NEUROTENSIN-EXPRESSING LATERAL HYPOTHALAMIC NEURONS ALLEVIATE PAIN VIA NEUROTENSIN RECEPTOR SIGNALING

By

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ABSTRACT

Chronic pain and obesity frequently occur together. An ideal therapy would alleviate pain without weight gain, and most optimally, could promote weight loss. The neuropeptide neurotensin (Nts) is implicated in reducing weight and pain, but the endogenous mechanisms underlying this physiology were unknown. We previously showed that activating lateral hypothalamic area neurons expressing Nts (LHA^{Nts} neurons) suppresses feeding and promotes weight loss. Here we hypothesized that LHA^{Nts} neurons are an endogenous source of Nts that can provide antinociception, and hence, that activating LHA^{Nts} neurons would alleviate pain dependent on Nts signaling via NtsRs. We first used Designer Receptor Exclusively Activated by Designer Drug (DREADDs) to activate LHA^{Nts} neurons in normal weight and diet-induced obese (DIO) Nts^{Cre} mice. Activating LHA^{Nts} neurons had no effect on thermal pain and mechanical responses in naïve normal weight mice. By contrast, both spared nerve injury- (SNI) and complete Freund's Adjuvant (CFA)-induced mechanical hypersensitivity was completely reversed by DREADD ligand clozapine N-oxide (CNO)-mediated stimulation of LHA^{Nts} neurons compared to control naïve normal weight mice. Similarly, activating LHA^{Nts} neurons had no effect on thermal pain responses in DIO mice. By contrast, obesity-induced pain hypersensitivity as well as CFA-induced inflammatory pain was completely reversed by CNO-mediated activation of LHA^{Nts} neurons compared to VEH control. However, pretreatment with the brain permeable Nts receptor pan-antagonist SR142948 (1mg/kg, i.p, 30 min before VEH/CNO) blocked CNO-mediated analgesia, indicating that LHA^{Nts} neurons alleviate chronic pain in an Nts-dependent manner. Furthermore, Nts deletion from the LHA by injecting AAV-Cre into the LHA of $Nts^{flox/flox}$ mice further exacerbated hyperalgesia in DIO mice compare to normal weight mice. Taken together

these data suggest that augmenting signaling via LHA^{Nts} neurons may be a common actionable target to treat comorbid obesity pain.

To the courageous Afghan women advocating for their right to education, and to my younger self for having the stubborn tenacity to see this through.

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LIST OF ABBREVIATIONS

AMPK	AMP-Activating Protein Kinase
ANOVA	Analysis of Variance
β-LT	Beta Lactotensin
CaMMK2	Ca^{2+}/Calmodulin Kinase II
CNO	Clozapine N-Oxide
CAR	Campus Animal Resources
CRH	Corticotropin-Releasing Hormone
CTCF	Corrected Total Cell Fluorescence
CGX	Cantulakin-G
CFA	Complete Freund's Adjuvant
DIO	Diet-Induced Obese
DRG	Dorsal Root Ganglion
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
DA	Dopamine
FFAR4	Free Fatty Acid Receptor 4
GPCR	G-Protein Coupled Receptor
Glp1	Glucagon-like Peptide Receptor
HFD	High Fat Diet
IACUC	Institutional Animal Care and Use Committee
ICV	Intracerebroventricular

IPAC	Posterior Limb of the Anterior Commissure
KO	Knock Out
LHA	Lateral Hypothalamic Area
LepRb	Long-form Leptin Receptor isoform
LS	Lateral Septum
MPP	Medial Preoptic Area
MAPK	Mitogen-Activated Protein Kinase
MOR	μ-Opioid Receptor
Nts	Neurotensin
NtsR1	Neurotensin Receptor 1
NtsR2	Neurotensin Receptor 2
NtsR3	Neurotensin Receptor 3
NtsR4	Neurotensin Receptor 4
NAc	Nucleus Accumbens
NTS	Nucleus of the Tractus Solitarius
PAG	Periaqueductal Gray
PVN	Paraventricular Hypothalamic Nucleus
PWL	Paw Withdrawal Latency
ROI	Region of Interest
RVM	Rostroventral Medulla
SDH	Spinal Dorsal Horn

SCN	Suprachiasmatic Nucleus
SNc	Substantia Nigra Pars Compacta
SNI	Sciatic Nerve Injury
VEH	Vehicle
VTA	Ventral Tegmental Area

CHAPTER 1: HUNGRY FOR RELIEF: POTENTIAL FOR NEUROTENSIN TO ADDRESS COMORBID OBESITY AND PAIN

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ABSTRACT

Chronic pain and obesity frequently occur together. An ideal therapy would alleviate pain without weight gain, and most optimally, could promote weight loss. The neuropeptide neurotensin (Nts) has been separately implicated in reducing weight and pain but could it be a common actionable target for both pain and obesity? Here we review the current knowledge of Nts signaling via its receptors in modulating body weight and pain processing. Evaluating the mechanism by which Nts impacts ingestive behavior, body weight, and analgesia has potential to identify common physiologic mechanisms underlying weight and pain comorbidities, and whether Nts may be common actionable targets for both.

1.1 THE COMORBIDITY OF OBESITY AND CHRONIC PAIN

Chronic pain is a major health issue globally[1,2]. In the United States alone 20.5% of the adult population experiences chronic pain[3,4], causing substantial economic burden[5,6] and negatively impacting quality of life[7]. Acute pain is a short-term unpleasant experience that occurs with injury and is important to prevent further tissue damage. In contrast, chronic pain is persistent, lasting beyond the injury healing period[8–10], which can negatively impact individuals' productivity, leading to disability and financial ramifications. While chronic pain may arise after injuries and surgical procedures or can be idiopathic, its prevalence strongly increases with chronic metabolic diseases, including diabetes[11] and obesity[12–14]. This presents a significant public

health challenge, given that one-third of Americans are obese and at increased risk to develop chronic pain[15–20]. Indeed, the Institute of Medicine identifies obesity as one of five major contributors of chronic pain[21]. Obesity increases the likelihood of developing various chronic pain conditions, such as diabetic neuropathy[22,23], osteoarthritis[24], as well as joint and back pain[25].

Although obesity and pain are comorbid, they have largely been considered as independent problems and treated separately. Few treatments have been evaluated to simultaneously address obesity and pain. Notably, life style changes such as exercise and diet-induced weight loss can stabilize pain[26,27]. However, sustaining weight reduction is challenging, and even with maintained diet and exercise most individuals regain lost weight due to metabolic adaptations[28], making them once again subject to chronic pain. Despite the high and increasing prevalence of obesity there are few effective pharmacologic strategies to elicit sustained weight reduction, as needed to prevent or alleviate pain. Likewise, treatment of pain itself is challenging and the presence of obesity complicates pain treatment. A pitfall of current non-opioid pain medications is that they induce weight gain with prolonged use[29]. For example, long-term use of one such non-opioid pain therapeutic, pregabalin is reported to increase body weight up to 11 pounds[30]. Opioids can be useful to treat acute pain but they are less effective in most chronic pain conditions[31–35]. Additionally, their reinforcing side effects have led to over-use and abuse, and the current opioid crisis[31,36]. The rising prevalence in obesity-related chronic pain has only exacerbated this public health issue, with over 14% of opioids prescribed to individuals classified as obese[36]. As such, neither opioid nor non-opioid treatments are ideal for individuals with comorbid obesity and pain. Development of alternative treatment strategies has been hampered by the lack of basic understanding of the underlying neurobiology mediating both energy balance

and nociception. However, an ideal therapy would be one that can alleviate pain without weight gain, and most optimally, could promote weight loss.

Neuropeptides have emerged as important players in regulating either obesity or pain, with some promoting weight loss (anorexigenic), and others providing pain relief (analgesic). Although the field has separately characterized anorexic and analgesic functions of certain neuropeptides, some neuropeptides may be beneficial in both domains, with potential to combat the comorbid chronic pain-obesity epidemic. One such multifaceted neuropeptide is neurotensin (Nts), which has been implicated as a regulator of body weight and pain in separate studies. In this review we will focus on the growing understanding of how and where Nts signals within the brain to regulate energy balance and pain, and the potential of modulating the Nts system to address comorbid obesity and pain simultaneously.

1.2 NEUROTENSIN AND ITS RECEPTORS

Neurotensin (Nts) is a 13 amino acid peptide first isolated from the bovine hypothalamus so it was initially characterized as a neuropeptide[37–39]. *In situ* hybridization and radio-immunolabelling later revealed that Nts is widely distributed throughout the central nervous system, including the spinal cord and brain regions such as the hindbrain (specifically the nucleus of solitary tract, locus coeruleus, the dorsal raphé nucleus, and parabrachial nucleus), the midbrain (ventral tegmental area, periaqueductal gray area, substantia nigra)[40], limbic system (amygdala, nucleus accumbens, septal area, hippocampus)[41–43], and the forebrain (caudate putamen, nucleus accumbens)[44,45]. Yet Nts is also expressed in peripheral tissues, including the enteroendocrine N-cells of the intestinal jejunum and ileum[46,47]. Nts also binds with varying affinity in the brain vs periphery suggesting an association with physiologically relevant

receptors[48,49] and the existence of Nts receptor subtypes[50,51]. Subsequently four types of Neurotensin Receptors (NtsRs) have been characterized, including Neurotensin Receptors -1, -2, -3, and -4.

1.2.1 Neurotensin Receptor 1 (NtsR1)

NtsR1 was discovered first when Barnard and colleagues isolated it from bovine cerebral cortex membrane[52]. Shortly thereafter Nakanishi and colleagues cloned NtsR1 (then referred to as NtS1) permitting further structural characterization[53]. NtsR1 is a seven transmembrane domain receptor that belongs to the G-protein-coupled receptor family. NtsR1 binds Nts with high affinity (K_d = 0.1-0.3 nM), and is widely distributed throughout the central and peripheral system in adult rodents and humans[54]. NtsR1 is found in diverse brain regions such as the ventral tegmental area (VTA), substantia nigra (SN), suprachiasmatic nucleus (SCN), supramammillary area, cortex, striatum, hypothalamus, and periaqueductal gray (PAG)[55,56], where it is expressed by neurons. NtsR1 is also present in the small intestine and colon although the specific cell types utilizing NtsR1 and its functions there remain elusive[57]. NtsR1 is reported to transduce intracellular signaling via Gα q/11, Gα i/o, Gα s, and/or β-arrestins[58–61], which transduce very different downstream signaling cascades. The site-specific actions and physiological outcomes attributed to Nts-NtsR1 signaling may vary depending on the coupled pathway, which could differ by the cell type expressing NtsR1 and/or where in the brain the cells reside. However, NtsR1 is well established to couple to Gα q/11 signaling in some specific brain regions, notably the VTA[60,62].

1.2.2 Neurotensin Receptor 2 (NtsR2)

NtsR2 was identified shortly thereafter, cloned from an adenocarcinoma cell line in 1993. Like NtsR1, NtsR2 belongs to the G protein-coupled receptor superfamily but exhibits lower

binding affinity (K_d = 3.7±0.2 nM) for Nts[63,64]. In contrast to NtsR1's robust expression on neurons, NtsR2 is predominantly expressed on astrocytes and epithelial cells distributed throughout the brain[65,66]. NtsR2 is also expressed within the cardiovascular system, brown adipose tissue, gastrointestinal tract[67,68] and ependymal cells[69–72]. NtsR2 has been reported to couple to various Gα signaling pathways, which may vary depending on the cell type and region.

1.2.3 Neurotensin Receptor 3 and 4 (NtsR3/4)

The receptor characterized as NtsR3 is also known as sortilin, a receptor sorting protein[73,74]. NtsR3 is structurally different from the NtsR1 and NtsR2 GPCRs in that it has single transmembrane domain and an intracellular domain with low affinity Nts binding [K_d = 10nM][73]. NtsR4 was identified in 2001 and is an intracellular protein related to the yeast sorting receptors-NtsR4/sortLA. Like NtsR3, the NtsR4 is a low affinity, single transmembrane domain receptor[75].

1.3 NEUROTENSIN'S PHYSIOLOGIC ROLES

Neurotensin modulates a diverse array of physiological processes through actions in different brain regions and the periphery. Pharmacological injection of Nts in the brain or periphery induces hypothermia[76–78], vasodilation, hypotension[37,79], hyperglycemia[80,81], and muscle contraction[82,83]. Nts is also linked to modulation of neuroendocrine function, sleep and arousal[84–86], body temperature, appetite, blood pressure, reward behaviors, regulation of ingestive behavior and body weight, and analgesia[87–90]**.** The roles of Nts in these aspects of physiology are yet under investigation and we point the reader to several excellent recent reviews concerning these topics[90–94]. Here we focus on the role of Nts in the physiology relevant to comorbid obesity and pain: how it modulates energy balance and analgesia.

1.4 NEUROTENSIN IN ENERGY BALANCE

Living organisms have dedicated physiological systems to preserve energy balance, a critical factor for their survival. These systems operate through two crucial mechanisms: first, by sensing the body's energy requirements to gauge its needs, and second, by prompting relevant behavioral responses from the brain to acquire the necessary energy (seeking and ingesting food). The ability to adjust behaviors based on energy status is vital to prevent starvation during deficiency and to curb excessive intake when reserves are full. Achieving this balanced regulation relies on peripheral cues that communicate current energy state to the brain (such as glucose and satiety hormones), which can thereby coordinate appropriate feeding and/or energetic responses. Hence, a well-functioning feedback loop between the body's metabolic condition and the brain's behavioral decisions is indispensable for organisms to maintain energy equilibrium and, consequently, to ensure their survival. Here we review the current knowledge on Nts' role in metabolism, specifically on lipid absorption, water and food intake, energy expenditure, and body temperature that impact energy balance and body weight. Given that Nts is produced in both the brain and periphery, with distinct actions attributed to these different pools of Nts, we will separately examine these sources and their effects.

1.4.1 Peripheral Neurotensin in Energy Balance

1.4.1.1 Neurotensin Facilitates Fat Absorption in the Periphery

The high level of Nts in plasma originates from chromaffin cells in the adrenal gland[95,96] and enteroendocrine N-cells in the distal small intestine that secrete gut hormones[97,98]. Given the crucial role of the intestine for energy absorption, the discovery of Nts in the intestine hinted that it might modulate body weight via modifying nutrient uptake[38,99,100]. Indeed, there is an increase

in circulating Nts levels following the ingestion of fat[101], which is thought to be released from the N-enteroendocrine cells[102]. The elevation in Nts is rapid (detectable in the circulation within 20 minutes of the meal initiation) and persistent, with a steady secretion rate persisting for at least 180 minutes[103]. The current interpretation of these data is that Nts is released from enteroendocrine cells after a meal to support lipid absorption. Indeed, knockout mice with developmental deletion of Nts have significantly reduced intestinal fat absorption. These Nts-deficient mice are even protected from developing obesity when on a high-fat diet (HFD), and exhibit attenuated obesity-associated insulin resistance and other metabolic improvements compared to wildtype mice that develop severe diet-induced obesity and metabolic complications[57]. Collectively, these findings support that peripheral Nts signaling mediates fat absorption that could potentiate weight gain in individuals consuming modern fat-rich diets[103,104].

The signaling mechanism by which intestinal Nts mediates fat absorption is an active area of inquiry, since it could identify strategies to mitigate fat uptake and weight gain. Intestinal Nts acts through NtsR1 and NtsR3, enhancing fatty acid absorption by suppressing Ca^{2+}/calmodulin kinase II complex CaMKK2-mediated AMP-activating protein kinase (AMPK) phosphorylation[57]. Conversely, increased AMPK phosphorylation, achieved through the inhibition of mitogen-activated protein kinase (MAPK/ERK1/2), decreases fatty acid-stimulated Nts release. These results establish an inhibitory crosstalk between MAPK and AMPK signaling pathways downstream of free fatty acid receptor 4 (FFAR4) to regulate Nts secretion in response to fat consumption[102]. Due to the difficulties in labeling Nts receptors, the specific location of intestinal cells expressing NtsR1 and NtsR3 remains unclear. Future work to identify these cells and to

understand how they work will provide vital information about the fundamental control of nutrient absorption.

Recent work has begun to investigate the role of peripherally-produced Nts in altered states of energy balance. Given Nts' role in fat absorption, loss of Nts action may exacerbate the degree of weight loss in individuals with anorexia nervosa, as it reduces fat absorption in additional to self-restricted dieting[105]. On the flipside, both rodents and humans with obesity and insulin resistance have elevated circulating levels of stable long-form Nts (proNts)[106], and lower circulating levels of processed (active) Nts after ingesting fatty meal[57,107]. A recent study demonstrated that fasting plasma levels of both mature and pro-Nts collectively decrease in obese mice and humans undergoing weight loss through food restriction. This indicates that the production of peripheral Nts is coordinated with body weight[108]. Moreover, the amount of circulating pro-Nts correlates with the degree of obesity and has been suggested as a potential diagnostic marker for risk of developing obesity and metabolic disease[57,106]. Interestingly, in obese individuals, circulating Nts levels are further elevated after Roux-en-Y gastric bypass (RYGB) surgery[109,110]. A recent study also observed increased number of Nts-expressing cells in the small intestine following RYGB surgery among individuals with type-2 diabetes. This increase in Nts-expressing cells is positively associated with the extent of improvement in their insulin sensitivity[111]. However, there is no correlation between circulating Nts levels and changes in body weight in patients undergoing gastric banding[112]. Consistent with clinical findings, a recent rodent study reported elevated Nts levels in plasma and the gastrointestinal tract after RYGB surgery[113]. The simultaneous elevation of Nts levels and reduction in body weight and insulin resistance suggests an important role of Nts in obesity treatment. One might question, if peripheral Nts

increases fat absorption that in turn increases body weight, then how it can improve the degree of weight loss post-bariatric surgery? While most peripheral studies have focused on how Nts acts within the intestine to modulate fat absorption, Nts may indirectly modulate brain signaling to alter food intake. A potential explanation for the paradox of elevated Nts post bariatric surgery and weight loss is that when peripheral Nts is present in high concentrations it signals through the vagal nerve to activate hypothalamic regions, inducing a feeling of satiety via hypothalamic anorectic pathways[113]. This could support reduced food intake, and accordingly, lower available fat to be absorbed by the intestine. Indeed, circulating Nts can access hypothalamic regions just beyond the blood brain barrier that are important for modulating feeding, such as the arcuate nucleus.

1.4.1.2 Effect of Systemic Neurotensin on Food Intake

Rodent studies support that peripheral Nts may impact food intake. Acute systemic injection of Nts modestly suppresses feeding in rodents, particularly during the dark phase when they consume the majority of their daily food[114,115] and pretreatment with an Nts receptor antagonist (SR-142948) diminishes the anorectic effect[113]. In mice with genetic obesity (due to deletion of the anorectic hormone leptin or the leptin receptor), systemic treatment with the Nts analog NT69L reduced food intake and body weight[116]. Systemic Nts treatment may mimic the endogenous release and action of Nts in response to meals (food intake), and coincide with the induction of the anorexic hormone leptin[117]. This effect is attributed, in part, to elevated plasma Nts being able to pass through the blood brain barrier to access immediately adjacent hypothalamic regions that modulate feeding[113]. However, there are other brain areas that contribute to energy balance that lie far from the blood brain barrier, such as extended

hypothalamic nuclei and the mesolimbic dopamine (DA) system. Given their location deeper in the brain and the short half-life of Nts in the circulation[104], it is unlikely that peripherally produced Nts is still biologically active if and when it reaches these brain regions. Such deeper brain structures are more likely modulated by the central pool of Nts that is produced within the brain and released as a neuropeptide transmitter to specific brain regions[101,104].

1.4.2 Central Neurotensin Signaling in Energy Balance

In contrast to Nts action in the periphery, where it promotes fat accumulation and weight gain, Nts serves as a satiety signal in the brain. In rodents, systemic Nts treatment causes a modest, transient suppression of feeding whereas intracerebroventricular (ICV) administration produces a more pronounced anorectic effect, particularly in diet-induced obese mice[114,115]. These data suggest that at least some of the anorectic effects of Nts are mediated via brain regions that may not have access to peripherally-produced circulating Nts, and perhaps are modulated by brain-produced Nts. Indeed, Nts neurons in the brain synthesize pro-Nts, and pro-hormone convertase cleaves it into a biologically active form[118]. Nts is released via dense core granules such that it can act as a peptide transmitter and bind to Nts receptors on other cells to modulate their activity and actions. Thus, identifying the specific neurons that produce Nts, which cells they release it to, and their Nts receptors is essential to understand the underlying mechanisms governing energy homeostasis and the potent anorectic effect of Nts in the brain. However, identifying endogenous Nts-expressing neurons has been technically challenging. Early studies used immunoreactive labeling to visualize Nts neurons, yet this method solely labeled Nts fibers and was unable to identify Nts-expressing soma, hindering the identification of the neurons' origin. To address this, animals can be pre-treated with the axonal transport inhibitor

colchicine, enabling sufficient Nts accumulation in the soma for immunohistochemical detection[40,119–122]. However, the drawback of the colchicine method is that it globally impairs neuronal signaling, as it blocks neurotransmitter release and ultimately leads to death within less than 48 hours. *In situ* hybridization labels *Nts* mRNA but this labor and cost intensive method is not optimal for routine detection in physiologic studies. Genetic strategies to label Nts have eased some of these barriers, including generation of knock-in mice that express recombinases in Nts-expressing cells such as Nts^{Cre} and Nts^{Flp} mice[123,124]. These models permit routine labeling of Nts-expressing cells and, using recombinase-mediated genetic tools, dissection of their physiological roles.

While central Nts does suppress feeding and weight loss, not all Nts-expressing neurons in the brain produce these effects. Instead, distinct Nts populations exhibit diverse and/or overlapping functions, ranging from ingestive behavior, ambulatory activity, social behavior, memory, and ethanol consumption[125–127]. Similarly, pharmacological administration of Nts into different brain regions elicits site-specific effects. ICV or intra-nucleus accumbens **(**NAc) treatment with Nts or an NtsR1 agonist attenuates baseline physical activity as well as cocaine- and amphetamine induced-psychostimulant hyperlocomotion, promoting resting behavior without affecting feeding. This effect is, in part, mediated through NtsR1/Dopamine receptor 2-expressing glutamatergic terminals in the NAc[93,128,129], which activate GABAergic medium spiny neurons known to inhibit NAc DA signaling[93,130,131]. Conversely, injecting Nts into the VTA reduces feeding but increases locomotor activity, at least in part via NtsR1-expressing DA neurons that release DA in the NAc[130,132,133]. Besides hyperactivity, microinjection of Nts into other parts of the limbic system and hypothalamic nuclei also reduces food intake in both food-deprived and satiated

rodents in a dose-dependent manner[134–137]. These findings underscore the necessity to systematically probe the roles of Nts cells distributed throughout the brain, to identify the endogenously producing Nts neurons that modify energy balance.

1.4.2.1 Neurotensin-Expressing Lateral Hypothalamic Area Neurons Regulate Ingestive Behavior and Physical Activity

One such population that has emerged in this regard is the Nts-expressing neurons in the Lateral Hypothalamic Area (LHA) or "LHA^{Nts} neurons"[138–142]. The LHA, known as a "hub" of energy homeostasis, governs various homeostatic processes through its heterogeneous populations of neurons[143,144]. The first data implicating LHA Nts in suppressing feeding came from dehydration-induced anorexia studies, in which water-deprived rodents stop consuming food until they have regained normal plasma fluid levels and only then do they resume feeding. During the dehydration anorexia there is upregulation of *Nts* expression in the LHA, suggesting that it may play a role in coordinating energy and osmotic needs[123,145,146]. Later genetic studies also supported a role for LHA^{Nts} neurons in these ingestive behaviors. Genetic ablation of LHA^{Nts} neurons in adult mice promotes weight gain[147]. Conversely, chemogenetic stimulation of all LHA^{Nts} neurons non-aversively restrains feeding even in hungry mice and increases their physical activity to decrease body weight[144]. However, activating all LHA^{Nts} neurons also invokes a burst of voracious drinking that temporarily increases body weight due to the water consumption, although once drinking normalizes the LHA^{Nts}-mediated feeding suppression and increased energy expenditure leads to weight loss[144]. Together these data suggest that LHA^{Nts} neurons can oppositely modulate drinking and feeding. This has prompted speculation whether all LHA^{Nts} neurons uniformly mediate feeding and drinking behaviors, or whether different subsets

modulate feeding restraint vs. drinking[123]. For instance, approximately 15% of LHA^{Nts} neurons co-express LepRb (long form leptin receptor isoform) that is necessary to mediate the anorectic response to leptin[139,141] and maintain proper energy balance (referred as LHA$^{Nts+LepRb}$ neurons). Leptin action via this LepRb-expressing subset of LHA^{Nts} neurons is important for body weight, as deleting LepR from these neurons results in weight gain without disrupting drinking. Intra-LHA infusion of leptin reduces food intake and body weight[148] and loss-of-function mutation of leptin signaling via LHA^{LepRb} neurons induces feeding and weight gain[124,149]. Similarly, stimulation of LHA^{LepRb} neurons prevents fasting-induced refeeding in food deprived mice but also reduces water seeking and water intake behavior[150]. These data suggest that the LHA$^{Nts+LepRb}$ neurons may influence feeding and body weight whereas drinking might be regulated via different subsets of LHA^{Nts} neurons[141]. Nonetheless, the convergence of Nts and leptin signaling implies that these two systems act synergistically to promote anorectic behavior. Indeed, the anorectic effect of leptin is mitigated in NtsR1 Knockout mice or wild-type mice treated with NtsR1 antagonists[117,151]. These effects may be mediated partly via the LHA^{Nts} neuronal projections to the VTA, where there are abundant NtsR1-expressing VTA^{DA} neurons[142] known to modulate mesolimbic DA signaling and motivated intake. For example, loss of leptin action from the LepRb-expressing subset of LHA^{Nts} neurons disrupts VTA DA signaling and weight loss behavior[148]. Similarly, *ob/ob* mice with a loss-of-function mutation in leptin also have reduced central Nts expression, disrupted DA signaling, increased food consumption, and these effects are reversed by leptin administration[152–154]. While leptin action via LHA^{LepRb} neurons regulates food-directed behaviors, this activity is heterogenous, perhaps due to their varied molecular composition. Intra-LHA leptin administration was shown to affect LHA^{LepRb} activity, specifically influencing appetitive behaviors

without impacting food intake or locomotor activity[155]. *In vivo* calcium imaging revealed that LHA^{LepRb} neurons displayed critical activity during reward-predictive and non-predictive cues during Pavlovian conditioning, and optogenetic manipulation of these neurons alters acute appetitive responses via projections to the VTA[155]. These effects might be partially mediated by overlapping $LHA^{Nts+LepRb}$ neurons projecting to the VTA, however this paper did not look into specific subsets of LHA^{LepRb} neurons. Together these data suggest that, to some extent, weight loss behavior is mediated by the collaborative action of leptin and Nts signaling through $LHA^{Nts+LepRb}$ neurons. Future work is required to provide resolution for how LHA^{Nts} neurons might be modulated to bias behaviors that support weight loss.

1.4.2.2 Other Neurotensin-Expressing Neuronal Population Involved in Energy Balance

Other populations of Nts-expressing neurons can mediate feeding suppression that could support weight loss. Nts injection into the paraventricular hypothalamic nucleus (PVN) significantly reduces food intake[156] without altering water intake and locomotion activity in food-deprived mice[157]. Nts action via the dorsomedial and ventromedial hypothalamus, as well as the nucleus of the tractus solitarius (NTS) also suppresses feeding[156,158]. Additionally, a population of Nts neurons in the lateral septum (LS) was recently characterized and linked with reducing feeding in stressful situations. *In vivo* calcium imaging revealed that LS Nts neurons are active during physical restraint and tail suspension tests, and when stimulated, these neurons reduce feeding and body weight without changing ambulatory activity[143]. Circuit based optogenetic stimulation of LS Nts neurons projecting to the LHA reduces food intake suggesting that this circuit is actively involved in regulating food intake under stressful situations[143]. However, it is yet to be elucidated if this anorectic response is Nts-dependent, as these neurons also co-express glucagon-like

peptide receptor (Glp1R) that is an important player in suppressing feeding and activation of LS Glp1R neurons leads to aphagia in mice.[133] Conversely, a population of Nts-expressing cells in the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), which is part of the extended amygdala, were recently implicated in promoting feeding and obesity[159]. These IPAC^{Nts} neurons are activated by cues of palatable foods to promote intake, whereas inhibiting the IPAC^{Nts} neurons protected mice from diet-induced obesity. Here again the role of Nts signaling via these neurons remains unclear, and it remains to be determined if Nts vs. other signals expressed by these neurons mediate their regulation of energy balance. Still other populations of Nts neurons do not appear to modify feeding, such as those in the medial preoptic area (MPO) or PAG, although they mediate important roles in social behavior[126] and sleep[160], respectively. These findings underscore the fact that "Nts neurons" across the brain are not homogeneous in function, and the need for population-specific studies to discern their respective contributions to physiology.

1.4.2.3 Involvement of NtsR1 in Energy Balance

Nts-mediated modulation of energy balance is currently thought to occur via NtsR1. The support for this comes from pharmacological augmentation of Nts-NtsR1 signaling, which promotes weight loss behavior[161]. Conversely, Nts-mediated feeding suppression is attenuated by pretreatment with the NtsR1 antagonist (SR48692) and in NtsR1 knockout mice[117,144,162]. However, systemic and brain-wide administration of Nts or first generation NtsR1 agonists also causes life-threatening hypothermia[76] and vasodepression[163], dampening interest in clinical applications for the Nts-NtsR1 system. Excitingly, new NtsR1 and NtsR2 agonists, specifically β-arrestin-biased NtsR1 agonists like SBI-553, circumvent the adverse side effects associated with

standard NtsR1 agonists that activate both Gq and β-arrestin pathways[164,165], and may have potential to safely modulate the Nts-NtsR1 system. Whether β-arrestin biased agonism is sufficient to modulate feeding and body weight is currently under investigation. However, it is also possible that NtsR1-expressing cells in certain brain regions may induce feeding restraint without eliciting hypotension and hypothermia. For example, the VTA contains a large population of NtsR1-expressing cells that couple to Gq pathways[60,62], where Nts action is linked with producing aphagia and hyperlocomotion but not other physiology associated with systemic Nts treatment. The VTA primarily consists of dopaminergic (DA-ergic) neurons that project to and release DA to the NAc (the mesolimbic system) or the prefrontal cortex (the mesocortical system). The majority of the VTA DA neurons co-express NtsR1[132,142], which are activated by Nts treatment and release DA exclusively to the NAc[166,167]. This Nts-NtsR1 mediated mesolimbic DA signaling suppresses feeding and increases ambulatory behavior, particularly in fasted or highly motivated mice with an augmented appetitive drive[134,135]. Better understanding of the mechanisms by which NtsR1 in the VTA, and throughout the brain, modifies feeding may suggest approaches to engage this system to bias for feeding suppression.

1.4.2.4 Involvement of NtsR2 in Energy Balance

Initial findings from NtsR2 knockout mice indicated normal food intake and body weight[168], leading to the preliminary conclusion that NtsR2 is not a participant in energy balance. However, concerns have been raised across the neuroscience field about the use of constitutive knockout mice, as they may be subject to developmental compensation such that they do not accurately reflect normal physiology. Furthermore, early studies presumed NtsR2 was expressed on neurons, when it is now recognized that is primarily expressed on astrocytes[65,68,70,72]. Recent

work has established important roles for astrocytes in modulating feeding and body weight[169,170], hence it is possible that NtsR2 expression on these cells may contribute to energy balance in yet unappreciated ways. Indeed, NtsR2 is expressed in brain regions where Nts facilitates weight loss behavior, including the VTA[132,144]. Further studies are needed to thoroughly evaluate the role of NtsR2 before its role in energy balance can be ruled out. This is all the more important given that there are preclinical NtsR2 agonists that do not promote hypotension and hypothermia, and so may be safe options to engage the Nts system.

1.5 CENTRAL NEUROTENSIN AND PAIN

Nts has been characterized as an analgesic peptide, exerting at least part of this effect within the brain. Clineschmidt and colleagues were the first to report an antinociceptive response of Nts in rodents that operates independently of μ-opioid receptor (MOR) signaling, initiating excitement about the analgesic potential of the Nts system[171]. Specifically, intracisternal administration of Nts caused a prolonged analgesic effect against noxious thermal and chemical stimuli. Naloxone, a MOR antagonist, did not alter the antinociceptive effect induced by Nts[171–173]. Excitement for Nts as a new analgesic was quickly dampened when subsequent studies revealed that even a very low dose of intracisternal Nts produced hypothermia that would be disadvantageous in the clinical setting. This led the field to scrutinize the central mechanisms by which Nts modifies pain processing, and whether there might be specific brain sites and/or Nts receptors to support analgesia without adverse effects.

Pain is sensed in the periphery and conveyed to the brain, and in response the descending inhibitory pain circuit controls the perception and regulation of pain[174]. The descending inhibitory pain circuit originates in the periaqueductal gray (PAG), which projects to the rostroventral

medulla (RVM) that in turn projects to the spinal dorsal horn (SDH)[175]**.** Nts and its receptors were identified in each of these areas[122]. Furthermore, microinjections of Nts into the PAG[122], RVM[176] and spinal cord[177] elicit profound analgesic responses. Given the important roles of each of these parts of the descending pain control system it is worth examining how Nts signaling engages them, and if any stand out as potential targets for Nts-ergic modulation of pain.

1.5.1 Periaqueductal Gray (PAG)

The PAG contains Nts-expressing neurons that can directly release Nts to the RVM[178], which were recently implicated in modulating sleep[179]. PAG Nts-expressing cells might also contribute to pain processing, supported by findings that visceral and inflammatory pain transiently increase Nts expression in the PAG[180]. It is conceivable that the endogenous Nts system might be upregulated during persistent pain in an attempt to provide relief. In addition, the PAG also receives indirect Nts via projections from hypothalamic nuclei and other brain regions[181]. These endogenous sources of Nts to the PAG may be important in pain processing, as pharmacological studies clearly demonstrate that Nts in the PAG diminishes pain, at least in part, via dis-inhibiting the downstream RVM[182]. Indeed, the antinociceptive effect of Nts in the PAG is nullified by electrolytic lesions of the RVM, indicating the critical interplay of these regions for Nts-mediated analgesia[183]. Nts may act via NtsR1 and NtsR2 expressed within in the PAG[168,184] including on cells that project to the RVM and the dorsal raphe nucleus[185]. Collectively these findings support the existence of Nts-mediated signaling in the PAG that could contribute to analgesia but the precise mechanisms governing this require further study.

1.5.2 Rostroventral Medulla (RVM)

Nts signaling indirectly modulates the RVM via projections from the PAG. The RVM contains at least two neuronal subsets with contrasting responses: “ON” cells that increase activity and “OFF” cells that decrease activity during noxious thermal stimuli[186]. Nts modulates the activity of both populations via NtsR1- and NtsR2-expressing RVM neurons, and in a dose-dependent manner[177,187]. Low dose Nts stimulates the “ON” cells, which has been associated with a hyper-reflexive response suggestive of increased nociceptive sensitivity[53,188]. Conversely, high-dose intra-RVM Nts application stimulates both populations, alleviating pain[177,189,190]. These findings imply that the response to noxious stimuli depends on the intensity of the Nts signal. One possible explanation for this dose-dependent effect may involve the differential engagement of high-affinity NtsR1 and low-affinity NtsR2 receptors. It is conceivable that a low dose of Nts activates only NtsR1, targeting the "ON" cells, while a high dose activates both the "ON" cells and the "OFF" cells expressing NtsR2. In support of this interpretation, treatment with the NtsR1 selective antagonist SR48692 mildly prolongs tail-flick latency; this suggests that blocking low-level endogenous Nts-NtsR1 signaling decreases nociceptive responding[189]. In fact, Nts actions in the RVM are likely mediated by both NtsR1 and NtsR2, as inhibiting either individually does not fully eliminate the antinociceptive response[162,176,177]. This could be due to NtsR1 and NtsR2 expressing cells of the RVM mediating different neural circuitry. For example, NtsR1-expressing neurons are serotonergic and Nts-NtsR1 action in the RVM neurons results in the release of serotonin in the SDH[177]. In contrast, NtsR2-expressing RVM neurons release norepinephrine via an indirect pathway and antinociception produced by NtsR2 agonist beta-lactotensin (β-LT) is reduced by intrathecal injection of yohimbine, an α2-adrenoceptor antagonist[176]. Therefore, both NtsR1 and

NtsR2 play a role in transmitting antinociceptive signals from the RVM to the SDH. Additionally, some RVM neurons may express and release Nts directly the spinal cord to modulate signaling there[191].

1.5.3 Spinal Dorsal Horn (SDH)

Treatment with Nts or Nts analogs into the SDH (e.g. intrathecal injection) alleviates pain[192–194]. The SDH contains local Nts-expressing interneurons and receives afferent indirect Nts input from RVM, evidenced by the dense Nts-ergic fibers in this region. The Nts-expressing afferent neurons are glutamatergic and form excitatory synaptic connections with NtsR2-expressing GABAergic neurons This inhibitory transmission serves to suppress nociceptive signals at the level of the SDH [195]. Nts is also present in dorsal root ganglion (DRG) neurons[196]. Additionally, a subset of DRG neurons also expresses NtsR1, which is also implicated in pain regulation at the SDH level, although the precise underlying mechanism remains unclear[197].

1.5.4 Does Neurotensin Facilitate or/and Attenuate Nociception?

While Nts has been generally characterized as having analgesic effects, the impact of Nts on nociception may vary, contingent on the intensity of Nts signaling and the Nts receptor isoform involved (NtsR1 vs NtsR2). As discussed above, low-level Nts signaling in the RVM is associated with increased nociception, which can be improved by inhibiting NtsR1. Similarly, knockout mice lacking NtsR2 (NtsR2-KO) have increased jump latency on the hot plate suggestive of reduced thermal nociception[168,198]. Some have interpreted this as evidence that NtsR2 may also play a facilitating role in nociception. Yet the jump response to thermal stimuli is often associated with a flight response to escape danger or acute pain, ensuring safety. Beyond the reduced

nociception, the increased jump latency in NtsR2-KO mice may suggest that NtsR2 signaling is involved in promoting behavior aimed at escaping danger during acute pain testing.

Yet, if Nts were to enhance nociception, one would expect that NtsR1 knockout mice and NtsR2 knockout mice would display a decreased paw lick response during the hot plate test for thermal pain responding compared to wildtype mice (indicative of reduced pain). However, no differences in paw lick response were observed between the knockout groups and wildtype mice in this regard. When considering both the paw lick response and jump latency data together, it suggests that Nts might not potentiate nociception but rather act as an alarm system to detect and facilitate the escape from acute pain. This interpretation is further supported by another study indicating that mice lacking Nts (Nts-KO) exhibit significantly less visceral nociception than wildtype mice[199], emphasizing the importance of Nts in pain perception. A caveat is that knockout mouse studies should be interpreted with caution. Constitutive loss of Nts or NtsRs may cause developmental alterations such that they no longer model normal physiology, including possibly exacerbating the function of the remaining Nts receptor isoforms. More work is required to untangle how the endogenous Nts system modulates pain processing to understand if the system can be pharmacologically harnessed for analgesia.

1.5.5 Neurotensin Receptors in Analgesia

NtsR1 has received greater focus for modulation of body weight, but NtsR2 has been proposed as the major receptor isoform mediating analgesia, largely based on experiments using first-generation pharmacological agonists and antagonists of the Nts system. Initial antagonist studies reported that SR142849, the non-selective antagonist that acts at NtsR1 and NtsR2, decreased Nts-mediated analgesia[189]. Moreover, levocabastine, a purported NtsR2-selective

ligand, reduced Nts-mediated analgesia[200], whereas the NtsR1-specific antagonist SR48692 did not[201,202]. Likewise, antisense oligonucleotide inhibition of NtsR2, but not NtsR1, attenuated Nts-induced analgesia in acetic acid-mediated pain after repeated brain microinjections[184,202]. This led to the conclusion that Nts promotes antinociception primarily through signaling via NtsR2. Recent advances in pharmacological understanding have challenged this conclusion. SR142849, SR48692, and levocabastine were revealed to function as partial agonists for NtsR2 under certain conditions, such that they cannot truly evaluate NtsR2 signaling, nor discriminate NtsR1 and NtsR2 signaling[64,203] For instance, levocabastine treatment in the brain reduces visceral pain[200], but if it is co-administered with Nts it diminishes the overall Nts-mediated antinociception. This reduction in analgesic response may be attributed to the fact that, as a partial agonist, levocabastine competes with Nts (a full agonist), resulting in a competitive agonism that overall diminishes the analgesic effect. Recent genetic studies also question the primacy of NtsR2 over NtsR1 for antinociception. In a model of thermal pain, antisense oligonucleotides against NtsR1 that were administered prior to Nts treatment notably diminished Nts-induced analgesia[88]. Remarkably, as little as 35-45 % reduction in NtsR1 expression in the PAG and hypothalamus was adequate to abolish Nts-induced analgesia. In alignment with the genetic inhibition studies, mice lacking NtsR1 exhibited heightened sensitivity (hyperalgesia) to thermal pain[198]. Furthermore, a newer NtsR1 agonist (PD149163) alleviates thermal pain and formalin-induced inflammatory pain in a dose-dependent manner. Importantly, this NtsR1 agonist-mediated antinociception is hindered by the NtsR1 selective antagonist SR48692[177,193]. Taken together, these investigations support roles for NtsR1 and NtsR2 in modulating nociception, and that both receptors warrant attention as targets to provide analgesia. Although very little is known about the role of NtsR3

and NtsR4 in nociception, a relatively recent study revealed that Nts and NtsR2 are upregulated in the brain of NTSR3/sortilin deficient mice leading to increased antinociception in thermal and chemical-induced pain. While NtsR1 levels remained unchanged, these data suggest that NtsR3 interacts with other Nts receptors and perhaps indirectly modulates nociception[204].

1.5.6 Pharmacological Efforts to Design a Neurotensin Analog for Treating Pain

For years, researchers have explored Nts and NtsR agonists as a potential alternative to opioids for pain relief. Yet, leveraging the Nts system for pain relief presents challenges due to the very short half-life of Nts and its metabolic instability in the circulation[101]. More critically, central treatment with Nts or the first-generation NtsR agonists can provide analgesia, but also accompanying hypothermia and vasodilation. These caveats have fueled an active drug discovery effort to develop Nts system modulators that can effectively ease pain without causing unwanted side effects. Initially, studies mainly investigated the analgesic effects of Nts and its receptor agonists in acute pain scenarios. However, patients commonly endure chronic pain in clinical settings, including visceral pain, neuropathic pain and inflammatory pain. Therefore, recent research has expanded to explore the potential nociceptive role of Nts using various models, including inflammatory and neuropathic conditions that are linked with chronic pain. In this section, we will explore various Nts agonists that have been developed thus far and assess their capacity to relieve diverse physiological and chronic pain conditions.

1.5.6.1 Acetic Acid-Induced Visceral Pain Model

Visceral pain can be modeled in rodents by injecting acetic acid and measuring their resulting writhing behavior. Preclinical NtsR2 agonists have been developed that show promise in providing analgesia in this model of chronic visceral pain without producing hypothermia and

hypotension[205]. The beneficial effect of NT79 may be attributed, at least in part, to the modulation of serotonin expression, which tends to increase in visceral pain[206]. Additionally, the analgesic effect of NT79 against acetic acid-induced writhing was also observed in NtsR1 homozygous knockout mice, which suggests that NtsR2 signaling alone is sufficient to regulate nociception. Interestingly, the NtsR1-specific agonist NT72 and the non-selective agonist for NtsR1/2, NT69L, also provide analgesia a mouse model of chronic visceral pain, even in mice that lack NtsR2. Taken together, these data support that both NtsR1 and NtsR2 are individually sufficient, even in the absence of the other, to relief visceral pain[207].

1.5.6.2 Neuropathic Pain Model

Surgical sciatic nerve injury (SNI) is commonly used to induce and model neuropathic pain in rodents. In this procedure, the sciatic nerve is exposed, and the tibial and common peroneal branches are transected while the sural branch is left intact[208,209]. Mice undergoing SNI surgery typically develop chronic pain characterized by heightened thermal sensitivity and mechanical allodynia in laboratory settings. Sciatic rodent injury models also exhibit mechanical allodynia measured with the von Frey filament test. Intriguingly, spinal cord expression of Nts was normal 3 days after sciatic nerve injury, but is elevated with sustained injury[210,211]. This implies that the gradual accumulation of endogenous Nts over several days might be involved in influencing chronic pain. Pharmacologically, intrathecal administration of the NtsR1-selective agonist PD 149163 attenuates nerve-injury-induced thermal and mechanical hyperalgesia[192]. A more recent analogue of Nts, contulakin-G (CGX), derived from snail venom, has demonstrated safety in clinical trials. Administering CGX intrathecally has been shown to alleviate thermal and mechanical hypersensitivity in both neuropathic and inflammatory pain models of both sexes.

Despite CGX's ability to bind to both NtsR1 and NtsR2, its antinociceptive effects are entirely blocked in the absence of NtsR2[212]. This suggests that CGX could avoid adverse physiological side effects that have been attributed to NtsR1 signaling, though more work is needed to assess its full potential in modulating pain and other behaviors.

1.5.6.3 Inflammatory Pain Model

To induce inflammatory pain, mice receive trans-plantar injection of either formalin or complete Freund Adjuvant (CFA). Similar to the SNI model, inflammatory pain is associated with increased Nts expression[213]. Consistent with anatomical data, pharmacological studies confirmed that Nts actions in the spinal cord are effective in alleviating inflammatory pain. For instance, the intrathecal administration of the NtsR1-selective agonist PD 149163, the non-selective NtsR1/2 agonist NT69L[193], and the selective NtsR2 agonist effectively alleviate formalin-induced hypersensitivity in dose-dependent manner[214]. Additionally, the NtsR2 agonist NT79 is a promising candidate for regulating persistent pain, particularly since it synergized with morphine-induced antinociception in a formalin-induced inflammatory pain model. Consequently, a combination therapy involving NT79 could potentially reduce the amount of morphine required for pain management, and reduce the risk of developing life-threatening morphine-related side effects[206]. With this goal in mind, there is an active effort to design hybrid compounds that target both MOR and NtsR2 while avoiding NtsR1-associated side effects[215,216] but metabolic stability and brain accessibility remain challenging[217]. At least one preclinical hybrid compound has been reported that alleviated acute and persistent inflammatory pain in opioid-independent manner without causing hypothermia, but also caused a mild hypotensive response[218]. Excitingly, this

hybrid compound also synergized morphine-induced analgesia, indicating promise for a combined NtsR/MOR targeted approach to maximize analgesic response.

1.5.6.4 Thermal Pain Model

Thermal pain is often modeled by placing rodents on a hot surface and assessing the time it takes for them to withdraw their paw or jump away from the surface. Newer Nts analogs such as NT69L and JMV2012 have improved metabolic stability compared to native Nts, can bind to both NtsR1 and NtsR2 with high affinity, and effectively diminish thermal pain responses 90 minutes post-injection. The analgesic effect persists for up to 5 hours[163,214,219]. However, despite their promise in alleviating thermal pain, these Nts agonists still induce hypothermia, rendering them less than ideal as potential analgesics

1.6 FUTURE CHALLENGES: IDENTIFICATION OF COMMON SITES TO TREAT OBESITY AND PAIN

The existence of peripheral vs. centrally produced pools of Nts raises the question of where and how Nts might simultaneously support weight loss and analgesia, as needed to address comorbid obesity-pain. Both pools of Nts have promise in mitigating pain. Enhancing Nts signaling, whether in the peripheral or central nervous system, has been shown to provide pain relief across various modalities[89,173,220], including the mitigation of neuropathic pain resulting from peripheral nerve injury[192,221]. However, contrasting effects are observed when comparing the peripheral vs. central Nts systems on body weight. While Nts promotes weight gain in the periphery, most data suggest that it acts centrally to facilitate weight loss by suppressing appetite and encouraging physical activity[114,161,222]. Moreover, disruptions in central endogenous Nts signaling may contribute to the onset or perpetuation of obesity, as both genetically and diet-induced obese rodent models exhibit reduced Nts expression in the brain[154,223]. Taken together,

these data suggest that modulating central Nts signaling may be the better target to achieve simultaneous weight loss and pain relief, as needed to effectively treat comorbid obesity-pain.

Yet, there are many unknowns that must be addressed to understand if and how the central Nts system may be used to address obesity-pain. While there has been ample research directed toward pharmacologically leveraging the Nts system to reduce feeding or pain, much less is known about how the endogenous Nts system is altered in linked obesity-pain. This information could have implications for understanding the origins of obesity-pain and, hopefully in the future, on how to pharmacologically leverage the Nts system to treat it. For instance, is there is a common central Nts system to modulate body weight and pain processing, or are these mediated by separate populations of Nts cells, signaling, and circuitry? Given the diverse physiology mediated by various Nts and NtsR-expressing populations throughout the brain, it is very possible that there are separate, dedicated central Nts systems devoted to regulation of feeding vs. nociception. This seems, on its face, to be supported by the fact that no published studies to date have documented brain sites that simultaneously modulate feeding and pain processing. Yet it is possible that there may be common nodes of central Nts signaling that they have been overlooked because Nts mediated effects have only been studied in separate physiological contexts. A goal of future Nts system studies should be to assess pain and ingestive behaviors together, so as not to miss potential connections. However, synthesizing the published findings to date (Table 1.1) indicates at least two brain sites linked with Nts-modulated energy balance and pain processing: the amygdala and the LHA. Intriguingly, while both regions are implicated in Nts-mediated analgesia they may oppositely modulate feeding and body weight. Activating the Nts-expessing IPAC neurons of the extended amygdala promotes mice to consume

highly palatable, obesogenic food. In contrast, activating Nts-expressing neurons in the LHA suppresses food intake, including blunting operant responding for palatable food. Further study of each area is necessary to determine whether they can simultaneously modulate body weight and pain and may reveal the reason for their opposing effects on food intake. Indeed, the variability of amygdala and LHA actions on feeding aligns with the documented pleiotropism of the central Nts system, where distinct neural pathways produce and release Nts to target cells expressing NtsR, resulting in diverse physiological and behavioral effects. Going forward, it will be important to characterize the contributions of all specific populations of Nts-expressing cells, their projection targets, and the complement of Nts receptors expressed there in both energy balance and pain processing. Additionally, there is ongoing debate over whether NtsR1 or NtsR2 mediate the anorectic and analgesic effects. This has proven challenging to resolve due to the limited specificity of previously available pharmacological tools or tools to identify precisely where in the brain these receptors are expressed. Resolving the receptor and signaling systems underlying Nts-modulation of energy balance and analgesia is critical to inform the drug-discovery pipeline and hone in on the most effective targets. By utilizing the array of new pharmacological and genetic tools available to the filed, there is hope to reveal the intricacies of the Nts signaling system and its potential to support weight loss and pain.

1.7 GOALS OF THE DISSERTATION

Chronic pain and obesity are prevalent, costly health problems that frequently occur together[12,13]. There are few interventions for obesity and a pitfall of current non-opioid pain medications is that they induce weight gain[29]. Obesity also contributes to use of opioids that alleviate pain but increase risk for dependence and fuel the opioid crisis[36], creating need for pain

therapeutics that do not cause dependence. Our limited understanding of the neurobiology underlying obesity and pain comorbidity is a major knowledge gap hindering the design of effective treatment strategies needed to improve and save lives.

Brain injections of the Nts or Nts receptor agonists promotes weight loss[114,151,158,161,222,224], and analgesia[171,225,226], suggesting that augmenting central Nts has potential to treat obesity and pain. Yet, the source(s) of endogenous Nts that could mediate these effects has remained elusive. Our lab has previously shown that a large population of Lateral Hypothalamic Area (LHA) neurons express Nts (referred to as LHA^{Nts} neurons) that support weight loss[147]. Excitingly, activating all LHA^{Nts} neurons restrains feeding and increases physical activity even in hungry mice that reduced their body weight, indicating their importance for supporting weight loss. However, the role of LHA^{Nts} neurons in pain processing, and whether they might also have potential to relieve pain had yet to be explored. If LHA^{Nts} neurons could simultaneously support weight loss behaviors and provide analgesia they could be promising targets to address comorbid obesity-pain. I examined this via my dissertation research, testing the role of LHA^{Nts} neurons in pain processing of normal weight mice (Chapter 2) and in diet-induced obese mice (Chapter 3). Hence, the goals of this dissertation are:

1. Detemine whether specifically activating LHA^{Nts} neurons alleviate thermal, neuropathic, and inflammatory pain via neurotensin receptor signaling in normal weight mice (Chapter 2)

Hypothesis: LHA^{Nts} neurons are an endogenous source of Nts that can provide antinociception, and hence, that activating LHA^{Nts} neurons would alleviate pain dependent on Nts signaling via NtsRs.

Method: I combined *Nts*^{Cre} mice and Cre-mediated excitatory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to selectively activate LHA^{Nts} neurons in thermal, neuropathic and inflammatory pain models, so as to decipher their role in pain regulation. Additionally, I pretreated these mice with the brain permeable Nts receptor pan-antagonist SR142948 before CNO-mediated activation of LHA^{Nts} to assess the role of Nts signaling in pain regulation. These data revealed whether activating LHA^{Nts} neurons in normal weight alleviates pain and if analgesia mediated by LHA^{Nts} neurons requires Nts-NtsR signaling.

2. Establish whether activating LHA^{Nts} neurons alleviates obesity-induced pain and its dependency on Nts-NtsR signaling (Chapter 3)

Hypothesis: Activating LHA^{Nts} neurons alleviates obesity-induced pain in an Nts-NtsR dependent manner, such that they are an endogenous source of Nts to mediate analgesia.

Method: I injected Cre-dependent excitatory DREADDs AAVs in the LHA of diet-induced obese *Nts*^{Cre} mice to selectively activate their LHA^{Nts} neurons. I then performed pain testing to study the role of LHA^{Nts} neurons in pain processing during obesity. To determine if Nts is a key player in LHA^{Nts} neurons-induced analgesia I injected newly generated *Nts*$^{flox/flox}$ mice with AAV-Cre directly into the LHA to enable Cre-mediated, site-specific deletion of Nts. Together, these data revealed if activating LHA^{Nts} neurons in obese mice alleviates obesity-induced sensitivity and inflammatory pain and whether specific LHA Nts deletion is needed for pain relief in normal weight and obese mice. Studying these mice will establish the specific role of LHA^{Nts} neurons in comorbid obesity and pain.

By completing this research, I revealed that the potential of LHA^{Nts} neurons in pain reducing pain and how endogenous stimuli modulate LHA^{Nts} neurons. Specifically, activating

LHA^{Nts} neurons alleviates inflammatory and neuropathic pain and this analgesic effect diminishes when mice are pretreated with NtsR1/2 pan-antagonist suggesting that Nts signaling is required for antinociception. Our work also showed that LHA^{Nts} neurons do not regulate acute thermal pain in naïve healthy mice. Moreover, we also established that LHA^{Nts} neurons regulate obesity-induced pain that is dependent on Nts signaling. Furthermore, deleting Nts specifically from LHA neurons in adulthood revealed that Nts signaling is important for pain relief in hypersensitive obese mice but not in normal weight naïve mice. These collective findings support the premise that augmenting activation of all LHA^{Nts} neurons holds promise in modulating body weight and pain. This work advances understanding of LHA^{Nts} neurons and endogenous roles of Nts that, in the future, might be leveraged to address comorbid obesity-pain.

Table 1.1: Central Nervous System Sites of Nts System Effects on Pain Processing and Energy Balance. NR = not reported. IPAC = Interstitial Nucleus of the Posterior Limb of the anterior commissure, a nucleus of the central extended amygdala. Shading indicates sites in which the Nts system has been documented to modulate pain and energy balance, though via separate studies.

Brain Region	Type of Pain	Analgesia	Food Intake	Water Intake	Locomotion	Body Weight	Receptor	Nts, and/or Nts analogs	Ref
DRG	Acute Pain	Increase	NR	NR	NR	NR	NtsR2	Nts, levocabastine, JMV-431, and β-lactotensin, (NtsR2-specific agonists)	194, 214
	Thermal Pain	Increase	NR	NR	NR	NR	NtsR2	CGX (Nts analog)	212
	Mechanical Nociception	Increase	NR	NR	NR	NR	NtsR2	CGX (Nts analog)	212
Spinal Cord	Persistent Inflammatory Pain	Increase	NR	NR	NR	NR	NtsR1 and NtsR2	Nts and NT69L (non-selective NtsR1/2 agonist)	177, 193, 214,
	Formalin-Induced Pain	Increase	NR	NR	NR	NR	NtsR1	Nts and PD149163 (NtsR1-specific agonist)	193
	Persistent Neuropathic Pain	Increase	NR	NR	NR	NR	NtsR1	Nts and PD149163 (NtsR1-specific agonist)	192, 210
	Thermal Pain	Increase	NR	NR	NR	NR	NtsR2	Nts, levocabastine, JMV-431, and β-lactotensin, (NtsR2-specific agonists) NtsNT69L, JMV-431	194

Table 1.1 (cont'd)

Amyg-dala	Thermal Pain	Increase	NR	NR	NR	NR	Not Specified	Nts injection in Central Nucleus of the Amygdala	224
	NR	NR	Increase	NR	NR	Increase	NR	Activation of Nts-expressing IPAC neurons in Amygdala	159
PAG	Thermal and Visceral Pain	Increase	NR	NR	NR	NR	NtsR1 and R2	NT79, NTS2 agonist	168, 183
RVM	Acute	Increase	NR	NR	NR	NR	NtsR1 and NtsR2	Nts, PD149163 (NtsR1-specific agonist), and levocabastine, (NtsR2-specific agonists)	176, 177
	Stress-Induced Nociception	Increase	NR	NR	NR	NR	NtsR1	Blocked by SR48692 (NtsR1-specific antagonist)	199
LHA	Stress-Induced Nociception	Increase	NR	NR	NR	NR	Not Specified	Nts expression increased in LHA	225
	NR	NR	Decrease	Increase	Increase	Decrease		Activation of Nts-expressing LHA Neurons	144
VTA	NR	NR	Decrease	Unchanged	Increase	Decrease	NtsR1	Activation of NtsR1-expressing VTA Neurons	133
VMH	NR	NR	Decrease	NR	NR	NR	NR	Nts Injection	158
PVN	NR	NR	Decrease	NR	NR	NR	NR	Nts injection	156
NAc	NR	NR	Unchanged	NR	Decrease	NR	NtsR1	Nts Injection in the NAc	93, 128, 129

Table 1.1 (cont'd)

NTS	NR	Increase	Decrease	NR	NR	NR	Not Specified	Nts	156
LS	NR	NR	Decrease	NR	Unchanged	Decrease	Not Specified	Activation of Nts-expressing LS neurons	143

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CHAPTER 2: NEUROTENSIN-EXPRESSING LATERAL HYPOTHALAMIC NEURONS ALLEVIATE NEUROPATHIC AND INFLAMMATORY PAIN VIA NEUROTENSIN RECEPTOR SIGNALING

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ABSTRACT

Persistent, severe pain negatively impacts health and wellbeing, but half of patients do not receive adequate relief from current pain treatments. Understanding signals that modulate central pain processing could point to new strategies to manage severe pain and the development of new treatments. Prior work has shown that administering Neurotensin (Nts) or Nts receptor (NtsR) agonists into the brain provides analgesia comparable to pharmacologic opioids. However, the endogenous sources of Nts that modify pain processing and might be leveraged for pain relief remained unknown. We previously characterized a large population of Nts-expressing neurons in the lateral hypothalamic area (LHA^{Nts} neurons) that project to brain regions that participate in descending control of pain processing. We hypothesized that LHA^{Nts} neurons are an endogenous source of Nts that can provide antinociception, and hence, that activating LHA^{Nts} neurons would alleviate pain dependent on Nts signaling via NtsRs. To test this, we injected Nts^{Cre} mice in the LHA with AAVs to Cre-dependently express either mCherry (Control) or the excitatory hM3Dq Designer Receptor Exclusively Activated by Designer Drugs in LHA^{Nts} neurons, permitting their stimulation after treatment with the hM3Dq ligand clozapine N-oxide (CNO, 0.3 mg/kg, i.p.). Activating LHA^{Nts} neurons had no effect on thermal pain and mechanical responses in naïve mice.

By contrast, both spared nerve injury- (SNI) and complete Freund's Adjuvant (CFA)-induced mechanical hypersensitivity was completely reversed by CNO-mediated stimulation of LHA^{Nts} neurons compared to control. However, pretreatment with the brain permeable Nts receptor pan-antagonist SR142948 reduced CNO-mediated analgesia, indicating that LHA^{Nts} neurons alleviate chronic pain in an Nts receptor-dependent manner. Taken together these data identify LHA^{Nts} neurons as an endogenous source of Nts that modulates central pain processing and may inform future development of Nts-based targets to treat severe pain.

2.1 INTRODUCTION

Severe, persistent pain is a prevalent serious health problem worldwide, including in the United States where 1 out of every 5 adults reports experiencing chronic pain[1-3]. Severe chronic pain differs from acute pain (short-term, local sensation) in that it continues past the normal healing time, often persisting for months or years[4]. As a result, severe chronic pain has a sustained negative impact on quality of life and can cause substantial economic burden due to cost of treatment, lost productivity, and disability[5-7]. Despite the high and increasing prevalence of chronic pain there are few effective interventions. Opioid analgesics have increased risk for dependence and abuse and have fueled the opioid epidemic[8]. Non-opioid pain medications have adverse effects that often preclude their use, such as weight gain that is not tractable for the nearly 60% of Americans already struggling with overweight and obesity[9-12]. Hence, management of severe, persistent pain remains a major health challenge. Better understanding of the brain cells and endogenous signals that mediate antinociception could point to strategies to leverage such systems and inform the development of effective pain treatments.

Neurotensin (Nts) is a 13 amino acid peptide that is produced endogenously in the periphery and brain and has promise to modulate pain. Nts signals via the high-affinity neurotensin receptor-1 (NtsR1) and low affinity neurotensin receptor-2 (NtsR2), both of which are G-protein coupled receptors[13-15]. Nts signaling in the peripheral nervous system and spinal cord is well established to provide analgesia via yet unclear mechanisms[16-19]. The central Nts system also modulates pain and augmenting Nts signaling in the brain provides analgesia in a variety of pain modalities[20-22]. Furthermore, pharmacological stimulation of NtsR1 or -2 in the brain alleviates neuropathic pain induced by peripheral nerve injury[17,19,23]. However, the previous difficulty in visualizing or modulating Nts or NtsR1/2-expressing cells prevented verification of which brain cells release endogenous Nts to mediate the analgesic effect. Pharmacological studies were not able to resolve this question since administration of Nts or NtsR agonists into different brain regions causes varying effects, from suppression of feeding, invoking drinking, hyper or hypo-locomotion, vasodepression, and analgesia[24]. This is, in fact, consistent with the role of central Nts as a neuropeptide transmitter; specific neural pathways may produce and release Nts to regionally and neurochemically distinct NtsR-expressing target cells, which can result in different physiologic and behavioral effects. Defining the brain site and mechanism of Nts-mediated analgesia is necessary to determine how to bias the Nts system for pain relief and avoid the adverse vasodepression and hypothermia attributed to systemic/hindbrain Nts treatment[25,26].

One notable source of Nts is the large population of Nts-expressing neurons in the lateral hypothalamic area (LHA) of the brain, referred to as LHA^{Nts} neurons. While the physiologic roles LHA^{Nts} neurons and the Nts released from them are just beginning to be appreciated[27,28], their

location within the LHA makes them plausible contributors to Nts-mediated antinociception. Indeed, the LHA has long been recognized as one of the brain regions responsive to noxious stimuli[29-31]. Several studies in animals have shown that electric stimulation of LHA neurons provides analgesia in both acute and chronic pain states[31-33]. However, the LHA is very heterogeneous, containing multiple subsets of molecularly defined neuronal populations that mediate distinct aspects of physiology[34]. Some LHA cell types have been implicated in pain modulation, including those that express glutamate or the hypocretin/orexin peptide[35-39], but there is not yet a comprehensive understanding of which LHA neuronal subsets and specific signals released from them modulate nociception. Intriguingly, stress increases Nts expression in the LHA which has been suggested to contribute to stress-induced analgesia[40]. However, it was unknown whether LHA^{Nts} neurons, and the Nts released from them, mediate antinociception in other pain modalities. Here we used chemogenetics to site specifically activate LHA^{Nts} neurons in naïve, inflamed, and neuropathic mice, along with antagonism of NtsR1 and NtsR2 to reveal the necessity of Nts signaling in the antinociceptive effects. Our data identify a role for LHA^{Nts} neurons in persistent pain hypersensitivity models that depends on intact Nts signaling, and hence, reveal an endogenous source of Nts that contributes to pain processing.

2.2 RESEARCH DESIGN AND METHODS

2.2.1 Mice

All mice used in this study were bred and housed in 12 hours light/ 12 hours dark cycles and were cared for by Campus Animal Resources (CAR). We produced and studied male and female heterozygous $Nts^{Cre/+}$ mice[41] on the C57/BI6J background. All experimental protocols were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at

Michigan State University and with the Association for Assessment and Accreditation of Laboratory Animal Care and the National Institutes of Health. For experiments, 4- to 6-week-old mice were randomly assigned to control or experimental groups with littermate, age-matched, and gender-matched controls. Following stereotaxic surgeries mice were individually housed with *ad libitum* access to water and chow (Harlan Teklad # 7913) for the duration of experiment, unless specified otherwise.

2.2.2 Surgical Procedures

Nts^{Cre} mice were anesthetized with a 2-4% isoflurane/oxygen mixture, fitted into a stereotaxic apparatus, and given Meloxicam (5 mg/kg, i.p.) prior to using a scalpel to expose the skull. Small holes were drilled in each hemisphere of the skull to permit injection of either AAV2-hSyn-DIO-mCherry (control, Addgene, 50459-AAV2) or AAV2-hSyn-DIO-hM3Dq (Gq)-mCherry (excitatory DREADDq, Addgene, 44361-AAV2) into the LHA (300nL per side, bregma: - 1.34 mm, midline: ±0.95 mm, skull surface: -5.25 mm) per the Franklin and Paxinos brain atlas. The injector was left in place for 10 minutes to ensure absorption of the AAV, then after retracting the hole in the skull was filled with bone wax and the incision was closed. Mice were allowed to recover from surgery for at least 2 weeks prior to testing.

2.2.3 Treatments

The hM3Dq DREADD-ligand clozapine N-oxide (CNO, 0.3 mg/kg) or vehicle (VEH, in this case phosphate buffered saline) were administered to mice via i.p. injection. CNO (Sigma Aldrich, Cat # C0832) was dissolved in 10% β-cyclodextrin (Sigma Aldrich, Cat # C0926) in sterile PBS to make 20X CNO stock aliquots (1.2 mg/ml). A 20X aliquot was diluted with sterile PBS just prior to the experiment to make the 1X working solution (60 μg/mL). We then administered 5µL of 1X

CNO per gram of body weight. Unless specified otherwise, mice were treated once 30 min prior to pain testing, 1- 2 hrs after onset of light cycle, via a crossover design. Thus, all mice received both VEH and CNO on different days.

For experiments examining the involvement of Nts signaling in pain regulation, 90 min before each test mice from each AAV group received i.p. pretreatment with either saline or the NtsR pan-antagonist, SR142948 (1 mg/kg) that has been reported to block signaling via NtsR1 and NtsR2[42,43], followed 30 min later by VEH or CNO (to activate LHA^{Nts} neurons). SR142948 (TOCRIS Bioscience, 2309/1) was dissolved in 0.1% Tween 80 in sterile PBS to make the 20x stock solution (2 mg/ml), which was diluted with sterile PBS prior to the experiment to make the 1x working solution (0.1 mg/ml). We administered 10 μL of 1X SR142948 per gram of body weight.

2.2.4 Perfusion and Immunofluorescence

After completion of all behavioral experiments, mice received i.p. injection of VEH or CNO 90 minutes before perfusion. Mice were then given a lethal i.p. dose of pentobarbital sodium (Fatal Plus C II, Vortech, SKU # 035946), and transcardially perfused with 1x PBS (pH 7.4) followed by 10% neutral buffered formalin (Epredia, Cat # 5725). Whole brains were extracted and post-fixed in 10% formalin overnight at 4°C then transferred to 30% sucrose in 1x PBS for storage at 4°C until further processing.

For immunostaining, brains were coronally sectioned (30µm thick) using a Leica SM2010R microtome (Leica Biosystems) and collected in 1% formalin/PBS solution. For cFOS immunohistochemistry, brain sections were blocked with 3% normal donkey serum in 1x PBS with 0.3% Triton X for an hour followed by incubation in goat anti-cFOS primary antibody (1:1000, Santa Cruz Biotechnology, sc-52-G). The following day the sections were washed with PBS prior

to incubation with secondary antibodies conjugated to donkey anti-goat biotin (1:100, Jackson ImmunoResearch, Cat # 705-065-147), then the immunolabeled cFOS was visualized using a DAB peroxidase substrate kit per the manufacturer's protocol (Thermo Scientific, Cat # 34065). Sections were then washed in PBS prior to overnight incubation at room temperature with rabbit-dsRed/mCherry (1:1000, Takara, 632496). The next day the sections were rinsed and incubated for 2 hr in donkey anti-rabbit Alexa-568 (1:200, Invitrogen, AB10042) to visualize DREADD-mCherry-expressing LHA^{Nts} neurons. Brain sections were mounted on slides for analysis using an Olympus BX53 fluorescence microscope. LHA images were obtained at 4x and 20x resolution via Cell Sens software. Images were analyzed using Photoshop (Adobe) to assess co-localization and to count cells. For DAB-labeled cFOS-expressing cells, images were taken with transmitted light and later pseudo-colored blue for ease of visualization and counting using Adobe Photoshop.

mCherry expression was used for posthoc evaluation of AAV targeting in the LHA. Mice were only included in the final data set if mCherry-expressing soma were bilaterally confined within the LHA. Mice with mCherry soma located outside of the LHA were excluded, as were mice found to only have unilateral LHA targeting

2.2.5 Thermal Nociception (Hot Plate Test)

A cylindrical plexiglass enclosure was placed on a hot plate (Ugo Basile, Hot/Cold plate). Mice were *i.p.* injected with VEH or CNO prior to thermal pain testing. After 30 mins, they were gently placed on the hot plate and their paw withdrawal latency (PWL), jump latency, and number of jumps were measured at 48°C, 50°C, and 52°C. A latency of 60 secs was used as a cutoff time and mice were removed from the hot plate after cutoff time to avoid tissue damage, as described previously[44].

2.2.6 Mechanical Nociception (von Frey test)

Mice were acclimatized for 30 mins in clear plexiglass enclosure (10×10×13 cm^3) on a wire mesh grid floor. Von Frey filaments (Aesthesio, Cat # 514000-20C) ranging from 0.008 g – 2.0 g were used to measure mechanical allodynia, which was defined as the hind paw withdrawal response to von Frey hair stimulation, by a blinded investigator using the "up and down" method, as described previously[45,46] Nociception responses were recorded when mice exhibited paw withdrawal, licking, and shaking upon filament application. Briefly, starting with the 0.4 g von Frey filament, pressure was applied perpendicular to the midplantar surface of the hind paw. Filament strength was increased until there was a paw withdrawal response or decreased until mice no longer exhibited a response.

2.2.7 Inflammatory Pain (Complete Freund's Adjuvant, CFA)

To induce inflammatory pain, mice were briefly anesthetized under 2% isoflurane and paw thickness was recorded using vernier caliper. Mice then received intra-plantar injection of 5 µl CFA (Sigma Aldrich Cat # F5881) in one hind paw and saline injection (control) in the other hind paw. Mice were returned to their home cages and inflammation was confirmed by redness and increased paw thickness due to swelling 24 hours after CFA administration. Pain testing was conducted between 2-, and 6-days post-CFA injection.

2.2.8 Neuropathic Pain (Spared Nerve Injury, SNI)

To induce neuropathic pain, mice underwent SNI surgery[45,47]. The sciatic nerve in the left hind leg was exposed under isoflurane anesthesia, then the tibial and common peroneal nerves were transected, whereas the sural branch was left intact. In sham-operated mice, the nerves

were exposed but remained untouched. Mice were returned to their home cages. Pain testing was conducted 7- and 28- days post-nerve injury.

2.2.9 RNAscope

Three LHA- containing sections (30µm thickness) were picked per mouse brain, as per[48]. RNAScope single-plex assay (Advanced Cell Diagnostics, catalog # 322360) was performed using the manufacturer's protocol. Free floating sections were washed with 1x PBS, and incubated in Pretreatment I (RNAScope H_2O_2, Advanced Cell Diagnostics, Cat # 322335) at room temperature until bubbling stopped (45-60 min). Slices were then washed with 0.5x PBS before carefully mounted on positively charged slides (Superfrost Plus Microscope slide, Cat # 12-550-15). Once mounted, sections were washed with dH_2O and dried overnight at 60°C in HybEZ II oven. Dried sections were incubated in 1x Pretreatment II (Target Retrieval Agent) at 99–104°C for 5-10 min, and then washed with dH_2O, dried at room temperature followed by 100% EtOH wash. Dried sections were then incubated in Pretreatment III (RNAScope Protease Plus, Advanced Cell Diagnostics, catalog # 322331) for 15 min at 40°C in HybEZ II oven and then washed with dH_2O. Sections were incubated with Nts probe (Advanced Cell Diagnostics, catalog # 420441) for 2 hrs at 40°C. For target-specific hybridization, sections were incubated in Amp1-6, followed by application of Fast-Red-A and Red-B for 10 min for visualization. Sections were washed with dH_2O then dried by briefly dipping in xylene and cover slipped using antifade mounting media.

2.2.10 Cell Counting

For cFOS and mCherry immunolabeling, three LHA sections representing the extent of the LHA (Bregma -1.22, -1.34 and -1.46) were selected for cell counting analysis. 20x images of the left and right LHA hemisphere were collected from the chosen sections and were given a coded

name to ensure blinded, unbiased analysis. Using Photoshop software, a blinded investigator counted the number of red (mCherry) and blue- (DAB-labeled cFos, pseudocolored blue in Photoshop) labeled cells in each image. Co-labeling was recorded only if mCherry-labelled soma encompassed a cFOS-labeled nucleus.

For quantitative analysis of *Nts* RNA after RNAScope, 10x LHA images were analyzed by a blinded investigator using FIJI ImageJ. Corrected Total Cell Fluorescence (CTCF) was calculated by taking the integrated density (IntDen) of the region of interest (ROI) and subtracting from it the background mean (BGMean) times the area of the ROI. The formula used for this calculation is CFCF = IntDen-Area x BGMean. A Grubbs outlier test identified an outlier in the Saline-treated control group of the CFA experiment (Figure 5F-H), so it was removed from the data set. Graphed data represent mean ± SEM.

2.2.11 Statistics

Student's t-tests and 2-way ANOVA were calculated using Prism 7 (GraphPad). Repeated measures 2-way ANOVA with Sidak's multiple comparisons test was used when each mouse received both VEH and CNO, and when data from the same mice were collected at different time points. Ordinary 2-way ANOVA with Tukey post-test was used if mice did not receive both treatments. A p-value of <0.05 was considered statistically significant. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

2.3 RESULTS

2.3.1 Chemogenetic Strategy Successfully Activates LHA^{Nts} Neurons

Nts^{Cre} mice were injected bilaterally in the LHA with cre-inducible AAV-hSyn-DIO-mCherry (control) or AAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express the excitatory DREADD hM3Dq in

LHA^{Nts} neurons (Fig. 1A). Posthoc analysis for mCherry fluorescence confirmed LHA targeting and expression of mCherry in LHA soma and fibers of control mice (Fig. 1B) and mCherry-hM3Dq expressing soma and processes in experimental mice (Fig. 1C). To confirm CNO-mediated stimulation of hM3Dq-expressing LHA^{Nts} neurons mice were treated with CNO 90 min prior to perfusion, then brains were analyzed for mCherry/mCherry-hM3Dq (red) and cFOS (a marker of neuronal stimulation, blue). Few neurons with co-localized cFos and mCherry were observed in control mCherry-expressing neurons (Fig. 1D and quantified in Fig. 1F) but CNO significantly increased cFOS expression in hM3Dq-mCherry expressing LHA^{Nts} neurons (Fig. 1E, 1F). This effect was specific to CNO-mediated treatment in DREADDq-expressing neurons and was not observed in PBS-treated hM3Dq-mCherry or mCherry-expressing *Nts^{Cre}* mice (Supplemental Fig. 1). Together, these data confirm that CNO treatment selectively activates hM3Dq-mCherry-expressing LHA^{Nts} neurons, and henceforth we used this chemogenetic method to determine whether activating them impacts nociception

2.3.2 Chemogenetic Stimulation of LHA^{Nts} Neurons Does not Regulate Thermal Pain in Naïve Mice

Early studies reported that intracisternal administration of Nts reduces acute thermal nociception[49-51], but did not verify the endogenous source of Nts mediating thermal antinociception. Here we asked whether stimulating LHA^{Nts} neurons can alter responses to thermal noxious stimuli. *Nts^{Cre}* mice were bilaterally injected in the LHA with Cre-recombinase dependent AAVs to express the fluorophore mCherry (Controls) or hM3Dq-mCherry (as in Fig. 1A), then were treated with VEH or CNO prior to hot plate testing at 48°C, 50°C and 52°C (Fig. 2A, Supplemental Fig. 2). Thermal responses in female and male mice were similar in the

LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq groups, indicating no underlying sex differences (data not shown) so data from each sex were pooled for analysis. LHA^{Nts}:mCherry mice exhibited similar thermal responses after treatment with VEH and CNO, indicating that CNO does not cause any off-target regulation in mice lacking the hM3Dq receptor. (Supplemental Fig. 2). In LHA^{Nts}:hM3Dq mice, CNO-mediated stimulation of LHA^{Nts} neurons did not alter paw withdrawal latency (Fig. 2B), jump latency (Fig. 2C), or the number of jumps (Fig. 2D) in response to any of the tested temperatures. These data suggest that stimulation of LHA^{Nts} neurons does not regulate acute thermal nociception in naïve mice.

2.3.3 Chemogenetic Stimulation of LHA^{Nts} Neurons Does Not Alter Mechanical Sensitivity in Naïve Mice

We next tested whether activating LHA^{Nts} neurons altered mechanical sensitivity thresholds in naïve healthy mice. Mice expressing mCherry (Controls) or hM3Dq in LHA^{Nts} neurons were treated with VEH and CNO prior to measuring paw withdrawal threshold via the von Frey test (Fig. 3A). CNO- treatment had no significant effect on paw withdrawal in LHA^{Nts}:hM3Dq mice compared to VEH over 5 hours, nor compared to LHA^{Nts}:mCherry control mice treated with VEH or CNO (Fig. 3B). These data suggest that stimulation of LHA^{Nts} neurons does not alter mechanical sensitivity in healthy mice.

2.3.4 Chemogenetic Stimulation of LHA^{Nts} Neurons Attenuates Persistent Neuropathic Pain-Associated Behaviors

Previous studies have shown that chemical[37,52] or electrical[29,31] stimulation of the LHA alleviates neuropathic pain in animal models. Given that LHA^{Nts} neurons are a subset of LHA neurons we asked whether specifically activating them after nerve injury would be sufficient to

reverse mechanical hypersensitivity associated with neuropathic pain. To investigate this, *Nts*^{Cre} mice expressing either mCherry or hM3Dq in LHA^{Nts} neurons underwent a spared nerve injury (SNI) procedure to invoke neuropathic pain-associated behavior (Fig. 4A-C). LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq mice had similar paw withdrawal thresholds prior to nerve injury (Fig. 4D, Pre Injury). Consistent with prior reports[45,53], SNI induces acute and persistent pain hypersensitivity indicated by reduced paw withdrawal threshold. CNO treatment has no effect on nerve injured controls lacking hM3Dq (Fig. 4D, black data points and line). However, CNO-mediated stimulation of LHA^{Nts}-hM3Dq expressing neurons completely reversed injury-induced reduction in paw withdrawal threshold at 7- and 28-days post-injury (Fig. 4D, red data points and line). This effect was specific to CNO-mediated effects at LHA^{Nts}-hM3Dq expressing neurons, as treating these mice with VEH elicited no change in pain hypersensitivity in LHA^{Nts}-mCherry control or LHA^{Nts}-hM3Dq mice (Supplemental Fig. 2.8). At the molecular level, sham control mice have ample *Nts* expression in the LHA (Fig. 4E) but, intriguingly, *Nts* expression is elevated after SNI (Fig. 4F, G). No sex differences were observed in *Nts* expression between females and males nor in pain-like behaviors after activating LHA^{Nts} neurons in SNI mice (data not shown). Overall, these data support that nerve injury upregulates *Nts* expression in both sexes but elevated *Nts* expression, by itself, is insufficient to attenuate nerve injury pain. Activating LHA^{Nts} neurons in this context did alleviate acute and persistent nerve injury-induced hypersensitivity, suggesting that increasing LHA^{Nts} neuronal mediated transmitter release is required to modulate neuropathic pain-induced hypersensitivity.

2.3.5 Chemogenetic Stimulation of LHA^{Nts} Neurons Attenuates Inflammatory Pain-Associate

To determine whether activating LHA^{Nts} neurons modulates inflammation-induced pain hypersensitivity, mice with mCherry or hM3Dq in LHA^{Nts} neurons (as in Fig 1A) were injected with saline in one hind paw (Supplemental Fig. 4) and Complete Freund's Adjuvant (CFA) in the other hind paw (Fig. 5A, B). Saline injection had no impact on paw thickness in LHA^{Nts}:mCherry or LHA^{Nts}:hM3Dq mice, nor did VEH or CNO treatment alter the paw withdrawal response of the saline-injected paw (Supplemental Fig. 4). By contrast, CFA induced an equivalent inflammatory response in both groups of mice, confirmed by increased paw thickness after CFA injection (Fig. 5C). Mice were then assessed for inflammation-induced pain hypersensitivity using the von Frey filament test. As expected, CFA treatment induced pain hypersensitivity in the LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq mice, indicated by their reduction in paw withdrawal threshold from pre-treatment baseline (Fig. 5D, Day 0 vs. CFA). Excitingly, the reduction in paw withdrawal threshold was completely reversed by CNO-mediated stimulation of the hM3Dq-expressing LHA^{Nts} neurons for at least 2 hr, as compared to VEH-treated mice (Fig. 5D). By contrast, CNO treatment had no analgesic effect on the LHA^{Nts}:mCherry control mice, which looked identical to VEH-treated mice of both groups.

LHA^{Nts} neurons express and, when activated, release multiple transmitters including the fast neurotransmitter GABA[54-56] and peptides such as galanin, corticotropin-releasing hormone (CRH)[56.57], and Nts itself. Any of these released signals might mediate the antinociception observed in mice with inflammatory pain after stimulation of LHA^{Nts} neurons (Fig. 5D). To evaluate whether Nts signaling via its receptors is specifically required for the antinociceptive effect, we

pretreated LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq mice with either saline (control) or the brain permeable NtsR1 & NtsR2 pan-antagonist SR142948 followed by CNO to activate h3MDq-expressing LHA^{Nts} neurons (see the timeline of treatments in Fig 5A). LHA^{Nts}:mCherry mice had low paw withdrawal threshold that was not modified by saline+CNO or SR142948+CNO treatment (Fig. 5E). In contrast, LHA^{Nts}:hM3Dq mice pretreated with saline and then CNO to activate LHA^{Nts} neurons increased their paw withdrawal threshold (Fig. 5E), consistent with the observations in Fig. 5D. Excitingly, pretreatment with the SR142948 pan-antagonist for NtsR1 and NtsR2 significantly blocked the antinociceptive effect of CNO-stimulated LHA^{Nts} neurons (Fig. 5E). The lack of CNO-mediated antinociception or NtsR antagonist effects in LHA^{Nts}:mCherry control mice confirms the absence of off-target drug effects and supports that chemogenetic-stimulation of LHA^{Nts} neurons mediates analgesia that depends on Nts signaling via NtsR1 and/or NtsR2. These data support that activating LHA^{Nts} neurons mediates antinociception in response to inflammatory pain that depends on Nts signaling.

Lastly, we assessed whether CFA-induced inflammatory pain modulates *Nts* expression in the LHA of wildtype mice treated with saline or CFA in one of the hind paws. The mice were perfused 24 hours after treatment and *Nts* expression was measured in the LHA via RNAScope. Saline-treated control and CFA-treated mice express *Nts* in the LHA (Fig. 5F-H) with no discernable sex difference (data not shown). However, *Nts* expression is elevated in the LHA of CFA-treated mice compared to Saline-treated controls. Taken together, these data indicate that CFA-mediated inflammatory-induced pain upregulates *Nts* expression but activating the Nts-expressing LHA^{Nts} neurons is necessary to alleviate inflammatory pain in mice. Moreover, the analgesic effect

mediated via activating LHA^{Nts} neurons is due, at least in part, to Nts signaling via NtsR1 and/or NtsR2.

2.4 DISCUSSION

Despite the large body of work describing the analgesic effects of exogenous Nts treatment[49.50] little is known about the endogenous sources of Nts capable of modifying pain processing. Here we explored whether the population of neurons in the LHA that produce and release Nts, the LHA^{Nts} neurons[54], modulate acute and persistent pain behavior. We found that chemogenetic stimulation of LHA^{Nts} neurons had no impact on acute thermal nociception and mechanical sensitivity in naïve mice over a timescale of hours. However, in models of persistent pain such as CFA- and SNI-induced mechanical hypersensitivity chemogenetic stimulation of LHA^{Nts} neurons significantly attenuated pain hypersensitivity and restored paw withdrawal threshold back to baseline levels. Furthermore, pretreatment with the NtsR1 and NtsR2 pan-antagonist (SR142948) prior to chemogenetic stimulation of LHA^{Nts} neurons blocked the antinociceptive effect in both inflammatory and neuropathic models. Upregulation of *Nts* expression in the LHA alone was not sufficient to attenuate pain behaviors, suggesting that the LHA neurons containing Nts must be stimulated to release Nts to target regions to modify pain processing. Taken together our findings suggest that LHA^{Nts} neurons are an endogenous source of Nts and that activating them to release Nts can alleviate mechanical hypersensitivity in the context of persistent pain. In the future, augmenting endogenous LHA^{Nts} neuron-mediated Nts signaling could hold promise for treating pain.

Here we focused on the LHA because it contains a large population of Nts neurons and is anatomically positioned to coordinate many behaviors important for survival and homeostasis,

including nociception. Indeed, stimulation of the entire LHA regulates descending pain processing in both acute[36,37,52], and persistent pain[37,58-61]. Yet, these bulk modulations may have obscured the role that molecularly defined subsets of LHA neurons play in modulating pain[62]. Among the many types of LHA neurons, some LHA glutamatergic and hypocretin-orexin expressing populations contribute to pain processing[36,37,52,61]. In contrast, LHA^{Nts} neurons are exclusively GABAergic, and presumably mediate distinct physiology[34,55]. However, LHA^{Nts} neurons are connected with brain areas implicated in modulating pain behavior, including the ventral tegmental area (VTA), substantial nigra compacta (SNc), lateral preoptic area (LPO), and the periaqueductal grey area (PAG)[63,64], an important brain region that processes noxious thermal and mechanical stimuli via descending pain pathways[65-70]. Given that LHA^{Nts} neurons interface with brain regions that modulate pain processing and that Nts injected into these areas produces analgesia[71-73] there is strong premise that LHA^{Nts} neurons are important endogenous players in pain behavior.

Intriguingly, chemogenetic stimulation of LHA^{Nts} neurons lessened nociceptive responses in the context of severe, persistent pain (inflammatory and nerve injury pain) but had no impact on thermal or mechanical nociception in naïve mice. These data suggest that the LHA^{Nts} neurons are biological modulators of persistent pain, not of acute nociception. Why this distinction? One possibility is that LHA^{Nts}-mediated influence on pain requires long-term changes to occur, such as the increased expression of *Nts* observed days after SNI or one day after CFA treatment. Prolonged stress-induced analgesia has also been associated with increased *Nts* expression in the LHA, suggesting a potential role for LHA^{Nts} neurons in pain processing[74]. Collectively, these findings support that persistent pain may biologically increase *Nts* expression in the LHA.

Interestingly, upregulated *Nts* expression alone is not sufficient to provide analgesia. For example, the SNI and CFA models exhibit significantly elevated *Nts* in the LHA, yet these mice experience hypersensitivity (FIG 4D-4G and FIG 5D-H, respectively). Activation of LHA^{Nts} neurons was necessary to reverse the hypersensitivity. This suggests that some activation-mediated release of a signal from the LHA^{Nts} neurons is necessary for modifying the pain processing. Nts is a strong candidate for the antinociceptive effect, given that it is released from LHA^{Nts} neurons and has been linked with pain relief[75]. Indeed, pretreatment with the pan NtsR1-NtsR2 antagonist prior to stimulation of LHA^{Nts} neurons in mice with CFA-induced inflammatory pain blocked analgesia, implicating released Nts that signals via NtsR1 and/or NtsR2 as the critical mediator of pain behavior. Taken together, these data indicate that endogenous Nts released from LHA^{Nts} neurons is sufficient to reduce pain in persistent pain contexts. Whether increased expression of *Nts* within the neurons modulates the degree of pain or is required for analgesia is a question for further exploration. Given that previous studies have shown that pharmacologically augmenting Nts signaling in the brain provides antinociception after peripheral nerve injury[16.17], and that LHA^{Nts} neurons elicited similar antinociception in our studies, augmenting LHA^{Nts} endogenous signaling could hold potential for treating persistent pain states.

We acknowledge that there are caveats with this study and remaining questions that should be addressed with future work. One vital question is where the Nts released from LHA^{Nts} neurons acts in the brain to alleviate pain and via which receptor/s. As discussed above, LHA^{Nts} neurons project to many regions implicated in pain processing, any of which could conceivably contribute to the analgesic effects. Circuit-based analysis will be important to define the critical pathways by which the LHA^{Nts} neurons engage and modulate pain, and to identify the

downstream cells of this endogenous signaling that might be pharmacologic targets for controlling pain. Moreover, further work is needed to clarify how LHA^{Nts} neuron-released Nts mediates its effects, be that signaling via NtsR1, NtsR2, or both receptor isoforms. Likewise, although our data supports an important role for Nts released from LHA^{Nts} neurons in mitigating nociception, the chemogenetic method used here causes release of all LHA^{Nts} neuronal signals and so we cannot exclude that signals other than Nts also contribute to pain processing. Given that LHA^{Nts} neurons express and release Nts as well as other neuropeptides such as galanin and CRH[56,57], along with the classical neurotransmitter GABA[54-56], these could play important roles as well. Our data support that LHA^{Nts} induced signaling via NtsR1/NtsR2 contributes to pain processing to some degree (Fig. 5D) but the other released signals also merit investigation. While there is yet much to learn about the precise mechanisms by which LHA^{Nts} neurons contribute to biological pain processing, our data reveal them as an important node in the endogenous Nts system and control of pain processing. Going forward, understanding how the LHA^{Nts} neurons engage the descending pain system and modulate it may point to design of novel analgesic approaches to provide relief for severe, persistent pain.

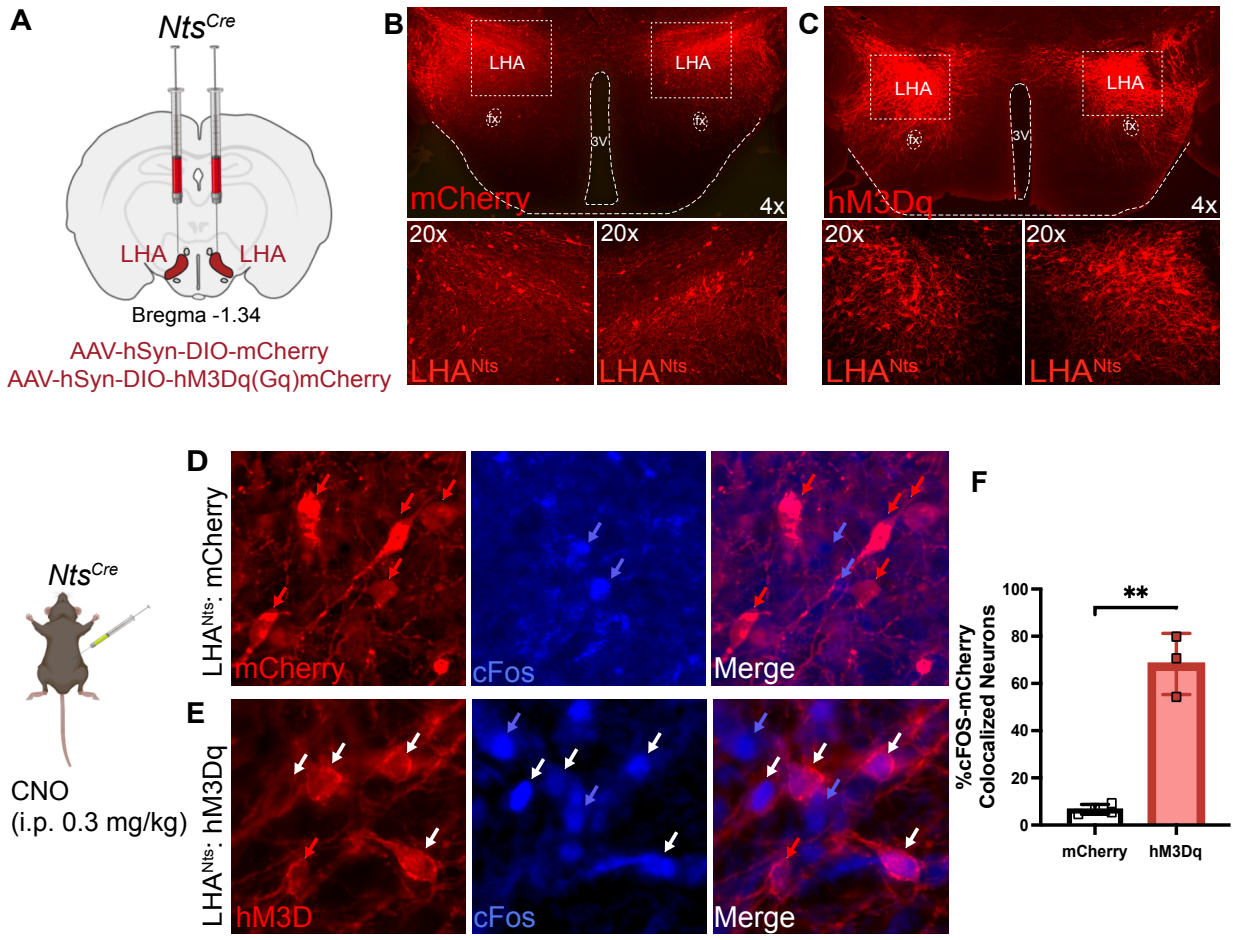

Figure 2.1 Chemogenetic Stimulation of LHA^{Nts} Neurons. A) 12 week-old male and female *Nts^{Cre}* mice were bilaterally injected in the LHA with either pAAV-hSyn-DIO- mCherry to express mCherry (LHA^{Nts}:mCherry Control mice) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). B, C) mCherry fluorescence (red) in the LHA of B) LHA^{Nts}:mCherry control mice and C) LHA^{Nts}:hM3Dq mice. Top row: 4x magnification images to show AAV injection sites. Bottom row: 20x magnification images showing mCherry-expressing soma. Images representative of 6 mice per group. D-F) Immunoreactivity for mCherry (red) and cFOS (a marker of neuronal depolarization, blue) in the LHA of D) LHA^{Nts}:mCherry control mice (n=4) and E) LHA^{Nts}:hM3Dq mice (n=3) treated with CNO 90 min before brain collection. Red arrows indicate Nts neurons that only express mCherry, blue arrows indicate neurons that only express cFOS-only neurons (middle) and white arrows indicate neurons that co-express mCherry and cFOS. Scale bars = 200µm. F) Average percentage of LHA mCherry neurons co-expressing cFOS, error bars indicate SEM. **p < 0.05 via unpaired t-test with Welch's correction.

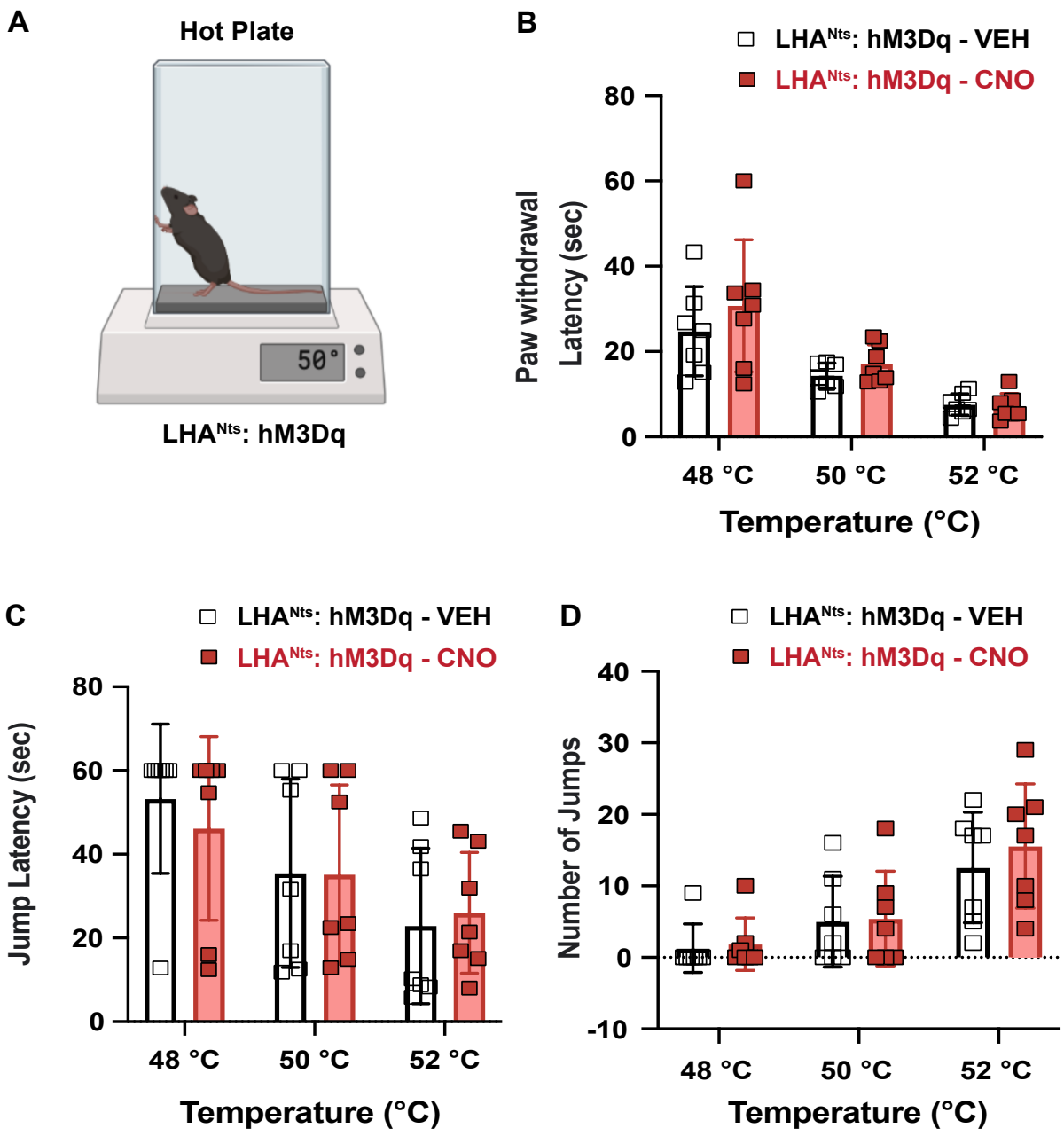

Figure 2.2 Chemogenetic Stimulation of LHA^{Nts} Neurons Does not Regulate Thermal Pain in Naïve Mice. Adult *Nts^{Cre}* mice were injected in the LHA with pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice) and assessed for thermal pain responses via hot plate test (n = 7). A) Schematic of hot plate test. Mice were placed on hot plate 30 min after i.p. treatment with VEH (control) or CNO (to activate LHA^{Nts} neurons). B-D) Paw withdrawal latency (B), jump latency (C) and number of jumps (D) were measured at 48℃, 50℃, and 52℃ temperature. No statistically significant differences were found between treatments as analyzed via two-way ANOVA with Sidak post-tests.

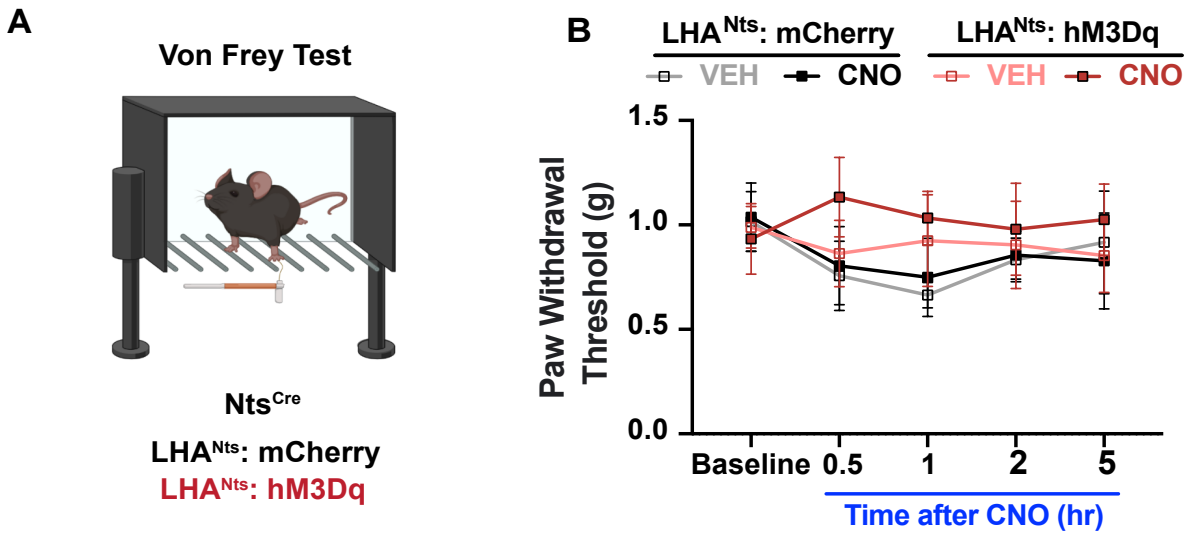

Figure 2.3 Chemogenetic Stimulation of LHA^{Nts} Neurons Does not Alter Mechanical Sensitivity in Naïve Mice. Adult *Nts^{Cre}* naïve mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). A) Mice were assessed for mechanical sensitivity via von Frey filament test. Mice received i.p. VEH/CNO injection prior to test. B) Paw withdrawal threshold in LHA^{Nts}:mCherry mice (black, n=4) and LHA^{Nts}:hM3Dq mice (red, n=5) before (baseline) and after treatment with VEH and CNO. Error bars indicate SEM. No significant differences between groups as analyzed by Two-Way ANOVA with Tukey post-test.

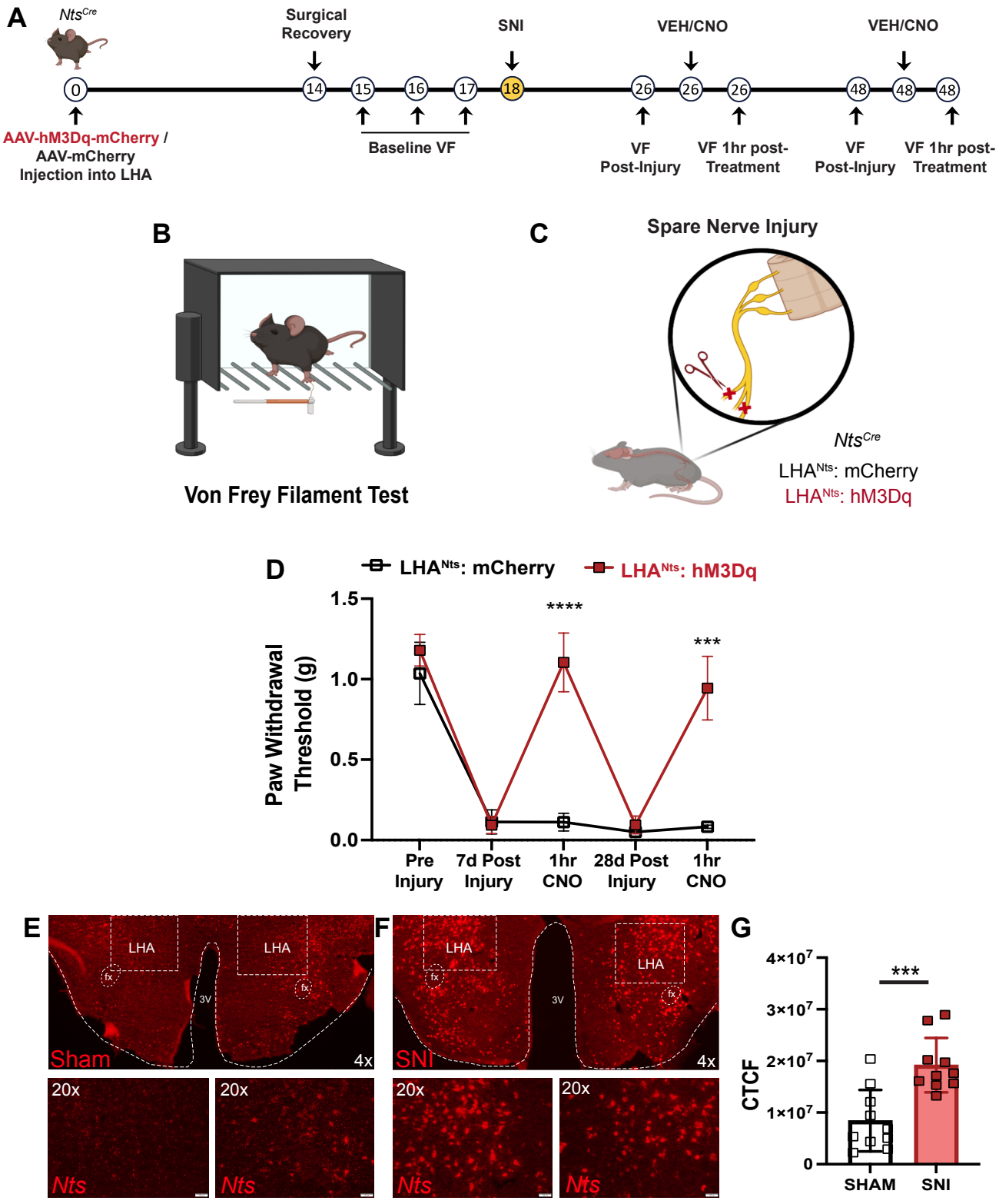

Figure 2.4 Chemogenetic Stimulation of LHA^{Nts} Neurons Attenuates Persistent Neuropathic Pain-Induced Mechanical Hypersensitivity. A) Adult *Nts^{Cre}* naïve mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry) or pAAV-hSyn-DIO-

Figure 2.4 (cont'd)
hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). A) Timeline of experiment, numbers indicate days. B) Mechanical pain sensitivity was measured via von Frey filament test in naïve mice. C) Mice underwent spared nerve injury (SNI) surgery to invoke neuropathic pain. D) Paw withdrawal threshold in LHA^{Nts}:mCherry (black, n=6) or LHA^{Nts}:hM3Dq mice (red, n=7) pre- and post-injury. Mice were assessed after 1 hour of CNO treatment on Day 7, and 28 post nerve injury via Von Frey filament test. ***p<0.001, ****p<0.0001 via Two-Way ANOVA with Sidak post-tests. E-G) *Nts* mRNA expression in the LHA of E) sham nerve-injured mice (n=10) and F) SNI mice (n=10). Top images: 4x magnification. Bottom: digital magnification of boxed regions from top row, white arrows indicate LHA neurons expressing *Nts*. fx = fornix. To quantify *Nts* expression, Corrected Total Cell Fluorescence (CTCF) was measured in the LHA of sham and SNI mice G) SNI significantly increased *Nts* expression in LHA neurons as compared to sham injury. Graph represents mean ± SEM. ***p<0.001 via unpaired Student's t-test.

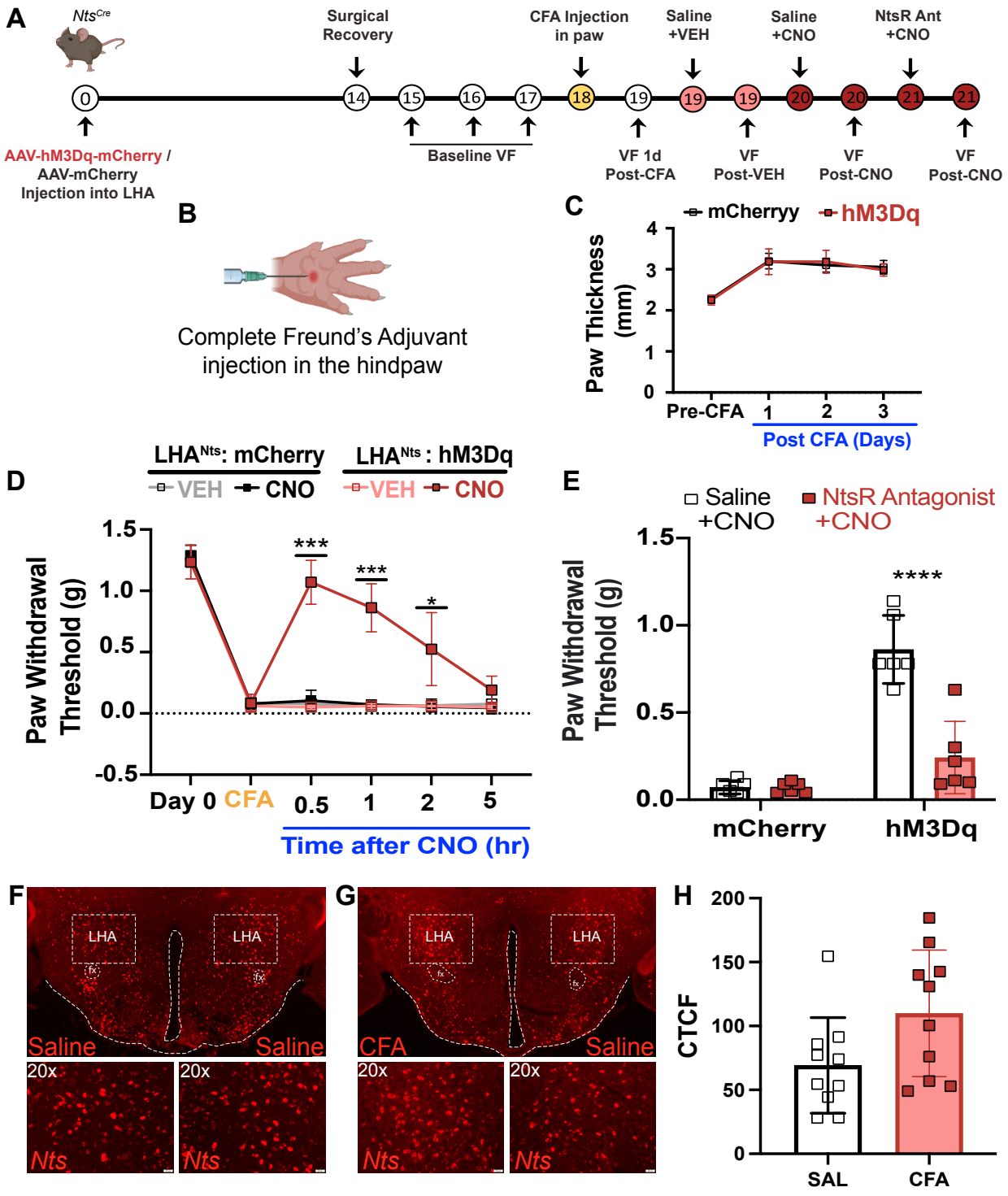

Figure 2.5. Chemogenetic Stimulation of LHA^{Nts} neurons Attenuates Inflammatory Pain-Induced Mechanical Hypersensitivity Via Nts Signaling. Adult *Nts^{Cre}* naïve mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). A) Timeline of experiment in days. B) Mice were injected with Complete Freund's Adjuvant

Figure 2.5 (cont'd)
(CFA) in the hind paw to induce inflammation. C) Paw thickness was measured before and after CFA injection. D) von Frey filament test to measure paw withdrawal threshold before CFA injection (day 0), 24 hr after CFA (CFA in yellow), and 0.5, 1, 2, or 5 hr after treatment with VEH or CNO to stimulate LHA^{Nts} neurons in LHA^{Nts}:mCherry (gray/black) and LHA^{Nts}:hM3Dq mice (pink/red) mice. CNO has an analgesic effect in hM3Dq-mCherry mice but not Controls. Graphs represent mean ± SEM. *p<0.05, ***p<0.001, ****p<0.0001, Two-Way ANOVA with Tukey's multiple comparisons test. E) Pretreatment with Saline or NtsR pan-antagonist SR142948 (red) 1 hr prior to CNO treatment. ****p<0.0001, Two-Way ANOVA with Sidak's multiple comparisons test. F, G) Representative images from wild type mice injected with F) Saline or G) CFA in hind paws (5 males, 5 females per treatment) and assessed for *Nts* mRNA expression (red) via RNA-Scope. Top images: 4x magnification. Bottom: digital magnification of boxed regions from top row. fx = fornix. H) Quantification of *Nts* mRNA from mice in F, G) via Corrected Total Cell Fluorescence (CTCF). Grubbs outlier test identified an outlier in the Saline-treated group, which was removed. Graphed data represent mean ± SEM, Saline n=9, CFA n=10, *p<0.01, unpaired Student's t-test.

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APPENDIX

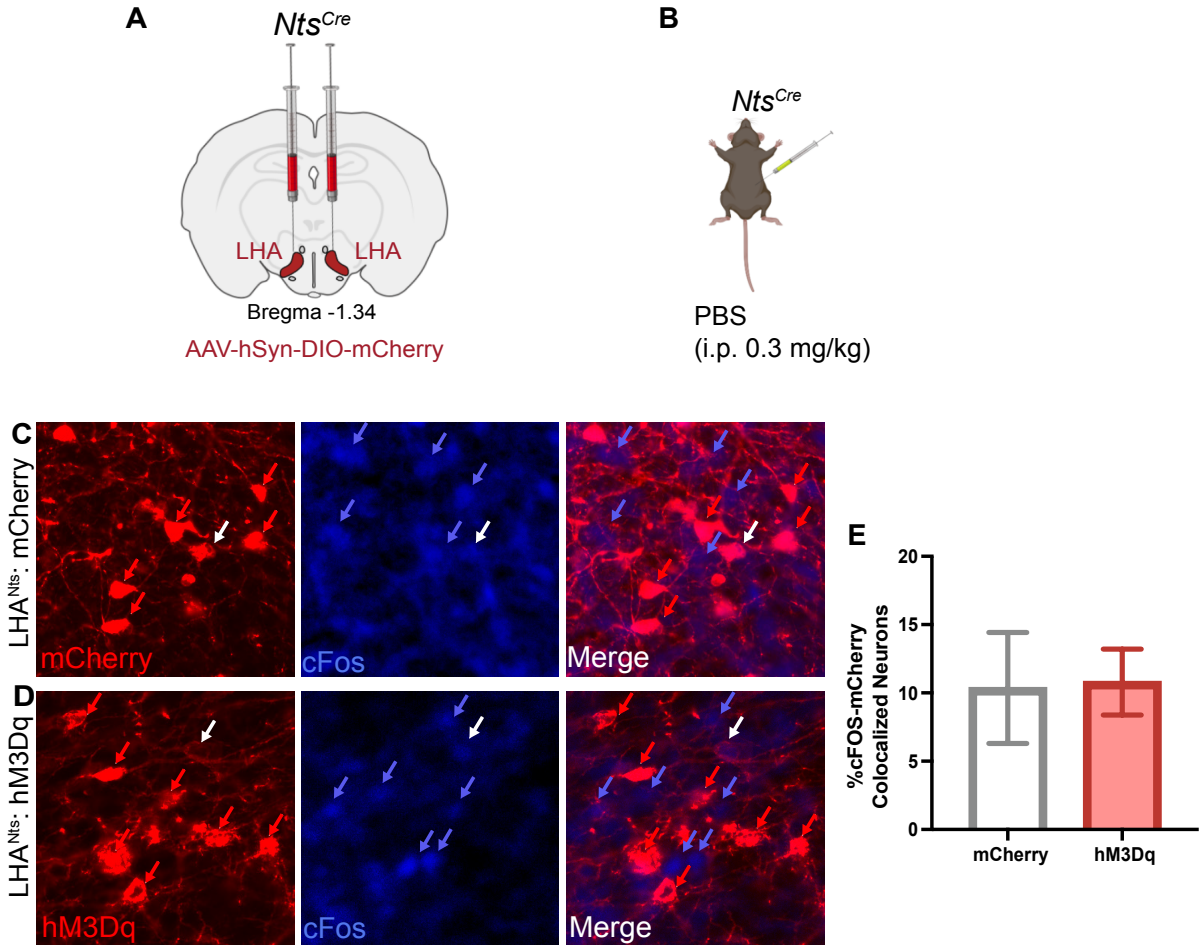

Figure 2.6. VEH Control Treatment Does Not Stimulate h3MDq-Expressing LHA^{Nts} Neurons. A) 12 week-old male and female *Nts^{Cre}* mice were bilaterally injected in the LHA with pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry Control mice) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). B) Mice were treated with VEH (PBS) 90 min before brain collection. C-D) Immunoreactivity for mCherry (red) and cFOS (marker of neuronal depolarization, blue) in the LHA of C) LHA^{Nts}:mCherry control mice (n=3) and D) LHA^{Nts}:hM3Dq mice (n=3) treated with PBS. Blue arrows indicate neurons that only express cFOS-only neurons and white arrows indicate neurons that co-express mCherry and cFOS. E) Average percentage of LHA neurons co-expressing mCherry and cFOS, error bars indicate SEM. No significant differences between groups as analyzed by unpaired t-test with Welch's correction.

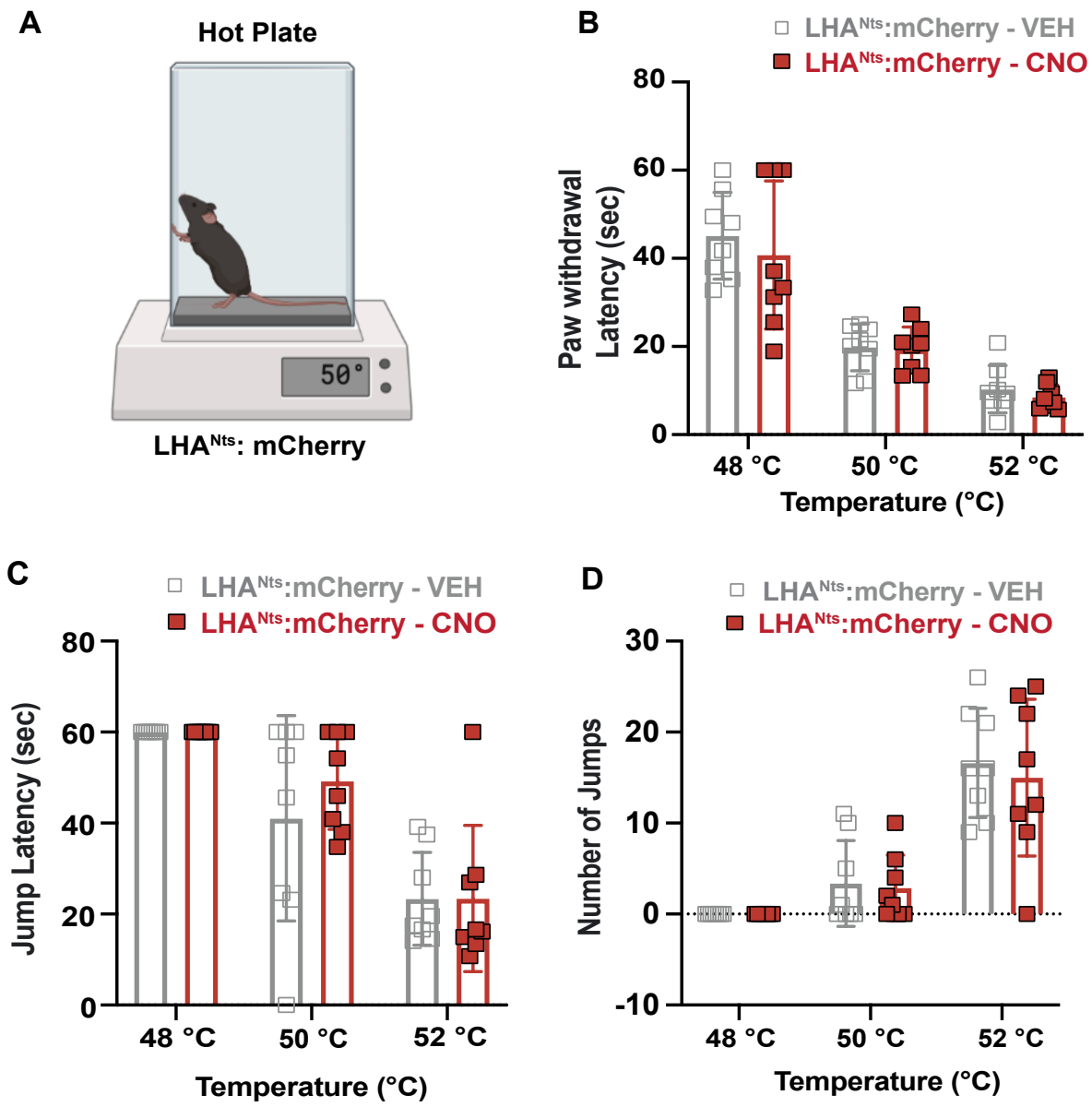

Figure 2.7 Treatment with VEH Control Does Not Alter Thermal Pain in Control and DREADDq Mice. Adult NtsCre mice were injected in the LHA with pAAV-hSyn-DIO-hM3Dq (Gq)-mCherry to express mCherry in LHANts neurons (LHANts:mCherry mice, n=8) and assessed for thermal pain responses via hot plate test. A) Schematic of hot plate test. Mice were placed on hot plate 30 min after i.p. treatment with VEH (control, PBS) or CNO. B-D) Neither VEH or CNO has any effect on paw withdrawal latency (B), jump latency (C) and number of jumps (D) measured at 48℃, 50℃, and 52℃ in these control mice lacking the h3MDq receptor. No statistically significant differences between treatments were found via two-way ANOVA with Sidak post-tests.

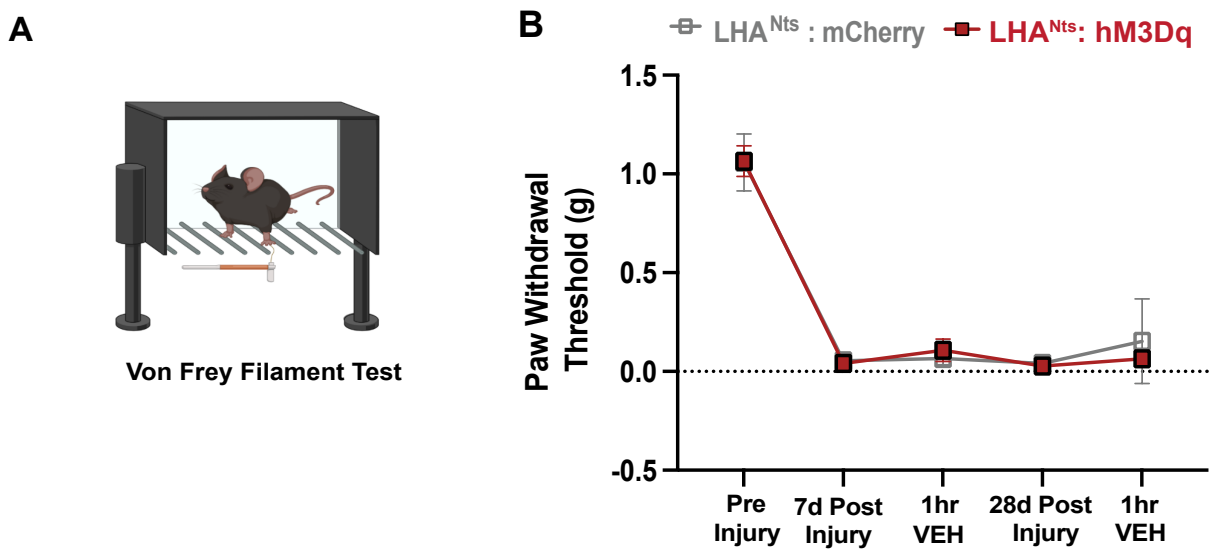

Figure 2.8 Treatment with VEH Control Does Not Alter Persistent Neuropathic Pain-Associated Behavior in Control and hM3Dq Mice. A) Adult NtsCre naïve mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHANts:mCherry) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHANts neurons (LHANts:hM3Dq mice). A) Mechanical pain sensitivity was measured via von Frey filament test in mice before and after spared nerve injury (SNI) surgery. B) Paw withdrawal threshold in LHANts:mCherry (Grey, n=6) or LHANts:hM3Dq mice (red, n=7) pre- and post-injury, and after 1 hour of VEH treatment on day 7- and 29-post SNI. No significant differences identified between VEH-treated LHANts:mCherry or LHANts:hM3Dq mice, analyzed via Two-Way ANOVA with Sidak post-test..

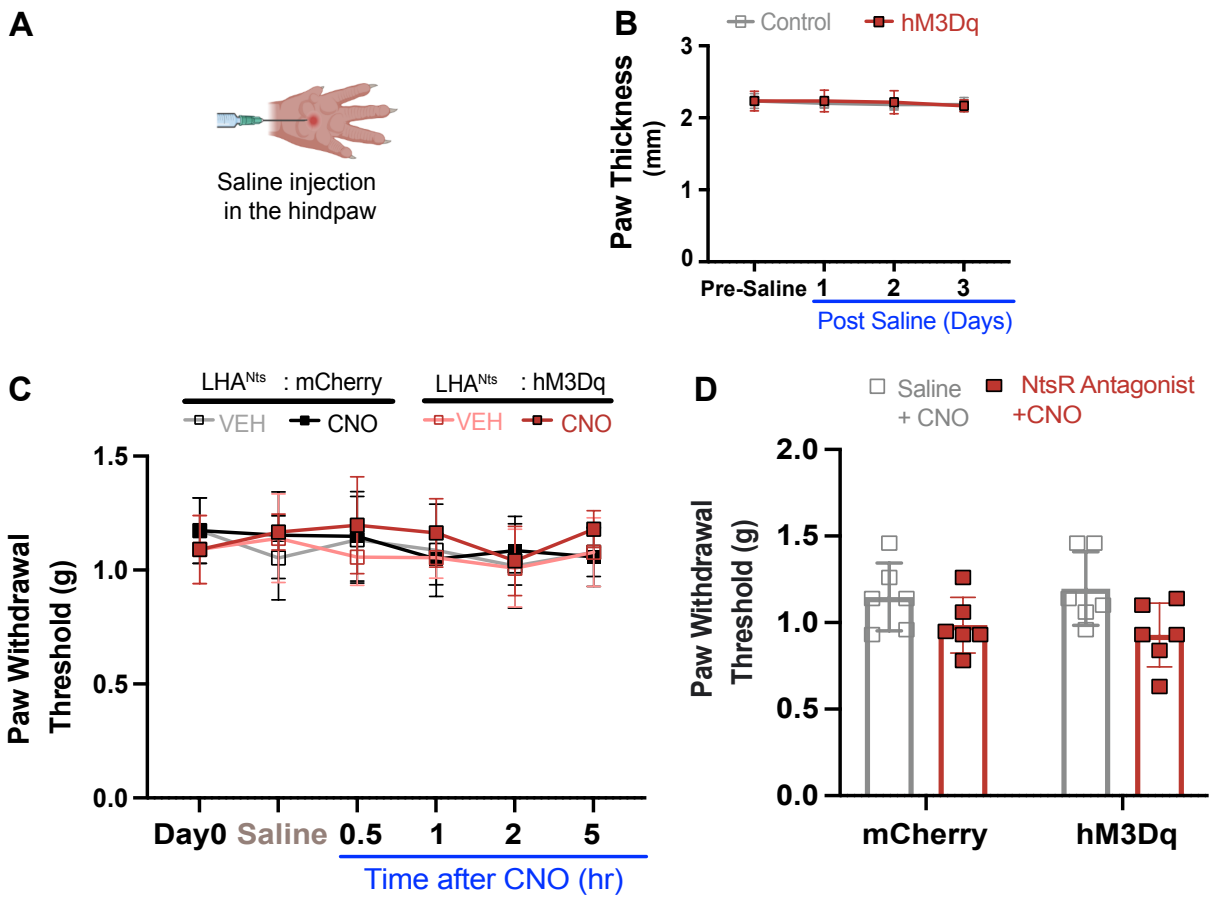

Figure 2.9 Control Saline Injection in the Hind Paw Does Not Induce Inflammatory Pain. Adult *Nts*^{Cre} naïve mice were injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice), then A) were injected with saline in the hind paw as control. B) Paw thickness measured before and after saline injection. C) von Frey filament test to measure paw withdrawal threshold before saline injection (day 0), 24 hr after saline, and 0.5, 1, 2, or 5 hr after treatment with VEH or CNO to stimulate LHA^{Nts} neurons in LHA^{Nts}:mCherry (gray/black) and LHA^{Nts}:hM3Dq mice (pink/red) mice. Injection with saline did not alter mechanical sensitivity in LHA^{Nts}:hM3Dq or in LHA^{Nts}:mCherry mice. Graphs represent mean ± SEM. Data analyzed via Two-Way ANOVA with Tukey's multiple comparisons test. D) Pretreatment with Saline or NtsR pan-antagonist SR142948 (red) 1 hr prior to CNO has no effect on paw withdrawal threshold. Data analyzed via-Way ANOVA with Sidak's multiple comparisons test.

CHAPTER 3: LATERAL HYPOTHALAMIC AREA NEURONS EXPRESSING NEUROTENSIN ALLEVIATE COMORBID OBESITY-PAIN

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ABSTRACT

Chronic pain and obesity frequently occur together. An ideal therapy would alleviate pain without weight gain, and most optimally, could promote weight loss. The neuropeptide neurotensin (Nts) is implicated in reducing weight and pain, but the endogenous mechanisms underlying this physiology were unknown. We previously showed that activating lateral hypothalamic area neurons expressing Nts (LHA^{Nts} neurons) suppresses feeding and promotes weight loss. Here we hypothesized that activating LHA^{Nts} neurons can also alleviate comorbid obesity pain. To test this, we injected normal weight and diet-induced obese (DIO) Nts^{Cre} mice in the LHA with AAVs to Cre-dependently express either mCherry (Control) or excitatory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in LHA^{Nts} neurons, permitting their activation after treatment with the DREADD ligand clozapine N-oxide (CNO, 0.3mg/kg, *i.p.*). Activating LHA^{Nts} neurons had no effect on thermal pain responses in DIO mice. By contrast, obesity-induced pain hypersensitivity was completely reversed by CNO-mediated activation of LHA^{Nts} neurons compared to VEH control. In DIO mice treated with complete Freund's adjuvant (which induces inflammatory pain), activating LHA^{Nts} neurons also relieved pain hypersensitivity. However, pretreatment with the brain permeable Nts receptor pan-antagonist SR142948 (1mg/kg, i.p, 30 min before VEH/CNO) blocked CNO-mediated analgesia, indicating that LHA^{Nts} neurons alleviate chronic pain in an Nts-dependent manner. Furthermore, Nts deletion from the LHA by injecting AAV-Cre into the LHA of $Nts^{flox/flox}$ mice further exacerbated hyperalgesia in DIO

mice compare to normal weight mice. Taken together these data suggest that augmenting signaling via LHA^{Nts} neurons may be a common actionable target to treat comorbid obesity pain.

3.1 INTRODUCTION

Chronic pain and obesity are common and expensive health issues that frequently coincide[1,2]. About a third of Americans are obese, putting them at a higher risk of developing chronic pain[1-6]. Studies with animals have shown that consuming a high-fat diet and being obese can lead to increased sensitivity to pain[7-9]. Although obesity and pain are comorbid, they have largely been considered as independent problems and treated separately. Notably, diet-induced weight loss and exercise can stabilize pain[10,11] but sustaining weight reduction is challenging, and even with maintained diet and exercise most individuals regain lost weight[12]. Despite the growing rates of obesity, there are few effective medications to help people sustain weight loss, which is crucial for preventing or alleviating pain[13-15]. On the flip side, the presence of obesity complicates pain treatment. Non-opioid pain medications induce weight gain[16], and opioids may do more harm than good[17,18] and lead to dependence, making them less than ideal for those dealing with both obesity and pain. Consequently, managing both conditions simultaneously remains a significant challenge, owing to our limited understanding of the neurobiology underlying obesity and pain, which hinders the development of effective treatment strategies necessary for improving and saving lives.

The majority of current research exploring the connection between obesity and pain concentrates on peripheral mechanisms, such as the function of primary sensory neurons. The neurotensin (Nts) peptide is present both in the periphery and centrally[19] and has been shown to act in both sites to reduce pain[20-22]. However, peripherally-produced Nts promotes weight

gain[23,24], whereas Nts produced in the brain acts locally to suppress appetite and promote weight loss[25-28]. We have focused on the central Nts system because it is where Nts can simultaneously regulate pain relief and weight reduction, which is essential for addressing the concurrent issues of pain and obesity.

A significant source of central Nts originates from a population of Nts-producing neurons situated in the lateral hypothalamic area (LHA) of the brain, referred to as LHA^{Nts} neurons. While the physiologic roles LHA^{Nts} neurons and the Nts released from them are just beginning to be appreciated[29.30], prior research from our lab has revealed their important role in regulating body weight. Loss of LHA^{Nts} neurons causes obesity but activating LHA^{Nts} neurons suppresses feeding and promotes weight loss, without invoking any of the adverse consequences associated with central or systemic Nts treatment[25]. Additionally, our findings in Chapter 2 show that activating LHA^{Nts} neurons alleviates severe pain in animal models of neuropathic and inflammatory pain. Together, these data suggest that LHA^{Nts} neurons could be a promising target for simultaneously addressing obesity and pain. Indeed, the LHA has also long been recognized as an important brain region for modulating feeding[31-33] and body weight and responses to noxious stimuli[34-36]. Electrically stimulating the entire LHA in rodents invokes feeding[37], and stimulating LHA neurons chemically[38] or electrically[36] provides pain relief in both acute and chronic pain conditions[36,39,40]. However, the LHA is very diverse, containing multiple subsets of molecularly defined neuronal populations that mediate distinct, and sometimes opposing, aspects of physiology. As a result, bulk stimulation or ablation experiment obscured the roles of the distinct LHA subsets[41]. This is well characterized for feeding, where subsets of LHA neurons expressing the peptides hypocretin/orexin or melanin concentrating hormone promote feeding, whereas separate,

adjacent LHA^{Nts} neurons suppress feeding[42]. Orexin and melanin concentrating hormone-expressing LHA neurons have also been implicated in modulating pain and itch[43-45]. Intriguingly, stress has been shown to elevate Nts expression in the LHA, potentially contributing to stress-induced pain relief[46]. Yet, it remained unclear whether LHA^{Nts} neurons, and the Nts they release play a role in alleviating different types of pain, including obesity-pain. To test this, we selectively activated LHA^{Nts} neurons using chemogenetics in both normal and diet-induced obese (DIO) mice. We also blocked Nts receptors to determine the importance of Nts signaling in the pain-relieving effects. Our findings indicate that LHA^{Nts} neurons play a part in models of obesity-related pain hypersensitivity and this effect relies on intact Nts signaling. Thus, this work unveils an endogenous source of Nts within the brain that influences pain processing in obesity-related conditions.

3.2 RESEARCH DESIGN AND METHODS

3.2.1 Mice

All mice used in this study were bred and housed in 12 hours light/ 12 hours dark cycles and were cared for by Campus Animal Resources (CAR). We produced and studied male and female heterozygous *Nts*$^{Cre/+}$ [47], and homozygous *Nts*$^{flox/flox}$ mice on the C57/BI6J background. For experiments, 4- to 6-week-old *Nts*$^{Cre/+}$ mice were weaned onto 45% high-fat diet (HFD, Research Diets D12451) to develop diet induced obesity (DIO), and *Nts*$^{flox/flox}$ mice were either put on chow (Harlan Teklad # 7913) or 45% high-fat diet (HFD, Research Diets D12451) for the duration of experiment, unless specified otherwise. At 12 wks, mice from both groups were randomly assigned to either control or experimental groups with littermate, age-matched, and gender-matched controls. Following stereotaxic surgeries mice were individually housed with *ad libitum*

access to water and their respective diet for the duration of experiment, unless specified otherwise. Since we did not observe any metabolic or behavioral difference between males and females in our test, both sexes were pooled. All experimental protocols were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at Michigan State University and with the Association for Assessment and Accreditation of Laboratory Animal Care and the National Institutes of Health.

3.2.2 Surgical Procedures

At 12 wks, *Nts*^{Cre} mice were anesthetized with 2-4% isoflurane/oxygen mixture, fitted into a stereotaxic apparatus, and given Meloxicam (5 mg/kg, i.p.) prior to using a scalpel to expose the skull. Small holes were drilled in each hemisphere of the skull to permit injection of either AAV2-hSyn-DIO-hM3Dq (Gq)-mCherry (excitatory DREADDq, Addgene, 44361-AAV2) to express DREADDq-mCheryy in LHA^{Nts} neurons or AAV2-hSyn-DIO-mCherry (control, Addgene, 50459-AAV2) as controls into the LHA (300nL per side, bregma: - 1.34 mm, midline: ±0.95 mm, skull surface: -5.25 mm) per the Franklin and Paxinos brain atlas. The injector was left in place for 10 minutes to ensure absorption of the AAVs, then after retracting the hole in the skull was filled with bone wax and the incision was closed.

Neurotensin Conditional Knock-out Mice: *Nts*$^{flox/flox}$ mice were injected at ~8wks of age in the LHA (bilaterally) with either rAAV2-hSyn-eGFP (control) or rAAV2-hSyn-GFP-Cre to generate LHA$^{Nts\text{-}GFP}$, and LHA$^{Nts\text{-}KO}$ mice, respectively. LHA$^{Nts\text{-}KO}$ mice thus have an adult-onset deletion of Nts specifically from LHA neurons.

3.2.3 Treatments

The hM3Dq DREADD-ligand clozapine N-oxide (CNO, 0.3mg/kg) or vehicle (VEH, in this case phosphate buffered saline) were administered to mice via *i.p.* injection. CNO (Sigma Aldrich, Cat # C0832) was dissolved in 10% β-cyclodextrin (Sigma Aldrich, Cat # C0926) in sterile PBS to make 20X CNO stock aliquots (1.2mg/ml). A 20X aliquot was diluted with sterile PBS just prior to the experiment to make the 1X working solution (60µg/mL). We then administered 5µL of 1X CNO per gram of body weight. Unless, specified otherwise, mice were treated once 30 mins prior to pain testing, 1- 2 hrs after onset of light cycle, via a crossover design. Thus, all mice received both VEH and CNO on different days.

For experiments examining the involvement of Nts signaling in pain regulation, mice from hM3Dq DREADD and mCherry control group were randomized into 4 separate treatment groups. 90 mins before each behavioral test, mice received *i.p.* pretreatment with either Saline or the NtsR pan-antagonist, SR142948 (1mg/kg) that has been reported to block signaling via NtsR1 and NtsR2[48,49], followed 30 min later by VEH or CNO (to activate LHA^{Nts} neurons). SR142948 (TOCRIS Bioscience, 2309/1) was dissolved in 0.1% Tween 80 in sterile PBS to make the 20x stock solution (2mg/ml), which was diluted with sterile PBS prior to the experiment to make the 1x working solution (0.1mg/ml). We administered 10µL of 1X SR142948 per gram of body weight.

3.2.4 Perfusion and Immunofluorescence

After completion of all behavioral experiments, mice received *i.p.* injection of VEH or CNO 90 minutes before perfusion. Mice were then given a lethal *i.p.* dose of pentobarbital sodium (Fatal Plus C II, Vortech, SKU # 035946), and transcardially perfused with 1x PBS (pH 7.4) followed by 10% neutral buffered formalin (Epredia, Cat # 5725). Whole brains were extracted and post-

fixed in 10% formalin overnight at 4°C then transferred to 30% sucrose in 1x PBS for storage at 4°C until further processing.

For immunostaining, brains were coronally sectioned (30µm thick) using a Leica SM2010R microtome (Leica Biosystems) and collected in 1% formalin/PBS solution. For cFOS immunohistochemistry, and detection of bilateral DREADD injections, brain sections were blocked with 3% normal donkey serum in 1x PBS with 0.3% Triton X for an hour followed by incubation in goat anti-cFOS primary antibody (1:1000, Santa Cruz Biotechnology, sc-52-G). The following day the sections were washed with PBS prior to incubation with secondary antibodies conjugated to donkey anti-goat biotin (1:100, Jackson ImmunoResearch, Cat # 705-065-147), then the immunolabeled cFOS was visualized using a DAB peroxidase substrate kit per the manufacturer's protocol (Thermo Scientific, Cat # 34065). Sections were then washed in PBS prior to overnight incubation at room temperature with rabbit-dsRed/mCherry (1:1000, Takara, 632496). The next day the sections were rinsed and incubated for 2 hr in donkey anti-rabbit Alexa-568 (1:200, Invitrogen, AB10042), followed by washes and 1 hr incubation with donkey anti-rabbit Alexa-568 (1:200, Invitrogen, AB10042) to visualize DREADD-mCherry-expressing LHA^{Nts} neurons. Brain sections were mounted on slides for analysis using an Olympus BX53 fluorescence microscope. LHA images were obtained at 4x and 20x resolution via Cell Sens software. Images were analyzed using Photoshop (Adobe) to assess co-localization and count cells. For DAB-labeled cFOS-expressing cells, images were taken with transmitted light and later pseudo-colored blue for ease of visualization and counting using Adobe Photoshop.

mCherry expression was used for posthoc evaluation of AAV targeting in the LHA. Mice were only included in the final data set if mCherry-expressing soma were bilaterally confined within the LHA. Mice with mCherry soma located outside of the LHA were excluded, as were mice found to only have unilateral LHA targeting.

3.2.5 Thermal Nociception (Hot Plate Test)

A cylindrical plexiglass enclosure was placed on a hot plate (Ugo Basile, Hot/Cold plate). DIO mice were *i.p.* injected with VEH or CNO prior to thermal pain testing. After 30 mins, they were gently placed on the hot plate and their first lick latency, and jump latency were measures at 48°C, 50°C, and 52°C. A latency of 60 secs was used as a cutoff time and mice were removed from the hot plate after cutoff time to avoid tissue damage.

3.2.6 Mechanical Nociception (von Frey Test)

Mice were acclimatized for 30 mins in a clear plexiglass enclosure (10×10×13 cm3) on a wire mesh grid floor. Von Frey filaments (Aesthesio, Cat # 514000-20C) ranging from 0.008 g – 2.0 g were used to measure mechanical allodynia, which was defined as the hind paw withdrawal response to von Frey hair stimulation by a blinded investigator using an “up and down” method, as described previously[50,51]. Nociception responses were recorded when mice exhibited paw withdrawal, licking, and shaking upon filament application. Briefly, starting with the 0.4 g von Frey filament, pressure was applied perpendicular to the midplantar surface of the hind paw. Filament strength was increased until there was a paw withdrawal response or decreased until mice no longer exhibited a response.

3.2.7 Inflammatory Pain

To induce inflammatory pain, mice were briefly anesthetized under 2% isoflurane and paw thickness was recorded using vernier caliper. Mice then received trans-plantar injection of 5µl CFA (Sigma Aldrich Cat # F5881) in one hind paw and saline injection (control) in the other hind paw. Mice were returned to their home cages and inflammation was confirmed by redness and increased paw thickness due to swelling 24 hours after CFA administration. Pain testing was conducted between 2-, and 6-days post-CFA injection.

3.2.8 Conditioned Place Preference (CPP)

Single-housed DIO male and female *Nts*^{Cre} mice expressing excitatory DREADDs (n=6) or control mCherry (n=6) were tested in CPP boxes (San Diego Instruments) consisting of two compartments with different visual and tactile cues separated by a narrow central intermediate area. On the pre-conditioning day DIO mice were allowed to explore the box freely for 20 min, and the difference between the time spent in both compartments was calculated. According to pre-conditioning data, 8 out of 12 mice showed strong bias for one side over the other. Bias is indicated by greater than 25% difference between time spent in each compartment. Taking pre-existing preference in account, we used a biased pairing method. In this method, we paired CNO with less preferred side and VEH treatment was pair with more preferred side. Mice were then conditioned for 4 consecutive days. In the morning they were given *i.p.* injection of VEH 30 mins prior to placing them in the VEH-paired chamber and were placed in CNO-paired compartment after CNO injection in the afternoon. While conditioning, mice were only allowed to stay in the compartment paired with the respective treatment for 30 mins. After each session, mice were returned to their home cage with *ad libitum* food and water. On post-conditioning day (test day),

mice were placed into the intermediate chamber with access to both main compartments of the chamber and were allowed to explore freely for 20 mins. Time-spent and locomotor activity in both VEH- and CNO-paired chambers were recorded by the manufacturer's software, and compared with time spent during pre-conditioning.

3.2.9 RNAscope

Three LHA- containing sections (30µm thickness) were picked per mouse brain, as per[65]. RNAScope single-plex assay (Advanced Cell Diagnostics, catalog # 322360) was performed using manufacturer's protocol. Free floating sections were washed with 1x PBS, and incubated in Pretreatment I (RNAScope H_2O_2, Advanced Cell Diagnostics, Cat # 322335) at RT until bubbling stopped (45-60 min). Slices were then washed with 0.5x PBS then were carefully mounted on positively charged slides (Superfrost Plus Microscope slide, Cat # 12-550-15). Once mounted, sections were washed with dH_2O and dried overnight at 60°C in HybEZ II oven. Dried sections were incubated in 1x Pretreatment II (Target Retrieval Agent) at 99–104°C for 5-10 min, and then washed with dH_2O, dried at RT followed by 100% EtOH wash. Dried sections were then incubated in Pretreatment III (RNAScope Protease Plus, Advanced Cell Diagnostics, catalog # 322331) for 15 min at 40°C in HybEZ II oven and then washed with dH_2O. Sections were incubated with *Nts* probe (Advanced Cell Diagnostics, catalog # 420441) for 2 hrs at 40°C. For target-specific hybridization, sections were incubated in Amp1-6, followed by application of Fast-Red-A and Red-B for 10 mins for visualization. Sections were washed with dH_2O then dried by briefly dipping in xylene and cover slipped using antifade mounting media.

For quantitative analysis of RNA after RNAScope, 10x LHA images were analyzed by a blinded investigator using FIJI ImageJ. Corrected Total Cell Fluorescence (CTCF) was calculated by

taking the integrated density (IntDen) of the region of interest (ROI) and subtracting from it the background mean (BGMean) times the area of the ROI. The formula used for this calculation is CFCF = IntDen-Area x BGMean.

3.2.10 Statistics

Student's t-tests and 2-way ANOVA were calculated using Prism 7 (GraphPad). Repeated measures 2-way ANOVA with Sidak's multiple comparisons test was used when each mouse received both VEH and CNO, and when data from the same mice were collected at different time points. Ordinary 2-way ANOVA with Tukey post-test was used if mice did not receive both treatments. A p-value of <0.05 was considered statistically significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.3 RESULTS

3.3.1 Chemogenetic Stimulation of LHA^{Nts} Neurons in Diet Induced Obese Mice

Diet induced obese (DIO) Nts^{Cre} mice were injected bilaterally in the LHA with Cre-inducible AAV-hSyn-DIO-mCherry (control) or AAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express the excitatory DREADD hM3Dq in LHA^{Nts} neurons (Fig. 3.1A). Posthoc analysis for mCherry fluorescence confirmed LHA targeting and expression of mCherry in LHA soma and fibers of control mice (Fig. 3.1B) and hM3Dq-mCherry expressing soma and processes in experimental mice (Fig. 3.1C). To confirm CNO-mediated stimulation of only hM3Dq-expressing LHA^{Nts} neurons mice from both control and experimental groups were treated with VEH/CNO 90 min prior to perfusion, then brains were analyzed for mCherry/mCherry-hM3Dq (red) and cFOS (a marker of neuronal stimulation, blue). Minimal cFOS was present in the LHA of VEH treated mice (data not shown). Few neurons with co-localized cFOS and mCherry were observed in the LHA of LHA^{Nts}:mCherry

control mice (Fig. 3.1D, white arrows). However, in LHA^{Nts}:hM3Dq mice CNO treatment increased cFOS expression in hM3Dq-mCherry-expressing LHA^{Nts} neurons (Fig. 3.1E, white arrows). Together, these data confirm that CNO treatment selectively activates hM3Dq-mCherry-expressing LHA^{Nts} neurons in DIO mice, and henceforth we used this chemogenetic method to determine whether activating them impacted nociception in obese animals.

3.3.2 Chemogenetic Stimulation of LHA^{Nts} Neurons Does not Regulate Thermal Pain in Naïve DIO Mice

In Chapter 2 we showed that LHA^{Nts} neurons do not regulate thermal pain in normal weight healthy mice. Here we asked whether LHA^{Nts} neurons stimulation can alter thermal pain responses in the context of obesity. *Nts^{Cre}* mice were bilaterally injected in the LHA with Cre-recombinase dependent AAVs to express the fluorophore mCherry (Controls) or hM3Dq (DREADDq-expressing) then were provided *ad libitum* 45% HFD for 12 weeks to induce obesity (as in Fig. 3.1A). DIO mice were treated with VEH or CNO prior to hot plate testing at 48°C, 50°C and 52°C (Fig. 3.2A). Thermal responses were similar between female and male mice indicating no underlying sex differences (data not shown) so data from each sex were pooled for analysis. In the LHA^{Nts}:hM3Dq group, CNO-mediated stimulation of LHA^{Nts} neurons did not alter lick latency (Fig. 3.2B) or jump latency (Fig. 3.2C) in response to any of the tested temperatures. Neither was any change in thermal pain-associated behaviors observed in CNO-treated LHA^{Nts}:mCherry control mice as compared to VEH treatment, confirming the absence of any off-target effect of CNO on thermal nociception (Fig 3.2D, E). These data suggest that stimulation of LHA^{Nts} neurons does not regulate acute thermal nociception in naïve DIO mice.

3.3.3 Chemogenetic Stimulation of LHA^{Nts} Neurons Alleviates Obesity-Induced Pain via Nts Signaling

We next examined whether activating LHA^{Nts} neurons can address obesity-induced pain itself. Many groups have demonstrated that high fat diet (HFD) produces diet-induced obesity, pain hypersensitivity, and peripheral neuropathy in wild type rodents, similar to humans[53,54]; this confirms that they can be used to model the comorbid obesity-pain disease state. Since induction of obesity can vary by mouse strain, and we require *Nts^{Cre}* mice and chemogenetics to selectively modulate LHA^{Nts} neurons, it was important to verify that HFD-induced obese *Nts^{Cre}* mice exhibit pain hypersensitivity using the von Frey test (Fig 3.3A). To test this we provided *Nts^{Cre}* mice with *ad libitum* 45% HFD to induce obesity (DIO) then injected them in the LHA with AAVs to prepare LHA^{Nts}:mCherry control and LHA^{Nts}:hM3Dq groups as described previously (Fig 3.3B). As expected, 45% HFD-fed DIO *Nts^{Cre}* mice weighed significantly more than chow-fed *Nts^{Cre}* mice (Fig 3.3C). Additionally, von Frey testing verified that DIO *Nts^{Cre}* mice develop pain hypersensitivity compared to chow-fed counterparts (Fig 3.3D). We next tested whether activating LHA^{Nts} neurons alters mechanical sensitivity thresholds in obese mice. We found that DIO LHA^{Nts}:mCherry control and LHA^{Nts}:hM3Dq mice exhibit hypersensitivity indicated by reduced paw withdrawal threshold at baseline (Fig 3.3E, baseline). Mice were then treated with VEH or CNO prior to measuring paw withdrawal threshold. Neither VEH or CNO treatment had any effect on paw withdrawal threshold in DIO LHA^{Nts}:mCherry control mice lacking hM3Dq (Fig 3.3E, black and grey data points and lines). Neither did VEH-treated LHA^{Nts}:hM3Dq mice exhibit any improvement in paw withdrawal threshold from baseline. However, CNO-mediated stimulation of LHA^{Nts}:hM3Dq neurons in the

same mice completely reversed obesity-induced reduction in paw withdrawal threshold over 5 hours as compared to VEH over 5 hours (Fig. 3.3E, blue data points and line).

When activated, LHA^{Nts} neurons express, and release multiple neurotransmitters including the fast neurotransmitter GABA[55-57] and peptides such as galanin, corticotropin-releasing hormone (CRH), and Nts itself[57,58]. Any of these released signals might mediate the analgesic effect observed in obese mice after stimulation of LHA^{Nts} neurons (Fig 3.3E). To test whether Nts acting via Nts receptors is the mediating signal from LHA^{Nts} neurons that controls pain relief, we pretreated LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq DIO mice with either saline (control) or the brain permeable NtsR1 & NtsR2 pan-antagonist SR142948. Mice then received CNO to activate h3MDq-expressing LHA^{Nts} neurons (see the timeline of treatments in Fig 3.3B). LHA^{Nts}:mCherry mice had low paw withdrawal threshold that was not modified by Saline + CNO or NtsR Antagonist (SR142948) + CNO (Fig. 3.3F). In contrast, LHA^{Nts}:hM3Dq mice pretreated with Saline + CNO to activate LHA^{Nts} neurons increased their paw withdrawal threshold (Fig. 3.3F). However, pretreatment with NtsR antagonist followed by CNO treatment significantly reduced the paw withdrawal response (Fig. 3.2F). The lack of CNO-mediated antinociception or NtsR antagonist effects in LHA^{Nts}:mCherry control mice confirms the absence of off-target drug effects. These data indicate that chemogenetic-mediated stimulation of LHA^{Nts} neurons in obese mice mediates analgesia that depends on Nts released from them and acting via NtsRs to impact pain.

Genetically obese rodents have reduced *Nts* expression in the brain, which has been suggested may contribute to their development of obesity[59,60]. However, it is not clear if DIO similarly disrupts *Nts* expression. We assessed whether obesity-induced pain modulates *Nts* expression in the LHA of DIO wildtype mice compared to age- and gender-match normal weight

naïve mice. Mice from both groups were perfused and *Nts* expression was measured in the LHA via RNAscope. Chow-fed controls and DIO mice exhibit similar *Nts* expression in the LHA (Fig. 3.3G-I) with no discernable sex difference (data not shown). Taken together with Fig. 3.3E and Fig. 3.3F these data indicate that comorbid obesity-pain occurs despite intact *Nts* expression in the LHA. Moreover, activating the Nts-expressing LHA^{Nts} neurons is necessary to alleviate obesity pain in mice, which is due, at least in part, to Nts signaling via NtsR1 and/or NtsR2.

3.3.4 Chemogenetic Stimulation of LHA^{Nts} Neurons Attenuates Inflammatory Pain Hypersensitivity via Nts Signaling in Obese Mice

Individuals with obesity are also prone to develop inflammatory pain[61,62]. To test if activating LHA^{Nts} neurons can relieve inflammatory pain, we combined the well-established complete Freund's adjuvant (CFA)-inflammatory model with our obesity-pain model. We used the von Frey filament test to assess paw withdrawal threshold (as in Fig 3.4A) in DIO LHA^{Nts}:mCherry and LHA^{Nts}:hM3Dq mice that were injected with Saline in one hind paw, and complete Freund's Adjuvant (CFA) in another hind paw (Fig 3.4B). DIO mice exhibit mechanical hypersensitivity in the saline-injected paw before and after saline administration (Fig 3.4C, Day 0 vs. Saline). We reason that it is because of their existing obesity-induced pain at baseline. Control LHA^{Nts}:mCherry mice showed no change with CNO treatment, nor did VEH treatment alter mechanical hypersensitivity in the LHA^{Nts}:mCherry or LHA^{Nts}:h3MDq groups (Fig 3.4C). However, as in Figure 3.3C, CNO-mediated stimulation of LHA^{Nts} neurons in DIO-LHA^{Nts}:hM3Dq mice reversed reduced paw withdrawal threshold, which is indicative of pain relief of the DIO state. Furthermore, CNO-induced antinociception was reduced by pretreatment with NtsR antagonist

(SR142948) only in DIO-LHA^{Nts}:hM3Dq mice (Fig 3.4D), supporting our previous results that Nts signaling is involved in LHA^{Nts}-mediated analgesia.

CFA-induced inflammation of DIO LHA^{Nts}:mCherry and DIO LHA^{Nts}:hM3Dq mice, equivalently lowered their paw withdrawal threshold from pre-treatment baseline (Fig 3.4E, Day 0 vs. CFA). Excitingly, the reduction in paw withdrawal threshold was reversed by CNO-mediated stimulation of the hM3Dq-expressing LHA^{Nts} neurons for at least 2 hr, as compared to VEH-treated mice (Fig 3.4E). By contrast, CNO treatment had no analgesic effect on the DIO LHA^{Nts}:mCherry mice, which looked identical to VEH-treated mice. These data support that activating LHA^{Nts} neurons can mediate antinociception in response to inflammatory pain. However, pretreatment with NtsR antagonist SR142948 to block Nts signaling via NtsR1 and NtsR2 significantly blocked the antinociceptive effect of CNO-mediated stimulation of LHA^{Nts} neurons (Fig 3.4F). The lack of CNO-mediated antinociception or NtsR antagonist effects in LHA^{Nts}:mCherry control mice confirms the absence of off-target drug effects. In sum, these data indicate that chemogenetic-mediated stimulation of LHA^{Nts} neurons in obese mice is sufficient to reduce inflammatory pain, but the effect depends on Nts signaling via NtsR1 and/or NtsR2.

3.3.5 Stimulating LHA^{Nts} Neurons Does Not Elicit Reward-Like Behavior in Diet-Induced Obese Mice

Relief of pain is rewarding. We therefore asked if LHA^{Nts} neuron-mediated relief from obesity-induced pain is, in itself, rewarding using a 3-chamber conditioned place preference paradigm. DIO-LHA^{Nts}:mCherry and DIO-LHA^{Nts}:hM3Dq mice were conditioned daily for 4 days with VEH-treatment received in one chamber and CNO-mediated activation of LHA^{Nts} neurons in another (Fig 3.5B, experiment paradigm). On test day, no treatment was given, and the mice were

given free access to both chambers. The time spent in each chamber was recorded for the duration of the test. LHA^{Nts}:mCherry controls and LHA^{Nts}:hM3Dq mice spent similar amounts of time in the CNO-paired chamber (Fig 3.5C). These results suggest that chemogenetic stimulation of LHA^{Nts} neurons does not increase elicit reward-like behavior.

3.3.6 LHA Specific Deletion of Nts Increases Pain Hypersensitivity in Obese Mice but Not in Normal Weight Mice

We next tested if Nts expression in LHA neurons is necessary for their analgesic effect in obesity-induced pain. We studied cohorts of *Nts*$^{flox/flox}$ mice maintained on chow or HFD to invoke DIO. Mice were bilaterally injected in the LHA with either rAAV2-hSyn-eGFP to generate mice with intact Nts ($LHA^{Nts\text{-}GFP}$) or rAAV2-hSyn-GFP-Cre to specifically deplete Nts from the LHA ($LHA^{Nts\text{-}KO}$ mice) (Fig 3.6A). Mice were assessed with von Frey filament tests 6 weeks after AAV injection (Fig 3.6B). Adult-onset deletion of *Nts* from LHA neurons in chow-fed normal weight mice did not change mechanical sensitivity in the $LHA^{Nts\text{-}KO}$ mice when compared with $LHA^{Nts\text{-}GFP}$ controls (Fig 3.6C). However, DIO $LHA^{Nts\text{-}KO}$ mice exhibited a reduction in paw withdrawal threshold compared to DIO $LHA^{Nts\text{-}GFP}$ controls with intact *Nts* in the LHA (Fig 3.6D, purple bars). These results confirm that endogenous *Nts* expression in the LHA is required for pain processing, and that loss of it worsens obesity-induced pain.

3.4 DISCUSSION

Despite the common occurrence of chronic pain and obesity together, few studies have investigated their shared neurobiological mechanisms. In particular, the Nts system has been studied independently regarding pain relief and body weight regulation, their intersection has been largely overlooked. We previously showed that the population of neurons in the LHA that

produce and release Nts, the LHA^{Nts} neurons[55], regulate energy balance and promote weight loss[25]. In Chapter 2 we described how LHA^{Nts} neurons contribute to the regulation of acute and persistent pain in normal weight mice. Here, we explored whether this population of neurons produced analgesia in obesity-induced chronic pain. We verified that mice fed HFD develop both diet-induced obesity (DIO) and heightened pain sensitivity, and that mice can be used to model the presence of comorbid conditions (Fig 3.3C, D). Activating LHA^{Nts} neurons had no impact on acute thermal nociception in DIO mice. However, DIO mice displayed increased sensitivity to mechanical stimuli compared to normal-weight mice (Fig 3.3D) that is exacerbated by CFA-induced inflammation (Fig 3.4E). Chemogenetic stimulation of LHA^{Nts} neurons significantly attenuated obesity-related pain hypersensitivity and restored paw withdrawal threshold back to baseline level equivalent to healthy responses (Fig 3.3E, 3.4E). These data suggest that LHA^{Nts} neurons play a role in regulating persistent pain over time, such as presented by obesity, as opposed to acute pain such as thermal pain.

In contrast to neuropathic (Fig 2.4E-G) and inflammatory pain models (Fig 2.5F-H), we did not observe any difference in *Nts* expression in the LHA between normal weight and DIO mice (Fig 3.3G-I). Our results diverge from previous reports from genetically-obese rodents, which have reduced *Nts* expression[59,60]. It remains to be determined if these differences are due to differing causes of obesity (diet vs. genetics), and if/how Nts contributes to weight gain. However, our current data show that despite DIO mice retaining normal *Nts* expression this was not sufficient to alleviate obesity-induced pain. Activating LHA^{Nts} neurons of DIO mice was required to provide analgesia from obesity-induced and inflammatory pain. This implies that intact or increasing *Nts* expression is not necessary for the pain-relieving effects induced by LHA^{Nts} neurons in obese mice,

but activating the neurons is[58,59]. On the flip side, previous studies have demonstrated activating all LHA^{Nts} neurons in normal weight mice reduces food intake and body weight, but not in obese mice[25]. The underlying cause of this alteration is yet to be determined. One possibility for the differing ability of LHA^{Nts} neurons to mediate analgesia but not feeding suppression in DIO mice could be due to differing demands of Nts signaling. Perhaps elevated *Nts* is required for anorexia, but the levels present in obese mice are sufficient for analgesia. Future work modulating Nts expression levels has potential to address this possibility. It is also possible that obesity shifts the relative expression of Nts vs. other signals released from LHA^{Nts} neurons. While LHA^{Nts} neurons contain and release Nts, they also release the classical inhibitory neurotransmitter GABA[55-57]. Additionally, subsets of LHA^{Nts} neurons express various molecular markers and other neuropeptides (galanin, corticotropin-releasing hormone, etc)[57,58], whose release may enable them to contribute to distinct aspects of physiology[58,63-65]. Hence, DREADD-activation of LHA^{Nts} neurons leads to release of Nts, GABA and other signals, any of which could potentially contribute to the observed pain relief and weight loss effects. Nts clearly has a role in both but it is possible that other signals may counterbalance its regulation of feeding more so than pain processing. Understanding how the balance of Nts and other signals released from LHA^{Nts} neurons change in obesity could provide insight into how the neurons might be leveraged for simultaneous analgesia and weight loss.

In this study we hypothesized that LHA^{Nts} neurons releasing Nts are chief players in the centrally-mediated analgesic action of Nts, and loss of Nts signaling from these neurons would hasten obesity-pain. We tested this hypothesis using two different approaches: pharmacological inhibition of Nts signaling via NtsR-pan antagonist (SR142948), and genetic deletion of Nts

specifically from LHA^{Nts} neurons using the Cre-lox system using in *Nts$^{flox/flox}$* mice. Pretreatment with the SR142948 prior to chemogenetic stimulation of LHA^{Nts} neurons diminished the antinociceptive effect in both obesity-pain and CFA-induced inflammatory pain models, suggesting that Nts signaling via NtsR1 and/or NtsR2 is required for pain regulation. However, it's worth noting that systemic administration of the antagonist likely inhibits Nts signaling globally rather than specifically in LHA^{Nts} neurons. To address this limitation, and to confirm that Nts from the LHA is indeed a source of Nts-induced analgesia, we deleted Nts site-specifically from the LHA and tested mechanical sensitivity in normal weight and DIO mice. Deletion of Nts from the LHA of normal weight mice (chow-fed LHA$^{Nts\text{-}KO}$ mice) did not alter their mechanical responses compared to control LHA$^{Nts\text{-}GFP}$ mice with intact Nts in the LHA. These data suggest that loss of Nts alone is not sufficient to invoke pain. However, in DIO mice the absence of Nts in LHA^{Nts} neurons exacerbated pain hypersensitivity. One possibility to explain these findings is that Nts itself does not determine the presence or absence of pain, but that its increased expression can alleviate pain induced by other sources, such as obesity. Nonetheless, our findings reveal, for the first time, that activating LHA^{Nts} neurons in obese mice can alleviate obesity-induced pain, even when coupled with inflammatory pain. In the future, strategies to augment Nts release or Nts-NtsR signaling may be useful to treat obesity-induced pain.

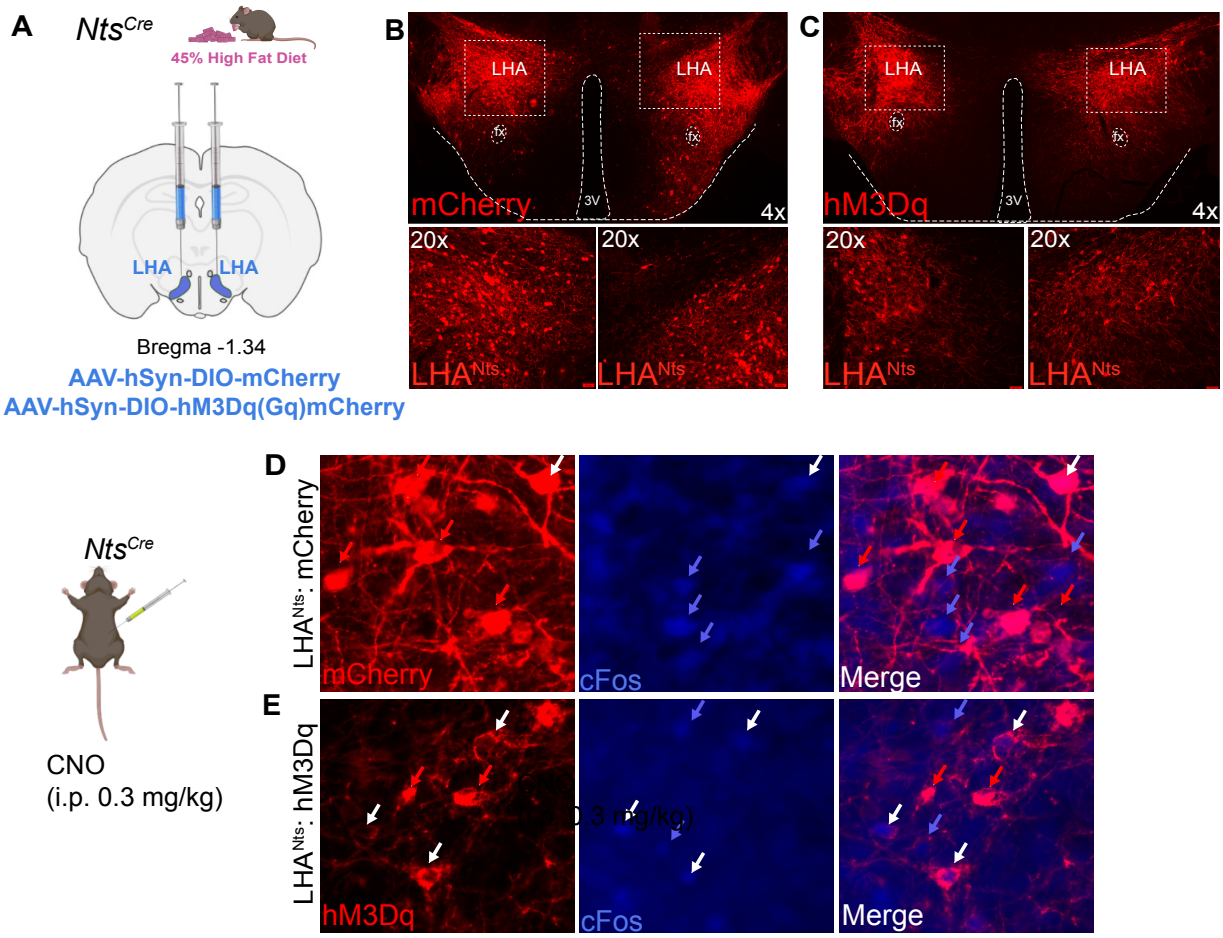

Figure 3.1 Chemogenetic Stimulation of LHA^{Nts} Neurons in Diet Induced Obese Mice. A) 12 week-old male and female *Nts^{Cre}* mice were bilaterally injected in the LHA with either pAAV-hSyn-DIO-mCherry to express mCherry (LHA^{Nts}:mCherry Control mice) or pAAV-hSyn-DIO-hM3Dq(Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice). B, C) mCherry fluorescence (red) in the LHA of B) LHA^{Nts}:mCherry control mice and C) LHA^{Nts}:hM3Dq mice. Top row: 4x magnification images to show AAV injection sites. Bottom row: 20x magnification images showing mCherry-expressing soma. Images representative of 6 mice per group. D-E) Immunoreactivity for mCherry (red) and cFOS (a marker of neuronal depolarization, blue) in the LHA of D) LHA^{Nts}:mCherry control mice (n=4) and E) LHA^{Nts}:hM3Dq mice (n=3) treated with CNO 90 min before brain collection. Red arrows indicate Nts neurons that only express mCherry, blue arrows indicate neurons that only express cFOS-only neurons (middle) and white arrows indicate neurons that co-express mCherry and cFOS. Scale bars = 200µm.

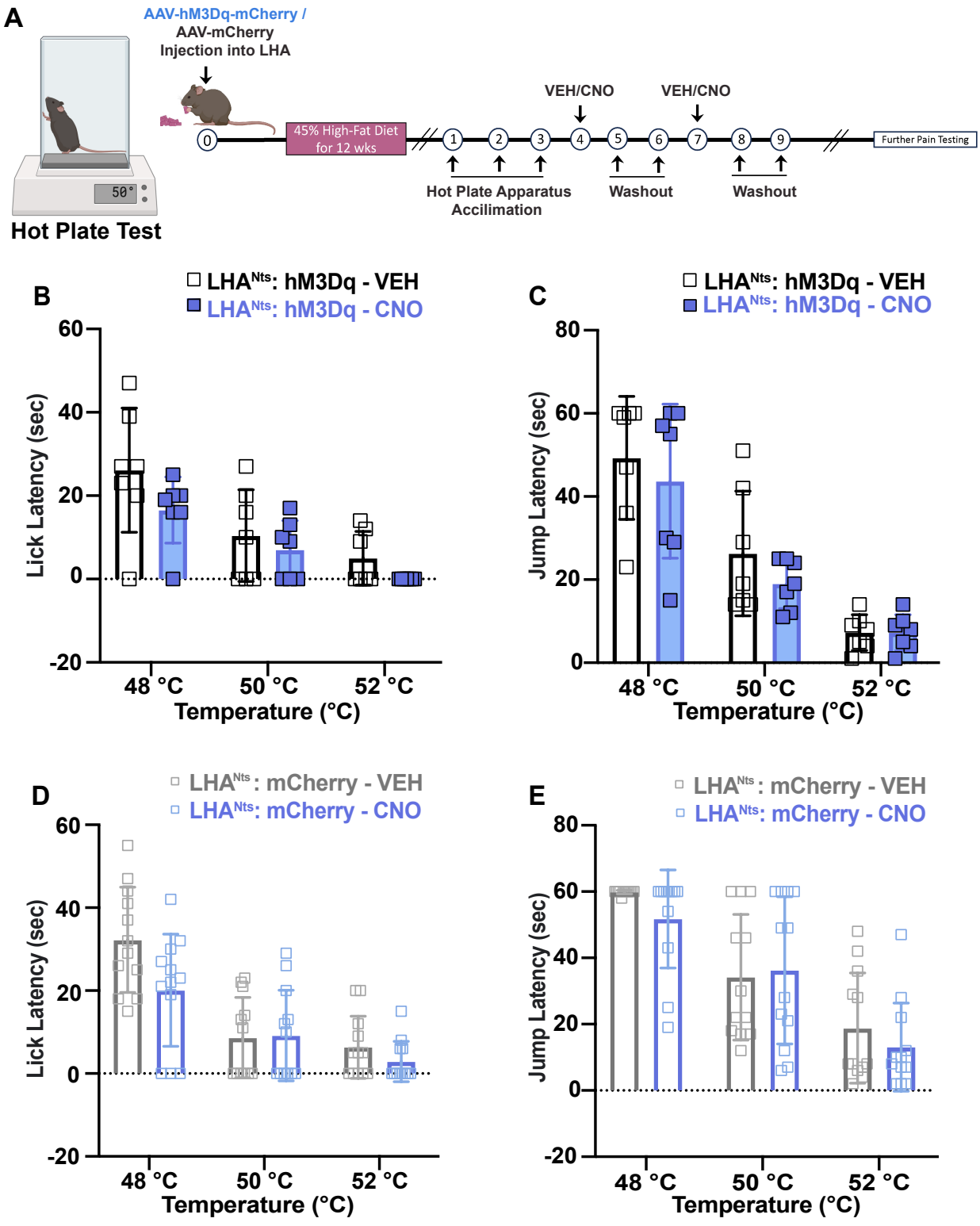

Figure 3.2 Chemogenetic Stimulation of LHA^{Nts} Neurons Does not Regulate Thermal Pain in Naïve DIO Mice. Adult *Nts^{Cre}* mice were injected in the LHA with pAAV-hSyn-DIO-hM3Dq (Gq)-mCherry to express excitatory hM3Dq-mCherry in LHA^{Nts} neurons (LHA^{Nts}:hM3Dq mice) and were put on a 45% high-fat diet to develop diet induced obesity to assessed for thermal pain responses

Figure 3.2 (cont'd)
via hot plate test in obesity. A) Schematic of hot plate test. Mice were placed on hot plate 30 mins after i.p. treatment with VEH (control) or CNO (to activate LHA^{Nts} neurons). B-E) Paw lick latency (B, D), and jump latency (C,E) of LHA^{Nts}:hM3Dq (n=7), and LHA^{Nts}:mCherry (n=12) were measured at 48℃, 50 ℃, and 52 ℃, temperature. Data was analyzed using two-way repeated measure ANOVA with Sidak post-tests. No statistically significant differences were found between treatments.

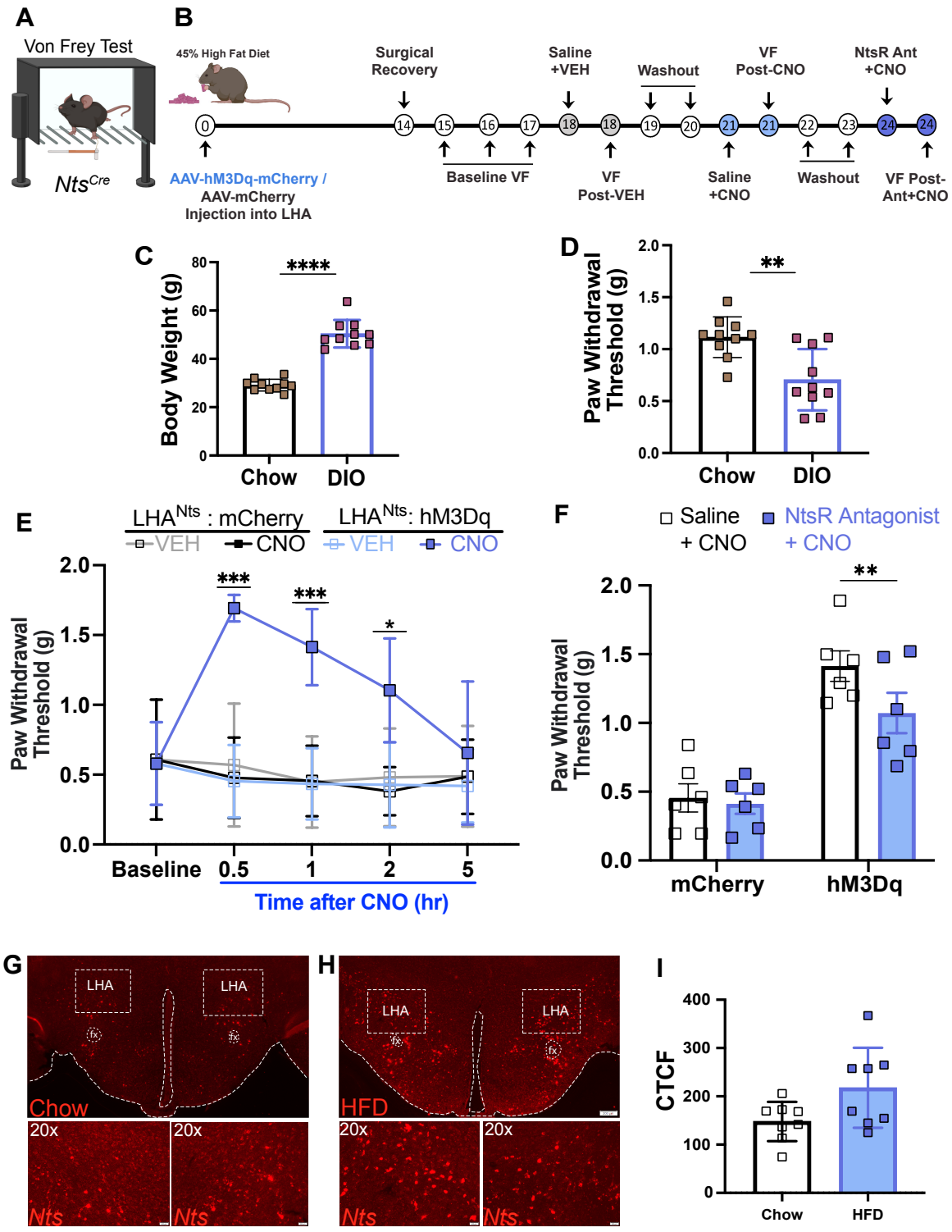

Figure 3.3 Chemogenetic Stimulation of LHA^{Nts} Neurons Alleviated Obesity-Induced Pain via Nts Signaling. Adult *Nts^{Cre}* -mCherry and -hM3Dq mice were fed either Chow or 45% high fat diet for 16 wk to invoke diet-induced obesity (DIO). A) Schematic of von Frey filament test. B) Plan of

Figure 3.3 (cont'd)
experiment. C) Body weight of age-match, sex-match mice on chow (Chow, brown data points, n=10) is significantly less than mice on 45% high-fat diet (DIO, pink data points, n=10). Graphs represent mean ± SEM. ****p<0.0001, unpaired Student's t-test. Mice were assessed for baseline mechanical sensitivity via von Frey filament test. D) Baseline paw withdrawal threshold of mice on chow (Chow, brown data points, n=10) is significantly higher than DIO mice (DIO, pink data points, n=10). Graphs represent mean ± SEM. **p<0.001, unpaired Student's t-test. E) DIO mice received i.p. VEH/CNO injection prior to test. Paw withdrawal threshold in DIO LHA^{Nts}:mCherry mice (black and grey, n=6) and DIO LHA^{Nts}:hM3Dq mice (Blue, n=6) before (baseline) and after treatment with VEH and CNO. CNO has an analgesic effect only in LHA^{Nts}:hM3Dq mice but not in Controls. Graphs represent mean ± SEM. *p<0.05, ***p<0.001, ***p=0.0001, Two-Way ANOVA with Tukey's multiple comparisons test. F) Pretreatment with Saline (black) or NtsR pan-antagonist (SR142948) (blue) 1 hr prior to CNO. **p<0.01, Two-Way ANOVA with Sidak's multiple comparisons test. G-I) Representative images from G) wild type chow- or H) HFD-fed mice (4 males, 4 females per group) and assessed for *Nts* mRNA expression (red) via RNA-Scope. Top images: 4x magnification. Bottom: digital magnification of boxed regions from top row. fx = fornix. I) Quantification of *Nts* mRNA from mice in F, G) via Corrected Total Cell Fluorescence (CTCF). Graphed data represent mean ± SEM. No significant differences via unpaired Student's t-test.

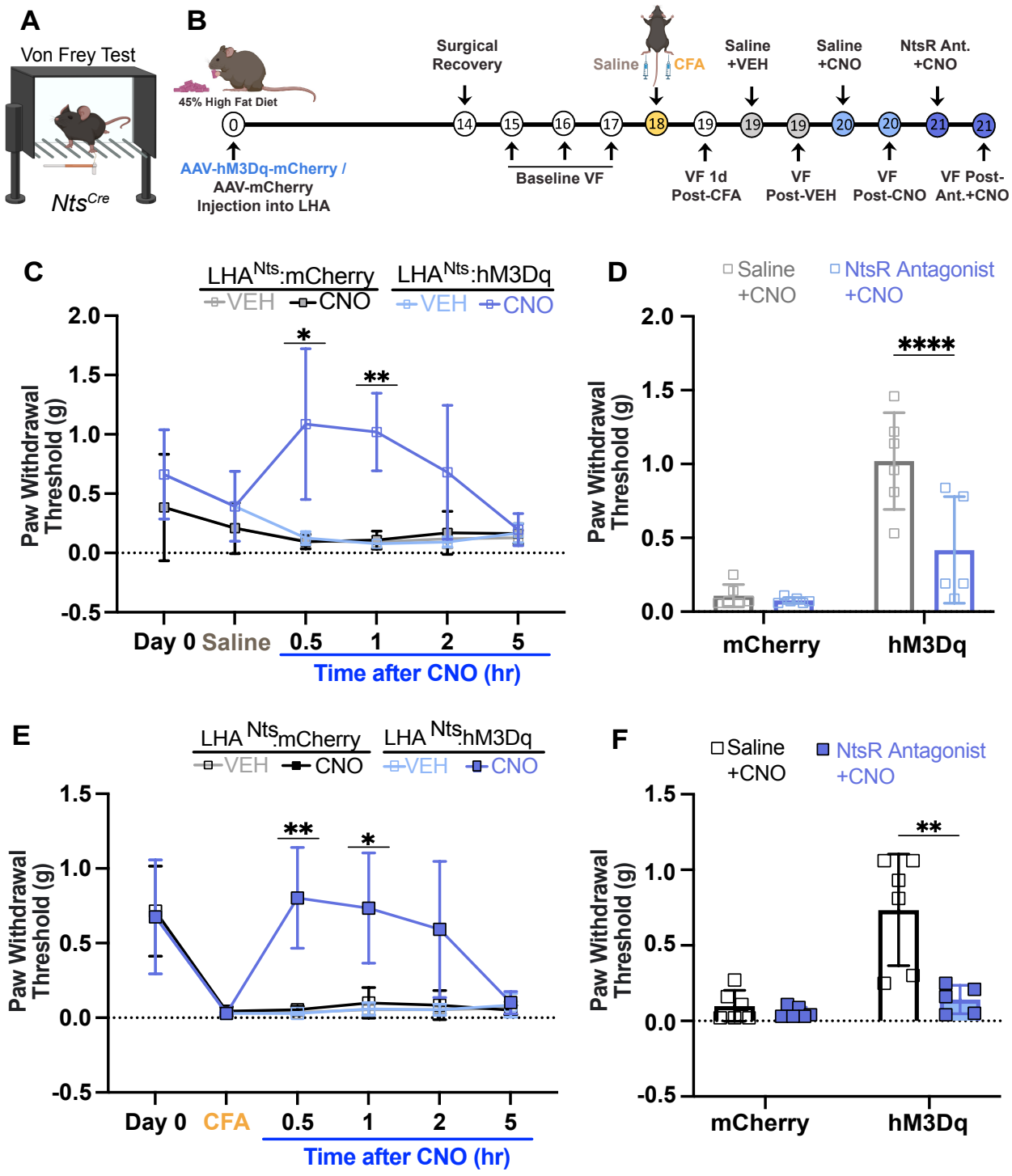

Figure 3.4 Chemogenetic Stimulation of LHA^{Nts} Neurons Attenuates Inflammatory Pain Hypersensitivity via Nts Signaling in Obese Mice. Adult *Nts^{Cre}* -mCherry and -hM3Dq mice were fed 45% high fat diet to invoke diet-induced obesity (DIO). A) Schematic of von Frey filament test. B) Experiment timeline. C, E) Paw withdrawal threshold before C) Saline injection and E) CFA (day 0), 24 hr after Saline/CFA (Saline in grey, CFA in yellow), and 0.5, 1, 2, or 5 hr after treatment with

Figure 3.4 (cont'd)
VEH or CNO to stimulate LHA^{Nts} neurons in LHA^{Nts}:mCherry (gray/black) and LHA^{Nts}:hM3Dq mice (blue) mice. CNO has an analgesic effect in LHA^{Nts}:hM3Dq mice but not LHA^{Nts}:mCherry mice. Graphs represent mean ± SEM. *p<0.05, **p<0.01, Two-Way ANOVA with Tukey's multiple comparisons test. D, F) Pretreatment with Saline or NtsR pan-antagonist SR142948 (Blue) 1 hr prior to CNO in D) saline injected paw and F) CFA injected paw. **p<0.01, ****p<0.0001, Two-Way ANOVA with Sidak's multiple comparisons test.

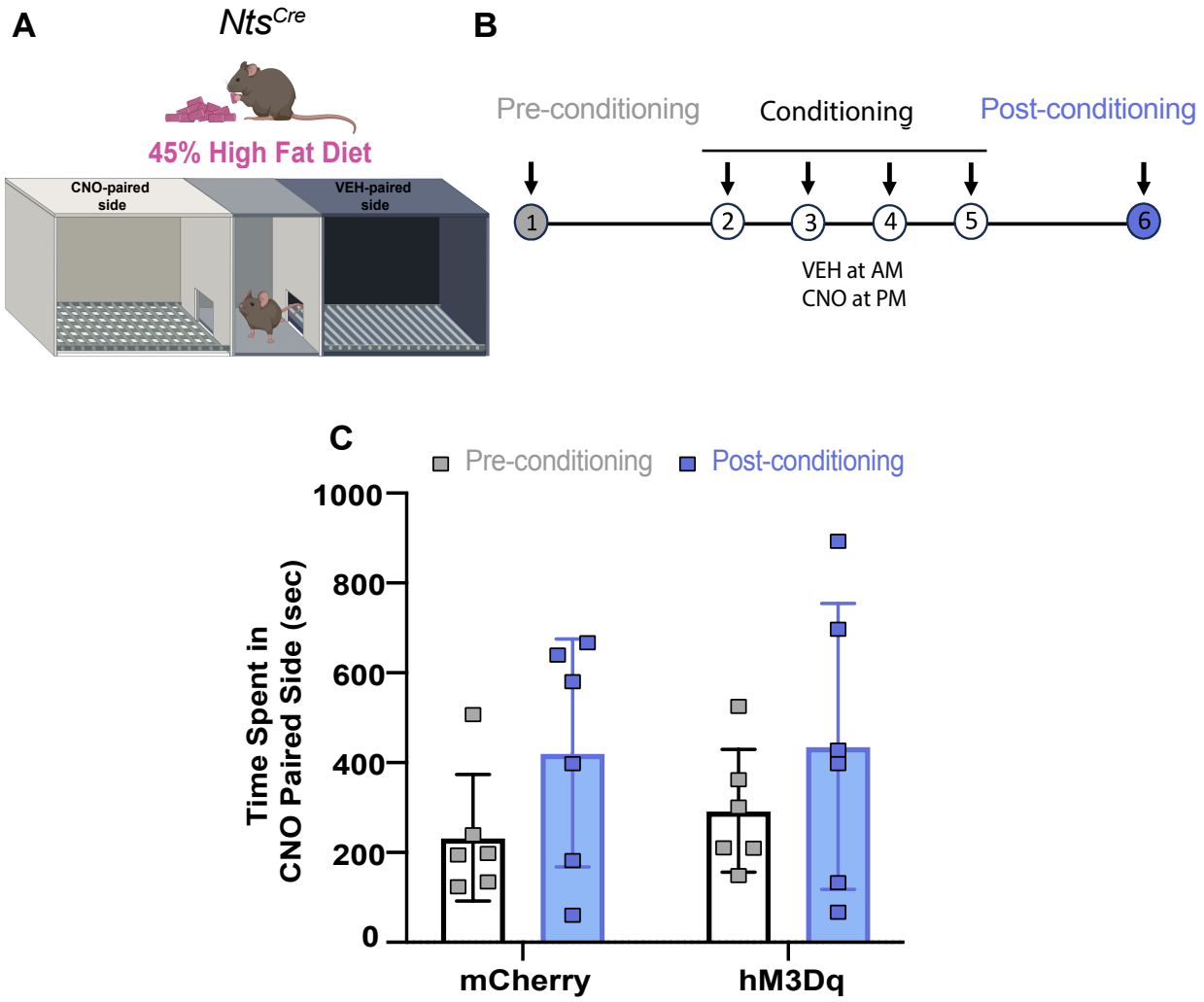

Figure 3.5 Stimulating LHA^{Nts} Neurons Does Not Elicit Reward-Like Behavior in Diet-Induced Obese Mice. A) Schematic of conditioned place preference apparatus use to test adult DIO LHA^{Nts}:mCherry and DIO-LHA^{Nts}: hM3Dq mice. B) Experiment timeline. C) Time spent in CNO-paired side by mCherry- (control) and hM3Dq-expressing mice before (pre-conditioning, black), and after (post-conditioning, blue) conditioning. No significant difference was found using Two-Way ANOVA with Sidak's multiple comparisons test.

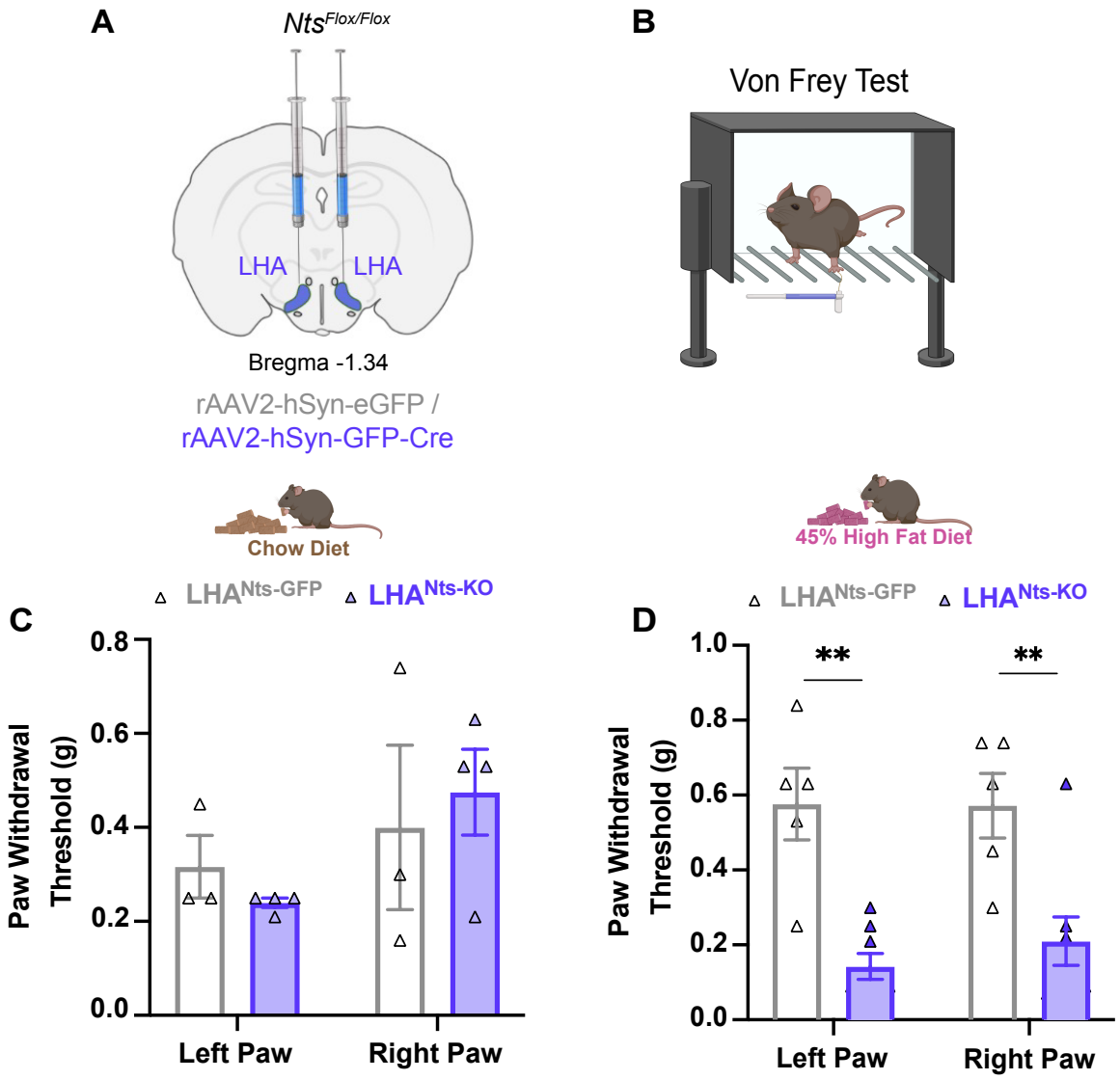

Figure 3.6 LHA-Specific Deletion of Nts Increases Pain Hypersensitivity in Obese Mice but Not in Normal Weight Mice. A) Adult male and female *Nts*$^{flox/flox}$ mice were bilaterally injected in the LHA with either rAAV2-hSyn-eGFP to express GFP (LHA$^{Nts\text{-}GFP}$ mice, controls with intact *Nts*) or rAAV-hSyn-GFP-Cre (LHA$^{Nts\text{-}KO}$ mice, in which *Nts* is deleted from LHA^{Nts} neurons). Mice were either fed chow or 45% HFD for 16 wk to invoke diet-induced obesity (DIO). B) Mechanical sensitivity was measure using von Frey test in C) in chow-, and D) HFD-fed LHA$^{Nts\text{-}GFP}$, and LHA$^{Nts\text{-}KO}$. **p<0.01, ****p<0.0001, Two-Way ANOVA with Sidak's multiple comparisons test.

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CHAPTER 4: SUMMARY, DISCUSSION, AND TRANSLATIONAL IMPLICATIONS

4.1 SUMMARY OF RESULTS

The overall goal of this PhD dissertation was to understand the role of LHA^{Nts} neurons in nociception. The main hypothesis of the work was that central Nts signaling can, and is necessary to, alleviate obesity induced pain. Moreover, we hypothesized that this can be achieved via augmenting endogenous Nts release from LHA^{Nts} neurons.

In chapter 2, we used Nts^{Cre} mice and chemogenetic/Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology to study how experimental activation of LHA^{Nts} neurons altered nociceptive responses in acute and chronic pain modalities. Our hypothesis for this chapter was that LHA^{Nts} neurons are an endogenous source of Nts that can provide antinociception, and hence, that activating LHA^{Nts} neurons would alleviate pain dependent on Nts signaling via NtsRs. We injected Nts^{Cre} mice in the LHA with AAVs to enable Cre-mediated expression of excitatory DREADDs, thereby selectively expressing DREADDs in LHA^{Nts} neurons. Systemic administration of the DREADD ligand CNO activated these neurons on command. We discovered that acute stimulation of LHA^{Nts} neurons does not regulate acute thermal and mechanical nociception in chow-fed, normal weight naïve mice. However, DREADD-stimulation of LHA^{Nts} neurons alleviated nerve injury-induced hypersensitivity. Moreover, CNO-mediated stimulation of LHA^{Nts} neurons also alleviated complete Freund's adjuvant (CFA)-induced inflammatory pain and the analgesic effect lasted up to 5 hours. These data reveal that activating LHA^{Nts} neurons can alleviate severe, persistent pain, prompting the question of how they mechanistically do so.

LHA^{Nts} neurons express, and release multiple signals in addition to Nts, including the fast neurotransmitter GABA[1-3] and peptides such as galanin, CRH[3,4]. Any of these released signals might mediate the analgesic effect observed so we also examined whether LHA^{Nts} neurons-induced analgesia is dependent on Nts signaling via NtsR1 and/or NtsR2. We found that administering the NtsR antagonist SR142948 prior to CNO-mediated stimulation of LHA^{Nts} neurons significantly reduced antinociception. We also found that *Nts* expression is significantly upregulated in the LHA in mice with persistent neuropathic pain and CFA-induced inflammatory pain compared to sham control mice. These results highlight the importance of LHA Nts signaling in pain regulation and suggest that LHA^{Nts} neurons may be a biological target that could be leveraged to treat chronic pain.

In chapter 3, we assessed the analgesic role of LHA^{Nts} neurons in the context of obesity. We reasoned that since activation of LHA^{Nts} neurons promotes weight loss[5] and provides analgesia in persistent pain models (chapter 2), then perhaps they can be a common target to treat comorbid obesity-pain. For these studies, we injected normal weight and diet-induced obese (DIO) *Nts^{Cre}* mice in the LHA with AAVs to express DREADDs in LHA^{Nts} neurons, permitting their activation after treatment with the DREADD ligand CNO. Activating LHA^{Nts} neurons had no effect on thermal pain responses in DIO mice. By contrast, obesity invoked mechanical hypersensitivity that was completely reversed by CNO-mediated activation of LHA^{Nts} neurons compared to control. Activating LHA^{Nts} neurons also relieved pain hypersensitivity beyond the DIO-induced pain state, including CFA-mediated inflammatory pain. However, pretreatment with the brain permeable SR142948 NtsR1/2 antagonist blocked CNO-mediated analgesia, indicating that LHA^{Nts} neurons alleviate obesity induced pain in an Nts signaling-dependent manner. Despite

the pain relief, and in contrast to neuropathic and inflammatory pain models, we did not observe any difference in *Nts* expression in the LHA between normal weight and DIO mice. These data, thus, suggest that although LHA^{Nts} neuronal activation was sufficient to alleviate obesity-induced hyperalgesia, increased expression of *Nts* in the LHA is not necessary for execution of LHA^{Nts}-mediated pain at baseline compared to severe chronic pain models. Furthermore, to define the significance of endogenous LHA-produced Nts in pain regulation, we used $Nts^{flox/flox}$ mice to generate mice with adult-onset Nts deletion from LHA neurons. Interestingly, we did not observe any difference in mechanical sensitivity between chow-fed, normal weight $LHA^{Nts\text{-}KO}$ (lacking Nts), and $LHA^{Nts\text{-}GFP}$ mice (intact Nts). However, in DIO mice Nts deletion from the LHA further exacerbated hyperalgesia. Altogether, these data suggest that augmenting Nts signaling via LHA^{Nts} neurons can alleviate pain in normal weight and obese mice. Prior work established the Nts as an anorectic peptide and that activating LHA^{Nts} neurons supports weight loss in an Nts-dependent manner. Taken together, these findings suggest that augmenting signaling via LHA^{Nts} neurons may be a common actionable target to treat comorbid obesity-pain.

4.2 DISCUSSION

4.2.1 Limitations and Technical Considerations of this work

We leveraged viral and Cre-lox mediated tools throughout this dissertation to specifically modulate LHA^{Nts} neurons as needed to test our hypotheses. Therefore, our results are limited by the efficacy of the mouse models and viral tools used in these experiments. To address potential caveats of these reagents we used several controls for each mouse strains, virally mediated tool and techniques that modulated gene expression or neuronal activity. For experiments in Chapters 2 and 3, we used a knock in Nts^{Cre} model because knock-in lines are reliable reporters of

endogenous gene expression[6]. However, it is recognized that introducing IRES Cre after the stop codon (as is the cause in *Nts*^{Cre} mice) can sometimes influence expression of the upstream coding sequence[7]. To control for this possibility, we used a within-subjects treatment approach, so that *Nts*^{Cre} mice could be used as their own controls. However, it would be valuable to verify our findings using approaches to activate LHA^{Nts} neurons that do not rely on use of the *Nts*^{Cre} line. This may be possible in the future with development of CRISPR-Cas9 reagents to modulate the Nts system in wild type mice[8]. However, while we cannot verify that *Nts*^{Cre} mice are fully equivalent to wild type mice, our within-subjects chemogenetics studies verifies that activating LHA^{Nts} neurons in this model is sufficient to provide analgesia that depends on Nts-NtsR signaling.

We also used well-established viral tools for chemogenetic studies (DREADDs) to modify the activity of LHA^{Nts} neurons and understand their function in pain regulation. Even through DREADDS have been enormously useful to dissect how specific neuronal populations contribute to physiology and behavior, there are still limitations in using them. The DREADD ligand CNO has been reported to be inert, but it may have off target effects due to the action of its metabolite, clozapine[9]. Therefore, it was important to verify that the CNO dosage used for our chemogenetics studies does not exert any effects when injected into non-DREADD expression mice. For this reason, we injected *Nts*^{Cre} mice with Cre-inducible mCherry control AAV lacking DREADD receptor, and these mice received the same CNO treatments as DREADD-expressing *Nts*^{Cre} mice. We also used a low concentration of CNO (0.3 mg/kg) that does not produce off-target effects in contrast to higher concentrations[9]. These experimental controls support that the dose of CNO used here has no significant off-target effects on nociception-associated behaviors in mice lacking DREADD receptors, consistent with previous reports from our lab that it does not impact physiology and

ingestive behavior[5,10,11]. Thus, the CNO-treatment induced alterations in behaviors reported in this dissertation represent genuine physiological responses attributed to the stimulation of LHA^{Nts} neurons. However, future studies can fully avoid the possibility of off-target effects via switching to use of newly developed DREADD agonists such as JHU 37152 or Compound 21, which are inert and are proven not to undergo back-metabolism[12]. Our preliminary data show that CNO and Compound 21 work at equivalent doses to activate DREADD-expressing LHA^{Nts} neurons and result in the same behavioral and physiological changes (data not shown).

An intriguing finding in Chapters 2 and 3 is that chemogenetic stimulation of LHA^{Nts} neurons does not change thermal nociception responses in normal weight and obese mice, and only lessens nociceptive responses in the context of severe, persistent pain (inflammatory, nerve injury and obesity-induced pain). These data suggest that the LHA^{Nts} neurons are biological modulators of persistent pain, not of unpleasant sensation per se. Why this distinction? One possibility is that LHA^{Nts}-mediated influence on pain requires long-term changes to occur, such as perhaps increased expression of *Nts*. For example, prolonged stress-induced analgesia increased *Nts* expression in the LHA, suggesting a potential role for LHA^{Nts} neurons in pain processing[13]. It is also worth noting that while Nts has been generally characterized as having analgesic effects, the impact of Nts on nociception may vary by concentration and/or site of action. For instance, administration of a low dose of Nts into the rostral ventral medulla results in a hyper-reflexive thermal response (a sign of increased nociception), as measured by the tail-flick test[14,15], and treatment with NtsR1 antagonist SR48692 mildly prolonged the tail-flick latency suggestive of decreased nociceptive responding[16]. Similarly, knockout mice lacking NtsR2 (NtsR2-KO) have increased jump latency on the hot plate suggestive of reduced thermal nociception[17,18]. Some

have interpreted this as evidence that NtsR1 and R2 play a facilitating role in mechanical and thermal nociception. Yet, the jump response to thermal stimuli is often associated with a flight response to escape danger or acute pain, ensuring safety. If Nts enhances thermal nociception, one would expect that NtsR1 knockout mice and NtsR2 knockout mice would display a decreased paw lick response during the hot plate test, indicative of reduced thermal nociception. However, no differences in paw lick response were observed between the knockout groups and wildtype mice in this regard. When considering both the paw lick response and jump latency data together, it suggests that Nts might not potentiate thermal nociception but rather act as an alarm system to detect and facilitate the escape from acute thermal pain. Partially in line with this interpretation, our LHA^{Nts} neurons DREADD-activation data suggest that activation of LHA^{Nts} neurons does not potentiate thermal nociception, as we found no change in thermal responses between DREADDs-expressing groups and control mice. Future studies will be needed to explore if genetically removing Nts or pharmacologically inhibiting Nts signaling via LHA neurons attenuates thermal and mechanical sensitivity.

Another caveat in Chapters 2 and 3 is that we used systemic injections of the brain-permeable NtsR-pan antagonist SR142948 for pharmacological inhibition of Nts signaling to study Nts-mediated analgesia. Although pretreatment with the SR142948 prior to stimulation of LHA^{Nts} neurons significantly diminished the antinociceptive effect in all persistent pain models, the systemic route of administration of NtsR antagonist does not guarantee that we specifically inhibited LHA^{Nts} neuron-mediated downstream signaling. To address this limitation, and to confirm that Nts from the LHA is indeed a source of Nts-induced analgesia, we used $Nts^{flox/flox}$ mice to selectively delete *Nts* from the LHA. This technique provides more spatial control to target

Nts-expressing neurons in the LHA, and to elucidate the role of this specific population of LHA neurons in pain. Although our results have consistently shown that pharmacological and genetic inhibition of Nts significantly diminishes anticonception, future studies should explore the necessity of other signals, such as GABA, releasing from the "LHA^{Nts} neurons"[1-3]. For example, recent work supports that Nts and GABA released from LHA^{Nts} neuron may mediate distinct reward behaviors and thermoregulatory responses, and it is possible that they may divergently modulate pain responses as well[18,19].

Additionally, in Chapter 3, we observed that pharmacological inhibition of Nts signaling using NtsR pan antagonist, SR-142948 significantly reduced the analgesic response induced by activation of LHA^{Nts} neurons in obese mice (Chapter 3, Figure 3.3F), but interestingly, the effect of antagonist is not as pronounced as was observed in normal weight mice with inflammatory pain (Chapter 2, Figure 2.5E). One possible reason can be that the same dose of antagonist as in normal weight mice is not enough in obese mice as drug distribution is highly impacted in obesity due to increase in fat mass ratio to lean body weight. As a result of this volume of distribution is increased for both hydrophilic and lipophilic drugs. In light of this, our future studies will account for changes in the distribution volume by adjusting the dose of drug.

A unique aspect of our work in Chapter 3 was assessing whether activating LHA^{Nts} neurons impact obesity-induced hypersensitivity. Obesity is one of the greatest risk factors for development of chronic pain[20-22], including painful peripheral neuropathy[23-25]. Animal models confirm that consumption of high fat diet and obesity is sufficient to cause peripheral neuropathy and support a direct link between obesity and chronic pain[26,27]. The hypothalamus is one of the brain regions known to mediate energy balance[28-30] as well as nociception via the actions of

multiple subsets of molecularly defined neuronal populations. For example, neuropeptides like hypocretin/orexin and melanin concentrating hormone in the LHA promote feeding, whereas adjacent neurons expressing Nts suppress feeding[31]. Moreover, both orexigenic and anorexigenic peptides have been shown to attenuate and/or facilitate nociception[32-34]. Among these different subsets of LHA neurons, what makes Nts-expressing LHA neurons of great interest is the large, separate bodies of literature supporting its role in weight loss[5,6,10,35] and pain regulation[36-42], respectively. However, studies of the effect of Nts on energy balance and pain regulation using separate animal models and routes of peptide administration (central vs peripheral) has hindered appreciation of whether energy balance and nociception are regulated by common or separate Nts systems. Our previous[5] and current work combined, addressed this gap in the Nts field, and identify LHA^{Nts} neurons as a common node of action whereby Nts signaling promotes weight loss as well as antinociception. Intriguingly, LHA^{Nts} neurons are connected with brain areas implicated in pain and energy regulation, including the VTA[11,43], and the PAG[44-49]. Taken together, study of the central Nts system holds unique potential to address both obesity and chronic pain.

In Chapter 2, we found that *Nts* expression is significantly increased in neuropathic and inflammatory pain models suggesting that persistent pain may biologically increase *Nts* expression in the LHA. In contrast, RNAscope analysis in Chapter 3 revealed that DIO mice have similar *Nts* expression as chow-fed control mice. On the contrary, monogenetic obesity rodent models have reduced *Nts* expression in the brain[50,51]. It remains to be determined if these differences are due to differing causes of obesity such as diet vs. genetics. However, our current data show that despite DIO mice retaining normal *Nts* expression and upregulated *Nts* expression in persistent pain models, that this elevated expression alone is not sufficient to provide

analgesia. Activating LHA^{Nts} neurons was necessary to reverse hypersensitivity. This implies that some activation-mediated release of a signal from the LHA^{Nts} neurons is necessary for modifying the pain processing. Additionally, it is worth highlighting that our quantitative *Nts* expression analysis in RNAscope experiments was not specific to just LHA neurons. We measured total florescence in the LHA which included *Nts* present in cells other that neurons and in extracellular matrix and cannot distinguish the cellular source of the *Nts*. Hence, it is possible that during DIO Nts levels are differentially regulated in neurons vs. other cell types in the LHA. If true, it is possible that *Nts* expression is indeed reduced in DIO LHA neurons as previously shown in genetic obesity models but increased expression in other cell types such as perhaps microglia, ependymal cells, or vasculature cells might mask the reduction. In the future this may be addressed via dual-label RNAScope with markers of various cell types to enable cell-type specific expression measurements or possibly single cell RNASeq analysis of LHA tissue from normal weight and DIO mice.

Another important question to draw from these findings is that does the upregulated Nts mRNA translate into a peptide and released outside the cells. To address this question, microdialysis technique will help us understand if persistent pain upregulate Nts expression, translation and peptide is produced. Furthermore, microdialysis will also provide us insights whether Nts is produced locally in the cell or accumulation occur in the extracellular surroundings from projections coming from other brain regions.

Finally, for all experiments in this dissertation, we have included males and female mice since we recognize that it is important to investigate potential sex-specific pain regulation via

LHA^{Nts} neurons. We did not find any significant difference between sexes in any of our experiments, so data obtained from both sexes were pooled for analysis.

4.2.2 Future Directions

The culmination of this dissertation shows that LHA^{Nts} neurons play a role in pain regulation. Although this work provides preliminary evidence that LHA Nts signaling is a potential candidate for treating pain, including in the context of obesity, several questions and challenges need to be addressed to fully understand the function of LHA^{Nts} neurons in comorbid obesity and pain. One vital question is where the Nts released from LHA^{Nts} neurons acts in the brain to alleviate pain and via which receptor/s. As discussed above, LHA^{Nts} neurons project to many regions implicated in pain processing, any of which could conceivably contribute to the analgesic effects. Circuit-based analyses will be important to define the critical pathways by which the LHA^{Nts} neurons engage and modulate pain, and to identify the downstream cells of this endogenous signaling that might be pharmacologic targets for controlling pain.

Moreover, further work is needed to clarify how LHA^{Nts} neuron-released Nts mediates its effects, be that signaling via NtsR1, NtsR2, or both receptor isoforms. NtsR1 and NtsR2 agonists and antagonists have been used to identify the role of Nts receptors in regulating nociception[16,18,52-56] (as discussed in Chapter 1). For example, NtsR1 agonist (PD149163) alleviates thermal pain and formalin-induced inflammatory pain in a dose-dependent manner and agonist-mediated antinociception is hindered by the NtsR1 selective antagonist SR48692[40,57]. Similarly, NtsR2 has been proposed as the major receptor isoform mediating analgesia, largely based on experiments using first-generation pharmacological agonists and antagonists of the Nts system. Thus, it is feasible to pharmacologically modulate Nts system. However, systemic or central

agonism of the receptors could bind to NtsRs in tissue that does not exclusively modulate pain and weight loss behaviors. Therefore, the potential for this system for treating obesity pain depends on whether NtsR agonists can be selectively targeted to act via the sites where LHA^{Nts} neurons mediate their actions. This may be possible in the future with further understanding of the LHA^{Nts} neuronal targets that mediate feeding and/or pain processing, the NtsR isoforms the express, and their neurochemistry. For example, NtsRs can heterodimerize with other receptors, which might permit dual-agonist strategies to direct NtsR agonists to specific sites[58]. Moreover, it still remains to be understood how LHA^{Nts} neurons engage the descending pain pathway. One possibility is via direct LHA^{Nts} neuronal projections to the PAG or VTA, but it is equally possible that LHA^{Nts} neurons modulate pain processing via multisynaptic pathways that ultimately engage the descending pain system. Here again is where more detail of the connectivity and signaling of LHA^{Nts} neurons is required to understand their endogenous control of pain behavior and how it might be modulated for analgesic benefit. This information is a crucial precursor to follow-up questions of whether such mechanisms are targetable *in vivo* with current or newly designed pharmacological reagents. Lastly, it remains possible that LHA^{Nts} neuronal modulation of feeding and pain may occur via separate circuit and signaling mechanisms, and hence that distinct pharmacological strategies would be required to mimic them. This knowledge might also suggest how to bias Nts action for certain biology (e.g. feeding vs. analgesia) that might be beneficial for addressing other conditions, such as eating disorders or numbness and itch. Lastly, better understanding of LHA^{Nts} neurons' Nts-mediated signaling may identify strategies to promote beneficial behaviors without invoking adverse physiological effects attributed to Nts such as hypothermia and vasodilation[163] that previously drew interest away from Nts-mediated analgesia

and energy balance. Answering these questions is important to understand the fundamental mechanisms by which LHA^{Nts} neurons contributes to energy balance and pain.

Likewise, although our data supports an important role for Nts released from LHA^{Nts} neurons in mitigating nociception, the chemogenetic method used here causes release of all LHA^{Nts} neuronal signals and so we cannot exclude that signals other than Nts also contribute to pain processing. Given that LHA^{Nts} neurons express and release Nts as well as other neuropeptides such as galanin and CRH, along with the classical neurotransmitter GABA, these could play important roles as well. Our data support that LHA^{Nts} induced signaling via NtsR1/NtsR2 contributes to pain processing to some degree but the other released signals also merit investigation. While there is yet much to learn about the precise mechanisms by which LHA^{Nts} neurons contribute to biological pain processing, our data reveal them as an important node in the endogenous Nts system and control of pain processing. Going forward, understanding how the LHA^{Nts} neurons engage the descending pain system and modulate it may point to design of novel analgesic approaches to provide relief for severe, persistent pain.

4.3 TRANSLATIONAL IMPLICATIONS OF THIS RESEARCH

The Nts system has been linked to nociception and energy balance before, but how it mediates antinociception remains unclear. This is in part due to systemic or central administration of Nts that manipulates the Nts system globally, and the pleiotropic effects of Nts throughout different brain areas and in the periphery. Defining and understanding the brain circuits through which Nts mediates these processes, as well as how disruptions in obesity contribute to disease pathogenesis could help identify treatments for chronic pain in obesity. Although we identified the LHA as a brain source of Nts that promotes analgesia in mice, this was

only possible due to the powerful array of Cre/Lox mouse lines and site-specific tools that permits us to identify and manipulate specific neurons and circuitry. Such mechanistic studies are simply not possible in humans. Studies in humans to identify the role of Nts in pain have been limited to testing the effect Nts and Nts analogs on human NtsRs expressed in animal models[63] that cannot provide information about how Nts-NtsRs system effects overall humans physiology. Even though there are not yet as many human studies about the role of Nts in pain regulation, these studies still support decades of accumulated results from rodents, signifying the translational value of rodent studies to decipher the central circuit controlling nociception. However, recent data suggests similarities in the Nts system of mice and humans, and hence, that preclinical research in mouse models may be relevant to understand human biology and disease states[61].

In summary, the basic science work of this dissertation, while confined to rodents, advances understanding of the physiological pain processing that promotes analgesia in different pain modalities. As such, this work could, in the future, have implications for common treatment targets for obesity-pain hypersensitivity in humans. Our findings that the activation of LHA^{Nts} neurons in normal weight and obese mice are sufficient to provide analgesia refines the field's knowledge of how and where to leverage the Nts system to mediate energy balance and pain relief. We showed that Nts is upregulated in the LHA in chronic pain models, but just the increased *Nts* expression is not enough to alleviate pain. Moreover, we showed that HFD induced obese mice exhibit mechanical hypersensitivity compared to chow-fed normal weight mice, and that activation of LHA^{Nts} neurons completely reverses this pain hypersensitivity in an Nts signaling-dependent manner. We also showed that specifically deleting Nts from LHA^{Nts} neurons in adulthood promotes hyperalgesia in mechanical test. From a translational perspective, these

results suggest promise for augmenting LHA^{Nts} neuronal activity for restraining feeding and pain, as may be relevant for future treatment of comorbid obesity pain.

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