appetite suppression.

In general, the LHA GABA → VTA circuit employs GABA signaling to regulate motivated feeding [286], suggesting that GABA-modulation may be a potential therapeutic avenue. In fact, GABA-targeted pharmacotherapy is currently used for weight loss. Phentermine is the most widely prescribed weight loss drug on the market [337] and combination therapy with Bupropion and zonisamide is being investigated [338]. This LHA→ VTA GABA circuit has potential to modify feeding to promote weight loss and should be further characterized. LHA Nts^{LepRb} neurons that project to the VTA are a subpopulation of LHA GABA neurons (Figures 2-8, 2-9), and thorough characterization of the specific role this circuit plays in regulation of ingestion could prove to be an effective target for therapy.

5.2.5 Toxicological Implications

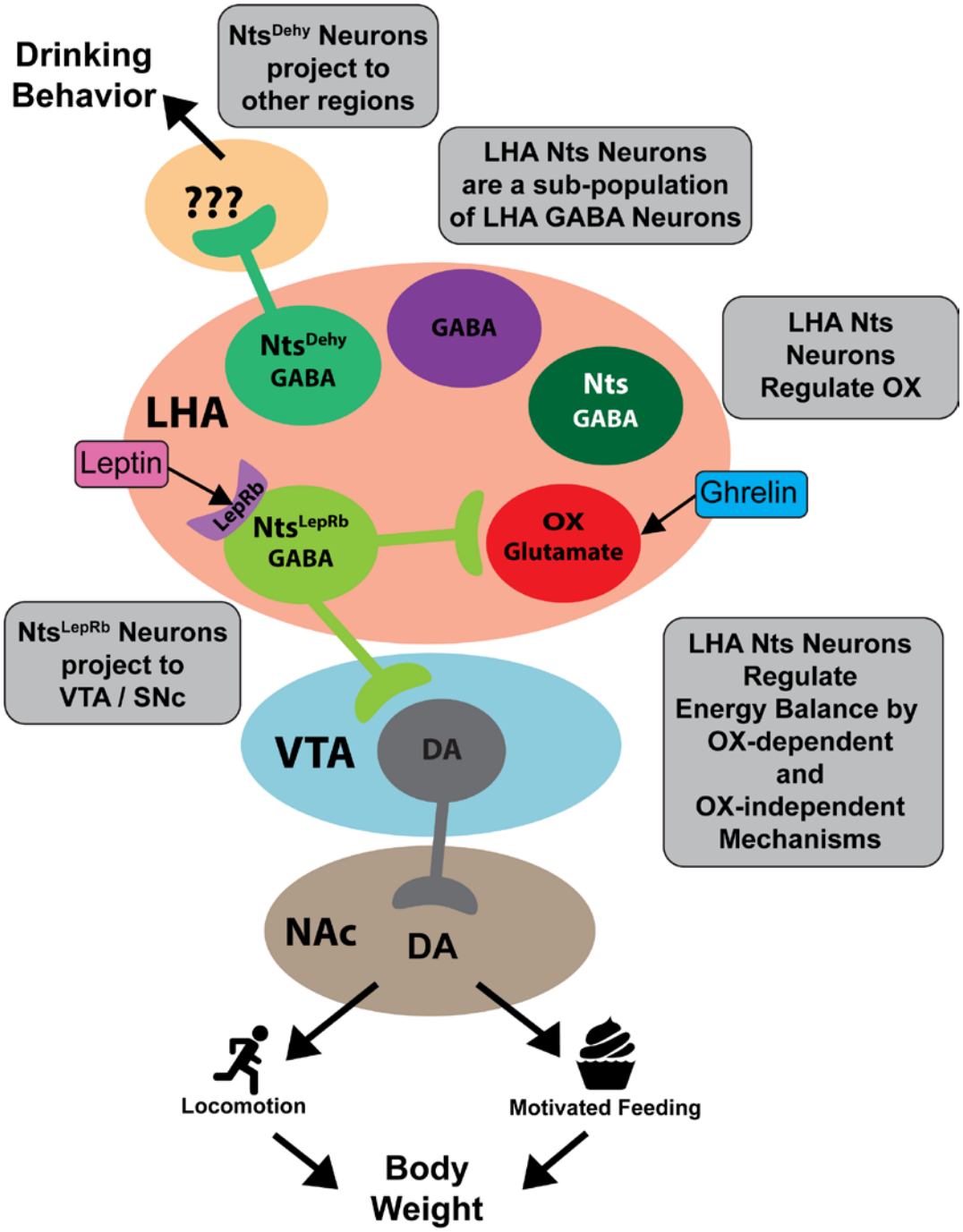
The relationship between LHA Nts neurons and OX neurons is both functional and potentially fragile. It has been established that LHA Nts neurons project to and regulate OX neuron within the

LHA [200], and this work as has reaffirmed that relationship. Nts neurons fill the role of 'command neuron' in the LHA; they regulate energy balance both directly, and by modulation of OX neurons. This arrangement allows for control and versatility, but it also puts the OX population at the mercy of the Nts population. In these studies, a modified DTA virus was used to selectively target and ablate Nts neurons. After loss of the Nts population, OX neurons exhibited dysregulation with a reduction in cell number as well as a decrease in OX mRNA expression by the cells that remain. Furthermore, DREADD-mediated chronic inhibition of LHA Nts neurons lead to dimming of OX immunofluorescence, suggesting a decrease in peptide expression. What this means, is that, if Nts neurons suffer some insult, OX neurons will too, and loss of OX neurons or loss of OX peptide in intact neurons, gives rise to narcolepsy symptoms and changes in behavior [140;339]. While there is not currently a known environmental toxicant that targets Nts neurons, this interdependent Nts – OX relationship must be considered when targeting LHA Nts neurons. Even when considering development of a drug that might inhibit Nts neurons, the potential impact on OX neurons must be considered.

Development of effective therapeutic strategies to support sustained weight loss are sorely needed for the millions of adults and children with obesity, as current obesity treatments, even when used in concert with diet and exercise have not proven sufficient to manage the disease. Other diseases resulting from dysregulation of energy balance could also benefit from understanding of the role that LHA Nts neurons play in adaptive feeding and activity. Manipulation of these circuits to increase feeding and decrease motivation to exercise, could be used to develop treatments for anorexia nervosa and bulimia. Better understanding of specific neural circuits that support weight management behaviors, as we have potentially uncovered in LHA Nts neurons, may help to develop disease modifying treatment and improve human health.

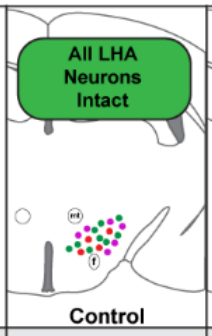
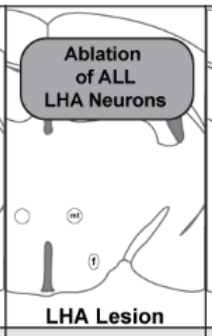
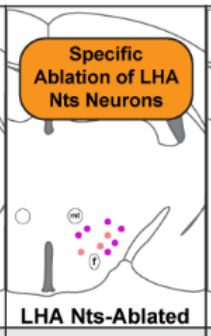
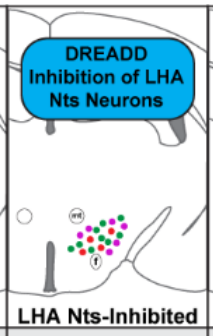
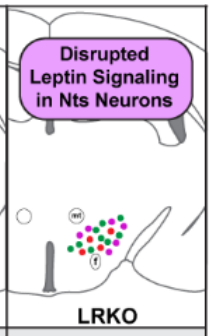
5.3 Figure

Figure 5-1: Model Showing Major Study Findings



A subpopulation of LHA Nts neurons is activated by dehydration, and does not project to the VTA. However separate LHA Nts neurons are leptin-sensitive and project to the VTA where they can manage leptin-mediated regulation of activity. LHA Nts neurons also regulate OX neurons and loss of all LHA Nts neurons decreases drinking and activity resulting in obesity. Here we have shown that LHA Nts neurons regulate energy balance by OX-dependent and independent mechanisms, and that distinct subpopulation regulate feeding and drinking discreetly.

Figure 5-2: Table Comparing Animal Models Used in This Research

Comparison: Animal Models Used to Disrupt LHA Nts Neurons • MCH Neuron • Nts Neuron • OX Neuron • Downregulaed OX Neuron	 Control	 LHA Lesion	 LHA Nts-Ablated	 LHA Nts-Inhibited	 LRKO
Body Weight	Average	Lean	No Change	No Change	Increase
Body Fat	Average	-	Increased	No Change	Increased
Drinking	Average	None	Decreased	No Change	No Change
Feeding	Average	Decrease	No Change	No Change	No Change
Ambulatory Activity	Average	Decreased	Decreased	Decreased	Decreased
Motivated Activity	Average	-	Decreased	-	Decreased
Sucrose Preference	Average	-	No Change	No Change	No Change
Sucrose Preference w/ Leptin/Ghrelin	Leptin: Decrease Ghrelin: Increase	-	-	-	No response to Leptin/Ghrelin
Operant Responding For Sucrose Reward	Leptin: Decrease Ghrelin: Increase	-	-	-	No response to Leptin/Ghrelin
Fasting-Induced Re-Feeding	Decreases with Leptin-Treatment	-	-	No Response to Leptin	No Response to Leptin

To facilitate the study of the functional role for LHA Nts neurons in adaptive energy balance, several mouse models were employed. Shown in this table are the experimental models along with a control animal and an LHA lesion model to illustrate the phenotypic changes that arise from each.

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